Principles of Laboratory Animal Science

A contribution to the humane use and care of animals and to the quality of experimental results

REVISED EDITION

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2001
ELSEVIER
AMSTERDAM • LONDON • NEW YORK • OXFORD • PARIS • SHANNON • TOKYO
It is widely recognized now that education and training in laboratory animal science are essential both for the quality of research and for the welfare of animals.

Scientists who are responsible for the design and conduct of animal experiments must not only be educated in one of the biomedical sciences (biology, medicine, veterinary medicine, pharmacy, etc.), but should also have taken an introductory course in laboratory animal science, encompassing welfare issues, ethical aspects and animal alternatives. Indeed, in some countries such requirements have been made compulsory by law.

This book contains basic facts and principles covering the main theoretical aspects of such a course. More than 50 authors, all experts in their fields have contributed relevant topics for the graduate student or young scientist who wishes to become a “competent” researcher. After a general introduction (chapter 1) and a glimpse into legislation (chapter 2), information is presented on the biology and husbandry of the most frequently used animal species (chapter 3) and on the relationship between behaviour, stress and wellbeing (chapter 4). Standardization can contribute to a reduction of animal use and several aspects are covered in chapters 5–8. Chapter 9 deals with procedures for the diagnosis of diseases in laboratory animals and the consequences brought about by impaired health on both the welfare of the animals and the results of the experiments. Chapters 10–13 outline several factors that should be taken into account when designing and conducting animal experiments. Persons involved in animal experimentation must be able to recognize signals of pain and distress (chapter 14). The researcher must also have some basic knowledge of anaesthesia (chapter 15) particularly when invasive techniques are part of the experiment (chapter 16). The book concludes by outlining the possibilities and limitations of the use of alternatives (chapter 17), together with a chapter on the ethical aspects of animal experimentation (chapter 18).
For further reading, a list of recommended literature is given at the end of each chapter.

It should be emphasized that the completion of a biomedical training programme that includes a course in laboratory animal science, may provide a basis for a humane and responsible use of animals, but does certainly not provide full competence. The great diversity of biomedical disciplines and the wide range of animal experiments performed by scientists, working in the field of biomedical science, implies that a standard training programme in which every requirement for the various kinds of animal experiments is fully met, is not feasible. There is no substitute for learning in the field, and a close co-operation with conscientious and experienced investigators, animal caretakers and animal technicians remains essential in order to fill any gaps in skill and knowledge.

The second edition of this book is largely the same as the previous edition. Also the list of contributors is only slightly changed. We would like to thank Dr. A.P.M.G. Bertens, Prof. Dr. W.J.I. van der Gulden, Dr. P.G.C. Hermans, Dr. B.C. Kruijt, Prof. Dr. E. Lagerweij, Dr. G.W. Meijer, Dr. J.W.M.A. Mullink (†), Prof. Dr. K.J. Öbrink (†) and Dr. G.A. van Oortmerssen for their contribution to the first edition and are grateful to Dr. A.E.J.M. van den Bogaard, Dr. H.A. van Lith, Prof. Dr. R. Remie and Dr. J.B.F. van der Valk for their willingness to contribute to this second edition.

We have prepared this second edition as careful as possible but would welcome critique and suggestions for further improvements.

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1 Introduction

L. F. M. van Zutphen

Laboratory animal science

Laboratory animal science can be defined as a multidisciplinary branch of science, contributing to the humane use of animals in biomedical research and to the collection of informative, unbiased and reproducible data. Laboratory animal science encompasses the study of the biology of laboratory animals, their husbandry and environmental requirements, genetic and microbiological standardization procedures, the prevention and treatment of diseases, the optimization of experimental techniques, and the improvement of anaesthesia, analgesia and euthanasia. Ethical aspects of animal experimentation, together with the search for alternatives, also fall within the domain of laboratory animal science.

The primary objectives of laboratory animal science are to contribute to the quality of animal experimentation and to the welfare of the animals.

The term “animal experiment” can be applied to any scientific procedure involving animals, irrespective of whether the animal used is vertebrate or invertebrate. In most legislative regulations on the protection of experimental animals, the term is restricted to experiments with vertebrates. In this book the description, “animal experiment” will be applicable to both vertebrates and invertebrates.

Most animal experiments are performed in the fields of medical, biological, veterinary and agricultural science.

In biology, veterinary science or agricultural science, experiments are frequently designed to collect information that is relevant to, or meaningful for, the animal or animal species in which the experiment has been performed. The vast majority of animals, however, is used for medical research and safety testing. Here the animal is almost exclusively used as a substitute or model for man.
History of animal use

The development of the use of animals as a model for man runs, to a great extent, parallel to the development of medical science. The basis of Western medicine stems from Greece, where philosophers were amongst the first to practise vivisection (literally: cutting into living organisms) for scientific purposes. Several examples of animal use are described in the first medical handbook, the *Corpus Hippocraticum* (about 400 B.C.). In this period medical science was mainly descriptive, with an emphasis on anatomy, whereas later physiological experiments were also performed. Galen (130–201 A.D.), who worked in Rome, was a physician and medical physiologist, and his experiments with pigs, monkeys and dogs have provided a basis for medical practices not only during that period but for many centuries thereafter.

With Galen the first era of medical research ended. Roman culture did not provide a good environment for the further development of medicine and biology. After the emergence of Christianity, experimental science ceased almost completely. Empirical studies were banned for more than a millennium, and no animal experiments worth mentioning were carried out. This situation lasted until the fifteenth century, the beginning of the Renaissance period. The revival of experimental medicine and biology, was part of the total revival of learning. The empirical approach rapidly gained ground. Initially, the emphasis which prevailed in medicine and biology was mainly on the anatomy (Vesalius, *De Humani Corporis Fabrica*, 1543) but, from the seventeenth century, physiological processes (Harvey, *Anatomica de Motu Cordis et Sanguinis in Animalibus*, Exercitatio, 1628) were also subjects of study.

At this time animals were not regarded as sentient creatures. This view was nourished by the publications of the French philosopher René (1596–1650) who proposed that living systems could be understood on purely mechanical principles. The main difference between man and animals was believed to be the fact that animals have no soul and therefore no consciousness. Man is able to think and to feel pain but the animal acts as an insensate machine.

From the eighteenth century onwards, it was gradually accepted that the results of experimental medicine contributed to man’s welfare and living conditions. It also became evident that the further development of medicine was dependent upon the results of animal experiments.

This, however, does not mean that, in those days, everyone was applauding the use of animals for this purpose. In 1789 an impressive contribution to the debate on the justification of animal experimentation was presented by Jeremy Bentham. In his famous *Introduction to the Principles of Morals and Legislation* he rejected the Cartesian view that animals have no capacity to feel pain. His view on the moral states of animals can hardly be expressed
more concisely than in the frequently quoted sentence: “The question is not, can they reason? nor, can they talk? but, can they suffer?”

In the nineteenth century, a major movement began against animal experimentation, first in Victorian England, but later also in France, where physiologists such as François Magendie and Claude Bernard were targets of criticism for the anti-vivisectionists. The first anti-vivisection organisation, “The Victoria Street Society”, was established in 1875 in England. It was also in England that the first law on the protection of experimental animals (Cruelty to Animals Act, 1876) was introduced.

The viewpoint of some extremists, demanding the total abolition of animal experiments, was not generally supported in society. On the contrary, from the end of the nineteenth century animal experimentation increased and became an integral part of biomedical research.

There are several points to be considered, as contributory factors to this increase:

– The discovery of anaesthetics in the first half of the nineteenth century enabled scientists to anaesthetize animals before exposure to painful experiments.
– In 1859 Charles Darwin published “The origin of species”, in which he gave a scientific basis for the concept of evolution. The evidence that the similarity between man and animals is based on homology, provided a rational basis for the use of animals as a model for man.
– In 1865 Claude Bernard’s “Introduction à l’étude de la médecine expérimentale” was published. In this book Bernard introduced methodology as a tool for the design of physiological experiments. He strongly emphasized the need of animal usage for furthering the development of experimental medicine.
– Development of the field of microbiology also greatly influenced the increase of animal use. In 1884, Koch’s “Postulates” were published, wherein it states that, amongst other things, the evidence for the pathogenicity of a micro-organism can be obtained, after successfully infecting healthy susceptible animals. Thus, the experimental animal became an indispensable substitute for man in microbiology. The need for experimental animals in microbiology was further increased when the production of antiserum and vaccines began, and their potency and safety was tested in animals.
– The development of several biomedical disciplines (pharmacology, toxicology, virology, immunology, etc.), and in particular the development of the pharmaceutical industry, caused a rapid increase in animal usage in the 20th century.

Not only has the total number of animals used increased, but also the number of different animal species. Until the end of the last century mainly domestic animals were used, but from the beginning of this century researchers have taken advantage of the availability of inbred strains of mice and rats. More
recently several other mammalian species are being used, as are also birds, reptiles, amphibians and fish species.

In 1940 about 1 million laboratory animals were used in the UK, in 1960 3.5 million and in 1970 5.5 million. More than 90% of these animals were rodents. No reliable figures on animal use in other countries are available for that period. According to a rough estimate, in 1960 world-wide about 30 million vertebrates were used and in 1970 between 100 and 200 million. In the 70’s several countries outside the UK also started to collect data on animal experimentation and to register the number of animals. Animal use seemed to stabilize during the late 70’s and has started to decrease from the early 80’s: e.g. in the UK from 5.5 million in 1980 to about 2.5 million in 2000, and in the Netherlands from 1.5 million in 1978 to less than 0.7 million in 2000.

During this period animal experimentation has become a major political issue. In several Western countries legislative regulations on the protection of experimental animals have been proposed and have taken effect during the last decades (see chapter 2).

Not only governments are dealing with the question on how animal use should be regulated. Scientific societies are also preparing their own guidelines. In this respect it is worth to mention the guidelines that have been adopted by the European Science Foundation (ESF), an association of 67 major national science-funding organizations in 23 European countries. In 2000 ESF has set out its views on the use of animals in research. These can be summarized as follows (see also http://222.esf.org):

- ESF recognises that laboratory animals not only have an instrumental value, but also an intrinsic value in themselves, which must be respected.
- While accepting the need of animal use for the advancement of scientific knowledge and for human and animal health and well-being, ESF strongly endorses the principles of the “Three Rs” (see below). Research aiming at improving the welfare of animals should be encouraged and actively supported.
- Prior to the performance of a programme of research, animal use should be subjected to independent expert review, for both scientific and animal welfare considerations. The assessment and weighing of the likely benefit and likely animal suffering should be an essential part of the review process.
- Investigators should assume that procedures that would cause pain in humans also cause pain in other vertebrates, unless there is evidence to the contrary.
- The best practical living conditions should be maintained for animals kept for research purposes. The care and health monitoring of the animals should be under the supervision of veterinarians or specialists in the field of laboratory animal science.
— Investigators and other personnel involved in the design and performance of animal-based experiments should be adequately educated and trained. ESF Member Organisations should encourage the development and organisation of accredited courses on laboratory animal science, including information on animal alternatives, welfare and ethics.
— ESF encourages the editorial board of journals publishing the results of animal-based research to include in “the instructions to authors” a statement on the ethical use of animals.

These guidelines clearly illustrate the position of ESF towards the use of animals. Much emphasis is put on the requirements for animal welfare, alternatives and ethics and on the responsibility of the researcher to meet these requirements.

Animal use: species and purposes

The registration of animal experiments has not only provided insight into numbers of animals but also into the variability of species and into the purpose of animal usage.

Figures 1-1 and 1-2 illustrate the distribution of animal species used in

![ANIMAL USE IN THE UNITED KINGDOM (1998: 2,660,000 procedures/year)](image)

Fig. 1-1. Distribution of animal species (vertebrates) used for research and education in the United Kingdom (1998)
Introduction

1998 in the UK and in the Netherlands, respectively. It can be seen that in both countries rodents (mouse and rat) are the most frequently used animal species.

Animals are used for a wide range of purposes. In the Netherlands in 1998 major areas are drug research (22%), vaccine testings (22%), toxicity testings (10%), cancer research (10%) and heart/circulation research (5%). Approximately 30% of the animals are used for other purposes, e.g. for basic biomedical research, genetic studies, diagnosis, experimental surgery, education etc.

The number of animals used for the various purposes may vary between countries, depending upon the degree of development of biomedical research.

Laboratory animal science associations

In many countries, scientific associations in the field of laboratory animal science have been founded. In the USA, the American Association for Laboratory Animal Science (AALAS) is most prominent whereas in Europe several national associations have established the Federation of European Laboratory Animal Science Associations (FELASA).

A world-wide organization in the field of laboratory animal science is ICLAS (International Council for Laboratory Animal Science), with national mem-

![ANIMAL USE IN THE NETHERLANDS (1998: 675,000 animals/year)](image)
bers from about 40 different countries. The aim of ICLAS is to promote international collaboration in the field of laboratory animal science and, in particular, to support developing countries in achieving the standard required for both high quality research and humane use of animals.

**Laboratory animal science and internet**

The world wide web is providing easy access to a wealth of information on laboratory animal science and animal welfare. There are numerous excellent sites that can be visited. E.g. starting with [http://www.aalas.org](http://www.aalas.org), the site of the American Association of Laboratory Animal Science (AALAS) or [http://oslovet.vet.hk.no](http://oslovet.vet.hk.no), the site of the Norwegian reference centre for laboratory animal science and alternatives (Norina), it is possible, through linking, to visit most of the relevant sites that are presently available on the web.

It is also possible to search databases for inbred strains (see chapter 7) or for transgenic animal models e.g. [http://tbase.jax.org/](http://tbase.jax.org/). Other sources are [http://ncbi.nlm.nih.gov/PubMed/](http://ncbi.nlm.nih.gov/PubMed/) for biomedical literature and [http://prex.vet.uu.nl](http://prex.vet.uu.nl) for specific veterinary and biological literature. For some of the databases a login/password combination is required.

For interactive exchange of information the Comparative Medicine Discussion List (COMPMED) is highly recommended. For a subscription an e-mail can be sent to: listerv@listeredv.aalas.org. The body of the e-mail message must contain: sub COMPMED FirstnameLastname.

**Russell and Burch: The Three R’s**

Legislative regulations, recently introduced by several countries, have a large influence on the development of laboratory animal science. The approach of Russell and Burch as elaborated in the late fifties in their book, “The principles of humane experimental technique”, has become a central theme in laboratory animal science. This book deals with the question of how the inhumane aspects of animal experimentation can be diminished or removed. The authors have introduced the “Three R concept” (Replacement, Reduction, Refinement) as a main guideline for the responsible use of animals in experiments.

*Replacement* refers to the substitution of living animals by *in vitro* techniques, computerized models, videos, film, etc. The experiment is replaced by an alternative procedure that yields the same result without the use of live animals.

*Reduction* refers to a decrease in the number of animals required for a given experiment. This can be achieved by choosing suitable experimental
Introduction

procedures, by controlling environmental factors and by standardizing the animal population. The introduction of standardization reduces the variation in the results. Chapter 12 explains in detail how the reduction of variation will lead to a decrease in the number of experimental animals required. During recent decades, significant developments in the field of health monitoring and standardization of genotype and environmental conditions, including nutrition, have contributed to a further reduction of variance. In particular, the improved organization within and between animal research institutes has contributed to the reduction of animal use.

Refinement refers to any decrease in the incidence or severity of painful or distressing procedures applied to animals. Refinement can be realized prior to experimentation on the animal e.g. by a better “reading” of the biological needs of the animal and translating them into adequate husbandry and environmental conditions. Adjustment of the environment to suit the behavioural and physiological needs of the animal, is a prerequisite for the animal’s homeostasis, whereas prolonged deviation from homeostasis may result in abnormal behaviour and disease. Refinement can also be realized during the course of the experiment, e.g. by improving experimental procedures or methods of anaesthesia, which may reduce distress.

The researcher should be aware of the fact that refinement not only contributes to the welfare of animals but also to the quality of the animal experiment. The Three Rs thus provide a concept for animal alternatives (see chapter 17). The search for animal alternatives meets an increasing interest. Specific journals are being published and major congresses are organized. In 1993 the first World Congress on Animal Alternatives was organized in Baltimore. Similar meetings have been organized in Utrecht (1996) and in Bologna (1999) all with more than 800 participants. At each of these meetings the Three Rs concept of Russell and Burch was a central theme.

Education and training

Competence, originating from adequate education and training, is a main condition for the quality of research and for a careful and responsible use of animals. The Council of Europe Convention for the protection of vertebrate animals used for experimental and other scientific purposes (1985), and the EC Council Directive on the protection of vertebrate animals used for experimental and other scientific purposes (1986) both state that “persons who carry out experiments or take part in them or take care of animals used for experiments, including supervision, shall have the appropriate education and training” (see chapter 2). Also, according to the US Animal Welfare Act (1986), persons involved in the use and care of animals in research must be trained properly.
Several categories of people are involved in animal experimentation e.g. the animal caretaker; the animal technician; the laboratory animal specialist/animal welfare officer; and the scientist.

In several countries training courses are being organised for the animal caretaker and animal technician. This type of training may take 2–3 years and most of these courses have a major practical component.

Usually the laboratory animal specialist/animal welfare officer is a veterinarian who has specialized in one of the areas of laboratory animal science, and who may be the authorized person for supervising the welfare of the laboratory animals at institutional level. Some countries are organising a postgraduate course in laboratory animal science for this group. This course may take 6 months–4 years, depending upon the graduate training programme and previous experience of the trainee.

The scientist, as the designer of the animal experiment, has the ultimate responsibility for the humane treatment of the animals. The welfare of the animals and the quality of animal research depends upon an understanding of animals. And, as stated by Sir Peter Medawar in *The hope of progress* (1979), “one does not come by this understanding intuitively; it must be learned”.

Education in the field of laboratory animal science is a *conditio sine qua non* for the researcher. This has been neglected for a long period of time. Only recently in some countries has education in this field been made mandatory for “new” scientists. FELASA has addressed the question of how the competence of the scientist must be defined. According to FELASA a scientist may be considered competent for the design or performance of animal experiments after having completed a graduate training in one of the biomedical disciplines (e.g. biology, medicine, veterinary medicine) and, in addition, having taken a course in laboratory animal science. The minimum duration for such a course being not less than 80 hours. Besides scientific and technical information necessary for the proper design of an animal experiment, the course should also deal with welfare issues and ethical aspects of animal experimentation. It must provide the tools for improving the quality of research, as well as for the careful and responsible use of animals. Replacement, reduction and refinement should be the guiding principles for the course.

This handbook has been compiled with the objective of covering the theoretical components of such a course.

Education and training, though essential, do not provide a free permit for animal experimentation. It should be noted that the performance of an animal experiment is acceptable and justified only if:

- the experiment is necessary (no alternatives available)
- the benefit outweighs the animal’s suffering (according to the judgement of an ethical committee)
the experiment is carefully designed and performed with maximum attention for the welfare of the animals and by persons who are fully competent.

Literature

2 Legislation and animal experimentation

P. C. M. de Greeve, J. Hampson and L. F. M. van Zutphen

Introduction

As already indicated in chapter 1, the first legislation concerning animal experimentation was enacted in the United Kingdom in 1876 in the form of the Cruelty to Animals Act. This statute emerged as a result of a long debate between scientists and animal protectionists.

The UK was thus the first and, for many years, the only country with legislation protecting animals used for scientific purposes. However, concern for laboratory animals has now grown and become a focus of major political interest in many countries.

In the USA, the principal federal law concerning the protection of laboratory animals, the Animal Welfare Act, was amended significantly in 1985 through the Improved Standard for the Laboratory Animals Act. Originally this law focused on preventing illegal transfer of pet animals to research institutions and on the humane care and treatment of non-human primates, dogs, cats, rabbits, guinea pigs and hamsters used in research, for exhibition, or as pets. The amendments include provisions for the use of anaesthetics and analgesics, and environmental enrichment for dogs and non-human primates.

Subsequent amendments have been adopted, requiring the review, by Institutional Animal Committees, of animal facilities, protocols for animal studies and proper veterinary care and qualifications for laboratory personnel. It is a responsibility of the Committees to ensure that the institution is providing training on relevant aspects of laboratory animal science to investigators and other personnel involved in the care and treatment of animals.

The Animal Welfare Act was extended in 1990 to cover horses and farm animals and is likely to include rats, mice and birds in the near future.
Legislation and animal experimentation

In addition to these Animal Welfare Act regulations, the Health Research Extension Act was introduced in 1985. As a result of the latter, institutions receiving grants from the Public Health Service (PHS) are required to comply with PHS policy on the humane care and use of laboratory animals. Principal features of PHS policy are the adoption of guidelines set out in the Guide for the Care and Use of Laboratory Animals and the establishment of an Institutional Animal Care and Use Committee. The composition and responsibilities of this Committee are consonant with those defined for Institutional Animal Committees in the Animal Welfare Act. All vertebrate animals are covered by the PHS policy.

In the 1980s two important documents controlling the use of animals in experiments were issued in Europe. In 1985, after several years’ discussion, the 26 countries of the Council of Europe in Strasbourg reached agreement on the Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS 123). This Convention, however, is not a binding document and has no legislative force. It becomes effective when signed and ratified by a Member State, i.e., when its Parliament has approved the instrument. The Member State is then legally bound under international law to implement the Convention.

The Convention contains the provision that Parties should hold Multilateral Consultations to examine the progress of its implementation and the need for revision or extension of any of its provisions on the basis of new facts or developments. During the last decade three Multilateral Consultations have been held.

In 1986 the Directive for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (86/609/EEC) was adopted by the Council of Ministers of the European Community (EC directive, see Annex 1). This document was based upon the Convention although its text is more concise and its requirements more stringent.

All EC Member States are compelled to implement the provisions of the EC Directive through their national legislation. These provisions must be seen, however, as minimum requirements. Member States are free to regulate specific issues more strictly if they wish.

All such laboratory animal protection legislation is based on the premise that, under certain conditions, it is morally acceptable to use animals for experimental and other scientific purposes. Most laws, however, contain provisions ensuring that the number of animals used is kept to a minimum. In addition, most regulatory systems have the following general objectives:

– to define legitimate purposes for which laboratory animals may be used;
– to ensure competence of all laboratory personnel and researchers;
– to limit animal use where alternatives are practicably available;
The EC Directive

Scope

The provisions of the Directive apply to vertebrate animals used in experiments likely to cause pain, suffering, distress or lasting harm. The Directive also covers the development of genetically modified animals likely to suffer pain and distress. The killing or marking of an animal using the least painful method is not considered to be an experiment.

The Directive is restricted in its application to experiments undertaken for the development and safety testing of drugs and other products, together with the protection of the natural environment (Article 3). However, in 1987, the European Parliament passed a Resolution stating that its provisions should also apply, through national legislation, to all animal experiments, including those undertaken for fundamental or basic research and for educational purposes.

Most national laws are put into operation by a government-controlled ‘authority’, as described by the Directive. The mechanism of control may rest with the authority itself, through a central licensing system such as that operated in the UK, or may be (partially) deployed at an institutional level, i.e. through institutional (ethics) committees.

Accommodation and animal care

Article 5 of the Directive contains provisions to ensure that animals are humanely treated not only prior to, but also during and after, any experimental procedure. Detailed guidelines for the implementations of these provisions are set out in Annex II of the Directive. These guidelines are mainly based upon common laboratory practice. They can be amended as new scientific or other evidence emerges with improved methods for the housing and care of animals. Article 5 states that the well-being and state of health of the animals must be overseen by a competent person. In Article 19 it is indicated that a veterinarian or other competent person should be charged with advisory duties in relation to the well-being of animals.
Legislation and animal experimentation

Competence

The provisions on competence warrant special attention. Laws and regulations are poor tools if not based upon an understanding of what constitutes humane and responsible animal use. Education and training provide the opportunity for gaining this understanding and also for an evaluation of the ethical considerations.

Article 7 of the Directive states that animal experiments should be performed solely by a person considered to be competent or under the direct responsibility of such a person. This provision is amplified by Article 14, which states that persons undertaking experiments, taking part in procedures, or caring for experimental animals (including supervision) should have appropriate education and training. It is essential that the people involved in the design and conduct of experiments should have received an education in a scientific discipline relevant to the experimental work. They also need to be capable of handling and taking care of laboratory animals.

Each Member State must specify how the provision of competence is to be implemented within national legislation. A proposal concerning educational and training requirements for scientists has been prepared by FELASA (see chapter 1). Some countries have already introduced strict legislative regulations regarding competence.

Alternatives to animal experiments

The concept of possible alternatives to experiments involving the use of animals is briefly described in chapter 1 and will be discussed further in chapter 17. Article 7 of the Directive not only deals with competence but also with alternatives. Performance of an experiment is not permissible if the result can be reasonably and practically obtained without the use of animals. If there is no alternative to animal use then animals with the lowest degree of neuro-physiological sensitivity (or the least capacity for suffering), compatible with the scientific objective, must be selected. Animals taken from the wild may not be used unless other animals would not fulfil the aims of the experiment.

All experiments must be designed to avoid distress and suffering as far as possible. Article 23 of the Directive states that the Commission of the EC and the Member States should encourage research into the development and validation of alternatives.

Anaesthesia

All experiments must be carried out under general or local anaesthesia (Article 8), unless anaesthesia is judged to be more traumatic than the experiment
The EC Directive

itself, or is incompatible with the aims of the experiment. If anaesthesia is not possible, then pain, distress or suffering must be limited and analgesics or other appropriate methods should be used. No animal should be subjected to severe pain, distress or suffering.

Euthanasia

At the end of the experiment, a veterinarian or other competent person must decide whether the animal should be kept alive or humanely killed. No animal is to be kept alive if it is likely to remain in permanent pain or distress, or if its well-being is otherwise jeopardised (Article 9). It is not permissible to use animals more than once in experiments entailing severe pain, distress or equivalent suffering (Article 10).

Registration

There is an obligation to notify the (governmental) authority in advance about the proposed experiments and who will be conducting them. If an animal is expected to experience severe pain that is likely to be prolonged, the experiment must specifically be declared and justified to, or specifically authorised by, the authority. Such an experiment is only permitted if it is of sufficient importance in meeting the essential needs of man or animal (Article 12).

Statistics

The authority must collect information on the total number of animals used, and statistics detailing the number used for specific purposes. As far as possible this must be made available to the public in the form of published statistics.

In 1997, after several years of negotiation, the European Commission and the Member States reached agreement about those (scientific) purposes for which data on animal use should be collected. Specific EU tables have been designed and distributed for the collection of such data. As from 1999, data on animals used for scientific purposes is collected in all Member States. Every two years, the European Commission will send a report on animals used for scientific purposes in Europe to the European Parliament.

Supply of animals

Only establishments approved by the authority in each Member State are allowed to breed or supply animals for research. Such establishments must keep records of the number and species of animals sold or supplied, and the
names and addresses of the recipients. Dogs, cats, and non-human primates must be supplied with an individual identification mark (Article 18).

Animal facilities

Establishments for animal use must be registered with or approved by the authority. Each user establishment must have sufficient numbers of trained staff and provision for adequate veterinary support. Only animals bred within the animal facility or from authorised breeding or supplying establishments may be used. The use of stray animals is not allowed. Records must be kept of all animals used.

Inspectorate

In most countries, the authority for supervising the observance of the regulations is a governmental inspectorate. The inspectors are mainly veterinarians or biologists, with experience in research and training in laboratory animal science.

Ethics committees

Several countries have institutional committees in operation specifically dedicated to review ethical aspects of animal experimentation. There is, however, no specific provision in the Directive demanding the establishment of such committees. As in the USA, some European countries have mandated the review of scientific protocols by a committee, prior to the commencement of the animal experiments.

Ethical aspects of animal experimentation are considered in more detail in chapter 18.

Impact and limitations of legislation

The central purpose of laws introduced to control animal experiments was set out by a UK Royal Commission as long ago as 1875: “to reconcile the needs of science with the just claims of humanity”. This principle was enshrined in the British Cruelty to Animals Act (1876) and is implicit in all laws, Conventions and Directives that have been passed since.

The extent to which these systems are effective in their stated aims of controlling animal research will depend not only on the implementation machinery (such as effective inspection or an in-house institutional monitoring system) but also on the attitudes and sensitivities of those conducting the research and caring for the animals. Thus good education and training are essential compo-
Impact and limitations of legislation

Components of the control system; this must include the acquisition of relevant practical skills and also a background in alternative methodologies and the ethical issues surrounding animal use.

No enforcement system could be effective if it were forced upon an insensitive research community that was hostile to the provisions of the law. In fact, what we have seen developing over the last decade as controls have been debated, is an increasing awareness in the scientific community both of practical and ethical issues, and the increasing involvement of the researcher in the devising and implementation of these controlling systems.

The law can set up the rules but rules cannot be effective if the institutional monitoring and implementation is inadequate.

Legislation can only ever be as effective as the strategy of measures to implement it on a day-to-day basis. Adequate daily care and proper conduct of experiments must be effected within the institution itself.

What centralised control does do, however, is to ensure that research work is not authorised unless it is shown to be valuable and necessary and that only competent persons in institutions with adequate facilities for the care of animals can carry it out. The authorisation procedure itself, i.e., applying for a licence and discussing it with a government inspector, or explaining a research proposal to an institutional ethics committee, focuses the mind of the researcher on the need to keep animal use to the minimum, and also encourages a consideration of alternatives and of ways to minimise pain or distress both during and after the experiment. Legislation has opened up a whole new debate on these issues between researchers, veterinarians, animal technicians and government inspectors.

Through all these effects, new legislation brings about improvements to animal welfare not immediately apparent in the content of the law itself. These improvements can be seen in anaesthetic technique, in better employment of analgesics or in refinements of experimental procedure, all of which might result from discussions about authorisation of a research project, or at symposia discussing implementation of the law.

It is at the authorisation level that the question of the purpose of the experiment might also be addressed, either by a governmental authority, or by an institutional committee appointed under the control system.

Some national laws state that the licensing authority or the institutional committee should weigh up the proposed benefits likely to flow from the research (such as new medical or scientific knowledge) against the likely degree of suffering to which animals will be subjected. Thus there may be cases where a proposed experiment is disallowed on the grounds that its scientific merit is particularly poor or because the proposed suffering is in excess of what is allowed under the law or of that which can be justified by its scientific importance.
This does not, however, imply that the use of animals for research purposes is being fundamentally questioned by these laws. Rather, justification of animal use is implicit in them. Critics of animal experiments see this as a major failing of all legislation and argue that, as the law stands at present, the central question of whether certain types of experiment (such as the testing of novel but non-essential consumer products) should be done at all, is not even addressed.

It is arguable that if this issue of the legitimacy of the research is not debated at some level, then the performance of any kind of cost/benefit analysis will be severely limited. Such limitation may be one of the reasons why legislation to date has not succeeded in fully satisfying public demand for greater accountability of what research is allowed to be performed on animals in the name of public interest.

It seems reasonable to assume that institutional ethics committees are in the best position to debate such matters. When these committees incorporate lay people from the local community among their membership they do go some way towards satisfying the demand for public accountability.

Public perception of the use of animals in research will continue to change as the moral consensus over such issues evolves in society as a whole. This can be seen as one aspect of a developing societal consciousness relating to the wider issues of medical ethics. Although strict laws now control the use of animals, and the conditions of their use and care do continue to improve, these deeper questions still remain to be addressed.

**Literature**


3 Biology and husbandry of laboratory animals


Introduction

One of the prerequisites for the responsible use of animals in biomedical research is a thorough knowledge of the biological characteristics and husbandry requirements of the species to be used. The choice of animal species, sex and age and the particular strain largely depend on whether their anatomical, physiological and behavioural characteristics are suited to the research demands. Housing, feeding, care, and transport must be appropriate to the requirements of the animal species that has been selected. There is an increasing awareness of the importance of optimal housing conditions, including enrichment of the environment, for all animal species. This benefits the well-being of animals and has a positive effect on the ability to adapt to unfamiliar (experimental) conditions. Therefore, it is of importance for the researcher and the science as well. Intensified international co-operation of companies and research institutes stimulates the transport of animals. Thus optimalisation and international regulation on requirements to transport should have special emphasis.

In this chapter some biological characteristics and zootechnical requirements of the most widely used vertebrates will be discussed.

Mammals

Of all laboratory animals, rodents are the most frequently used, accounting for approximately 70–85% of all vertebrate laboratory animals. This category boasts the largest order among mammals, and consists of approximately 1800 species. In general they are nocturnal animals, rather unspecialized and can adapt readily to their environment. Due to their extensive use within biomedical research, a great deal of biological data concerning them is currently
available. The table which follows shows the most important suborders of rodents used in biomedical research.

<table>
<thead>
<tr>
<th>Order</th>
<th>Suborder</th>
<th>Family</th>
<th>Genus</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rodentia</td>
<td>- - - -</td>
<td>- - -</td>
<td>Mus</td>
<td>M. musculus (house mouse)</td>
</tr>
<tr>
<td></td>
<td>Muridae</td>
<td>- -</td>
<td>Rattus</td>
<td>R. norvegicus (Norwegian rat)</td>
</tr>
<tr>
<td></td>
<td>Myomorpha</td>
<td>- -</td>
<td>Mesocricetus</td>
<td>M. auratus (Syrian hamster)</td>
</tr>
<tr>
<td></td>
<td>Cricetidae</td>
<td>- -</td>
<td>Meriones</td>
<td>M. unguiculatus (gerbil)</td>
</tr>
<tr>
<td></td>
<td>Hysteromorpha</td>
<td>- -</td>
<td>Caviidae</td>
<td>Cavia</td>
</tr>
</tbody>
</table>

Together with rodents, the rabbit is also frequently used in biomedical research. The rabbit is classified with the hare within the order Lagomorpha. They can be distinguished from rodents by, amongst other things, the presence of a pair of small incisors placed just behind the larger upper incisors.

Other mammals, for example non-human primates, dogs, cats, pigs, sheep and goats are less frequently used and account for around 3% of vertebrates used in research.

**Mice**

*Use.* In biomedical research the house mouse (*Mus musculus*) is the most widely used vertebrate species, with more than 400 genetically defined inbred strains and many transgenic strains (see chapter 7). Considerable differences in both anatomical and physiological characteristics exist amongst the inbred strains of mice. Due to the presence of these distinctive characteristics some strains are used as specific animal models, e.g. the athymic nude mouse. Mice are mainly used for cancer and drug research, vaccine production and safety tests.

*Physiology and anatomy.* Taking the small size of the mouse into account a number of physiological parameters such as heart rate and respiratory frequency, are relatively fast (table 3-1), but these can vary considerably depending on strain, age, environmental conditions and microbial status.

Like most other rodents, the mouse is a nocturnal animal. The diurnal rhythm has peaks of activity during the dark period. These activity patterns can be strain specific.

The dental formula for the mouse is 2(1003/1003), which means that each half of each jaw contains one incisor and three molars. Canine teeth and premolars are absent, resulting in an open space, the diastema. The incisors grow continuously whilst the animal lives, and are kept short by chewing. The
Table 3-1
Environmental requirements and physiological parameters of mice, rats and Syrian or golden hamsters

<table>
<thead>
<tr>
<th>Environmental requirements</th>
<th>Mice</th>
<th>Rats</th>
<th>Golden hamsters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (°C)</td>
<td>20–24</td>
<td>20–24</td>
<td>20–24</td>
</tr>
<tr>
<td>Relative humidity (%)</td>
<td>50–60</td>
<td>60</td>
<td>50–60</td>
</tr>
<tr>
<td>Ventilation (changes/hour)</td>
<td>15</td>
<td>10–15</td>
<td>10–15</td>
</tr>
<tr>
<td>Light/dark (hours)</td>
<td>14/10</td>
<td>12–14/12–10</td>
<td>12–14/12–10</td>
</tr>
<tr>
<td>Minimum cage floor size</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>One individually housed adult (cm²)</td>
<td>180</td>
<td>350</td>
<td>180</td>
</tr>
<tr>
<td>Breeding animal with pups (cm²)</td>
<td>200</td>
<td>800</td>
<td>650</td>
</tr>
<tr>
<td>Group (cm²/adult)</td>
<td>80</td>
<td>250</td>
<td>n/a</td>
</tr>
<tr>
<td>Minimum cage height (cm)</td>
<td>12</td>
<td>14</td>
<td>12</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>General physiological parameters</th>
<th>Mice</th>
<th>Rats</th>
<th>Golden hamsters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult weight (g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>20–40</td>
<td>300–500</td>
<td>120–140</td>
</tr>
<tr>
<td>Female</td>
<td>25–40</td>
<td>250–300</td>
<td>140–160</td>
</tr>
<tr>
<td>Life span (years)</td>
<td>1–2</td>
<td>2–3</td>
<td>2–3</td>
</tr>
<tr>
<td>Heart rate (/min)</td>
<td>300–800</td>
<td>300–500</td>
<td>250–500</td>
</tr>
<tr>
<td>Respiration rate (/min)</td>
<td>100–200</td>
<td>70–110</td>
<td>40–120</td>
</tr>
<tr>
<td>Body temperature (°C)</td>
<td>36.5–38.0</td>
<td>37.5–38.5</td>
<td>37–38</td>
</tr>
<tr>
<td>Number of chromosomes (2n)</td>
<td>40</td>
<td>42</td>
<td>44</td>
</tr>
<tr>
<td>Body surface (cm²)</td>
<td>20 g: 36</td>
<td>50 g: 130</td>
<td>125 g: 260</td>
</tr>
<tr>
<td></td>
<td>130g: 250</td>
<td>200g: 325</td>
<td></td>
</tr>
<tr>
<td>Water intake (ml/100g/day)</td>
<td>15</td>
<td>10–12</td>
<td>8–10</td>
</tr>
<tr>
<td>Puberty (weeks)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>5</td>
<td>6–8</td>
<td>4–6</td>
</tr>
<tr>
<td>Male</td>
<td>—</td>
<td>7–9</td>
<td></td>
</tr>
<tr>
<td>Breeding age (weeks)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>8–10</td>
<td>12–16</td>
<td>6–8</td>
</tr>
<tr>
<td>Male</td>
<td>8–10</td>
<td>12–16</td>
<td>10–12</td>
</tr>
<tr>
<td>Oestrous cycle (days)</td>
<td>4(2–9)</td>
<td>4–5</td>
<td>4</td>
</tr>
<tr>
<td>Duration of oestrus (hours)</td>
<td>14</td>
<td>14</td>
<td>2–24</td>
</tr>
<tr>
<td>Duration of pregnancy (days)</td>
<td>19(18–21)</td>
<td>21–23</td>
<td>15–17</td>
</tr>
<tr>
<td>Litter size</td>
<td>6–12</td>
<td>6–12</td>
<td>6–8</td>
</tr>
<tr>
<td>Weight at birth (g)</td>
<td>0.5–1.5</td>
<td>5</td>
<td>2–3</td>
</tr>
<tr>
<td>Weight at weaning (g)</td>
<td>10</td>
<td>40–50</td>
<td>30–40</td>
</tr>
<tr>
<td>Weaning age (days)</td>
<td>21–28</td>
<td>21</td>
<td>20–22</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Blood parameters</th>
<th>Mice</th>
<th>Rats</th>
<th>Golden hamsters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood volume (ml/kg)</td>
<td>76–80</td>
<td>60</td>
<td>80</td>
</tr>
<tr>
<td>Haemoglobin (g/100 ml)*</td>
<td>10–17</td>
<td>14–20</td>
<td>10–18</td>
</tr>
<tr>
<td>Haematocrit (vol%)</td>
<td>39–49</td>
<td>36–48</td>
<td>36–60</td>
</tr>
<tr>
<td>Leucocytes (×1000/mm³)</td>
<td>5–12</td>
<td>6–17</td>
<td>3–11</td>
</tr>
<tr>
<td>Glucose (mg/100 ml)**</td>
<td>124–262</td>
<td>134–219</td>
<td>60–150</td>
</tr>
</tbody>
</table>

¹ Under stress conditions this value may rise to 2 × normal.
² n/a = not applicable.
*mmol/l haemoglobin: g/100 ml × 0.62; ** mmol/l glucose: mg/100 ml × 0.0556
labial surface of the incisors is covered with a thick layer of enamel. The mouse is omnivorous and possesses molars for grinding.

The sex of the animal can be determined by comparing the anogenital distance (fig. 3-1), which is twice as long in males than in females. In females this area is also hairless. The urethra enters the floor of the vagina shortly before the external opening. Testicles can be present in the scrotum, but they can also be retracted through the inguinal canal into the abdomen.

**Housing.** Wild mice live in groups with an evident social hierarchy. The behaviour of the laboratory mouse has partially adapted to life in captivity, but there are still some similarities with its wild counterpart. In the design of caging systems considerable attention has been paid to economic and hygienic aspects but the animals’ requirement to carry out natural behaviours has hardly been taken into account. Mice can be housed in transparent macrolon cages with a tightly fitting lid. In 1986, guidelines for minimum cage sizes per animal were issued by the EU (table 3-1). The maximum number of mice that may be housed in type 1 cage (200 cm²) is one female with pups or one breeding pair. However, the use of type 1 cages is under discussion. It is uncertain whether in the revised European guidelines these cages will still be considered acceptable. Larger numbers can be housed in cages types 2 (450 cm²) and 3 (900 cm²).

Fig. 3-1. The distance between anus and genital opening is larger in a male (r) than in a female (l) mouse.
When males are housed together, aggressive behaviour may occur. This depends upon the strain, the space available for each animal and the “furniture” within the cage. Individually housed mice exhibit more aggression when re-housed in groups, than animals permanently housed in groups. However, (male) litter mates housed together from weaning show a reduced level of aggression. In the case of group housing, no more than 30 animals should be put together in one cage, as overcrowding may lead to the death of individual animals by hyperthermia or hypoxaemia.

Sawdust is commonly used as bedding material and it should be free of fine dust and microbial and chemical contaminants. Shredded paper or cotton wool can be used as nesting material. Sometimes mice are housed on wire mesh floors, for example in toxicological studies. This is to facilitate cleaning and prevents animals from being in contact with the bedding material, but this type of housing should be used only when strictly necessary for the experiment. Breeding animals with neonate pups should not be housed on wire mesh as thermoregulation in neonates would be adversely affected due to the absence of nest material. In metabolism cages, animals are housed individually in order accurately to measure individual feed and water intake, and in order to collect and quantify urine and faeces production separately.

Absence of environmental enrichment can have a serious adverse affect on the development of the central nervous system and on the animal’s ability to perform learning tasks. It is, therefore, often of benefit to science as well as to the animal, to increase the complexity of the cage by adding objects such as wood blocks, guttering or some form of shelter, and cardboard or paper to shred.

The optimal values for the environmental conditions in the animal room (macroclimate) are set out in table 3-1. Large fluctuations in temperature and draught must be avoided, as these can increase susceptibility towards airborne infections, especially if the humidity is high. The microclimate (inside the cage) will depend upon for example, the cage type (e.g. macrolon cages versus wire mesh cages), the location of the cage in the animal room, the ventilation system, the number of animals in the cage, the use of a filter top, and the frequency of cage cleaning. A regular day/night light cycle is essential for establishing normal behaviour patterns, and for the normal expression of physiological processes, such as reproduction. Light intensity, which should be moderate, especially for albino animals (less than 50 lux), and noise can have a distinct influence on breeding and on the results of experiments.

Food and drink. Usually the same type of diet is fed to all mice, irrespective of age, pregnancy or lactation. It is usually administered *ad libitum* in the form of pellets. On average, depending upon the energy content of the diet, approximately 3–4 g is eaten per mouse per day. The food rack must be well
filled as it is difficult for the animals to gnaw the food when there are only a few pellets in the food-hopper. Microbial digestion and the subsequent consumption of faeces (coprophagy) make certain nutrients available to the animal in an indirect manner (see rabbit).

Fresh drinking water must be available at all times. Water requirements will largely depend on the water content of the diet and environmental circumstances. It can be made available via bottles or an automatic watering system. If drugs have to be supplied in the drinking water, bottles have to be used. Acidification (to pH 3.0 with 1N HCl) or chlorination (0.2–0.5 ml free chloride per litre) of the drinking water inhibits microbial growth without lowering the water consumption. To prevent the risk of drowning caused by leakage into the solid bottomed cage, the drinking nipple of an automatic watering system should be situated just outside the cage and the nipple checked regularly for any obstruction.

Reproduction. Sexual maturity in mice occurs very early. Females are polyoestrous. When an animal is individually housed, its cycle is more regular than when group housed. When females are housed in groups without males, there is a tendency for them to be anoestrous. The introduction of a male into such a colony will lead to synchronization of their oestrous cycles (Whitten-effect). The different stages of the oestrous cycle can be identified by means of a May–Grünwald Giemsa staining of vaginal swabs (fig. 3-2). If the female is housed with a second male within 24 hours after a successful mating, implantation of fertile egg cells will not take place, and therefore no pregnancy will occur (Bruce-effect). The Bruce and Whitten effects are induced by pheromones present in the urine of male mice. For 12–24 hours after mating, a coagulation plug (secretion of the accessory glands in the male genital tract) can be detected in the vagina. This is a simple, though not very reliable, way to detect that mating has taken place. Another way is by determining the presence of spermatozoa microscopically in fluid which has been used to flush the vagina.

Pups are born both blind and naked after a pregnancy period of 19–21 days. Passive immunity is passed via the placenta (placenta haemochorialis) and the colostrum to the pups. Pups are weaned 3 weeks after birth. If mating takes place during a postpartum oestrus, lactation and pregnancy may occur simultaneously. Lactation may delay implantation, and prolong the gestation period by 3–5 days.

Different mating systems can be used. In the case of monogamous pairs (one male with one female) and with “trios” (one male with two females), the male remains constantly with the female(s), so that postpartum matings can take place. Pups must be weaned prior to the next nest being born. In the case of polygamous breeding (“harem-system”), 3–6 females are housed with one
Handling and simple techniques. The best way to lift a mouse up is to hold it firmly at the base of the tail. If the animal needs to be restrained, it should be placed on a surface where it can obtain a grip (e.g. the cage lid). Then the skin of the neck is taken between the thumb and index finger of the other hand. The mouse should then be lifted and the tail held between the ring finger and the palm of the hand (fig. 3-3a,b). For determining sex, the hind part of the mouse can be lifted slightly by the tail (fig. 3-3c). The marking of animals for identification should be as least invasive as possible and not cause them to experience pain or lasting harm. Mice can be marked by means of punching small holes in the ear(s) at a young age or by toe amputation. The latter method is not recommended and, if inevitable, should be carried out before the animal is three days old in order to minimize trauma. The combination of ear marking with toe amputation results in a possible numeration scale from 0 to 12,999. The application of coloured marks on the fur or on the tail(base) provides a means of identification within small groups on a temporary basis (fig. 3-3d). Tattooing of ears and tails is also possible. Of increasing impor-

Fig. 3-2. Microscopical preparations of vaginal smears of a mouse showing different stages of the oestrous cycle. 1. pro-oestrus: many nucleated and few keratinized epithelial cells; 2. oestrus: many keratinized epithelial cells; 3. met-oestrus: a few keratinized cells and leukocytes; 4. di-oestrus: mainly leukocytes are present.
Fig. 3-3. Lifting (a) and restraining (b) of a mouse.
Fig. 3-3 (continued). Handling to determine the sex (c). Identification by staining the tail (d).
tance is the use of microchips because this permits extensive individual identification as well as the ability to record physiological data such as body temperature and heart rate (see chapter 16).

Transport. When transporting mice, solid boxes must be used, and good ventilation must be ensured even when the boxes are stacked (fig. 3-4). For SPF animals (see chapter 8) infection should be prevented by HEPA filters in the ventilation openings. Wire netting should be fixed to the inside of the boxes, so that the mice cannot gnaw holes and escape. Bedding material must also be provided. For long periods of transportation, food and water must be supplied in the form of wet cotton wool, agar gel or vegetables which contain large amount of water (cooked potatoes 80%, carrots 90%, cucumber 97%).

Transport always causes considerable stress to animals and may result in loss of weight. The animals may need a week or more in order to acclimatize to their new surroundings.

Detailed guidelines on transportation of laboratory animals can be expected to be issued by the EU in the near future.
Rats

Use. The laboratory rat has descended from the Norway or brown rat (*Rattus norvegicus*). The name “brown rat” is misleading, as the colour may vary. The first rat strains for biomedical research were developed at the Wistar Institute in Philadelphia. Many of the inbred strains which are used at present are descendants of these albino Wistar rat strains.

The rat is the most commonly used vertebrate after the mouse, and is used mainly in medicine, food, behaviour and toxicity research. Currently there are more than 200 genetically defined inbred strains and about 50 outbred strains on record.

Physiology and anatomy. The physiology and anatomy of the rat resemble those of the mouse (table 3-1).

The nephrons in the kidney cortex are quite near the surface, and therefore reasonably accessible. The adrenal glands are located away from the major blood vessels, which makes adrenalectomy in the rat less risky than for example, in the rabbit.

Within the eye-socket lies the Harderian gland which produces a brown-red secretion which contains porphyrins. This secretion is removed by grooming and the presence of reddish secretion around the eyes and nose indicates a diminished well-being (less grooming or excess of secretion due to an infection of the upper respiration tract). Between the eye and the base of the ear an extra-orbital lacrimal gland is located. In contrast to mice, rats have no gall bladder.

The body-weight of the adult rat is about 10–15 times the body-weight of the adult mouse (see fig. 3-5).

![Graph](image-url)

Fig. 3-5. The average body-weight of the mouse, rat, (Syrian) hamster and gerbil at different ages.
Housing. There are many similarities between the housing of mice and rats. Rats are usually housed in macrolon cages or stainless steel cages with wire-mesh grids or bases. In these cages there is a lack of environmental stimuli which can have a serious affect on the development of the central nervous system and on the animal’s ability to perform learning tasks. It is therefore advised to increase the complexity of the cage by adding objects such as wood blocks, guttering or some form of shelter, and cardboard or paper to shred.

Rats are less aggressive than mice and usually male rats can be housed in groups without any problems, depending on the strain. Different groups of adults, however, should not be housed together in one cage, as this will lead to aggressive behaviour.

Group housed rats have significantly higher plasma corticosteroid concentrations than their individually housed counterparts. The social behaviour of grouped animals changes when a solitary housed rat is introduced into the group, and this may influence the level of some of their reference values.

Overcrowding can cause an unacceptable rise in body temperature and may even lead to deaths. However, this is extremely unlikely if the guidelines on stocking density as laid out in the EU guidelines are followed. Body temperature is regulated in rats and mice by sweating from the foot pads and vasodilatation of the tail, and rats have been known to cover their body with saliva in an effort to reduce their temperature.

When relative humidity remains lower than 45% for a long period of time, “ringtail” (localized constrictions of the tail) can develop in young animals. Rats are sensitive to disease of the respiratory tract, and therefore ventilation has to be well controlled, avoiding draughts and high cage levels of ammonia.

Rats and mice are sensitive to ultrasonic sounds, and sudden noises can evoke audiogenic seizures in some strains. Light is an important factor in the regulation of physiological activities, but a too high intensity will cause retinal atrophy in albino animals.

Food and drinking water. The supply of food and drinking water is on the whole comparable with the mouse. Under ad libitum conditions, the feed intake of rats, in contrast to mice, occurs mainly during the dark period.

Reproduction. The breeding of the rat is, to a large extent, comparable with that of the mouse. However, the Bruce-effect does not occur, and oestrous cycle synchronization due to male pheromones is not as apparent. Synchronization of oestrus and pregnancy can be achieved by administering progesterone for a period of 4 days (induces anoestrus), followed by an injection of FSH or PMSG (pregnant mare serum gonadotrophin). The oestrous cycle responds to variation in the length of the light cycle. A period of 12–16 hours
of light per day results in the best breeding performance. Exposure to continuous light seriously disturbs fertility, even after only 3 days.

After mating has taken place, a vaginal plug is usually present during the following 12–24 hours. Its presence in the vagina or passed onto the gridded cage floor can be used to time matings. The breeding systems for rats are similar to those for mice. When the female is mated during postpartum oestrus, the period before implantation, and therefore the length of pregnancy, will be extended by a few days.

**Handling and simple techniques.** Most strains of rats are easy to handle. The rat should be lifted by placing a hand around the chest with the thumb placed under the chin and the index finger around the neck. In this way the head is secured firmly. Another way of lifting a rat is by taking it around the shoulders and supporting it in the full hand (fig. 3-6 a,b). The other hand should be used to support the hind part of the body in the event of the animal being large or pregnant. A less preferable way is to pick it up by the base of the tail and place it on a solid surface. For oral administration of fluids or for injections the rat can also be restrained by grasping the loose skin at the back of the neck.

When housed on wire mesh bases, rats should not be picked up quickly or unexpectedly by their tails, as they will try to hold on to the wire mesh and their nails could be torn off.

Plastic cylinders may be useful for restraining the rat for a short period of time. For identification and transport: see the mouse.

**Syrian hamsters**

*Origin and use.* The Syrian or golden hamster (*Mesocricetus auratus*) and the Chinese hamster (*Cricetulus griseus*) are the most widely used hamster species (fig. 7). The natural habitat of the Syrian hamster is Southeast Europe and the Middle East. All of the domesticated Syrian hamsters have probably originated from one male and two females which were captured in the neighbourhood of Allepo in Syria, in 1930. The wild Chinese hamster lives in the area between the Caspian Sea and the east coast of China. The number of hamsters used in biomedical research is relatively small (less than 1%). The Syrian hamster is mainly used for reproduction and teratogenicity studies, as well as for tumour and blood circulation research. Due to its hibernating habit, the hamster is also used to study effects of hypothermia. Hamsters are not very susceptible to common spontaneous infections, but are rather sensitive to experimentally-induced infectious diseases such as leptospirosis, influenza, and canine distemper. The Chinese hamster is used for karyotype research and as a model for diabetes mellitus in humans.
Fig. 3-6. Handling (a) and restraining (b) of a rat.
Physiology and anatomy. The Syrian hamster hibernates when the environmental temperature falls below 5–6°C and when the light period is less than 8 hours per day. During hibernation body temperature, heart and respiration rate decrease considerably. Hamsters which are in hibernation can be awakened by stimuli such as touch. During hibernation, hamsters sleep for periods of approximately 2–3 days, and then wake for periods of 12 hours or less. During these “awake periods”, physiological parameters reach normal levels. This implies that food and water must be available during the hibernating period.

On both flanks of the animals there are dark coloured areas of the skin with coarse hair, containing sebaceous glands. These hip glands, which are well developed in sexually mature males, secrete pheromones, and one of their functions is to mark out territory. The Syrian hamster has cheek pouches that are distensible and stretch from the cheeks to the shoulder blades. They are used for the transport of food and sometimes of pups as well. The fundus of the stomach is rather large and has a thin wall. It is connected to the rest of the stomach by a very narrow passage. Adrenal glands are larger in males than in females. The urine has a pH of 8 and often has a turbid, milky appearance.

Compared with most other rodent species, hamsters are less sensitive to morphine, and they have a greater tolerance to pentobarbitone. They do not readily develop anaphylactic shock, however, they are more sensitive to corticosteroids. Some antibiotics such as penicillin, erythromycin and tetracyclines are indirectly toxic, because they may cause lethal endotoxaemia due
to a decrease of Gram-positive and an increase of Gram-negative gut bacteria in the intestinal tract (dysbacteriosis). Other antibiotics can also lead to an excessive growth of *Clostridium difficile* which can cause serious inflammation of the intestinal wall, toxæmia and death (so called ‘wet tail disease’).

Caecotrophy, directly from the anus, will take place even when the hamsters are housed on wire mesh, which is also the case for rats and mice.

**Housing.** Hamsters are solitary, nocturnal animals and they are normally housed individually in type 2 cages, but litter mates can be housed together for long periods in larger cages. Females are more aggressive than males and sometimes it may be necessary to house females individually from the onset of puberty (around 40 days). Hamsters should be housed in cages with solid floors, bedding material (sawdust) and a lid that can be closed tightly. The types of cages used for rats and mice are also suitable for hamsters. When hamsters are disturbed during the daytime i.e. their resting period, they may become aggressive and try to bite. By reversing the day/night cycle, hamsters will become active during the day-time and will react less aggressively when handled.

Solid food intake commences at the age of 7–10 days. Pellets must be deposited near the nest inside the cage, so that the pups can reach the food. When they start eating solid food, they must be able to drink water as well, otherwise disturbances of the intestinal tract may develop. To allow for this, the drinking nipple should be of a sufficient length so as to be accessible to the young animals. It has to be checked daily for obstruction.

**Food and drink.** The hamster is omnivorous, just like the rat and the mouse. It is common practice to give mouse and rat pellets to hamsters and care has to be taken to ensure that the hamster can actually reach the food, as they have a flatter face than mice and rats. It is advisable to put some pellets on the cage floor, so that they can practise their natural behaviour of hoarding. The drinking nipple should not be made of glass, as it can be easily broken by biting.

**Reproduction.** The oestrous cycle is very regular in hamsters and lasts exactly 4 days. The morning after ovulation, a vaginal secretion can be seen that has a milky appearance with a high viscosity and a distinctive smell. On observing this secretion, mating can be successfully achieved in the evening three days later. When a female in oestrus is placed into a cage with a male, she will respond to the sniffing of the male by lordosis. Copulation will be repeated several times within 20–60 minutes. After mating has taken place the female must be removed, otherwise she will attack the male. If 5 or 9 days after the mating a vaginal excretion is observed, then the female is not pregnant. Compared with the Chinese hamster, with a pregnancy period of
21 days, the Syrian hamster has a shorter duration of pregnancy of 15–17 days (table 3-1). The hamster has a postpartum oestrus just like the mouse and the rat; the difference being that in the hamster it is not fertile.

For reproduction, both monogamous and polygamous mating systems can be used. After weaning it is possible to form monogamous pairs which can stay together for the entire reproductive phase. However, it is usually the case that the animals are housed individually due to the risk of aggressive behaviour. In the case of “hand mating”, the female is placed into the cage of the male just after dark. If applying the harem system then 1–4 males are housed with 5–15 females. Pregnant females are housed separately until after weaning. When these female hamsters are returned into the colony, fighting will often occur, therefore polygamous systems are best be avoided.

Cannibalism, carried out by the mother on the pups, occurs primarily in primiparous animals during the first postpartum week. Possible causes are thought to be lack of experience, too much excitement, disturbance and/or insufficient lactation. The pups should not be touched during the first week, especially when the female is primiparous. In contrast to rats and mice, the fostering of newborns is hardly ever successful.

Handling and simple techniques. Hamsters should not be picked up until you are sure that they are awake. The easiest way is to grasp the animal around the head and thorax. It is also possible to pick up a hamster by placing the hand over the animal with the thumb near the head and then grasping as much as possible of the loose skin of the neck and back region firmly. Another possibility is to place the thumb on the inside of the femur with the index and middle fingers around the rear, securely holding the head and front legs with the remaining free fingers.

Hamsters can be marked by ear punch holes or ear clips, by subcutaneously implanted microchips, and by tattooing the shaved skin, for example on the hind leg. (For more details: see mouse).

Transport. If hamsters are very aggressive, they can be caught and transported from one cage to another using a tin. The tin should be placed into the cage and, as a rule, the animals will crawl into the tin. (For more details: see mouse).

Gerbils

Origin and use. The gerbil (Meriones unguiculatus) is indigenous to the deserts of Mongolia and northern China. In captivity the gerbil will reproduce well and is relatively free of the normal “spontaneous” diseases. These factors have favoured the use of the gerbil in biomedical research. Epilepsy in
### Table 3-2

Environmental requirements and physiological parameters of gerbils, guinea-pigs and rabbits

<table>
<thead>
<tr>
<th>Environmental requirements</th>
<th>Gerbils</th>
<th>Guinea-pigs</th>
<th>Rabbits</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Temperature (°C)</strong></td>
<td>20–24</td>
<td>20–24</td>
<td>15–21</td>
</tr>
<tr>
<td><strong>Relative humidity (%)</strong></td>
<td>35–45</td>
<td>50</td>
<td>50–60</td>
</tr>
<tr>
<td><strong>Ventilation (changes/hour)</strong></td>
<td>15–20</td>
<td>10–15</td>
<td>5–15</td>
</tr>
<tr>
<td><strong>Light/dark (hours)</strong></td>
<td>12/12</td>
<td>14/10</td>
<td>12/12</td>
</tr>
<tr>
<td>Minimum cage floor size</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>One individually housed adult (cm²)</td>
<td>230</td>
<td>600</td>
<td>1 kg: 1400</td>
</tr>
<tr>
<td>2 kg: 2000</td>
<td></td>
<td></td>
<td>3 kg: 2500</td>
</tr>
<tr>
<td>3 kg: 3000</td>
<td></td>
<td></td>
<td>4 kg: 3000</td>
</tr>
<tr>
<td>4 kg: 3000</td>
<td></td>
<td></td>
<td>5 kg: 3600</td>
</tr>
<tr>
<td>One breeder with nest (cm²)</td>
<td></td>
<td></td>
<td>1 kg: 3000</td>
</tr>
<tr>
<td>(couple)</td>
<td></td>
<td></td>
<td>3 kg: 4000</td>
</tr>
<tr>
<td>4 kg: 5000</td>
<td></td>
<td></td>
<td>5 kg: 5000</td>
</tr>
<tr>
<td>Group (cm²/adult)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minimum cage height (cm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adult weight (g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>80–110</td>
<td>900–1000</td>
<td>2–5 (kg)</td>
</tr>
<tr>
<td>Female</td>
<td>70–100</td>
<td>700–900</td>
<td>2–6 (kg)</td>
</tr>
<tr>
<td>Life span (years)</td>
<td>3–4</td>
<td>5–6</td>
<td>5–6</td>
</tr>
<tr>
<td>Heart rate (/min)</td>
<td>360</td>
<td>230–380</td>
<td>130–325</td>
</tr>
<tr>
<td>Respiration rate (/min)</td>
<td>90</td>
<td>42–104</td>
<td>30–60</td>
</tr>
<tr>
<td>Body temperature (°C)</td>
<td>38.1–38.4</td>
<td>38–40</td>
<td>38.5–39.5</td>
</tr>
<tr>
<td>Number of chromosomes (2n)</td>
<td>44</td>
<td>64</td>
<td>44</td>
</tr>
<tr>
<td>Body surface (cm²)</td>
<td>190g: 205</td>
<td>400g: 565</td>
<td>2–5kg: 1270</td>
</tr>
<tr>
<td>800g: 720</td>
<td></td>
<td></td>
<td>4–8kg: 3040</td>
</tr>
<tr>
<td>Water intake (ml/100 g/day)</td>
<td>4–7</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>Puberty (weeks)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>9–12</td>
<td>4–5</td>
<td>16</td>
</tr>
<tr>
<td>Male</td>
<td>9–12</td>
<td>8–10</td>
<td>20</td>
</tr>
<tr>
<td>Breeding age (weeks)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>9–12</td>
<td>9–10</td>
<td>20–36(^1)</td>
</tr>
<tr>
<td>Male</td>
<td>9–12</td>
<td>9–10</td>
<td>24–40(^1)</td>
</tr>
<tr>
<td>Oestrous cycle (days)</td>
<td>4–6</td>
<td>14–18</td>
<td>n/a</td>
</tr>
<tr>
<td>Duration oestrus (hours)</td>
<td></td>
<td></td>
<td>n/a</td>
</tr>
<tr>
<td>Duration pregnancy (days)</td>
<td>25–26</td>
<td>68(59–72)</td>
<td>30(28–35)</td>
</tr>
<tr>
<td>Litter size</td>
<td>4–6</td>
<td>1–6</td>
<td>4–10(^1)</td>
</tr>
<tr>
<td>Birth weight (g)</td>
<td>2.5–3.0</td>
<td>70–100</td>
<td>30–100(^1)</td>
</tr>
<tr>
<td>Weight at weaning (g)</td>
<td></td>
<td>180–240</td>
<td></td>
</tr>
<tr>
<td>Weaning age (days)</td>
<td>20–30</td>
<td>15–28</td>
<td>35–56</td>
</tr>
</tbody>
</table>

**Blood parameters**

- Blood volume (ml/kg)
- Haemoglobin (g/100 ml)
- Haematocrit (vol%)
- Leucocytes (×1000/mm³)
- Glucose (mg/100 ml)

- 66–78 69–75 60
- 13–16 12–15 10–16
- 44–47 38–48 36–48
- 7–12 7–13 5–11
- 50–135 60–125 78–155

\(^1\)Dependent upon the strain: small breeds reach breeding age at an earlier age and have a smaller nest size than larger breeds.

\(^2\)These values apply to young adult New Zealand White rabbits.
gerbils has a genetic background and it is used in neurological research as a model for human idiopathic epilepsy. Gerbils develop high serum and liver cholesterol concentrations, even when on a diet with relatively low amounts of fat. They are therefore used for studying cholesterol metabolism and experimental atherosclerosis. Some other research areas in which gerbils are used are obesity, filariasis and brain infarcts.

**Physiology and anatomy.** In the wild, gerbils live as monogamous pairs in a self-built system of tunnels. Periods of activity alternate with resting periods both during the day as well as the night, but, the highest activity takes place at night. The gerbil usually excretes only a few drops of urine per day as their nephrons have adapted to desert life. In the wild the gerbil is capable of compensating for a lack of drinking water for several weeks. They possess a considerable adaptability to fluctuations in environmental temperature and few signs of discomfort are seen between 0–35°C. When the relative humidity exceeds 50%, the fur may then have a rough and greasy appearance. The fur of the gerbil is tight, short-haired and agouti-coloured. In contrast to most other rodents, the ears and footpads are covered with hair. The skin of the tail is loose and can be easily stripped if the animal is not handled properly. A well-developed sebaceous gland is present around the umbilicus.

**Housing.** The use of solid bottomed cages is preferable for the housing of gerbils. Their general condition improves when they are housed on solid floors with a thick layer of bedding rather than when they are housed on wire mesh floors. Reproduction is also more successful when there is a shelter in the cage. The height of the cage must be at least 15 cm to allow the gerbil to stand upright on its hind legs. Bedding usually remains dry and odourless for long periods of time and will need to be cleaned once every two weeks, depending on the stocking density.

When introducing animals into an unfamiliar environment epileptiform attacks may occur. Overpopulation, or the housing together of unfamiliar individuals, can lead to serious fighting, but this will not usually be a problem when gerbils are housed together from weaning age.

**Food and drink.** It is common practice to feed gerbils *ad libitum* with rat and mice pellets. It is not advisable to feed the animals a diet with a high fat content, as they may develop obesity and atherosclerosis. In females, fat will accumulate around the ovaries, which will have a negative influence on fertility. Young gerbils, between 2–5 weeks old, may sometimes have problems in eating pellets which are too hard.

Gerbils drink approximately 4–5 ml of water per day per 100 g of body weight, when they are fed on a pellet diet. However, they are able to maintain
their body weight when they drink only 2 ml per day. In the wild, gerbils can survive on seeds and roots, but when fed dry pellets in the laboratory, water must be offered *ad libitum*, taking care to ensure that young animals are able to reach the drinking nipple.

**Reproduction.** The gerbil shows monogamous sexual behaviour. An adult gerbil will usually accept only its own partner. Gerbils are polyoestrus. If the lighting regime is kept constant with 12 hours light, they can breed all year round. Since breeding couples are usually kept permanently together, oestrus detection is not necessary. Vaginal swabs are not reliable for oestrus detection and behaviour patterns of the two sexes are a better indicator. Mating usually takes place in the late evening. A copulation plug is generally not noticeable, as it is small and retained deep inside the vagina. When the female is mated whilst suckling a large number of pups, the implantation is delayed and the duration of the pregnancy will be lengthened by more than 2 weeks.

Monogamous pairing is the most successful way and breeding pairs can be formed preferably around puberty (8 weeks). If the male and female start to fight, it is better to find another partner for both of them. Pairing new partners in a neutral, clean cage will decrease aggressive behaviour. The male remains in the cage when the pups are born and helps to take care of the young. In the event of a fight occurring, for example when the male rejects the pups or even attacks them, the breeding pair must be separated temporarily. Removing the male from the cage during the 2 weeks post partum, in order to prevent mating and disturbing the nest, may be done as a matter of routine. This period should not last longer than 2 weeks, as otherwise the two partners may start fighting again when reunited.

If a polygamous breeding system (trio) is used it is necessary to house the gerbils together before puberty.

**Handling and simple techniques.** The best way to lift a gerbil is by holding it in one hand. Less preferable is grasping it by the base of its tail. Grasping the distal part of the tail must be prevented, as the skin can easily be torn off. To restrain the animal, it is necessary to hold it by the base of the tail and the other hand should hold the skin of the neck or its back. It is not advisable to turn a gerbil on its back.

**Guinea-pigs**

**Origin and use.** The guinea-pig or cavy (*Cavia porcellus*) originates from the Andes in South America. The wild type (*Cavia cutleri*) has been domes-
ticated by the Indians for meat production. The Spaniards probably introduced the guinea-pig into Europe in the 16th century.

Of the three hair-types (short-haired or English guinea-pig, rough-haired or Abyssinian and long-haired or Peruvian) the short-haired variety is used almost exclusively in biomedical research, accounting for approximately 2–3% of all registered laboratory animals. The main purpose for using guinea-pigs is for the production and control of sera, vaccines and other biological products. The guinea-pig can be sensitised easily and is achieved by repeated injections resulting in a hyper-sensitivity reaction. Due to its high susceptibility to infectious diseases, such as tuberculosis, diphtheria, leptospirosis and brucellosis, the guinea-pig is important for diagnostic reasons. The guinea-pig is a useful animal model for immunological research as for example its plasma-complement has a high activity level, for otology experiments due to the favourable anatomy of its middle and inner ear, and for nutritional studies into vitamin C, folic acid, thiamine, arginine and calcium.

There are a limited number of inbred strains available. Most work is carried out using out-bred strains; many of them originating since 1926 from the Dunkin–Hartley line.

**Physiology and behaviour.** The guinea-pig is strictly herbivorous, has transversely inclined molars, and the jaw movements are made from front to back. The incisors and the molars grow continuously. The animals are active for roughly 20 hours a day. There is no clear circadian rhythm; periods of activity alternate with short sleeping periods which last for about 10 minutes. Guinea-pigs eat their own faeces (coprophagy) directly from the anus, but obese and pregnant animals eat their droppings from the floor, because they can not reach the anal opening. Newborn animals eat their mother’s droppings, from which they obtain the same intestinal microflora.

The skin around the anus is naked and invaginated (fossa perinealis) and has many sebaceous glands. Just above the rudimentary tail there are some large sebaceous glands (coccygeal glands). Males (boars) as well as females (sows) have one pair of nipples in the inguinal region. Males can be distinguished by extruding the penis using gentle pressure on the abdomen just cranial to the genital opening. The vagina of a female which is not in oestrus and not delivering, is closed by a membrane. The caecum is large with a crenated edge and fills a large part of the abdomen. There are many mononuclear leucocytes carrying an oval inclusion body in the blood of guinea-pigs, known as Kurloff’s Bodies, and they are present especially during pregnancy. These leucocytes probably protect the fetus against immunocompetent cells of the mother, because they are present in high concentrations within the placenta.

Guinea-pigs are very sensitive to antibiotics, even more so than hamsters. In particular, antibiotics against Gram-positive bacteria such as penicillin and
erythromycin can be fatal. The animals are relatively unaffected by cortico-
steroids. On the other hand, histamine can lead to a lethal constriction of the
smooth muscular tissue of the bronchioles.

**Housing.** Guinea-pigs are social animals and therefore should preferably be
housed in groups in pens or large cages. They are hardly ever aggressive
towards each other, and only when unfamiliar male adult animals are put
together does fighting take place. A solid bottomed cage with bedding mate-
rial is better than a cage with a wire bottom, as the animals lose hair and body
weight on wire and there is also a risk of bone fracture and footpad inflamma-
tion. Guinea-pigs usually do not climb and, if the cage sides are at least 20–25
cm high, they can be housed without a lid. Cages with a lid must have a
minimum height of 18 cm, although some cm more should be preferred, due
to the height they need for caecotrophy. Care must be taken with sawdust as
bedding material; it can accumulate inside the preputium of males and cause
an infection and even obstruct the erection of the penis. In females it can
cause vaginitis.

High environmental temperatures (above 28°C) in combination with high
relative humidities (above 70%) are not well-tolerated by guinea-pigs. In preg-
nant animals this can result in abortion. The guinea-pig is very sensitive to
temperature fluctuations and draughts. Sudden changes in environmental con-
ditions, such as during transportation, can lead to dramatic weight loss.

Guinea-pigs are nervous and easily liable to panic, for example, if sub-
jected to sudden unfamiliar noises. They will emit loud squeals and possibly
run in circles. Over-crowding, boredom and stress can induce behaviour dis-
orders, notably hair-biting.

**Food and drink.** Guinea-pigs have a good appetite and eat regularly during
the day as well as at night. Within a few days after birth they should be intro-
duced to solid food. In general guinea-pig refuse bitter, salty or sweet-tasting
food and synthetic diets. Restricting the intake of food and drinking water
can be harmful. Vitamin C cannot be synthesized by guinea-pigs and is there-
fore, in most cases, added to commercial guinea-pig feed. However, the shelf-
life of feed containing vitamin C may be less than 3 months, even if stored in
a cool and dark place. Vitamin C can also be given via the drinking water
(220 mg/litre); the water should be free from chlorinate, otherwise the vitamin
will be inactivated. Any swelling of the joints or salivation or lethargy could
be indicative of a vitamin C deficiency.

The supplementation of good quality hay to the diet provides a good source
of crude fibre, and provides the animals with a distraction which also helps to
prevent hair-biting.

Guinea-pigs do not lick the drinking nipple but gnaw on it. That is why the
nipple must be made of stainless steel. In a breeding colony the nipple must be long enough to allow the newborns to reach it as well. During drinking, guinea-pigs blow mouth-fluid and food particles back into the bottle and therefore the water becomes polluted very quickly. The water must be frequently changed and the bottle must be cleaned and disinfected regularly.

Reproduction. The guinea-pig is polyoestrous. The oestrous period is recognizable because at this time the vaginal membrane is open. There are also typical behavioural patterns exhibited by the sow; for example the sow will attempt to mount cage mates just like a boar. When a sow on heat is approached by a male, she will show lordosis (hollow back) and will ‘shoot’ small quantities of urine in the direction of the male. The act of mating shortens the oestrous period and can be recognized afterwards by the presence of a copulation plug in the vagina. The average length of pregnancy of the guinea-pig is 68 days, which is significantly longer than that of other rodent species. Just prior to delivery the pelvic bones on the ventral side (pubic symphysis) separate, providing a spacious birth canal. During oestrus and parturition the vaginal membrane is open.

Guinea-pigs are well developed at birth: they possess a complete coat of hair, their eyes and ears are open, and they can walk almost immediately. Within a few hours after birth, the young guinea-pigs can eat solid food. Fostering by other females can also be successful. The sow should have her first litter before she herself is fully grown, thereby preventing the firm fusion of the pubic symphysis may results in dystocia. Older animals develop obesity and ovarian cysts which can lead to diminished fertility and a greater risk of toxaemia or stillborn of the young.

Both monogamous and polygamous breeding systems are used. Monogamous pairs stay together during the reproductive period. Postpartum mating is common and can result in approximately five litters per sow per year. In polygamous groups one male is housed with 8–10 females. In this situation it is usual for pregnant females to be housed separately until some weeks after delivery.

Handling and simple techniques. Guinea-pigs should be lifted by grasping them gently but firmly around the shoulders and thorax, whilst at the same time supporting the back and hind legs with the other hand (fig. 3-8). If the animal is gripped too tightly around the trunk and belly, this can cause shock or possibly lead to liver and lung injuries.

The identification of multi-coloured guinea-pigs using sketch cards can be useful. However, as the majority of laboratory guinea-pigs are white, the best method for long term marking is micro-chipping, tattooing of the ear(s) or the naked skin behind the ear. Ear clipping, punching or tagging should not be applied, as they can be easily torn out. Temporary marking can be achieved
by the staining of the coat with dyes such as fuchsin, acriflavine or gentian violet.

Rabbits

Origin and use. The origin of the European wild rabbit (*Oryctolagus cuniculus*) is thought to begin on the Iberian Peninsula, from whence it spread to the Mediterranean area. There are many different breeds, which are selected by body weight, coat type and colour. As a laboratory animal, the most commonly used breeds are the Dutch breed which weigh less than 2 kg, and the New Zealand White which weigh 2–5 kg. There are a limited number of inbred strains. Compared with the numbers of mice and rats used in biomedical research, the number of rabbits used is rather low, being approximately 2% of all registered laboratory animals. The areas of research where they are used are toxicity (teratogens) tests, the production of antiserum, the calibration of biologically active products, eye and skin irritation tests and studies on atherosclerosis.

Physiology, anatomy and behaviour. Rabbits are classified according to their body weight i.e. large breeds weigh more than 5 kg, medium breeds (2–5 kg) and small breeds less than 2 kg. Small breeds are sometimes sub-divided to cover dwarf breeds of less than 1 kg. The rabbit is herbivorous and produces two types of faeces: the soft ‘night’ faeces and the hard, dry ‘day’ faeces. During the second half of the night and early morning, the content of the
caecum is transported virtually unaltered to the colon and rectum in small spherical particles surrounded by a mucous layer. These soft droppings, which have a relatively low fibre content but contain high levels of proteins and vitamins B and K, are eaten by the rabbit directly from the anus (coprophagy or caecotrophy).

The rabbit’s urine may vary in colour from turbid yellow to reddish brown, depending upon the composition of the feed. The sebaceous glands under the skin of the chin and around the anus (fossa perinealis) secrete odorous products (pheromones) used by the rabbit when marking out its territory. Hares and rabbits belong to the order Lagomorpha, and have one pair of small incisors just behind the large incisors of the upper jaw. At the outlet of the ileum into the caecum there is a dilation known as sacculus rotundus, the walls of which contain lymphatic tissue (tonsilla ileocaecalis). The large caecum is coiled up like a spiral, the blind end of which also contains a large amount of lymphoid tissue (appendix caeci). The female rabbit (doe) has a double cervix. The male rabbit (buck) can be distinguished from the female by protruding the penis. When determining the sex of young animals experience is needed.

When a rabbit is resting it breathes using its diaphragma which causes the abdomen to move in and out.

The neutrophilic leucocytes contain many eosinophilic granules in the cytoplasm (pseudo-eosinophilic leucocytes).

Approximately 30% of rabbits react atypically to atropine, as this drug is inactivated by atropine-esterase in the blood.

**Housing.** Stainless steel cages with a wire floor were commonly used for housing laboratory rabbits, but now plastic cages are used more frequently, or animals are kept in groups in pens (fig. 3-9). Cages should be constructed in such a way that young animals do not get their legs trapped in the wire floor, and the adult animals do not acquire damage to their foot pads. The toe nails must be clipped at regular intervals. The cage must be of a sufficient size to enable an adult rabbit to stretch to its full length and to sit upright on its hind legs. The minimum requirements for cage dimensions for rabbits are set out in the European guidelines and are related to body weight but do not seem to meet their behavioural requirements. In these guidelines no attention is paid to age or environmental enrichment.

Male rabbits can be housed individually immediately after weaning, and female rabbits at the age of 12 weeks. Group housing in relatively small pens is preferable, if the experimental protocol permits it. However, group housing of sexually mature animals (males) can lead to severe aggression, as the animals will defend their territory.

Due to the high concentration of crystals present in the urine of rabbits which stick to the floor of the cage, the cleaning of the cages is made rather difficult. They should therefore be treated periodically with descalants.
The optimal temperature for rabbits is between 15–19°C. In contrast to low temperatures, heat and draught are not well tolerated. Temperatures above 30°C in combination with high relative humidity leads to a risk of heat stress, which can cause infertility and mortality.

Food and drink. Rabbits are fed on pellets of a diameter of approximately 3 mm as rabbits grind the pellets between the (pre)molars. Young animals and does with a litter are fed *ad libitum*, whilst other animals should be rationed in order to prevent obesity. The crude fibre content of the diet must not be lower than 10%. It is advisable to supplement the feed with hay as this prevents intestinal disturbances and obstructions caused by hairballs, and also enriches their environment. Any changes to the composition of the feed, such as after weaning, should always be introduced gradually, at least over a period of 4–5 days, in order to give the intestinal microflora the opportunity to adapt to the new conditions. Fresh drinking water must be available *ad libitum*.

Reproduction. Although a true oestrous cycle with spontaneous ovulation does not occur in does, there are certain periods during which she will not accept the buck. As result of mating the pituitary gland secretes luteinising hormone (LH) which induces ovulation some 10 hours later. Ovulation can
also be induced by injecting LH, and is done for artificial insemination and for research in the field of reproduction such as studies on maturation of germ-cells, fertilization, cleavage and implantation. When the induction of ovulation is not followed by fertilization, pseudopregnancy occurs which lasts 16–18 days. If the animals are not subjected to artificial lighting, then the activity of the ovaries will be lower during the autumn and the winter, due to the shorter periods of light. Some days prior to parturition, the doe gathers hair from her abdominal region. This exposes her nipples (4 pairs) and provides material for the nest. The newborn are naked and remain in the nest for approximately 3 weeks. If a pup accidentally falls out of the nest, it will not be retrieved by the mother. The young pups are only suckled once or twice a day for a period of 4–5 minutes. This is sufficient because doe milk is extremely concentrated, containing 13% fat and 10% protein. After the suckling period is over, the nest is closed.

The optimal breeding age is between 5 months and 3 years. One buck is sufficient for 10–20 does, providing the buck is used for mating no more than 5 times a week. When mating is to take place, the doe is brought to the buck for 15–20 minutes. If the female will not mate or the animals start fighting, it is possible to try another buck or to reintroduce the same buck 1–2 days later. Ten to 14 days after mating, pregnancy can be confirmed by abdominal palpation. A nesting box and nesting materials should be provided during the last week of pregnancy. Newborns do not need to receive colostrum, due to the passage of immunoglobulins across the placenta. Newborns can only be fostered if they are not older than 2 weeks, and if the adopting litter is younger than 3 days. The smell of the nest should be masked by sprinkling the young pups and the nose of the foster mother with perfume. The mortality of fostered pups is, however, high.

Does can be mated again after weaning (after 4–5 weeks), although mating immediately after parturition is also possible.

Handling and simple techniques. Lifting rabbits out of their cage can be done by grasping the skin at the back of the neck and the back firmly with one hand, while supporting the belly and the hind legs with the other (fig. 3-10). When carrying the rabbit, the head of the animal is firmly held between one arm and the body of the handler. The rabbit should now be lying quietly on the handler’s arm. Struggling animals risk breaking their backs. Should this occur then euthanasia should be carried out immediately. For long term restraint, a rabbit can be placed in a restraining box. This should be done with care because when struggling back damage can occur. For the permanent identification of a rabbit, microchips or ear tattoos are recommended. Ear tags are not suitable. Short-term marking can be done using dyes such as fuchsin, acriflavine, or gentian violet.
**Transport.** Rabbits should not be transported without adequate ventilation as they cannot tolerate heat. The transportation boxes must have sufficient room, with a minimum specifications $40 \times 25 \times 35$ cm ($l \times b \times h$) and good ventilation openings. It is recommended that animals fast for 12 hours prior to transportation, in order to prevent the risk of gastric ruptures.

**Monkeys**

*Origins and use.* Various species of monkeys (non-human primate) have different origins. The lower monkeys (Prosimiae) are found on the Indonesian islands and in Madagascar. The true monkeys (Simiae) are found in Central and South America, such as the squirrel monkey (Saimiri) and marmoset (Callithrix); in Africa, such as the chimpanzee (*Pan troglodytes*), and in South and East Asia, where the rhesus monkey (*Macaca mulatta*) and the cynomolgus monkey (*Macaca fascicularis*) are found. Years ago, it was common practice to use wild monkeys for research, but nowadays, most are bred and reared under laboratory conditions. The number of monkeys used as experimental animals is declining. Most monkeys are used for the testing of vaccines and medicines, but are also used for a variety of scientific investigations, such as behavioural research and specific studies into (infectious) diseases which occur in man.
Physiology, anatomy and behaviour. Besides possessing the general characteristics of primates, each species has its own physiological and anatomical peculiarities. This is also the case for nutritional requirements and behavioural patterns. To illustrate this point, lower monkeys are active during the dark period, have large eyes and ear flaps. Most of them are insectivores or omnivores, the most primitive amongst them being the tupaias (e.g. *Tupaia glis*). The true monkeys are more highly developed and some, notably the Homidae, bear a strong resemblance to man.

Detailed biological knowledge and practical experience in relation to the particular species of monkey used for research is crucial when using them as laboratory animals. The section which follows gives only some general information in terms of housing conditions, nutrition and handling of the frequently used species.

Housing. Many primate species, such as the macaques and the chimpanzees, live in groups which have a very distinct social order. These animals should preferably be housed, therefore, in large rooms or pens. When forming these groups the species, age, sex etc. of the monkeys must be taken into account. The behaviour within the group should be under continual observation, so that the handler can intervene in the case of extreme aggression.

The minimum dimensions regarding floor area and cage height vary according to the species and the size of the animals. The European guidelines divide monkeys into seven groups according to body weight. The height of the cage must at the minimum give the monkeys the possibility of standing upright. For spider monkeys there must be sufficient room for them to hang freely from the ceiling extended to their full length. Apart from actual floor area and height, the shape of the cage is also important. Whenever possible there should be small shelves in the cage so that the animals can sit and rest near the top of the cage. Enrichment of the environment is important for all species. This can be achieved by all kinds of materials, such as furniture, and car tyres (but beware the metal rims). It is sometimes necessary to house monkeys individually on a temporary basis, for example during quarantine or whilst carrying out experimental procedure or during recovery after anaesthesia. The cages should then be arranged in such a way that the animals can still see their group mates. Long term solitary confinement may lead to abnormal behaviour. However, some primates, such as the male orang-utan live alone, and marmosets live as monogamous couples.

The optimal environmental temperature for most of the monkey species is 20–24°C, although marmosets prefer a somewhat higher temperature of between 22–28°C.

Nutrition. Monkeys can be fed commercially produced monkey food. How-
Table 3-3
Environmental requirements and physiological parameters of marmosets, cynomolgus, rhesus monkeys and chimpanzees

<table>
<thead>
<tr>
<th>Environmental requirements</th>
<th>Marmosets</th>
<th>Cynomolgus</th>
<th>Rhesus m.</th>
<th>Chimpanzees</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (°C)</td>
<td>22–28</td>
<td>20–24</td>
<td>20–24</td>
<td>20–24</td>
</tr>
<tr>
<td>Relative humidity (%)</td>
<td>40–60</td>
<td>50–70</td>
<td>50–70</td>
<td>50–70</td>
</tr>
<tr>
<td>Ventilation (changes/h.)</td>
<td>—</td>
<td>9–12</td>
<td>9–12</td>
<td>9–12</td>
</tr>
<tr>
<td>Light/dark (hours)</td>
<td>13/11</td>
<td>13/11</td>
<td>13/11</td>
<td>13/11</td>
</tr>
<tr>
<td>Minimum cage floor size</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>One individually housed adult (m²)</td>
<td>0.25</td>
<td>0.7–0.9</td>
<td>0.9–1.1</td>
<td>2.5</td>
</tr>
<tr>
<td>Breeding animal with young (m²)</td>
<td>0.25</td>
<td>0.9</td>
<td>1.1</td>
<td>—</td>
</tr>
<tr>
<td>Group (m²/adult)</td>
<td>0.25</td>
<td>0.7</td>
<td>0.9</td>
<td>—</td>
</tr>
<tr>
<td>Minimum cage height (cm)</td>
<td>60</td>
<td>90</td>
<td>90–120</td>
<td>200</td>
</tr>
</tbody>
</table>

General physiological parameters

<table>
<thead>
<tr>
<th>Adult weight (kg)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>0.4–0.6</td>
<td>2.5–6</td>
<td>4–9</td>
<td>35–45</td>
</tr>
<tr>
<td>Female</td>
<td>0.4–0.5</td>
<td>4–8</td>
<td>6–11</td>
<td>45–60</td>
</tr>
<tr>
<td>Life span (years)</td>
<td>10–16</td>
<td>15–25</td>
<td>20–30</td>
<td>40–50</td>
</tr>
<tr>
<td>Heart rate (/min)</td>
<td>—</td>
<td>100–150</td>
<td>100–150</td>
<td>85–90</td>
</tr>
<tr>
<td>Respiration rate (/min)</td>
<td>—</td>
<td>40–65</td>
<td>40–65</td>
<td>30–60</td>
</tr>
<tr>
<td>Body temperature (°C)</td>
<td>—</td>
<td>37–40</td>
<td>36–40</td>
<td>36–39</td>
</tr>
<tr>
<td>Number of chromosomes (2n)</td>
<td>—</td>
<td>—</td>
<td>42</td>
<td>48</td>
</tr>
<tr>
<td>Body surface (cm²)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Water intake (ml/100g/day)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Puberty (years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>0.8–1</td>
<td>3–4</td>
<td>3–4</td>
<td>6–8</td>
</tr>
<tr>
<td>Male</td>
<td>0.8–1</td>
<td>3–4</td>
<td>3–4</td>
<td>8–10</td>
</tr>
<tr>
<td>Breeding age (years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>1.5–2</td>
<td>4–5</td>
<td>3–4</td>
<td>9–11</td>
</tr>
<tr>
<td>Male</td>
<td>1.5–2</td>
<td>4–5</td>
<td>4–5</td>
<td>10–12</td>
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<tr>
<td>Oestrous cycle (days)</td>
<td>27–29</td>
<td>31</td>
<td>29</td>
<td>32–38</td>
</tr>
<tr>
<td>Duration of menstruation(d)</td>
<td>none</td>
<td>4</td>
<td>3</td>
<td>—</td>
</tr>
<tr>
<td>Duration of pregnancy (d)</td>
<td>142–146</td>
<td>161</td>
<td>155–170</td>
<td>210–250</td>
</tr>
<tr>
<td>Litter size</td>
<td>2–3</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Weight at birth (g)</td>
<td>25–35</td>
<td>300–400</td>
<td>450–500</td>
<td>1500</td>
</tr>
<tr>
<td>Weight at weaning (g)</td>
<td>80–120</td>
<td>800–1200</td>
<td>1000–1500</td>
<td>—</td>
</tr>
<tr>
<td>Weaning age (months)</td>
<td>3–6</td>
<td>12–16</td>
<td>12–16</td>
<td>36</td>
</tr>
</tbody>
</table>

Blood parameters

| Blood volume (ml/kg) | 70        | —         | 50–90     | 62–65       |
| Haemoglobin (g/100 ml) | —        | —         | 11–12.5   | 10–14       |
| Haematocrit (vol%)   | —         | —         | 39–43     | 38–43       |
| Leucocytes ($\times$ 1000/mm³) | —     | —         | 7–13      | 10–14       |
| Glucose (mg/100 ml)  | —         | —         | 60–160    | 80–95       |

ever, the exact composition must be adapted to the specific requirements of any given species. For example South American monkeys, such as marmosets, cannot absorb vitamin D2, whereas macaques can use both vitamin D2
and D3 as a source of vitamin D. All primates need vitamin C (1–5 mg/kg b.w./day). Food is primarily a source of nutrients, but is also a means of keeping the animal occupied. The composition of the menu greatly influences the amount of time the animals spend on both the collection and eating of food. Selection of food by the animals should be avoided to prevent deficiencies.

Reproduction. The breeding of monkeys should be carried out in specialized centres, as it requires not only optimal housing but also specific knowledge and training.

The reproductive cycle of marmosets is of a shorter duration than that of macaques and chimpanzees. Macaques have visible bleeding (menstruation) and ovulation occurs during oestrus in the middle of the menstrual cycle. During oestrus, hyperaemia of the perineal skin occurs. In order to ascertain whether mating has taken place or not, the vagina is checked for the presence of a coagulation plug or spermatozoa. After approximately four weeks of gestation, pregnancy can be diagnosed by rectal palpation of the uterus. Towards the end of pregnancy the position of the fetus should be checked regularly as in the event of an abnormal position natural delivery may not take place. In some situations it is necessary to perform a Caesarean section. Most monkey species produce only one offspring at a time, twins are exceptional. After approximately 12–16 months the young can be weaned and housed in groups with animals of equal age and size to prevent molestation by stronger animals. Weaning at a younger age can result in socialisation problems.

Handling. Appropriate handling is very important and staff must be trained by a competent supervisor. This is especially true in the case of the larger monkey species, as these animals are intelligent, strong, fast and occasionally aggressive. Their long, sharp canine teeth can be dangerous, and, in some cases, it may be necessary to tranquillize the animal (e.g. with ketamine) before handling it. Whilst administering such an injection the animal can be restrained against the front of the cage by means of a movable cage wall (squeeze back). A small or medium sized monkey which is housed within a pen can be captured using a net. Whilst the animal is captive in the net, the arms should be secured behind its back. During such handling and securing it is advisable to wear thick gloves. A variety of harnesses and chairs are available for restraint, but care must be taken to avoid physical damage to the animal.
Dogs

Origin and use. The dog (Canis familiaris) has a long history of domestication and is a probable descendent of the wolf (Canis lupus). Nowadays there are approximately 300 breeds, ranging from the very small (± 18 cm high and 1–2 kg) to very large and heavy varieties (± 80 cm high, ± 90 kg body weight). Next to beagles (10–12 kg body weight), mongrel dogs are the most frequently used as laboratory dogs. Beagles are particularly suitable, because they are docile and can be kept both in groups or individually. The number of dogs used in experiments has been steadily decreasing over the last 15 years. Dogs are mainly used for the testing of vaccines and medicines, for developing surgical techniques and for research into cardiovascular disease.

Physiology and behaviour. Many physiological parameters of the dog are well known; a number of them are shown in table 3-4. Given the extreme variation between breeds, it goes without saying that some biological differences will also be found.

When using dogs as experimental animals, detailed knowledge of their behaviour is required. The dog is a social animal and a complicated social ranking exists within any group, of which the hierarchy depends largely upon the sex of the animal. In general male dogs are more aggressive than females (bitches), especially towards other males and unfamiliar adult dogs. As a rule beagles tend to be more tolerant and do not show many signs of aggression. Male dogs mark out their territorial boundary by regularly spraying urine on different places. Working in a uniform and consistent way in close and regular contact with the animals, especially during the socialisation period between the age of 4–8 weeks, is extremely important. The animals become used to both humans and other dogs and it prevents nervous behaviour.

Housing. Dogs can be housed indoors as well as outdoors and should preferably be housed in small groups with an indoor sleeping area and an outdoor run. The indoor pens should have a solid floor, a ‘tender-foot’ floor, or metal grid floor, and must be kept warm with no draughts. There should also be a dry insulated sleeping area possibly with under-floor heating. The floor of the outside run should have a suitable slope (approximately 5%) to ensure adequate drainage. If an animal has to be housed temporarily on its own, then cages can be used. According to the European guidelines the minimum dimensions of the cage are relative to the size of the dog. For example, a beagle needs a minimum floor area of 1 m² and a height of 80 cm for a short stay. When reintroducing an animal that has been temporarily away from the group, extra care must be taken to ensure that the animal is not rejected. Beagles grouped together require an indoor area of 1.2 m² with a run of at least 1.6 m².
per animal. The environmental temperature for a group of dogs could be lower than that for dogs housed individually. Newborn pups depend upon external heat for regulating their body temperature. In order to achieve the ambient temperature of between 26–29°C for the first 5–10 days, a heating lamp should be placed at a height of at least 1 metre.

**Food and drink.** The dog is a carnivore. Adult dogs should normally be fed once a day (large breed twice a day) with commercial dog chow, either in a dry or wet form. Variation in the type of food in the diet is advisable. This food should be given in durable and easy-to-clean pans. For dogs housed in groups, it is essential to check that all the animals get sufficient food. Water should be provided *ad libitum*, either in a drinking dish or by means of an automatic watering system.

**Reproduction.** In general, the breeding of dogs takes place in a laboratory animal breeding station, as it is very time consuming. The physiological data related to reproduction in dogs are shown in table 3-4. The (pro-)oestrus of the bitch can be detected by the swelling of the vulva, followed by a bloody to serous discharge lasting 6–14 days. The best time for mating to occur is around the 10th to 12th day after the start of vulval bleeding and usually when the discharge is serous or less bloody. Pregnancy can be confirmed by palpation between day 21 and 28 after mating or by echoscopy after about 14 days. After the 25th day diagnosis by palpation becomes more difficult. In the last week prior to parturition, the bitch should be provided with a special box to make a nest. The interval between the delivery of the pups is usually not much longer than 1 hour. After the birth of each pup, the umbilical cord is bitten through and the placenta is eaten by the bitch.

During the first week of life, the pups will be suckled every 2 hours, after which the frequency will gradually diminish. The eyes and ears of the pups open between the 10th and 14th day and the pups can walk and start eating puppy feed after the 20th day. The use of artificial milk is possible, but one must take into account that dog milk has a higher percentage of fat, protein, calcium and phosphorus than cow milk. There are high quality milk substitutes available for dogs.

**Handling and simple techniques.** A dog should always be approached quietly from the front, whilst talking to it. When in contact with dogs, the handler must always be the dominant party, but this should always be achieved by reward rather than by aversive means. Experimental handling must never be carried out within the animal’s territory.

The animal can be lifted up with one hand supporting the belly with the other hand holding the scruff. The most successful way to hold a dog securely is to put it on its side, whilst firmly fixing its underlying legs, and using the arms
of the handler to push down the neck and the pelvis of the dog. Animals which have a nervous and aggressive disposition, which is often induced by fear, should be calmed. As a last resort one can use a sedative or apply a bandage (> 7 cm broad) around the nose and jaws of the dog knotted once under the lower jaw with a second knot tied firmly at the back of the neck. Dogs can be

<table>
<thead>
<tr>
<th>Environmental factors</th>
<th>Dogs</th>
<th>Cats</th>
<th>Pigs</th>
<th>Sheep/goats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (°C)</td>
<td>15–21</td>
<td>15–21</td>
<td>17–24</td>
<td>10–24</td>
</tr>
<tr>
<td>Relative humidity (%)</td>
<td>40–60</td>
<td>40–60</td>
<td>40–60</td>
<td>40–60</td>
</tr>
<tr>
<td>Ventilation (m³/h/animal)</td>
<td>20–80</td>
<td>20–50</td>
<td>100–180</td>
<td>100–150</td>
</tr>
<tr>
<td>Light/dark (hours)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minimum cage floor size¹</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>One individually housed adult (m²)</td>
<td>0.75–1.75</td>
<td>0.2–0.6</td>
<td>0.35–0.8</td>
<td>1.4/1.6</td>
</tr>
<tr>
<td>Breeding animal with young (m²)</td>
<td></td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group (m²/adult)</td>
<td>1–4</td>
<td>0.2–0.6</td>
<td>0.2–2.5</td>
<td>0.7/0.8</td>
</tr>
<tr>
<td>Minimum cage height (cm)¹</td>
<td>60–180</td>
<td>50</td>
<td>50–80</td>
<td>1200/2000</td>
</tr>
</tbody>
</table>

### General physiological parameters

#### Adult weight (kg)

<table>
<thead>
<tr>
<th>Sex</th>
<th>Dogs</th>
<th>Cats</th>
<th>Pigs</th>
<th>Sheep/goats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>10–80</td>
<td>3–7</td>
<td>200–300</td>
<td>50–70</td>
</tr>
<tr>
<td>Female</td>
<td>10–60</td>
<td>3–4</td>
<td>150–220</td>
<td>50–60</td>
</tr>
</tbody>
</table>

#### Duration of life (years)

<table>
<thead>
<tr>
<th>Sex</th>
<th>Dogs</th>
<th>Cats</th>
<th>Pigs</th>
<th>Sheep/goats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>10–15</td>
<td>10–17</td>
<td>14–18</td>
<td>10–15</td>
</tr>
<tr>
<td>Female</td>
<td>10–15</td>
<td>10–17</td>
<td>14–18</td>
<td>10–15</td>
</tr>
</tbody>
</table>

#### Heart rate (/min)

<table>
<thead>
<tr>
<th>Sex</th>
<th>Dogs</th>
<th>Cats</th>
<th>Pigs</th>
<th>Sheep/goats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>80–150</td>
<td>100–120</td>
<td>60–90</td>
<td>70–80</td>
</tr>
<tr>
<td>Female</td>
<td>20–30</td>
<td>20–40</td>
<td>8–18</td>
<td>12–25</td>
</tr>
</tbody>
</table>

#### Body temperature (°C)

<table>
<thead>
<tr>
<th>Sex</th>
<th>Dogs</th>
<th>Cats</th>
<th>Pigs</th>
<th>Sheep/goats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>38–39</td>
<td>38–39.5</td>
<td>38–40</td>
<td>38.5–40</td>
</tr>
<tr>
<td>Female</td>
<td>78</td>
<td>38</td>
<td>38</td>
<td></td>
</tr>
</tbody>
</table>

#### Number of chromosomes (2n)

<table>
<thead>
<tr>
<th>Sex</th>
<th>Dogs</th>
<th>Cats</th>
<th>Pigs</th>
<th>Sheep/goats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>78</td>
<td>38</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>78</td>
<td>38</td>
<td>38</td>
<td></td>
</tr>
</tbody>
</table>

#### Water intake (ml/100g/day)

<table>
<thead>
<tr>
<th>Sex</th>
<th>Dogs</th>
<th>Cats</th>
<th>Pigs</th>
<th>Sheep/goats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>0.03–0.25</td>
<td>2–6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>0.03–0.25</td>
<td>2–6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### Puberty (months)

<table>
<thead>
<tr>
<th>Sex</th>
<th>Dogs</th>
<th>Cats</th>
<th>Pigs</th>
<th>Sheep/goats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>8–14</td>
<td>6–8</td>
<td>5–7</td>
<td>6–10</td>
</tr>
<tr>
<td>Female</td>
<td>7–8</td>
<td>6.5–7</td>
<td>5–7</td>
<td>6–10</td>
</tr>
</tbody>
</table>

#### Breeding age (months)

<table>
<thead>
<tr>
<th>Sex</th>
<th>Dogs</th>
<th>Cats</th>
<th>Pigs</th>
<th>Sheep/goats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>&gt;12</td>
<td>10–12</td>
<td>&gt;7</td>
<td>&gt;10</td>
</tr>
<tr>
<td>Female</td>
<td>&gt;12</td>
<td>&gt;12</td>
<td>&gt;7</td>
<td>&gt;10</td>
</tr>
</tbody>
</table>

#### Oestrous cycle (days)

<table>
<thead>
<tr>
<th>Sex</th>
<th>Dogs</th>
<th>Cats</th>
<th>Pigs</th>
<th>Sheep/goats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>4–8 mths</td>
<td>15–18</td>
<td>18–24</td>
<td>14–20/15–24</td>
</tr>
<tr>
<td>Female</td>
<td>4–8 mths</td>
<td>15–18</td>
<td>18–24</td>
<td>14–20/15–24</td>
</tr>
</tbody>
</table>

#### Litter size

<table>
<thead>
<tr>
<th>Sex</th>
<th>Dogs</th>
<th>Cats</th>
<th>Pigs</th>
<th>Sheep/goats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>63–67</td>
<td>60–65</td>
<td>110–118</td>
<td>144–155</td>
</tr>
<tr>
<td>Female</td>
<td>3–6</td>
<td>3–5</td>
<td>11–16</td>
<td>1–2</td>
</tr>
</tbody>
</table>

#### Weight at birth (g)

<table>
<thead>
<tr>
<th>Sex</th>
<th>Dogs</th>
<th>Cats</th>
<th>Pigs</th>
<th>Sheep/goats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>200–500</td>
<td>90–130</td>
<td>900–1600</td>
<td>–</td>
</tr>
<tr>
<td>Female</td>
<td>200–500</td>
<td>90–130</td>
<td>900–1600</td>
<td>–</td>
</tr>
</tbody>
</table>

#### Weight at weaning (g)

<table>
<thead>
<tr>
<th>Sex</th>
<th>Dogs</th>
<th>Cats</th>
<th>Pigs</th>
<th>Sheep/goats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>1.5–4</td>
<td>0.6–0.8</td>
<td>6–8</td>
<td>–</td>
</tr>
<tr>
<td>Female</td>
<td>1.5–4</td>
<td>0.6–0.8</td>
<td>6–8</td>
<td>–</td>
</tr>
</tbody>
</table>

#### Weaning age (months)

<table>
<thead>
<tr>
<th>Sex</th>
<th>Dogs</th>
<th>Cats</th>
<th>Pigs</th>
<th>Sheep/goats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>6–7</td>
<td>7</td>
<td>4–7</td>
<td>4–8</td>
</tr>
<tr>
<td>Female</td>
<td>6–7</td>
<td>7</td>
<td>4–7</td>
<td>4–8</td>
</tr>
</tbody>
</table>

### Blood parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Dogs</th>
<th>Cats</th>
<th>Pigs</th>
<th>Sheep/goats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood volume (ml/kg)</td>
<td>72–74</td>
<td>65–75</td>
<td>74</td>
<td>80/60–70</td>
</tr>
<tr>
<td>Haemoglobin (g/100 ml)</td>
<td>12–17</td>
<td>11–14</td>
<td>11–13</td>
<td>11–13/8–12</td>
</tr>
<tr>
<td>Haematocrit (vol%)</td>
<td>37–55</td>
<td>24–55</td>
<td>41</td>
<td>32/34</td>
</tr>
<tr>
<td>Leucocytes (x1000/mm³)</td>
<td>7–17</td>
<td>9–20</td>
<td>8–16</td>
<td>15–20/8–12</td>
</tr>
<tr>
<td>Glucose (mg/100 ml)</td>
<td>60–80</td>
<td>75–110</td>
<td>60–90</td>
<td>30–60</td>
</tr>
</tbody>
</table>

¹Dependent upon body weight and/or height.
marked for identification by tattooing the ear or by a subcutaneous chip in
the neck. Identification is mandatory in several countries. Temporary iden-
tifications can best be done by the use of numbered or named collars.

To prevent infectious disease, dogs are vaccinated according to a vaccina-
tion schedule. Regular treatment must be given to prevent ecto- and endo-
parasitic infections. Scale on teeth should be removed regularly and can (partly)
prevented by giving them regularly a marrow bone to gnaw. In order to pre-
vent oestrus, bitches can be injected with progestagens every 5–6 months.
However, these treatments may affect the results of experiments, depending
upon the type of study being undertaken.

Cats

Origin and use. The European domestic cat (*Felis catus*) has been domesti-
cated for a long time. It has probably developed as a result of cross breeding
between two wild cats, *Felis silvestris* and *Felis lybica*. At the moment there
are more than 100 breeds in existence. A variety of differently coloured Eu-
ropean short-haired cats are used as laboratory animals. The number of cats
used in experiments has strongly declined during the last decade, but they are
still used for the development of medicines, mainly short-term experiments
under general anaesthesia, neurological investigations and research into car-
diovascular diseases.

Physiology and behaviour. Cats are solitary hunting animals with good eye-
sight and acute hearing. They communicate with each other, with other ani-
mals and with humans, by producing different sounds, such as miaowing and
purring, by facial expressions using their ears and teeth and by body position-
ing i.e. hairs on end and an arched back). Generally speaking female animals
(queens) and castrated males will behave in a friendly way towards each other.
Male cats (toms) are more aggressive, especially towards unfamiliar males.
Both queen and tom cats are strong territorial predators. The territory is marked
by an excretion from their circum-anal glands and by their urine.

Housing. Female cats can be housed in groups of up to 20 animals. Group
housing for tom cats is much more difficult and from the age of 4–6 months
onwards, they have to be separated, unless they are litter mates. It is impossi-
to introduce new tom cats in an existing group. Castrated toms can be
treated as females, providing it is carried out before sexual maturity.

Cats can be housed in an indoor pen, preferably with an outdoor run. The
indoor area for a group of 10–20 cats should have a minimum floor size of
12–25 m² with a height of 2–3 m. The floor must have a slope of 3–5% for
drainage purposes. The pen must be provided with scratching and climbing
facilities, places for sleeping other than on the ground, a number of dirt-trays with cat litter and with a double door to prevent escape. It should be possible to see inside the cat area to observe their behaviour. An outdoor run, accessible via two or more doors, is advisable. If there are only a few cats located in one large area, it is possible that two sub-groups will be formed, each with its own territory, which can result in fighting.

It is possible to house cats individually on a temporary basis in cages with a minimum floor area of 0.4–0.6 m² for animals of 3–4 and 4–5 kg body weight, respectively, and with a height of at least 50 cm. There should be a shelf installed above the ground for the cat to sleep upon, a litter tray and both scratching and climbing facilities following the European guidelines. Other recommendations (UK Home Office) require a minimum floor area of 0.75 m² and a height of 80 cm for cats with a body weight of 3 kg and more. Cats that are housed on their own are generally more nervous and less easy to handle, and their reintroduction into a group can cause serious problems.

Food and drink. Adult cats can be fed once a day with a variety of commercial cat food. Kittens up to 2 months old must be fed at least three times a day, and kittens up to six months old, twice a day. When cats are housed in groups it is necessary to check that each animal gets sufficient food. Cats are rather choosy, and additions to the feed can easily lead to them refusing it. The cat has a relatively high requirement for protein, vitamin B5 (nicotinic acid), vitamin A, arachidonic acid and taurine. The drinking water can be chlorinated (0.2–1.0 ml free chlorine per litre), but should not be acidified. The intake of drinking water will vary according to the water content of the food.

Reproduction. The queen cat is seasonally polyoestrous and this usually occurs from February to October. The duration of the cycle is approximately 14 days with an inter-oestrous period of between 3–6 days. Oestrus is generally obvious due to the behaviour of the female cat: the tail will be held erect, the body stretched and the back sunken, and she will roll over and miaow a lot. In order to guarantee pregnancy, mating should take place 2–3 times, mainly as ovulation is induced by copulation (post copulatory ovulation). To avoid territorially defensive behaviour, it is better to bring the female to the male for mating rather than the other way around. Queen cats who are in a late stage of pregnancy should be removed from the group and housed in, for example, a breeding cage. Approximately 10 days prior to delivery, the cage must be provided with a kittening box. Kittens are born at close intervals, normally about 30 minutes apart. When the queen cat does not produce sufficient milk, kittens can be fostered by another queen. Bottle feeding is possi-
ble and must be given 4 times per day, but the high protein content of the cat milk must be taken into account. After 3–4 weeks, the kittens can start to eat solid food. During parturition and the first weeks of rearing, the ambient temperature should be higher than normal (23 ± 2°C).

Handling and simple techniques. The handling and caring of cats should ideally be carried out by one person. The approach of, and handling by, unfamiliar people can have an influence on the results of experiments. The cat can be taken out of its cage by firmly holding the scruff, resulting in the occurrence of a rigidifying reflex. The animal can now be supported under the arm of the technician using the hand to support the chest and the fingers to fix the forelegs of the cat. A cat can be most easily restrained on a table lying on its side. The fore and hind legs should be held firmly between the fingers, and the head is pushed onto the table by the wrist of the handler. Intractable cats can be contained in a cloth. Wild cats should be dealt with using protective gloves and given a tranquillizer.

To prevent infectious diseases, cats can be vaccinated and, if necessary, treated against ecto- and endoparasites at regular intervals. In order to prevent oestrus, queens can be treated with hormones, but, these treatments may affect some experimental results.

Pigs

Origin and use. The European wild swine (Sus scrofa) probably originated from the Asian swine (Sus vitatus). Many varieties and breeds have been developed from the European swine. In biomedical research there are many commercial breeds in use, such as the Large White Landrace and Yorkshire pig, along with the miniature Göttingen pig from Germany, and Pittmoore, Minnesota and Yucatan varieties from the USA and Mexico. Even smaller pigs (micropigs), are becoming commercially available. The number of pigs which are used in research, e.g. for vaccine development and food research, is limited in comparison with the total numbers of laboratory animals used (less than 1%). The pig is used as an animal model for humans as it shows many biological similarities in areas such as the skin, skeleton, gastro-intestinal tract, pancreas, kidneys and cardiovascular system.

Physiology and behaviour. Physiological data related to pigs are shown in table 3-4. Miniature pigs will, in some instances, show different values for some of the parameters. Pigs live in social groups and in the wild there is a distinct hierarchical ranking. Adult male pigs (boars) either live a solitary existence or are the leader of a group.

When it is necessary to use groups of piglets for a biomedical study, these
must be formed at a very early age, i.e. immediately after weaning. When older piglets are placed into a group, fighting will occur which can last 1–2 days.

Young piglets are by nature very active and enjoy to play. If bored they will exhibit abnormal behaviour in the form of tail and ear biting, or the sucking of the umbilicus, the vulva or prepuce. Although piglets have the tendency to get excited, to struggle, to resist and to scream, they become tractable and docile with time when being properly handled and trained (with the exception of the Vietnamese minipig).

**Housing.** Young pigs can be housed in groups in indoor pens on a metal grid or wooden slatted floor without litter, or better still on a concrete floor with straw or sawdust. Straw provides the animals with an opportunity to play, which keeps them distracted, and they will also eat it. According to the European guidelines the minimum floor area must be 0.3–0.8 m² per animal of 50 to 100 kg bodyweight, respectively. The best way to establish new groups of piglets is to bring the animals together simultaneously into a clean pen just prior to feeding or the dark period. The individual body odours can be camouflaged by eau de Cologne or a low concentration of chloric solution. If problems remain, then tranquilizers will have to be used. If it is necessary, pigs of up to 15, 20 or 40 kg can be housed individually in cages with a floor area of at least 0.35, 0.55, or 0.8 m² and a height of at least 50, 60, or 80 cm, respectively.

Pigs can be humanely restrained in a frame with a sling with holes cut out for the legs which then suspends them above the ground.

The recommended environmental temperature for adult pigs is between 17–24°C, with a relative humidity of 40–60%. Newborn piglets need an ambient temperature of 30–32°C. This can be achieved by positioning a heating lamp in one corner of the pen. The temperature can then be gradually reduced to 22°C over a 3 week period. Good ventilation is also necessary. The velocity of the air stream has to remain low; for adult animals lower than 0.3 m/sec., for newborn piglets lower than 0.1 m/sec.

**Food and drink.** Pigs are omnivores. Piglets, as well as adult pigs, can be fed with commercially available food, which can be adapted to suit the requirements of different ages. The feed can be supplied as pellets or meal. There must be sufficient feeding places per cage or pen, which, according to the European Guidelines, must be at least 20–30 cm per animal between 10–100 kg body weight. Feeding troughs should be constructed in such a way that they can be fixed to the wall or the floor, ensuring that no feed is wasted. Drinking water is provided *ad libitum* by means of automatic drinking nipples or troughs. Young animals frequently waste a good deal of water through playing with the nipple.
Reproduction. The physiological data concerning reproduction are shown in table 3-4. The first signs of a pig being on heat are seen on the 2nd and 3rd days of pro-oestrus and are characterized by restlessness, diminished food intake and by reddening and swelling of the vulva. A sow in oestrus reacts to pressure on the back or to the smell of a boar (for example from a spray) by showing the so called standing-reflex. At this point the sow will allow natural mating. However, it is more often the case that artificial insemination will be used. In general, the piglets will be delivered every 10–30 minutes. The placentae either come out during the delivery of the piglets, or 2–3 hours after the last piglet is born. Suckling of colostrum should begin within 45 minutes of birth and, after that, the newborns should drink every hour. A high environmental temperature is essential during the first days post-partum (see housing). Piglets can be fostered very easily during the first week of life. This may be necessary in order to equalize the number or to share out the maternal effects amongst the experimental groups. Between the 2nd and 5th day after birth, the piglets are injected with iron (150–200 mg/piglet, i.m.); this is to counteract an iron deficiency which may otherwise develop due to the low concentration of iron in the sow’s milk and the high growth rate of the piglets. After the 3rd week, piglets will start to eat pig meal or baby pellets, and can be weaned after the 4th or 5th week.

Handling and simple techniques. The method for handling pigs depends upon the age of the animals. Young piglets of up to 5 kg can be caught by a hind leg and then carried in both arms. Heavier animals need to be supported with one arm beneath the trunk and the belly, thus directing the head backwards and fixing it by the elbow against the body of the handler. Pigs can scream loudly and at a high frequency. It is therefore recommended to use ear defenders. Adult animals, in particular unfamiliar boars or sows with piglets, have to be treated with caution, as they can react aggressively. Pigs can be marked for identification with ear tags, by ear tattooing, or with microchips.

To prevent infectious diseases, pigs can be vaccinated, for example against Aujeszky’s disease, swine erysipelas, and atrophic rhinitis, and if necessary, they can be treated for ecto- and endoparasites. Pigs with an SPF status and housed in a containment room are generally not vaccinated.

Transport. Pigs are very sensitive to stress which can even be fatal during transportation. To help overcome this, gentle handling and a ventilated transport vehicle with a rough floor is recommended. If thought necessary the food can be withdrawn for a period of 12–24 hours prior to transportation. Very sensitive animals, such as the Pietrain breed, can be tranquillized using a beta-blocker.
Sheep and goats

Origin and use. The number of sheep (Ovis aries) and goats (Capra hircus) used in biomedical research is limited (less than 0.2% of the total number of vertebrates), except in New Zealand and Australia. These animals are used for a variety of purposes, such as blood donors for microbiological culture media, for the induction of antibodies, for obtaining surgical experience, and for various scientific research topics. Pregnant sheep are used for fetal and perinatal research.

Physiology and behaviour. Sheep and goats are ruminants. In addition to a true stomach (abomasum) they have three pro-ventriculi: the rumen, the reticulum and the omasum. The microbial fermentation of vegetable material takes place in the rumen. This process begins as soon as the animal changes from a milk diet to solid feed rich in fibre. The content of the rumen consist of three layers: a fluid mass fills the base above which is a structured porous layer consisting of just eaten or ruminated food, above which is a layer of gas (methane). Regular contractions of the rumen cause some mixing of the liquid and the structured layers. By a process of microbial fermentation, the crude cell materials are broken down; proteins are transformed into other proteins and certain vitamins are synthesised (mainly of the B-complex). Every 24 hours, approximately 15 ruminating periods will take place, which vary drastically in length from between 1–120 minutes. Altogether approximately 8–10 hours per day are spent in rumination. Sheep belong in herds, whereas goats are more ‘loners’. Although these animals are rather timid whilst in the field, they do get used to people who take regular care of them.

Housing. Sheep and goats can be housed outdoors as well as indoors. The choice made will depend upon the facilities available and the purpose for which they are to be used. For individual housing in pens, which should not be encouraged, the European guidelines prescribe the following minimum dimensions of a pen for sheep or goats: 1.4 m² or 1.6 m², respectively with a minimum length of 1.8 m for one of the sides. These dimensions are smaller than those suggested by the UK Home Office, which are 2.0 (< 35 kg) or 2.8 m² (> 35 kg). When animals have to be housed individually, they should be able to have contact with each other. If no other sheep are present, a mirror can help to relax the animal. For group housing (6–12 animals) the guidelines require 0.7 or 0.8 m² per sheep or goat, respectively (fig. 3-11). Special attention must be paid to (in)tolerance between the animals of a group and to the behaviour of horned animals. When planning the floor area it must always be taken into account that the animals should be able to lie down and get up easily. The floor should be covered with straw and sawdust, which should be
replaced every other day. The animals can also be housed on a metal grid floor or a ‘tender-foot’ floor with no ground cover, which reduces the labour required.

The recommended environmental temperature for sheep and goats is between 10 and 24°C with a relative humidity of 55% ± 10%. The ventilation capacity should be around 3 m$^3$/hour/kg body weight, in order to prevent the accumulation of rumen gas (methane) and ammonia.

**Food and drink.** Sheep and goats should be fed with a good quality hay, provided in a rack fixed to the wall or the front grill, with sheep pellets (with a low copper level) given as supplementary feed. Drinking water should be provided *ad libitum* from automatic drinking trays.

**Reproduction.** Sheep and goats are seasonally polyoestrous, with active oestrous cycles from September to December, with the lambs being born between February and June. Oestrus synchronization can be induced by administering two injections of synthetic prostaglandin with an interval of 10 days. Outside the breeding season, oestrus can be induced and synchronized using progesterone, followed by an injection of FSH or PMSG (pregnant mare serum gonadotrophin). Depending upon the breed, 1–2 offspring are born per litter, and breech presentations are quite frequent. Immediately after birth, the umbilical cord should be disinfected (e.g. with iodine) to prevent infection.
Newborns can walk unaided within one hour of being born and start looking immediately for the nipple and take in colostrum. Only weak animals will need external heat after birth. The fostering of lambs is not always successful. Ewes recognize their own lambs by smell, therefore it sometimes helps to transfer the smell of the new ewe or her own lambs onto the lamb to be fostered. Bottle feeding is possible, but it must be taken into account that sheep milk contains 1.5 times more dry matter than cow milk (220–250 g milk powder for calves per litre water at 40°C). After 2½–3 weeks lambs start to eat solid food.

Handling and simple techniques. Sheep and goats which are being used in biomedical research should be socialised to people early on. When sheep and goats are to be housed under experimental conditions, they will need a period of at least 3 weeks to adapt. Young lambs can be caught by their hind legs and carried in the arms of the handler. In order to secure an adult sheep firmly it should be put in a sitting posture or laid on its back. This can be achieved by bending the head sidewards and then by pushing them in the side. Due to the production of gas in the rumen they should be held in this position only for a few minutes. The male sheep or goats must be treated carefully.

The tails of lambs are often docked (not necessary if kept indoors and monitored daily) and male lambs are generally castrated (not necessary if going to be kept for a short time, or it is out of the breeding season). The animals can be marked by tattooing the ears, or by fixing an ear tag soon after birth, or by microchipping.

To prevent infectious diseases the animals can be vaccinated (for example against Clostridium infection, foot-rot) and treated for ecto- and endoparasites, such as gastro-intestinal helminths and lungworms. In the case of the hooves becoming too long, or horn-edges curled or crumbly, these should be clipped. Sheep housed outdoors need to be shorn once a year, whilst those housed indoors should be shorn more often.

Birds

Introduction

Approximately 8–10% of the vertebrates used annually, are birds. The chicken (Gallus domesticus) is the most widely used whereas the pigeon (Columba livia), the dove (Streptopelia risoria) and Japanese quail (Coturnix coturnix japonica) are also commonly used.

Physiology and anatomy. Birds have specific physiological and anatomical characteristics when compared with mammals. For example, their skin is
devoid of sweat and sebaceous glands. In order to facilitate preening, birds possess either powdery, downy feathers and/or two preening glands. Birds have no diaphragm; the lungs are connected to the rib cage and they possess a system of air sacs which are essential structures in the process of respiration.

The abdominal cavity is rather small and includes the caudal part of the gizzard, the intestine, the spleen, and either the ovaries with the oviduct, or the testicles with the ducti deferentes. Birds have no urinary bladder; urine is transported via the ureters, which empty into the cloaca.

Erythrocytes of birds are oval and nucleated. Platelets are absent and ‘replaced’ by nucleated thrombocytes. In birds the female is heterogametic (2 different sex chromosomes (ZW)), whereas the male is the homogametic sex (ZZ).

Compared with mammals, birds have a high metabolic rate. This means that a continual intake of food of a high quality must be guaranteed in order for their energetic and nutritional requirements to be met. The absorption, metabolism and elimination of nutritional elements and waste are performed at a high rate. These aspects need to be considered when using birds as experimental animals.

The chicken, the pigeon and the Japanese quail are herbivorous (grain-vorous). Food is mixed with the saliva in the mouth, which contains amylase, and then it is passed down to the crop where it is predigested. Further digestion takes place in the proventriculus, where it is mixed with gastric juice, which contains pepsin and hydrochloric acid. Grinding of seeds and food takes place in the gizzard. Nutrients are subsequently mainly digested and absorbed in the relatively short small intestine, and a certain amount of cellulose will be fermented in the two caeca by the intestinal microflora.

**Chickens**

*Origin and use.* The various breeds of the chickens now in existence stem from the red jungle-chicken (*Gallus gallus*), which was domesticated in Burma many centuries before Christ. Centuries of careful selection has led to two basic types of chickens: the light breeds which are used for egg production (the layers) and the heavy breeds which are used for meat (the broilers).

Of particular use in laboratory experiments are the embryos which are used in areas such as virology, embryology and toxicology. When conducting a series of experiments it is important to use eggs which have the same origin, preferably from SPF stock. This is to avoid variations in response caused by the different strains or infections. Chickens are mainly used for research into feeding, vaccination/immunology and toxicology.
Housing. Chickens are frequently kept in wire cages, usually stacked in two or more levels. This, however, must be considered as a poor housing system in which the behavioural needs cannot be exercised. It is possible to house them individually, but also in pairs or groups. Chickens, when grouped together, are probably better able to meet their behavioural needs but may show a marked social hierarchy (pecking order), which can lead to the violent pluck-
Chickens

ing of each others feathers, and even cannibalism. There are minimum mea-
urements for cages which are laid down as European guidelines. There are 5
iations, depending upon the weight of the animals and the number of ani-
mals per cage (3–5). The wiremesh floors should have a maximum mesh
ith width of 10 mm when they are to be used for chicks and 25 mm width when
structed for young and adult animals. The thickness of the wire must be at
least 2 mm in every case. The floors must be well supported and should be
ngthened by 6 mm rods. Laying hens should be provided with a tilted
ire mesh floor with maximum 14% tilt. When carrying out experiments of a
atively long duration involving young chicks, the fast rate of growth of the
imals must be taken into account. This is necessary to minimize or exclude
allocation and regrouping of the animals during the experiment.

The amount of ventilation which is required depends upon the number of
imals, their size and the environmental temperature. The standard is 7 m³ of
fresh air/hour/kg bodyweight, which needs to be of a low velocity. The opti-
um temperature for chicks which are between 1–3 days old is 35°C which
should be provided by one or more heating lamps. After this age temperatures
be lowered gradually over the next 4 days at a rate of 0.5°C per day, and
thereafter at 1°C per day, until the lower temperature of 18–21°C is reached.

Food and drink. Food must be available immediately as the chicks hatch out,
and needs to be supplied on flat dishes. The older animals can have their food in
eeding troughs rationed according to the size of the chicks. Generally com-
ercial feed is given, and the presence of grit is essential in order to optimize
the grinding of the food in the gizzard. Drinking water is usually provided via
an automatic system with nipples, which require frequent checking.

Reproduction. After copulation, several eggs will be fertilized, and therefore
there is no need for copulation to occur daily. The eggs may be collected 2–4
times a day and, after disinfection, are incubated at 37°C with a relative hu-
midity of 60%. During the first 18 days the eggs should be turned 3–5 times
per day.

Handling and techniques. Young chicks should be picked up in one hand
with the thumb and index finger placed gently around the neck. When remov-
ing a fully grown hen from its cage, both hands will be necessary, with the
ingers spread widely and the thumbs placed dorsally over the wings. These
eed to be positioned around the body of the hen in such a way that the bird’s
head is facing the handler. The hen can then be held under the handler’s arm,
facing backwards, whilst the handler fixes the legs with one hand. Restraint is
possible by taking the wings together on its back. Thereafter the hen can be
placed on its side.
Determining the sex of chicks is the work of a specialist. When they reach the age of 4–6 weeks hens and cocks can be differentiated by their external features. The cocks develop a prominent comb, their legs are thicker and they possess spurs. Hens can be marked for identification with (multi-colored) rings on their legs or with numbers clipped to their wings or by microchipping.

**Pigeons and doves**

*Origin and use.* There are approximately 650 species of pigeons. Over the centuries more than 800 breeds have developed. Pigeons (*Columba livia livia*) and doves (*Streptopelia risoria*) account for less than 0.1% of the total of experimental animals. They are used for research in the field of physiology, behaviour, pharmacokinetics and toxicology. The White Carneau, which is mainly used for its meat, is also used for research concerning atherosclerosis.

*Housing.* Pigeons are best kept in large flocks. Given sufficient space and sufficient nest boxes or sitting places, rivalry will hardly ever occur in the indoor enclosures, even when one sex may predominate. This can, however, not be applied across the board for pigeons and doves, for example with turtle doves persistent quarrels will occur when the number of males exceeds that of females. Doves which are to be used as experimental animals can be accommodated individually in cages which are 40 × 40 × 40 cm, the front being made from metal bars, with provisions made for containers for feed and water. The floor can be made from metal bars which need to be 3 mm in thickness and 2 cm apart to permit faeces to pass through onto sand or paper. Hygiene is very important. Faecal droppings should be removed daily. The racing pigeon can survive extremely low temperatures, but housing should provide an adequate shelter against rain and wind. The thermo-neutral zone is between 20 and 25°C. The day/night cycle can be 12/12 h, but 14 h light can improve the reproduction.

*Food and drink.* Food for pigeons is commercially available as is food for turtle doves, although they require specialized feed. Drinking water is provided *ad libitum.* The containers for feed and water should preferably be situated outside the cages with access from the inside, in order to prevent contamination by faeces. Containers for water should be cleaned on a daily basis. Pigeons whose regime is undisturbed by human activity and kept under a light/dark schedule of 14:10 hours, have their maximum intake of food between 17.00 and 20.00 hours. The effect on blood values of substances administered via the food or the drinking water will be markedly influenced by this physiological aspect.
Reproduction. The breeding of pigeons is not difficult. Pigeons will breed all year round when kept indoors under appropriate light conditions. The females are monogamous as a rule and only break this if their own partner is unavailable. Egg production is initiated by copulation. In a normal and complete reproductive cycle, the female produces clutches of 2 eggs every 34–40 days. Veins from tobacco leaves can be used as nesting material which helps to reduce feather lice and is available from pet shops. Both parents are active in caring for and raising the squabs. They both produce crop milk which is the only food given to the young during the first 5–7 days of life. Young squabs grow very quickly and within 48 hours their birth weight has doubled, and by the 28th day the squabs are virtually fully grown. Artificial incubation of eggs is possible, but there are still problems encountered when trying to handrear the squabs. They must be kept at a temperature of about 32°C and frequently fed with a high protein and fat diet.

Handling and simple techniques. The daily handling of pigeons and doves should be performed in a peaceful way. In the dark they can easily be picked up when dazzled with a light beam. Pigeons can either be held in one or both hands. When held in one hand, they should be placed in the palm of the hand, with their feet held between the middle and 4th finger, the thumb being free to hold the tail and the tips of the wings (fig. 3-12).
There is no sexual dimorphism in pigeons. Determining the sex of doves and pigeons requires a great deal of experience. In the male squab the eyes are generally further apart than in the female, and the head is flatter. Adult male pigeons in general have a heavier build than the females and the head is larger. If there is an element of doubt, then endoscopy can be performed to determine the sexual anatomy. Marking pigeons for identification can be done with microchips or by fixing a ring permanently around the leg. This ring should be put on when the pigeons are only a few days old, and the size is determined by the species. Temporary marking is possible using rings which are simply bent around one of the legs (fig. 3-13).

**Quails**

*Origin and use.* Approximately 600 years ago the quail (*Coturnix coturnix*) was domesticated in Japan. Since about 1950, the quail has been in use as an experimental animal, mainly due to its rapid reproduction of up to five generations per year, and its high output of eggs which can number 80–90 in 100 days. Quails are used in embryological, pharmacological and toxicological research.

*Housing.* Quail can be housed in large groups, in cages with sawdust. A disadvantage with this type of husbandry is that quail will not make a nest and, as a result, the eggs will be spread over the cage floor. It is better, therefore, to house quails in small groups in separate cages. According to the European guidelines, adult quail should have a minimum area of 200 cm² per animal, if they are kept in groups of three or more. In small breeding groups, for instance one male and 4 females, the females may be aggressive towards the male. Individual housing is possible in small closed cages or wire mesh cages with a minimum floor measurement of 850 cm² and a minimum height of 15 cm, but this type of housing is poor and does not meet their behavioural needs.

The minimum environmental temperature depends upon the age of the animals. Day-old chicks should be kept at 35–37°C, with localised heating provided by an electric heating bulb. During the next few weeks, the temperature should be lowered gradually by 4–5°C per week, until a temperature of 19–23°C is reached. A good ventilation system is necessary, both for the handlers and for the animals, as quails produce a penetrating smell. A prescribed ventilation rate of 1.7 m³/hour/animal in growing animals is recommended and 30–35 m³/hour/animal in the case of adult quails. The relative humidity does not appear greatly to influence quail. The day/night rhythm is important within breeding units, the norm being 14 hours of light.

*Food and drink.* Due to the high growth rate and their egg production, quail
require a food rich in protein and energy; e.g. turkey starter-crumbs having a crude protein content of 25–28% and 2 kcal/ g. Food and water can be provided in containers used for chicks.

Reproduction. Egg production starts at an age of 40–45 days. This diminishes after the quail reach 26 weeks. In total they may lay up to 300 eggs per year. Eggs are primarily laid during the last 6 hours of the light period. In order to guarantee that eggs are fertilized, 20 males and 40–50 females should be grouped and permanently housed together. For effective fertilization one male is necessary for every 2–3 females. Under these circumstances a fertilization rate of 80–90% can be expected. The best results are obtained using animals between 8–20 weeks old. The eggs will hatch after an (artificial) incubation period of 16 days.

Handling and simple techniques. Quail tend to be nervous and need therefore to be approached quietly. The young chicks are very tiny and should be caught between the thumb and index finger. Fully grown quail need to be taken in one hand with the thumb on the breast, the index and middle finger over the wings, and the legs held between the ring and the little finger.

Sexing of young quail on the basis of anatomical differences in the cloaca.
requires a great deal of experience. Between the ages of 2–3 weeks, animals can be sexed by the colour of the breast feathers: in males these feathers are brown, whilst in the female they are grey with black spots.

Eggs and young quails are very vulnerable and should not be handled or transported if possible. If transportation is necessary then it should preferably be done when the quail is at least 4 weeks old. They are transported in cardboard boxes with a layer of sawdust. Marking quails for identification can be done as described for pigeons.

**Poikilothermic vertebrates**

**Reptiles**

*Use.* The use of reptiles, such as chelonians (turtles), snakes and lizards, in research is very limited. Main areas of research for which they are used are in zoology, comparative virology, immunology and endocrinology. Only some general features of reptiles will be discussed here.

*Anatomy and physiology.* Reptiles are unable to regulate their body temperature by endothermic processes. The body temperature and related physiological processes depend on the environmental temperature and the presence of heating devices. The skin is thick and cornified and, in many reptiles (the squamata), provided with scales. The horny layer prevents dehydration to a large extent. The skin is, with a few exceptions, devoid of glands, which further minimizes evaporation and the loss of body fluids.

*Housing and feeding.* Their anatomical and physiological characteristics imply that the reptiles’ environment must be constructed in such a way that they can maintain their normal physiology. In a vivarium, reptiles must be given the opportunity of varying their distance from the heating device, thereby maintaining their own physiologically preferred body temperature (see Guidelines for the keeping of reptiles).

Male lizards should be housed outside visual contact of each other, otherwise their instinct to defend their territory will keep them under constant stress. This also leads to dysregulation of body temperature and loss of appetite.

Many reptiles catch their prey and are very selective and adapted for this task. Some snakes will only eat (live) vertebrates, others can be fed with freshly killed mice and/or rats; other species will only eat birds, whilst others will eat insects, snails or fish. For omnivorous and insectivorous reptiles it is essential to add a multivitamin and mineral mixture to the fruit, vegeta-
bles or insects. For meat-eating reptiles, such as terrapins, the meat should have added calcium and vitamin A. Young animals should be provided with plenty of food.

Reproduction, sexing and identification. Reptiles kept in captivity may breed if exposed to seasonal fluctuations, with regard to day-length either by natural or artificial means. The majority of reptiles are oviparous. Under laboratory conditions the eggs are incubated artificially. A few species are ovoviviparous and in these species the young develop and hatch within the female’s oviducts.

Sexing of reptiles requires experience. Chelonian males have a relatively large tail, both with regard to width and length. The opening of the cloaca is
situated near the tip of the tail. With male lizards and snakes, the two hemi-
penises are retracted they form two pouches in the base of the tail, which can
be examined by inserting a probe.

Marking of chelonians for identification purposes can be achieved by
painting, or by putting a tab through a hole in the carapace or by making
cuts in the non-living edge of the carapace. Snakes and lizards can be marked
by branding them with an appropriately shaped piece of metal, cooled in
liquid nitrogen.

**Amphibians**

*Use.* The African clawed toad (*Xenopus laevis*) and the axolotl or Mexican
salamander (*Ambystoma mexicanum*) are the most generally used amphib-
ians for experimental purposes. Both species lay fairly large fertilized eggs
into water, which makes them of particular use in research concerning orga-
nogenesis and molecular genetics.

*Physiology.* Amphibians are not able to maintain an elevated body tempera-
ture via the endogenic production of energy. The skin is moist and contains
many secretion glands. Amphibians have no firm cornified epidermis, and
are therefore even more dependent upon and sensitive to environmental fac-
tors than reptiles. Water can pass through the skin in both directions. The
aquatic species, such as the clawed toad and the axolotl are particularly sus-
ceptible to dehydration. Respiration in amphibians is either via (external)
gills at the larval stage or via lungs at the adult stage after metamorphosis has
taken place. In both cases intake of oxygen across the skin is an additional
route. In the axolotl, which is kept in vivaria in its larval stage, the external
gills are a permanent feature. Larvae of the clawed toad metamorphosize at the
age of 4 months. From that time onwards they depend upon the lungs for the
exchange of gas. When metamorphosized a clawed toad will drown if respira-
tion is blocked, because the intake of oxygen via the skin will be insufficient.

Axolotls are fully grown at around 18 months, and they may live to reach an
age of 15 years. There are three known varieties according to colour: the colour
which occurs in the wild which is browny-blackish, the white coloured form
which has black eyes, and the albino variety which is yellowish with red eyes.

The clawed toad is fully grown at 12 months and they may live to reach a
maximum age of 25 years. There are two known colour varieties: the colour
which occurs in the wild which is brown-grey with black areas, and the albino
which is white with red eyes, which also has three black claws on its hind legs.
The lateral line system of clawed toads is visible with the naked eye and is seen
as a symmetrical pattern of elongated greyish dots, each 2–3 mm in length. In
both these species di-, tri- and tetraploid animals are in existence.
Housing and feeding. Both of the species which have been discussed are strictly aquatic. They need to be kept in clean water, free from chlorine and copper, preferably in containers attached to running tap water to prevent the accumulation of waste products. Water depth should be approximately 30 cm, the pH value between 7.5 and 8.5, and the temperature between 18–20°C. Variations in the ambient temperature in excess of 5°C per 24 h may harm the animals. Exposure of animals to direct sunlight should be avoided. Some shelter should be provided in the form of Elodea canadensis or a flower pot. When using an artificial source of light for a ratio of day to night of 12:12 hours, fluorescent tubes colour 33 (Philips), 40 W are appropriate.

Animals should be fed ad libitum, 3 times per week, with slices of meat into which a multivitamin and mineral mixture should have been intensively rubbed; it is not sufficient to merely powder the mixture over the meat. It is essential to leave the food in the container for one and a half hours, in order to ensure that each animal has had sufficient time to eat; any remnants remaining should be removed. Animals which are housed individually can be fed using a blunt pair of forceps. Overfeeding will lead to vomiting.

Reproduction. For reproduction purposes animals aged between 2–5 years are used. The reproduction period of the axolotl is restricted to between December and July. One week prior to mating the water temperature should be kept at 22°C. The female will produce pheromones which stimulates the male into depositing spermatophores. The female positions herself over the

Fig. 3-15. Handling and restraining of a Xenopus.
cone-shaped spermatophores and takes them into her cloaca. When this has taken place the water temperature should be lowered to 12°C and oviposition will take place within 12 hours. If plastic threads are put into water the eggs will deposited on them, or otherwise the eggs are deposited on the floor of the vivarium. Eggs should be kept isolated in subdued lighting in order to create a restful environment. Females can be used every three months for reproduction.

Female clawed toads are about twice the size of the males. Males can be identified by the copulatory pads on their thumbs, which are a blackish colour, and are found on the inner side of the fore feet.

To induce oviposition, the males are injected with gonadotrophin; 24 hours later the females are given the same injection. The temperature of the water at that time should be 10°C. Eight hours after the hormonal treatment of the females, the water temperature should be gradually increased over an 8 hour period to 23°C. Prior to mating, the lips of the female’s cloaca will become noticeable swollen. Amplexus then occurs, during which the male embraces the female around the loins with his forelegs. The female will then deposit eggs which can then be (artificially) fertilized. If the eggs should be harvested directly, the female can be ‘stripped’ manually.

Handling and identification. Axolotls and clawed toads should be lifted out of the water by hand, as the use of a net can damage them, in particular the axolotls. To hold the clawed toad securely it should be grasped around the hind
legs. The handler’s fingers should be placed at the top of the legs, near the body, while the body section should rest in the palm of the hand (fig. 3-15). Axolotls should be approached from the front with the thumb and index finger of one hand placed around the body, just behind the gills, being careful not to damage them. The thumb and index finger of the second hand should be positioned just cranially to the hind legs with the tail in the palm of the second hand (fig. 3-16).

Marking of clawed toads for identification purposes can be performed by means of an autotransplant (under MS 222 anaesthesia), in which a piece of the lighter coloured abdominal wall is implanted into the animals back, or a piece of the darker coloured skin is implanted onto the abdomen (fig 3-17).

Fish

Use. Approximately 4–8% of experimental vertebrates are fish (cf. fig. 1-1). They have particular use in (environmental) toxicological research such as drinking water control. The main species which are used are the guppy (*Poecilia reticulata*), the zebra fish (*Brachydanio rerio*), the trout (*Salmo trutta*), and members of the carp family.

Physiology. Fish are poikilotherms. This means that the body temperature and all physiological functions depend upon the temperature of the water. For example, both the body temperature and the oxygen consumption will diminish
directly in relation to a lowering of the water temperature. Temperature stress, 
educed by rapid changes in the water temperature, will result in a rise of 
oxygen consumption. When the water temperature stabilizes after such a 
change, the fish will need approximately one week to adjust their oxygen 
consumption to the normal level for that specific temperature. Lifting a fish 
out of the water or transporting the fish will result in stress, which may lead 
to behavioural disturbances. The time required for restoration of normal 
behaviour will vary, depending upon the severity of the stress. Recovery from 
severe stress, such as being imported from tropical areas, may require an 8– 
12 week recovery period. Extreme temperatures may also lead to stress which 
will lower the animal’s resistance to infection.

The skin of fish is covered with a mucous layer. Damage to this layer, by 
drying up, rough handling, or by placing the fish on a dry, instead of a wet, 
piece of cloth or paper tissue, will destroy its first defensive barrier and may 
facilitate local or even systemic infections.

The growth and life span of a fish depends on the species, but is also re-
lated to the circumstances under which the animals are kept. The goldfish 
(*Carassius auratus*) can reach 8 cm in length within 9 months, given a water 
temperature of 22°C. There can, however, be marked variations in growth 
within one clutch of eggs of externally identical parents. In outdoor ponds, a 
length of 8 cm can be reached after the fishes’ second summer, at an age of 
12–15 months. The goldfish has a maximum life span of 15 years; by contrast 
the zebrafish has a life span of approximately 4 years.

**Housing.** For most freshwater fish originating from moderate climatic zones, 
optimal temperatures range from 8–16°C. Variations in temperature should 
not exceed 5°C at a speed of more than 1°C per hour. The pH of the water 
should be between 6.5 and 8.5. When tap water is used, it should be free from 
chlorine and copper. In general, the best way to keep fish is in a system with 
flowing water. The temperature-controlled water should be put into the tank 
in the form of a shower, with a flow length of 20–30 cm. This is to ensure that 
there is an exchange of gases which were under pressure in the pipeline sys-
tem, and to ensure a minimum O₂ content of 7–10 ppm. The outlet should be 
situated at water level. A separator, usually made of perforated aluminium, 
will prevent the escape of fish via the drain. Both the aquarium and the filter-
ing system, should be installed at least 2 weeks prior to the actual start of the 
experiment. This is particularly important with biological filtering systems, 
to ensure an optimal detoxification and denitrification of the tank water. The 
number of fish kept per volume of water will vary according to the species. In 
a closed system, with a biological filter the general rule is 1 gram of fish per 3 
litres of water. In general, 12–16 hours of daylight and/or artificial illumina-
tion should be given.
Food. Commercial fish food is available for carp, trout, catfish, and eel. Such food can also be used for related fish species. In general, fish should be fed once a day, but the amount of food has to be adapted according to the circumstances. A general rule is to provide the amount of food which can be eaten within 15 minutes. An excess of unused food will lead to putrefaction, to the production of toxic substances such as ammonia and nitrite, to a shortage in oxygen, and to changes in the pH of the water. Such changes may lead to the death of the fish. Consequently any remnants of food together with faeces should be removed.

Reproduction. There are wide variations in the reproductive system of fish; the majority are oviparous, some, such as the guppy, are viviparous. The methods of depositing and fertilizing the eggs are species specific. Some species, such as the eel cannot be bred in captivity, whereas the guppy (*Poecilia reticulata*) is bred with relative ease. Female guppies are placed in a “birth receptacle”, which should be at least 40 cm in length. This birth receptacle is constructed in such a way that the young can escape and will not therefore be eaten by the mother. An optimal temperature for young guppies is between 25–28°C, at a pH of 7. The largest females produce the largest number of offspring. The average period between births is approximately 31 days.

Handling and identification. For movement of a single fish to another aquarium, the fish should be lifted carefully out of the water by hand or caught with a net. When fish are taken out of the water, they should be placed on a moist paper or cloth surface, in order to prevent damage to the mucous layer covering the skin.

Determination of sex can be very difficult depending on species. In the case of the guppy, however, males are smaller than females, their dorsal fin is elongated caudally and their abdominal fin is almost as long as the copulatory organ (gonopodium). The female has a rounded or an only slightly elongated dorsal fin.

For the temporary identification of fish a small piece of a fin can be clipped away.

Literature

Biology and husbandry of laboratory animals


4 Behaviour, stress and well-being

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Introduction

During the 20th century, the nature of the relationship between human beings and animals has changed considerably. In mankind’s early history, cattle were used to provide food, clothes and labour. By a process of gradual domestication, wild animals have been turned into domestic animals and later into pet animals as well. This process has taken many centuries to develop. The first animals used for experimental purposes were, as a rule, from the domesticated groups i.e. pigs, dogs, cats, chickens, rabbits, etc. Industrialization in the second half of the 19th century caused an important change in the traditional relationship between animals and man. With the commencement of intensive livestock farming and the breeding of animals specifically for experimental purposes, both housing conditions and care for the animals changed rapidly. In particular during recent decades, changes in size, structure and the interior of housing systems along with density and group composition are such that animals frequently have problems to adapt to these conditions. Modern housing systems for farm and experimental animals often give rise to abnormalities in behaviour and physiology, mainly due to the fact that these conditions do not appear to fulfil the minimal environmental requirements.

It is man’s moral obligation to keep animals in a state of optimal well-being. Also, from a practical standpoint, it is essential to avoid causing serious suffering to animals during experimentation, as this may give rise to deviations in experimental results.

With this in mind, the EC has formulated the “Directive for the protection of vertebrate animals used for experimental and other scientific purposes” (see chapter 2). One of the fundamental paragraphs within the Directive states that all experimental animals must be provided with suitable housing, environment, adequate freedom of movement, food, water and care appropriate to their health and well-being. Any constraints on the physiological needs of the
animal or ethological needs should be kept to the absolute minimum (art. 5). This paragraph has wide consequences on a practical level, and demands a detailed and thorough knowledge of the biology of the various animal species being used. As seen in chapter 3, many uncertainties along with a lack of sufficient knowledge, do still exist.

What then are the environmental desiderata, which of them are essential, and to what extent are behaviour, physiology, and well-being affected when animals are deprived of some of their needs? This chapter will discuss in general terms the behavioural and physiological mechanisms which allow mammals to adapt to environmental challenges. Attention will also be given to the constraints within these adaptation mechanisms, to the development of stress-pathology, and to the consequences for the animals’ well-being.

**Basic mechanisms**

*Homeostasis*

A useful concept when discussing stress, adaptation and well-being in animals is “homeostasis”. This implies that aspects of both the internal milieu (such as body temperature, blood glucose, water content of the body, etc.) and those of the environment (such as positioning within a social group etc.) will be kept at a constant or at least a predictable level for a certain period of time. An animal can only maintain homeostasis when it can compare its actual situation with the norm for certain internal or external factors, and when it has the behavioural and physiological means to realize the norm. Fig. 4-1 depicts the most basic form of such a homeostatic regulatory mechanism.

Environmental factors may challenge the biological balance. So long as the animal is able to adapt to these changes without any problems, it is said to

![Fig. 4-1. Principle of a homeostatic mechanism. The actual state of the internal milieu is detected by a receptor mechanism (R) and compared with an internal standard. A difference between the observed situation and the expectation will activate mechanisms (F), which will normalize the situation.](image-url)
be in homeostasis or in harmony. However, when an animal is unable to maintain homeostasis, stress will develop in the course of time. This can manifest itself as abnormal behaviour or disease. Clearly, in such a situation the animal will suffer or its well-being will be reduced. Accordingly, the lawyer Albert Lorz in his comments to the German Tierschutzgesetz (Animal Protection Act) defined well-being as ‘living in physical and psychological harmony with the environment’ a definition which is not far from a state of homeostasis. Later in this chapter we will discuss the issue of animal well-being in greater detail.

Two quite distinctive mechanisms are involved when considering the capacity of animals to adapt to the environmental challenges in everyday life within a natural habitat. Firstly, in the course of evolution the animals have been selected from amongst a population of genetically different organisms, because they can cope the best with the environmental demands morphologically, physiologically, and behaviourally. This type of selection forms the basis of speciation (phylogeny). The second mechanism is selection by the individual itself, i.e. the selection from a variety of potential behavioural solutions to the demands of the environment. This mechanism (which is learning) is especially important during the development of the individual (ontogeny) but also in the adult. These two selection mechanisms lead to behaviour in animals which is adapted to the environment.

**Phylogeny**

Many studies have shown that behaviour has a firm genetic basis. This is due to the fact that, in the course of evolution, the more poorly genetically adapted individuals have been eliminated. This form of selection is the basis of species-specific behaviour, as well as the species-specific morphology and physiology of animals, including that of present day experimental animals. In their original, natural habitat, animals are characterized according to the adequacy of their morphology, behaviour, and physiology. The regulatory range of homeostatic mechanisms is therefore geared up to and restricted to the environmental variation which the animal might meet in its everyday life. Therefore, the adequacy of homeostatic mechanisms may fail, should changes in the environment suddenly require completely different characteristics of these mechanisms. For example, when a wild animal is kept in a cage which radically differs from its natural habitat, it may repeatedly exhibit maladaptive behaviour. By so doing it will be indicating the feelings of constraint due to obstruction of the inherited adaptive capacity.

During the process of domestication, experimental animals have acquired new specific characteristics. This does not mean, however, that species-specific behaviour has disappeared; generally speaking only minor aspects will
change. Behaviour which is essential for survival (i.e. feeding, nest building and social behaviour such as offensive, defensive, sexual, and parental behaviour) is strongly genetically determined. This will be present in the offspring even when selection is not in its favour. These species-specific behaviours cannot, or at least not without great difficulty, be changed by experience, and they will be performed even though the environment does not allow for their full expression (fig. 4-2).

Selection by the environment is an important factor in the phylogeny of adaptive behaviour. Animals are, however, not fully dependent upon genetically programmed behaviour. Individual animals can, within their genetic limits change their behavioural repertoire by learning during both ontogeny and adulthood.

Learning

The phenotype of an individual is the result of a complex interaction between the genotype and a specific environmental problem. One important, and therefore an intensively studied aspect of the phenotype, is the ability of learning based upon genotype and specific experiences. Learning is a mechanism which
Basic mechanisms allows both flexible and rapid behavioural adaptation to short-term changes in the environment. There are, however, considerable species-specific constraints with regard to learning abilities. One of these constraints involves the stimuli which any given animal is able to perceive. Behavioural studies have shown that certain stimuli will elicit species-specific behaviour even in the absence of any previous experience. These stimuli, known as sign stimuli, represent environmental aspects which have species-specific significance to the animal. Given certain internal conditions, this significance will not change and will have a fixed relationship to certain behaviours. A classic example is the red belly of a male stickleback during the reproductive season. This red belly acts as a sign stimulus in eliciting aggressive behaviour from other male sticklebacks.

The remaining stimuli can be associated with behaviour via learning processes, and as such may gain certain significance or may change in significance (Pavlovian stimulus conditioning). The first response of an animal to an unknown stimulus (for example a noise of certain intensity) is arousal and — in many cases — an orientation response. If the stimulus has no consequences (either positive or negative) to the individual, it will no longer respond after repeated presentations. This process of habituation thus prevents the organism from reacting to irrelevant stimuli. If, however, the stimulus is associated with positive or negative consequences, learning takes place, in this case stimulus conditioning. Both habituation and conditioning, however, may disappear. When a stimulus to which the animal has become habituated is absent for a period of time, then the original orientation response will emerge again. Similarly, when a conditioned stimulus frequently occurs in the absence of the associated unconditioned stimulus, the reaction to this stimulus will also disappear. The organism will have learned that the stimulus is of no further significance. However, sign stimuli will neither totally habituate nor will the response to these stimuli disappear totally. If either does occur, it will only be for a very short period of time.

An organism can also learn that behaviour has pleasant or unpleasant consequences. This conditioning of behaviour or operant conditioning can lead to an increase or decrease in frequency of performance. For example, rats generally prefer the dark. When put into the illuminated side of a two-chambered box, they will usually move to the dark side immediately. If this response is followed by a punishment however (e.g. a weak electric shock), then the next time they are placed into the light side, they will stay there, i.e. they have learned to avoid the “unpleasant” situation.

The same mechanisms which are involved in stimulus conditioning are also involved in the conditioning of behaviour. These learning processes can be thought of as mechanisms involved in the capacity of animals to distinguish causal relationships within their environment, i.e. to distinguish information from noise.
Usually both stimulus and operant conditioning will occur simultaneously, and will take place with respect to both pleasant and aversive situations.

There are large variations between species with regard to learning processes, not only in the degree to which learning is important in behavioural adaptation, but also to the types of behaviour that can be changed through learning. It is not possible to associate every stimulus with all subsequent events in the same way that not every type of behaviour can be used to obtain the desired effect. With rats, for example, it is almost impossible to associate light or sound stimuli with a subsequent illness induced by poisoned water, but an association can very easily be made between the taste of the water and the illness. It is virtually impossible to teach hamsters to obtain food by using grooming behaviour, whereas this can easily be taught using other operants such as digging or exploring. When certain housing systems require a learning process, for example to obtain food or water, it has to be realized that the particular combination of stimulus and operant is one that can easily be learned by the animals. Many chickens kept in battery cages have died due to their inability to learn that to obtain water they had to peck at the water nipple.

Knowledge of the environment, in combination with the available behavioural and physiological tools, allows the animal to maintain homeostasis. While this knowledge is based upon learning processes, there are considerable constraints in learning. Species have specific programmes when obtaining food and water, but also with regard to reproduction and social contacts etc. Many of these programmes have within them a certain degree of rigidity and cannot be changed through learning, i.e., certain components cannot be omitted without there being consequences for the animal. For example, in young mammals, food has to be obtained by sucking. Similarly, feeding has to be accompanied by rooting in pigs, or by digging and scratching in chickens or by gnawing in rodents. When these behaviours become redundant due to the supply, for example, of ready made food, they may be redirected to other objects or individuals, manifesting itself in feather pecking in chickens, or mutual sucking in calves. A good housing system should therefore suit the species-specific behaviour programmes of the animals.

**Ontogeny**

Ontogeny makes a large contribution with regard to adaptation capacities of the organism during adulthood, within its genetic limits of course. Both prenatal and postnatal factors have considerable influence on both the behavioural and the physiological characteristics of the adult offspring. In mammals the internal milieu of the mother will affect the development of the offspring from the moment of conception. For example, offspring from
mothers which experienced stress during pregnancy or nursing will show different social behaviour in adulthood. Not only is the relationship with the mother important, but also experiences with conspecific animals will be important in the development of adult social behaviour. The complexity and variability of the environment will also largely affect the development of exploratory behaviour and learning capacity. These are two important aspects of behaviour that determine the adult capacity to adapt to environmental challenges.

The periods in ontogeny during which certain behaviours can be affected by environmental factors are more or less limited. A clear example of the significance of such sensitive periods in life is imprinting. This is the learning and long-term consolidation of the significance of stimuli (conspecific animals, food), and the orientation of behaviour towards the stimulus. Imprinting is an important phenomenon in many birds and mammals in which the young are fully developed after hatching or birth and can follow their mother from the beginning. In such precocial or nidifugous species, the young are imprinted to the mother. In many precocial species of mammals, the mother becomes attached to her young during a very short sensitive period immediately after giving birth. Pet animals which have contacts both with conspecific animals as well as with human beings during a critical period of life, may develop a double bond to their own species as well as to human beings.

The period during which behaviour patterns develop is generally limited. Modulating behaviour beyond the sensitive period is difficult and sometimes even impossible. For example, male rhesus monkeys which had no playful mounting experiences with conspecifics in their first year of life will have serious difficulties in performing normal sexual behaviour in adult life. The species-specific performance of agonistic behaviour in these monkeys also appears to depend upon the playful, non-violent type of aggression, performed in the early years. Deprivation of conspecifics, or insufficient space for normal social relationships, may result in abnormal behaviour patterns which may seriously disrupt social groups.

An important question for animal research is how to house experimental animals, taking not only the requirements of the experiment into account, but also the environmental conditions which are necessary for the animal itself with regard to its species, and specifically individual habits. Experiments on deprivation show that animals raised under conditions which lack certain complex stimuli, will have serious problems when coping with more complex (social) situations as an adult. It seems that conditions of rearing must fulfil certain species and strain-specific requirements. Only under these conditions will animals develop the full capacity to cope with environmental challenges.
Interaction between the animal and its environment

The relationship of animals with regard to aspects of their environment is such that certain behaviour is strongly linked to certain specific locations. For example, Calhoun in a study of wild rats describes that the incidence of aggression is largely area-specific. He observed a male rat transporting a piece of cake it had found outside its territory. During this process the animal dropped some cake, which meant it had to run back and forth several times. At a given moment, the animal noticed that an unfamiliar male had intruded into its territory. Immediately an aggressive interaction followed and the intruder was chased away from the territory. However, only a few minutes later the same male which intruded was eating the pieces of cake which were dropped outside the territory and the territorial animal peacefully joined the meal. Such examples demonstrate that within a certain area intruders are not acceptable, whereas outside this area a certain degree of tolerance exists.

An animal will familiarize itself with its environment by means of exploratory behaviour before it will start feeding in, or defending the area. In fact, an unfamiliar environment will elicit physiological (endocrine) responses which cannot be distinguished from responses to other arousing or even painful stimuli such as weak electric shocks. By exploring its environment, the animal develops a kind of neural map. This can be illustrated by a classical experiment using a maze in which there were three routes leading from the starting point to the finish, where there was some food. The three routes differed in length: a short, a medium, and a long route. Hungry rats were placed inside the maze. After a period of exploration all the rats used the short route to the reward. As part of the experiment, the short route was barred using a sliding door (see fig. 4-3). Having discovered the blockade, most rats used the longest route instead of first trying the medium one. It seems as if they chose immediately the secure side of the design.

The concept of cognitive maps was introduced to explain this phenomenon, indicating that the behaviour of animals in their home territory is not only guided by simple reflexes and habits, but also by knowledge of the environment. Subsequent experiments confirmed these ideas which were at first disputed. Now it is widely recognized that animals develop a detailed knowledge of the spatial structure of their environment. In vertebrates, the main central nervous structure involved in this cognitive mapping is the hippocampus. In this structure of the brain, neurons respond in relation to the location of the animal in its environment, irrespective of its behaviour at that location. Other neurons are active when the animal is at another location.

This knowledge of the environment depends, of course, on the sensory capacities of the animal. In this respect there are large differences across the
species. Consider, for example, the ability of rodents or bats when detecting ultrasonic sounds, the ability of mammals detecting minimal amounts of specific odours, or the sensitivity of pigeons and chickens to the flicker-frequency of ordinary fluorescent light. It is very important then to realize that each animal species (or individual, depending on experience, sex, and age) has its own way of life. Von Uexküll called all the aspects of the environment which can be perceived (Merkwelt) and those which can be influenced (Wirkwelt) by an individual, the Umwelt of the organism (fig. 4-4). This Umwelt includes both an internal and an external component, which are closely interconnected. Any question concerning the housing and care of a certain species should first consider the Umwelt of males, females, and juveniles of the species.

The Umwelt of an animal is not comparable to ours, and therefore seemingly small changes in a housing system (i.e. ventilation, hygiene, care, etc.), may often have a drastic effect on the animals. For experimental animals, such as rats and mice, odours are an essential component of their Umwelt. Changes in the pattern of scentmarks in their environment may elicit reactions in the animals which are similar to those elicited by other unfamiliar factors. Not only physical but also social aspects, such as the relationship
between individuals of a species, belong to their Umwelt. Such relationships are apparent as some animals will seek contact with each other whilst avoiding others. The way in which this works often communicates the idea that animals know each other individually, as illustrated by the mother–child relationship. Clearly a bond exists between the two that not only covers the nursing and safety of the child, but is also stress reducing. When such a bond is disrupted, several emotional expressions such as behavioural agitation and neuroendocrine stress responses will emerge, both in the mother and in the child.

Apart from the mother–child interactions, relationships may exist between individual animals of a certain species which are not only strongly emotional in nature, but are also strongly stabilizing. For example, specific male–female combinations of tree shrews (*Tupaia*) almost immediately form a harmonious pair-bond. In such a pair, both partners show a persistent reduction in heart rate and catecholamine levels in their blood, an improved immunological resistance, decreased physiological responses to external stressful stimuli and optimal reproductive success. This is in direct contrast to the non-harmonious pairs which have to be separated to prevent serious stress-related pathology. Clearly individuals of a certain species may like or dislike certain conspecifics. It is vital to take this into account when groups of animals are formed and put into a stable or cage. The individual differences in a group may be so great that it cannot be maintained in that specific composition. So far, little knowledge is available on the factors involved in the stability of social groups. Possibly the distinction between types of individuals (such as the active and passive copers mentioned later on) plays a role. Such a distinction can be important to the development of a social hierarchy, be-
Interaction between the animal and its environment

cause the two types of animals seem to have a different predisposition to be either the dominant or the subordinates in a social group.

It is often questioned to what an extent such stabilizing or disrupting factors exist in the relationship between animals and human beings. There is evidence to show that they do exist, for instance, in pig husbandry, a human factor appears to be involved in the growth and reproduction of the animals. In laboratory rats, handling has also been shown to affect age-related changes in behaviour and physiology. Although more experiments are needed in this field, these results would seem to indicate that the way humans interact with animals may affect the health of these animals, and hence the quality of the experimental results.

To summarize, the social contacts amongst animals as well as those between animals and human beings may be important to the health and well-being of the animals. With this in mind, animals living in social groups should only be housed in isolation when this is unavoidable due to the nature of the experiment. It should also be taken into account that animals are individuals and come to the experimental procedures with their own, unique background.

Animals have not only knowledge of spatial aspects of their environment, but also of the way in which events precede or follow on from each other. As mentioned in the paragraph on learning, this knowledge is based on stimulus and operant conditioning. Stimulus conditioning allows the animal to predict certain events in its environment, for example the entrance of the animal caretaker predicts food. By means of operant conditioning the animal learns which behaviour will cause significant changes in its Umwelt, e.g. obtaining food or avoiding a rival. This implies that animals have expectations about future events. These expectations are not, however, always realized. It is possible to observe emotional expressions when the reality appears to differ from what was expected. Emotional expressions can be seen for example in dogs by tail wagging, piloerection, various facial expressions, vocalizations such as yelping or screaming, along with physiological reactions such as heart-rate changes. These emotional expressions will emerge when the organism is less certain of its environment. One good example of this is given in fig. 4-5 which illustrates the fact that whenever an expected event does not occur, chickens will cackle as a form of emotional expression. These emotional expressions also sometimes have a signalling function with regard to conspecifics.

Predictability and controllability

Central to modern stress research are the terms controllability and predictability. The importance of these terms can be illustrated by a classical experiment. The experimental conditions are given in fig. 4-6. Three rats are put into three identical cages with their tails extending through the back wall.
Electrodes are connected to the tails. For the animals 1 and 2 the electrodes are then connected to a shock generator. The wiring of the system is such, that for these two rats weak electric shocks can be given which are identical in frequency, duration, intensity and moment in time. Rat number 3 serves as a

Fig. 4-5. Below: whenever hungry chickens do not get the expected food, they perform “cackling”. Upper figure: sonogram of one “cackle”.

Fig. 4-6. Layout of the experiments by Weiss. See text.
Interaction between the animal and its environment

control animal and therefore does not receive any electric shocks. Each rat has in front of it a light which can be switched on and off and a wheel. In the case of rat 1 the light is presented at certain intervals followed by a shock after 10 seconds (stimulus conditioning, see above). The same number of signals and shocks are also presented to rat 2, but without a clear relationship between the signals and the shocks. In a different experiment, rat 1 can switch off the shock by turning the wheel (operant conditioning, see above), whereas rat 2 also has a wheel, but one which is not connected to the shock generator. With these conditions the predictability and the controllability of the stressor can be manipulated during the experiment.

Although rats 1 and 2 receive exactly the same number and intensity of shocks, the stress symptoms vary considerably. Rat number 2 that can not predict and has no control over the electric shock, suffers from severe damage to its stomach wall, showed signs of immunosuppression, and has the largest rise of plasma corticosterone (see also below). In fact rat 1, that can control or predict the stressor shows only minor differences in stress symptoms compared with rat number 3 that receives no shocks at all. It can be concluded then, that it is the degree to which a stressor can be controlled or predicted which will determine the severity of stress symptoms, and not the stressor in itself.

Using this type of data as a basis, the following definitions of stress can be formulated:

- acute stress is the state of an organism after a sudden decrease in the predictability and/or the controllability of relevant environmental changes.
- chronic stress is the state of an organism which occurs when relevant environmental aspects have a low predictability and/or are not or not very well controllable over a long period of time.

The distinction between predictability and controllability is significant. While predictability is closely associated with stimulus conditioning, controllability is associated with operant conditioning. Controllability itself implies predictability, whereas the reverse is not necessarily true. For example, animals in certain housing systems may easily be able to predict the delivery of food, but this event may be totally beyond their control. The absence of control over an aspect of life as important as food can be an important stress-eliciting factor within some housing systems. This does not mean that an ideal environment should be totally predictable and controllable. There is accumulating evidence that a certain degree of unpredictability is required to avoid the negative aspects of boredom.

Conflict behaviour and behavioural pathology

Uncertainty may induce a state of stress, and it is conceivable that such a situation may induce a conflict. Conflict situations are part of daily life, and
the accompanying conflict behaviour has been described extensively in ethology. Conflict behaviours fall in a group which includes certain agonistic behaviours (a mixture of aggression and flight), ambivalent behaviour, redirected behaviour, and adjunctive or displacement behaviour.

— Agonistic behaviour may occur when an organism is prevented from reaching its goal. An animal may, for example, attack an innocent conspecific when a lever-press, which usually results in a food reward, suddenly does not deliver food any more.

— Ambivalent behaviour may occur when an organism has both a tendency to approach or to avoid a goal situation. When the two tendencies are equally strong this may result in circling movements around the goal.

— Redirected behaviour can be observed when an organism cannot reach the goal or when it is afraid of the goal. In this situation, behaviour may be directed towards a replacement object. For example, during an aggressive interaction, birds may vigorously peck at inanimate objects rather than at the conflict partner.

— Adjunctive behaviour is characterized by the fact that it has seemingly little or no relationship to the conflict. For example, during a fight, cocks may suddenly start grooming or pecking the ground as if to start feeding. Adjunctive behaviour is often situation specific and may therefore have a signal value to conspecifics.

Conflict behaviours, which occur during acute stress situations, are often characterized by the fact that their duration is short. The intensity of this behaviour may be exaggerated.

In a situation where such conflicts cannot be resolved, and are of a more permanent nature, chronic stress will occur. The evidence available indicates that the original conflict behaviour will modify into deviant behaviour. The best known pathological forms of behaviour are behavioural stereotypes and behaviour which causes damage to the subject or to its conspecifics. Damaging behaviours such as feather pecking in chickens housed on grid floors, tail biting of pigs housed on a concrete floor, and the grazing of one-another’s fur in mice, seems to stem from redirected behaviour. Similarly, socially isolated monkeys may bite their fingers, and mink in commercial fur farming often seriously damage their tails, by continuous sucking. Although the origin of this deviant behaviour is not always known, the conclusion can be drawn that it indicates serious shortcomings with regard to housing conditions and care.

A second group of behavioural pathologies, the stereotypes, are frequently observed in various species kept in captivity, for example zoo animals, farm animals, pets and laboratory animals. Stereotypes are characterized as follows:

— they have a relatively simple and constant construction
Interaction between the animal and its environment

– they are repeated frequently
– they seem to have no specific purpose
– the form and expression of stereotypes are characteristic to the individual.

Stereotypes can be considered to be ritualizations of conflict behaviours. Studies using tethered sows, or voles kept in small cages showed that these stereotypes can be reduced through the use of the opioid antagonist naloxone. This would suggest that the occurrence of stereotypes is in some way associated with the release from the central nervous system of endorphin, which has an analgesic action. This finding may elucidate the functional significance of stereotypes. When taking this view, stereotypes may be biologically significant to the animal but one has to realize that the incidence of stereotypes indicates that the animals have been (or still are) in a state of chronic stress. Hence, housing conditions within which stereotypes develop, should be avoided.

The ability to cope and individual differentiation

The question as to whether an animal can control the situation successfully, i.e. can cope with the environmental demands or not, depends upon the mechanisms the animal has available to deal with the environmental challenge and upon the individual appraisal of the situation. When the situation is considered to threaten homeostasis (controllability), mechanisms are activated to cope with that the situation. Both the appraisal and the available coping mechanisms will depend upon the genotype and the phenotype of the individual. Not only the reaction to a stressor will vary, but also the type of reaction seems to differ amongst individuals. In a number of animal species, including human beings, different coping styles can be seen.

In rats and mice, and in a variety of other species, two extremes of reactions can be seen. One group of animals react with an active coping style, characterized by attempts to control the environment actively. This can be seen, for example, in the form of the active defence of the home territory, flight in the presence of a dominant and active avoidance of shock. This type of stress response was originally described as the fight/flight response. A second type of coping style is characterized by a predominantly passive acceptance of the situation. For example, these animals will not defend territory, will freeze when approached by a dominant and are poor at avoiding aversive stimuli. This passive style of coping is also called the conservation-withdrawal response. Recent studies indicate that these two styles may appear to be alternative solutions to a comparable problem but that both may lead to some kind of environmental control. For example, as far as the avoidance of attacks by a dominant male is concerned, active escape has in the short term the same result as quietly hiding in the corner of a cage.
Animals employing an active coping style are usually the ones that are active in social groups and may be found in the dominant and subdominant positions. Animals with a passive coping style are usually the subordinate animals in a given group. It can be presumed that individual differentiation is in principle present in every socially living vertebrate. The degree to which this differentiation will be expressed in captivity will depend upon the degree to which the housing system allows for the formation of social groups. In this context, factors such as group size and composition, sex-ratio, and available space are important.

Social relationships will always contribute to the variation between experimental animals. It is important to realize that this variation is not only of a quantitative but also of a qualitative nature. Reduction of this natural variation will implicate a form of selection of the experimental results.

Interaction between environment and physiology

Behaviour is an important way to obtain and maintain homeostasis of both the internal and external milieu. Physiological processes, however, also play an important role. For example, when an animal is put into a cold environment, it will reduce its heat loss not only by building a nest, but also by reducing its peripheral blood flow. In addition to this, it will increase heat production by shivering and by increasing its metabolism. An environmental challenge (stressor) will therefore always induce a strongly integrated behavioural and physiological response. This integrated response is initiated and coordinated by the central nervous system (CNS). With regard to the physiological response, the CNS has two major pathways at its disposal; the autonomic nervous system and the endocrine system (see fig. 4-7).

The autonomic nervous system

The autonomic nervous system has two major subdivisions; the (ortho-) sympathetic and the parasympathetic branches. With only a few exceptions, all organs are innervated by these two systems. At the beginning of this century, it was Cannon who recognized the importance of the sympathetic nervous system and its innervation of the adrenal medulla in the physiological response to stressors. A stressor may result in a sympathetic mass discharge, i.e. a virtually total activation of the whole sympathetic nervous system. Due to the widespread distribution of sympathetic nerve fibres, this mass discharge will be seen in a wide variety of physiological parameters such as an increase in plasma adrenaline and noradrenaline levels, an increase in heart-rate and blood pressure, an elevation of body temperature and changes in the immune system. Cannon called this pattern of reactions the fight-flight response be-
cause the whole pattern is consistent with the physiological preparation for physical activity. The sympathetic mass discharge will only emerge in the most extreme cases. Recent studies show that sympathetic activity can also be restricted to certain parts of the system. For example psychological stressors usually result in an increase in plasma adrenaline due to the selective activation of the adrenal medulla, whereas noradrenaline released from the sympathetic nerve endings is generally associated with physical activity.

Both the sympathetic branch and the parasympathetic branch of the autonomic nervous system are active in reaction to a stressor. The fact that the two systems are generally balanced in their activity can be seen by the cardiovascular response to a stressor. The handling of an animal usually results in an increase in heart-rate and an increase in plasma catecholamines, which indicates an increase in sympathetic activity. When the animal is subsequently placed into a cage where it had previously experienced an aversive event, a sudden drop in heart-rate may occur even though the levels of plasma catecholamines may have continued rising. This relative decrease in heart-rate or bradycardia is due to an increased parasympathetic activity. This can be seen by the disappearance of the bradycardia response, having blocked cholinergic neurotransmission by means of atropine. This example illustrates the general presence of a balance in activity between the two components of the autonomic nervous system, and also how the two can be activated separately by specific stressors.
The hypothalamus-pituitary system generally determines the neuroendocrine response to stressors. The system that is classically involved in the neuroendocrine stress response is the hypothalamus-pituitary-adrenocortical (HPA) axis. Selye was the first to demonstrate that a wide variety of stressors such as heat, cold, or tissue damage are able to activate this system. This led to the formulation of the General Adaptation Syndrome (GAS) in 1950. Three different phases are distinguishable when an animal is chronically exposed to stressors. The first is the alarm phase which is the direct physiological reaction to a stressor. After a while this reaction modifies into the second phase, that of resistance. In this phase, the animal adapts its physiology to the continuous presence of the stressor. The increased excretion of adrenocortical hormones (mainly cortisol and corticosterone) may result in a hypertrophy of the adrenal cortex. In this situation, according to the Selyean stress concept, the animal has an enhanced resistance to the stressor. In the third phase, a state of exhaustion may occur, particularly when the animal is continuously exposed to rather severe stressors. In this phase, the physiological capacity of the animal is not sufficient in meeting the environmental demands, and the animal will die of stomach ulcers, infections, etc. In case of chronically extreme stress, the symptoms of the GAS, as elaborated by Selye, are clearly distinguishable. However, in present day housing conditions for laboratory animals, such extreme situations are rare. For this reason a great deal of research focuses on the activity of the pituitary-adrenocortical system in reaction to less severe environmental challenges. It would appear that the activity of the HPA axis is related to the predictability and controllability of the stressor. In the experiment which involved either predictable or controllable electric shocks in rats as described above, the plasma levels of corticosterone were highest in the animals which could not predict or control the stressor.

The pituitary hormone which activates the adrenal cortex in secreting corticosterone is ACTH (adrenocorticotropic hormone). This hormone is synthesized from a large precursor molecule known as pro-opiomelanocortin. A number of other stress hormones are derived from this precursor molecule. One of them is β-endorphin, an endogenous opiate. Studies taking place in recent decades have revealed that many neuroendocrine systems respond in reaction to a stressor. These include not only systems involved in the regulation of the adrenals (corticotropin-releasing hormone, vasopressin, ACTH), but also in reproduction (FSH, LH, testosterone, oestrogen, prolactin), in metabolism (growth hormone, thyrotropin, thyroxine, insulin) and in the regulation of blood pressure and body fluids (vasopressin, oxytocin). The effects of stress on these neuroendocrine systems may be direct, but they can also be indirect through the interaction with other neuroendocrine systems.
Body temperature

One of the consequences of the combined activation of the autonomic nervous system and the neuroendocrine system during a stressor, is an increase in body temperature. A stressor may induce an acute rise in body temperature of about one degree Celsius. A major stressor, such as social defeat, may induce an increase in resting phase body temperature, lasting for more than ten days. This stress-induced hyperthermia shares important central nervous mechanisms with infection-induced fever. For that reason, it is also known by the term ‘emotional fever’. Recent evidence suggests that an increase in body temperature may be a sensitive indicator for reduced well-being.

To summarize, it seems that a stressor induces a complex pattern of physiological changes. This complexity is only partially understood in terms of the underlying mechanisms and its functional significance. An important consequence, however, is that a wide variety of neuroendocrine and physiological parameters depend upon the degree to which an animal is exposed to stressors, i.e. they depend upon the predictability and controllability of the environment. This phenomenon can be seen as an important source of variation in animal experiments.

Functional significance of physiological stress responses

Physiological parameters, such as enhanced plasma levels of catecholamines or corticosteroids, are frequently used as indicators of an animal’s lack of well-being. However, it should be noted that these parameters are adaptive and necessary for the organism to cope with the present (stressful) conditions, and to survive in the situation.

The physiological changes in reaction to a stressor are important on two organizational levels of the organism (see fig. 4-7). Both the autonomic nervous system, as well as the different neuroendocrine systems, affect peripheral organs such as the heart, blood vessels, immune system, etc. It can be stated, in general, that the function of this is the preparation of the peripheral physiological processes such as blood pressure, blood distribution, metabolism, immunocompetence, etc. in producing an adequate behavioural and physiological stress response. Not only peripheral organs are affected, the central nervous system is also an important organ targeted by the products of neuroendocrine systems. Several hormones, such as the steroids, cross the blood–brain barrier and bind themselves to specific receptors in certain areas of the brain. Other hormones affect the CNS via pathways which are still unknown, possibly through specific receptors on afferent autonomic nerve fibres.

This feedback of hormones to the CNS has several functions. With regard
to the hypothalamus and the pituitary, it is involved in classical neuroendocrine feedback mechanisms. When looking at the higher limbic structures such as the hippocampus and the amygdala, however, hormones may affect behaviour as well. Many hormones which are released in reaction to a stressor, such as adrenaline, CRH, ACTH, corticosterone, vasopressin, β-endorphin, etc., are reported to affect learning and memory processes. For example, the plasma concentration of adrenaline immediately after the acquisition of a learning task will greatly affect memory consolidation.

In summary, it can be said that the acute physiological stress responses have two functions: They organize the organism to cope behaviourally and physiologically with the challenge and, at the same time, they facilitate learning and memory processes which allow the animal to react more adequately to a similar stressor in the future. In fact, these physiological and neuroendocrine mechanisms can be considered as the basic mechanisms underlying all types of behaviour. Therefore, they can only be used as parameters indicative of the disturbed well-being of the organism when they show long term deviations from normal values.

Pathophysiology

The previous section was mainly devoted to acute stress responses and their significance for homeostasis. An organism may, however, reach a state of chronic stress when the homeostatic mechanisms are insufficient; in other words when relevant aspects of the environment are hardly, or are not, predictable and/or controllable.

The significance of chronic stress to the condition of laboratory animals has been illustrated by some experiments on male tree shrews. The males of this animal species live a solitary existence in large territories, where they may occasionally meet their neighbours. In a laboratory experiment, two males were allowed to have a short fight leading to a dominance relationship between the two. If the animals were separated for the rest of the day by a wooden partition, this situation had no negative consequences on physiology and behaviour of the winner and the loser, even when the daily fights were repeated for weeks. If, however, both animals were separated after the fight by a wire-mesh partition, so that the loser could not be attacked by the winner but could see him constantly, he rapidly lost bodyweight and died after a few days as the result of dramatic physiological stress responses, while the winner even gained weight and improved its health. This means that the dramatic physiological responses of the losers and their death are not the consequences of the physical exertions during the fights or of wounding, but of the constant presence of the potentially dangerous rival. It would
seem that the possibility to actively escape from the presence of the dominant (controllability), outweighed the high number of fights to which the subordinate male was subjected.

Pathophysiological changes are not only observed in laboratory or animal husbandry conditions. In natural conditions, signs of disease can also be observed. In a study carried out on wild Australian marsupials (Antechinus) a high incidence of lethal infectious disease was observed in aggressive, territorial males during the mating season. These infectious diseases were due to the immunosuppressive action of high plasma levels of corticosteroids. These levels were enhanced during the mating season, mainly due to the intensive aggressive interactions of the territorial males. Similarly, high blood pressure and atherosclerosis were found in dominant or subdominant males in colonies of mice, rats, or monkeys, depending upon the stability of the social structure. The relationship between position in the social structure and the incidence of stress-related pathology, appears also to depend upon an individual differentiation in coping style as mentioned above. Animals which react with an active coping style, predominantly react with a sympathetic response, whereas animals using a passive coping style have a more reactive HPA system and show parasympathetic stress responses. Due to the differential involvement of these physiological systems in various types of pathology, this may also indicate an individual differentiation in susceptibility to stress pathology.

The distinction between physiology and pathophysiology is very difficult to define. In the event of less extreme circumstances, it is better to think in terms of susceptibility to certain stress-related diseases. As has been previously mentioned, the phenotype of an individual is the result of a continuous interaction between the genotype and its own life history. Many events which occur in life can account for some more or less permanent changes in behaviour and physiology. For example, the levels of plasma corticosterone during nursing will determine the activity and reactivity of the HPA axis at an adult age. Variation in corticosterone levels during nursing in the rat may either be due to the fact that the pups are confronted with a stressor, or by stress in the mother, because corticosteroids can be transmitted from the mother to the pup via the milk. During adulthood, permanent changes in the HPA axis may also be induced. A strongly activated HPA axis may lead to a permanent decrease of corticosterone receptors in the hippocampus. Due to the fact that these CNS corticosterone receptors are not only involved in behaviour, but also in the neuroendocrine feedback, i.e. the height and the duration of the response, a sort of spiral will develop in which a period of elevated adreno-cortical activity will increase the likelihood of subsequent elevations. It seems, in general, to be the case for both neuroendocrine and neurotransmitter systems that the receptors will adjust to the concentration of the ligand (receptor up- and down-regulation). This is an important fundamental principle in the...
dynamic adaptation mechanisms of the organism. It means that every individual, along with its appraisal of the situation, is a product of its own history. This is one of the factors involved in the individual differentiation of behavioural and physiological reactivity to stressors.

The dynamic interaction between environment, central nervous system and peripheral physiology, determines vulnerability to certain diseases. Long term sympathetic activation will, for example, enhance the risks of cardiovascular diseases such as hypertension and atherosclerosis, whereas a more chronic increase in adrenocortical activity will increase the risk of infection. A large parasympathetic activation will enhance the risks of heart rhythm disturbances and of sudden cardiac death. A serious stressor is, as a rule, not pathogenic in itself, but merely enhances the vulnerability and greatly potentiates pathogenic processes when other risk factors are also present. In stress-induced cardiovascular pathology, this may be seen in a high level of serum cholesterol or a reduced glucose tolerance, whereas stress-induced immunosuppression will only ever lead to pathology when infectious agents or malignant cells are also present.

It may therefore be concluded that the individual history of animals determines their behavioural, physiological and neuroendocrine state. This “state” determines the reactivity of an animal to certain challenges and the outcome of animal experiments, as well as its susceptibility to stress-related diseases. Therefore, it is important to know the degree to which the processes mentioned above play a role not only in the housing and care of experimental animals but also during the actual animal experiments.

A summary of the processes leading to pathology is schematically shown in fig. 4-8. In this model the response to a stressor is divided into three categories: the recognition or appraisal of a stimulus as a threat to homeostasis, the behavioural and physiological response, and the consequences of this response.

Well-being

Clearly, adaptation can only occur within the genotypic and phenotypic limits of the individual. The wider the regulatory range of homeostatic mechanisms, in terms of both behaviour and physiology, the better the guarantee for the well-being of the animals. Extreme conditions, such as low environmental temperatures, will not affect the well-being of the animal, as long as the conditions are controllable by means of the full repertoire of adaptation mechanisms i.e. nestbuilding, metabolism, shivering, etc. A judgement of the degree to which well-being may be affected under certain conditions has to be based, therefore, on a thorough knowledge of the animal species involved. Depending on the animal species and its normal social organization, environ-
mental factors such as cage size and structure, light (intensity, wavelength, photoperiod, flicker-frequency), sounds, ventilation, etc. are as important as the presence or absence of conspecifics, their sex, and the predictability and controllability of the environment. There is a certain danger of anthropomorphism in the judgement of the relative importance of these factors. Conditions that are good for human well-being are not necessarily good for animals as well. This also holds for a comparison between different animal species, and between the strains of a single species.

This problem can be tackled by measuring the preference of an animal for certain environmental conditions, using a preference test. In this system, test animals are offered a choice of various conditions such as housing systems, bedding material, food, etc. (see fig. 4-9). When the choices are combined with detailed behavioural observations, information can be obtained on the relative importance of environmental factors. Care must be taken, however, when interpreting the obtained results in terms of well-being. The choice may, in fact, be biased due to previous experience, or an animal may not be able to judge which choice might be good for its own well-being in the long term.
Another way to approach animal well-being is by using a scoring system for signs of stress or suffering, based upon behavioural and clinical parameters. Morton and Griffiths were the first to develop a list of indicators such as piloerection, reduced body weight, increased heart rate, diarrhoea, etc. Additional scoring systems for animal suffering have been developed since then. Each of these systems is aimed at estimating the degree of suffering based upon clinical, physiological and behavioural parameters. Although most of these parameters can be determined objectively, the question of to what extent they are indicators of well-being will remain a matter of interpretation.

**Concluding remarks**

Animals are highly dynamic, emotionally reacting and information-processing organisms, which continuously try to adapt themselves to the environmental conditions using behavioural and physiological mechanisms. Due to the nature of these mechanisms, each individual is a product of its own unique history. The regulatory range of these adaptive, homeostatic mechanisms is limited and strongly determined by both genotypic and phenotypic constraints. Animals, used either as pets, in animal experimentation or in animal husbandry, are subject to the conditions offered by the responsible persons. Inevitably this will mean that their decisions on the housing and care greatly influence the well-being of the animals. In view of the dynamics in behaviour...
and physiology, as mentioned above, this also implies that in animal research these decisions influence the quality of the experimental results at the same time. Due to the fact that the laboratory or husbandry conditions are unavoidably limited in comparison with the richness and the variations in nature, it has to be considered as to which part of the wide spectrum of natural variation is studied in the particular species under the limited and standardized experimental conditions. Standardization of housing and care of experimental animals is essential for the reliability and reproducibility of experimental results. However, very often at the same time, standardization also means restricting the extent to which animals can fulfil their behavioural and physiological needs. In every experiment, therefore, one should consider the question whether and/or to what extent the chosen housing and experimental conditions will affect the state of the animals in terms of their behaviour, physiology and well-being.

Literature


Weerd H A van de, Loo P L P van, Zutphen L F M van, Koolhaas J M. Preferences for nesting material as environmental enrichment for laboratory mice. Lab Animals 1997; 31: 133–143.


5 Standardization of animal experimentation

A. C. Beynen, K. Gärtner and L. F. M. van Zutphen

Introduction

Standardization of animal experimentation can be taken in this context to mean the defining of the properties of any given animal (or animal population) and its environment, together with the subsequent task of keeping the properties constant or regulating them. The aim of standardization is to increase the reproducibility of group mean results from one experiment to another within the limits set out in chapter 4. This will improve comparability of results within and between laboratories. Standardization also often aims to reduce the variation in quantitative measurement values in apparently identical animals within a given experiment. From a statistical point of view, a reduced inter-individual variation of measured values lowers the number of animals needed per experiment (see chapter 12). It is implicit within standardization that reported results should be accompanied by a careful description of the potential sources of variation, such as the animals themselves, their environment and the experimental procedures employed.

Standardization of animal experiments can only involve the potential sources of variation that are known at the time. Variation in measured values can occur at two levels, the first being between apparently identical experiments known as between-experiment variation. Secondly it may occur between apparently identical animals within a given experiment known as within-experiment variation or inter-individual variation. In essence, both types of variation can be as a result of the same sources, i.e. the variable properties of animals and their environment along with other influences including experimental procedures. The observed inter-individual variation may be divided into various components (see chapter 12). What can be achieved by the standardization of potential sources of variation along with its limitations will be discussed here.
Between-experiment variation

Repetition of a given experiment, with the same or a different group of animals, will result in varied group mean measured values. The treatment effect, which refers to differences between the group averages for the control group and the test group, will vary therefore between experiments. This between-experiment variation is subject to two fluctuating components; the variation in measurement values between individual animals and the differences in experimental conditions. If interaction takes place between the treatment effect and the experimental conditions, then there is an extra cause for the treatment effect to vary per experiment. This may lead to a false interpretation of the results. In order accurately to assess the treatment effect, it is necessary to repeat experiments. The treatment effect is thus composed of the true effect either reduced or augmented by a systematic error and/or noise term.

Decreasing the between-experiment variation of treatment effects will reduce the need repeatedly to perform the same experiments and will therefore contribute to a reduction of the use of laboratory animals. From a scientific point of view, experimental results need to be reproducible and therefore independent of both time and place. A hypothesis for further research must be based upon reproducible experimental results.

Within-experiment variation

Quantitative measurements between apparently identical animals within an experiment will, however, be subject to inter-individual variation. This within-experiment variation can be summed up as the variation in the execution of experimental procedures, the analytical variation, the intra-individual variation and the intrinsic inter-individual variation (see chapter 12). The intrinsic inter-individual variation is a result of the contribution of each individual animal to the measured value. The intrinsic contribution will differ per animal and will basically be independent of the type of treatment to which the animal will be subjected. The intra-individual variation consists of non-standardizable variations within an animal which will result in day-to-day fluctuations of the measured value.

If the statistical power (i.e. the chance of detecting a true effect) is to remain constant given an increasing inter-individual variation in measured values (i.e. increasing standard deviation), then the number of animals required per experiment will increase. It is possible to reduce this number by decreasing the apparent inter-individual variation of results. This has important economical, practical and ethical consequences.
Sources of between- and within-experiment variations

An important source of variation within animal experiments is the animal itself. Differences between animals in one treatment group or between two groups of animals could involve differences in age, body weight, number of litter mates during the suckling period and other differences that may exist prior to the experiment. Such differences can increase either the within- and/or the between-experiment variations in measured values. Differences in characteristics (other than the intentional treatment) between control and test group may influence the treatment effect. Inter-individual differences in genotype, including differences in gender, can also increase the variation in measured values. Uncontrolled fluctuations in (micro)biological (pressure of infections, oestrus cycle etc.), physical (light, temperature etc.), chemical (nutrition, bedding etc.) and social (number of animals per cage, interactions with personnel etc.) environmental factors can increase the between- and within-experiment variations of the results. Such fluctuations may thus also affect the treatment effect.

The measured value obtained in an animal experiment is determined fundamentally by genotype-environment interactions. The response to treatment will also be influenced by this interaction. There are various degrees to which the environment can interact with the genotype of the animal. Environmental influences from the fertilization of an egg and the fetal stage through to sexual maturity are referred to as the primary milieu. The interaction between this milieu and the genotype will give rise to the phenotype. The phenotypical properties will subsequently be influenced by the pre-experiment conditions which are referred to as the secondary milieu. As a result, the dramatype is formed. Furthermore the laboratory animal will be affected by experimental procedures and treatments known as the tertiary milieu.

The diagram below illustrates the potential sources of variation within the results of animal experimentation.

```
genotype ← primary milieu
    ↓
phenotype ← secondary milieu
    ↓
dramatype ← tertiary milieu
    ↓
results of animal experiment
```
Depending on the type of measured values, the interactions between genotype and milieu, can have a variable influence upon the within- and between-experiment variations, including the treatment effect. Chapter 12 describes how the systematic errors and noise terms within observed treatment effects can be diminished.

**Standardization of the animal and its environment**

In theory, inter-individual differences in the genotype can be eliminated by using genetically uniform animals. The individuals of an inbred strain or F1 hybrid (see chapter 7) are virtually genetically identical and as a result will on the whole show a smaller inter-individual variation in measured values (before and after treatment) than genetically non-uniform animals (i.e. random-bred or outbred animals). The interactions as depicted in the above diagram, however, illustrate that the genetic background of the animals is only one of the factors influencing the measured value. For instance, for the variation in body weight of mice of the same sex and age it has been shown that about 20% of the total variance may result from differences in pre- and post-natal environment and about 30% of the total variation may relate to analytical error and so-called intangible variance. The latter remains in genetically uniform animals, even after equalizing the controllable environment for each animal. The intangible variance is caused by uncontrollable genotype-environment interactions at various developmental levels, including that of ontogenesis.

Given adequate genetic quality control, it is possible to work with animals with a constant genotype when moving from one experiment to another, but not all experiments require animals to react identically. Certain experiments (e.g. toxicity tests) rather require some degree of genetic variation (see chapter 7).

The microbiological quality of laboratory animals can be of influence upon various types of measured values. Pressure of infection (latent infections) can markedly increase the inter-individual variation within an experiment and can also vary between experiments. Differences in microbiological environment of control and test animals can affect the treatment effect. This type of variation can be eliminated to a certain extent by using microbiologically defined animals. The status of these animals must be maintained by adequate hygienic measures. Microbiological quality control must be seen here as a prerequisite (see chapter 8).

Transportation involves changes in environment that can have a major impact on laboratory animals. Prior to arrival in the animal house, animals purchased from commercial breeders may have been transported by car, train
and/or aircraft for many hours. Transportation by itself causes endocrine and metabolic responses that require up to one week for a new steady state to be reached. This so-called transportation stress may affect different individuals to a different extent and thus raises inter-individual variation of certain measured values, at least until a new steady state is reached. Transporting of animals from one place to another may involve changes in chemical, physical, microbiological and social environmental factors. The altered environment will cause changes in certain measured values, whether transient or not, and will at the same time raise inter-individual variation. Depending on the type of measurements to be carried out in projected experiments, transported animals must generally be given a period of adaptation of up to three weeks before being used in an experiment.

In practice, it is not possible to house individual animals under identical environmental conditions. Within any given animal house there are as a rule a number of animals housed either individually or in groups. Local differences in environmental conditions are inevitable and a few are mentioned here. Depending upon the type of ventilation system, the temperature at 1.5 m can be 3–4°C higher than it is at 0.5 m. The location of a cage in a rack will as a result influence the temperature within the cage (micro-climate). The micro-climate will also be affected by the number of animals per cage, relative humidity and the type of bedding material. Group housing of rats or mice can raise the temperature within the cage by up to 5°C, compared with room temperature. Fluorescent tubes, which are commonly used for lighting suspended from the ceiling, will cause a higher light intensity in the cages at the top of the rack. The difference between light intensity between cages at the top or the bottom of a rack can be four-fold.

Table 5-1 illustrates the influence of the number of animals per cage on food intake. The food intake of the mice falls markedly as cage occupation increases. Mice housed in groups generally huddle together which reduces heat loss, resulting in a depressed energy requirement.

<table>
<thead>
<tr>
<th>Number of animals per cage</th>
<th>Food intake, g/mouse/day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Males</td>
</tr>
<tr>
<td>1</td>
<td>7.6</td>
</tr>
<tr>
<td>2</td>
<td>6.2</td>
</tr>
<tr>
<td>4</td>
<td>4.9</td>
</tr>
<tr>
<td>8</td>
<td>4.2</td>
</tr>
</tbody>
</table>

After Chvédoff et al. (1980).
Standardization of animal experimentation

Fig. 5-1 shows another example of an environmental factor which will affect experimental results, i.e. the effect of environmental temperature on the toxicity of the pharmacological agents, salicylate and ephedrine. Toxicity of salicylate, expressed as a lethal dose, shows a U-shaped correlation with room temperature. Similar relationships have been shown in connection with atropin, chlorpromazine and pentobarbital. There is a direct relationship between ephedrine toxicity and room temperature, i.e. toxicity rises with temperature increase. Other correlations can be made between temperature and diphenyl-hydramine, pentachlorophenol and kinidin-sulphate. Therefore, in order, to reduce between-experiment variation in toxicity of drugs, room temperature should be standardized.

Differences between individual housing conditions should be equally distributed between control and test groups during an experiment. As a consequence, the treatment effect will not be biased by differences in housing conditions between the control and the test groups. The local differences in environmental conditions within animal rooms should be constant for the different experiments. Ideally, all environmental factors should be constant from one experiment to another.

Standardization of environmental factors is essential when justifying the use of laboratory animals. The practical realization of such standardization is not so straightforward and questions need to be answered. To which degree should environmental factors be standardized? Should the level be constant or rhythmic? Upon which criteria should the ideal level of environmental factors be based? These criteria may be a combination of optimal welfare of the animals and ergonomic considerations. The level of a given environmental factor formed on the basis of these criteria is not, by definition, associated with a
small within-experiment variation of measured values. The Council of Europe and the E.C., in their attempt to stay ahead, have already formulated recommendations for the housing and care of laboratory animals despite the lack of sufficient experimental data. These recommendations are based upon current knowledge, whether or not scientifically substantiated, and upon common practice.

**Standardization of experiments and extrapolation of results**

In principle, experimental results only hold for the conditions (animals, environmental factors) under which the experiment has been carried out. For validated routine testing, e.g. for the control of vaccines, this is not a problem. However, there are experiments from which it should be possible to generalize the outcome. Standardization of experiments implies a specialization of experimental conditions. Therefore, standardization is not identifiable with generalization of experimental results. The first question to be answered is to what extent are the animals used representatives of all comparable animals? Even if it is acceptable that the animals used are indeed representing a given population, the results will still only have a bearing upon this (limited) population (animals of the same strain, of the same sex, with the same body weight etc.) and upon limited conditions (the biological, physical and chemical properties of the environment). It becomes even more complicated when the results obtained from working with laboratory animals must be "extrapolated" to other species, in particular to man. Clearly, caution must be the watchword here. The range over which results can be extrapolated from experiment to practice or, in particular, from one species to another, is actually rather wide in relation to any limitation of generalization of results due to standardization. Therefore the need for extrapolating results should not be an excuse for ignoring standardization.

**Efficacy of standardization**

The aim of standardization, i.e. the reduction of between-experiment variation and often also of within-experiment variation, is only to a certain extent empirically substantiated and then only for specific, quantitative values. In certain cases, standardization can have an opposite effect, i.e. an increase in within-experiment variation. Nevertheless it is a fact that standardization improves the comparability of results between laboratories, because, by its very nature, standardization implies careful reporting of experimental conditions.
Concluding remarks

It is clear that many questions concerning standardization remain unanswered. In particular, it is not known which aspects of standardization require the most attention and exactly how standardization should be implemented in practice. It is of course essential that standardization does not have a negative influence on the animal’s welfare. This implies that in striving towards standardization, the physiological and ethological needs of the animal should be taken into account. If this is not the case then standardization could even be counterproductive.

The experimenter generally has to rely upon commercial suppliers when acquiring animals, and cannot therefore determine and/or control standardization prior to the experiment. However, certain demands can be placed upon the supplier in the areas of genetic quality or microbiological status with regard to the animals. A reliable supplier will inform the researcher about changes which have been introduced regarding housing and care of the animals, as well as their genetic and microbiological status.

In the following chapters, the influence of nutrition upon experimental results and the possibilities of genetic and microbiological standardization of laboratory animals will be described in detail.

Literature


6 Nutrition and experimental results

A. C. Beynen and M. E. Coates

Introduction

The health status, performance and metabolism of experimental animals are influenced by the composition of the diet and the feeding practice. Thus, nutrition not only affects the well-being of the animals but also the outcome of experiments. Nutritional studies focus upon deliberate alterations to diets and the effects this has. The results, however, of some animal experiments can be affected unintentionally by the composition of the diet. Such effects can be brought about, for example, by undesirable and unknown variations in the dietary constituents, which can adversely affect the accuracy and precision of experimental results. This can lead to unnecessary sacrifice of animals and to wastage of time and resources. As part of an experiment food intake may differ between control and experimental groups. This can create biased results and lead to a false interpretation of the data. Should this occur, the use of animals can be considered unwarranted. This chapter focuses on nutrition as a potentially interfering factor within animal experimentation.

Nutrient requirements

The most critical aspect of formulating experimental diets is ensuring the presence of all the essential nutrients in the required concentrations. There is a considerable amount of published data regarding the nutrient requirements of laboratory animals. These data are periodically reviewed by committees established by the U.S. National Research Council, and reports are issued containing the estimated nutrient requirements. One such report in the series (National Research Council 1978, 1995) contains extensive estimates of the nutrient requirements for normal growth and reproduction. Absolute figures
Table 6-1
Recommended nutrient allowances for growing animals fed *ad libitum* (expressed per kg feed consisting of 90% dry matter)³

<table>
<thead>
<tr>
<th></th>
<th>Mouse</th>
<th>Rat</th>
<th>Hamster</th>
<th>Guinea pig</th>
<th>Rabbit</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Digestible energy</strong>² (kJ/g)</td>
<td>16.8</td>
<td>16.0</td>
<td>17.6</td>
<td>12.6</td>
<td>10.5</td>
</tr>
<tr>
<td><strong>Fat</strong> (g/kg)</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>s.u.</td>
<td>20</td>
</tr>
<tr>
<td><strong>Fibre</strong> (g/kg)</td>
<td>r.u.</td>
<td>r.u.</td>
<td>s.u.</td>
<td>150</td>
<td>110</td>
</tr>
<tr>
<td><strong>Protein</strong> (g/kg)</td>
<td>180</td>
<td>150</td>
<td>150</td>
<td>180</td>
<td>160</td>
</tr>
<tr>
<td><strong>Arginine</strong> (g/kg)</td>
<td>3</td>
<td>4.3</td>
<td>7.6</td>
<td>12</td>
<td>6</td>
</tr>
<tr>
<td><strong>Asparagine</strong> (g/kg)</td>
<td>s.u.</td>
<td>4</td>
<td>s.u.</td>
<td>s.u.</td>
<td>s.u.</td>
</tr>
<tr>
<td><strong>Glutamic acid</strong> (g/kg)</td>
<td>s.u.</td>
<td>40</td>
<td>s.u.</td>
<td>s.u.</td>
<td>s.u.</td>
</tr>
<tr>
<td><strong>Histidine</strong> (g/kg)</td>
<td>2</td>
<td>2.8</td>
<td>4</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td><strong>Isoleucine</strong> (g/kg)</td>
<td>4</td>
<td>6.2</td>
<td>8.9</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td><strong>Leucine</strong> (g/kg)</td>
<td>7</td>
<td>10.7</td>
<td>13.9</td>
<td>10.8</td>
<td>11</td>
</tr>
<tr>
<td><strong>Lysine</strong> (g/kg)</td>
<td>4</td>
<td>9.2</td>
<td>12</td>
<td>8.4</td>
<td>6.5</td>
</tr>
<tr>
<td><strong>Methionine + Cystine</strong> (g/kg)</td>
<td>5</td>
<td>9.8</td>
<td>3.2</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td><strong>Phenylalanine + Tyrosine</strong> (g/kg)</td>
<td>7.6</td>
<td>10.2</td>
<td>14</td>
<td>10.8</td>
<td>11</td>
</tr>
<tr>
<td><strong>Proline</strong> (g/kg)</td>
<td>s.u.</td>
<td>4</td>
<td>s.u.</td>
<td>s.u.</td>
<td>s.u.</td>
</tr>
<tr>
<td><strong>Threonine</strong> (g/kg)</td>
<td>4</td>
<td>6.2</td>
<td>7</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td><strong>Tryptophan</strong> (g/kg)</td>
<td>1</td>
<td>2.0</td>
<td>3.4</td>
<td>1.8</td>
<td>2</td>
</tr>
<tr>
<td><strong>Valine</strong> (g/kg)</td>
<td>5</td>
<td>7.4</td>
<td>9.1</td>
<td>8.4</td>
<td>7</td>
</tr>
<tr>
<td><strong>Glycine</strong> (g/kg)</td>
<td>s.u.</td>
<td>s.u.</td>
<td>s.u.</td>
<td>s.u.</td>
<td>r.u.</td>
</tr>
</tbody>
</table>

**Minerals and trace elements**

<table>
<thead>
<tr>
<th></th>
<th>Mouse</th>
<th>Rat</th>
<th>Hamster</th>
<th>Guinea pig</th>
<th>Rabbit</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Calcium</strong> (g/kg)</td>
<td>5</td>
<td>5</td>
<td>5.9</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td><strong>Chloride</strong> (g/kg)</td>
<td>0.5</td>
<td>0.5</td>
<td>s.u.</td>
<td>0.5</td>
<td>3</td>
</tr>
<tr>
<td><strong>Magnesium</strong> (g/kg)</td>
<td>0.5</td>
<td>0.5</td>
<td>0.6</td>
<td>1</td>
<td>0.35</td>
</tr>
<tr>
<td><strong>Phosphorus</strong> (g/kg)</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>4</td>
<td>2.2</td>
</tr>
<tr>
<td><strong>Potassium</strong> (g/kg)</td>
<td>2</td>
<td>3.6</td>
<td>6.1</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td><strong>Sodium</strong> (g/kg)</td>
<td>0.5</td>
<td>0.5</td>
<td>1.5</td>
<td>0.5</td>
<td>2</td>
</tr>
<tr>
<td><strong>Sulphur</strong> (g/kg)</td>
<td>s.u.</td>
<td>r.u.</td>
<td>s.u.</td>
<td>s.u.</td>
<td>s.u.</td>
</tr>
<tr>
<td><strong>Chromium</strong> (mg/kg)</td>
<td>2</td>
<td>r.u.</td>
<td>s.u.</td>
<td>0.6</td>
<td>s.u.</td>
</tr>
<tr>
<td><strong>Copper</strong> (mg/kg)</td>
<td>6</td>
<td>5</td>
<td>1.6</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td><strong>Fluoride</strong> (mg/kg)</td>
<td>s.u.</td>
<td>r.u.</td>
<td>0.024</td>
<td>s.u.</td>
<td>s.u.</td>
</tr>
<tr>
<td><strong>Iodine</strong> (mg/kg)</td>
<td>0.15</td>
<td>0.15</td>
<td>1.6</td>
<td>0.15</td>
<td>0.2</td>
</tr>
<tr>
<td><strong>Iron</strong> (mg/kg)</td>
<td>35</td>
<td>35</td>
<td>140</td>
<td>50</td>
<td>r.u.</td>
</tr>
<tr>
<td><strong>Manganese</strong> (mg/kg)</td>
<td>10</td>
<td>10</td>
<td>3.65</td>
<td>40</td>
<td>8.5</td>
</tr>
<tr>
<td><strong>Selenium</strong> (mg/kg)</td>
<td>0.15</td>
<td>0.15</td>
<td>0.1</td>
<td>0.15</td>
<td>s.u.</td>
</tr>
<tr>
<td><strong>Zinc</strong> (mg/kg)</td>
<td>10</td>
<td>12</td>
<td>9.2</td>
<td>20</td>
<td>r.u.</td>
</tr>
</tbody>
</table>

**Vitamins**

<table>
<thead>
<tr>
<th></th>
<th>Mouse</th>
<th>Rat</th>
<th>Hamster</th>
<th>Guinea pig</th>
<th>Rabbit</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Retinol</strong> (mg/kg)</td>
<td>0.15</td>
<td>1.2</td>
<td>1.1</td>
<td>7.0</td>
<td>0.17</td>
</tr>
<tr>
<td><strong>Cholecalciferol</strong> (mg/kg)</td>
<td>4</td>
<td>25</td>
<td>62</td>
<td>25</td>
<td>r.u.</td>
</tr>
<tr>
<td><strong>DL-α-tocopheryl acetate</strong> (mg/kg)</td>
<td>32</td>
<td>27</td>
<td>3</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td><strong>Menadione</strong> (mg/kg)</td>
<td>1</td>
<td>1</td>
<td>4</td>
<td>5</td>
<td>r.u.</td>
</tr>
<tr>
<td><strong>Thiamin</strong> (mg/kg)</td>
<td>5</td>
<td>4</td>
<td>20</td>
<td>2</td>
<td>r.u.</td>
</tr>
<tr>
<td><strong>Riboflavin</strong> (mg/kg)</td>
<td>7</td>
<td>3</td>
<td>15</td>
<td>3</td>
<td>s.u.</td>
</tr>
<tr>
<td><strong>Pyridoxine</strong> (mg/kg)</td>
<td>8</td>
<td>6</td>
<td>3</td>
<td>3</td>
<td>39</td>
</tr>
<tr>
<td><strong>Cyanocobalamin</strong> (µg/kg)</td>
<td>10</td>
<td>50</td>
<td>10</td>
<td>10</td>
<td>n.r.</td>
</tr>
<tr>
<td><strong>Nicotinic acid</strong> (mg/kg)</td>
<td>15</td>
<td>15</td>
<td>90</td>
<td>10</td>
<td>180</td>
</tr>
<tr>
<td><strong>Folic acid</strong> (mg/kg)</td>
<td>0.5</td>
<td>1</td>
<td>2</td>
<td>4</td>
<td>s.u.</td>
</tr>
<tr>
<td><strong>Biotin</strong> (mg/kg)</td>
<td>0.2</td>
<td>0.2</td>
<td>0.6</td>
<td>0.2</td>
<td>r.u.</td>
</tr>
<tr>
<td><strong>Pantothenic acid</strong> (mg/kg)</td>
<td>16</td>
<td>10</td>
<td>40</td>
<td>20</td>
<td>s.u.</td>
</tr>
<tr>
<td><strong>Choline</strong> (mg/kg)</td>
<td>2000</td>
<td>750</td>
<td>2000</td>
<td>1800</td>
<td>1200</td>
</tr>
<tr>
<td><strong>Inositol</strong> (mg/kg)</td>
<td>r.u.</td>
<td>n.r.</td>
<td>100</td>
<td>n.r.</td>
<td>s.u.</td>
</tr>
<tr>
<td><strong>Ascorbic acid</strong> (mg/kg)</td>
<td>n.r.</td>
<td>n.r.</td>
<td>n.r.</td>
<td>200</td>
<td>n.r.</td>
</tr>
</tbody>
</table>

---

³ Elsevier/Castlefield Press/VAN ZUTPHEN: Ch 6
Nutrient requirements

Notes to table 1-1

1 As described in the text, absolute figures for nutrient allowances cannot be given, and different committees propose different allowances (see also Clarke et al., 1977).

2 Metabolizable energy generally varies from 90–95% of digestible energy.

n.r. = not required; r.u. = required, but requirement unknown; s.u. = status unknown.

Based on NRC Nutrient Requirements of Laboratory Animals (1978, 1995) and NRC Nutrient Requirements of Rabbits (1977).

for nutrient requirements cannot be established and thus any requirement set always remains debatable.

When determining nutrient requirements, criteria such as growth, reproduction, nutrient storage, enzyme activity and morphological and histological characteristics are taken into account. In general it is only possible to establish the minimum requirement of any given nutrient. The minimum requirement is the lowest amount of a given nutrient which can be present without showing signs of deficiency, or it can refer to the amount which just allows equilibrium between intake and excretion, or to the maintenance of normal levels of certain metabolites in the blood or urine. The various criteria for determining the minimum requirement do not necessarily reflect the same nutritional status. Therefore, the given minimum requirement for a nutrient is related to the criterion which was used when it was established.

When the minimum requirement of a nutrient is being ingested, the animal is in a critical state. A small increase in need, for instance due to sub-clinical infection, may induce deficiency. When formulating laboratory animal diets, recommended nutrient allowances are followed, which differ according to the animal species. The recommended allowances refer to the assessed amounts of nutrients to be ingested guaranteeing the optimum functioning of most individual animals. Clearly, the recommended allowance is greater than the minimum requirement. Maximum growth is often used as the criterion for determining the recommended requirements for laboratory animals. However, maximum growth has a negative impact on health at advanced age and certainly in the case of rodents, it can also reduce longevity.

Table 6-1 shows recommended nutrient allowances for the mouse, rat, hamster, guinea pig and rabbit. The recommendations are given for growing animals, as these are the most frequently used in experiments. Adult animal values are less well established or even unknown, but are likely to be lower than those for growing or breeding animals. The nutrient requirements are expressed in amounts (g or mg) per kg diet. The recommended nutrient requirements are given as guidelines and must be treated as such. The values are based upon observations made at different laboratories operating different conditions for the preparation and storage of diets, housing of the animals, etc.

The amount of food ingested by the majority of laboratory animals will be determined by the animals’ energy requirements and therefore the energy
density of the diet is crucial. Dietary energy is in the form of fats, carbohydrates, protein and fermentable fibre. The animal’s energy requirement is expressed in terms of a unit of heat, the MJ (1 MJ = 1000 kJ = 240 kcal). Not all of the energy in the diet, i.e. total combustible energy, can be utilized by the animal. Some of the dietary energy will remain undigested or will be lost in the form of flatulence or urinary products. Metabolizable energy is the available energy left after faecal, urinary, and gaseous losses have been accounted for. The energy value biologically available to the body is 16.8 kJ per g for carbohydrates and protein, whilst dietary fats provide 37.8 kJ per g. The quantity required of diets with high energy density, expressed as g of feed, is lower than that of low-energy diets. Table 6-1 shows, therefore, the recommended nutrient allowances of diets for specified energy densities.

Care must be taken when using the recommended nutrient allowances. There are, for example, differences in minimum requirements between inbred strains of rats and mice. These differences have not been taken into account by the recommendations. It must be stressed that the recommendations do not necessarily hold for germ-free animals. Vitamins K and B12, for example, will be synthesized by the gut flora of conventional rats and mice and will be sufficiently ingested as a result of coprophagy. For SPF animals it is a wise precaution to include higher supplements of vitamins B and K, as their microflora may not contain the full complement of vitamin-synthesizing organisms.

Under certain conditions, the recommended requirement of a given nutrient in a diet may be insufficient. This may relate to the availability of the nutrient, i.e. the extent to which the nutrient is actually absorbed. Nutrient availability may be depressed when it is present in a poorly digestible form or when it interacts with other dietary components. The recommendations do not take these factors into account. For example, if a diet contains large amounts of soya bean protein concentrate and/or cereal products, the required concentration of zinc in the diet increases. These products contain phytate which forms an insoluble compound with zinc in the intestinal lumen so that the availability of zinc is reduced. Housing conditions can also influence the required concentration of zinc in the diet. The recommended requirement of zinc for the rat is 12 mg/kg diet (energy density = 16 kJ/g diet). When rats are housed in galvanized cages, they will lick the zinc and therefore, the required concentration of zinc in the diet will fall to 2–4 mg/kg.

Types of laboratory animal diets

Diets for laboratory animals are classified according to the degree of the refinement of the ingredients. They are known as: natural-ingredient, purified, or chemically defined diets.
Types of laboratory animal diets

Natural-ingredient diets

These are formulated with natural ingredients such as oats, corn, soya bean meal, and fish meal. They are the most economical and the most widely used diets. Nutrient concentration can vary considerably due to changes in the source of ingredients used in the manufacture of the diet. This is due to the fact that nutrient concentrations of natural ingredients are not fixed. Information on the ingredient composition of most commercial food is not readily available as it is the property of the manufacturer marketing the product under a trade name. The commercial closed-formula diets are the most readily available, the most economical and the most widely used. They are formulated with the aim of providing all known nutrient requirements for the growth of the species. The quantitative nutrient concentration range of a closed-formula diet is generally available from the manufacturer. Diets of known ingredient composition, open-formula diets, have been published for rodents kept in conventional environments, as well as for those reared in environments requiring sterilized food (National Research Council 1978). Formulations of existing open-formula diets can be modified, or new diets made, to meet the requirements of specific research programmes.

Chemically defined diets

These are formulated with pure chemicals. For instance, individual amino acids are supplied in place of whole proteins and carbohydrates are provided by specific sugars. Chemically defined diets are, however, expensive. At the time of manufacture, the nutrient concentrations in these diets are theoretically fixed; but just as for natural ingredients, the availability of nutrients may be altered due to, for instance, oxidation or interactions between nutrients. The formulation of these diets is dependent upon the knowledge of the nutrient requirements of the animals involved. Problems can occur associated with palatability.

Purified diets

These are formulated with a combination of natural ingredients, pure chemicals, and ingredients of varying degrees of refinement (e.g. protein may be supplied by casein). These diets are relatively inexpensive and, under most conditions, their nutrient concentrations are more stable than those of chemically defined diets.

The diet is a potential source of microorganisms that can be pathogenic to laboratory animals. Food for gnotobionts must be sterilized by either autoclaving or by gamma-radiation, and diets for SPF animals (see chapter 8) are
usually sterilized. Autoclaving causes some destruction of most of the vitamins and may denature proteins. Radiation is less damaging, although it causes some loss of vitamins, particularly vitamin K. Losses on irradiation are much greater in the presence of water. Moist diets or aqueous solutions should not be sterilized by gamma-radiation.

**Variation in diet composition**

Variation in the concentration of dietary components, nutrients and contaminants, can cause clinical signs of deficiency or toxicity, which can be readily observed. When such a variation occurs, although it may be harmful to the animals, no biased results will appear in the literature as the experiment will be stopped. Comparatively small variations in the diet, which occur more frequently, however, are not always so obvious. Nevertheless, the metabolism on the cellular level of the animals may be affected, which in turn may influence the outcome of the experiment. Small variations in diet might unknowingly cause undesirable and biased results to appear in scientific journals.

Two types of diet variation need to be distinguished: one being differences occurring between diets from different manufacturers and the other where differences occur between batches of one particular brand. Table 6-2 illustrates the magnitude of the between-brand variation that may occur between commercial rat diets based upon natural ingredients. Essentially all diets were of the closed-formula type. It is clear from the table that commercial rat diets can differ considerably with respect to their composition.

Different batches of one brand of diet based on natural ingredients can also differ markedly in their composition (see table 6-3). It is important to note that the variation in protein concentration can be much higher than that indicated on the table. It has been found in certain cases, after correction for analytical error, to be as high as 12% (expressed as relative standard deviation). Different batches of an open-formula diet also show considerable variation in composition. Fluctuations in the composition of dietary components are likely to be minimal when using purified diets based on refined, standardized ingredients.

Variations in the diet of one brand, as shown in table 6-3, can be caused by errors occurring during the preparation of the diets, by changing the source and quality of the ingredients and/or by differences in the processing and storage conditions. Different manufacturers use different formulas and therefore produce foods with different nutrient compositions as shown in table 6-2. Researchers should not choose a brand of commercial diet purely on the basis of catalogue values, since such values generally do not conform with chemical analyses (see table 6-4).
Variation in diet composition

Impact of variation in diet composition

Commercial laboratory animal diets can vary markedly between brands. If these differences in diet composition affect experimental results, the use of different brands may introduce a bias in the results. The use of a certain diet

Table 6-2
Table showing the variation in the composition of natural-ingredient, commercial rat diets from different manufacturers

<table>
<thead>
<tr>
<th>Component</th>
<th>No. of manufacturers</th>
<th>Mean ± S.D.</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (g/kg)</td>
<td>4</td>
<td>206 ± 40</td>
<td>155–268</td>
</tr>
<tr>
<td>Riboflavin (mg/kg)</td>
<td>4</td>
<td>9–56</td>
<td></td>
</tr>
<tr>
<td>Calcium (g/kg)</td>
<td>4</td>
<td>8.9 ± 4.1</td>
<td>4.1–13.0</td>
</tr>
<tr>
<td>Zinc (mg/kg)</td>
<td>4</td>
<td>149 ± 67</td>
<td>56–233</td>
</tr>
<tr>
<td>Selenium (mg/kg)</td>
<td>3</td>
<td>0.08 ± 0.04</td>
<td>0.03–0.11</td>
</tr>
<tr>
<td>Lignin (g/kg)</td>
<td>5</td>
<td>18.5 ± 3.4</td>
<td>14.5–22.4</td>
</tr>
<tr>
<td>DDT (mg/kg)</td>
<td>3</td>
<td>1.2 ± 1.0</td>
<td>0–2.5</td>
</tr>
</tbody>
</table>

Based on Beynen (1987).

Table 6-3
Table showing the variation in the composition of different batches of one brand of a natural-ingredient, commercial rat diet

<table>
<thead>
<tr>
<th>Component</th>
<th>No. of batches</th>
<th>Mean ± S.D.</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (g/kg)</td>
<td>65</td>
<td>198 ± 0.7</td>
<td>182–213</td>
</tr>
<tr>
<td>Calcium (g/kg)</td>
<td>65</td>
<td>10.4 ± 0.1</td>
<td>8.0–14.0</td>
</tr>
<tr>
<td>Zinc (mg/kg)</td>
<td>65</td>
<td>50 ± 10</td>
<td>24–77</td>
</tr>
<tr>
<td>Selenium (mg/kg)</td>
<td>148</td>
<td>0.34 ± 0.15</td>
<td>0.04–0.66</td>
</tr>
<tr>
<td>DDT (mg/kg)</td>
<td>148</td>
<td>0.03 ± 0.05</td>
<td>0–0.3</td>
</tr>
<tr>
<td>Cadmium (mg/kg)</td>
<td>65</td>
<td>0.43 ± 0.16</td>
<td>0–0.9</td>
</tr>
<tr>
<td>Nitrosodimethylamine (g/kg)</td>
<td>6</td>
<td>0.2–21.3</td>
<td></td>
</tr>
</tbody>
</table>

Based on Beynen (1987).

Table 6-4
Zinc concentrations in four different commercial rat diets according to chemical analysis and catalogue values

<table>
<thead>
<tr>
<th>Diet</th>
<th>Zinc (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Catalogue</td>
</tr>
<tr>
<td>1</td>
<td>38</td>
</tr>
<tr>
<td>2</td>
<td>84</td>
</tr>
<tr>
<td>3</td>
<td>39</td>
</tr>
<tr>
<td>4</td>
<td>36</td>
</tr>
</tbody>
</table>

Based on Wise and Gilburt (1981).

Impact of variation in diet composition

Commercial laboratory animal diets can vary markedly between brands. If these differences in diet composition affect experimental results, the use of different brands may introduce a bias in the results. The use of a certain diet
can either enhance or mask the response of animals to a given stimulus. This in turn can lead to incorrect interpretation of the results. From a scientific point of view this is undesirable. A variable diet-induced bias of experimental results implies that the results of different experiments are not comparable. This will increase the need to repeat experiments. A variable diet-induced bias of results essentially invalidates the comparison of experiments between different laboratories. If, however, during the course of a particular study or research programme the brand of the diet is altered, this bias can be reflected in experimental results obtained within one laboratory. Between-batch variation within a series of one type of experiment can decrease the precision of results. In other words, the standard deviation increases, which lowers the statistical power. This will result in the use of more animals in order to obtain valid information.

The feeding of different commercial diets to animals is likely to give rise to different results. For instance, with rats growth rates may differ, and the brand of diet has also been shown to affect the outcome of potency tests of bacterial vaccines and toxicity tests. Biological effects can be evoked by altering the concentrations of dietary components within their range of fluctuation in practical situations. For example, the protein content of the food (within the range 30–300 g/kg) affects demethylation and hydroxylation of certain xenobiotics by liver homogenates from the rat. Dietary selenium (within the range 0–0.1 mg/kg) affects growth performance and glutathione peroxidase activity in erythrocytes and liver of rats. Cadmium intake (within the range 0–1 mg/kg of diet) affects systolic blood pressure and renal vasculature in rats. Residues of DDT in the diet (within the range 0–1 mg/kg) influence the activity of certain hepatic microsomal enzymes of the rat. Thus, unknown fluctuations of diet within certain studies can lead to an incorrect interpretation of the experimental results.

Diet standardization?

Clearly a standard diet for rats (and other laboratory animals) does not exist. It is also the case that many researchers do not specify the diet of their rats, probably due to the fact that they believe it to be standard. Table 6-5 highlights this problem. Should the diet affect the experimental results, it is difficult, if not impossible, to duplicate the experiments with comparable results without adequately describing the diet.

This leads to the question of whether efforts should be made to design guidelines in order to produce an international, standardized diet. However, when using natural ingredients it is not possible to produce a standard diet. Even with the use of purified ingredients, a standard diet over any period of
time cannot be reliable. The constantly changing source and quality of ingredients excludes the possibility of a true standard diet, and of course specific research programmes may require specific diets. The standardization of laboratory animal diets should involve the concept of reference diets rather than a single set diet. This would allow for flexibility with regard to changing the concentrations of one or more components, whilst keeping the remainder of the diet relatively constant.

Practical approach to diet variation

Depending upon the parameters being studied, the results in many experiments may not be affected by small changes in the concentrations of dietary components. However, one must be aware of the possibility of adverse influences caused by variations in the composition of animal diets. How then should this problem be addressed? Researchers should initially search through the literature and identify components of the diet which may affect the parameters to be measured in the experiment. The diet to be used should also be analysed to measure the concentrations of the components which have been identified. The concentrations of these components should then be kept constant throughout the experiment. In addition the diets of experimental animals should be described as extensively as possible in scientific papers. Repetition of experiments can only be meaningful if these guidelines are followed. It would then be possible to track down the part played by diet, if any, when it is apparent that experiments cannot be reproduced.

Diet forms

Diets for laboratory rodents can be provided in different physical forms. Experimental procedures will generally determine the most advantageous form for any given study. For example, when highly toxic compounds are added to
the diet, a form should be selected that will produce a minimum amount of dust.

Generally speaking, pellets are the most efficient form of food for laboratory rodents as they are easy to handle, to store and to administer. Only minimal amounts of these products are wasted by the animals. Food additives or test compounds cannot however be added after completion of the pelleting process, unless the pellets are reground.

Meal is a very inefficient form of food for rodents because they have a tendency to waste large amounts. When storage conditions are less than ideal, there is a tendency for meal to cake together and special equipment is required for feeding. Diets will be fed in meal form when food additives or test compounds are incorporated after the manufacturing process has been completed.

Semi-moist or gel form diets are efficient to use when dusty or highly toxic test compounds are to be incorporated. They are usually much more palatable than dry food. Diets in this form are, however, more susceptible to bacterial growth, they must be administered frequently, and large quantities are bulky and hard to handle.

Energy requirements and feed intake

Table 6-6 lists the energy requirements of rats under various physiological conditions. The values are not absolute and deviations will occur in practice. Growth and lactation for example, impose heavy demands on energy. As energy requirements are related to metabolic weight, i.e. body weight $w^{0.75}$, individual animals and strains with different body weights will have different energy requirements. The estimated energy requirements are related to metabolic body weight and metabolizable energy in the following way (Clarke et al., 1977):

- Maintenance requirement $= 0.45 \times w^{0.75}$
- Growth requirement $= 1.20 \times w^{0.75}$
- Pregnancy requirement $= 0.60 \times w^{0.75}$
- Lactation requirement $= 1.30 \times w^{0.75}$

All requirements are expressed as a daily intake of metabolizable energy in MJ; body weight is expressed in kg. It should be stressed here that the estimates obtained assume a minimal expenditure of energy for physical activity, and are not applicable to animals from which any sustained work output is expected. Furthermore, no extra heat generation has been allowed for to compensate for low environmental temperature as it has been assumed that the animals are housed under conditions of thermal comfort or thermal neutrality.
Natural-ingredient diets may contain the following amounts of energy sources (expressed as weight percentages): 50% carbohydrates, 25% protein and 5% fat. The energy density of this diet is then 14.5 kJ/g. The contribution made by fibre to dietary energy is negligible. On the basis of the energy requirement of the animal and the energy density of the diet, the expected *ad libitum* food intake can be calculated (Table 6-6).

Along with body weight and physiological status, the condition of the animal must also be taken into consideration when considering food intake e.g. sick animals generally consume less food. The energy density of a diet is an important determinant of food intake. Dietary characteristics which influence palatability may also determine food intake.

### Variable energy density of diets

Various groups of animals showing the same gender and breed or strain with similar body weight, age, health status etc., will have a similar energy requirement. Regardless of the composition of the diet, the animals will generally consume an almost constant amount of energy, provided that they are fed *ad libitum*. This is because laboratory animals tend to adjust their food intake to satisfy their energy requirements. Therefore, if the energy density of the diet increases, the animals will consume less food and *vice versa*. This implies that, when diets are being used which have different compositions within one experiment, the concentrations of the components, which must not vary between experimental groups, must be identical when expressed relative to dietary energy. If this is not done, then the intakes of these components will differ between the control and the test animals when they have free access to food. This must be taken into account when formulating diets with different compositions.

It is of course the case that researchers will sometimes wish to make variations to the content of a nutrient or ingredient in an experimental diet. Such

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**Table 6-6**

Estimated requirements of metabolizable energy and food intake in rats

<table>
<thead>
<tr>
<th>Physiological status</th>
<th>Body weight (g)</th>
<th>Energy requirement (MJ/day)</th>
<th><em>Ad libitum</em> food intake1 (g/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth</td>
<td>100</td>
<td>0.21</td>
<td>15</td>
</tr>
<tr>
<td>Growth</td>
<td>200</td>
<td>0.36</td>
<td>25</td>
</tr>
<tr>
<td>Maintenance</td>
<td>400</td>
<td>0.23</td>
<td>16</td>
</tr>
<tr>
<td>Pregnancy</td>
<td>400</td>
<td>0.30</td>
<td>21</td>
</tr>
<tr>
<td>Lactation</td>
<td>400</td>
<td>0.65</td>
<td>46</td>
</tr>
</tbody>
</table>

1Energy density of diet: 14.5 kJ/g
modification may result in changes in the consumption of other components of the diet. Natural-ingredient diets can be misused in studies where test diets are formulated by adding large amounts of one ingredient such as fat or sugar. What generally then occurs is that the natural-ingredient diet, which was originally formulated as a complete diet, is considered to be the control diet. This causes the problem that the test and control diets contain different concentrations of all nutrients, because those in the test diet have been diluted by the addition which has been made. When the concentrations of energy are different, then different amounts of diet will be consumed by animals fed with the test or the control diets. It is impossible to achieve a valid interpretation of data collected from such a study, since observed differences in animal response could be due to the matter of interest in, or to other differences in the constitution of the test and the control diets. This problem can be kept to an absolute minimum when the test diet is formulated by adding minute amounts of a compound to the complete, natural-ingredient diet.

Control and test diets can both be prepared by adding control and test supplements to a commercial, natural-ingredient diet. However, the problem still exists of reducing the intake of essential nutrients. Commercial, natural-ingredient diets generally contain sufficiently high amounts of essential nutrients to permit dilution without producing serious imbalances. As a rule of thumb, natural-ingredient diets can be diluted by 10–20% with supplements. The possible effects of such dilutions must be evaluated carefully for any given experiment. It must be taken into account that the background composition of the diet, i.e. the natural-ingredient diet, is poorly characterized and subject to variation. Accurate nutritional control includes both standardization and characterization of the ingredients used to make up the experimental diets. For this reason, purified diets are generally used for nutritional experiments with small animals.

Table 6-7 shows the potential effects from certain low-fat and high-fat dietary formulations. In this example, the low-fat diet (Diet 1) contains 10% fat, 20% protein, 60% carbohydrates, fibre, vitamin and mineral mixes, and a test compound. The high-fat diet (Diet 2), is formulated by adding 20% fat, deleting an equal weight of carbohydrates, whilst maintaining the same level of other components. The expected effects on nutrient intake are shown on the table underneath the diet formulation. The high-fat diet will have a 24% higher energy concentration than the low-fat diet. It can be presumed that the caloric intake of rats consuming these diets under ad libitum conditions would be essentially the same i.e. that the rats will consume a constant amount of energy. One would therefore presume that the weight of food consumed by rats fed the high-fat diet would be considerably lower than that consumed by rats fed the low-fat diet. In addition to the changes in fat and carbohydrate intake, the intake of protein, mineral mix, vitamin mix, fibre and the test
compound will subsequently all be lower with the rats fed on the high-fat diet. Thus, the change in food intake is also important when a test compound is being administered in the diet. In the example given, there would be a 20% difference in the intake of the test compound.

One method which would eliminate some of these problems is illustrated by Diet 3. Fat and carbohydrates have been exchanged isocalorically: some carbohydrate is omitted to compensate for the increase in calories resulting from the addition of extra fat. This adjusted high-fat diet ensures an intake of protein, vitamins, minerals, and fibre comparable with that of Diet 1. Although the components of Diet 3 do not add up to 100 g, and the expected food intake is lower than with Diet 1, the intake of the test compound and of all nutrients except for fat and carbohydrate will be the same.

Apart from the fat and carbohydrate intake, rats fed Diets 1 and 3 would also show differences in actual food intake, i.e. the quantity of the intake. This alone could produce a difference in experimental results between the

<table>
<thead>
<tr>
<th>Diet ingredient</th>
<th>Diet 1 Low-fat</th>
<th>Diet 2 High-fat</th>
<th>Diet 3 High-fat, adjusted</th>
<th>Diet 4 High-fat, adjusted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (g)</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Carbohydrate (g)</td>
<td>60</td>
<td>40</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Fat (g)</td>
<td>10</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Fibre (g)</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Mineral mix (g)</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Vitamin mix (g)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Test compound (g)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>“Inert” compound (g)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>25</td>
</tr>
<tr>
<td>TOTAL (g)</td>
<td>100</td>
<td>100</td>
<td>75</td>
<td>100</td>
</tr>
<tr>
<td>Energy value (kcal/g)</td>
<td>4.10</td>
<td>5.10</td>
<td>5.47</td>
<td>4.10</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Expected intake</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (kcal/day)</td>
<td>82</td>
<td>82</td>
<td>82</td>
<td>82</td>
</tr>
<tr>
<td>Food (g/day)</td>
<td>20</td>
<td>16</td>
<td>15</td>
<td>20</td>
</tr>
<tr>
<td>Protein (g/day)</td>
<td>4</td>
<td>3.2</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Carbohydrate (g/day)</td>
<td>12</td>
<td>6.4</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Fat (g/day)</td>
<td>2</td>
<td>4.8</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Fibre (g)</td>
<td>0.8</td>
<td>0.64</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td>Mineral mix (g/day)</td>
<td>0.8</td>
<td>0.64</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td>Vitamin mix (g/day)</td>
<td>0.2</td>
<td>0.16</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Test compound (g/day)</td>
<td>0.2</td>
<td>0.16</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>“Inert” compound (g/day)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>5</td>
</tr>
</tbody>
</table>
groups. In another approach to diet formulation (Diet 4), a relatively inert ingredient, such as cellulose, may be added to bring the ingredients up to 100 g. Diet 4 is isocaloric with Diet 1, and the nutrient intake should also be the same. This approach would appear to be satisfactory, but for the fact that inert compounds do not exist. Therefore, one would have to elect for the formulation of a high-fat diet as illustrated by Diet 3.

A similar example could be given for fibre-enriched diets at the expense of carbohydrates. The high-fibre, low-carbohydrate diet would be expected to have a lower energy value than the low-fibre, high-carbohydrate diet. The consumption, therefore, of the high-fibre diet could be expected to be greater. Here again, there would be changes in the intake of other nutrients, and comparisons between animals fed the high- and low-fibre diets would become very difficult.

In the above discussion of the impact of variable energy density of diets, it is assumed that animals are able to keep constant with mathematical accuracy their energy intake. However, in practice when animals have free access to diets with variable energy density there can be differences in energy intake. When energy intake is to be kept constant among different dietary treatments, restricted feeding as described below can be applied.

**Feeding regimes**

Various feeding regimes can be applied to laboratory animals. The choice of feeding regime will depend upon practical and scientific criteria.

*Ad libitum feeding.* With this regime the animals have free access to food any time of the day or night. Under *ad libitum* conditions, rodents and rabbits consume most of their food during the dark phase. A rat will take approximately 12 meals per day of which 8 meals will be taken during the dark phase.

*Meal feeding.* During fixed time periods, one or more periods per day, the animals are allowed to consume as much food as they like. Meal feeding can be controlled by a feeding apparatus. This feeding regime is used for experiments requiring a strictly controlled nutritional state, such as a certain number of hours postprandially.

*Restricted feeding.* This regime involves limiting the food intake or underfeeding, but is not equivalent to malnutrition or the induction of nutrient deficiency. Restricted feeding generally refers to both nutrient and energy restriction. It can be used to equalize food intake of different animals, for instance those in control and test groups.

*Pair feeding.* This is a specific form of restricted feeding. It involves the measurement of the amount of food consumed by animals with depressed
Feeding regimes

food intake, (e.g. treated animals), whilst giving the same amount to animals in the non-treated control group. By doing this, the food intake of control and test animals will be equalized. When employing this procedure, each animal in the test group must be assigned its own control counterpart. The amount of food consumed by the treated group will be offered to the control group one day later.

Restricted feeding brings with it various practical implications. Under ad libitum conditions, the animals may be given food once every two to four days. When animals are fed restrictedly, food has to be provided daily either manually or automatically, and animals have to be housed individually. If the animals are housed in groups, the dominant ones may almost be able to carry out ad libitum-feeding. It is a fact that restricted feeding reduces diet costs.

When carrying out either pair feeding or restricted feeding studies, a third group should always be fed the identical control diet ad libitum, as an additional control. Researchers should take into account that pair-fed control animals are usually underfed and will therefore consume their food faster than animals with an unrestricted intake. Various techniques can be used to partially compensate for this difference in consumption pattern, e.g. feeding control animals their daily ration in divided batches. Each experiment, should have its food consumption determined and have it reported on periodically.

Variation in food intake within experiments

In certain experiments with animals fed ad libitum, food intake of the test animals may be lower than that of the control animals. Reduced food intake in the test animals can be due to intoxication with the compound under study, irrespective of whether it is given with the food or not, or to deficiency of a nutrient. Food intake can also be depressed due to the fact that the compound under study has of itself anorexic activity. The difference of food intake between the control and the test groups would imply that comparison of the measured parameters is not straightforward. If and when both the treatment and the food intake exert an independent influence upon the parameter under study, then the effect of the treatment cannot be interpreted unequivocally. The observed effect will have been caused partly by the difference between the control and the test treatment, and partly by the difference in food intake between the control and the test group. If the research requires insight into a specific effect of the test treatment, then the variation in food intake should be overcome. This can either be done by the administration of restricted amounts of food or by employing the paired feeding technique.

It has been demonstrated repeatedly that food restriction inhibits the development of spontaneous tumours and prolongs life in rats and mice. The
impact, therefore, of depressed food intake in test animals on the interpretation of results has been especially relevant in cancer bioassay feeding studies with rats. When rats are treated with a carcinogenic substance together with a reduced food intake, then the carcinogenic activity of the test compound will be underestimated. In fact, this has occurred frequently in cancer bioassay studies in which the rats were fed *ad libitum*. Table 6-8 shows hypothetical outcomes of a carcinogenesis bioassay in which control and test rats were fed *ad libitum*. The test compound reduces food intake by 20%, which frequently occurs in practice. Of the control rats, 30% will develop tumours, whilst a food restriction of 20% will reduce tumour incidence to 10%. In experiment A, the test compound increases tumour incidence to 40%. When this is compared with the incidence rate in control rats fed *ad libitum*, one could presume that the compound causes cancer. However, the carcinogenic activity of the compound is underestimated, and this becomes clear when test animals are compared with control animals which are fed restrictedly. In experiments B and C with both control and test rats fed *ad libitum*, the incorrect conclusions would be drawn that the test compound is not carcinogenic, or that it indeed protects against carcinogenesis. The latter has occurred in practice. It is clear that carcinogenicity testing with rats fed *ad libitum* will not yield results from which conclusive interpretations can be drawn. Ideally, both control and test rats should be fed restrictedly so that feed intakes are identical, or the technique of paired feeding should be applied.

### Table 6-8

Hypothetical results of a cancer bioassay

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Feeding regime</th>
<th>Food intake</th>
<th>Percentage of rats with tumours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>Control</td>
<td><em>Ad libitum</em></td>
<td>100%</td>
<td>30</td>
</tr>
<tr>
<td>Test</td>
<td><em>Ad libitum</em></td>
<td>80%</td>
<td>40</td>
</tr>
<tr>
<td>Control</td>
<td>Restricted</td>
<td>80%</td>
<td>10</td>
</tr>
</tbody>
</table>

**Literature**

Variation in food intake within experiments

7 Genetic standardization

L. F. M. van Zutphen, H. J. Hedrich, H. A. van Lith and J. B. Prins

Introduction

The genotype of the animals is a major factor contributing to the variation of results in animal experiments. Genetic variability among individuals within test and control groups may be acceptable in certain experiments, e.g. when results are to be extrapolated to other species, such as in toxicity tests. The majority of experiments, however, requires a more uniform animal population. Whatever the requirements of the tests, experimental results should be reproducible. Reproducibility of the results benefits from standardization of the genetic background of the animals. This can be achieved with specific breeding programmes or by biotechnological manipulations. Both techniques have resulted in the production of animal populations with a standardized genetic variation. In this chapter, the breeding systems applied for generating groups of genetically standardized animals and the biotechnological procedures applied for generating transgenic animals will be discussed. Different aspects of genetic quality control, when dealing with these populations will also be covered.

Genetic uniformity

Monozygotic animals

Usually, littermates have different genotypes. There are, however, certain species where only genetically uniform littermates are born. Siblings of the nine-banded armadillo (*Dasypus novemcinctus*) are genetically identical. This primitive mammal, found in the Southern and Central Americas, is mainly used as a test animal for leprosy research. The armadillo produces four monozy-
Genetic standardization

Monogytic offspring per litter (i.e. derived from one fertilized egg). This phenomenon is rather uncommon, but the birth of monogytic twins is not an unusual event in several other mammalian species.

Genetic uniformity, however, does not mean that these animals are phenotypically identical, or that their response, when submitted to the same experimental procedure, will be exactly the same. When comparing monogytic twin calves, minor differences may be visible in their coat colour patterns. Subtle differences in the environment during the intra-uterine development may have caused variation in the migration of melanocytes from the neural tube to the peripheral parts of the body, resulting in differences in pigmentation. Slight differences may also be found in other morphological and physiological characteristics of genetically identical twins. But these differences are usually smaller than those between littermates with different genotypes. Thus, if genetic uniformity is required, the use of monogytic individuals would be ideal. However, only a limited number of monogytic animals are available for use in research. Although it is possible to produce genetically uniform animals by cloning, the traditional and still the most frequently used method for the production of genetically uniform animals is inbreeding. A large number of inbred strains of both mice and rats are available at present.

Inbred strains

Inbreeding, or the crossing of closely related animals, increases homozygosity in the offspring. The degree of inbreeding is expressed by the coefficient of inbreeding (F). F is the fraction of the original heterozygous genes that have become fixed in a homozygous state during the breeding process. F will increase with the number of generations of inbreeding. The increase in F per generation (ΔF) is dependent upon the degree of consanguinity of the ancestors (see fig. 7-1).

The production of inbred strains commenced at the beginning of this century, mainly because of the need for genetically identical animals for cancer research. Tumour tissues were maintained by successively transplanting (passaging) the neoplasm onto different individuals from the same species. This method was sometimes successful, but very often the recipients rejected the tissue shortly after grafting. The reason for this remained unclear until 1903, when Carl Jensen succeeded in maintaining a lung tumour in a population of closely related albino mice for 15 generations. The tolerance or the rejection of transplants seemed to be a genetically controlled process. This discovery led to an increase in the production of inbred strains. At present there are more than 400 inbred strains of the mouse and 200 inbred strains of the rat available. According to the “Committee on Standardized Nomenclature for Inbred Strains of Mice” a population may only be defined as an inbred strain after a minimum of 20 successive
generations of either brother-sister (bxs) mating, or mating between offspring and the youngest parent. At that point (i.e. 21st generation) the coefficient of inbreeding will be 98.4%, which means that, on average, 98.4% of the originally heterozygous loci have become fixed in a homozygous state. Although it is possible that small variations still occur within an inbred strain, it may be considered to be a genetically uniform group of animals, and therefore regarded as isogenic.

According to the rules for the nomenclature of genetically defined strains (see: http://informatics.jax.org/mgihome/nomen/strains.shtml#Inbred) an inbred strain is designated by a code that consists of one to four capital letters (e.g. A; DBA; WAG). The only exceptions to this rule are names of some old strains (e.g. C3H; C57BL; 129) which were already widely accepted and recognized before the rules were established.

Inbred strains can be separated into substrains. An established inbred strain is considered to be divided into substrains when branches are separated before generation 40 (i.e. after 20 to 40 generations of bxs matings) or if the branch (substrain) has been maintained separately from the ancestral strain for 100 or more generations. Furthermore, strains shall be regarded as substrains if differences are detected between branches. Substrain designations are appended to the strain’s name. Strain symbol and substrain designations are separated by a slanted line and an appropriate symbol (e.g. C57BL/6J or C57BL/1OScSn).

Fig. 7-1. The increase of inbreeding per generation depends upon the relationship between the individual partners of a breeding pair. Brother-sister matings (cf. A) result in a steep increase of the breeding coefficient. With random matings the increase depends upon the size of the population, or more specifically upon the number of breeding pairs (cf. B and C).
In order to facilitate a true comparison of experimental results between laboratories, it is essential to use the correct strain nomenclature when reporting experiments.

Inbred strains are frequently used in biomedical research, not only because of their uniformity, but also because several strains carry specific genetic characteristics or disorders that also occur in man. (For an overview of the specific strain characteristics see: http://informatics.jax.org/external/festing/search_form.cgi).

Inbreeding can result in decreased vigour, having a negative effect upon growth, survival and/or fertility. However, most inbred strains are not seriously affected and possess normal viability. This can be explained as follows. In any natural population, most animals carry a number of detrimental recessive alleles, which, in heterozygous condition, are not apparent in the phenotype. Usually such alleles occur in low frequency. Thus the chances of a descendant being homozygous for one or more of these recessive alleles is rather small in the case of random matings. This chance will increase, however, when the parents are related. Inbreeding depression due to an increased chance of homozygosity of harmful alleles will occur mainly during the first 4–10 generations of the inbreeding process. Gradually, the less favourable combinations of alleles will be eliminated (i.e. the respective breeding lines will decease), whereas the more favourable combinations will be propagated (i.e. will survive). Therefore, a fully inbred strain usually possesses a gene pool, which is balanced, enabling normal development of the animals.

It was long presumed that genetic uniformity of an inbred strain would also guarantee a uniform response when the animals were submitted to the same experimental conditions. However, as long ago as 1956, McLaren and Michie demonstrated otherwise. They showed that offspring from mice of a cross between two inbred strains (F1) responded to exposure of the anaesthetic pentobarbital, more uniformly than either of the two parental inbred strains or the F2 population. There have been many other examples reported since that date to support this phenomenon.

F1 hybrids

F1 hybrids are animals resulting from a cross between two inbred strains. All F1 hybrids are genetically uniform and heterozygous for all the genes for which the two parental strains differ (fig. 7-2). The finding that a more uniform response to an experimental procedure is more likely to occur with a F1 hybrid, than with the parental strains, may be explained by the variation of alleles present in the F1 hybrids. A larger pool of alleles provides the animal with a sturdier buffer to compensate for minor environmental fluctuations.
This decreased sensitivity to environmental influences may not always be desirable. Certain responses of F₁ hybrids are less uniform than those of individuals from the parental inbred strains. This may be due to the improved ability of the F₁ hybrids to adapt. This phenomenon is known as the “Tryon-effect” after R.C. Tryon who was the first to describe this variation, using F₁ animals of “maze-bright” and “maze-dull” rats.

F₁ hybrids generally show a more uniform response when a morphological/physiological parameter is being studied, and a more variable response when behavioural characteristics are under investigation. There are, however, no general rules, which can be applied. For example, when studying communication between animals, behavioural responses are often rather uniform, like a morphological trait. This seems logical, since variability in communicative behaviour could easily lead to misinterpretation.

Co-isogenic strains

Occasionally mutants are found among the individuals of an established inbred strain. If the mutant represents a genetic model for a human disease, or if the mutation involves a gene of general interest, then a subline of the inbred strain carrying the mutant gene may be established. A strain that differs from the established inbred strain by only one differentiating gene, is called a co-isogenic strain. Such a strain may be extremely useful as it enables the comparison of two isogenic strains that only differ at the locus of interest. The symbol for a co-isogenic strain must consist of the full strain and substrain designation, followed by a hyphen and the symbol of the mutant gene (e.g. BALB/cRij-nunu, which means a BALB/cRij substrain carrying the “nude” allele (nu) in homozygous condition).
Sometimes it may prove difficult to maintain a co-isogenic strain. This is, of course, particularly so when the mutation involves a recessive allele, which leads to infertility or is lethal in homozygous condition. In such cases, the mutant gene can only be maintained in the colony by crossing of animals that are heterozygous for the mutant gene. When the mutant gene is maintained in heterozygous condition, this should be indicated by including a + sign in the symbol (e.g. BALB/cRij-\textit{nu}+).

**Congenic strains**

It is possible to introduce a genetic trait into an inbred strain by repeatedly backcrossing. Several breeding schemes, using repeated backcrossing, have been designed. The choice of which scheme to use will depend upon whether the differential locus is dominant, co-dominant or recessive. A system (cross-intercross-backcross system), which is used for the introduction of a recessive allele, is shown in fig. 7-3. Here, after an initial cross, a cycle of intercrossing-backcrossing is repeated at least 8 to 10 times, whilst ensuring that the animals selected for the backcross carry the gene of interest (donor gene, \textit{mm}). As a result of crossing-over (recombination) during meiosis, and the use of the homozygous (\textit{mm}) animals for breeding to strain A, the donor gene is selectively introduced into the genome of strain A (recipient strain). The resultant strain is congenic. If the mutant allele is detectable in heterozygous condition, a simple backcross system can replace the cross-intercross-backcross system. In both situations the resulting congenic strain is genetically similar to the original strain, but contains the donor gene \textit{mm} as well as some flanking ‘passenger’ DNA on either side of the locus of interest. This amount of DNA and the number of retained adjacent genes will be reduced with successive generations of backcrossing.

In the mainstream of ‘genomics’ projects and production of transgenic animals (see below) an increasing number of congenic (and double-congenic) strains has been produced and still is being produced now. The production of these strains is, however, time consuming. The number of backcrosses can be reduced by using a DNA-marker-assisted breeding system.

The designation of congenic strains is similar to the designation of co-isogenic strains. However, if congenic strains differ at the major histocompatibility locus, it is acceptable to give an abbreviated strain name. The full or abbreviated symbol of the recipient strain is separated by a full stop from the abbreviation symbol of the donor strain (e.g. B10.D2, which refers to a C57BL/10 carrying the H-2 haplotype of the DBA/2 inbred strain).
Consomic strains (chromosome substitution strains)

Instead of exchanging a small chromosomal segment, which is carrying a specific gene of interest, it is also possible to exchange a whole chromosome. An inbred strain in which a chromosome is exchanged by a homologous chromosome of another inbred strain is called a consomic strain or a chromosome substitution strain. Such an exchange can be relatively easy performed with the Y chromosome, but for introgressing the X chromosome or an autosome, chromosome-specific DNA markers are needed for the selection of the target chromosome.

Once a consomic strain has been established, this strain can be backcrossed to the original inbred strain and, by intercrossing of the progeny, chromosome-specific recombinants can be produced. The designation for consomic strains is host strain-chromosome-(superscript) donor strain (e.g. C57BL/6J – YAKR) is a consomic strain with the Y-chromosome of strain AKR backcrossed onto 57BL/6J.

Recombinant inbred strains

Recombinant inbred (RI) strains are produced by bxs mating of individuals from the F2 generation of a cross between two (unrelated) inbred strains, termed progenitor strains. Preferably a large number of strains is derived...
Genetic standardization

from these two progenitor strains in parallel and independently (see fig. 7-4). The RI strains can be regarded as established after a minimum of 20 generations of bxs matings. They form a set or series of RI strains. They are designated by combining abbreviated names of both the parental strains which are separated by a capital X. The parallel lines are given numbers (e.g. B6XH1 which is the first recombinant inbred strain derived from the progenitor strains C57BL/6J and C3H/HeJ). RI strains represent a fixed set of randomly assorted genes of both progenitors. These strains are very valuable for genetic research, in particular for studies on linkage analysis and for the identification and genetic analysis of complex genetic traits.

Recombinant congenic strains

Recombinant congenic (RC) strains represent a series of inbred strains, which are derived from the second or third backcross generation of two unrelated inbred progenitor strains, one serving as background (recipient), the other as donor strain. After 20 generations of bxs mating, the genome of each of the resulting recombinant congenic inbred strains will primarily contain genomic material from the background strain, plus a small proportion of the donor genome. When the process of inbreeding starts after two backcrosses this is, on average, the equivalent of 12.5% of the donor genome. The genomic material of the donor strain is unevenly dispersed over the genome of the background strain. When comparing recombinant congenic strains, the various chromosomal segments of the donor strain will show some overlap. Therefore, some
Transgenic animals

20–25 parallel lines will be necessary in order to cover at least 95% of the donor strain’s genome.

Recombinant congenic strains are usually indicated by the prefix RCS and the abbreviated names of the progenitor inbred strains (with the recipient strain given first, followed by the donor strain), separated by a lower case ‘c’ (e.g. CcS, a set of recombinant congenic strains from a cross between BALB/c and STS, backcrossed to BALB/c). Individual strains of the series are distinguished by appending numbers to the strain symbols. The number of generations of backcrossing may be indicated in parentheses when more than two backcross generations were used (e.g. CcS1(N4)).

Recombinant congenic strains have been developed for the study of the genetic background of quantitative traits, such as susceptibility to tumour development or disease resistance.

Transgenic animals (transgenesis and targeted gene transfer)

By means of transgenesis and targeted gene transfer (targeted mutation), the genome of an organism is altered biotechnologically in such a way that the alterations are transmitted through the germ line. Additional genes (of the same or another species) may be introduced or existing genes (including expression regulating sequences) may be altered. These methods are frequently employed to study the functionality of known or newly identified genes at the level of the organism. In this way, deeper understanding of complex biological processes e.g. growth, differentiation, reproduction and ageing can be obtained. Also, genetically modified animals can serve as model systems of human diseases (e.g. cancer, vascular diseases or metabolic diseases) and can be employed to explore treatment strategies (e.g. radiation, enzyme therapy, gene therapy). Transgenesis has also been applied to improve farm animals (e.g. resistance to diseases, increasing meat mass) or for the production of bio-drugs (e.g. antithrombin III in goats).

In biomedical research the mouse is the species of choice for the application of transgenesis and targeted mutation. One of the reasons is that there are many genetically uniform strains available. Also, a lot of knowledge has been acquired about genetics, reproduction, in vitro fertilisation, embryo manipulation and transplantation. And the mouse is a good breeder (see chapter 3). Furthermore, lines of ES cells, which are most frequently used for targeted mutagenesis, are so far only available for the mouse.

The number of genetically modified animals used in research has increased substantially in recent years. In the UK the number of procedures involving genetically modified animals increased from 267,000 in 1995 to 511,000 in 1999.

The two main methods to change the genome of mammals (transgenesis and targeted gene transfer) are described in some detail.
Transgenesis

The most widely used method to add DNA to the genome is by microinjecting DNA-constructs into fertilised oocytes (zygotes). One or more copies of the injected DNA will integrate at random sites of the genome, usually as head to tail concatamers. (By adding loxP sites to the DNA constructs, multi-copy transgenes can be transferred into single-copy transgenes, simply by crossing the mice carrying multiple copies with mice which are transgenic for Cre recombinase. This enzyme recognises loxP sites and deletes or inverts intermediate DNA sequences). In order to produce a protein, the DNA construct needs to contain a promoter sequence, the coding sequence for the protein (cDNA), and the 3' translated region of the gene, inclusive transcription stop signals. Often, expression levels of transgenes are enhanced through the introduction of intron sequences. Depending upon the purpose of the experiment, one can choose for a strong or a weak promoter, for a universal promoter (stimulates expression in all tissues), or for a tissue-specific promoter. There is also the choice of an inducible promoter that can be switched on or off, for example by feeding or withdrawing tetracycline (Tet-VP16 transactivator systems) or Zn++ (metallothionein promoter systems). If one wishes to analyse when and where a certain gene is expressed, promoter and other expression regulating sequences of the gene of interest can be linked to the gene coding for β-galactosidase or GFP (‘green fluorescent protein’).

Fertilised oocytes (zygotes), which are used for the injection of the gene construct, are obtained from female mice, treated with hormones to stimulate superovulation. DNA is injected into the male pro-nucleus of the fertilised oocyte. Subsequently, these zygotes are implanted into another female (‘foster or surrogate mother’) which has been made pseudo-pregnant by mating to a vasectomised male. DNA, obtained from the tip of the tail, is used in Southern blotting and/or PCR in order to detect the presence of the transgene in the offspring (see fig. 7-5a). When the transgene has integrated into the genome before the first cell division, all cells of the resulting animal will contain the transgene. Mosaics are animals in which the transgene is expressed only in part of the body’s cells. They arise when the transgene has integrated into the genome after the first cell division. An animal that transmits the transgene to their offspring is called a ‘founder’. These animals form the basis of a transgenic strain.

The production of transgenic animals by applying the microinjection technique depends on the integration of the DNA construct into one or more chromosomes. This is a random process, which may lead to insertional mutations, and, as a consequence, to undesirable and harmful side effects. On the other hand, insertional mutation may also provide opportunities to study the function of previously unknown genes.
Transgenic animals

Targeted gene transfer (targeted mutation)

In contrast to the previously described method, with targeted mutation a specifically defined locus is altered. This technique is most often used to generate knock-out mice. In these animals, an entire gene (or parts of a gene) has been silenced. With this method it is possible to introduce subtle mutations or ‘tags’ into genes or to integrate genes at defined loci. To achieve targeting, constructs are used in which genetically modified DNA sequences are flanked by stretches of DNA with a sequence of nucleotides identical (homologous) to the DNA sequences present on either side of the targeted DNA segment. Exchange of the native DNA sequence with its mutated DNA sequence is achieved through homologous recombination. Since homologous recombination is a 1000 × less efficient process than random integration of DNA, knock-out and knock-in constructs are not injected in oocytes, but transfected into pluripotent embryonic stem (ES) cells. These cells are easily cultured in vitro and maintain their capacity to partake in embryogenesis after injection into blastocysts. ES cells in which homologous recombination has taken place, can be selected in vitro if the DNA construct contains a selectable marker gene (e.g. neomycin, hygromycin or puromycin resistance genes). These cells can be analysed through Southern blotting and/ or PCR.

Fig. 7-5a. Production of transgenic animals through micro-injection of a DNA construct.
After injection into the cavity of blastocysts, obtained from a donor animal, ES cells may contribute to the genesis of the embryo. When the coat-colour of the blastocyst donor strain (e.g. C57B46; black, \(aAP\)) is different from that of the ES cell donor strain (e.g. 129/Ola; beige, \(Aapp\)), animals in which ES cells have contributed to the development of the embryo can be recognised by their multicoloured coat (chimera). The lighter the coat the greater the contribution of modified ES cells. Since ES cell lines are derived from a male animal, chimeric male mice are mated with wild type C57BL/6 female mice to determine whether the genetic modification is transmitted to the next generation (germ line transmission). If this is the case, pups can easily be

Fig. 7-5b. Production of transgenic animals (knock-in or knock-out) through targeted gene transfer.
identified by their agouti coat colour. DNA of agouti \((AaPp)\) mice is screened for the presence of the genetic modification (50%). Heterozygous mice are then mated to generate homozygous offspring (see fig. 7-5b).

Transgenesis and targeted gene transfer technology may be combined. LoxP sites can be introduced on either side of a specific gene or a cluster of exons by targeted gene transfer. This way, the gene remains intact. These loxP transgenic mice can be mated with transgenic mice expressing Cre-recombinase constitutively or inducibly in all tissues or in a tissue specific fashion. This technology allows for the generation of mice in which genes can be switched off, i.e. deleted in specific tissues at any time. Since ES cell lines are not available from species other than the mouse, the production of homologous recombinants through injection of ES cells is presently only feasible in mice (see also next section on ‘Cloning’).

**Cloning through nuclear transfer**

In 1997 scientists from the Roslin Institute in Scotland, in collaboration with scientists from PPL Therapeutics succeeded, for the first time, in the breeding of a sheep, cloned by nuclear transfer. Nuclei from a cell line, created from the udder of an adult ewe were transplanted into enucleated oocytes. This transplantation took place through fusion of the cells in an electric field. After fusion, the oocytes with a diploid nucleus were implanted into a ‘foster mother’, thus leading to the birth of ‘Dolly’, a healthy lamb with the DNA of the six year old ewe, from which the mammary cells were taken. This technique of cloning can be used for multiplying animals that have best production results (e.g. in farm animal production) or have special characteristics that are of interest for their use as animal model. The technique can also be used for targeted gene transfer in species other than the mouse. The process of genetic modification through homologous recombination can take place in foetal fibroblasts or other cultured somatic cells instead of in ES cells. The genetically modified somatic cells can be selected and used for fusion with enucleated oocytes.

**Genetic variation**

*Random bred colonies and outbred stocks*

As mentioned above, it may be preferable to have a certain degree of genetic heterogeneity within a group of animals, e.g. as the base population in selection experiments, or if the animals are used to obtain results that are to be extrapolated to other species. It is, however, more difficult to maintain a given
level of variation within an animal colony than it is to maintain its uniformity. If less than an infinite number of breeding animals are used for propagation of the colony, inbreeding is inevitable, causing a loss of alleles in subsequent generations. When mating occurs at random, ∆F is \(1/(8nf) + 1/(8nm)\), where \(nf\) is the number of female breeders and \(nm\) the number of male breeders. If \(nf = nm\) then \(∆F = 1/(2N)\), where \(N\) is the total number of breeding animals. This means that if a population consists of 50 female and 50 male breeding animals \(∆F = 0.5\%\), and if there are only 10 breeding pairs \(∆F = 2.5\%\).

With increasing inbreeding coefficient the gene pool of a random bred colony will correspondingly decrease. The rate of this process is accelerated when the breeding population of the colony has been temporarily reduced in size, e.g. when a conventional colony is rederived into an SPF colony by hysterectomies or embryo transfers (bottle-neck effect).

If offspring of as many parents as possible is being used for future breeding purposes, then the reduction of the colony’s gene pool will be kept to a minimum. Mating may be at random, or better, should be arranged in such a way that breeding partners are only distantly related. Rotation schemes, which have been designed for this purpose, may reduce the increase of the inbreeding coefficient by 50%. Thus, consistent breeding according to a rotation scheme may result in a reduction of \(∆F (1/(4N))\) instead of \(1/(2N)\) per generation.

It will be clear that the reproducibility of the results of successive experiments will be limited when animals are used from a random bred colony, or from a population, which has been propagated by a system of minimal inbreeding. The gene pool will gradually change over time and eventually it will become impossible to compile a group of test animals with (exactly) the same genetic background as previous experimental groups.

If a random bred colony is maintained as a closed population for at least four generations, and if the number of breeding animals is sufficient to guarantee that \(∆F < 1\%\), then the colony may be designated as an outbred stock. An outbred stock is not subject to artificial directional selection for any characteristic, apart from breeding performance. Following the rules of nomenclature, the designation of an outbred stock consists of a symbol indicating the current breeder/holder of the stock, followed by a colon and the stock symbol consisting of between one and four capital letters (e.g. Ola:SPRD; Han:NMRI).

Mosaic population: inbred strains and their \(F_1\) hybrids

In case of experiments that require consistent genetic variation but take place over an extended period of time, the use of an outbred stock is inadequate due to the gradual change of the gene pool. The genetic variation can be standardized by using reciprocal crosses of a number of inbred strains (see table 7-1 and Cholnoky et al.). At least 10 different genotypes can be produced with
Genetic variation

four inbred strains A, B, C and D. In general terms, \( n + \frac{n^2 - n}{2} \) different genotypes can be produced (\( n \) = number of inbred strains). Reciprocal F\(_1\) hybrids such as (AB)\(_{F1}\) and (BA)\(_{F1}\) are considered identical, unless we are dealing with sex-linked genes or with genes of which the phenotypic expression depends upon the parent transmitting it (parental imprinting). A “mosaic population” (population of inbred strains and their F\(_1\) hybrids) will result. A group of test animals and a group of control animals with identical genetic variation can be established by taking one or two (one of each sex) animals from each entry of the breeding scheme. The same genetic variation can be generated at any later stage, provided that the authenticity of the inbred strain is guaranteed. This requires an accurate breeding programme, and a monitoring system for controlling the genetic quality of the inbred strains.

Genetic quality control

Genetic contamination, which is the inadvertent out-crossing (miscegenation) of an inbred strain, can go undetected for some time. In the case of miscegenation occurring between two different albino inbred strains, the phenotypes of the offspring cannot be distinguished from the parental phenotypes. Several reports have been published indicating that certain inbred strains have become genetically contaminated over time. It has been found that up to 20% of the strains which had been tested were genetically contaminated, or were designated with wrong names.

It is imperative to check both uniformity and authenticity of inbred strains regularly. Genetic monitoring should be a common practice to all who breed and maintain defined strains. Quite often, researchers rely merely on published data of strains with identical names.

Table 7-1

Standardization of the genetic variation by hybridizing inbred strains (mosaic population). A, B, C and D are inbred strains.

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Strain uniformity can be tested by skin grafting. The acceptance or rejection of grafts will largely depend upon the compatibility of the MHC (major histocompatibility complex) haplotypes of the donor and the recipient. Although isohistogenicity is a sensitive method to verify uniformity, it is certainly not the method of choice to determine authenticity.

The establishment of appropriate systems for monitoring both uniformity and authenticity requires the description of genetic strain profiles. Originally, such profiles were based on biochemical/immunological markers and, depending upon the strains maintained, also on morphological, pathophysiological and cytogenetic markers.

However, in recent years, DNA markers, based on variation (polymorphism) in the sequence of nucleotides, have widely replaced the former markers. DNA markers can be subdivided into Type I and Type II markers. Type I markers are based on polymorphisms in functional genes that have been localized in different mammalian species, whereas Type II markers are based on polymorphisms that occur in parts of the DNA of which the function is not (yet) known. For the genetic characterization of inbred strains Type II markers and, in particular, those markers that are polymorphic in the introns (non-coding regions of the gene) are most frequently used. There are several kinds of Type II markers e.g. SNPs (single nucleotide polymorphisms), RAPDs (random amplified polymorphic DNA) and AFLPs (amplified fragment length polymorphisms), but for the genetic characterization and quality control SSLPs (simple sequence length polymorphisms) are presently the markers of choice. The polymorphism of SSLP markers is based on variation in the length of microsatellites. These are short stretches of DNA, each consisting of multiple repeats of 2–5 nucleotides. In most mammalian species there are many microsatellites (up to 100,000) randomly distributed over the genome. Since most of these microsatellites are located in non-(protein)coding regions, they are highly polymorphic (differences in length, due to different number of repeats). Combined with the fact that, for most microsatellites, this polymorphism is rather stable, these are perfectly suited markers for use in genetic monitoring programmes. The differences in length (allelic variants) are visualized through PCR amplification of the microsatellite and subsequent electrophoretic separation of the amplified fragments (see fig. 7-6). The fragments (PCR products) can be visualized through fluorescence or through autoradiography.

Simultaneous amplification and electrophoresis of multiple microsatellites (‘multiplexing’) can increase the efficiency of this process.

It is thus possible to establish a genetic profile, unique for a given strain, based on the allele distribution of a given set of protein and/or DNA markers. These profiles can then be used to test for authenticity and uniformity of the strains.
Genetic quality control

Monitoring procedures for testing uniformity and authenticity of inbred strains need to be repeated at regular intervals. The genetic stability of a colony can be determined through routine verification of the genetic profile of old breeding pairs, or by screening a representative sample of an inbred strain every third or fourth generation.

The genetic profile should ideally include markers covering the entire genome. In reality, only part of the genome will be covered in genetic monitoring programmes. Therefore, the stability of a genetic profile should not be interpreted as if the entire genome had remained unchanged. Genetic quality control is not aimed at tracing minor changes, but is executed primarily to detect genetic contamination at an early stage.

Genetic relationship between inbred strains

The origin of an inbred strain and the interrelationship of different inbred strains is not always known. However, it may be essential to know the origin of a
strain or to be able to determine the genetic distance between strains, e.g. in comparative tests, or when crosses between distantly related strains are to be set up. In general, strains with similar genetic profiles (strain distribution patterns, SDPs) are likely to be more closely related than strains with SDPs that differ to a large extent. Therefore, comparison of the SDP of inbred strains may provide information that can be allowed to estimate the relationship between inbred strains (fig. 7-7).

**Cryopreservation**

Mammalian embryos can be preserved through the use of special techniques involving freezing and storing in liquid nitrogen. This is referred to as cryopreservation. This technique offers the possibility of storing embryos in a “bank”. Embryo banking is considered to prevent the extinction of valuable inbred strains that could occur as a result of a fatal disease or an accident. It is also a mean to circumvent genetic drift and to ensure constant availability of strains without the need for maintaining these strains as vital breeding nuclei. It is presently even more important as a procedure to cope with the growing number of genetically modified strains and shortage of housing facilities to maintain all strains that carry a transgene or a targeted mutation.
Cryopreservation

After mating, pre-implantation embryos are collected from pregnant females and are subjected to a defined procedure of freezing (slow freezing; fast two-step freezing; vitrification). Embryos are then stored in liquid nitrogen (–196°C). After thawing, the embryos may be implanted into a recipient female. The survival rate of embryos depends upon the vigour of the strain, and on the procedures employed for freezing and thawing. Generally speaking, not more than 20–30% of frozen embryos will develop into live foetuses following implantation.

This technique can also be used to start a germ-free or SPF-colony (see chapter 8) or to transfer a strain from one laboratory to another. Re-derivation of a strain via embryo transfer excludes the chance of intrauterine contamination during pregnancy, which is reported to occur for a variety of bacterial and viral infections.

Literature

Genetic standardization
8 Microbiological standardization

R. Boot, J. P Koopman and I. Kunstýř

Introduction

Microbiological quality assurance of laboratory animals aims to produce animals that meet with preset requirements of microbiological quality, and the maintenance of this quality during the experiments. Microbiological quality assurance is a prerequisite for microbiological standardization of laboratory animals.

Reasons for microbiological quality assurance of laboratory animals

Disease and mortality in laboratory animals

The occurrence of outbreaks of infectious diseases in groups of animals makes apparent a reason for paying attention to their microbiological quality. In rodents and rabbits, the most commonly used laboratory animal species, infectious pathology is frequently found in the respiratory tract, the intestinal tract and the liver. These organ systems can be affected by several groups of microorganisms such as viruses, mycoplasmas, bacteria and parasites. Individual species, or combinations of microorganisms, can cause high morbidity and mortality. Different strains of an animal species can show genetically based differences in their susceptibility to infectious pathology from any given microorganism. Contamination by mousepox virus is, in some mouse strains, followed by lethal infection, but other strains are almost totally resistant to clinical disease (see chapter 9). Lewis rats for example are more susceptible to *Mycoplasma pulmonis* infection than are Fisher (F344) rats. Several genetic factors (Ity, Lsh and xid) influence the susceptibility of mice to *Salmonella* infection. Similarly, C57BL/6 mice are more susceptible to *Streptobacillus moniliformis* infection than other strains.
Contamination is not necessarily followed by clinical symptoms (latent or subclinical infection). The number of microorganisms considered to be (potentially) pathogenic for laboratory animals, amounts to several dozens (table 8-1) and every year ‘new’ pathogens, notably viruses are being discovered. Studies on the pathogenesis of viral and bacterial respiratory and intestinal infections, have shown that, generally, contamination with both a virus and a bacterium is necessary before a clinical disease will develop. Rodents can carry *Pasteurella pneumotropica* or *Mycoplasma pulmonis* for their whole life without showing clinical symptoms. During Sendai virus infection, however, they are at serious risk of dying from secondary bacterial pneumonia. Respiratory viruses presumably inhibit various activities of the lung macrophages, which as a result are no longer able to remove inhaled bacteria from the lower airways. Severe pneumonia is likely to develop. Non-respiratory viruses can also induce a secondary bacterial infection in the respiratory tract. Reo 3 virus infection, which causes among other things hepatitis in rodents, can also lead to an impairment of the local immunological defense system of the lungs. Non-microbiological factors may also have a role in the development of clinical disease. Relatively high concentrations of ammonia can inhibit ciliary movement in the respiratory tract, and thereby limit the elimination of microorganisms.

The association of microorganisms with disease and mortality in laboratory animals, has made a considerable contribution to the introduction and use of animals that are free of specified (potentially) pathogenic microorganisms (Specified Pathogen Free or SPF animals; see page 160).

### Interference with results of experiments

Most viral, bacterial and parasitic contaminations do not lead to overt clinical symptoms, but to latent infection. However, these latent infections can have a considerable impact upon the outcome of animal experiments. The subtle effects of latent infections became apparent with the increase in the use of SPF animals. This reduced the frequency with which disease outbreaks occurred.
There are numerous examples of influences of microorganisms on the physiology of the laboratory animal and hence of the interference of latent infections with results of animal experiments. For instance, Sendai virus infection, which is associated with a decreased B-cell and T-cell response after antigenic stimulation. The virus enhances the production of interferon, and decreases the serum level of the 3rd complement factor (C3). Infection by mouse hepatitis virus (MHV) is, amongst other things, associated with the suppression of the phagocytic activity of the reticulo-endothelial system. The virus inhibits the cytotoxic activity of lymphoid cells, and induces a clear increase in the serum levels of a number of liver enzymes such as aspartate-transaminase (ASAT) and alanine-transaminase (ALAT). Lactate dehydrogenase elevating virus (LDHV), which is one of the most frequently observed contaminating viruses of transplantable tumours of laboratory animals, induces a serious increase in the lactate dehydrogenase and corticosteroid levels in plasma, and delays the rejection of skin transplants. These examples illustrate that latent infections can influence the outcome of animal experiments. It is essential, therefore, that the presence of microorganisms which might modulate the animal’s response during experimentation, is known.

Zoonoses

Some of the microorganisms which may be present in laboratory animals, can also affect man (zoonoses). Zoonoses can stem from members of all groups of microorganisms and zoonoses can present themselves along the range from subclinical to lethal infections. Conventional animals are the most likely to harbour microorganisms which can cause disease in man. Laboratory animals that have never been rederived by hysterectomy and animals that have been trapped in the wild, should be considered as potential reservoirs of zoonotic microorganisms. It is rather unlikely for SPF animals to be contaminated by these microorganisms. However, zoonotic microorganisms have been detected in rederived animal populations, e.g. \textit{Streptobacillus moniliformis}, a bacterial species which can be cultivated from the nasopharynx of healthy conventional rats. In man, \textit{S. moniliformis} can cause rat-bite fever if transmitted through an animal bite, or Haverhill fever if transmitted via contaminated water or milk. Untreated rat-bite fever can be a fatal disease. The occurrence of the bacterium in laboratory animals is possibly underestimated. \textit{S. moniliformis} is quite difficult to cultivate and serological methods for the detection of antibodies to the bacterium in rats are not yet in common use, although they are available.

Trichophytosis which occurs quite frequently, is a fungal zoonosis, mainly caused by \textit{Trichophyton} sp. and \textit{Microsporum} sp., and can be present in a variety of mammals. The infection is mostly subclinical. In man, trichophytosis
manifests itself as a localized skin disease, with circular or ringlike skin lesions (ringworm).

An important latent infection in rats is Hanta virus infection. The virus is transmitted via respiratory and intestinal secretions and excretions, and via the urine. Man can become easily contaminated through both direct and indirect contact with contaminated animals, biological products, materials and equipment. Hanta virus infection in humans can present itself as a serious acute interstitial nephritis (haemorrhagic fever with renal syndrome – HFRS), causing the complete loss of the functional capacity of the kidneys, and can occasionally lead to death.

Laboratory animals that have been experimentally inoculated with zoonotic microorganisms, are certainly a source of human pathogenic microorganisms, but risks are limited by the use of preventive hygienic measures (see below).

**Quality of biological products**

The risk of zoonotic infections has led to quality requirements for products that are made with the use of laboratory animals. All sera, vaccines and other biological products to be used in humans have to be safe. It is obvious that the administration of, for example, live or attenuated viral vaccines, which are produced in cells of contaminated animals, can result in zoonotic infections, despite having taken stringent purification steps. Therefore, the production of vaccines and other biological products is governed by strict precautionary regulations. These ‘Good Manufacturing Practise’ (GMP) rules, aim to fully control all production steps, finally resulting in a product of a good, i.e. safe, quality. Laboratory animals that are used for production purposes, must originate from an animal colony that is free from a number of specified pathogenic microorganisms, notably those which have zoonotic potential.

SPF animals are also used in vaccine control experiments. These experiments are performed to assess the potency and safety of the given product. Safety testing of, for example, viral vaccines is amongst other things, aimed at excluding the presence of extraneous viruses, including human pathogenic ones. The experiments are performed by injecting laboratory animals with the vaccine and examining their sera for antibodies to various undesired viruses. During the study, intercurrent contamination of the animals from other sources must be excluded.

**Sources and paths of contamination**

Laboratory animals can become contaminated with (potentially) pathogenic microorganisms from several sources and through different paths. The most
Important sources of contamination are other laboratory animals, biological materials derived from them, pet animals, staff and materials and equipment used for breeding and experimentation.

**Laboratory animals**

Contaminated laboratory animals are an important source of undesired microorganisms. Despite all the efforts made to maintain their microbiological quality, buying animals from breeding colonies frequently causes the introduction of (potentially) pathogenic microorganisms into a laboratory. The frequency with which the various (potentially) pathogenic microorganisms occur in animal colonies varies with time. However, in general, two groups of undesired viruses can be distinguished: those which are only rarely reported, and those which occur widely in animal colonies. Examination of animals for the presence of microorganisms belonging to the latter group, is very useful for gaining a quick first impression of the animal’s microbiological quality.

In comparison with breeding colonies, in experimental colonies less attention is generally paid to preventive hygienic measures. In experimental colonies, animals from different sources are often housed together. In addition, experimental laboratories, as a rule harbour experiments which overlap each other in time, thereby maintaining infections which have been introduced. This implies, therefore, that animals in an experimental laboratory are quite likely to contain several (potentially) pathogenic microorganisms. The introduction, therefore, of animals from another experimental colony brings with it the serious risk of introducing contamination. Most of the experimental colonies are generally not monitored as of yet, for the presence of undesired microorganisms.

**Biological material**

In modern biomedical research, laboratory animals are frequently used as a source of biological material such as sera, ascites, cells, tissues and organs. Animals are also used as a source of microorganisms, that can to date not be cultivated *in vitro*. Laboratory animals can become contaminated by the administration of biological materials which have been derived from contaminated animals.

In principle, biological materials can contain the same, notably intracellular microorganisms, that are present in live animals. Most contaminations are viral, but also mycoplasmas and intracellular bacteria, including *Clostridium piliforme*, have been found to be contaminants. Studies in the USA showed that more than 90% of the transplantable tumours and more than 70% of the viral reagents examined, were contaminated by one or more
viruses. Contamination with several infectious microorganisms can result from serial passage of biological materials in animals. As a result of serial passage, a tumour cell line can become consecutively contaminated with several contaminants present in the animals used. Serial passage of a *Treponema pallidum* strain, for example, that was presumably contaminated with a Coronavirus, was accompanied by intercurrent death (up to 40%) in rabbits. The introduction of biological materials is presently the most important cause of outbreaks of Ectromelia virus infection.

The risk of a zoonosis also exists when biological materials are used *in vitro*. Homogenization of contaminated tumour material has led to Hanta virus infection. Contamination by Lymphocytic choriomeningitis (LCM) virus has been detected in *Toxoplasma* strains that were passed on serially in mice.

The presence of contaminating microorganisms in biological materials can be detected by the examination of sera from animals that were previously injected with the material (e.g. Mouse Antibody Production test or MAP test). Contamination of the animals from other sources during the study must be excluded. Therefore, antibody production tests should be performed preferably within isolators.

*Pet animals*

Pet animals can constitute a serious risk of contamination for laboratory animals. For those individuals involved in the care and treatment of laboratory animals, the keeping of pet animals, especially of the common laboratory animal species, must be discouraged. In some breeding colonies, the keeping of pet animals by animal technicians and staff and their families is forbidden. This seems reasonable, given that contamination has serious (financial) consequences for the breeder.

*Personnel*

Despite the above mentioned preventive hygienic measures, man is, without doubt, the most important factor in the transmission of contaminating microorganisms between groups of animals. Man can act as a vector after contact with contaminated animals, and can also temporarily be the host of microorganisms that are (potentially) pathogenic for laboratory animals.

It is advisable to have separate personnel working with laboratory animals of different microbiological qualities. For instance, those involved in breeding should not enter experimental laboratories. Even personnel who do not have contact with contaminated laboratory animals, can be a source of contamination. Tuberculosis for example can be transmitted to almost all mammalian species. The transmission of, for example, *Salmonella* species and
Sources and paths of contamination

*Campylobacter jejuni*, as a result of food poisoning, is more likely. Several conditions in man, especially those showing an increased spread of (potentially) pathogenic microorganisms, increase the risk of contaminating laboratory animals, e.g. diarrhoea, skin rashes, chronic respiratory disease.

Individuals who carry and shed *Salmonella* species and *Staphylococcus aureus* can initiate some problems. SPF animals are more susceptible to staphylococcal disease than conventional ones, due to their rederivation and the consequent loss of their ‘normal’ microflora (see below).

One may presume that various microorganisms which are pathogenic to man are not able to propagate well in laboratory animals on account of the species barrier. However, contamination by these microorganisms can cause a small group of the animals to temporarily develop antibodies against them. These antibodies may react with antigens that are used for the serological monitoring of laboratory animals (see below).

Materials and equipment

Materials and equipment can serve as a vector in the contamination process. Food and bedding can become contaminated by wild rodents at times of production, harvesting and storage. The animals’ bedding can be sterilized by autoclaving. Their food is usually supplied in pelleted form. During the pellet-making process, the temperature rises up to 70–80°C, which kills off most of the microorganisms. In short-term experiments, pelleted diets are used without further treatment. Food to be used in SPF breeding units has to satisfy more stringent demands and is often additionally treated by gamma-irradiation or by autoclaving, in order to eliminate all (potentially) pathogenic microorganisms (pasteurization).

Water can also be a source of contamination for laboratory animals, for example *Pseudomonas aeruginosa*, which can lead to infection, whilst the animals’ resistance is low as a result of for example sublethal total body-irradiation. Bacterial growth in water bottles can be prevented by acidifying (pH 2–3) or by chlorinating (15–20 ppm active chloride) the drinking water.

The cages and surgical equipment used, can also act as a vector for contamination, but they can be sterilized or disinfected (see below). Generally speaking the air is filtrated. There are various types of filters in use. When air has to be sterilized, the so called HEPA filters offer the best results.

Microbiological qualities

Laboratory animals can be arbitrarily classified according to a number of different microbiological qualities (fig. 8-1). The first animal experiments
Microbiological standardization were performed upon conventional (CV) animals. These animals can harbour ‘by nature’ the whole range of infectious microorganisms, since they are kept without the application of preventive hygienic measures. At the other end of the quality scale, germfree (GF) animals exist. These animals can be obtained from CV counterparts by performing a hysterectomy (rederivation) and are kept under sterile conditions within isolators. GF animals are very susceptible to infections by microorganisms that are only rarely the cause of disease in CV animals. Therefore GF animals can not be housed without problems under conventional conditions. To provide GF animals with some resistance to opportunistic infections, they are deliberately given a flora, which provides the formerly GF animal with a general resistance to the growth of other bacteria (colonization resistance). An example of this approach is the use of mouse derived enteric colonization resistant (mCRF) flora. CRF animals are housed as GF animals within isolators and some animal species will even breed in this microbiological status. Frequently CRF animals are used to start SPF breeding colonies (see

Fig. 8-1. Quality-barrier system: relationship between microbiological qualities of laboratory animals and their barrier systems.
Microbiological qualities

For large-scale breeding, CRF animals are introduced into units where a wide range of protective hygienic measures, aiming at preventing undesired microbial contaminations (barrier system), are operational.

Gnotobiotic animals

Gnotobiotic animals harbour a ‘fully’ known microflora and/or fauna (gnotos = known; biota = flora and fauna), and are therefore microbiologically standardized animals. If no detectable microorganisms are present, the animal is considered to be GF. Both GF animals and CRF animals having one or more known microorganisms, are gnotobiotic animals. CRF associated animals are also said to be gnotobiotic animals. The exact composition of the flora of CRF animals is unknown, but they are housed under the same conditions as their GF counterparts.

Rederivation.

This is a technique which is used to create a nucleus of animals for starting a colony free from (potentially) pathogenic microorganisms. Rederivation of laboratory animals is mainly performed by an aseptic hysterectomy on pregnant mothers, prior to normal delivery. During this procedure the closed uterus is removed from the donor-animal, and aseptically introduced into a sterile isolator passing through a dunk tank, containing a disinfecting solution. Finally the young are born by opening the uterus. Rederivation can also be performed by Caesarean section, in which the uterus is opened in situ and the young are isolated immediately after operative birth. Animals obtained by hysterectomy or through Caesarean section are reared by hand or are fostered by lactating animals (foster mothers). Rederivation is also possible through embryo transfer to uninfected animals of the same species.

Since the early sixties the most common species, i.e. mouse and rat, and related strains, have been rederived. Rederivation of other animal species has been done on a limited scale only. This is due partly to economic reasons but also to zootechnical problems. Rabbits for instance, have to be raised by hand. Rederivation of hamsters and gerbils has been found to be extremely difficult, and many attempts have failed due to enteric infections.

Vertical transmission of microorganisms.

Hysterectomy or Caesarean section mostly yields GF animals. However occasionally the offspring is found to be contaminated with one or (more rarely) several microbial species, which have been transmitted during pregnancy to the young, through the placenta or via another means (vertical contamination). Vertical transmission, i.e. passing on to the next generation, can occur if the mother suffers from an active infection during pregnancy, accompanied by the presence of microorganisms in the bloodstream and concomitant penetration of the placental barrier.
Autochthonous or normal microflora. In conventional animals, the skin and the mucous membranes of the mouth, the respiratory tract, the urogenital system and the gastro-intestinal tract, harbour apathogenic, so called ‘normal’ or autochthonous flora. This flora contributes to the animals’ resistance against (potentially) pathogenic microorganisms. This microbial based resistance to colonization by pathogens has been described as bacterial antagonism, bacterial interference and colonization resistance.

The number of bacterial species which make up the normal flora is unknown. It is estimated that the mouse intestinal flora may contain 500 species. Bacterial counts in the intestinal contents can be up to $10^{10}$ to $10^{11}$ per gram. Which bacterial species inhabit other parts of the body is largely unknown. The intestinal bacterial flora lives in a very intimate fashion with its host (fig. 8-2), and both host and bacterial flora benefit from this relationship.

Fig. 8-2. Mutualism between intestinal bacterium and enterocyte.
Microbiological qualities

The importance of this mutualism for the host is apparent when making a comparison between CV and GF animals.

Anatomical and physiological characteristics. GF animals show a variety of morphological and physiological ‘abnormalities’, when compared with CV animals. Intestinal parameters show striking differences between the two groups. In GF animals the caecum is considerably enlarged and its contents are rather liquid. The intestinal wall is thin and less well developed than that of CV animals. GF animals produce faeces which have a soft consistency.

An inevitable consequence of rederivation via hysterectomy, is the loss of all ‘normal’ flora. To compensate for this loss of autochthonous (notably intestinal) flora, GF animals were administered with aerobic and facultatively anaerobic bacterial species. This approach very often gave bad results. In GF guinea pigs that were associated with *Escherichia coli*, *Streptococcus fecalis* and lactobacilli, 75% of the animals died. Most animals were lost from enteric infections due to the bacterial species administered to them.

Since more than 99% of the ‘normal’ enteric flora of animals consists of strictly anaerobic bacteria, GF animals have also been associated with such anaerobes. The so called Schaedler flora, which has been given to mice and rats, partly consists of such anaerobic bacteria. Animals given such a flora have, however, been found to be rather susceptible to enteric infections. The bacteria which can be cultivated from the affected gut are aerobic and facultatively anaerobic species, which are only rarely present in infections in CV animals. Infections by these opportunistic pathogens, e.g. *S. aureus* and *P. aeruginosa*, can be found in CV animals, but only after debilitating treatments such as sublethal body-irradiation or the administration of antibiotics, have taken place.

In SPF breeding colonies, opportunistic infections usually increase in frequency some time after the establishment of the colony. The number of infectious processes can increase rapidly, but problems usually gradually disappear. This is presumably due to the introduction into the colony of bacterial species which partly contribute to colonization resistance in the intestinal tract and other parts of the body.

Strictly anaerobic flora has been used in different laboratory animal species. For mice and rats, a mouse derived enteric flora (mCRF), was sufficient to ‘normalize’ the GF animals. In both species the size of the caecum was clearly reduced and an acceptable colonization resistance was also obtained.

The autochthonous enteric flora is at least partly species specific. In rodents and rabbits, species specific flora has been found to ‘normalize’ GF abnormalities better than flora from another animal species. The next few
years will certainly see more attention paid to the development and improvement of species specific (enteric) flora. Such flora is a prerequisite for the development of microbiologically fully standardized animals.

*Use.* Gnotobiotic animals are used for different purposes. One such an application is the production of viral vaccines. Animals which are to be used as a donor of cells to be used for the production of vaccines for use in humans, have to be taken from animal colonies that are free from adventitious infections. Gnotobiotic animals meet with this requirement. Gnotobiotic animals which have been associated with a more or less complex enteric flora, can be used to study the role of bacteria or bacterial species in the transformation of orally administered compounds (biotransformation). Another example is the study of the effects of sublethal total body-irradiation or other immunosuppressive methods applied during cancer research. This type of research is almost impossible to perform using conventional or sometimes even SPF animals, due to disturbing opportunistic infections. Research on intestinal ecology, including the pathogenesis of infections, the role of the local intestinal immune system and the ‘normal’ flora, would not be possible without the use of gnotobiotic animals.

*SPF-animals*

Specified pathogen free (SPF) animals are those which have been found to be free from a number of specified (potentially) pathogenic microorganisms. SPF animals are not kept under conditions guaranteeing a full separation from the environment. Therefore, the animals used to start the colony (mostly CRF), will be colonized in time by a wide variety of microorganisms. Some of these microorganisms will inevitably be introduced by animal caretakers. Colonization is not of itself a problem, as long as the animals are not colonized by undesired microorganisms. It could be argued that the more heavily the animals are colonized by apathogenic species, the more likely it is that species will be present which contribute to the resistance of the animal to opportunistic pathogens.

SPF animals can hardly be considered to be standardized animals. The most one can expect is that examination of the animals for the presence of (potentially) pathogenic microorganisms, will show which species are presumably not present within the colony. With the lifetime of the SPF colony, the anatomical and physiological characteristics of the animals will gradually ‘normalize’ towards those of the CV animal.

*Use.* There are various reasons why SPF animals are used; for example, the testing for the safety of products, and the performance of animal experi-
Microbiological qualities

ments with no interference from infections (ultimately all experiments). The duration of experiments partly determines the likelihood of contamination. Long-term experiments are at greater risk than short-term ones. The use of SPF animals in very expensive (semi)chronic toxicological studies is strongly advised. Ageing-research (gerontology) clearly is also a field in which SPF animals have to be used. The median survival time (MST) of rats, and hence the frequency of tumours in the animals, can be seriously affected by intercurrent infections. The life-span of SPF animals generally exceeds that of conventional animals. SPF animals are also used in studies in which the animals’ immunological capacity is decreased by immunosuppressive regimes, or by its genetic constitution, as in T-cell deficient nude animals and both T- and B-cell deficient SCID (Severe Combined Immuno Deficient) mice.

Conventional animals

Conventional animals are still widely used in biomedical research. They are suitable for particular types of experiments. Animals should be considered as conventional animals, if their microbiological status is unknown or questionable. As a rule, these animals (notably rodents and rabbits), are ‘quarantined’ for a period of time. The length of this period is based upon the longest incubation time required for excluding infections. This implies the expectation that the ‘quarantine’ period would reveal certain clinical symptoms in the animals. However, most infections in laboratory animals are latent. ‘Quarantining’ of animals could be used as part of a strict quality assurance programme. Release of the animals from ‘quarantine’ should then be based upon the results of screening and diagnostic examinations performed on them, not yielding any indication of the presence of specified pathogens. Screening is time-consuming and costly, and often animals have to be sacrificed for a thorough examination. Therefore this approach is only rarely applied. A better alternative seems to be to perform experiments in ‘quarantine-units’ (isolators), or to use the period merely for acclimatization. Dogs and cats for example could be vaccinated and de-wormed during such a period.

Barrier systems

The barrier concept is a basic principle in the quality assurance of laboratory animals. A barrier consists of a wider or lesser range of preventive hygienic measures. The range will depend upon the microbiological requirements set for the animals.
Microbiological standardization

Absolute barrier (isolator)

The microbiological status of gnotobiotic animals can be maintained only if the animals are kept fully separated from the environment. For this an absolute barrier (isolator), is required. Several types of isolators have been developed. All types, including the most commonly used Trexler (plastic) and Gustafsson (steel) isolators, have a similar construction (fig. 8-3). All equipment, such as cages and materials, food and bedding, are sterilized and introduced through a dunk tank or via a lock which can be sterilized using peracetic acid vapour.

Although CRF animals are not gnotobiotic, since the composition of their flora is not exactly known, they must be kept under gnotobiotic conditions to prevent the introduction of other flora. CRF animals that are maintained in isolators for any length of time, seem for some reason, to loose part of their flora.

Classical SPF-barrier

The preventive hygienic measures taken to protect SPF animals in breeding units, are less strict than those for gnotobiotic animals. Here only the introduction of (potentially) pathogenic microorganisms has to be prevented, so
Barrier systems

Disinfection instead of sterilization, of materials and equipment will (theoretically) be sufficient. Generally, however, sterilization of materials and equipment is carried out, since it is by far the safest. Special care is taken with personnel. The number of people entering the unit, is limited to the absolute minimum. Taking a shower is considered essential before commencing work within the ‘clean’ area. Although showering increases the shedding of resident bacteria from the skin, it also removes the far more important transient (potentially) pathogenic microorganisms. Sterilized working clothes, a mouth mask, gloves etc., should be worn. Within the ‘clean’ area, positive pressure is maintained towards the environment.

Modified barrier systems

The preventive measures which aim at protecting SPF animals during experimentation, are derived from those of the classical barrier system. Which measures are taken will depend upon insights into the risks of contamination and according to the consequences of a barrier breakdown. For long-term toxicological experiments, the barrier system may imitate the classical SPF-barrier, but in short-term pharmacological experiments, preventive measures need hardly be taken. Animals can be housed in cages provided with protective hoods (filter top, fig. 8-4), which are only removed when the animals have to

Fig. 8-4. Filter top cage.
be handled. These cages are preferably only opened within Laminar Air Flow (LAF) cabinets.

**Reversed classical barrier (isolation-units)**

Sometimes the environment has to be protected against contamination by microorganisms carried by the animal. Animals can be housed in a barrier system (infection unit), which is actually a ‘reversed’ version of the classical barrier system. Waste material is disinfected and personnel take a shower upon leaving the area. Microorganisms can be classified into ‘pathogen groups’. There are different classifications in use, but all are based upon the potential consequences of contamination in man. For each pathogen group, a series of containment or restrictive measures are operational (physical containment level). For work with harmless microorganisms, no special measures are necessary. Animal experiments in which microorganisms are involved, that can have serious consequences for individuals, but not for the community, such as HIV, can be performed within a gnotobiology department. Animals are kept within isolators which have a negative pressure compared with the environment. Exhaustive air is filtered until sterile. If very dangerous pathogens are involved, i.e. microorganisms that constitute a serious risk to the community, there must be absolute certainty that no single microorganism reaches the environment. Experiments with, for example, the hemorrhagic fever associated viruses, must be performed in separate buildings. Laboratory animals used for these experiments are housed within isolators, in order to guarantee a full physical separation between the research personnel and the microorganisms.

Experimental infections with serious laboratory animal pathogens, such as Ectromelia virus, are also performed under strict conditions providing a containment of the microorganisms (isolators).

**Individual barrier-measures**

Basically all measures are aimed at reducing, as far as possible, the number of (potentially) pathogenic microorganisms that could be passed to the animals through contact with personnel, materials or equipment. A variety of methods involving one of the following approaches such as cleaning, disinfection and sterilization, can be used.

*Cleaning.* Animal rooms, cages, drinking bottles etc., should be periodically cleaned. Animal rooms are cleaned using large quantities of hot water, if possible with the aid of high-pressure equipment. Soiled bedding is removed from cages, which are then cleaned in a special type of washing machine. The washing-steps, using hot water and detergents, are aimed at removing any
remaining dirty material. Drinking bottles are thoroughly cleaned by rinsing and scale is mechanically removed. The most important purpose of the cleaning is a reduction in the number of microorganisms. Cleaning makes disinfection and sterilization more effective.

Disinfection and sterilization-methods. For the elimination of (potentially) pathogenic microorganisms, i.e. disinfection, or of all living microorganisms, i.e. sterilization, essentially the same approaches are used. Disinfection needs less intensive treatment than sterilization. If it is possible to apply both approaches, then sterilization is preferable. The disinfection methods do not guarantee the killing of all (potentially) pathogenic microorganisms.

Physical and chemical methods exist for disinfection and sterilization. Food can be disinfected using 0.9 Mrad gamma-irradiation, whereas 2.5 Mrad is needed for sterilization. Whether the intended goal can be achieved or not, will depend upon the number of microorganisms initially present, since a particular treatment will lead to a fixed decimal reduction in the number of microorganisms. Upon heating, the intensity, i.e. the combination of time and temperature, will determine whether disinfection or sterilization results. Disinfection will result from short-term heating at, for example, 70°C, whereas sterilization will need more intensive heating under positive pressure at 121°C.

The ease with which microorganisms are killed varies according to the particular treatment. The decimal reduction time is the time needed to reduce the number of microorganisms by a factor of 10. This time also differs according to the method applied.

In chemical disinfection (decontamination), the killing of microorganisms is determined by several factors, such as the concentration of the disinfectant, the temperature and pH during treatment and the presence of organic material. The disinfectant of choice, will depend upon the nature of the material that has to be treated, and on the groups and/or species of microorganisms to be eliminated. The working spectrum of the various groups of disinfectants such as alcoholic solutions, halogens, phenols, aldehydes and biguanides, differs. Halogens whose activity is based, for example, on free chlorine, are effective against several groups of microorganisms, but biguanides, such as chlorhexidin, are only active against vegetative bacteria (except mycobacteria). Peracetic acid is a very potent antimicrobial substance and can be considered as a sterilant. Peracetic acid vapour is commonly used in gnotobiotic technology. Chemical disinfectants and sterilizing agents have to be used with care as all substances (of course depending on their concentration), are basically harmful to mammalian species, including man.

The effect of cleaning and disinfection can be monitored using agar plates, on which the number of bacterial colonies can be counted, after an appropriate incubation. Autoclaving can be monitored by recording temperature,
Microbiological standardization

pressure and humidity which were present during treatment. The process can also be monitored by the simultaneous autoclaving of strips showing a temperature dependant colour-change. Spore strips which are incubated after processing, can also be used.

Quality control

Microbiological quality control can be divided into the control of the barrier system and the control of the laboratory animal. The latter is dominant in many laboratories, where it is assumed that the absence of contamination in the animals will indicate that the integrity of the barrier system has been fully maintained.

Control of the barrier system (process control)

The series of preventive hygienic measures constituting the barrier, can be monitored using both microbiological and physical methods. Autoclaving can be monitored by examining whether test organisms were killed by the treatment or not, but also by recording the time, temperature and pressure. The number of microorganisms present in food, air and on surfaces can be estimated. Air pressure in different areas can be recorded and can, in the case of deviation from preset requirements, be reported and even be automatically adjusted.

Control of the laboratory animal (product control)

The examination of animals for the presence of (potentially) pathogenic microorganisms is of considerable importance. Most attention is paid to the periodic examination of healthy animals (screening). Postmortem examinations are performed on diseased and dead animals (diagnosis), to clarify the various causes. In the event of a presumed microbial involvement in disease problems observed, the microorganism(s) could be sought for using a ‘direct’ approach, e.g. by microscopy or cultivation. An alternative approach could be the examination of sera for antibodies to these microorganisms (serology). The various methods used for screening and diagnosis in laboratory animals, are summarized in table 8-2. The examination of laboratory animals for the presence of the various (potentially) pathogenic microorganisms should preferably be left to the specialist.

Gnotobiotic animals. Gnotobiotic animals are animals with a ‘completely’ known microflora. This is an operational definition, since this ‘complete-
Quality control

ness’ depends upon the examination methods used. This means that, if the methods used did not reveal the presence of microorganisms, or only revealed those deliberately given to the animal, the animal is considered to be gnotobiotic.

The microbiological examination of gnotobiotic animals which are to be used to start SPF colonies, can vary. If animals have been obtained by rederivation from an SPF colony that did not contain undesired microorganisms, the likelihood of vertical transmission of these microorganisms is, of course, very low. If rederivation was performed on CV animals, the examination must be much broader to detect a wide range of microorganisms, since numerous species can be present and vertically transmitted. If there are no indications showing that vertical contamination exists, then further examinations could be limited to those microorganisms which are most likely to be introduced in the event of a barrier breakdown (e.g. spore forming bacteria).

In the sera of hysterectomy-derived animals, antibodies to various microorganisms that were present in their mothers, can be detected in the first two to three months of life. These antibodies will be mostly of maternal origin, but these have to be differentiated from antibodies produced by the young themselves. The levels of the latter, one may presume, increase with time or remain more or less stable if the young are contaminated. On the contrary, maternal antibodies will gradually disappear. The examination therefore of paired sera, taken with an interval of at least two weeks from individual animals, is sometimes necessary in order to reach definite conclusions.

Table 8-2

Methods for diagnosis in and screening of laboratory animals for the presence of (potentially) pathogenic microorganisms

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Material</th>
<th>Screening</th>
<th>Diagnostics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viruses</td>
<td>serum</td>
<td>CF, HAI, SN</td>
<td>isolation</td>
</tr>
<tr>
<td></td>
<td>organs</td>
<td>IF, ELISA</td>
<td>IF on histologic preparations</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>IEM</td>
</tr>
<tr>
<td>Mycoplasmas</td>
<td>serum</td>
<td>ELISA</td>
<td>selective cultivation</td>
</tr>
<tr>
<td></td>
<td>organs</td>
<td>selective cultivation</td>
<td></td>
</tr>
<tr>
<td>Bacteria</td>
<td>serum</td>
<td>MA, IF, ELISA</td>
<td>cultivation</td>
</tr>
<tr>
<td></td>
<td>organs</td>
<td>selective cultivation</td>
<td></td>
</tr>
<tr>
<td>Parasites</td>
<td>serum</td>
<td>IF, ELISA</td>
<td>microscopy on native or fixated material</td>
</tr>
<tr>
<td></td>
<td>organs/feces</td>
<td>microscopy of native</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>material</td>
</tr>
</tbody>
</table>

CF = complement fixation test; HAI = hemagglutination inhibition test; SN = serum neutralization test; IF = immunofluorescence test; ELISA = enzyme-linked immunosorbent assay; MA = micro-agglutination test; IEM = immuno-electron microscopy; PCR = polymerase chain reaction.
SPF-breeding populations. SPF breeding colonies are monitored periodically for the presence of (potentially) pathogenic microorganisms. The frequency of examination ranges between 2 and 12 times per year. The number of animals that are examined on each occasion also varies, but falls mostly between 5 and 25. The sample size seems to be on average 10 animals or sera. The size of the sample taken will basically determine the likelihood with which a contamination present in the colony, will be detected (table 8-3). A sample size of 5 implies that the detection level is only about 50%. This means that contaminations having a prevalence of lesser than 50% will, with 95% probability, not be detected.

The list of microorganisms sought for during routine monitoring, varies between colonies, and contains only part of the microorganisms that are associated with disease, mortality or other interferences during research. This seems reasonable since, in rederived colonies, maintained under classical SPF conditions, a wide range of (potentially) pathogenic microorganisms will never arise. A number of microorganisms will be very difficult, if not impossible, to detect in healthy animals. The presence of other microorganisms will probably be detected on postmortem examination, and not by the examination of healthy animals. Laboratory methods used show a wide range of variability, and studies to outline the suitability of different methods in detecting particular microorganisms, are rather scarce.

The above mentioned reasons point to the fact that records of health monitoring data, indicating the microbiological status of the animals, are very difficult to interpret. When analyzing the data, one has to take into account all

<table>
<thead>
<tr>
<th>Percentage contaminated animals (prevalence)</th>
<th>Sample size</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>59</td>
</tr>
<tr>
<td>10</td>
<td>29</td>
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<tr>
<td>20</td>
<td>14</td>
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<td>9</td>
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<td>40</td>
<td>6</td>
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<tr>
<td>50</td>
<td>5</td>
</tr>
<tr>
<td>70</td>
<td>3</td>
</tr>
<tr>
<td>100</td>
<td>1</td>
</tr>
</tbody>
</table>

Calculated as

$$S = \frac{\log p}{\log N}$$

where $S$ is the sample size, $p$ the likelihood of error of result (here 5%) and $N$ is the percentage of uncontaminated animals.
Quality control

the barrier measures which apply to the colony. Breeding records can give some information on the possible presence of adventitious microorganisms within the colony. Infections can be accompanied or followed by a dip in production. The interpretation of health monitoring data is complicated and should therefore be left to the specialist.

SPF-animals in experiments. In general, animal experiments follow a less strict hygienic regimen than that of breeding colonies. Therefore, contaminations occur more frequently during experiments than within breeding stock. During long-term experiments, SPF animals can become more or less conventional. This ‘conventionalization’ can have various effects on the physiology of the animal, and thereby influence the outcome of the experiments.

The degree of conventionalization of animals during experiments, can be estimated by examining them repeatedly during the study, using the methods described for within the breeding colonies. On the whole, the examinations are limited to a small number of microorganisms. The values of various physiological parameters in control animals during successive experiments, can provide very useful additional information on the possible interferences upon the outcome of the experiment. During (immuno)toxicological research, counts of the total number of white blood cells and the various cell types, immunoglobulin levels (IgM and IgG) and the relative weight of lymphoid organs (thymus, spleen), are assessed regularly. If there is a considerable variation in the values for these parameters in animals of control groups of successive experiments, there is a clear indication of the fact that intercurrent infections are present.

Vaccination and therapy

Vaccination of laboratory animals is, as a rule, limited to the larger laboratory animals, i.e. cats and dogs. It is possible to vaccinate rodents and rabbits against a small number of viral diseases, for example mousepox, Sendai and myxomatosis, but there is some doubt as to its effectiveness. Clinical disease can occur in spite of the fact that vaccination has been performed. Vaccination leads to the development of antibodies, which can seriously interfere with serological monitoring. It is virtually impossible to differentiate between antibodies which have been caused as a result of vaccination or contamination.

In the case of an outbreak of disease, the administration of antibiotics should be considered in order to save a valuable experiment. The use of antibiotics, however, is certainly not without risks. In some rodents, for example guinea pigs and in rabbits, the normal intestinal flora is easily disturbed, leading to
severe enteric pathology caused by toxigenic bacteria. Preventive medicine can be given to animals via their food or drinking water, but any medication can, of course, influence the results of experiments (see chapter 9).

The taking of therapeutic measures in animals that are infected by a zoonotic microorganism, is to be discouraged. It is also the case that animals which are contaminated by microorganisms, that can cause devastating problems within laboratory animal colonies, will usually have to be destroyed. The implications of, for example, Sendai- or mousepox virus infection is always more important to the research community as a whole, than for any individual study.

Organization of quality assurance and quality control

Microbiological quality assurance of laboratory animals and animal experiments is primarily based on spatial and personal separation of animals possessing different microbiological qualities. Classification of animals according to different qualities is, of course, arbitrary. It should be based upon a thorough knowledge of the microbiological quality of all the sources of animals and groups of animals in experimental laboratories.

The ordering of animals should be centrally based and carried out under the guidance of a veterinary microbiologist. The microbiologist must have insight into all planned experiments, since the introduction of infectious microorganisms or contaminated biological materials into animals, can have serious risks of infection for other laboratory animals. Finally he/she can give advice on methods of preventive hygiene and the interpretation of results of the microbiological monitoring of laboratory animals.

Literature

Vaccination and therapy


9 Diseases in laboratory animals

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Introduction

Diseases and pathological lesions in laboratory animals can be divided into two groups:

– Induced diseases (induced pathology):
  Generally speaking these experimentally induced diseases are the subject of a particular study. Genetically selected diseases in inbred strains (genetic models), diseases in genetically modified animals (e.g., transgenic and knockout mouse models), and diseases induced by toxicity testing also belong to this group.

– Non-induced diseases (spontaneous pathology):
  In reality these are not diseases that occur spontaneously but are abnormalities which occur unintentionally, for example with ageing, and in many cases their cause is unknown.

This chapter only deals with non-induced lesions that generally may have a negative effect upon the quality of the animal experiment. The main focus of this chapter is an overview of the procedures and available methods that are suitable to detect whether disease is present in individual animals and groups of animals and to determine the cause(s) of this disease. First some definitions pertaining to disease and pathology are presented and the potential impact of disease on animal experiments and the causes of disease are briefly discussed. The chapter ends with a short description of possibilities and limitations of therapeutic intervention in diseased experimental animals.
Definitions

Disease

A normal healthy organism (man, animal) is confined to a narrow range of functional and structural characteristics, whereby it is in a steady state balance with all environmental influences known as homeostasis. In order to maintain this homeostatic balance, all physiological processes within the organism are subject to feedback and compensatory regulating mechanisms. This results in the continuous adaptation of the organism to environmental changes. When circumstances are normal, these physiological adaptations are in a dynamic balance with the external factors. When external factors change, for example due to the occurrence of pathogenic microorganisms, a new balance is achieved, brought about by adaptations within the organism. Changes or abnormalities within the organism itself may influence its adaptability and will therefore have an effect on the homeostatic balance between the organism and its environment. Despite the fact that in such situations potentially harmful conditions are present within the organism, clinical manifestation of disease may be absent (subclinical disease). Manifest disease, as a result of organ dysfunction, will only occur when the adaptive capacity of the organism to cope with a harmful external factor, or combination of harmful external influences, is exceeded and/or when the regulating mechanism itself is defective or alterations within the organism itself result in a decreasing capacity to adapt.

Depending upon the degree of organ dysfunction, there may be clinical signs of disease and associated macroscopic or histological lesions may be present in the affected organs. Such lesions are not always visible, even by microscopic examination; for example, there may be alterations in biochemical or physiological processes without any histologically detectable associated lesions.

Pathology

Pathology is the study of disease, including the causes of cell and tissue injury, the mechanisms of such injury, and the responses of cells, tissues and organisms to injury. Pathology as a science is concerned with disease processes from the onset of injury and cellular and tissue abnormalities through their evolution to their final outcome ranging from death to complete repair and disappearance; this sequence of events is known as pathogenesis. This study encompasses functional, metabolic, molecular, physiological, and morphological phenomena. This complexity of disease processes necessitates a multidisciplinary approach. Thus, disease can not be understood purely by a
Definitions

Clinical, histological, biochemical or microbiological approach. Pathology, therefore, includes not only the study of the living animal (clinical studies, cytology, clinical chemistry, haematology) but also includes post-mortem studies (gross or microscopic pathology, histopathology, microbiology, biochemistry, molecular biology). In order to understand the pathophysiological processes that lead to or that are the result of disease, knowledge from all these different areas has to be integrated. The term “pathology” is also often used when referring to a specific area of pathology, for example clinical pathology, histopathology, background pathology etc.

Intercurrent disease

Non-induced diseases that manifest themselves during the course of a given experiment are called intercurrent diseases. The course intercurrent disease can take varies:

– If the disease results in the death of the animal, its significance is recorded as mortality, a figure that indicates the number of animals lost, in this case due to intercurrent disease.
– If the disease is clinically manifest, but does not result in mortality, then the term morbidity is applied. This refers to the percentage of clinically diseased animals in a colony of animals or and experiment. Such a disease may be transient, but could also result in persistent lesions. For example, corneal inflammation in rats caused by a sialo-dacryo-adenitis virus (SDAV) infection will disappear after a short period. Retarded cerebellar development (hypocerebellism) in rats neonatally infected with Kilham Rat Virus (KRV), on the other hand, is an example of a persistent lesion.
– Often an intercurrent disease takes a subclinical course and no manifest clinical symptoms are present. For instance, many bacterial and viral infections occur in rodents without any detectable signs of disease. However, in the field of animal experimentation, it is important to be aware of the presence of such subclinical or latent infections, since experiment-associated stress or immunosuppression can result in the clinical manifestation of a disease due to such an infection. Tyzzer’s disease, caused by Clostridium piliformis, is a well documented complication occurring due to experimental stress in a variety of laboratory animal species. Most importantly, subclinical diseases often have an influence on immunological, biochemical and/or haematological parameters that are measured in an experiment. Examples of subclinical diseases that interfere with experimental parameters are viral infections with Sendai virus, mouse hepatitis virus (MHV) and lactate-dehydrogenase (LDH) virus. When faced with unexpected or aberrant experimental results, one must consider the possibility that these are the result of such a subclinical infection. Microbiological monitoring of the animals may lead to recognition of
such infections. Apparently healthy carriers of infectious agents may cause
disease in other, more susceptible, animals or even man (investigator or ani-
mal technician). Finally, also non-infectious diseases often remain subclini-
cal, unless experimental or other stress causes them to become clinically
apparent.

**Background pathology**

Besides manifest diseases, pathological lesions may also occur that have no
detectable adverse influence on animal health or on experimental results or
only occur in ageing animals. Yet it is of great value to the researcher when
animal breeders keep records of the frequency of macroscopic and histologi-
cal lesions, as part of their animal quality monitoring programs. This enables
the researcher to anticipate the spectrum of lesions and the incidence, with
which they can be expected. In other words, the researcher will know what is
“normal” for the species and strain at the particular age that is under study.
Background pathology is therefore the study of the nature and incidence of
spontaneously occurring lesions. It may range from bald spots in rats or mice
acquired due to excessive grooming, to congenital lesions, such as atrophy of
the optical nerve associated with microphthalmia (underdevelopment and
malformation of eyes) in rats, to chronic degenerative renal disease or hy-
dronephrosis (dilated pelvis of the kidney, fig. 9-1). Another important aspect
Definitions

of background pathology is the recording of the onset and course of development and pathophysiological significance of these lesions. An example of this would be the age at which in rats of a certain strain degenerative and inflammatory renal lesions are first histologically observed and at what stage of their development these lesions result in functional alterations. The same applies to the development of tumours. The importance of the availability of background pathology data when selecting animal species and strains for specific experimental purposes, can not be overemphasised. Due to genetic drift and dynamic interactions between the animal and its environment, background pathology will not be entirely consistent in every detail, and variations will occur between experiments. Therefore, necropsies and complete histopathologic examination are always carried out in the context of subchronic and chronic toxicity tests on animals of the exposed as well as the control groups. These control animals also provide some insight into the background pathology of the strain. Laboratory animal breeders and users typically invest a great deal of effort in keeping the animal’s environment as constant as possible. A shift in the pattern of lesions may be an indication that a change in environmental conditions has occurred. For instance, an increased incidence of biting injuries may be associated with disturbances in the animal room, which could, for example, be attributable to the start of nearby construction activities. An increased incidence of renal calcification in rats may indicate that a change in diet composition has occurred. Similarly, a change in the incidence of congenital lesions, such as microphthalmia, may be a result of inaccurate breeding or selection procedures. Thus, background pathology information is often valuable when evaluating the influence of environmental factors and changes therein, on laboratory animals and on experimental parameters measured in these animals.

Causes of disease

The causes of disease (etiology) may have a genetic, environmental and/or infectious basis. Disease is rarely a result of a single causal factor, i.e., the cause of most diseases is multifactorial.

Genetic factors are important determinants of the susceptibility of animals to disease. For example, there are large differences amongst mouse strains with regard to the occurrence of tumours, renal disease and myocardial calcification to name but a few lesions. Infection with mouse pox virus results in high mortality in the DBA/1, DBA/2, BALB/c and C3H strains, for example, whereas the susceptibility of strains such as C57BL/6 and AKR is much lower. Genetic factors are also involved in heritable diseases and some, but not all, congenital abnormalities. Increasingly important are pathological changes in
genetically modified animals. In addition to expected lesions, be it primary or secondary, also non-expected lesions can frequently be found, often at not-expected sites of the body of these transgenic or knock-out animals. Recognition of these lesions is very important, not only for the interpretation of experimental results, but also for estimating the degree of discomfort to the animal by the introduced change in the genome.

Environmental factors such as infections, chemical and physical agents, as well as nutrition, husbandry, and housing, have an enormous influence on health and disease. Errors in animal care and nutrition, alone or in combination with active or latent infections, can have serious consequences for animal and experiment. The handling and treatment of the animal during the experiment always has either a direct or an indirect influence on the physiological balance of the animal. For example, pathologic lesions may also be caused by inadequate experimental technique. Blood sampling by inexperienced personnel can result in haemorrhage (bleeding) and local inflammation, complications of blood sampling by puncturing the orbital sinus may occasionally result in the complete loss of the eye. Adverse side effects of cardiac puncture may be haemopericardium (presence of blood within the pericardial cavity), haemothorax (presence of blood within the pleural cavity) or damage to lung tissue. If injection fluid is administered extra-vascularly during an attempted intravenous injection, tissue necrosis (cell death) and concomitant inflammation can occur. All such complications may not only have a local effect but can also lead to generalized reactions, for instance in the immune and/or the circulatory systems. Many diseases and lesions in laboratory animals are caused by infectious environmental factors. Due to improvements in both standards of husbandry hygiene and the microbiological status of the animals, the incidence of infectious diseases has greatly declined in recent decades. As a result, non-infectious environmental and genetic factors that can lead to disease and/or affect results of experiments have become more prominent and are requiring more attention from the researcher.

**Diagnosis of disease**

The aim of diagnostic examination is to detect subclinical disease and lesions and to define clinically manifest pathological problems. Having made a correct diagnosis, it is often possible to estimate morbidity and mortality together with the potential spread of the problem to other (groups of) animals and the possible risks involved to exposed personnel. Thus, it will be possible to assess the consequences of the disease for the animal as well as for the experiment and the animal facility of a research institute, and to define therapeutic and preventive interventions and other remedies. Precautions can be
taken to prevent spreading of the disease and against its reoccurrence. Inci-
dental morbidity and mortality must be given as much attention as massive
outbreaks of disease in laboratory animal breeding colonies and in experi-
mental groups. A single diseased animal may be the indicator of an impend-
ing massive disease outbreak, or it may indicate the presence of subclinical
disease. Making a correct diagnosis requires considerable experience, and
the assistance of specialists in the field of laboratory animal pathology and
medicine is invaluable. Both the scientist responsible for the experiment and
the animal technician involved in it need to be able to recognize signs of
disease/abnormality at an early stage in order to alert veterinary staff to pos-
sible problems. To do this, they must have a basic knowledge of the diagnos-
tic procedures and methods available for application in laboratory animals.

**Diagnostic examination**

A complete diagnostic investigation must include the history of the disease
and the background of the animal as well as physical, laboratory and post-
mortem examinations. For the successful completion of such examinations,
appropriate laboratory facilities are needed. Sometimes it is possible to make
a correct diagnosis with a simple physical examination, whilst in other cases,
even after carrying out all possible investigations and tests, it is not possible
to arrive at a definitive diagnosis. In the next sections, the various diagnostic
procedures and methods will be discussed. Special attention is given to infec-
tious diseases as they can easily lead to severe problems within a laboratory
animal colony. However, the detection of the presence of an infectious agent
does not necessarily imply that this agent is the causal factor of the disease
problem. The infection may be secondary to the disease that itself is brought
about by other factors.

An in-depth review of the diagnostic techniques that are available and the
interpretation of the results is beyond the scope of this chapter. For such highly
specialised information the reader is referred to references at the end of this
chapter.

**History**

At the first sign of a problem, as much information as possible should be
collected about the history of the disease and the background of the animals
before starting any examinations. This is to avoid loss of information that
may be important to the diagnostic process. The following checklist gives an
overview of some of the important disease history and background features
(anamnesis).
General information about the group of animals:
- species; strain; sex; age
- microbiological status (germ-free; gnotobiotic; specified pathogen free; conventional; microbiological monitoring procedures used)
- origin and source; date of delivery
- use (breeding; stock; type of experiment)

Information about the environment, both present and past:
- animal room
- quarantine and barrier procedures
- type and size of cage; number of animals per cage
- diet and drinking water (special diets; automatic watering vs. water bottles)
- bedding (nature; amount; chemical quality; microbiological quality)
- hygiene and sanitation (procedures and actual situation); vermin control
- possible recent disturbances in cage, cage rack, or room; recent other changes (e.g. new animals)
- light-dark regime; ventilation; air quality; relative humidity; temperature
- possible relationship between diseased animals and specific cages, position on the racks, position of the rack in the room, animal technicians, or investigators.

History of the disease and accompanying clinical signs, reason for submission of the animal:
- nature of clinical signs and observed abnormalities (onset and duration; estimation of severity; number of animals affected; morbidity; mortality; age, sex, and experimental treatment of the affected animals vs. unaffected animals)
- previous disease and possible treatment
- breeding results (fertility; litter size; litter viability)
- possible seasonal associations
- abnormalities in experimental results.

Information about experimental procedures:
- (surgical) interventions
- treatments with experimental substances or microorganisms

Clinical examination

First and foremost in clinically examining a living animal, is the close observation of the animal’s appearance and behaviour. Any abnormalities recorded at this stage will give the first and often critical leads for making a diagnosis. Further examinations can then be carried out targeted at presumed causes of
Diagnosis of disease

the observed abnormalities. It is essential to be familiar with the normal appearance and behaviour of animals of the species and strain in question. Otherwise distinguishing normal and abnormal in terms of general appearance and behaviour is not possible. In general, animal technicians who have daily contact with the animals will often be the first to notice that something is wrong. Clinical examination consists of a systematic review of the various organ systems in order to ascertain which system has been affected and to narrow down the number of possible diseases or lesions.

The following list outlines a number of important aspects to check:

- general condition (behaviour; appearance and development; body weight; body temperature; temperature (and colour) of extremities and ears)
- respiratory system (frequency and type of respiratory movements)
- circulatory system (heart beat frequency; colour of mucous membranes; presence of oedema)
- digestive system (food intake; production of faeces and its appearance; inspection of the mouth; possible malocclusion; abdominal palpation; presence of wetness or faecal matter around anus)
- urinary system (drinking; production of urine and its colour and appearance; abdominal palpation of kidneys and/or bladder)
- skin (inspection; determination of turgor to assess hydration status; presence of wounds or lesions)
- musculoskeletal system (posture; locomotion; presence of muscular weakness or fractures; abnormalities of joints or feet and toes)
- nervous system (behaviour; possible paralysis or seizures; reaction to environmental stimuli).

Clinical examination is more difficult to carry out in small animal species such as rodents than it is in larger animals. Normal reference values of physiological parameters are often not well defined and have such a wide variation as to be only really useful when looking at marked abnormalities. Apart from this, the handling of these animals may cause considerable changes in the respiratory, cardiac and endocrine systems. The initial, and perhaps most important, contributions to a clinical examination of rodents is the careful observation of the animals in their own cages by experienced staff in combination with reliable information about the environmental conditions and the history of the animal group. First and easy to recognize signs of disease applicable to rodent species are reduction or cessation of food intake, reduced growth or weight loss, rough hair coat, loss of grooming behaviour (in rats resulting in red eyes and nose due to porphyrin containing tears), segregation from other animals, apathy.
Additional examinations

When the history of the illness together with a clinical examination, is insufficient to arrive at a diagnosis, additional examinations are necessary. Depending on the animal species, the type of problem and whether the animals are in experiment or not, it may be possible in some cases to collect blood and other samples from the animals. In other cases animals may need to be sacrificed for further diagnostic examination. Scrape samples of skin and samples of fur, faeces and urine can be collected for parasitological, mycological, bacteriological and (bio)chemical tests. Samples can be taken from the mucous surfaces of nose, throat, vagina and from the rectum for microbiological tests. Often very valuable haematological, biochemical and serological information can be obtained from blood samples. Other diagnostic tests used in veterinary medicine, such as X-rays and biopsies, can be used, depending on the animal species and the problem in question.

Post-mortem examination

Post-mortem examination is often an invaluable diagnostic tool for establishing the cause of disease or the cause of death of animals. An autopsy can reveal changes in tissues and/or organs that have as yet not manifested themselves in clinically apparent symptoms. Post-mortem examination is frequently used when problems occur in rodents.

The diagnostic possibilities of a post-mortem investigation will largely depend upon the interval between the death of the animal and the autopsy. The autopsy should be carried out as soon as possible after the death of the animal and by an experienced person.

Autolysis and bacterial putrefaction commence immediately after death and progress rapidly at room temperature. Within as little as one hour, the mucous membranes of the intestinal tract may be unusable for histological examination. In rabbits, the bacterial production of gases can cause a rupture of the stomach within a few hours after death. Autolysis and bacterial putrefaction can be delayed by keeping the carcasses cool. The core temperature of the dead animal should be lowered to 4°C as fast as possible, ideally by making it wet with/in (ice)cold water, which diminishes the insulation capacity of the fur. After cooling down the animal fast, it should be placed in a sealed plastic bag in a refrigerator. The plastic bag will retain the ectoparasites, which tend to leave a dead animal, and ensures that they are not missed during the post-mortem investigation.

When it is not possible to perform a complete post-mortem investigation the same day or the next, it may be advantageous to keep (certain parts of) the dead animal in some form of temporary storage. Fixation in a buffered 10%
Diagnosis of disease

(\text{v/v}) formalin (= 4\% (w/v) formaldehyde) solution of the entire animal (rodents, especially mice) following opening of the abdominal and thoracic cavities is one possibility. Formalin penetrates only slowly into the tissues, extracts water and disinfects; the thickness of samples should be 6–8 mm or less. Organs and tissues preserved in formalin can mainly be used for histological and immunohistochemical investigations. Freezing of the entire animal at –20°C (rodents) or small parts of larger animals is another means of preservation. Freezing permits some microbiological examinations. However, freezing causes destruction of the cellular plasma membrane due to crystallization of salts within the cells, which may impede macroscopic examination of these (parts of the) animals and markedly interferes with microscopic examination. In addition, small tissue samples can be frozen in liquid nitrogen using a cryopreservative procedure for enzyme- and immunohistochemical examination (cryostat sections) and for some microbiological examinations; the thickness of samples should again be 6–8 mm or less.

Problems caused by autolysis and/or bacterial putrefaction are not a factor when living animals are submitted for post-mortem investigations. The animals can be subjected to a clinical examination prior to the autopsy and blood can also be collected for further investigations. It must be taken into account here that transportation and a new environment (necropsy room) can cause behavioural changes and affect the clinical parameters that are to be assessed. The fresher a corpse is, the easier the autopsy, the higher the quality of the tissue samples preserved for histology, and the better the resemblance of the microbial flora to the flora of the living animal. It is, for example, much easier to find motile protozoa (flagellates) in the enteric contents of a fresh corpse than it is to find their cysts in the enteric contents of a corpse which has cooled down.

Finally, when submitting living animals for post-mortem investigation, it is of course possible to choose the time and the method for euthanizing the animal(s). It is important to realise that the method of euthanasia chosen will influence the appearance of tissues and organs at the post-mortem investigation and may affect the results of laboratory tests. The use of barbiturates, for example, causes dilatation of blood vessels and in dogs, cats and horses, it results in a severe splenomegaly (enlargement of the spleen). Injection of euthanasia drugs into the abdominal or thoracic cavity may cause acute inflammatory changes and/or haemorrhages if blood vessels are damaged. Exsanguination will cause a macroscopically visible anaemia, but may facilitate microscopical examination for the presence of subtle changes.

Submission of animals for a post-mortem investigation should be accompanied by a clear history of the disease (anamnesis) and, if possible, the results of a clinical investigation. This will often greatly facilitate the identification of the disease and/or cause of death.
A post-mortem investigation begins with a macroscopic inspection of the outside of the animal, in the course of which the fur should be checked for the presence of ectoparasites. A magnifying glass or a stereo-microscope may be necessary when examining animals for ectoparasites such as mites. Post-mortems should be conducted on a surface that can easily be cleaned and disinfected or discarded, small laboratory animals fixed belly side up and larger non-ruminant animals lying on the right side. The fur on the ventral surface of the animal should then be dampened from the chin to the tail with disinfectant. This is to diminish problems caused by loose hairs and to reduce the risk of bacterial contamination. The skin should then be removed from the ventral side commencing with a mid-line incision and working towards the dorsal side. The amount and colour of subcutaneous fat, the hydration status of the subcutis and the appearance of the subcutaneous lymph nodes and mammary glands should then be examined. The joints of the limbs need to be opened in order to inspect the articular surfaces and the synovia; this is difficult in small animals and is routinely only done in larger species. The thoracic, abdominal, oral and cranial cavities should be opened to check the size, colour and location of the organs and the presence of lesions should be determined, as well as the possible presence of abnormal contents and changes in the lining of the cavities. Finally, all organs should be removed from these cavities and examined individually.

In some cases, macroscopic examination of the corpse and the organs is sufficient to make a diagnosis. More often, however, additional microscopic and microbiological investigations will be necessary. For example, microscopic examination is needed to determine whether an observed tissue mass is a neoplasm, and, if so, what type of tumour it is. A correct diagnosis of most macroscopically visible alterations in laboratory animals requires microscopic examination. A number of diseases result in changes which are only visible through microscopic examination. Therefore, a complete post-mortem examination of laboratory animals should always include a microscopic examination of a selected number of organs, just as it is done in toxicological studies. Information collected from post-mortems of control groups and intercurrent deaths will give an indication of the expected range of background pathology within the researcher’s own laboratory animal colony.

The results obtained from the macroscopic examination often provide the information necessary to decide whether additional histological, microbiological, parasitological, clinical, chemical, haematological and/or serological investigations need to be performed. The organs which are to be used for (immuno)histochemical examination should be kept in a fixative or be frozen using liquid nitrogen. The choice of the fixative (formalin, Bouin’s solution, glutaraldehyde) will depend upon the kind of investigation to be performed. Materials for microbiological studies should be collected and sampled in such
Methods of microbiological examination

Microbiological investigations must be performed to determine if the cause of a disease is infection with a microorganism and, if so, which microorganism is involved. The more certain and detailed the diagnosis is, the greater the effectiveness will be of the measures that can be taken. Depending on which microorganism is involved, the following diagnostic techniques can be employed:

- Direct techniques: visualization of a causative agent by means of histological examination (Gram staining, immunofluorescence techniques, electron microscopy) or by isolation and cultivation.
- Indirect techniques: detection of the presence of antibodies against an infectious agent or the presence of products from a causative agent (e.g. toxins from Clostridium botulinum).

An important difference between these two groups of diagnostic techniques is that a positive result using an indirect test does not mean that the etiological agent is still present. It is even possible that it has never been present, for example, the presence of antibodies against parvo virus in cats does not necessarily mean that the cats are or have ever been infected with the virus. Vaccinations and antibodies present in young animals but received from the mother, may also be the cause of positive antibody titers. Negative results, in both direct and indirect tests do not always exclude the presence of an infection. Every technique employed has its own detection limit, below which an existing infection will not be detectable, which determines the sensitivity of the test. It takes some time (generally a number of days, sometimes much longer) after the initial contact with an antigen, before the amount of antibodies in the blood serum will exceed the detection limit of a test. By studying the amount (titer) and the type (IgG, IgM or IgE) of antibodies present, it is sometimes possible to ascertain whether an infection is recent or has been present for a while. To perform this type of investigation (known as “paired sera”), it is necessary to collect, over a two to three week period, a number of serum samples taken from the same animal.

A brief description of a number of microbiological methods for detection of infection with the various microbial agents is presented in the following (see also chapter 8).
In the absence of immunosuppression or immunodeficiency (e.g., athymic mice) or complicating secondary, often bacterial, infections, most viral infections do not cause clinical symptoms within otherwise healthy adult small laboratory animals (exceptions are the ectromelia virus in mice and the SDA virus in rats). They are more likely to cause disease in neonates (e.g. the Sendai virus in mice). Another reason for suspecting a viral infection would be a negative change in breeding results within an animal colony. The presence of a viral infection is, however, usually detected by means of serological examinations which are performed as part of a health-monitoring program or as a result of interference with experimental results (see chapter 8). In larger laboratory animal species, a number of viruses, for example, parvo virus in dogs and cats and viral haemorrhagic disease in rabbits, may cause severe clinical problems and even lead to death. When dealing with such clinically evident viral diseases, results of clinical and post-mortem investigations (macroscopic and/or microscopic) generally provide sufficient information to arrive at a preliminary diagnosis. Such an initial diagnosis can usually be confirmed relatively quickly, by means of a microbiological method, preferably a direct one by detecting the presence of viral antigen.

Intracellular or intranuclear inclusion bodies, detectable using histological examination, occur in the course of a number of viral infections. Detectability of such inclusion bodies can be increased by applying specific staining techniques. Viral antigens can be visualised in tissues using (group)-specific antisera in immuno-morphological methods. Viral RNA or DNA can be demonstrated by (in situ) molecular biological methods. Tissue that is to be investigated by these methods must be collected as soon as possible after death, frozen for histological examination in liquid nitrogen and stored at –70°C. The isolation of a virus can be performed using cell cultures, laboratory animals, or embryonated eggs. Virus isolation is generally only applied when the direct immunological detection techniques have not been satisfactory, or if the presence is suspected of viruses which have as yet not been characterized or for which there are no antibodies available. Materials which are to be used for such investigations should also be frozen at –70°C or should be stored in liquid nitrogen.

**Bacteria (including Mycoplasmata)**

The detection of bacterial infections can be done using a variety of techniques. Various direct techniques allow the determination of the specific morphology of bacteria and to their staining characteristics using Gram and other staining methods. Additional information can be derived from their growth
pattern on agar plates and to their metabolic characteristics (biochemical reactions) using selective culture media. Serology, an indirect technique, can also be employed and involves the detection of antibodies against bacterial antigens which provide evidence of (previous) infection. New specific methods for detecting an increasing number of bacteria are constantly being developed.

**Morphology of bacteria.** It is relatively quick and easy to demonstrate the presence of bacteria by microscopic examination of smears, tissue imprints or histological sections, which are stained using a variety of stains. Bacteria can be subdivided according to their shape into bacilli (rods), cocci, spirilla and vibrios. They can also be classified according to their staining properties, for example as positive or negative when using the Gram stain, their acid resistance in the Ziehl-Neelsen staining reaction, and whether or not they are encapsulated.

A variety of other characteristics can in addition be examined, such as endospore production, division pattern (budding or true branching) and motility. For a number of bacteria, that do not multiply in broth or on agar plates (e.g. *Clostridium piliformis*), histological examination of affected tissues and organs is the most important diagnostic approach (fig. 9-2). Although special histological staining techniques may be necessary, microscopic inspection of smears of organs that normally are free from bacteria, is generally both quick and satisfactory. This method provides direct information about the presence or absence of bacteria, and it will also give information about their quantity and morphology.

**Growth on culture media.** Most bacteria can be cultured in the laboratory employing appropriate techniques. Culture media contain the nutrients necessary for the bacteria to grow and multiply, and they exist in solid (agar plates) as well as liquid (broth) form. Cultivation on agar media (generally at 37°C for a period of at least 24 hours) should reveal characteristics of the resulting bacterial colonies such as growth rate, shape, colour and whether swarming occurs. Together with colony size and smell, all these factors are helpful in discriminating characteristics in identifying bacteria.

**Biochemical characteristics.** The growth of bacteria is a biological process which requires nutrients and which produces metabolites. Determination of the nutrients required, and identification of the metabolites produced, by cultured bacteria provides more useful information for identification purposes. This is often achieved by the addition of indicators to the medium which change colour when a particular type of bacteria grows. By varying the type and amount of components of a medium, it is possible to produce so-called
elective, selective or non-selective media. Non-selective media contain all the necessary nutrients for growth of many types of bacteria. The bacterial colonies that are suspected to be relevant, are isolated from a non-selective agar plate in order to grow a pure culture from which the bacterium can be identified. The composition of elective media allows certain (types of) bacteria to grow faster than others. Selective media only allow the growth of selected bacteria. Selective media can be produced, for example, by adding restricting antibiotics which inhibit growth of all except a few types of bacteria. An example of a selective medium is the medium used for isolation of Mycoplasma spp.

The isolation and identification of the most common pathogenic bacteria can usually be completed in a number of days, provided that the suitable culturing media and identification techniques are available. Multitest systems that require the inoculation of a limited number of media have been developed and are commercially available.
Immunological tests. For some bacteria, it is difficult or very time consuming to carry out direct identification techniques. The presence of some bacteria can more easily be determined using serological methods that detect the presence of antibodies. Such tests are carried out as a matter of routine when screening for among others Mycoplasma pulmonis in rodents, Corynebacterium pseudotuberculosis (causes caseous lymphadenitis) in goats and other species, and Leptospira spp. in various animal species. Serological methods are relatively cheap, fast and often reliable and, as such, this type of screening will probably be more widely used in the near future for the detection of a growing number of bacterial infections (see table 8-2). Frequently, however, the sensitivity and/or specificity of these serological methods are insufficient. If an animal or a colony is seropositive for a certain bacterium, it is advisable to obtain confirmation by isolating the bacterium in question.

Protozoa

Certain protozoan organisms can infect animals and cause lesions in different organs. For example Encephalitozoon cuniculi (Nosema) may cause inflammatory lesions in brain and kidney in both rabbits and guinea pigs. Toxoplasma gondii cysts can be found in almost any part of the body of infected laboratory animals, just as they can be present in other animals including man. Eimeria stiedei causes macroscopically visible changes of the bile ducts in rabbits. All these examples of protozoan infections can be diagnosed by conducting a careful post-mortem and a histopathological examination, supplemented where necessary by the use of immunological methods (immunofluorescence test or IFT; enzyme-linked immunosorbent assay or ELISA).

Most protozoan infections occur within the intestinal tract (e.g. coccidia, flagellates); a number of these will only appear in a specific part of the intestine. Flagellates, sarcodines and ciliates can be recognized by their motility characteristics in fresh, wet smears of the enteric contents, or in a fresh sample scraped off from the enteric wall for examination. These samples need to be kept at approximately body temperature. Samples can be preserved for further investigation and/or identification in Bouin’s fixative. Cysts of, for example, coccidia, can be detected in direct wet faecal preparations by microscopic examination at a magnification of 400×, provided that they are excreted via the faeces in sufficiently large numbers. It is possible to detect smaller numbers of cysts using an enrichment method, such as a sedimentation or flotation method with a saturated salt solution.
Diseases in laboratory animals

Fungi

Dermatophytes and deep fungi may be the cause of opportunistic infections in laboratory animals, but they are not primary pathogens. Lesions caused by fungi may, however, occur and can be divided into two groups: the superficial mycoses and the deep mycoses. The incidence of superficial, dermal mycosis in laboratory animals that are kept in adequate conditions, is minimal. If it does occur, it is generally caused by *Microsporum* spp. or *Trichophyton* spp. These infections may affect man (and are called zoonoses) and they are often persistent. Superficial mycoses can be diagnosed by the microscopic examination of hairs and skin scrapings which have been taken from the border line between the normal and the macroscopically altered skin regions. In the event of a *Microsporum canis* infection, the infected hairs and skin will show up bright green under the ultraviolet light of a Woods-lamp. If deep fungal diseases are suspected, microscopic examination of exudate and/or tissue biopsies will be necessary, using special staining techniques such as PAS (periodic acid Schiff) or methamine silver staining.

The tracing of carrier animals of subclinical fungal disease, and the identification of dermatophyte or deep fungus, requires the use of culture methods. The materials under investigation should be inoculated in specific media developed for fungi (Sabouraud or malt agar plates). Generally, two identical plates will be inoculated, one incubated at 37°C and the other at 20°C (room temperature). After inoculation, the plates should be kept in the dark. As soon as growth occurs, which can take anything from a number of days to a few weeks, the mould or yeast can be identified by examining morphological and biochemical characteristics of the cultured organism.

Helminths

Endoparasites, particularly helminths frequently occur in the intestinal tract of laboratory animals. Nematodes such as oxyurids (pinworms) found in rats, mice and rabbits and cestodes (tapeworms) can be detected by macroscopic and microscopic examination of the intestinal contents in the course of a post-mortem. The oxyurids of rats, mice and rabbits occur in the caecum and colon. Whilst the host animal is resting, the females of these worms deposit their eggs around the anus (rat or mouse), or in the mucosal crypts of the colon (rabbits). The eggs of the oxyurids hatch within the enteric crypts in the rabbit, whereas those of the rodents hatch outside the animal. Therefore, it may be difficult to detect eggs of oxyurids in the faeces of rats, mice or rabbits. An alternative diagnostic method, used for the screening of oxyurids in living rats and mice, is to press a piece of transparent adhesive tape against the anal region of the animal during the first half of the afternoon, i.e. the
middle of the host’s resting period, followed by mounting the tape on a glass
slide for microscopic detection of the eggs. The presence of other (egg pro-
ducing) intestinal helminths can be determined by searching for eggs in the
faeces by carrying out microscopic examinations (magnification 40×) of di-
rect or enriched (sedimentation/flotation) wet preparations.

Helminths can also occur in the blood, lungs, urinary bladder or indeed
anywhere in the body. Diagnostic techniques employed include those of mac-
roscopic and microscopic postmortem inspection, direct immunofluorescence
techniques for the detection of antigens, serology and isolation using cultur-
ing methods.

**Ectoparasites**

Ectoparasites, such as fleas, mites and lice, generally cause clinical abnor-
malities such as itching (pruritus), alopecia and/or inflammatory lesions of
the skin. Often, the parasites or their products, namely the faeces of fleas and
the eggs of lice or mites, are visible upon close inspection with the naked eye,
or can be seen with the aid of a magnifying glass or stereomicroscope.

There are also ectoparasites that do not live on the surface of the skin but,
for example, in the external auditory canal (such as *Psoroptes cuniculi* which
causes ear mange in the rabbit, see fig. 9-3a,b,c) or in the skin (such as *Demodex*
spp. in the hamster and dog or *Trixacarus caviae* found in the guinea pig). These
parasites can be diagnosed by microscopic examination of excreta
(earwax of the rabbit) and skin scrapings. The presence of ectoparasites
under the skin or inside the body, for example, in the respiratory or diges-
tive tract, can be detected in the course of a macroscopic post-mortem or a
histological examination.

**Interpretation of results of diagnostic examinations**

After all the relevant information has been obtained, a diagnostic decision
should be made combining and evaluating the results of the various diagno-
tic tests. This task is best carried out by specialists in laboratory animal medi-
cine, who will present their conclusions along with advice for action to the
breeder or user of the animals. The interpretation of the combined informa-
tion is a delicate process with many pitfalls. Many diseases are caused by a
combination of factors, and the overestimation of the importance of factors
such as opportunistic infections may lead to an incorrect diagnosis and inade-
quate remedial measures. The identification of a single pathogenic agent
does not mean that this is the only or primary cause of the disease. The inter-
action with and the importance of environmental factors should not be under-
estimated. In addition, invasion of enteric bacteria into mesenteric lymph nodes or liver just before death (agonal translocation) or contamination during sampling and the occurrence of commensal microorganisms can lead to diagnostic errors. Comparison of the data from the diagnostic tests with reference data obtained from healthy animals, kept under the same circumstances, is often of great value.

**Therapeutic and preventive interventions**

When the cause of the disease has been established, the choice has to be made of accepting the situation without further action, applying therapy or euthanasia of the affected animals or of the entire group, colony or experiment, or
isolation of the affected animals and institution of preventive measures for the remainder of the group or colony. Therapy is generally the first choice considered. However, negative effects may be associated with the proposed therapy, both for the animals (side effects) and for the experiment (influence on results). The risk of transmission of an infectious disease to other animal groups within the institute is another serious factor that has to be considered.
Diseases in laboratory animals

The fear of spread of the infection to other animal rooms, or to other experimental groups, is usually dominant when discussing what actions need to be taken, and often leads to the conclusion that groups of infected animals must be eliminated. In other cases, isolation of affected animals combined with preventive measures may be indicated. There are certain experiments, particularly toxicity testing studies, that fall under stringent guidelines, leaving little room for treating sick animals. In other cases, sick animals may only be treated if the treatment is proven not to interfere with the experiment. However, it is often difficult and sometimes even impossible to guarantee that a therapy will not influence test results. When the application of treatment is justified, the most effective therapy has to be selected. For larger animals such as dogs, cats and pigs, sufficient information to help in the decision making process is available from veterinary medicine. Information about the therapeutic effect and side effects of drugs is, however, not always readily available, especially regarding small laboratory animals. One often has to rely upon therapeutic information from other animal species or from man. However, differences in species-specific susceptibility may pose considerable problems. A particular oral dose of antibiotic may be used without much risk in man or dogs, whereas the same drug may cause disease or even death in rodents as a result of disturbing the microbial balance within the intestinal tract (the same may occur when an injected drug is excreted by way of the bile).

An important factor to be considered is the degree to which the treatment produces complete resolution of the disease without subclinical or clinically visible or functional remnants. Often persistent lesions may occur in one or more of the internal organs, which may influence experiments. An infectious disease often persists latently in clinically healthy animals which puts them and their environment at risk. All of the above mentioned factors and consequences must be considered before a decision is made about the type of treatment. Logistic considerations, such as the number of animals that need treatment, also need to be taken into account. Furthermore, it will have to be decided whether the treatment should be restricted to the diseased animals, whether it should include all animals (including controls) of the particular experiment, or whether it should be applied to the animal colony or facility as a whole. Treatment will be more acceptable towards the end of a long-term experiment with irreplaceable or very valuable animals, whereas at the start of an experiment with easy to replace animals the decision of “no treatment and stop the experiment” will be easier.

It is beyond the scope of this book to deal with the question what therapy is indicated in a specific situations. This decision should be left to the veterinarian who is specialized in the field of laboratory animal science.
The problems and limitations in therapeutic possibilities with regard to laboratory animals underscore the extraordinary importance of preventive measures and microbiological and husbandry standardization when breeding or experimenting with these animals.

Literature


10 Animal models

A. C. Beynen and J. Hau

Introduction

Important decisions have to be taken when planning experiments and selecting experimental methodology, equipment and materials. Medical and biological research projects will generally employ at least one of the following categories:

– human volunteers
– experimental animals
– embryos, organs, tissues or cells having either plant, animal or human origin
– bacteria, fungi, protozoa
– inanimate models such as computer programmes, physical or chemical products.

Experimental material should be chosen with the purpose of solving the case being studied in the simplest possible way. Together with the scientific considerations, there are also legal and ethical matters which have to be taken into account when selecting test material (see chapter 18).

This chapter deals with the general aspects involved when using laboratory animals as models of man or indeed of other animal species. It is often the case that ethical, practical and/or financial reasons prevent the use of human volunteers or the target animal species for these studies. This chapter does not set out to list the various animal models which are currently available. Rather, the aim here is to describe the basic strategy required when selecting an animal model.

The use of experimental animals

One way in which laboratory animals are used for research or medical purposes is as the providers of biological products, such as hormones or anti-
Animal models

bodies. Some examples here would be the production of the pregnant mare’s serum for the treatment of reduced fertility, and the production of polyclonal and monoclonal antibodies, which are mainly used for scientific purposes but which may also be used for therapeutic and diagnostic reasons. The researcher should aim to select the animal most suited to producing the required product in both sufficient quantities and in the purest possible form.

Animals are also used within science as models for the study of biological responses. Here the researcher employs physiological, pathophysiological or behavioural parameters to study the responses of the animal, having administered compounds or stimuli. This is necessary when monitoring the pharmacological and toxic effects of certain compounds, when measuring the concentration of drugs and when establishing the efficacy and safety of vaccines. Responses also need to be studied when testing the implantation of foreign elements, e.g. artificial organs and also in microbiological diagnostics. The animals used within these studies serve as a sentient “measuring device” or as a “biological instrument”.

The demands which the test animal has to fulfil, when employed as a measuring device, will depend upon the type of measurements required. Ideally the test animal should react unequivocally to the given stimulus. With regard to diagnostic procedures, for example the use of mice for toxoplasmosis testing, only a positive or a negative reaction is required. Neither false-positive nor false-negative reactions should occur. The test animal must be sufficiently sensitive to enable its reaction to be both specific and clearly discernible. In the case of other bioassays, such as calibration tests for the determination of hormone concentrations or for the efficacy testing of vaccines, the researcher should also select an animal which will show a consistent, dose-dependent relationship. The use of defined animals within a controlled environment is vitally important. When an experiment is undertaken with the aim of finding the pharmacological or toxic effects of compounds, it is of paramount importance that the test animal’s reactions can be extrapolated to those of other species, including man.

Animals are also used within research for the study of biological processes. In this area of study, scientists attempt to gain insight into the physiological and pathophysiological or the ethological processes within the animal. Examples here would include the development and growth of organs, regulatory processes such as circulation, respiration and the production of urine, immunological processes, the development of tumours, metabolic disorders, behaviour etc. With other processes, more specific, morphological, metabolic and/or pathophysiological functions may play a role (e.g. muscular dystrophy, hypertension, diabetes). In both these cases it should be possible to generalize the findings and extrapolate them to other animal species or man.
The concepts of animal models

Most of our knowledge regarding general biochemistry, physiology and endocrinology stems from animal experiments, which ideally should be extrapolated to man. In most experiments the animal, therefore, serves as a substitute for man and is referred to as an animal model. It is important here to define the term “laboratory animal model”. One definition would be a model in which normative biology or behaviour can be studied, or in which a spontaneous or induced pathological process can be investigated, and in which the phenomenon resembles in at least one respect, the same phenomenon in human or other species of animal. This definition includes the use of animals as models for studies of normative biology and behaviour, but most laboratory animal models are developed and used to study the cause, nature and cure of human disorders.

The significance of results from animal experiments is dependent upon the selection of a suitable animal model. The extent to which the results can be “extrapolated” depends upon the type of animal model and the nature of the research. There are no rules regarding the choice of a proper animal model, nor are there rules for the extrapolation of results from the model to another animal species or man. It is, however, very valuable to have knowledge of relevant comparative biomedical aspects.

Animal models used in the study of human disease can be divided into several groups: induced models (including transgenic models), spontaneous models, negative models and orphan models. The induced and spontaneous models are the most important.

With induced animal models, a disease or disorder is induced experimentally, either surgically or by the administration of biologically active substances, so that a corresponding likeness is obtained with regard to symptoms and etiology from those which would be expected in the target species. By interfering with the environmental, dietary, endocrine, immunological or infectious status of animals, models have been created for a plethora of human diseases and malfunctions. A new group of induced animal models in this field is transgenic animals. Developments in genetic engineering have rapidly made transgenic animals the perhaps most important category of disease models (see chapter 7). To date mice are the preferred transgenic animals for research purposes, although other species, including fish, are receiving considerable attention. The production of transgenic animals containing foreign genes may result in hitherto unrecognized welfare prob-
Animal models

lems, and researchers must be alert to signs of distress and pain in these animals.

*Spontaneous animal models* of human disease are those which exhibit naturally occurring genetic variants, and hundreds of strains/stocks of animals have been analyzed and categorized showing spontaneous diseases reflecting those of man. Migaki (1982) has listed 206 diseases in animals which are attributed to non-experimentally induced inborn errors of metabolism. It is possible to obtain spontaneous models either from inbred strains (genetically uniform) or from random-bred populations (heterogeneous) where a high percentage of the animals are affected by the disease.

*Negative models* are species, breeds or strains in which a certain disease does not develop. This term may also be given to a model which is insensitive to a certain stimulus which would usually have an effect on other species or strains. The underlying mechanisms of insensitivity can however be studied with a view to providing further insight into the given area of research.

*Orphan animal models* refer to models where a disease is initially recognized and studied in an animal species, with the knowledge that a human counterpart could be identified at a later stage. Papilloma viruses in malignant epithelial tumours, and Mareks disease virus as a lymphoproliferative agent, are two examples of orphan animal models.

Only rarely, however, do animal models fully mirror the human state in health or disease. It is often sufficient to determine the selection of an animal model given a similarity between man and animal with regard to only one aspect of the phenomenon under study. A number of different spontaneous and induced models of the same condition should be employed to scrutinize the different, possible mechanisms involved.

Extrapolation

When selecting an animal model for use, it is important to consider the desired range of generalization of the results to be obtained. The rationale for extrapolating results to other species is based on homology. Homology refers to the evolutionary similarity between morphological structures and physiological processes amongst different animal species but also between animals and man. Despite the fact that wide divergences have occurred during evolution, there are still many similarities amongst the varied animal species and between animals and man. When embarking upon the study of specific features, it is necessary to select the species or strain that displays total conformity with regard to the specific anatomical or physiological features with the species to which the results are to be extrapolated.

Extrapolation can take two forms; it may be qualitative or quantitative.
Extrapolation deals with an animal’s (pathophysiological) processes and its reactions to stimuli extrapolated to other animals or man. Quantitative extrapolation involves assessing, on the basis of animal tests, the dosage of a certain compound which would be beneficial or harmful to man or the target animal. Qualitative differences between species together with possible quantitative differences in physiological processes have a role to play here. Rates of basal metabolism and conversion of compounds in warm-blooded animals are proportional to body surface i.e. the smaller the organism, the larger its relative body surface and therefore the more intense its metabolism. In smaller animals, a higher percentage of the body weight is taken up by the liver, the kidneys and the heart. Therefore, it is advisable to relate dosage to body surface or metabolic weight (= body weight$^{0.75}$) rather than to body weight.

The extrapolatability of results may also be affected by the degree of discomfort felt by the test animal. Table 9-1 gives a general indication of the extrapolatability of results from animal experiments, taking into account the degree of similarity between animal model and target animal, and also the degree of discomfort inflicted by experimental procedures during the experiments. When extrapolating results within the same species, one of the main differences recorded will be discomfort due to experimental procedures. Extrapolation may also be hampered by differences in genotype, sex, age and physiological status. The effect of differences in genotypes will be even more pronounced when the data is to be extrapolated to another species or to man. This is due to the differences in morphological and biochemical characteristics, to the response to substances and other stimuli, or to differences in the pathophysiological reaction patterns which exist between species.

Extrapolation from animal to man should always be carried out with reservation. Test results obtained from animals will ultimately have to be verified in studies with humans. This means that it generally remains a matter of hindsight as to what extent extrapolation from animal to man was justified. It is often not possible to verify animal data in humans. It can only be suggested that, given this situation, animal tests can reduce risks imposed on man.

<table>
<thead>
<tr>
<th>Animal model and target animal</th>
<th>Discomfort during experiment</th>
<th>Type of extrapolation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Qualitative</td>
</tr>
<tr>
<td>Animal model matches target animal</td>
<td>slight</td>
<td>+ + +</td>
</tr>
<tr>
<td></td>
<td>severe</td>
<td>+ + +</td>
</tr>
<tr>
<td>Animal model does not match target animal</td>
<td>slight</td>
<td>+ +</td>
</tr>
<tr>
<td></td>
<td>severe</td>
<td>+ +</td>
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+ = low degree of extrapolatability; + + + = high degree of extrapolatability.
Animal experiments for example set up to assess the safety of drugs and synthetic substances employed in agriculture, industry and food processing, reduce the risks involved for humans, even though toxicity data from test animals can never guarantee complete safety for human beings. The risk of false extrapolation can be minimized by using several species of animals in the experiments. This is the case in toxicological screening, where the authorities usually require the use of two species, one of which is to be a non-rodent. When carrying out research into the etiology and therapy of diseases, extrapolatability from an animal model is enhanced when the diseases under study have a common origin in both man and the experimental animal. Animal tests can speed up the progress of research undertaken to combat sickness in man. Whilst observation of phenomena in animals provides ideas for directional research in humans, it also makes such research safer.

The selection of animal models

The selection of an animal model for research requires careful, indeed meticulous, planning. Before commencing, the key question or hypothesis must be clear, as this will determine the choice of animal model. Only when the key question has been clearly defined will it be possible to ascertain which “key substrate” is necessary to provide the answer i.e. a particular type of cell, tissue, an organ, or the interaction between organs. The substrate then has to be defined. Should the substrate be healthy or ill, growing, adult or old? If the decision is taken that the substrate should be ill, the question then arises as to whether an induced model or a spontaneous one would suffice. The key substrate might be a single affected cell type, but could equally be a complete system of organs.

Having clearly defined the key substrate, it is then possible to look for species or strains which are suitably endowed with the required characteristics. The next step is to determine whether the key substrate is to be examined as such (e.g. as an *in vitro* organ) or whether the intact live animal should be used as a carrier of the key substrate. The latter may allow studies of longer duration and will also permit the investigation of interactions between organs.

The steps taken in the selection of an animal model can be summed up briefly:
1. define the key question
2. decide on the key substrate
3. determine in which animal species/strains this key substrate is found
4. establish which animal species/strains possessing the key substrate are the most advantageous from the technical point of view and which cause the minimum discomfort in the animals
The selection of animal models

5. establish which practical factors should be decisive i.e. availability, accommodation, care, tractability, equipment, published information, expertise, expense

6. select the animal model on the basis of scientific, practical and ethical considerations.

Scanning available literature will indicate species used within the topic of interest, but often these species are used more out of habit than the fact that they are chosen on the basis of comparative studies. There are obvious practical reasons which favour the use of the common laboratory animal species such as mice and rats. Moreover, the mouse is of particular interest as animal model, because of the increasing number of transgenic lines that is presently available and because of the detailed knowledge of the genetic map of this species.

For the study of specific diseases, the available literature can disclose availability of spontaneous and/or induced models. When a suitable animal model is not readily available, the researcher may have to consider developing a model, but this is very time consuming. Apart from the model’s desired properties, the researcher has to study pathophysiological reactions and possible infringements on the animal’s welfare. This applies both to induced and to spontaneous models. The developed model then has to be validated to demonstrate that it can indeed act as a model for man or for the target animal.

The commonly used species of experimental animals, such as mice, rats, hamsters, guinea pigs and rabbits, usually present no problem as far as supply is concerned. They are often bred on the research premises, or can be purchased from commercial breeders. Random-bred animals and, generally speaking, the most widely used genetically defined animals can also be obtained from these sources. When planning research it must be taken into account that delivery periods grow longer as the specifications multiply or the number of animals on the order increases. With regard to inbred strains, which must show specific characteristics (genetic models), it is often necessary to turn to another research institute from where it is possible to purchase a few pairs of animals for breeding purposes.

The majority of inbred strains and genetic models have been registered in international catalogues and it is relatively easy, therefore, to locate any one particular inbred strain. Preference should be given, wherever possible, to specialized laboratory animal breeders; this is due to the fact that the animals on offer are generally kept under constant control with regard to microbiological status and genetic quality. Sometimes there are certain legal provisions which place restrictions on the methods of acquiring animals for experimental purposes. These provisions may differ between countries. This may also apply to the purchase of dogs and cats. These types of animals can be obtained from commercial laboratory animal breeders, but it may also be possible to
Animal models

buy them from authorised dealers. Non-human primates may be subject to certain jurisdictions, which means that permission is required from governmental authorities for trade, as well as for research purposes. The large-scale import of monkeys practised a few decades ago has all been but abolished. In several countries monkeys are now being bred in specially organized centres for primates.

More than a hundred animal species are being used for research and, apart from the more commonly used laboratory animals and farm animals, this includes a variety of birds, reptiles, amphibians and fish. In most countries the importation of animals is subject to a number of rules and regulations designed to control contagious diseases and to protect certain species. When animals are purchased with no background health knowledge, or have been caught in the wild, there is a risk that they are infected with pathogens. Depending upon the animal species, the researcher should place the acquired animals in quarantine (see chapter 8) before introducing them to rooms where animals are already resident. Supervision during this quarantine period should be carried out by a veterinarian.

Literature

Institute for Laboratory Animal Research. Unusual mammalian models. ILAR J 1997; 38(1).
Institute for Laboratory Animal Research. The role of computational models in animal research. ILAR J 1997; 38(2).
Institute for Laboratory Animal Research. Animal models of aging research. ILAR J 1997; 38(3).
Institute for Laboratory Animal Research. Mouse behavioral models in biomedical research. ILAR J 2000; 41(3).
The selection of animal models


11 Phases in an animal experiment

_A. C. Beynen and M. F. W. Festing_

**Introduction**

Animal experimentation plays an important part in many branches of natural science. Animals are used to investigate, describe, explain and predict biological phenomena and effects. Statements on the basis of experiments must be in keeping with the empirical data. These data must in turn be methodologically correct. This chapter presents an introduction to the philosophy and methodology of animal experimentation, discussed in relation to the phases in an animal experiment.

**Descriptive and experimental research**

Scientific information is gained in two ways: by systematic observation and by experimentation. In the former case, known as “descriptive research”, emphasis is placed on determination of amounts or relationships. For instance, estimation of the average age of people from different countries, or the number of people who die each year as a result of coronary heart disease, etc. With descriptive research, cause–effect relationships, such as those between smoking and lung cancer, can be inferred, but are difficult to prove beyond reasonable doubt.

In experimental research the approach is quite different. Here, research involves the design and analysis of experiments with populations to which different treatments are assigned during the course of the experiment. In this case causal relationships are much more easily demonstrated. This chapter is restricted to a discussion of experimental research, particularly that carried out with animals. Experimental research is usually based on the results of descriptive research or earlier experimental research. It may be basic research, aimed at a deeper understanding of biological systems, or it could be carried
out because of legal obligations. For instance, prior to launching a drug on the market, the risk of the drug causing harmful side effects at dose levels appropriate to human exposure must have been investigated and considered to be acceptably low.

Philosophers do not entirely agree on how new knowledge is acquired, and at least three different philosophies have been accepted by working scientists. Briefly, these are:

(1) The inductive-hypothetico-deductive philosophy. According to this philosophy new theories or hypotheses are formulated by observing all possible facts in order to gather relevant data from which an hypothesis is developed by a process of inductive reasoning. Inductive reasoning is a process whereby a general law is formulated on the basis of repeated observation of a particular phenomenon. Once the hypothesis has been formulated, experiments may be carried out with a view to finding support for it. The problem with this approach is that inductive reasoning does not seem to be an entirely logical process. The 18th century philosopher David Hume stated that we are not justified in reasoning from (repeated) instances of which we have experience to other instances (conclusions) of which we have no experience. “The reason why we do have expectations in which we have great confidence is custom or habit; that is because we are conditioned by repetitions and by the mechanism of association of ideas...”. This led him to the conclusion that argument or reason plays only a minor part in our understanding, and that “our knowledge is unmasked as being not only of the nature of a belief, but of rationally indefensible belief – of an irrational faith”. Thus the scientific method, the most powerful intellectual tool devised by humans, appeared to him to be logically unjustifiable.

(2) The deductive philosophy of Karl Popper. Popper put forward an alternative philosophical approach which he claims overcomes the problems associated with inductive reasoning and which he claims we do not need to justify because it is a process which we do not in fact use. His approach is now widely accepted by many working scientists.

According to Popper no hypothesis or theory can ever be shown to be true, but it can sometimes be shown to be false. If we are unable to falsify a theory, we can provisionally accept it. According to him, good theories are ones which can easily be falsified and, at any one time, there may be several competing theories, among which we must choose. Very briefly, he states that “the method of science is the method of bold conjectures and ingenious and severe attempts to refute them”.

Bold theories are ones which have a high level of generality and a high truth content. Such bold theories should be the most easily tested. If they can be shown to be false, then a competing theory would have to be developed. The more rigorously we can test a theory, the more confidence we
Descriptive and experimental research can have in it, but it can never be proved to be true. The most that we can claim is that the theory is a better approximation of the truth than any competing theory.

(3) The paradigm shift philosophy of Thomas Kuhn. According to Kuhn, science progresses by a process of sudden change, followed by a period of consolidation. Most “normal science” consists of experiments and observations based around existing hypotheses. As information gradually accumulates, it may become clear that these existing hypotheses are not entirely satisfactory. At some stage the existing hypothesis may become untenable, and an entirely new hypothesis may emerge. Kuhn terms this a “paradigm shift”. The emergence of this new hypothesis may lead to rapid scientific advance, but this eventually settles down again to another period of “normal science”, until the next paradigm shift.

With any of the three philosophies noted above, when experimental research is based on earlier investigations, the various observations are studied to develop one or more explanatory theories. Logic and intuition play an important role here. According to the inductive-hypothetico-deductive philosophy, it is at this stage that inductive reasoning is used. For instance, when a group of analogous chemicals fails to induce cancer in various laboratory animals species, inductive inference might lead to the general hypothesis that all similar chemicals are non-carcinogenic in all animal species, including man. However, it is possible that certain animal species do not fit into the generalization or that the new chemical does not comply with it. On the basis of the general hypothesis a specific or working hypothesis can be formulated. For instance, the new chemical does not cause cancer in humans, or it is non-carcinogenic in the rat. This hypothesis contains the inference (prediction) that administration of the compound to man or rats does not cause cancer. The inferring of predictions from an hypothesis is called the process of deduction.

However, according to Popper, a working hypothesis is arrived at not by a process of induction, but by a process of choosing among competing hypotheses, many of which can be discarded because they have already been shown to be false. With the above example, the hypothesis might be that the chemical is a non-carcinogen in all mammals. This is a testable hypothesis because it can be administered to mammals of various species and, if it causes cancer, then the hypothesis would be rejected. In practice, the two philosophies lead to a working hypothesis that appears quite similar and must usually be tested by experimentation. Here, Popper places great emphasis on the need to try to falsify the hypothesis, whereas other philosophical approaches tend to seek data to support rather than refute the hypothesis.

The hypothesis can now be put to the test in an experiment. When the hypothesis is not rejected on the basis of the experimental results, it is ac-
cepted for the time being. Experimental results, often also including the con-
current serendipitous observations, may contribute to a completely new spiral
of induction-hypothesizing-deduction-experimentation-induction (according to
one philosophy), or the rejection of the current hypothesis and the formula-
tion of a new one (according to Popper).

Below the various phases in an animal experiment are described in chrono-
logical order. The case is put that the objective of the experiment is to test a
certain hypothesis. The various phases illustrate the general course of such
an animal experiment. Clearly, depending on the actual problem, there will
be deviations from the following outline. However, prior to this, there is a
brief discussion of whether the philosophical underpinnings of the scien-
tific method are of any great importance to research workers working with
animals.

Does scientific philosophy matter?

The practical consequences of all three philosophies noted above superfi-
cially appear to be very similar. An hypothesis is arrived at by a process of (1)
inductive reasoning or (2) discarding competing hypotheses or (3) as a result
of the last paradigm shift in the particular discipline. Whatever the process,
the next step is the formulation of an appropriate set of experiments to test the
hypothesis and gather new data. Many scientists can spend their whole career
making a valuable contribution to their branch of science, without really be-
ing aware of any of the above philosophies.

However, there are some occasions on which a clear idea of the underlying
philosophy is useful. In animal research, a concept that is often poorly under-
stood is that of “extrapolation from animals to humans”. According to some
scientists, research is carried out on animals, and then the results are extrapo-
lated to humans. Exactly how this is done is rarely stated. However, the un-
derlying philosophy is that of inductive inference, or arguing from a particular
set of observations of which we have experience (for example that a drug is
safe in rats and dogs) to another set of which we have no experience (that it is
safe in humans). This is exactly the problem faced by Hume, who concluded
that the whole process is illogical. It also seems illogical to many anti-vivi-
sectionists who can point to specific instances where tests in animals gave
results which were not of predictive value in humans (e.g. the fact that peni-
cillin kills guinea-pigs). It would seem that the modern trend towards the use
of in vitro techniques would be even more difficult to justify. If it is difficult
to explain how one extrapolates from rats to humans, how much more diffi-
cult is it to explain how one can justify extrapolating from bacteria to hu-
Does scientific philosophy matter?

mans, as appears to be necessary in the case of the Ames test (see chapter 17).

However, according to Popper’s philosophy, there is no need to explain how to extrapolate from bacteria or animals to humans, because it does not happen. A toxicologist would set up a series of hypotheses, and proceed to test them experimentally. The first hypothesis might be that the compound is not genotoxic. This can be tested using the Ames test and other in vitro techniques. Failure to show that it is genotoxic would mean that the hypothesis is provisionally accepted. The testing would, of course, need to be as rigorous as possible. From this, another hypothesis might be developed that the compound is not a mammalian carcinogen. This could be tested in rats and other laboratory species. If it were shown to be a rat carcinogen then the hypothesis would have to be rejected. In most cases the compound would then be discarded. However, it would be possible to continue with another hypothesis that the chemical is not a carcinogen in animals which metabolize it by route X-Y-Z (as in humans), provided an animal which uses such metabolic pathways can be found. It will be seen that according to this philosophy, there is no extrapolation. A hypothesis suggests certain experiments which can be used to test it, and suitable action is decided before the experiment is even started.

There appear to be many advantages in considering animal experiments in the light of Popper’s philosophy. Extrapolation does not need to be explained, and the place of in vitro tests and lower organisms in testing hypotheses can be seen more clearly, so that pressure to use more primates could well be reduced.

Selection of research topic

The selection of the research topic, in its broadest sense, is determined by social scientific or practical relevance (for instance, environmental factors in relation to the development of cancer). In a narrower sense (for instance, the influence of specific dietary constituents on development of a specific form of cancer), the selection of research topic is also determined by the possibilities and objectives of the research institution and/or the interest of the investigator.

Animal tests may also be carried out by order of industries or government. There are legal guidelines for the admission of new chemicals (colouring matters, drugs etc.) on the market. Within this framework, testing on animals is compulsory before the compound is sold. The manufacturers of new compounds frequently commission research. In the course of the development of new chemicals, industries also carry out their own research.
Formulation of hypotheses

Ideally, the three following stages should precede the formulation of an hypothesis.
1. Observing and recording all relevant information.
2. Analysis and classification of this information.
3. The development of general hypotheses based on such information.

In practice, it is difficult to decide what information is relevant. This must be judged subjectively, and intuition may play an important role. A thorough search of the literature is usually necessary. This can be done efficiently with the use of computer programs and literature banks.

The relevant information has to be analyzed and classified. Provisional hypotheses will already have been developed. “The critical discussion of competing theories which is characteristic of good science goes (as a rule) far beyond the kind of thing with which we are perfectly satisfied in ordinary life” (Popper 1972). A creative, imaginative faculty is essential.

A general hypothesis involves making a guess at the relationship between observed phenomena and their possible causes. Great inventiveness is needed, and this is especially so when this hypothesis implies a radical break with conventional notions. Remember that, according to Popper, bold conjectures are preferred, because these are most easily tested. A bold conjecture is a theory with a greater truth content than the one which we are hoping that it will replace.

The ways of developing fruitful hypotheses are quite diverse and sometimes appear very unscientific. This is quite acceptable provided rigorous procedures are used to choose among, or test, competing theories.

Experiments are used to test the validity of the preferred hypothesis, and great care must be taken to ensure that the chosen experiment will in fact fulfil this function. The predicted experimental results should be carefully considered. If this is not done, it may be found that, after having obtained the experimental results, it is not possible to use them to refute the preferred hypothesis.

For instance, the duration of treatment for an effect to be perceptible should be indicated prior to execution of the experiment. Otherwise, it might be concluded by hindsight that the experiment did not last long enough, and that the experiment was incapable of doing what it was designed to do. Fortuitous observations are of scientific interest when they are used for the generation of hypotheses that are subsequently tested in experiments deliberately designed to do so. However, in rare cases, they may lead to a rejection of the current hypothesis, in which case they may be of critical importance.

The relevance of a prediction is determined by at least three factors.

(1) The degree of specialization that took place to render the hypothesis
Formulation of hypotheses

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testable. For instance, the experiment may involve using a specific strain of rats or duration of the treatment. A failure to disprove the hypothesis (for example that a compound is not toxic to rats) in this case may be because the wrong strain of rats was used. Well designed experiments may take account of such problems by including more than one species or strain of animals, exposure duration, different diets, husbandry conditions etc., in order to increase the generality of the results.

(2) The power of the experiment. A very small or badly designed experiment, using very variable material, may not be capable of detecting subtle effects which are still of biological importance and relevance to the hypothesis being tested. Remember that the method of science involves bold conjectures but also severe and ingenious attempts to refute them. This implies rigorous, well designed experiments with sufficient power to detect any treatment effect of sufficient magnitude to be of biological interest.

(3) How critical the experiment is to the hypothesis. If the results of the experiment lead to a total rejection of the current hypothesis, this is of much more interest than if all that is required is a minor adjustment in the scope of the hypothesis.

The hypothesis must be made operational. This is a purely practical point. At this stage, the parameters to be measured are defined and the measuring apparatus selected. Validity (lack of bias) and reproducibility (high precision) of the analytical methods are crucial here.

Selection of test material

When selecting suitable test material, many alternatives should be considered, including alternatives to living organisms and even humans. Here, we consider the situation in which an animal model is chosen. Within the framework of the inductive philosophy, the choice of animal is extremely important in relation to later generalization of the experimental results, especially for extrapolation of the results to another animal species, including humans (see chapter 10).

Preparation of research protocol

In the process of formulating the hypothesis, the practical possibilities of testing should be taken into account. The researcher is usually quite free to choose the most appropriate methods. However, the size of the experiment is determined by the statistical verification standards which have been set (see chapter 12). Limiting factors are often of a practical nature. For instance, the
choice of animal may be determined by experience concerning experimental techniques in a given animal species. The size of the animal or availability of a specific disease model may be critical.

The next step is to draft the research protocol. It may list the following:
1. Facts and inferences that led to the general hypothesis.
2. Formulation of the working hypothesis.
3. Exact deductive arguments that lead to the prediction to be verified.
4. Schematic design of the experiment, including treatments (see chapter 12).
5. Description of operational definitions and measuring methods. Criteria on which the choice of animal model is based should be indicated as well as the arguments speaking against the use of alternatives to living organisms. The validity and reproducibility of the analytical methods should be discussed, as well as the units in which the results are to be expressed.
6. A description of the anticipated discomfort of the animals (see chapter 14).
7. Number of animals to be used and statistical and methodological considerations on which this number is based should be given. This implies that the choice of effect considered to be relevant, statistical analyses, statistical power, acceptable chance of type I error and experimental design (see chapter 12) are discussed.
8. A description of logistics, i.e. the ways of collecting, processing and analyzing the data.
9. Practical aspects, such as personnel required, name of research leader, costs, etc. (see chapter 13).

The research protocol serves several purposes. It is useful for assessing the ethical acceptability of the proposed experiment (see chapter 18). It may be used as working paper to be scrutinized by colleagues. It can be submitted to potential funding bodies. It is useful in preparing a detailed protocol (see below) and it forms the basis for the evaluation and reporting of the experimental results (see below).

**Detailed protocol for the execution of the experiment**

What is needed next is a paper describing the practical actions to be undertaken. In a simple and clearly structured way, the course of the experiment must be described. Such aspects as choice of species, and strain, and the housing of the animals, must be noted. Their nutrition, the method and timing of the collection, processing and/or storage of biological samples and any euthanasia of the animals should be accurately described. The detailed proto-
Evaluation and reporting

Evaluation and reporting

On the basis of the experimental results and statistical analysis conclusions are drawn as to validity of the original working hypothesis. This may be difficult, especially for the more general hypothesis.

It is not always easy to be sure that any given experiment will give a rigorous test of the working hypothesis. In practice, an experiment will often be carried out in a specific animal house, using a particular species and strain of animals, under a particular husbandry regimen, using a particular diet, and measuring a particular set of end-points. If the experiment shows that the working hypothesis is clearly wrong, then the experiment can be judged to have been successful. However, if it fails to falsify the hypothesis, there is always the suspicion that had a different strain, diet, or set of end-points been measured, a different result may have been obtained. A really rigorous test of the working hypothesis might include testing under a wide range of different conditions, such as with different species and strains, different diets etc. This is particularly true if the hypothesis is one such as “this compound is non-carcinogenic in all mammals (including humans)”. This is a testable hypothesis in that, if it could be shown to be a carcinogen in rats, the hypothesis would be disproved. However, it is difficult to test severely without using several species, strains, modes of administration etc. This may require a whole series of experiments.

The final phase is reporting and interpreting the results of the experiment or series of experiments. This can be done in the form of an internal report or publication in a scientific journal. In the latter case, the layout of the publication is determined by the journal to which it will be submitted. A general layout is as follows.
1. Title and authors (with affiliation). Generally, individuals should not be included as authors unless they have made a substantial contribution to the work. All authors carry responsibility for the validity of the work.
2. Summary. Brief description of the aims of the experiment and the hypotheses tested, experimental design and conclusions.
3. Introduction. Summary of existing knowledge and possible working hy-
Phases in an animal experiment

1. Hypotheses, with a statement of the results to be expected if the hypotheses are not true.

4. Materials and methods. Results of animal experiments should be reproducible in other laboratories. Thus, when reporting the results, the animals used and their environment should be carefully described so that the experiment can be repeated under similar conditions (see chapter 5). The following experimental details should be given.

a. Animals:
   Origin, species, breed, strain (inbred, outbred, any genetic quality control) and proper nomenclature, specific properties as model, age, sex, body weight, microbial status (with indication of procedures used to test this).

b. Housing:
   Type of animal room (barrier, isolator, laminar flow), temperature, relative humidity, ventilation, lighting (intensity, day–night cycle), cage type (size, model, material, presence of a filter top), number of animals per cage, bedding (type, frequency of changing), transport, acclimatization period.

c. Nutrition:
   Diet (manufacturer, composition, form, sterilization, any quality control procedures), feeding regimen (ad libitum, restricted, pair feeding), drinking water (ad libitum, automatic supply, bottles, sterilization, quality).

d. Experimental procedures:
   Chemicals, drugs (dose, manufacturer, purity, additives), time point (time during the day, season, oestrus cycle, nutritional status), route of administration of drugs (orally, intravenously), sampling methods (blood, urine, faeces), anaesthesia (premedication, type of anaesthetic, dose, duration, route of administration), execution of experimental techniques, euthanasia (method), collection, processing and storage of organs.

e. The experimental design:
   Formal layout (completely randomized, randomized block, Latin square, factorial), measuring methods and method of statistical analysis (parametric; \( t \)-test, analysis of variance, regression, correlation; non-parametric) should also be stated. It may also be noted that the experimental protocol had been approved by an animal experiments committee (see chapter 18).

5. Results. Well-organized presentation of results in the form of figures, tables and words.

6. Discussion. Description of the results in the light of earlier work, implications for the working hypothesis being disproved or provisionally accepted, generalization of the outcome, suggestions for further research.
7. **Acknowledgements.** Names of persons and institutions that contributed to the investigation, but did not justify being an author, names of funding agencies.

8. **Literature cited.** List of literature cited in the text. No paper should be cited unless it has been studied by one or more of the authors.

A manuscript will not usually be published immediately. The editorial board of the journal refers it to a number of anonymous experts who judge its quality on the basis of the methods used and their relevance to the working hypothesis, the way that the experiment has been conducted, and the interpretation of the outcome. In most cases the manuscript will be returned to the authors with suggestions for ways in which it could be improved, or pointing out areas where the referee does not agree with the conclusions. It is then for the authors either to modify the manuscript, or defend their original submission.

This evaluation attempts to prevent publication of papers based on poorly conducted experiments. Moreover, the referee system will encourage investigators to design and execute their experiments properly; only then does the investigator have a reasonable chance that his/her work can be published in a respected journal.

After publication in an international journal, preferably in English, the results of the experiment are available to any interested person. The article can then either trigger the formulation of new hypotheses, or of experiments designed to test the existing hypotheses even more rigorously, or the results contribute to formulating aspects of policies in relation to public health.

**Literature**


Phases in an animal experiment
12 Design of animal experiments

A. C. Beynen, M. F. W. Festing and M. A. J. van Montfort

Introduction

Animal experiments should be designed and executed so that the results are as informative as possible. It is wrong to use too few animals as the experiment may lack the power to detect some biologically meaningful effects. Such experiments will be a waste of time and resources, and an unnecessary sacrifice of animals. Likewise, it is wrong to use more animals than are required to detect a treatment effect. In order to prevent the use of either too few or too many animals, it is essential to consider, prior to execution of the experiment, the experimental design and the number of animals needed.

In an animal experiment, treatments are applied to groups of laboratory animals. Despite accurate execution of the experiment, individual animals from identically treated groups will give different results or measured values. This is due to uncontrollable variation between individual animals.

Variation between apparently identical animals, i.e. animals belonging to the same treatment group, is caused by factors that cannot be made identical for all animals. These factors involve individually-determined genetic or microbial status, developmental accidents and environmental influences and interactions between them, including interactions with the experimental treatment. If an experiment is repeated with the same or other animals but under identical conditions different group mean values will be obtained. As a result, the observed treatment effects will also be different.

Statistical analysis of the results is usually required to account for this variability. The process of statistical analysis should ideally be done according to an experimental specification (see chapter 11) that was set prior to starting the experiment.

This specification should include:

a. The minimum magnitude of treatment effect that is considered to be of interest.
b. The chance of failing to detect such an effect, if it truly exists, i.e. the chance of obtaining a false-negative result.

c. The chance of obtaining a false-positive result (i.e. there may be no true treatment effect, but by chance the means may differ to such an extent that it appears as though there is). This is usually specified as the significance level that will be accepted.

This specification, together with data on expected inter-individual variability, makes it possible to estimate the number of animals needed to detect the specified treatment effect with a suitably low probability of obtaining a false-positive or false-negative result.

In most experiments, the objective is to determine the effect of different treatments. This has to be done against a background of uncontrollable variation which should be reduced as far as possible, and distributed at random among the treatment groups. As a result, any uncontrolled variation will have a minimal impact on the treatment effect.

This chapter presents an introduction to various elementary aspects of the design of animal experiments. Details and more complex aspects belong to the domain of statisticians rather than of biomedical researchers.

Testing of hypotheses

The testing of hypotheses is a formalized procedure for drawing conclusions. In its simplest form, an experiment consists of two groups of animals: a control and test group. The usual hypothesis to be tested is the “null hypothesis”, that there is no difference between treated and control groups. An “alternative hypothesis” should also be specified. Commonly, this would be that there is a difference between treated and control groups, though sometimes it would take the form that the treatment reduces (or increases) the effect of interest. A formal procedure, based on probability calculations, is used either to accept the null hypothesis, or reject it in favour of the alternative hypothesis.

The first step is to choose a “significance level”. This is the probability that the treated and control groups will be judged to be different when in fact there is no true treatment effect. Clearly, this probability should be quite low otherwise many differences will be judged to be due to a treatment effect when in fact they have risen simply by chance allocation of variable animals to the two groups. By convention, a significance level of 5% is often chosen, although other levels could also be justified.

The next step is to calculate the probability that the observed, or even larger, differences between the treated and control groups could have arisen by chance. This is done by means of a statistical test such as Student’s t-test or the analysis of variance. If this test is done using a computer it is likely that
Testing of hypotheses

the result will be a “p-value”, which is the probability that we are seeking. This is the probability that a difference as large as, or greater than, the observed difference could have arisen by chance if the two groups are in fact random samples from the same population. This p-value is then compared with the predetermined significance level. If the p-value is less than the significance level, then it is unlikely that the observed difference could have arisen by chance and the null hypothesis is rejected. If it is larger, then the observed difference may well have arisen by chance, and the null hypothesis is not rejected.

As an example, assume that one wishes to examine the effect of a drug on blood pressure in rats. Rats are allocated at random to the control group or to the test group, which receives the drug. Assume that the researcher predicts that the new drug affects blood pressure, and sets the significance level at the 5% probability level. At the end of the experiment the data is entered into an appropriate computer program and a t-test is used to compare the treated and control groups. Suppose a “p-value” of 0.03 is obtained. As this is less than the predetermined probability level, the null hypothesis would be rejected, and the alternative hypothesis accepted.

Suppose, however, that the “p-value” was 0.06. As this is larger than the 0.05 predetermined significance level, the null hypothesis would not be rejected, and it would be concluded that according to this experiment there is no evidence that the drug has an effect. In this case, the observed p-value was not very different from the significance level. It might be suspected that the treatment really does have an effect, but that this experiment failed to detect it, possibly because the sample size was too small, or the variability of the animals was too great. Failure to reject the null hypothesis does not necessarily mean that it is true, only that in a particular experiment there was no evidence for a particular treatment effect.

In most cases, this procedure will lead to the correct decision being made. However, false decisions may sometimes occur, as suspected in the above example. Table 12-1 illustrates this. A type I error (false positive result) is made when on the basis of statistical analysis a true null hypothesis is rejected. The probability of a type I error is identical to the significance of the test. In other words, by accepting a 5% significance level, the research worker is accepting that on 5% of occasions the experiment will lead to a false posi-

Table 12-1
Possible decisions when testing hypotheses

<table>
<thead>
<tr>
<th>Decision</th>
<th>Reality</th>
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<tbody>
<tr>
<td>Accept null hypothesis</td>
<td>Null hypothesis, correct</td>
<td>Right</td>
</tr>
<tr>
<td>Reject null hypothesis</td>
<td>Null hypothesis, incorrect</td>
<td>Type I error</td>
</tr>
<tr>
<td>Accept null hypothesis</td>
<td>Null hypothesis, correct</td>
<td>Right</td>
</tr>
<tr>
<td>Reject null hypothesis</td>
<td>Null hypothesis, incorrect</td>
<td>Type I error</td>
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</table>
tive result. A type II error (false negative result) is made when an invalid null hypothesis does not get rejected; this happens when a true treatment effect did not induce a sufficiently “clear” difference between control and test group in the experiment.

As noted above, rejection or acceptance of the null hypothesis is based on the p-value for the experiment (i.e. the chance of observing a treatment effect of this magnitude, or even larger, given that the null hypothesis is true) in relation to the chosen significance level. As a rule, significance levels of 5% or 1% are usually used. For multiple comparisons of treatments, the significance level should be lowered to take into account the increased probability of a type I error (Bonferroni’s or Hochberg’s procedure). If smaller significance levels are chosen it will increase the chance of a false negative result (failure to detect a true treatment effect, or type II error).

Experimental data can take many forms. Most commonly it arises as a result of a measurement such as body weight or the concentration of an enzyme in the blood. In other cases, it may be a count such as the number of animals with or without a tumour, or it may even involve some sort of subjective classification such as the degree of necrosis of a tissue, scored as 0, +, ++, and ++++. The type of statistical test used in assessing the treatment effect may depend critically on the type of data.

Assume, for the moment, that the experiment involves measurement data. Assume also that, under the null hypothesis, both the control and treated group represent a random sample of animals from a hypothetical infinite population in which the character of interest has a so-called normal, or Gaussian distribution. Such a distribution, which is extremely common in nature, is shown in fig. 12-1. It is specified by two “parameters”, the mean (a measure of location) and the standard deviation (a measure of the variation about the mean). These are the only two items of information that are needed to specify the distribution with complete accuracy. The distribution is symmetrical and bell-shaped.

The alternative hypothesis must be stated clearly, as it will determine whether a “two-sided” or a “one-sided” test is to be used. Sometimes it is not relevant or known whether the treatment should increase or decrease the mean value so the null hypothesis is that there is no treatment effect, and the alternative is that there is a treatment effect. As the treatment effect can go in either direction, a two-sided test is appropriate. However, if the alternative hypothesis is that the treatment reduces the mean (say), and it would be inconceivable that it could increase it, then a one-sided test is appropriate. This directly influences the required number of animals in test and control groups (see later).

There are many statistical tests to evaluate the results of experiments. The choice of test to be used is determined by the nature of the observations and design of the experiment. The most widely used statistical tests require theoretically that the deviations from the mean values are normally distributed.
Testing of hypotheses

However, small departures from this do not cause any problems. In practice, measured values seldom conform to a perfect normal distribution. Sometimes the distribution is skewed. Often the data can be transformed (e.g. conversion into their logarithmic values) to a more normal distribution. When this is not possible, so-called “non-parametric” statistical tests may be used. In certain cases, the experimental design, and consequently also the statistical analysis, can be very complex, so a statistician should be consulted prior to starting the experiment.

The probability of a type II error cannot easily be controlled as it depends on the true difference between control and test group, the variability (standard deviation) of the material and the number of animals used. When there is substantial variation between animals, and when the number of animals is small, then there is a high probability of a type II error. Such an experiment is said to lack “power”. This also holds true in case of a small difference in mean measured values between control and test group.

Identification of the “experimental unit”

So far, it has been assumed that the whole animal is the “experimental unit”, defined as the unit which can be assigned independently to one of the treat-
ments. This is the unit which will be subjected to statistical analysis. However, the experimental unit may be part of an animal, or a group of animals. The experimental unit must be identified precisely; failure to do so may result in an experiment which can not be statistically analyzed. This point can best be explained by giving some examples:

(a) A rat is used as a source of hepatocytes, obtained by perfusion of the liver with a collagenase solution. The hepatocytes are plated out in Petri dishes, and each dish is assigned at random to one of the treatments. The experiment is repeated on 6 rats.

In this case, the experimental unit is clearly the Petri dish. The individual rats represent replications of the experiment. The appropriate statistical analysis would recognise this structure.

(b) Twenty mice are used in a nutrition experiment, to compare two pelleted dietary formulations. The mice are assigned at random to two large cages with ten mice per cage. One cage is fed dietary formulation A and the other formulation B. Data is collected on growth and body composition of each mouse.

In this case, the cage (not the individual mouse) is the experimental unit. Although mice were assigned at random to the two cages, the two diets could not be assigned at random to individual mice, since all mice in the same cage had to have the same diet. Thus, once a diet had been allocated to the first mouse in the first cage, the diet of all other mice would automatically follow. All other mice in the same cage would have to have the same diet, and all mice in the other cage the other diet. The mice within a box are not independent replications of the treatment groups. As there are only two experimental units, and they have different treatments, this is an example of an experiment which can never be correctly analyzed, and is virtually useless, scientifically. True, at the end of the experiment it would be possible to use a t-test to compare body weight (say) in the two groups. But if this differs between the groups, it would never be known whether this is due to the treatment or to some other effect such as fighting among the animals in one cage, or to different locations of the two cages.

(c) Forty mice are used in a nutrition experiment to compare the effects of four different vitamin solutions given by intraperitoneal injection. The mice, identified by an ear punch, are housed four to a box. Within each box, a mouse is assigned at random to each of the four treatments.

In this case, the mouse is the experimental unit, as mice can be independently assigned to each treatment. However, the statistical analysis should take account of the particular structure of the experiment, with the boxes each representing a replication of the four experimental treatments.

(d) A pharmacologist uses anaesthetized rats to study the effects of various drugs, administered intravenously, on the heart. Ten compounds can be studied
Identification of the “experimental unit” sequentially on each rat, and these are tested in a random order. Five rats are used in a particular study.

In this case, the experimental unit is a rat for a period of time, so that each rat provides ten experimental units. The structure of the experiment using five rats would need to be taken into account in the final statistical analysis.

Determining the size of an experiment

There are several ways of determining how large an experiment needs to be. The three main methods are listed below; the method based on mathematical equations and the “resource equation” method are discussed in more detail in the following sections.

(a) Determining sample size from mathematical equations. For relatively simple experiments, it may be possible to calculate exactly the number of experimental units (usually, but not always whole animals) which are needed to attain specified objectives. With appropriate computer programs, even more complex designs may be planned in this way. This approach is particularly useful for long-term and expensive experiments such as clinical trials of new drugs.

(b) The “resource equation method”. With more complex experiments, the “resource equation” approach suggested by Mead (1988) is recommended. It is based on arbitrary rules for the number of treatments and the number of units to be assigned to the estimation of “experimental error”, but is a sensible and logical approach. It is the method which is probably used by most statisticians for short-term and complex experiments which are not particularly expensive to conduct.

(c) Methods based on previous experience. Frequently, the size of an experiment is determined by previous experience. The disadvantage of this method is that it may be too large (or too small), leading to a waste of resources and animals. In some cases, such as toxicity testing, the size of an experiment is determined by regulatory authorities, who also appear to have relied on past experience. An individual faced with this sort of experiment must follow the guidelines. However, a critical examination of the size of experiments based on past experience should be made periodically using method a or b above.

Determining sample size from mathematical equations

The explanations below are applicable to experiments consisting of a control and test group with the objective being to detect a specified treatment effect, if it exists, with a pre-established probability (power). The principles described
Design of animal experiments

Design of animal experiments

Here, however, hold for all types of experiments. The method is described in detail by Cohen (1988), and a computer program (Piantadosi, 1990) is available which covers a wide range of designs, though it has been written for the professional statistician rather than the experimenter.

Suppose that an experiment is to be designed to investigate the effect of a newly developed drug on blood pressure in rats. The difference between the blood pressure measurements at the start and end of the experiment is calculated so that for each animal there is a difference measurement. One group of animals receives the drug in between the initial and final measurement (test group) and the other group of equal size receives a placebo. How many animals per group have to be used?

Prior to starting the experiment the objectives must be defined. These include:

a. The significance level to be used (probability of a type I error), typically set at the 5% level.

b. The required statistical power (1 – the probability of a type II error), often set at 90%.

c. The minimum treatment effect (difference in mean of control and test group) considered to be of interest.

In addition, an estimate of the anticipated variation (standard deviation) between individual measurements with respect to difference of final and starting value is needed, and the type of statistical test must be specified. In this case, the data will consist of quantitative measures of blood pressure change, and a t-test would be the appropriate method of statistical analysis. From this information it is possible to calculate the number of animals needed in each group as follows:

(a) It is assumed that a type I error of 5% and power of 90% is to be specified (thus, the probability of a type II error is 10%).

(b) The size of the treatment effect of interest (the biologically meaningful difference between the treated and control group) is determined on the basis of physiological, clinical, public health or economical considerations. For example, it may be that the new drug should have a higher efficacy than those already being used. If current drugs lower blood pressure in rats by 25% when compared with controls, the new drug should lower blood pressure by more than 25%. A smaller effect, even if it truly exists, may not be of interest. The minimum effect to be detected now is 25%. At this stage, interest is not primarily directed towards a good estimate of the magnitude of the true effect. We merely wish to detect, as statistically significant, a treatment effect at least this large when it exists. Should the observed difference turn out not to be statistically significant, the conclusion will be that a possible treatment effect is less than 25%. It is possible, however, that there is a true treatment effect which is smaller.
Determining sample size from mathematical equations

(c) The anticipated individual variation (standard deviation, \( \sigma \)) must be estimated. This can be done from earlier experiments or data from the literature. When this is not possible, \( \sigma \) can be estimated from a pilot study with sufficient experimental units. Pilot studies are often necessary anyway in order to validate the experimental protocol. We will assume that the \( \sigma \) of the control and test group are identical.

When \( \sigma \) is known, the size \( n \) of each group (assuming equal numbers of treated and control subjects) with two-sided testing is given by the formula:

\[
n = \frac{2 \left( \frac{(z_{\alpha/2} - z_{\beta/2})^2}{(\mu_1 - \mu_2/\sigma)^2} \right)}{\left( \frac{(\mu_1 - \mu_2/\sigma)^2}{\left(\frac{\mu_1 - \mu_2}{\sigma}\right)^2} \right)}
\]

For one-sided testing the equation is:

\[
n = \frac{2 \left( \frac{(z_{\alpha} - z_{\beta})^2}{(\mu_1 - \mu_2/\sigma)^2} \right)}{\left( \frac{(\mu_1 - \mu_2/\sigma)^2}{\left(\frac{\mu_1 - \mu_2}{\sigma}\right)^2} \right)}
\]

The values of \( z \) come from the standardized normal distribution, depending on the chosen significance level and power. The right \( z \) points of the standardised normal distribution (fig. 12-1) are: \( z_{0.10} = 1.282; z_{0.05} = 1.645; z_{0.025} = 1.960; z_{0.01} = 2.326; z_{0.001} = 3.090 \). The complement of the right \( z \) point is negative; thus if \( z_{0.10} = 1.282 \) then \( z_{0.90} = -1.282 \), etc.

In the example above, the reference drug produces a blood pressure lowering of 25\% (= \( \mu_2 \)). Let us say that the effect of a new drug should be considered statistically significant with a high probability when its blood pressure lowering effect is at least 35\% (= \( \mu_1 \)). Assume that in a comparable experiment the coefficient of variation (\( \sigma/\mu \times 100 \)) of blood pressure reduction was 14\%. Suppose that the chance of a type I error is set at \( \alpha = 5\% \) and the chance of a type II error at \( \beta = 10\% \), so power (\( \pi \)) is 90\% The desired group size can then be calculated using the above formula, by using the following values:

\[
\alpha = 0.05; \quad z_{\alpha} = 1.645 \\
\pi = 0.9; \quad z_{\pi} = -1.282 \\
\frac{(\mu_1 - \mu_2)}{\sigma} = \frac{(35 - 25)}{14} 
\]

Therefore,

\[
n = \frac{2 \left( \frac{(1.645 + 1.282)^2}{(10/14)^2} \right)}{33.6}, \text{ that is } n = 34 \text{ per group.}
\]

Note that, in this example, the data is presented in terms of the percentage reduction in blood pressure, and the coefficient of variation, rather than the actual numerical reduction and standard deviation. However, the same result would have been obtained had the actual values been used.
If one cannot assume that $\sigma$ is known, the relationship between $n$, $(\mu_1 - \mu_2)/\sigma$, $\alpha$, and $\pi$ is given in fig. 12-2. This figure holds for $\alpha = 0.05$ (two-sided) and has the quantity $(\mu_1 - \mu_2)/\sigma$ on the horizontal scale, $n$ on the vertical scale and the probability of a type II error $(1 - \pi)$ with the lines.

For other combinations of $\alpha$ and $\pi$, when the question to be addressed is one-sided, $n$ has to be multiplied with the factor $(z_\alpha - z_\pi)^2/(z_{0.05} - z_{0.90})^2$. Table 12-2 illustrates the dependency of $\alpha$ and $\pi$ on this factor. In the above-mentioned example, lowering of $\alpha$ to a value of 1% and raising the desired power to 95% leads to a required number of animals per group of $n = 1.84 \times 33.6 = 62$.

When several parameters are measured on each animal, a different number of animals may be required for each parameter. The various group sizes should be combined, depending on the importance of each end-point, so that one number of animals is obtained. When more than two treatments are to be
Determining sample size from mathematical equations

Comparison of variance rather than the *t*-test is indicated, and calculation of the required group size becomes more complicated. However, for $\alpha = 0.05$ and $\pi = 0.90$, the number of animals estimated to be necessary to compare two groups will be a reasonable estimate.

To estimate the number of animals needed for a two-sample situation, Table 12-3 can be used. This table has been constructed for $\alpha = 5\%$ (two-sided testing) and $\pi = 90\%$. Suppose that it is considered meaningful from a physiological point of view, if the true treatment effect (difference between control and test group) is 20% or more. The coefficient of variation in the projected experiment is estimated to be 15%. According to the table a minimum of 26 animals (13 per group) is needed to detect, as statistically significant, an effect of 20%. The table shows that the group size required falls with decreasing coefficient of variation, at constant effect to be detected.

#### Table 12-2

Relationships between required numbers of animals per group with variable type I error probability ($\alpha$) and power ($\pi$) but with constant difference considered meaningful and expressed as multiple of the variation coefficient ($\sigma$)

<table>
<thead>
<tr>
<th>$\alpha$</th>
<th>$\pi = 0.50$</th>
<th>$\pi = 0.70$</th>
<th>$\pi = 0.80$</th>
<th>$\pi = 0.90$</th>
<th>$\pi = 0.95$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.10</td>
<td>0.19</td>
<td>0.38</td>
<td>0.53</td>
<td>0.77</td>
<td>1</td>
</tr>
<tr>
<td>0.05</td>
<td>0.32</td>
<td>0.55</td>
<td>0.72</td>
<td>1</td>
<td>1.26</td>
</tr>
<tr>
<td>0.02</td>
<td>0.49</td>
<td>0.78</td>
<td>0.98</td>
<td>1.30</td>
<td>1.60</td>
</tr>
<tr>
<td>0.01</td>
<td>0.63</td>
<td>0.95</td>
<td>1.17</td>
<td>1.52</td>
<td>1.84</td>
</tr>
</tbody>
</table>

The table is exact with a normal distribution and knowledge of $\sigma$ for one- and two-sided testing and is a good estimate with $\sigma$ to be assessed in combination with Student’s *t*-test. In the table values for $(z_{\alpha} - z_{\pi})^2/(z_{0.05} - z_{0.90})^2 = 0.1167(z_{\alpha} - z_{\pi})^2$ are presented.

#### Table 12-3

Required sample size ($n$; number of animals per group) to detect a statistically significant effect when using a two-sided Student’s *t*-test for two samples with type I error probability ($\alpha$) of 0.05 (or 0.025 for one-sided testing) and power ($\pi$) of 0.90

<table>
<thead>
<tr>
<th>Difference considered meaningful (%)</th>
<th>Anticipated coefficient of variation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>23</td>
</tr>
<tr>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td>15</td>
<td>4</td>
</tr>
<tr>
<td>20</td>
<td>3</td>
</tr>
<tr>
<td>25</td>
<td>3</td>
</tr>
<tr>
<td>30</td>
<td>3</td>
</tr>
</tbody>
</table>

The figures in the table ($n$) are based on the theory underlying fig. 12-2.
Methods of estimating the sample size from mathematical equations have certain disadvantages. If many end-points are involved, then:

a. It is often difficult to specify the size of treatment effect which would be of biological interest.

b. There may not be any useful estimates of the standard deviation of each character.

c. The calculations are difficult to perform and interpret with complex experiments such as those involving several treatments and factors.

d. Different sample sizes may be needed for each character, and these may be difficult to reconcile across all characters.

It may still be well worthwhile using above mentioned method for expensive and time-consuming experiments with important consequences, such as clinical trials of a new drug. However, for most short-term animal experiments, an easier method is needed.

The “resource equation” method proposed by Mead (1988) is probably the method used by most statisticians when designing relatively inexpensive experiments. What Mead has done is to express usual practice explicitly, and give some justification for it. In this discussion, it is assumed that the end-point is measured data (not discrete dead/alive data), and it is assumed that the experiment will be analyzed by the analysis of variance, though this would not always be essential.

If there are N experimental units, then Mead’s resource equation is:

\[ N - 1 = T + B + E \]

where T is the number of degrees of freedom associated with treatments (i.e. the number of treatments –1), B the number associated with blocks and other methods (e.g. the analysis of covariance, not discussed here) of allowing for environmental variation (i.e. B is the number of blocks plus covariates –1), and E is the number associated with error (obtained by subtraction of T and B from N).

All that this equation states is that the number of degrees of freedom can be added together. What Mead has done is to suggest the number of degrees of freedom that should be associated, in particular, with treatments and error.

Mead suggests that in a “good” experiment, the number of treatments or treatment combinations (e.g. with a factorial experiment, described later) should be between 10 and 50. He suggests that to have fewer than 10 treatments is wasteful of resources. To have more than 50 would be to make the experiment too complex. In fact, many biological experiments do have fewer than 10 treatments, and are therefore not as efficient in terms of the use of resources (animals) as they might be. Generally, research workers should be
Determining sample size from mathematical equations

asking many more questions in their experiments than they are doing at present. With factorial designs (see below) it is relatively easy to have a large number of treatment combinations.

Mead also suggests that $E$, the number of degrees of freedom associated with experimental error should be between about 10 and 20. This can be justified by examination of fig. 12-3, which shows the critical value of Student’s $t$ plotted against the degrees of freedom. Note that, as the number of degrees of freedom increases from one to ten, the critical value of $t$ declines steeply. This implies that the size of biological effect that can be detected declines rapidly and substantially as the error degrees of freedom increases from one to ten. In other words, the experiment becomes substantially more powerful, and sensitive to a small treatment difference. It is a matter of debate whether the curve is sufficiently flat at 10 degrees of freedom, or whether it would be better to draw the line at 15 degrees of freedom. However, there is

\[
t = \frac{\bar{x}_1 - \bar{x}_2}{\overline{S_{\bar{x}}}}
\]

Fig. 12-3. The critical value of Student’s $t$-test at the 5% probability level, plotted against degrees of freedom. Note the rapid decline as the degrees of freedom increase from one to about 10, followed by little further decline up to 40 degrees of freedom and beyond (not shown). Vertical lines have been drawn at 10 and 20 degrees of freedom, corresponding to the range suggested by Mead (1988) as being suitable for well-designed experiments. Below 10 degrees of freedom, experiments are likely to lack power. Above 20 degrees of freedom, there is little gain in precision without a substantial increase in sample size, so experiments will tend to be wasteful of resources.
very little further decline from that point onwards. The experiment would have to be made very substantially larger to gain any useful increase in statistical power. In many cases, this would not be worthwhile. In fact, Mead suggests that to have more than 20 degrees of freedom for error would, in most cases, be a waste of resources.

Mead points out that it would be possible to keep the number of degrees of freedom between 10 and 20 by performing several small experiments, rather than one large one. However, this would be inefficient because of the need to estimate the standard deviation within each experiment. He states that “The experimenter should identify clearly the questions he (or she) wishes the experiment to cover, and he should also consider carefully if he is asking enough questions to use the experimental resources efficiently”.

Note that Mead’s suggestions are only guidelines. Where experimental units and measurements are cheap (e.g. Petri dishes of hepatocytes when one rat may produce enough cells to fill hundreds of dishes, and the end-point is easily determined) it may be worthwhile doing larger experiments than he suggests. Also, in cases where very small biological effects may still be important (say in toxicology), it may be worth designing experiments with 25–40 degrees of freedom for error.

Mead makes no particular comments about B, the degrees of freedom associated with blocking. At first sight, it would seem that blocking would lead to an increase in the size of the experiment. However, the loss of a few error degrees of freedom for blocks is usually more than compensated for by the reduction in overall variation and the consequent increase in statistical power. The important point is to identify possible sources of variation that can be controlled by blocking.

In conclusion, using this method, all the experimenter has to do is decide on the number of treatments to be used, and whether any variation can usefully be eliminated by using blocks. He or she will then need a total number of experimental units to ensure that there are 10–20 degrees of freedom left over to estimate error. Such an experiment should detect all “large” treatment effects. It may not be able to detect “small” treatment effects, but these could, in any case, only be detected by a very large experiment.

As an example, consider a hypothetical experiment to determine whether low levels of heavy metals in the diet influence the growth, health and behaviour of rats. Four dose levels are to be given, and, as the response may be genetically determined, it has been decided to use three strains of rats. Thus there are $4 \times 3 = 12$ treatment combinations, which is within the range 10–50 suggested by Mead. The rats are to be fed the diets in individual cages, they are to be weighed regularly, blood samples are to be taken at certain intervals for estimation of heavy metal loads, and the rats are to be subjected to a battery of behavioural tests such as activity and learning ability. Finally, at
the end of the experiment the rats are to be killed, and organs are to be re-
moved for chemical analysis and histology.
In view of the range of different end-points to be determined, it is felt that
overall variation could be minimised by doing the experiment in blocks of 12
rats (corresponding to the 12 treatment combinations). How large should the
experiment be?
Using the resource equation, with 2 blocks, $N = 24$, $T = (12 - 1) = 11$, $B =
(2 - 1) = 1$, so $E = 24 - 11 - 1 - 1 = 11$. The experiment might just be large
enough with 24 rats in total. However, if some of the effects are quite subtle,
it may be worth using three blocks (36 rats in total), in which case $E$ is 23,
which is not far above the number recommended by Mead. Note that it is
essential that an experiment of this sort is analyzed by the correct method. It
would be quite wrong (and almost impossible) to attempt to use Student’s $t$-
test to compare the different treatments. It is essential that a single overall
estimate of the standard deviation is estimated for each character, and this can
be done most appropriately using the analysis of variance.

**Intra-individual variation and the value of multiple measurements**

The variation among individuals treated alike is measured by the within-group
standard deviation. This *inter-individual* variation or “error” is made up of
two components:
1. Variation due to analytical errors and “time” effects at the time that the
measurements are made.
2. Intrinsic individual variability
If the inter-individual variation is large, then the precision of the various treat-
ment means will be low, and the experiment will lack power. Obviously, the
experimental units should be as uniform as possible. Some ways of achieving
this, by controlling the intrinsic variability such as the use of disease-free and
inbred animals, as well as techniques such as stratification or blocking, are
discussed later. This section discusses briefly the reduction of variation due
to analytical errors and time effects.
Suppose that a fully developed rat is weighed daily. The recorded weight
will vary, depending on small errors in reading the balance and rounding the
numbers, and on whether or not the animal has recently eaten, drunk some
water, defecated or urinated. Thus, daily weighing will result in small fluc-
tuations in apparent body weight, which will be in addition to the intrinsic
“real” body weight differences between individuals. In the case of body weight,
such fluctuations are usually so small that they can be ignored. However, for
other characters, this within-unit variation may be substantial. In such cir-
cumstances, making the measurement two or more times may be well worth-
while. Thus, chemical determinations may be made on duplicate or triplicate samples, behaviour may be assessed by observing the animals or measuring their activity on several occasions etc. However, it is important to remember that the use of duplicate samples does not remove the intrinsic variation between individuals. It can only reduce that part of the variation associated with analytical errors and time effects.

Whether or not it is worthwhile making more than one measurement on each experimental unit will depend on the relative magnitude of the two types of variation, and the cost of measurement versus the cost of increasing the total size of the experiment. This is discussed in more detail by Snedecor and Cochran (1980). Generally, it is only worthwhile doing duplicate measurements if the measurement error is high in relation to the intrinsic error. However, if each measurement is expensive (e.g. in the use of radioactive reagents), but experimental units are cheap (e.g. Petri-dishes of cultured cells), then it may be better to have more experimental units, and do a single measurement on each. In contrast, if experimental units are expensive (e.g. dogs), and measurement is relatively cheap (e.g. simple clinical biochemistry), and measurement error is large, then multiple measurements may be well worthwhile.

Where two or more samples are taken from an individual, the results should be averaged before starting the statistical analysis. Variation within an individual should never be used as a measure of the within-group standard deviation. A small experiment involving, for example, five mice in two treatment groups should never be made into a larger experiment by doing duplicate determinations of the end-point in each mouse, and analyzing the 20 observations rather than the 10 that would result from averaging the duplicate measurements. Enlarging an experiment in this way will lead to too many false positive results.

In conclusion, if there is substantial intra-individual variation as a result of fluctuations over time, or analytical errors, then it may be worthwhile making several measurements of the chosen end-point. However, the use of resources in this way has to be balanced against the possibility of adding more experimental units, which may increase the power of the experiment even more by reducing the standard error associated with intrinsic differences between individuals.

**Intrinsic variation among individuals and errors in assessing the treatment effect**

Usually, the results of an animal experiment are expressed in the form of numbers, such as the difference between the means of a character in the control and test group. The importance of these numbers is determined by the
variation or “error” within the experiment. Low variation leads to high precision, so that smaller biological effects can be detected for a given size of experiment. The previous section dealt with reduction of the error using multiple measurements. This section deals with the reduction of intrinsic variability between experimental units. Having minimised both measurement and intrinsic variability, it may be possible to reduce variation still further by stratification or “blocking”. This is discussed in a separate section.

Intrinsic variability can be reduced in a number of ways, including the following (see also chapter 5):

(a) Use of isogenic strains.

Variability can often be reduced by using genetically uniform animals of an inbred strain or, an F1 hybrid, where they are available (e.g. particularly with mice and rats).

(b) Use of “SPF” animals.

Subclinical infection can drastically increase the variability of the animals because each animal is likely to be at a different stage in the disease process, and chance will play a large part in determining the exposure of each individual to the disease-causing organisms.

(c) Environmental and nutritional control.

The environmental conditions (temperature, humidity, lighting, bedding material, type of cage etc.) and nutrition should be well controlled and optimal for each species. Sub-optimal environments have been observed to lead to increased variability for a wide range of characters.

(d) Selection for uniformity.

Variation can be reduced by selecting animals which are uniform on the basis of observable characteristics, such as age, body weight etc., whether or not in combination with genetic and microbial status. Animals which are excessively large or small should always be eliminated as they may be intrinsically unhealthy.

(e) Acclimatization.

Moving animals from one environment to another may lead to increased variability, as each animal acclimatizes at its own individual rate. Animals should not be used for experimental purposes for 2–3 weeks after a major change of environment.

In conclusion, all research workers should aim to use animals of a defined (preferably isogenic) strain, free of pathogens, and maintained in a defined optimal environment. This will lead to low overall variability. The result is either that fewer animals are needed to achieve the same level of statistical precision or, if the same number of animals is used, then the precision of the experiment is increased. An added advantage is that an experiment of this sort should be much more repeatable, as the conditions under which it was carried out have been closely defined.
Reduction in error by stratification or blocking

Having reduced inter-individual variation as much as possible by using defined animals with a controlled environment, and having considered whether multiple measurements of the end-points is worthwhile, it may still be possible to increase precision by the use of stratification or blocking.

Stratification involves dividing the animals into groups (blocks) on the basis of certain known present or future characteristics. Within each block the animals are randomly assigned to treatment groups. For instance, the animals from one litter have a high degree of genetic and environmental similarity. If there are four treated groups, each litter could be divided into groups of four animals of the same sex, one of which could be assigned to each treated group at random. Each set of four would then represent a “block”. Within each block the animals would be as similar as possible except for any treatment effect.

One great advantage of blocking is that the experiment can be conveniently divided up into small sections, which can spread the work load while at the same time increasing precision. In the section describing the “resource equation” method of deciding on the size of an experiment, an example was given of an experiment with four dose levels of heavy metals, and three strains of rats, giving 12 treatment combinations. It was suggested that such an experiment could be done as either 2 or 3 blocks. In this case, the 12 animals of a block would be treated as a group. The first group of 12 animals may be put on to the experiment during the first week, the second on the next week, and so on. Measurements of the 12 animals within a block would all be done at the same time (though in random order), which may be convenient if one step involves, say, centrifugation of samples, and there are only 12 slots available in the centrifuge. Any differences that arise between blocks are eliminated during the statistical analysis, and do not inflate the overall variation. There are many occasions when splitting the experiment up in this way is administratively convenient, but without a stratified design of this sort, it is not always easy to see how this can be done.

Preventing bias in the treatment means

During the course of the experiment, care should be taken to prevent interfering factors having dissimilar influences on control and test groups, thereby causing biassed results. Control and test animals must be housed identically at least on a group basis. Cages of control and test animals should be distributed over the racks, either randomly or in blocks, according to some known or presumed environmental influence such as illumination or temperature.
When a test group receives a certain compound by intravenous administra-
tion, the control group should be given the solvent intravenously. This will
exclude bias due to any effects of the solvent and/or the procedure of intrave-
nous administration. With oral administration of a test compound, the control
group may be given a placebo, i.e. the carrier material without the test com-
| pound. When the influence of a surgical intervention is studied, the control
group should undergo a so-called sham operation. The control animals then
are subjected to all surgical procedures (anaesthesia, laparotomy, suturing,
after-care) except for the actual experimental procedure.

Generally, the experimental data for each individual is obtained through
objective laboratory techniques. However, to ensure that there can be no pos-
sibility of bias, the animals should still be processed in random order, prefer-
ably as numbered samples without any indication of which treatment group
they belong to. If the control animals were processed on one day, and the
treated animals on another, any differences in calibration of the equipment,
composition of the reagents or skill of the staff, could be misinterpreted as
being due to the effects of the treatment.

For measurements that are intrinsically subjective, this is even more im-
| portant. Suppose that the effect of a compound on hair loss is to be measured.
The degree of hair loss may be classified in four categories: none, little, mod-
erate, much. The categories must be defined as carefully as possible using
sketches or photographs. Nevertheless, it is often difficult to decide whether
a given animal has little or moderate hair loss. In such experiments, the con-
trol and test group must be evaluated by a person who is blinded to treatment
group. Thus, the assessor cannot be influenced by any prejudice (bias) which
would cause a systematic error. It is also important that the animals from the
two groups are evaluated in random order. This will prevent the influence of
any gradually changing standards during assessment, for instance due to chang-
ing attitude of the assessor, which could otherwise cause a systematic error in
the scores.

Determination of treatment effects with little or no systematic errors al-
| lows unequivocal interpretation. Cancelling out variable, interfering influ-
| ences, so as to obtain accurate and unbiased treatment effects, will enhance
| the reproducibility of results of animal experimentation within and between
| laboratories. The need to repeat animal experiments will thus be reduced,
| leading to responsible animal use.

**Randomization**

If the aim is to compare the effects of control and test treatments in animals,
and there is no information about individual responses, the available animals
Design of animal experiments

(or other experimental units) should be assigned randomly either to control or test group (randomization). This process will divide the inter-individual variation approximately equally between control and test group.

For each animal a piece of paper may be numbered and drawn blindly from a box producing man-made random numbers. The animals are allocated to control and test groups in rotation. Tables of random numbers or random allocation on the basis of computer-generated random numbers can also be used.

In some cases, it may be impossible to assign treatments to animals in a random manner. For example, if the aim is to see whether males and females, or animals of different strains have similar levels of a particular hormone, the treatment is the sex or strain of the animal, which clearly can not be assigned at random. In this case, the study is more of a survey of sex or strain characteristics, although in all other respects it should be regarded as a formal experiment.

Note that, following randomization, it would be possible to perform a statistical test for a character such as body weight and find a “significant” difference between groups. By definition, such a result could be expected 5% of the time if a 5% significance level is used, and 1% of the time with a 1% significance level, in the case of no real effect at all. If the measured character (e.g. body weight) is thought to be an important contributor to the final measured end-point (say liver weight), then a correction can be made using a technique such as the analysis of covariance. Strictly, one should accept the results of a randomization of this sort. In practice, if body weight were the end-point of interest, and the two groups differed significantly for body weight following randomization, then most individuals would make a second randomization.

In many cases, however, it will not be possible to measure the character of interest until the end of the experiment.

Even if the differences in a character, such as initial body weight, do not differ significantly between groups, research workers are sometimes tempted to “improve” on the random allocation of animals by rearranging individuals so that mean weights are exactly identical. However, this can not be recommended. By reducing the variability between groups, the variability within-groups is automatically increased. This reduces the power of the experiment. If variation in body weight, or some other measurable character, is believed to contribute to overall variability, and if the animals are more variable than would be ideal, then this effect should be removed by stratification or blocking.
**Replication**

The use of more than one experimental unit (for example animal, part of an animal or cage of animals) per group ensures that the magnitude of the treatment effect can be assessed more accurately. Replication is also essential in order to estimate the within-group variability of experimental units in terms of the standard deviation.

**Some experimental designs**

The design of an experiment is the way in which the experimental units (for example individual animals) are arranged with regard to the treatments and possible interfering influences. The accuracy of experimental results is greatly affected by experimental design. The researcher can select from a wide range of designs, but in each case the appropriate statistical tests must be used. However, the principle is always the same. The probability of observing a treatment effect as large as or greater than that which has been observed is calculated. If this is less than the pre-chosen significance level, then the null hypothesis is rejected and the alternative hypothesis is accepted. In most cases a test statistic such as Student’s $t$-test or a variance ratio is calculated and compared with the value in a table appropriate to the predetermined significance level. However, many computer programs give a direct estimate of the probability (p-value).

Most designs involving measurement data can be analyzed by the analysis of variance. The design and statistical analysis of certain experiments can be extremely complex. We restrict ourselves here to relatively simple designs which are commonly used. In most cases, these can be analyzed quite simply using widely available computer programs. However, it is not possible to explain the analysis of variance, or the assumptions (such as a normal distribution for measured values, independence between experimental units, and similar variances in each group) which are necessary if the data are to be correctly analyzed. Emphasis here is on the design of experiments. A well chosen design is the first step towards obtaining results which are unbiased and have high statistical precision, leading to a powerful experiment.

The statistical analysis of the data is carried out according to a fixed procedure. Any set of measured values can be analyzed statistically. However, the statement “statistically significant” is relevant only when the measured values had been collected using a proper experimental design. In this case, it is possible to assume a causal relationship between the imposition of a treatment, and the difference in treatment means.

Experimental designs may be divided into several categories. The main formal designs are described briefly below:
Completely randomized or “parallel” designs

These are designs in which each animal is subjected to one treatment only (though there may be several different treatments), with all animals undergoing the various treatments simultaneously. In this case, possible time influences cannot act as confounding factors: control and test groups are exposed to the same time influences. These simultaneous or parallel designs have a number of advantages. A large number of treatments can be investigated simultaneously while the duration of the experiment is limited to the duration of one treatment. For statistical analysis, inter-individual variation can be substantial.

In completely randomized designs the animals (or other experimental units) are randomly assigned to the various treatments (or vice versa). These designs may be modified in different ways. Figure 12-4 illustrates this for an experiment that compares three treatments (or two test treatments and one control treatment). Each treatment (A, B or C) is applied to a different set of animals; the number of animals per set is not necessarily identical. In scheme 1 (fig. 12-4), only at the end of the test period are individual measured values established (indicated with arrow).

In the statistical analysis, the values are averaged over each treatment and the differences between treatment means represents estimates of the treatment effects. The process of randomization makes it unlikely that animals with different intrinsic contributions to the measured value are unequally distributed between the treatment groups.

The disadvantage of scheme 1 is that inter-individual variation for a character, such as body weight, contributes to the standard deviation of the treatment means. This reduces the statistical power. In scheme 2 (fig. 12-4), individual measured values are established both at the beginning and end of the test period (clearly, this is not technically feasible for characters such as kidney weight which involve killing the animal). The change in measured value during the course of the test period can now be calculated for each animal and averaged for each treatment. The difference between the mean changes of two groups equals the treatment effect. When compared with scheme 1, the advantage of scheme 2 is that some inter-individual variation is eliminated from the standard deviation of the treatment means. In scheme 2, randomizing guarantees that inter-individual variation in the initial measurement, and thus also in the difference between initial and final measured value, is distributed with equal probability between all treatment groups. Part of the inter-individual variation in initial values may be caused by differences in past history between the animals. Scheme 3 takes into account such differences. The scheme is identical to scheme 2, but the test period is preceded by a run-in or acclimatization period X, which is identical for all animals. Even during this pre-experimental period, environmental conditions should be identical for each animal.
Some experimental designs

Randomized block designs

With parallel randomized blocks, the animals are grouped into blocks on the basis of certain characteristics such as initial body weight, or because they come from the same litter. It may also depend on the way that the data is collected or the cages are arranged on the animal house shelf. In some cases, blocks are separated in time. For example, four short-term treatments may be compared in four animals, repeated on six different days. The days would represent blocks.

Formation of blocks, on the basis of certain characteristics, is especially desirable when the measured values are known to be influenced by these characteristics. Typically, the size of the blocks is the same as the number of treatments, or some multiple of this number. In exceptional circumstances, blocks may need to be smaller than the number of treatments, leading to a
Design of animal experiments

class of design called “incomplete blocks”, which is not discussed here. This situation also shows up when, for example, the number of treatments exceeds the number of individuals within a litter. As far as possible, animals from one block should have almost identical characteristics (e.g. weight, same litter, concentrations of blood constituents, measurements taken on the same day, etc.) and are assigned randomly to the various treatments. In the absence of a treatment effect, all animals from one block would ideally give identical measured values; between the blocks the mean measured values may be different. Figure 12-5 illustrates this. In this example, each block contains three animals, and there are three treatments labelled A, B and C. Assuming that the design is balanced (i.e. there are equal numbers of animals on each treatment and within each block), the treatments are averaged across all blocks. The aver-

![Diagram](image)

Fig. 12-5. Example of an experimental design consisting of randomized blocks to compare three treatments: A, B and C. The time point of measurement and/or sampling is indicated by an arrow. The animals are assigned to the blocks on the basis of observed (e.g. body weight) or future (e.g. time at which the end-point is measured) characteristics; within each block the animals are as comparable as possible but between blocks there are differences. Within each block the animals are randomly allocated to one of the treatments.
Some experimental designs

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ages are estimates of the treatment effects. The between-block variation is eliminated because comparison of the treatments is always done within each block of animals. This is readily achieved using a 2-way analysis of variance without interaction. The precision of such designs is high because inter-individual variation within each block is relatively small due to the selective formation of the blocks.

The success of block designs is determined by proper block formation. It should be clear that block formation is based on obvious characteristics of the animals and/or in the way that the data is to be collected. Inter-individual variation cannot be eliminated completely. Randomized block designs are particularly useful for large experiments. In such cases a completely randomized design becomes inefficient because it is difficult to obtain enough animals which are in all respects reasonably identical, house them all under identical conditions, and process the samples or collect the data in a short period.

Cross-over designs

These are designs in which the animals undergo different treatments consecutively. In this case, the experimental unit is the animal for a certain time period. It is clear that such designs cannot be applied when the animals have to be killed to obtain the measured values, or when endpoints such as chronic diseases are to be studied. However, when, for instance, studying concentrations of blood components, these designs may be highly efficient. Inter-individual variation is eliminated automatically because each animal acts as its own control. An important advantage is that fewer animals are usually needed than in parallel designs, because treatments are compared within the same animal. Cross-over designs are in fact randomized block designs in which the individual animal is the block, and as noted above, the experimental unit is the animal for a brief time period.

The simplest form of cross-over design is presented in fig. 12-6. Each treatment (A, B or C) is applied (in random order) to one animal for a pre-defined

Fig. 12-6. Examples of cross-over designs. All animals are subjected to each treatment (A, B or C) in the same order. The arrows indicate the time points at which measurements are carried out and/or samples are taken.
Design of animal experiments

period, followed by the next treatment. The differences between measured values at the end of each test period are the estimates of the treatment effects. The treatment effect must not carry over into the next period. An advantage of the design is that intrinsic inter-individual variation is eliminated because the comparison of treatments is done within the same animal.

Latin square designs

In the example given above, one disadvantage of cross-over designs is that correction for time trends is not possible without linear regression or covariate analysis, as the treatments are applied in random order. To take account of both inter-individual variation and time influences, a Latin square design may be applied (fig. 12-7).

In such a design the number of treatments, time periods and minimum number of animals (using the above example) must be equal. Thus, a 4 × 4 Latin square might have four animals, four time periods per animal and four treatments. However, the treatments are applied to each animal in a pre-defined order so as to eliminate time trend effects. Although there is an element of randomization in Latin squares, it is restricted in such a way that time trends are still eliminated. Latin square designs might also be used to eliminate a time trend or heterogeneity within one day (say animals have to be measured consecutively over a period of a whole day) and across several days (say the experiment lasts a week). In the latter case, heterogeneity may arise over a period of several days if there is a subjective element in assessing the data and/or if the assessment is done by a different person each day.

Fig. 12-7. Latin squares for the comparison of two or three treatments (A, B, C). Each group of animals undergoes the treatments in a different order. These experimental designs can be used to eliminate inter-individual variation and time influences, given an appropriate experimental unit (see text). The animals are assigned to one of the sequences of treatments at random. The arrows indicate the time points at which measurements are carried out and/or samples are taken.
Some experimental designs

Although a Latin square design provides high precision due to the elimination of two sources of variation (inter-individual and between time periods in the above example), a major limitation is that squares with less than about five treatments (i.e. $5 \times 5$ squares) are too small and may lack statistical power. A $4 \times 4$ Latin square only has 6 degrees of freedom for the error term. Squares larger than $7 \times 7$ may be too large to manage easily. Smaller squares may be replicated, which leads to a more complex statistical analysis.

The designs discussed do not allow corrections for carry-over effects. These effects imply that treatments not only have effects during active treatment but that there is also an after-effect into the following test period. Such an after-effect contributes to the measuring value in the next period when another treatment is applied. In this case, a design should be used in which all different treatments follow one another an equal number of times during the entire course of the experiment. The $2 \times 2$ Latin square already satisfies this requirement: the sequences A–B and B–A each occur once. When three treatments are investigated, a Latin square is unsatisfactory. The sequences A–B and B–A now must occur twice each. It is necessary to extend the $3 \times 3$ Latin square (fig. 12-8) so that each measuring value will be influenced by the after-effect of each treatment except for the measuring values at the end of the first test period. The estimated treatments effects will be relatively accurate, but the standard deviation is overestimated because the after-effect goes in it. Carry-over effects are best eliminated by separating the test periods from one another by a wash-out period.

![Fig. 12-8. Cross-over design for comparing three treatments (A, B and C), while correcting for possible after-effects of the treatments. Each order of treatments occurs twice. The animals are assigned randomly to each sequence of treatments. The arrows indicate at which time points measurements are performed and/or samples are taken.](image-url)
mals receive a control treatment. The wash-out periods should be sufficiently long so that after-effects of the previous treatments are not present. By incorporation of wash-out periods, the duration of the experiment is prolonged. It is also possible to quantify and eliminate after-effects by applying multiple linear regression to the data.

**Factorial designs involving more than one treatment factor**

Factorial designs study the combined effect of two or more treatment effects (factors). Such designs are highly efficient. They are widely used in agricultural research, and should be more widely used in research involving laboratory animals.

Table 12-4 shows an example of a $2 \times 2 \times 2$ or $2^3$ factorial scheme: there are three factors (A, B and C) each at two levels (high or low), giving 8 treatment combinations. These could represent, for example, two drug treatments, two mouse strains and two time periods. Each square presents one combination of treatments which is applied to one or more experimental units.

One of the advantages of factorial designs is that they increase the generality of the results, without increasing the size of the experiment. In the above example, the main aim may be to compare the two drug treatments. However, by using two strains it is possible to determine whether the result is “general” across both strains, or whether it is “specific” to a single mouse strain, with the other strain showing no effect. If the effect can be generalised (i.e. both strains respond equally), then there will have been virtually no loss of precision in comparing the two drugs. However, if the strains behave differently, then this information may be of great importance, as it will show that the results obtained with one mouse strain cannot be generalised to other strains.

It is unfortunate that many research workers are taught to vary only a single factor at a time. In fact, experiments aimed at exploring the interrelationships between two or more factors are highly efficient in terms of use of experimental resources, provided they are carefully planned and correctly analyzed.

Table 12-4

**Example of a $2^3$ factorial scheme**

<table>
<thead>
<tr>
<th>A, low</th>
<th>A, high</th>
</tr>
</thead>
<tbody>
<tr>
<td>B, low</td>
<td>B, high</td>
</tr>
<tr>
<td>C, low</td>
<td>□</td>
</tr>
<tr>
<td>C, high</td>
<td>□</td>
</tr>
</tbody>
</table>

Each square indicates a treatment group of animals.
More factors and/or more levels can be used, though the number of animals needed may become rather large. An experiment with three factors at three levels \(3^3\) involves 27 treatment combinations, involving at least 54 animals if there are two animals in each treatment group to allow for replication to give an estimate of experimental error. Such an experiment may be too large to be entirely practical, although smaller factorial designs do not cause any particular problems.

Factorial experiments are often designed as randomized blocks: each set of treatment combinations is represented by one block, so this experiment would involve, say, two blocks each with 27 animals. Factorial schemes can also be carried out as completely randomized designs. The statistical analysis of factorial experiments is described in detail in most text books; also computer programs are readily available.

*Split-plot or repeated measures designs, with more than one type of experimental unit*

In the designs discussed, above the experimental unit is the same for each factor being studied. Often it is a cage of animals, a single animal, or part of an animal. However, in some circumstances, two different experimental units may be used in the same experiment.

Assume, for example, that the effect of two dermal administrated creams is to be investigated using animals with an induced skin disorder. The compounds are to be administered to each animal at symmetrical locations on the body (for example the left and right flank, assigned to treatment A or B at random). In this case, the experimental unit is the patch of skin on an animal and each animal acts as a homogeneous block. Suppose also that a factor only applicable to whole animals, for instance nutrition, is incorporated into the experiment (say diet X and Y). Whole animals will then act as the experimental unit for the factor nutrition and animal parts as the experimental unit for the factor skin cream.

Designs based on this principle were originally developed for agricultural research and are called split-plot or repeated measures designs. The main plot (i.e. one animal) can be assigned to a whole-plot treatment. It can also be split into parts to which the sub-plot treatments are randomly assigned. If the split plots represent a time factor, then the experiment is regarded as a repeated measures design. For example, animals may be assigned to four nutritional treatments. Growth rate may then be studied over a number of time periods, with a view to studying trends in growth rate during these periods. Each time period then represents a sub-plot. Admittedly, in this example “time” is not a variable that can be assigned at random. However, in some cases, the sub-plots may have the status of a crossover design, with genuine random allocation of
time periods to a treatment such as a psychometric drug. The interest in this case would be in comparing the drug effects on animals of different nutritional status.

For the factor applied to the whole plots, the inter-individual variation is present, while for the factor applied to the sub-plots the inter-individual variation is eliminated, leaving only the intra-individual variation. Thus the sub-plot treatments effects are usually measured with considerable precision, while comparisons among main-plot treatment effects are much less precise. For an extensive description of these designs and appropriate methods of statistical analysis see the list of recommended literature.

In some cases, the dividing line between characters, experimental units and measurements is not entirely clear. Consider body weight of a rat measured at, for example, 3, 6 and 9 months of age. It might be argued that these three body weights represent different characters in the same way as body weight and liver weight are clearly different characters. In this case, they should be analyzed separately, or by an appropriate multivariate analysis (discussion of which is beyond the scope of this chapter). On the other hand, it may be argued that these represent three determinations of the same character “body weight”. In this case, they should be averaged prior to statistical analysis. Finally, it may be that the aim is to study a trend in body weight over time. In this case, they would represent repeated measures, and a split-plot analysis would be used. Also, weight differences could be calculated for each rat, and analyzed accordingly. It is up to the individual research worker to make the appropriate interpretation, depending exactly on the circumstances.

Conclusion

The choice of experimental design depends on many factors, such as possibility of subjecting one animal to more than one treatment and/or measurement, the number of available animals, the number of treatments to be examined and available time and finances. The most important consideration should be the anticipated number and type of interfering influences, and the type of treatment structure (simple or factorial). Precision can usually be improved by better experimental design, so that the same result can be obtained with fewer animals. Thus, paying careful attention to the experimental design and statistical analysis prior to starting the experiment can contribute substantially to a responsible use of animals and to a reduction of animal use.
Conclusion

Literature

Piantadosi S. Clinical trials design program. Ferguson, Miss./Cambridge, UK: Biosoft, 1990.
13 Organization and management of animal experiments

A. E. J. M. van den Bogaard, R. T. Fosse, R. G. M. ten Berg and J. T. W. A. Strik

Introduction

Research is, as a rule, not conducted by individuals working in isolation. Biomedical research projects usually involve a great number of people and many kinds of processes and structures. All of these are interrelated and influence all the participants, including the researcher him/herself. If the researcher does not master the management of the processes, this will negatively effect his/her research: animals or materials will not be made correctly available, experimental results will be unreliable and the whole process will be to no avail.

A successful researcher, just like the successful leader and manager, uses the positive forces within the organization and knows how to limit those that are potentially negative. In order to achieve this, the researcher must have an understanding of the structures (organization) and the processes (management) that are important when carrying out research.

Organization

At some point, the workload becomes so extensive that it is impossible for one person to manage all aspects of the project by him/herself. It has so many aspects that it becomes expedient to subdivide the tasks and allocate them to persons with varying degrees of specialization. For example, within a growing research group, the ordering of chemicals will no longer be the task of each individual. One (preferably specialized) person will be put in charge of this task. Specialization implies that certain techniques will be mastered, and therefore carried out, by only one (or a few) of many co-workers. Along with the actual allocation of research tasks, there will also be a separation of competence. Financial and budgetary matters are often the responsibility of
persons without a direct scientific interest in the project. Another aspect of increasing importance is legislation requiring that only competent persons, qualified and/or authorised for particular procedures, may perform interventions on animals. Definition of competence inevitably brings about a division of responsibility. The researcher is responsible for the correct design and progress of the project. He/she publishes the results or reports to the research director. Laboratory technicians are responsible for correctly carrying out laboratory tests, while qualified animal technicians are responsible for animal procedures. Animal caretakers are responsible for the daily care and surveillance of the health and welfare of the animals. The tasks of the laboratory animal welfare officer (often a veterinarian, specialised in laboratory animal medicine) are: to advise the researchers, to oversee that all procedures that influence the welfare of the animals are carried as humanely as possible, and to answer to the governing board of the institution and the national authority.

Management

The term “management” embraces all activities within an organization which are necessary to allow an effective and efficient operation to function, using the personnel and materials that are available. This includes the administrative aspects as well as quality control (accuracy) and quality assurance (follow-up). Professional management is needed in order to enable a process to run smoothly, especially if an existing project is threatened with failure. Failure can be caused by many factors and include illness, sudden and unexpected changes in deliveries, product specification, or supplier. Division of tasks according to competence and level of responsibility requires procedural discipline on the part of all the participants. Everyone must know what to do and when to do it. The person responsible for the chemical stock must be informed sufficiently in time enabling the order to be fulfilled. As a rule, animals are not delivered on a daily basis. Numbers and specifications of animals must be given on time to the person in charge of co-ordinating the orders. It is clear that agreed fixed procedures will prevent mistakes and avoid frustration. Division of tasks necessitates agreements on the timing and execution of the different phases of the project. All agreements should be in writing and listed in a procedure protocol. It may seem that there is much paperwork (written protocols, grant application, animal order form, reagent order forms etc.) and that the number of different forms is excessive. However, if used correctly, they will facilitate co-ordination, lead to higher efficiency and prevent mistakes.
Organization of research as a dynamic process

More attention should be paid to management when an organization becomes more complex, when changes in procedures or in the structure of the organization occur more frequently, or when workload pressure increases. This is not uncommon when working in scientific research, particularly when working within university or similar research institutions, due to the rapidly expanding knowledge base, and development of new insights. Traditionally, these institutions have a research activity profile that is characterized by rapid changes in project types and staff turnover, and many short-term projects. In comparison, industrial research has a long history of working with project plans, in which goals, methods, time schedules, personnel requirements and material costs are defined in advance and then monitored during the project. In academic research environments, researchers are still used to a great deal of liberty, both in the choice of research topic and the scientific approach to its solution. Budget constraints within non-commercial research institutes have been the most important single driving force for change and have prompted analysis of whether planning and monitoring of research could lead to higher efficiency.

Changes in national funding policies have led to academic research being conducted according long-term projects plans that have perspectives lasting several years. Since the 1980s, there have been major changes in the way scientific research is structured within many universities. More than ever before, the financing of research and consequently, the survival of academic research groups is increasingly dependent upon scientific output and quality. Research projects are being designed in such a way that the risk of failure is minimized. These changes have their own drawbacks. Planning and rigidity may hamper the flexibility and creativity to move along with new ideas and could hamper the response to unexpected findings and results. The situation is by no means stable. There will always be pressure on research organizations, academic and industrial, continuously to adapt and adjust the organization of research.

Conditions specific to animal experiments

As stated above, the organization of research is changing, mainly due to efforts to optimise the use of personnel and material resources. Animal experiments have an extra dimension that extends beyond personnel and material needs. When planning experiments that use animals, in addition to scientific and efficiency requirements, every scientist has an obligation to limit the number of animals used, and to avoid pain and distress as much as possible.
under the circumstances of the experiment. These added requirements have brought about special legislation in most countries. Ethical committees that regulate the welfare aspect of research have been established (see chapters 2 and 18). The researcher is ethically bound to plan his/her experiment so that whenever possible alternatives to animals are used, the numbers of animals used are kept to a minimum, and the methods and techniques are refined wherever possible. There are many ways to achieve these goals. Improved exchange of information between institutes, the use of dead instead of live animals for training, and through the use of experienced and skilled animal caretakers and technicians. Log books, required by legislation in many countries, will prevent unnecessary and uncontrollable research being done. This will ensure that the progress of research is recorded, the objectives of the experiment and the numbers of animals registered, and the expected degree of distress estimated. The log book will also record the protocol for the experiment together with the amount of compound administered, all surgical interventions performed, all observations, and ultimately the results of the procedures.

Ethical committees have been established in several countries and are being proposed in others. Their purpose is to deal with the question of whether the scientific objectives of an experiment justify the use and possible suffering of animals. Protocols for animal experiments must be presented to, and approved by, an ethical committee beforehand. The project leader must there-

Table 13-1
Checklist of legal aspects that may be involved in animal experiments

1. Is the procedure an experiment according to the national law? (In several countries not all procedures involving animals are considered to be experiments.)
2. Is the institute accredited (licensed) by an authorised accreditation organisation to carry out experiments with animals?
3. Is the researcher qualified (competent) and authorised (licensed) to design and/or carry out an animal experiment?
4. Is there an adequate research plan whereby the inevitability of the use of animals, instead of alternatives, is clear?
5. Are there sufficiently qualified support staff (caretakers, technicians, analysts) to permit the project to be successfully carried out?
6. Are the animals obtained from an accredited source?
7. Does the animal facility meet the requirements? (Housing and husbandry conditions).
8. Is there appropriate equipment for anaesthesia and euthanasia and do all relevant personnel know how to use it?
9. Have the appropriate animal welfare authorities (local animal welfare officer, responsible person, or central authority) been informed about the experiment? Has this been done in good time before the experiment is to be started?
10. Has permission been obtained for performing the research project from an ethics committee (in those countries where this is appropriate)?
11. Is the use of laboratory animals recorded and is a log book being kept?
fore be aware of relevant legislation as well as undertake an ethical evaluation when planning an experiment (table 13-1).

Legal requirements in most countries are in a process of change as animal welfare concerns play an increasingly leading role. There is a general trend towards higher qualification requirements on the part of all persons engaged in experimentation with animals. This is true at all levels: designing, performing and supervising, and taking care of animals in experiments.

The factors described in table 13-1 place extensive demands on the organization and management of animal experiments. Compliance with most if not all of these will, however, also benefit the researcher *per se*. A well designed protocol will lead to good science as well as avoid the use of too many animals. This has an ethical as well as a time/cost benefit. Good and efficient research will automatically meet many of the demands indicated in table 13-1.

**Factors influencing the results of animal experiments**

The reproducibility of animal experiments is highly dependent upon the degree of standardization of the factors that can influence the reaction pattern of the animal (see chapter 5). When designing an experiment with animals, the factors mentioned in table 13-2 should match the requirements of the experiment and animals with as little variation as possible. Suitable animals with as little variation as possible should be used. Standardization implies, wherever possible, the same supplier of animals, the same caretakers and technicians, the same animal room, the same experimental procedure at the same time each day etc. This means tight and close co-operation between the researcher and support personnel. Support personnel (caretakers and technicians) can contribute substantially to the quality of research because of their direct and daily experience with the animals. They will often know where to place priorities, and how to deal with specific problems.

**Good Laboratory Practice (GLP) and accreditation**

One special field of research that involves the use of animals, and in which organization and management are of great importance, is the area of research, which has to meet the standards required for the registration of substances (chemicals, drugs, medical devices, dietary supplements etc.). This type of experimentation has to be carried out according to procedures set by governmental guidelines. A growing number of countries also demand that this research fulfils the management principles of GLP (good laboratory practice).
These GLP principles were first presented at the end of the 1970s by the American Food and Drug Administration (FDA). They refer exclusively to the methods of conduct and management of the research and to the circumstances under which it is carried out. They are there to stimulate accuracy in the conducting and reporting of animal experiments. For example, according to GLP, prior to commencing research, a clear-cut study plan must be available, detailing the nature and scope of the experiments including all the procedures designed to achieve the pre-set objective. It should also contain the name and qualifications of the researcher responsible for the experiment, together with who will carry out the procedures and when, plus a record of all the observations. All procedures must be described in detail, stating the work instructions.
Centralization of animal experiments

and the SOP’s (standard operating procedure). There must be SOP’s for animal maintenance, experimental techniques, laboratory measurements, hygienic procedures, examination of the animals, collection and processing of data, etc. GLP principles also take into account the lay-out and equipment of laboratories and animal rooms. GLP sets out guidelines regarding the construction of the animal rooms and the environmental conditions, under which the laboratory animals must be kept. The animals themselves must meet quality requirements, specified by the researcher. Documentation must be kept which confirms all these conditions.

Although GLP regulations are primarily meant to guarantee accuracy in toxicity research, their application to other areas of research involving the use of animals, can contribute towards standardization and hence towards the reproducibility of research results. The application of these rules does not, however, guarantee that the required level of standardization will be achieved for each experiment. Each type of experiment has its own specific demands with regard to standardization.

Experimental animal facilities or departments should obtain accreditation by an independent organisation for the accreditation and assessment of experimental animal care. This will ensure that they meet or exceed applicable standards when housing and using animals for scientific procedures.

Centralization of animal experiments

Many institutions have some form of centralization for both spatial and personnel facilities with regard to animal experiments. Centralization may include:

(a) Joint purchase and breeding, housing in quarantine and stocking of animals: the “laboratory animals supply department”.

(b) As (a) but also housing and maintenance of animals in experiments: “the laboratory animal hotel”. This concept may refer to joint facilities for less commonly used animals and/or animals which have special housing demands, such as sheep, dogs, cats and primates. It may also refer to the housing of all animals during experiments. In this case, the research departments do not have animal rooms in their own buildings, but make use of a centralized facility. Animals would still be removed for special interventions such as surgery, irradiation, X-ray, etc.

(c) The most extreme form of centralization is the one, in which not only the activities mentioned under (a) and (b) are centralized, but also the entire experiment (surgical) interventions are carried out at the central department. In this case all personnel, including the animal technicians, are employed by the facility: the central experimental animal institute.
For the researcher, centralization implies that co-workers, rooms, and other facilities no longer belong to his/her own department. This is often seen as a disadvantage and often implies an extra consultation step in that the facility management will have to be involved in the decisions and action process. It is essential that co-workers who work within the facility are motivated, informed and are consulted when contributing to animal experiments. Equal time and attention should be given to both animal facility co-workers and workers from the associated user research institute. Giving time and attention to the facility co-workers will ensure that they are informed about the demands placed on the care and health status requirements of the animals that are used in the project.

A central animal institute is faced with a great variety of research lines which differ from one institute to another, each with its own demands with regard to quality and health profiles of the animals that are needed. This inevitably will mean several suppliers, each of which may use different methods of quality assessment and control. Centralization also means that more personnel will be involved and consequently brings more problems with regard to the maintenance of hygienic conditions in the animal rooms. For practical reasons it is usually not possible to separate animals on the basis of supplier, demands of the experiment, etc. This means that the central institute, in the interest of all researchers, has to apply procedures and rules which may not always seem necessary to the individual researcher. This can have cost/budget implications and is sometimes the subject of debate and negotiations. Advantages of centralization lie in the greater volume of trained personnel available, which may lead to a more efficient allocation of tasks, to an increase in knowledge and skills over a wider range of techniques, to better opportunities for creating optimal spatial and technical facilities and to the more efficient use of animals. The concentration of expertise is also of benefit to the welfare of the animals. Within a central institute, researchers and research lines meet one another, and as a result researchers with related research questions, corresponding animal models and techniques come to know each other and can exchange information. The central institute management should actively encourage this.

**Optimal arrangement of research activities**

In the preceding paragraphs, a number of restrictive conditions have been mentioned which the researcher has to deal with. One of the most crucial factors is time planning. Research activities must be organized in such a way, that the different reports (internal reports, articles, and dissertations) are ready within a given period of time. This is conditional on all the research activities being carried out in the right sequence and in time. All the phases from the start, execution and end (the final report) of the research project must be
Optimal arrangement of research activities

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taken into account (see chapter 11). In addition to general factors which apply to all types of experimentation, there are those which apply more specifically to animal experiments:

– Time must be allocated to obtaining the right number of animals. These must comply with the protocol needs regarding biological status (sex, age, body weight, strain/stock). This is especially the case when ordering animals from less commonly used strains or from non-commercial suppliers. The same also applies when the demand for animals from own breeding colonies increases. The size of the breeding stock has to be enlarged before animals can be delivered for research. Supplying aged animals will also imply considerable delays;

– An adequate quarantine period necessary to determine potentially undesirable contamination of animals of unknown health status must be built in (see chapter 8). The quarantine period is also needed to allow newly transported animals to adapt to the new environment. This includes moving animals within the central institute;

– Delays caused by unexpected and spontaneous death of animals;

– Time must be allowed to learn and master new techniques;

– Time for delivery of special diets;

– Pilot experiments are always a prerequisite when new methods, techniques, diets etc. are introduced;

– Time needed for the submission to, and approval by ethical committees or regulatory authorities.

Logistic resources necessary for successfully carrying out the experiment can also influence time planning:

– space in the animal rooms

– availability of personnel (caretakers, technicians, analysts);

– funds for the purchase of animals, reagents etc.;

– availability of special equipment and apparatus (isolators, laminar airflow benches etc.).

The above mentioned factors all demand planning and co-ordination.

A well-founded estimate of costs and a plan of expenditure must be drafted. This is essential if appropriate funding is to be raised and budget constraints are met during the course of the research project. The animal technicians and animal institute leadership will make a valuable contribution to this process and must be consulted.

Co-operation – working with others

Almost every animal experiment involves interdisciplinary and cross-departmental co-operation. The researcher has to take the role of conductor and make sure that all the necessary actions take place in the right way and at the
right time by the designated persons (see fig. 13-1). There is a greater chance for success if the following rules are implemented:

1. Take decisions about activities that have to be performed by others only after deliberation with those concerned. They must be informed well in advance about what has to be done, how to do it, and when it has to be done. Their opinions should be taken into account when establishing policy that will influence a final decision.

2. Motivate co-workers. Inform them about the aims of the research. Give them specific information about the significance of the role that they will play. Tell them about the progress of the research and of the results as they appear (both positive and negative findings, as well as setbacks). Give reasons for changes to the protocol. Motivate also by asking advice and by giving arguments when asking them to perform a procedure in a particular way.

3. Keep a log and diary and minutes of all meetings:
   - make a short written report of every consultation meeting, about the agreements that were reached (who agreed to what, how and when);
   - make written protocols, signed by the technician concerned, covering the dates that techniques are to be carried out, the numbers of animals involved, doses to be administered, etc.;
   - develop forms for dated recording of research data including information on the strain, sex, numbers of animals, body weight development, health status, tumour dimension, dates of death, anaesthesia, euthanasia, etc.;
   - distribute copies of reports, protocols and forms to those concerned.

4. Make agreements on time and change them as seldom as possible. Much time is wasted by ill-prepared and hasty work. If changes in the protocol are absolutely necessary, discuss them well before they are due to take place, and note them down in writing. Make sure that all the people concerned are informed and understand the changes.

5. Keep your agreements and make sure that others do the same.
Safety in the animal house

The centralised management of an experimental animal facility also has implications for comprehensive and extensive personnel and in-house safety programmes. The manager of the institute, a specialised veterinarian or other competent and suitably qualified person, will also be able to maintain an overview of activities that play a role in personnel safety and prevention of accidents and infections. Planning scientific projects with animals involves factors not limited to the animals themselves. Researchers may bring in substances that could be hazardous to the technical or other staff. This is particularly the case when handling substances such as radioactive isotopes, carcinogens, cytostatics or other substances potentially hazardous to staff. The animals may be intentionally infected with infectious agents as a part of the experimental protocol or they may be infected with zoonoses that can cause disease in the members of staff. The protocol may require inoculating the animals with material of human origin. Alternatively the material could be of animal origin and carry undesirable infectious agents. Finally, the animals could represent a source of antigen causing allergic symptoms among sensitive staff members.

Planning a research project places demands on both researcher and the manager of the animal facility. The researcher could often be ignorant of factors that could influence the general environment of the institute. This is particularly the case when disease causing organisms (viruses, bacteria or parasites) are introduced. The degree of specialisation that may be necessary when planning research projects makes it difficult for an individual researcher to have a total overview of all microbiological, veterinary and medical aspects related to the chosen protocol. The staff of the central animal facility plays a crucial role in preventing inadvertent accidental contamination. There may be a need to contain or isolate animals for a period of time while special examinations are carried out and the microbiological quality of the animals or tissues established. Disease contamination is one of the most significant and serious setbacks in research with animals. It is therefore essential that projects are planned with this in mind and that the expertise of the institute’s laboratory animal science specialists is included in planning.

There is also a significant cross-flow of specialities in preventing zoonoses. The vast majority of the most commonly used laboratory animals are bred and supplied from sources that are free from zoonoses. The situation is different when working with wild caught animals, primates in particular. Close collaboration between veterinary and human medical expertise is necessary when planning projects with these animals. There are several serious diseases (zoonotic infections), which can be transferred from animals to humans. Some of these can have fatal consequences for the individuals concerned. It is therefore essential that the institute leadership plays an active role in a planning
and advisory capacity when these animals are used. It should not be the sole responsibility of the researcher to decide what type of housing, handling and procedures should be used when using this type of animal.

Similar precautions need to be applied when working with substances as carcinogens, cytostatics and radioisotopes. There is often a chain of decision making when planning a procedure. The person who decides to include a substance in a protocol — and at the same time has the most information regarding the potential hazards associated with the use of the substance — is often not the person actually responsible for administering the substance. There may also be a change in the toxicity of the substance in that metabolites excreted in the urine or faeces may be considerably more toxic than the parent substance. It is clear from this that each sub-stage in the administration of a compound needs to be analysed. Sets of written rules need to be prepared and safety procedures established with clear and easily understood standard operating procedures. At no time should there be any doubt as to what is being given, what the hazards are and who is or could be in contact with the substance. Responsible individuals must be appointed and they should be accessible in the case of accidents or doubt.

Immunisation procedures can have special hazards when administering antigens that could represent a hazard. An example worth noting is immunising animals with HIV or viral hepatitis antigens. Inadvertent self-injection with such antigens could at worst make a technician HIV or hepatitis positive with fatal results. It is therefore essential that there are clear descriptions of what is being given and what action to take in the event of an accidental self-injection. Such instructions should accompany all procedures involving any material of human origin.

Planning and experimental protocol should be extended to include factors that are of importance to safety planning. The leader of the animal facility should be included as a member of the team and there should be an interchange of expertise that covers this aspect of project planning. The institute’s project registration scheme could be used to register potential hazard information. Any chemical substance that is to be given to the animals must be described. Carcinogenicity, cytotoxicity or radioactivity must be noted in full detail and copies of the information made available to all members of the team. Standard operating procedures should be drawn up. The staff of the animal facility should play an active role in gathering this type of information and make sure that all co-workers are fully informed before they are exposed to potentially hazardous procedures. Lines of responsibility should be clearly established and each member of the team must be made aware of whom to report to in the event of an accident. Emergency medical personnel must be informed of procedures prior to an accident taking place. This is particularly necessary in the event of accidental self-injection. This type of accident often
Safety in the animal house

seems to be minor and superficial at the time of happening. Emergency department personnel must be trained as to what to do since the treatment that may be necessary might appear to be out of proportion to the degree of immediate damage. The special knowledge and expertise of the animal institute’s staff will play a significant role in ensuring that procedures can be carried out with a minimum of risk to personnel and animals in the facility.

**Literature**


Hayden C C. How to increase employee participation in the lab animal environment. Lab Anim 1987; 16, 47–49.


Organization and management of animal experiments
14 Recognition of pain and distress

V. Baumans and P. F. Brain

Introduction

Pain and distress are relatively vague concepts when applied to animals. As these terms refer to subjective states, they are strictly only applicable to humans. However, the sharing of common neural structures and physiological processes, and the existence of behavioural manifestations comparable to those seen in humans in states of pain and discomfort, make it plausible to assume that animals also experience pain and discomfort. It is ethically prudent to go one step further and to accept that animal suffering can at least be equivalent to the suffering of a human when both are subject to the same procedure. This analogy postulate should be accepted unless its invalidity has been proven in a specific case. The postulate can be the basis for making choices when using animals for research, although one should realize it has a weak scientific basis and should be used with caution. One cannot exclude the possibility that procedures, which are comparatively harmless for human subjects, are painful or stressful for certain animals, and vice versa.

It would be much more satisfactory if we could reliably estimate an animal’s suffering from behavioural and physiological signs. But, as yet, there is no guarantee that states of pain and discomfort are properly recognized on the basis of presumed analogous behaviours. For one thing, our estimation of the intensity of such subjective states may be incorrect because of interspecific differences in the relations between states and behavioural manifestations and because the context influences this relation. Does the deafening squealing of a piglet which is physically restrained indicate that it is subject to more intense pain and suffering than the comparatively mute way in which a wildebeest undergoes being torn apart by Cape hunting dogs? When we move away from mammals to other vertebrates, such as fish, and, especially, when we move to invertebrates, the inference of pain becomes progressively more
Recognition of pain and distress

The lack of recognized behavioural responses may be misleading, suggesting the absence of pain perception and suffering. Nevertheless, in spite of some drawbacks, the analogy postulate remains our first and best principle.

The behavioural criteria for the recognition of states of pain and discomfort are discussed in this chapter. An evaluation of these criteria should be based on insight into the biological function of these supposed states and their behavioural correlates. We can subsequently arrive at some guiding principles, by recognizing that, in nature, discomfort and pain form adaptive monitoring systems playing an important role in the motivational structuring of behaviour, rather than being meaningless sources of suffering.

Discomfort and suffering

Discomfort is integral to animal life and, in regulating the priorities of the various functions to be executed by the organism, emotional correlates undoubtedly play a role. Although we cannot know the subjective experiences associated with these emotions in other animals, we recognize expressions that are similar to ours, especially in species that are phylogenetically close to us.

We know personally that there are positive emotional states of pleasure, agreeability and satisfaction that are the rewarding experiences associated with the successful execution of biological functions. Conversely there are various “negative” states of want, need and discomfort, reflecting a discrepancy between what is actually the case and the norm value of certain internal conditions or environmental relations. Such discrepancies are the motivational factors, driving the behaviours serving to correct these discrepancies and to reach and maintain the respective norm situations. Hunger, thirst, fatigue, the absence of a mate, the presence of a threat are obvious examples. These states respectively motivate the behaviours of feeding, drinking, sleeping, searching for mates and courting, and defence or avoidance. Within the natural boundaries of variation, such states can hardly be considered suffering. Discomfort turns into distress and suffering when such states persist at high intensities for long durations. This seems likely to occur in higher animals especially when the prospects of performing the appropriate corrective functions are unavailable and when the expectations of coping are low. Then, components of fear (a tendency to avoid specific objects, events and situations) and stressful anxiety (a more general and unfocused form of fearful arousal) make their contribution. The important role of expectancies and the predictability and controllability of variables, which are relevant to the animal in the performance of its functions, has become evident since the pioneering experiments of Weiss (see chapter 4).
This leads to the conclusion that various forms of activity may be indicators of discomfort and distress. Appetitive behaviours associated with different functional systems may signal discomfort if:

- their performance is unnaturally intense and prolonged,
- they do not lead to the consummatory or goal situations characteristic for the respective functions, and
- they lead to severe (possibly detrimental) deregulation of the hierarchy of priorities and of the time budget relations of biological functions.

The experiences of discomfort, need and hindrance must be regarded as biologically meaningful phenomena. Such experiences provide the impulse for the execution of behaviours by which these states are removed. If this does not ensue as a result of the behaviour in accordance with the animal’s expectations, the animal may switch to other behavioural modes. As explained in chapter 4, pathological forms of behaviour, such as soothing stereotypies and directly damaging elements, may occur when animals are kept in a situation where conflicts cannot be solved and discomfort turns into stress. If no available behaviour delivers the “expected” result, the animal can also end up in a situation equivalent to depression or “learned helplessness”. The animal loses the impulse to act, becomes passive, apathetic and listless. The switch to a ‘depressive’ attitude may be seen as a biologically meaningful reaction. If coping attempts remain fruitless, or even are harmful, then a strategy may be preferable in which the animal resigns from the situation which is unmanageable, or suppresses all initiatives until the situation changes for the better. There is no point in wasting effort and running unnecessary risks. A similar apathetic attitude, characterized by depressed posture and unresponsiveness, can also be observed in cases of illness and chronic pain (see below).

Pain

Pain represents a special class of distress. A heterogeneous set of phenomena can be subsumed by this concept. It varies, for example, from the acute pain that is associated with certain forms of tissue damage, the aching of muscles associated with too much exercise leading to the accumulation of lactic acid, to various forms of often chronic neural pain. In some of its forms, at least, a sensoneural substrate specifically tuned to nociception can be distinguished.

Pain has always been regarded as a biologically-adaptive mechanism, the main function of which is to warn the organism that (part of) it is under potentially damaging stress or that damage has actually occurred. Learning psychologists have emphasized an additional function. In the conditioning of behaviour, two kinds of effects influence the future occurrence of a behaviour: rewards and punishments. The influence of the latter has customarily
Recognition of pain and distress

been studied by using mild electric shocks as a punishment. Here pain acts as an unconditional stimulus, releasing withdrawal and defensive responses. At the same time, learning psychologists point out, associations are formed with the stimulus characteristics of the pain-provoking situation. These then act as conditional stimuli, releasing avoidance responses and fear towards that situation. Thus the animal learns to recognize danger. However true, this role should not lead to a generalized statement that fear is a conditional response to pain. The ethological literature abounds with examples showing that flight and avoidance may also be evoked as unconditional responses, for instance, by sudden ‘startling’ stimuli, or, more specifically, to certain sign stimuli, such as those indicating a possible predator. Fear of predators cannot, as a rule, be a pain-conditioned response, because few animals have a second chance after a painful confrontation with a predator.

An important distinction is made between primary and secondary pain. This distinction is clearly recognisable in our own experience. The immediate sensation following a pain-inflicting stimulus is an acute, sharp and well-localized pain. This “primary pain” is mediated by thick, fast-conducting myelinated Aδ-fibres. The primary pain often subsides soon, to be followed later by “secondary pain”. This is less localized and is generally of a chronic (or “tonic”) nature, sometimes pulsating. Even though potentially hurting severely, its quality is “diffuse” and “aching” rather than “sharp”. It is mediated by thin, slow-conducting, unmyelinated C-fibres. The relation between primary and secondary pain is complex. In the “gating theory” of Melzack and Wall (1965), a mechanism is proposed for the short-term regulation of the transmission of incoming signals from nociceptive neurons via the spinal cord to the brain. The signals of the fast-conducting fibres, responsible for the initial acute pain, are, at the same time, supposed to build up a self-inhibiting influence by clogging the spinal gate through which these signals are transmitted upwards. This would explain that the primary pain abates soon after its sharp onset. When, later, the signals of the slow-conducting C-fibres begin to come in, this counteracts the inhibition and the blockade of the gate is raised. Thus the nociceptive signals are transmitted once more to the brain. In addition to this short-term regulatory mechanism, there are other regulatory mechanisms of pain sensation, operating at more central levels under the influence of a diversity of motivational factors, for example via enkephalins (fig. 14.1). The transmission from the C-fibres appears to be very sensitive to opiate blockade; such an inhibitive influence has not been found for the fast Aδ-fibres.

Bolles and Fanselow (1980) have argued that the different forms of pain, rather than being separate sensations, represent functionally differentiated aspects of an integral process of pain behaviour in response to tissue damage. They have distinguished three behavioural phases. The first two phases, the perceptive and defensive phases, are associated with the perception of
primary pain. This triggers quick withdrawal responses of the organism, or its affected body parts, as well as defensive responses directed at warding off the pain-provoking agent. Reflex-like responses of this kind can partly remain after brain connections have been severed. It seems that the more direct and reflex-like the reaction, the more easily it can be dissociated from subjective experience, and the less trustworthy these are as objective measures of the pain experience. This also means that, although we can experience this pain, such experience is not necessary for the adaptive response. It also means that the occurrence of such responses in animals does not necessarily imply perception and concurrent suffering. In the perceptive phase the animal is also alerted to the pain-inflicting agent and conditioned to its stimulus characteristics. The third phase, the recuperative phase, is characterised by the onset of secondary pain. This pain produces a motivational reorganization of inhibiting activities that might interfere with recovery and allowing the animal to recuperate. The animal may look for a place to hide, rest, and lick its wounds. Other functions are suspended. Posture and movements are depressed, just as in the human patient who is ill or suffers from chronic pain and displays a listless and apathetic attitude. Such “depression” can be seen
as a biologically-adaptive response enabling the animal to be quiet and wait until it has recovered or (in the case of “helplessness” after repeated failures) until the situation has improved. In the past, attention has been focused mainly on acute nociperception. Until recently, chronic pain in animals has received only limited attention. The LASA Working Party (1990) expressed their opinion that chronic pain or distress may often be more insidious, particularly in the early stages, than acute pain.

According to Bolles and Fanselow, pain behaviour is best regarded as a separate motivational system, interacting competitively with other motivational systems. This is in line with ethological theory, which sees behaviour as a hierarchical structure of motivational systems, competing for hegemony of expression. When a particular system achieves hegemony and its behavioural functions take place, this largely inhibits other systems. This inhibition ensures that the programme of the activated function can be brought to completion, largely without interruption. Just as the state of pain can suppress other motivations interfering with the recuperative process, the reverse can be true (other motivations, when strong, can temporarily suppress the state of pain).

The recognition of pain

The recognition of pain can occur in two contexts. Pain can be induced intentionally by applying noxious stimuli in order to establish the effect of some experimental manipulation (drugs, such as analgesics, or stress factors) on pain perception. The methods used have mainly been focused on primary and acute forms of nociception, and not so much on chronic pain. Stimuli used most often include pinching, heat stimuli and electric shocks. Pain can also occur as a consequence of both intended and unintended procedures. An indication of the level of suffering experienced by the animal is needed in order to judge the acceptability of the procedures.

Valid judgements about the degree of suffering and animal experiences must be based on behavioural responses of the animal. Here it is important to realize that different types of reaction may be expected, according to the phase of the pain behaviour process:

1. The reactions released by primary pain are generally withdrawal and protective responses. In addition, the sudden onset of a primary pain often evokes vocal responses in many species. The immediacy and conspicuousness of such responses makes them easily recognizable as symptoms. It is important to note that the threshold and intensity of responding can be influenced by a diversity of motivational factors, such as stimuli relating to predation risk, social conflict, territoriality, sex, etc. (see also below “Social facilitation and inhibition”).
The recognition of pain

(2) In the phase of recuperation, which follows when lasting damage has been inflicted, the responses are different and of a more heterogeneous nature:

a. Motor patterns and their co-ordination may be changed or performed in a slow and wary fashion to spare structures that have been damaged, e.g. limping. Such behavioural changes are not too difficult to recognize for the experienced observer, as most veterinarians can confirm. Changes in the locomotory activity of experimental animals may be valid criteria for estimating the degree of chronic pain. The same applies to behavioural elements such as cringing; these might be seen as attempts to escape from the pain sensation or to suppress it with "competing" stimulation.

b. Motivational changes, resulting, for instance, in withdrawal to a safe and quiet place and refraining from all but the most urgent actions. Low alertness, a "depressed" posture, subdued dynamics and low motivations for other, even vital behavioural functions (for example, anorexia) reflect a state of apathy and "depression".

Symptoms of apathy and "depression" are less easily appreciated than elements such as limping and cringing, and certainly not as easily as the primary pain responses. In addition, certain forms of chronic pain are not associated with any easily observable adjustments. Thus dental pains and certain abscesses may go unnoticed, even though we would expect them to be painful by analogy. Thus horses and pigs do not betray certain internal afflictions which we might consider painful on the basis of the analogy postulate.

(3) In addition, emotional expressions may occur. As indicated under 1. vocal reactions like screams and roars may be given in response to primary pain. Sighs, moans, groans and yelps may be responses to secondary pain. At least some of these are communicative signals, evolved to inform others, particularly conspecifics, about the state of the sender. Vocal signals are the most conspicuous to a human observer but many species also produce olfactory signals, for example the alarm substances effused by fish of certain species when their skin is damaged. Many laboratory workers fail to appreciate that rodents and lagomorphs can also communicate pain and distress by odour, for instance, to conspecifics in neighbouring cages. We may expect such displays of pain and suffering to occur in social species where such informing can be ultimately beneficial for the sender by adaptively influencing the behaviour of receivers.

In addition to the behavioural parameters discussed above, physiological features can also be important. We can distinguish immediate and long-term physiological responses. Examples of the first are changes in pupillary dilation, cardiac rate, respiratory pattern, salivation, sweating, gastrointestinal motility and urination and defecation. There are good reasons to believe that
these reflect the actual experience of the animal. In some cases, such as certain internal afflictions in horses, these are the only ones visible. In other cases of suffering, long-term indicators may include impaired immune system functioning and increased levels of corticosteroids and catecholamines, which can be measured both in the blood and in the urine. These endocrine changes can inhibit gonadal activity and suppress reproductive functions. This can manifest itself behaviourally in inhibited sexual and nursing behaviours. Finally, growth may be impaired and body weight may even decrease. The measurement of some of these parameters demands invasive techniques, which can add, *per se*, to the disturbance. For example, the stress involved in collecting blood samples for hormone measurements may modify the endocrine effects, which one wants to measure. In this instance, permanent cannulation of the jugular vein can be the solution, since it reduces the stress of handling during blood sampling.

Post-mortem parameters may provide clues about levels of discomfort, especially when these variables have been shown to correlate with clinical signs of discomfort in man and/or animals. They may include fatty depots, muscle volume, stomach ulcers, adrenal cortex size, lymphoid organs and fluid balance.

**Significance of pain signals**

In nature, screams given as a response to primary pain can, just as fear screams, alert conspecifics to the source of danger. This might evoke help in defence from these conspecifics. Even if the alarm brings no direct benefit to the sender, the sender can nevertheless profit in terms of “inclusive fitness”. This can occur when there are relatives with a similar genetic background, which can then avoid the danger more effectively, for instance, by learning about its nature.

Displays of pain and distress can also serve as signals of helplessness and need. Examples are the yelps and whines occurring in some species, such as dogs and wolves. They are derived from the infantile repertoire, where they release parental care and protection. In some species, care has come to be given also to certain adult animals. It has even become the concern of group members other than a parent. Examples include dolphins rendering support to incapacitated pod members in danger of drowning, or canids where pack members bring and regurgitate food, not only to cubs, but also to the adults staying with the cubs. In species such as these, signals of helplessness may release tolerance and even active support.
Species differences in pain expression

Varied forms of emotional expression of secondary pain are expected to be especially characteristic of species, which have evolved co-operation, sharing, and communal brood care, e.g. in socially co-operative carnivores rather than in ungulates.

Social signals of primary pain are expected, especially in species where conspecifics can immediately and effectively adjust and avert the danger. Thus pigs are very ‘touchy’ and react immediately and loudly when being squeezed. This is a very adaptive response in a species where body contact is common and the piglets need strong signals when in danger of being squashed by heavy adults. In contrast, many other ungulates, such as antelopes, are comparatively mute.

Social facilitation and inhibition

Little is known still about contextual and environmental factors that might influence the expression of pain in animals. Concealment of pain behaviours may be expected in species and in conditions where their display might be a hazard. Signs of injury or sickness might attract and direct the attention of predators to an easy prey; a limping individual in a herd of herbivores is an unmistakable signal. Also, in species with a social hierarchy, a display of weakness might tempt conspecifics to try and overthrow a dominant. Under such conditions of risk, other behavioural motivations might compete with the pain system and suppress it; this may consequently lead to increased pain thresholds. This reflects a trade-off in which certain short-term interests, for example momentary safety, are secured at the price of a retarded recuperation. The evidence for such phenomena in animals is still largely anecdotal and there is a need for systematic investigation.

There is more evidence that social facilitation of pain expression, well known in the human species, also occurs in some animal species. Such expression may be reinforced into “hypochondriac” behaviour when an animal has learned that this brings social relief, tolerance or affection. In humans, conditioning processes may influence not only pain expression but also the thresholds of pain perception. Phenomena, reported especially for dogs, have been interpreted in this way, such as “sympathetic lameness”, limping, asthmatic behaviour, and anorexia nervosa. Clearly this provides a further complication when judging signals of pain and distress, especially, when dealing with companion animals rather than experimental counterparts.
Recognition of pain and distress

Grading the severity of pain and distress

A variety of schemes for scoring pain and distress in laboratory animals have been reported. Such assessments may be important in three stages of experimental procedures, namely: in gaining approval of animal ethics committee; during the experiment per se; and in post-mortem examinations. The response to pain depends on age, sex, health status, species and strain of the animal. Criteria used in pain evaluation are applied differently in different schemes. All current methods are unsatisfactory in that they are relatively subjective but most workers agree that it is considerably better than do nothing, because the assessment of pain and distress implies that explicit attention on the (potential) suffering of the animals used in procedures.

In general, discomfort can either be assessed in a qualitative or in a – more or less – quantitative way. In both, assessment of discomfort contains two steps: collection of data, which can be regarded as an objective process, followed by ‘translation’ into a degree of discomfort, which is a subjective process. Morton and Griffiths (1985), Beynen et al. (1988), LASA (1990) and the Disturbance Index used by Barclay et al. (1988), all have tried to score signs of pain and distress. LASA (1990) identified components of severity and gave a numerical rating reflecting its potential range. Morton and Griffiths (1985) have correlated clinical signs and (severity of) pain and distress, whereas in the Disturbance Index the changes in the number of movements made by a rat or mouse, which is introduced into an unfamiliar cage, is used as a method for assessing severity of procedures.

The newly developed behaviour recording system LABORAS™, for the automatic registration of different behavioural elements of mice and rats, such as eating, drinking, grooming, climbing, resting and locomotion, can be used to assess, in a less laborious way, behavioural changes as indicator of discomfort.

Using healthy animals housed in comfortable, stimulating environments will benefit both animals and the experiment. Environmental enrichment can help to create such an environment. This can be achieved by e.g. giving opportunities to forage for food, social grouping, handling or training by humans and structuring of cages by providing e.g. nesting material, nest boxes, climbing devices (Van de Weerd and Baumans, 1995).

Providing anaesthesia during painful procedures, skilful experimenters, giving adequate care such as providing warmth, soft bedding and palatable food and analgesics post-invasively, will also reduce discomfort in the animal.

It is important to determine humane endpoints (for the humane killing of animals at a stage that the first signs of approaching death are recognized). The animal would benefit, as unnecessary suffering is eliminated or reduced. Also, researchers could benefit, as the experiment would be more valid, data being less variable and timely collection of samples being scheduled.
Clinical signs that can help to determine humane endpoints are: rapid weight loss (15–20% in a few days), extended period of weight loss, prolonged diarrhoea (3 days), nasal discharge, coughing, neoplasia accounting for 10–20% bodyweight, self-induced trauma, icterus, central nervous system signs, severe ulceration or bleeding, drop in body temperature > 4°C, prolonged inability to ambulate, laboured breathing and cyanosis.

A pilot study may help specify humane endpoints in particular experiments. Monitoring of the animals should be done at least twice daily.

**Concluding remarks**

We have discussed various behavioural manifestations of pain and distress. It is clear that these may lead to underestimation of an animal’s suffering in some cases and to overestimation in others. Our ethical obligation to the animals that may suffer as a result of our interference during experimentation requires us to err at the side of the latter.

Our moral obligation to animals placed in our custody is the most important factor in guiding decisions on whether or not to perform certain experiments, but avoidance or reduction of pain and distress in experimentation is also demanded for more practical reasons. Pain, fear, and anxiety, all have strong motivational and physiological consequences. These may substantially interfere with the results of the investigations. Also for this reason, a careful and thoughtful application of analgesics and anxiolytics is required (see also chapter 15). Such application should be informed, because effects may be complex. If the pain system competes with other motivational systems for its behavioural expression in terms of functional priorities, fear may inhibit pain, namely when the source of fear has nothing to do with the pain. Under different circumstances, namely when the fear is actually directed to a particular pain experience, the sensitivity to that stimulus may be enhanced. This complexity is exemplified nicely by the human experience of fearing the pain of the dentist’s treatment, abolishing the aching of the teeth that required the dental treatment in the first place. If pain is an unexpected result of a treatment, which causes fear, the fear may inhibit the pain perception. Reducing the fear, for instance by applying anxiolytics, may actually restore the pain sensitivity. Appropriate treatments should reflect a complete and integrated view of the motivational organisation of a particular animal and its activation in given situations.
Literature


15 Anaesthesia, analgesia and euthanasia

L. J. Hellebrekers, L. H. D. J. Booij and P. A. Flecknell

Introduction

Experiments in animals can result in pain and distress and, for both ethical and scientific reasons, this should be reduced to a minimum or even completely be eliminated. The ethical arguments for the humane treatment of animals are discussed in chapters 1 and 18. The scientific reasons become apparent when it is appreciated that pain and discomfort evoke a range of physiological responses affecting a large number of organ systems. Eliminating or reducing pain can reduce the magnitude of these effects, and so improve the validity of an animal model.

The experience of pain occurring during surgical procedures can be completely prevented by the use of appropriate anaesthetic techniques and these are discussed in detail below. It is important to realize that most anaesthetics affect many organ systems, and in doing so may interact with the experimental protocol. To minimize such interactions, anaesthetic regimens should be selected with care, after consideration of the pharmacology of the drugs involved.

Post-operative pain, and pain occurring as a result of some non-surgical experimental procedures, can be alleviated by the administration of analgesics. In order to control pain effectively, it is essential to be able to assess the degree of pain that is being experienced by the animal. Pain is a subjective sensory/emotional experience. Establishing the presence of pain in animals, which cannot communicate with us verbally, is difficult. Nevertheless, comparison of the structure and function of the central nervous system in animals and that in man indicates that the necessary mechanisms for pain sensation (nociception) are present in animals. In addition, analgesics modify the responses of animals to procedures that are known to be painful in man. These observations support the conclusion that noxious stimuli are likely to be unpleasant.
for animals, producing a sensation that is probably similar to pain in man. For further details on the recognition of pain in animals, see chapter 14.

**Anaesthesia**

Anaesthesia is a reversible and controllable condition in which the perception of noxious (painful) and other stimuli by the central nervous system are suppressed. Anaesthesia can be produced either by administration of drugs which produce a loss of consciousness as well as loss of pain perception (general anaesthetics), or make localized areas of the body insensitive (local anaesthetics). A variety of drugs can be used to anaesthetize animals. Tables 15-1 through 15-4 present dosage information for frequently used anaesthetics and related drugs in laboratory animals. The selection of a particular anaesthetic technique will depend on the animal species to be used, the type (invasiveness) of procedure to be carried out, the duration of the procedure, the experience of the research worker and the purpose of the experiment. If a non-invasive, non-painful procedure is to be carried out, then either a heavy sedation or light hypnosis (sleep) may be sufficient. For invasive procedures, immobilization and effective pain relief are also essential. If physiological data have to be collected, a very stable situation (steady state) will be required, in which changes in anaesthetic depth during the experiment are kept to a minimum. In addition, the anaesthetic selected should have minimal effects on the organ system of interest.

An “ideal” anaesthetic should have the following characteristics: it should be easy to administer, induce a sufficiently deep and stable state of anaesthesia, have no influence on physiological functions, be reversible and be safe for both animal and personnel. The recovery must be fast and uneventful. Unfortunately there is no anaesthetic technique available that meets all of these criteria. Thus compromises have to be made and for this reason it may be necessary to consult a specialist in animal anaesthesia for advice before starting an experiment. Besides, large differences exist in response to anaesthesia between the different animal species. Extrapolations from one species to another are absolutely invalid. Also between the different breeds in one species large differences may exist. Variability is also caused by sex, body weight, body composition and co-existing diseases.

**General anaesthesia**

Four components of general anaesthesia can be distinguished: loss of consciousness (hypnosis), loss of sensory function (analgesia), relaxation of skeletal muscles and suppression of reflex activity (autonomous stabilization).
General anaesthesia can be produced by the administration of one single drug, for example by the use of inhalational agents such as halothane or isoflurane, or by administration of injectable agents such as pentobarbitone or propofol. However, in any particular experiment, the degree of suppression of consciousness and reflex responses and the degree of analgesia that is required will vary. When only one drug is administered, it is impossible to adjust the separate components of anaesthesia independently. In contrast, if a separate drug is used for one or more components, then the degree of suppression can be tailored to the specific requirements of the particular experiment. Such an anaesthetic technique is called balanced anaesthesia. When using a balanced anaesthetic regimen, the dosage of each anaesthetic agent used can be relatively low, reducing the danger of an overdose and the undesirable side effects of each drug can be minimized. A potential disadvantage of balanced anaesthesia is that drug interactions are possible, and also each individual drug can have specific effects on the physiological processes of the animal. A thorough knowledge of the pharmacology of the drugs to be used is therefore needed.

Local anaesthetics

In contrast to general anaesthesia, local anaesthetics affect only a specific part of the body, while the animal remains conscious. Local anaesthetics can be applied in a variety of ways, to achieve different degrees of (local/regional) anaesthesia.

(i) Surface anaesthesia: Direct application of local anaesthetic gel or spray to mucous membranes, or application of local anaesthetic cream to intact skin, can produce localized areas of anaesthesia. This technique can be used for minor, superficial interventions, such as catheterization of the urethra, percutaneous placement of catheters into blood vessels or superficial skin incisions.

(ii) Local infiltration: It is possible to desensitize deeper layers of tissues by infiltration of local anaesthetic into the tissues. This is a useful technique for minor surgical procedures such as skin biopsy.

(iii) Local nerve block: Larger areas can be desensitized by injecting local anaesthetic around the nerves supplying that particular body part. This technique is especially useful for desensitizing the limbs or tail, and enables extensive surgical procedures to be undertaken.

(iv) Regional anaesthesia: Even larger areas can be desensitized by administration of the local anaesthetic close to the spinal cord, or in the plexus supplying one of the limbs. If the drug is administered intrathecally, into the cerebrospinal fluid, the technique is termed spinal anaesthesia. If the drug is administered into the epidural space, i.e. before the dura mater is pierced, it is
Anaesthesia, analgesia and euthanasia
termed epidural anaesthesia. With correct dosage, spinal and epidural anaesthesia can be used to allow surgery in the caudal part of the body, i.e. hind legs and abdomen. Although these techniques have been most widely used in larger animals, particularly sheep and cattle, they may also be used in smaller mammals such as dogs or rabbits. Plexus anaesthesia is used for procedures in one of the limbs.

The most widely used local anaesthetics are procaine, lidocaine, bupivacaine and prilocaine. In some cases, epinephrine or norepinephrine are added to cause local vasoconstriction and slow the absorption of the compound. This will prolong the anaesthetic effect, but may lead to the typical cardiovascular responses seen after administration of catecholamines. The advantage of local anaesthesia is that in general, it has less influence on normal physiological functions. However, especially following induction of spinal or epidural anaesthesia over a number of spinal segments, an extensive blockade of the sympathetic nervous system may occur, leading to vasodilatation in the blocked area and resulting in systemic hypotension and tachycardia. Local anaesthetic drugs may, depending on the concentration and volume administered, also exert toxic effects. During local or regional anaesthesia the animal remains conscious, and this will be a major limitation of the technique in animals which have not been habituated to handling and restraint. Administration of a sedative may resolve this problem. Alternatively, local anaesthesia can be used to provide analgesia in animals that have been rendered unconscious by administration of a low dose of a hypnotic or a general anaesthetic.

Pre-anaesthetic preparations

(For details on dose ranges of anaesthetics mentioned in this section: see Tables 15-1 through 15-4.)

Before inducing anaesthesia, a number of preparations are necessary. The most appropriate anaesthetic regimen must be selected, and all of the equipment that will be required must be checked to ensure that it is intact and functioning correctly. Also, ensure that sufficient quantities of anaesthetic and emergency drugs are readily available. The animals, which are to be anaesthetized, must have undergone one to two weeks acclimatization period and, during the immediate pre-operative period, their body weight and daily food and water consumption must have been recorded. The animals must be in good health and free from any clinical signs of disease. In some instances it may be appropriate to carry out laboratory investigations to confirm the health status of the subjects. Prior to major surgery, haematological and biochemical evaluations may be carried out in larger species. For example, knowledge of the pre-operative haemoglobin content or hae-
matocrit is helpful if these parameters are to be used to evaluate the degree of intra-operative haemorrhage.

Especially larger animals must be fasted to prevent regurgitation and aspiration of stomach contents. Also during recovery from anaesthesia, some species may vomit if they have been recently fed. To avoid this problem, dogs, cats, ferrets, pigs and non-human primates should be fasted for 12–16 hours prior to anaesthesia. Pre-anaesthetic fasting is unnecessary and undesirable in small rodents and rabbits.

If the animal is to recover from anaesthesia, then preparations for providing post-operative care must be made before commencing the procedure. A suitable recovery pen or incubator should be available, and equipment for maintaining body temperature post-operatively must be switched on to allow sufficient time for the temperature in the recovery area to stabilize.

When using an anaesthetic regimen for the first time, it is advisable to anaesthetize only one animal to ensure that an appropriate depth of anaesthesia is attained and that recovery is uneventful. The response of different inbred strains of rodents or rabbits to anaesthetic agents varies considerably, and the dosages quoted in this chapter may need to be modified for use with certain strains.

Pre-anaesthetic medication: It is sometimes useful to administer drugs prior to induction of anaesthesia, either to reduce possible side-effects of the anaesthetic agents, or to minimize the distress associated with induction and to ensure a smooth recovery from anaesthesia. Anticholinergic agents, such as atropine or glycopyrrolate, may be used to reduce the volume of bronchial and salivary secretions, and to block any undesirable autonomic response either drug-induced or due to surgical manipulation of the viscera. Sedatives or tranquilizers may be used to reduce stress and make animals easy to restrain. Use of sedatives will also smooth the induction and recovery from anaesthesia. The majority of commonly used sedatives and tranquillisers have no analgesic action, and so additional medication will be required to control per- or post-operative pain. Suggestions for pre-anaesthetic medication for different species are given in Tables 15.1 and 15.2.

Induction and maintenance of general anaesthesia

General anaesthesia can be induced either by injection of one or more compounds by the intravenous, subcutaneous, intramuscular or intraperitoneal routes, or by inhalation of volatile anaesthetics. In small animals (<1kg), induction with a volatile anaesthetic can be achieved using an induction chamber. With larger species, this is rarely practicable and the animal will need to be restrained with a facemask applied to administer the anaesthetic.
## Table 15-1

**Rodent and rabbit anaesthetic dose rates**

<table>
<thead>
<tr>
<th></th>
<th>Mouse</th>
<th>Rat</th>
<th>Hamster</th>
<th>Gerbil</th>
<th>Guinea pig</th>
<th>Rabbit</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Premedication (anticholinergics)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atropine</td>
<td>0.05 mg/kg s/c</td>
<td>0.05 mg/kg s/c</td>
<td>0.05 mg/kg s/c</td>
<td>0.05 mg/kg s/c</td>
<td>0.05 mg/kg s/c</td>
<td>0.05 mg/kg i/m</td>
</tr>
<tr>
<td>Glycopyrrolate</td>
<td>0.01 mg/kg s/c</td>
<td>0.01 mg/kg s/c</td>
<td>0.01 mg/kg s/c</td>
<td>0.01 mg/kg s/c</td>
<td>0.01 mg/kg s/c</td>
<td>0.1 mg/kg s/c</td>
</tr>
<tr>
<td><strong>Premedication (sedatives)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diazepam</td>
<td>2.5–5 mg/kg i/p</td>
<td>2.5 mg/kg i/p</td>
<td>5 mg/kg i/p</td>
<td>5 mg/kg i/p</td>
<td>5 mg/kg i/p</td>
<td>1–2 mg/kg i/v</td>
</tr>
<tr>
<td>Acepromazine</td>
<td>2–5 mg/kg s/c</td>
<td>2.5 mg/kg s/c</td>
<td>5 mg/kg s/c</td>
<td>3 mg/kg s/c</td>
<td>2.5 mg/kg s/c</td>
<td>1 mg/kg s/c</td>
</tr>
<tr>
<td>“Hypnorm” (fentanyl/fluanisone)</td>
<td>0.1–0.3 ml/kg i/p</td>
<td>0.3–0.5 ml/kg i/p</td>
<td>0.5 ml/kg i/p</td>
<td>0.5–1 ml/kg i/p</td>
<td>1 ml/kg i/p</td>
<td>0.2–0.5 ml/kg i/m</td>
</tr>
<tr>
<td>Xylazine</td>
<td>5–10 mg/kg i/p</td>
<td>1–5 mg/kg i/p</td>
<td>5–10 mg/kg i/p</td>
<td>2–3 mg/kg i/p</td>
<td>5 mg/kg i/p</td>
<td>2–5 mg/kg i/m</td>
</tr>
<tr>
<td>Medetomidine</td>
<td>0.1–0.3 mg/kg s/c</td>
<td>0.1–0.3 mg/kg s/c</td>
<td>0.1 mg/kg s/c, i/p</td>
<td>0.1–0.2 mg/kg i/p</td>
<td>0.3–0.5 mg/kg i/p</td>
<td>0.2–0.3 mg/kg i/m</td>
</tr>
<tr>
<td><strong>Anaesthesia (short duration, 5–10 minutes)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alphaxalone/alphadolone</td>
<td>10–15 mg/kg i/v</td>
<td>10–12 mg/kg i/v</td>
<td>150 mg/kg i/p</td>
<td>80–120 mg/kg i/p</td>
<td>40 mg/kg i/p</td>
<td>6–9 mg/kg i/v</td>
</tr>
<tr>
<td>Propofol</td>
<td>26 mg/kg i/v</td>
<td>10 mg/kg i/v</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>10 mg/kg i/v</td>
</tr>
<tr>
<td>Thiopentone</td>
<td>30–40 mg/kg i/v</td>
<td>30 mg/kg i/v</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>30 mg/kg i/v</td>
</tr>
<tr>
<td>Methohexitone</td>
<td>10 mg/kg i/v</td>
<td>7–10 mg/kg i/v</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>31 mg/kg i/p</td>
</tr>
</tbody>
</table>

(Continued)
<table>
<thead>
<tr>
<th>Anaesthesia (medium duration, 20–60 minutes)</th>
<th>Mouse</th>
<th>Rat</th>
<th>Hamster</th>
<th>Gerbil</th>
<th>Guinea pig</th>
<th>Rabbit</th>
</tr>
</thead>
<tbody>
<tr>
<td>“Hypnorm”/midazolam (1 part Hypnorm, 1 part midazolam and 2 parts water for injection)</td>
<td>0.1 ml/10 gr i/p</td>
<td>2.7 ml/kg i/p</td>
<td>4 ml/kg i/p</td>
<td>8 ml/kg i/p</td>
<td>8 ml/kg i/p</td>
<td>0.3 mg/kg i/m</td>
</tr>
<tr>
<td>Ketamine/acepromazine</td>
<td>100 mg/kg i/p</td>
<td>75 mg/kg i/p</td>
<td>150 mg/kg i/p</td>
<td>75 mg/kg i/p</td>
<td>125 mg/kg i/p</td>
<td>50 mg/kg i/m</td>
</tr>
<tr>
<td>Ketamine/diazepam</td>
<td>100 mg/kg i/p</td>
<td>75 mg/kg i/p</td>
<td>70 mg/kg i/p</td>
<td>50 mg/kg i/p</td>
<td>100 mg/kg i/p</td>
<td>25 mg/kg i/m</td>
</tr>
<tr>
<td>Ketamine/xylazine</td>
<td>100 mg/kg i/p</td>
<td>90 mg/kg i/p</td>
<td>200 mg/kg i/p</td>
<td>50 mg/kg i/p</td>
<td>40 mg/kg i/p</td>
<td>25–35 mg/kg/m</td>
</tr>
<tr>
<td>Ketamine/medetomidine</td>
<td>100 mg/kg i/p</td>
<td>75 mg/kg i/p</td>
<td>100 mg/kg i/p</td>
<td>75 mg/kg i/p</td>
<td>40 mg/kg i/p</td>
<td>5 mg/kg i/m</td>
</tr>
<tr>
<td>Pentobarbitone</td>
<td>40–60 mg/kg i/p</td>
<td>40–55 mg/kg i/p</td>
<td>50 mg/kg i/p</td>
<td>60 mg/kg i/p</td>
<td>37 mg/kg i/p</td>
<td>30–45 mg/kg i/v</td>
</tr>
<tr>
<td>Anaesthesia (long duration, non-recovery)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chloralose</td>
<td>50–100 mg/kg i/p</td>
<td>55–65 mg/kg i/p</td>
<td>50–100 mg/kg i/p</td>
<td>–</td>
<td>70 mg/kg i/p</td>
<td>80–100 mg/kg i/v</td>
</tr>
<tr>
<td>Urethane</td>
<td>1 g/kg i/p</td>
<td>1–2 g/kg i/p</td>
<td>1–2 g/kg i/p</td>
<td>–</td>
<td>1.5 g/kg i/p</td>
<td>1 g/kg i/p</td>
</tr>
<tr>
<td>Anaesthesia (inhalation agents short/medium/long duration)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ether</td>
<td>Induction concentration 15–20%</td>
<td></td>
<td></td>
<td>Maintenance concentration 5%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Halothane</td>
<td>Induction concentration 4–5%</td>
<td></td>
<td></td>
<td>Maintenance concentration 1–2%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isoflurane</td>
<td>Induction concentration 4%</td>
<td></td>
<td></td>
<td>Maintenance concentration 1.5–3%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sevoflurane</td>
<td>Induction concentration 8%</td>
<td></td>
<td></td>
<td>Maintenance concentration 3–4%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methoxyflurane</td>
<td>Induction concentration 4%</td>
<td></td>
<td></td>
<td>Maintenance concentration 0.5–1%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Table 15-2

Dog, cat, ferret and larger species anaesthetic dose rates

<table>
<thead>
<tr>
<th></th>
<th>Dog</th>
<th>Cat</th>
<th>Ferret</th>
<th>Sheep/goat</th>
<th>Pig</th>
<th>Primate</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Premedication</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(anticholinergics)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atropine</td>
<td>0.05 mg/kg s/c</td>
<td>0.05 mg/kg s/c</td>
<td>–</td>
<td>0.05 mg/kg s/c</td>
<td>0.05 mg/kg s/c</td>
<td>0.05 mg/kg s/c</td>
</tr>
<tr>
<td>Glycopyrrolate</td>
<td>0.01 mg/kg s/c</td>
<td>0.01 mg/kg s/c</td>
<td>0.1 mg/kg s/c</td>
<td>–</td>
<td>0.01 mg/kg s/c</td>
<td>0.01 mg/kg s/c</td>
</tr>
<tr>
<td><strong>Premedication</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(sedatives)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diazepam</td>
<td>–</td>
<td>–</td>
<td>2 mg/kg i/m</td>
<td>2 mg/kg i/m</td>
<td>1 mg/kg i/m</td>
<td>1 mg/kg i/m</td>
</tr>
<tr>
<td>Acepromazine</td>
<td>0.03–0.06 mg/kg i/m</td>
<td>0.5–0.1 mg/kg i/m</td>
<td>0.2 mg/kg i/m</td>
<td>0.1 mg/kg i/m</td>
<td>0.2 mg/kg i/m</td>
<td>0.2 mg/kg i/m</td>
</tr>
<tr>
<td>“Hypnorm” (fentanyl/fluanisone)</td>
<td>0.2–0.3 ml/kg i/m</td>
<td>–</td>
<td>–</td>
<td>0.5 ml/kg i/m</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Xylazine</td>
<td>0.5–1 mg/kg i/m</td>
<td>0.5–1 mg/kg i/m</td>
<td>–</td>
<td>1 mg/kg i/m (sheep)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Medetomidine</td>
<td>0.03–0.05 mg/kg i/m</td>
<td>0.04–0.08 mg/kg i/m</td>
<td>–</td>
<td>0.05 mg/kg i/m (goat)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>Anaesthesia</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(short duration, 5–10 minutes)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alphaxalone/alphadolone</td>
<td>–</td>
<td>9–12 mg/kg i/v</td>
<td>8–12 mg/kg i/v</td>
<td>2.2 mg/kg i/v</td>
<td>2 mg/kg i/v (then 2 mg/kg i/v)</td>
<td>6 mg/kg i/m</td>
</tr>
<tr>
<td>Propofol</td>
<td>5–7.5 mg/kg i/v</td>
<td>7.5 mg/kg i/v</td>
<td>–</td>
<td>3–4 mg/kg i/v</td>
<td>3 mg/kg i/v</td>
<td>–</td>
</tr>
<tr>
<td>Thiopentone</td>
<td>10–20 mg/kg i/v</td>
<td>10–15 mg/kg i/v</td>
<td>–</td>
<td>10–15 mg/kg i/v</td>
<td>6–9 mg/kg i/v</td>
<td>15–20 mg/kg i/v</td>
</tr>
<tr>
<td>Methohexitone</td>
<td>4–8 mg/kg i/v</td>
<td>4–8 mg/kg i/v</td>
<td>–</td>
<td>4 mg/kg i/v</td>
<td>5 mg/kg i/v</td>
<td>10 mg/kg i/v</td>
</tr>
</tbody>
</table>

(Continued)
Induction and maintenance of general anaesthesia

<table>
<thead>
<tr>
<th>Anaesthesia (medium duration, 20–60 minutes)</th>
<th>Dog</th>
<th>Cat</th>
<th>Ferret</th>
<th>Sheep/goat</th>
<th>Pig</th>
<th>Primate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ketamine/diazepam</td>
<td>10 mg/kg i/v</td>
<td>–</td>
<td>25 mg/kg i/m</td>
<td>4 mg/kg i/v</td>
<td>10 mg/kg i/m</td>
<td>15 mg/kg i/m</td>
</tr>
<tr>
<td></td>
<td>0.5 mg/kg i/v</td>
<td>–</td>
<td>2 mg/kg i/m</td>
<td>1 mg/kg i/v</td>
<td>2 mg/kg i/m</td>
<td>1 mg/kg i/m</td>
</tr>
<tr>
<td>Ketamine/xylazine</td>
<td>15 mg/kg i/v</td>
<td>15 mg/kg i/m</td>
<td>10 mg/kg i/m</td>
<td>4 mg/kg i/v</td>
<td>10 mg/kg i/m</td>
<td>10 mg/kg i/m</td>
</tr>
<tr>
<td></td>
<td>1 mg/kg i/v</td>
<td>1 mg/kg i/m</td>
<td>1 mg/kg i/m</td>
<td>1 mg/kg i/m</td>
<td>1 mg/kg i/m</td>
<td>0.5 mg/kg i/m</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>–</td>
<td>0.5 mg/kg i/m</td>
<td>1 mg/kg i/m</td>
<td>0.5 mg/kg i/m</td>
<td></td>
</tr>
<tr>
<td>Ketamine/medetomidine</td>
<td>5 mg/kg i/m</td>
<td>5–8 mg/kg i/m</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>0.03–0.05 mg/kg i/m</td>
<td>0.5–0.8 mg/kg i/m</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Pentobarbitone</td>
<td>20–30 mg/kg i/v</td>
<td>25 mg/kg i/v</td>
<td>25–30 mg/kg i/v</td>
<td>30 mg/kg i/v</td>
<td>30 mg/kg i/v</td>
<td>5–15 mg/kg i/v</td>
</tr>
</tbody>
</table>

Anaesthesia (long duration, non-recovery)

<table>
<thead>
<tr>
<th>Anaesthesia</th>
<th>Dog</th>
<th>Cat</th>
<th>Ferret</th>
<th>Sheep/goat</th>
<th>Pig</th>
<th>Primate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Choralose</td>
<td>80–110 mg/kg i/v</td>
<td>80–90 mg/kg i/v</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>60 mg/kg i/v</td>
</tr>
<tr>
<td>Urethane</td>
<td>1 g/kg i/v</td>
<td>1.25 g/kg i/v</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Anaesthesia (inhalation agents, short/medium/long duration)

<table>
<thead>
<tr>
<th>Anaesthesia</th>
<th>Dog</th>
<th>Cat</th>
<th>Ferret</th>
<th>Sheep/goat</th>
<th>Pig</th>
<th>Primate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ether</td>
<td>Induction concentration 15–20%</td>
<td>Maintenance concentration 5%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Halothane</td>
<td>Induction concentration 4–5%</td>
<td>Maintenance concentration 1–2%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isoflurane</td>
<td>Induction concentration 4%</td>
<td>Maintenance concentration 1.5–3%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sevoflurane</td>
<td>Induction concentration 8%</td>
<td>Maintenance concentration 3–4%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methoxyflurane</td>
<td>Induction concentration 4%</td>
<td>Maintenance concentration 0.5–1%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The animal may resent the procedure and so be difficult to restrain; especially the cardiovascular system may be influenced by this stress. To minimize this stress during induction it is advisable either to administer a sedative prior to mask induction, or, even better, to administer a short-acting injectable anaesthetic to produce loss of consciousness, followed by administration of an inhalational agent to maintain anaesthesia for the desired period.

Inhalational agents have the advantage of allowing easy adjustment of the depth of anaesthesia provided that a calibrated vaporizer (see fig. 15-2) is used. It is therefore easy to respond to variations in the level of invasiveness during the surgical procedure. Repeated administration of intravenous anaesthetics result in unstable depth of anaesthesia, unless a continuous infusion is used. Furthermore, accumulation may occur, resulting in a prolonged recovery period. Following short periods (<30min) of anaesthesia, recovery is very rapid. Because of their ease of administration to small rodents and the rapid recovery, inhalational agents are probably the agents of choice for most procedures in these species.

**Anaesthetic agents**

**Inhalational agents**

*Isoflurane:* This is a potent anaesthetic that produces rapid induction of anaesthesia, rapid recovery and can provide safe and effective anaesthesia in all laboratory animal species. Isoflurane is non-flammable and non-explosive. Isoflurane is irritating to the airways resulting in a slower induction of anaesthesia due to breath holding. This anaesthetic undergoes virtually no biotransformation and is almost entirely removed from the animal by exhalation from the lungs. Because of this, it does not induce liver enzymes, and this minimizes the risk of interference with experimental studies that involve drug metabolism. Because it is a potent anaesthetic, isoflurane should only be used with a calibrated vaporizer. Isoflurane does not depress the baroreceptor reflex greatly but results in vasodilatation and consequently, hypotension and tachycardia.

*Halothane:* Like isoflurane, halothane is a potent anaesthetic that must be used in a calibrated vaporizer. Induction and recovery are slightly less rapid in comparison with isoflurane. Halothane causes moderate hypotension at surgical planes of anaesthesia. The compound is extensively metabolized in addition to being exhaled through the lungs.

*Methoxyflurane:* Methoxyflurane is a potent anaesthetic but, because it is less easy to vaporize than halothane and isoflurane, it can be used safely in simple apparatus. If a vaporizer is unavailable, liquid methoxyflurane can be
Anaesthetic agents

poured onto a cotton wool pad placed in an induction chamber. It is important to ensure that contact between the cotton wool pad and the animal is prevented by use of a metal grid or similar device, as the liquid anaesthetic is an irritant. Methoxyflurane undergoes extensive metabolism, releasing inorganic fluoride ions that can cause damage to the kidney, and so should probably not be used for studies of renal function. In several countries methoxyflurane is no longer on the market, and is expected to become totally unavailable in the near future.

*Enflurane*: Enflurane resembles halothane, but induction and recovery are slightly more rapid. It is less extensively metabolized than halothane, but it offers no significant advantages in comparison to halothane and isoflurane, and is rarely used in laboratory animals.

*Sevoflurane*: Sevoflurane is a relatively new inhalation agent that will rapidly induce anaesthesia and lead to a fast recovery. Although it is extensively metabolized it does not lead to renal or hepatic dysfunction.

*Desflurane*: Desflurane is another new inhalation agent that is not metabolized in the body but does results in a sympathetic stimulation. It is not used much in laboratory animal anaesthesia.

*Ether*: This irritant and inflammable anaesthetic forms explosive gas mixtures with oxygen and air. The irritant properties of ether vapour result in profuse salivation, increased bronchial secretions and occasional laryngospasm in animals. Irritation of the respiratory tract by ether can exacerbate pre-existing respiratory disease in rodents and rabbits. Despite these obvious disadvantages, ether is still used to anaesthetize small rodents. It can be administered using simple apparatus, or by way of a cotton wool pad in an anaesthetic chamber. It is difficult to overdose an animal even when using such crude methods of induction. However, animal welfare concerns, and the significant safety hazard posed by ether, have resulted in many laboratories discarding this agent in favour of safer and more humane alternatives.

*Nitrous oxide*: This anaesthetic is supplied in liquid form in pressurized cylinders. It is delivered as a mixture with oxygen and, to avoid hypoxia, the relative proportions of the two gases should not exceed 65% nitrous oxide with 35% oxygen. In man, nitrous oxide is an effective analgesic. In laboratory mammals, it is considerably less potent, and will not produce unconsciousness, even when administered at high (>70%) concentrations. It is of limited use in laboratory animal anaesthesia, and is of practical benefit only as an adjunct to other anaesthetic agents in larger animals, such as the dog and cat.

Most inhalation agents result in bronchodilation, abolition of hypoxic pulmonary vasoconstriction and decrease of mucociliary function in the airways. This may result in ventilation-perfusion mismatch and an increased incidence of pulmonary infection.
<table>
<thead>
<tr>
<th></th>
<th>Ketamine</th>
<th>Pentobarbitone</th>
<th>MS–222</th>
<th>Urethane</th>
<th>Halothane</th>
<th>Isoflurane</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Birds</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2–4%</td>
<td>3–5%</td>
<td>combination in pigeons: ketamine 30 mg/kg + metomidate 10 mg/kg i.m. or pentobarbitone 10–20 mg/kg i.m. endotracheal intubation relatively easy</td>
</tr>
<tr>
<td>0.1 kg</td>
<td>10–20 mg/kg i.m.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1–0.5 kg</td>
<td>5–10 mg/kg i.m.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5–3 kg</td>
<td>2–5 mg/kg i.m.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Snakes</strong></td>
<td>20–80 mg/kg i.p.</td>
<td>15–30 mg/kg i.p.</td>
<td>200–400 mg/kg i.p.</td>
<td></td>
<td>3.5–6.5%</td>
<td>4.0–6.5%</td>
<td>metomidate 8–10 mg/kg i.p.; thiopentone 8–45 mg/kg i.p.</td>
</tr>
<tr>
<td><strong>Lizards</strong></td>
<td>15–70 mg/kg i.m.</td>
<td>10–25 mg/kg i.p.</td>
<td></td>
<td></td>
<td>4–5% for induction; 1–2% for maintenance</td>
<td>4–5% for induction; 2–3% for maintenance</td>
<td>induction lasts 10 min.</td>
</tr>
<tr>
<td><strong>Tortoises</strong></td>
<td>60–120 mg/kg i.m.</td>
<td>10–30 mg/kg i.p.</td>
<td></td>
<td></td>
<td>4% for induction; 1.5% for maintenance</td>
<td>4% for induction; 2.0% for maintenance</td>
<td>induction lasts 10 min.</td>
</tr>
<tr>
<td><strong>Frogs</strong></td>
<td>30–60 mg/kg in dorsal lymph sack</td>
<td>induction 1500 mg/l; maintenance 600 mg/l</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>chloralhydrate 10%; 1–2 ml dorsal lymph sack; MS–222 should be neutralised with 50 ml 0.5 M NaHCO₃/l</td>
</tr>
<tr>
<td><strong>Fish</strong></td>
<td></td>
<td></td>
<td>250–14000 mg/l water</td>
<td>10–90 g/l water</td>
<td></td>
<td></td>
<td>Propanidid 0.2–1.5 mg/l</td>
</tr>
</tbody>
</table>
Table 15-4
Dose rates of muscle relaxants and antagonists in mg/kg

<table>
<thead>
<tr>
<th></th>
<th>Mouse</th>
<th>Rat</th>
<th>Guinea pig</th>
<th>Rabbit</th>
<th>Cat</th>
<th>Dog</th>
<th>Sheep</th>
<th>Goat</th>
<th>Pig</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Muscle relaxants</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pancuronium</td>
<td>2</td>
<td>0.06</td>
<td>0.1</td>
<td>0.06</td>
<td>0.06</td>
<td>0.06</td>
<td>0.06</td>
<td>0.06</td>
<td>0.06</td>
</tr>
<tr>
<td>Alcuronium</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Atracurium</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Vecuronium</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Gallamine</td>
<td>1</td>
<td>1</td>
<td>1.0–0.2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>D-Tubocurarine</td>
<td>1</td>
<td>0.4</td>
<td>0.1–0.2</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>Guafenesin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Antagonists</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Opiate: Naloxone</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.05–0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Alpha-2-adrenergic:</td>
<td>Atipamezole</td>
<td>Dose based upon 2.5–5 × dose of medetomidine administered.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Inhalation anaesthetics also decrease myocardial contractility and cause vasodilatation. This results in hypotension. Most i.v. injectable anaesthetics have the same cardiovascular effects. However, ketamine increases contractility and causes vasoconstriction.

**Anaesthetic potency:** The required inspiratory concentration of an inhalational anaesthetic needed to induce unconsciousness and anaesthesia depends upon the species, the procedure to be performed and the anaesthetic agent used. The “minimal alveolar concentration” (MAC) is the concentration of an anaesthetic (as an inspired percentage concentration) at which 50% of the animals do not react to a standardized painful stimulus (Table 15-5). To provide a surgical level of anaesthesia, 1.5 MAC is usually required, although under specific circumstances certain individuals may require 2 MAC or greater. Administrations of injectable anaesthetics, analgesics and/or sedatives lower the required MAC-value of inhalational anaesthetics.

**Gas scavenging:** Chronic exposure to inhalational anaesthetics is detrimental to human subjects, and it is standard practice in most hospital operating rooms to use apparatus to remove waste anaesthetic gases. Not only do they affect the cognitive function, but they may also lead to an increased incidence of spontaneous abortion and teratogenic effects. Safety legislation in many countries now requires all users of inhalational anaesthetics to ensure effective scavenging of waste anaesthetic gas, and so reduce the degree of pollution of laboratories and operating rooms. A number of commercially produced devices are available, for example the “Fluovac” (International Market Supply). As a temporary measure, simple anaesthetic apparatus can be transferred to a fume cabinet, but this is rarely convenient in the long term.

**Injectable anaesthetics**

A large number of different injectable anaesthetic agents are available for use in laboratory animals. These are briefly summarized below, and suggested

<table>
<thead>
<tr>
<th>Table 15-5</th>
<th>MAC-values in percentages for inhalation anaesthetics in different species</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ether</td>
</tr>
<tr>
<td>Human</td>
<td>1.92</td>
</tr>
<tr>
<td>Primate</td>
<td>3.04</td>
</tr>
<tr>
<td>Dog</td>
<td>1.45</td>
</tr>
<tr>
<td>Pig</td>
<td>2.10</td>
</tr>
<tr>
<td>Sheep</td>
<td>3.20</td>
</tr>
<tr>
<td>Cat</td>
<td>3.20</td>
</tr>
<tr>
<td>Rat</td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td></td>
</tr>
</tbody>
</table>
Anaesthetic agents

Dosages are listed in tables 15-1 through 15-3. It is important to emphasize the variation in response to anaesthetics that occurs between individual animals, and the potentially even larger variation reported between animals of different strains. When first using a new anaesthetic regimen, it is essential to assess the regimen in one animal, before proceeding to anaesthetize larger groups. The variability in anaesthetic response is most frequently seen with small rodents, because anaesthetics are usually administered as a single bolus (intraperitoneal) injection. This does not enable the anaesthetic to be administered gradually (titrated) to achieve the desired effect. In rabbits and larger species, anaesthetics can be administered by the intravenous route and, after administration of approximately 50% of the predicted dose, the remaining drug can be injected more slowly to provide the appropriate depth of anaesthesia. Intravenous administration of anaesthetics in rats and mice is not especially difficult when required expertise is available. Consideration should then be given to using this route of administration, especially if short (<10 minute) periods of anaesthesia are required.

Barbiturates: Two short-acting agents (thiopentone and methohexitone) and a longer acting agent (pentobarbitone) are widely available for animal anaesthesia. All of these anaesthetics produce sleep (hypnosis), but have no (intrinsic) analgesic property. Surgical anaesthesia is only attained at high dose rates that cause significant cardiovascular and respiratory depression. If barbiturates are administered intravenously, the dose rate can be carefully adjusted and they can be used with relative safety, depending on the type of intervention. Intraperitoneal injection of short-acting agents has very unpredictable effects and is not recommended. In addition, i/p administration of thiopentone causes severe irritation because of its high pH. Intraperitoneal administration of pentobarbitone can produce a state of surgical anaesthesia, but the drug has a narrow safety margin and mortality may be high. A disadvantage of intraperitoneal administration with many drugs is related to the potential damage of organ tissue.

Dissociative anaesthetics: Ketamine and tiletamine are the most widely used dissociative anaesthetics. In larger species, particularly non-human primates, dissociative anaesthetics can provide light surgical anaesthesia, but the degree of muscle relaxation is poor. In small rodents they are relatively ineffective unless dangerously high dosages are used. If the dissociative anaesthetic is combined with a sedative or tranquilizer, the quality of anaesthesia is greatly improved. The commercial preparation of tiletamine consists of a mixture of tiletamine and zolazepam (a benzodiazepine). Ketamine can be combined with a tranquilizer, such as acepromazine or, alternatively, with midazolam or diazepam, but is most effective when administered in combination with a sedative-analgesic such as xylazine or medetomidine. A particular advantage of this latter combination is that the sedative-analgesic can
be reversed with a specific antagonist (atipamezole), thereby considerably reducing the duration of the recovery.

**Neuroleptanalgesics:** Neuroleptanalgesics are mixtures of a potent analgesic and a tranquilizer. The most widely used commercial preparations are fentanyl/fluanisone (Hypnorm); fentanyl/droperidol (Innovar Vet or Thalamonal); etorphine/methotrime prazine (Immobilon SA) and etorphine/acepromazine (Immobilon LA). When used alone, these compounds produce profound analgesia, but the degree of muscle relaxation is poor, and respiration may be depressed, depending on the dose used. In several species it is possible to combine the use of fentanyl/fluanisone with a benzodiazepine (diazepam or midazolam) and produce surgical anaesthesia, good muscle relaxation and only mild respiratory depression.

**Steroid anaesthetics:** Alphaxalone/alphadolone (Saffan) is a useful anaesthetic in most laboratory species. This anaesthetic should not be used in the dog, since the solubilizing agent (Cremophor) present in the commercial preparation causes histamine release. In the rabbit, surgical planes of anaesthesia are produced only at dose rates that can cause respiratory arrest. In most other species, if administered by intravenous injection, alphaxalone/alphadolone produces moderate surgical anaesthesia lasting 5–15 minutes. Repeated injections, or a continuous infusion, can be used to prolong anaesthesia without unduly extending the recovery time.

**Benzodiazepines:** Benzodiazepines such as diazepam, midazolam (Dormicum, Versed) and zolazepam (in Telazol, Zoletil) are regularly used as anaesthetic ‘adjunct’ drugs in animals. They have minimal cardiovascular and respiratory effects. For anaesthesia they are used in combination anaesthesia together with ketamine, opioids and inhalational agents.

**Other agents**

**Alpha-2 adrenergic drugs:** Alpha-2 adrenergic drugs such as xylazine and medetomidine (Domitor) have a central nervous system depressant effect and cause a centrally-induced muscle relaxation. These are frequently used in large animals such as ruminants and horses as sedative-analgiesic, and is used in numerous laboratory animal species in combination with for instance ketamine, as general anaesthetic combination.

**Medetomidine,** being the newer and the more potent alpha-2 agonist, provides good sedation and analgesia in dogs, cats, rabbits and most of the different rodent species. The use of this drug (greatly) reduces the dose of other anaesthetics used in the same protocol. For antagonization, a specific alpha-2 antagonist, atipamezole (Antisedan) is available.

**Propofol:** This anaesthetic can be used to produce surgical anaesthesia in non-human primates, dogs, cats, sheep, pigs, rats and mice when adminis-
tered intravenously. In rabbits the degree of analgesia produced is insufficient for major surgery. The duration of anaesthesia is short (<10 min) and recovery is rapid. Repeated doses of propofol, or administration of a continuous infusion can be used to prolong anaesthesia without unduly extending the recovery time.

**Tribromoethanol**: This anaesthetic can be used in rodents, and produces surgical anaesthesia with good muscle relaxation. Decomposition of the solution can cause severe irritation upon intraperitoneal administration, resulting in death of some animals. It is therefore essential that only a freshly prepared solution should be used. Administration of a second anaesthetic on a subsequent occasion can cause gastrointestinal disturbances and death, even if a freshly prepared solution is used.

Recent studies have suggested that even a freshly prepared solution can have irritant properties, and so this agent should be reconsidered.

**Alpha-chloralose**: Alpha-chloralose and chloral hydrate are both primarily hypnotic agents that cause minimal cardiovascular depression. Upon intraperitoneal administration, it causes irritation of the gastrointestinal tract, potentially resulting in adynamic ileus.

Alpha-chloralose is sometimes used in combination with urethane, which is carcinogenic and must only be used in terminal experiments.

**Assessment of depth of anaesthesia**

Whatever anaesthetic is selected, it is important to be able to monitor the depth of anaesthesia to ensure that the animal is not too lightly anaesthetized and perceives painful stimuli, nor is too deeply anaesthetized and so be in danger of death due to anaesthetic overdose. Reflex activity, changes in the pattern, the rate and the depth of respiration, alterations of heart rate and blood pressure and other reactions to (painful) stimuli are used to determine the depth of anaesthesia. Unfortunately the changes in these parameters at different depths of anaesthesia vary in different animal species and with different anaesthetic agents. It is important to recognize that the transition from awake consciousness to complete surgical anaesthesia is a continuum, and not a series of discrete steps. Nevertheless, anaesthesia has traditionally been divided arbitrarily into four stages. These stages can be recognized most easily when anaesthesia is induced without the use of any premedication and when only inhalational agents are used.

*Stage 1, the induction stage*: During this stage the animal is conscious and experiences a light state of analgesia and sedation. Responses to stimuli are slightly delayed.

*Stage 2, the excitation stage*: The animal is losing consciousness and shows
exaggerated reflex activity and muscle movements. The pupils become dilated and there is an increase in tear and mucous production, and the eyes may show uncoordinated movements.

Stage 3, the surgical stage: The frequency of respiration is reduced, and its depth increases. The eyelid and corneal reflexes disappear, muscle tone and reflex responses decrease. There is no response to surgical or other stimulation.

Stage 4, the hypoxic (toxic) stage: The vital brain centers are depressed to such an extent that respiration and the heart beat slows and eventually stops. The pupils become dilated and unresponsive to light. This stage is reached during euthanasia when an overdose of an anaesthetic is administered.

For most experiments the surgical stage is required. Unfortunately, there is considerable variation in the responses described above when different anaesthetic combinations are used. To determine whether the animal has sufficient anaesthesia, the following reflex responses can be assessed:

1. the righting reflex: the animal normally attempts to turn over to the sternal position after being placed on its back. Under anaesthesia the animal remains on its back.
2. the palpebral reflex: blinking when the inner or outer canthus of the eye is touched. The reflex is abolished during surgical anaesthesia.
3. the pedal reflex: withdrawal and flexion of a leg when a digit or the interdigital skin is pinched. This reflex disappears during anaesthesia.
4. the swallowing reflex: pulling the tongue or pressing the throat results in swallowing without anaesthesia.
5. the tail pinch reflex: pinching the tail with finger-nails or a haemostat results in a flick of the tail and occasionally in vocalization, when the animal is not deeply anaesthetized.
6. the ear pinch reflex: pinching the ear in rabbits and guinea pig produces a head shake response when awake.

Loss of the righting reflex and the pedal withdrawal reflex are the most practically useful assessments that can be made in most laboratory mammals. These reflex responses are lost in a graded manner. For example, vocalization in response to pinching the tail or digit is lost before the reflex twitch or withdrawal disappears. The magnitude of these withdrawal responses falls gradually, until they are completely absent at moderate to deep planes of anaesthesia during which surgical intervention is possible.

**Endotracheal intubation and artificial ventilation**

Endotracheal intubation is the insertion of a tube into the trachea. Intubation can only be carried out in anaesthetized animals. Intubation is necessary when
Endotracheal intubation and artificial ventilation

Respiration is to be maintained by artificial ventilation, but is also useful in spontaneously breathing animals since it ensures that the airway remains unobstructed and allows ventilation to be assisted if respiratory depression occurs.

The technique for endotracheal intubation varies in the different species, but is always easier if an appropriate laryngoscope is available. Blades suitable for larger species are available commercially and techniques for intubation are described in standard veterinary textbooks (e.g. Short, 1987; Hall and Clarke, 1991). Blades for small rodents need to be specially constructed, and those described by Costa et al. (1986) are simple to manufacture and easy to use. A guide to the sizes of endotracheal tube appropriate for each species is given in Table 15-6.

Table 15-6
Diameter and length of endotracheal tubes in different species

<table>
<thead>
<tr>
<th>Species</th>
<th>Outer diameter (mm)</th>
<th>Inner diameter (mm)</th>
<th>Length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>1.0</td>
<td>0.5</td>
<td>3</td>
</tr>
<tr>
<td>Rat</td>
<td>1.8</td>
<td>1.0</td>
<td>13</td>
</tr>
<tr>
<td>Hamster</td>
<td>1.6</td>
<td>1.0</td>
<td>8</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>2.0</td>
<td>1.5</td>
<td>10</td>
</tr>
<tr>
<td>Rabbit</td>
<td>2.0–3.5</td>
<td>1.8–3.3</td>
<td>15</td>
</tr>
</tbody>
</table>

In all species, the animal must be sufficiently deeply anaesthetized to allow the mouth to be opened and the tongue pulled forward without eliciting any chewing or swallowing responses. The tongue is extended and a laryngoscope (fig. 15-1a) is inserted. This allows the operator to see the epiglottis and vocal folds. In cats, pigs, rabbits and non-human primates, the larynx should be sprayed with local anaesthetic prior to intubation to minimize the risk of laryngospasm. The endotracheal tube should be lubricated with a local anaesthetic ointment prior to insertion into the trachea. For intubation of mice, rats, hamsters and guinea pigs, either a purpose-made laryngoscope can be constructed, or the anaesthetized animal should be placed in dorsal recumbency, the mouth opened and fixed with an eye speculum, or a purpose-made mouth gag. The tongue should be pulled out of the mouth and fixed with a vessel clip and a cotton wool plug. A strong light should be directed towards the ventral part of the neck. This light passes through the tissue. Using fine forceps, the base of the tongue should be pressed upwards and the opening of the larynx will become visible. It is also possible to carry out this procedure using an otoscope with a diameter of 4 mm (fig. 15-1b). For this the animal should be placed in sternal recumbency. Using cotton wool swabs moistened with local anaesthetic, the mucous membranes of the mouth and pharynx are desensitized. The head is lifted backwards, perpendicular to the table. The
tongue is then pulled out of the mouth using a cotton wool plug. The otoscope is inserted into the mouth. The ventral side of the epiglottis becomes visible. A stylet is passed through the otoscope (0.8 mm) and the soft palate is pushed dorsally. The epiglottis will fall ventrally, revealing the vocal folds. The stylet is now pushed between the vocal folds and the otoscope is carefully removed. The endotracheal tube is passed over the stylet and, when it is in the trachea, the stylet is removed. With both methods, the tube must not be inserted too far in order to avoid ventilation of only one lung.

Rabbits can be intubated using a Wisconsin laryngoscope blade, although it is also possible to intubate rabbits without visualizing the larynx. The rabbit must be placed in sternal recumbency. The head is raised and tilted backwards so that the mandible is perpendicular to the table. The head is held in one hand with the thumb and index finger placed in the corners of the upper and lower jaw, thus holding the mouth open. The tube is inserted along the palate. As the tube reaches the pharynx the operator should listen for respiration sounds while passing the tube between the vocal cords. To check the proper position of the tube, a small mirror or a few hairs can be placed close to the end of the tube. The mirror will become misty and the hairs will be blown away when the animal exhales. Great care must be taken to avoid pushing the endotracheal tube against the larynx, as this delicate structure is easily damaged. The resulting oedema and haemorrhage can cause post-operative airway obstruction and death.

If intubation is difficult, or impossible, a tracheotomy may be carried out, provided that the animal is not required to recover from the procedure.
Endotracheal intubation and artificial ventilation

Most birds and reptiles are easy to intubate. After induction of anaesthesia, the mouth is opened and the entrance to the trachea can easily be seen at the base of the tongue.

Anaesthetic breathing systems

For the administration of oxygen and inhalational anaesthetics, different breathing systems are used. An essential requirement for any breathing system is the provision of sufficient oxygen and the adequate removal of carbon dioxide. The simplest technique is to deliver oxygen and anaesthetic gases by means of a close-fitting facemask. The animal can be connected via a facemask or endotracheal tube to a T-piece or Bain’s circuit. Using this system, the fresh gas flow rates from the anaesthetic machine should exceed three times the animal’s minute volume, in order to prevent the rebreathing of dead space air. The minute volume is the quantity of gas inhaled in one minute and is calculated by multiplying the volume of one breath (the tidal volume, approximately 15 ml/kg of body weight) by the respiratory rate. Use of this mask system for gas delivery does not in all circumstances allow ventilation to be assisted adequately, and the high fresh gas flows needed may be uneconomic when anaesthetizing large animals. Detailed descriptions of the use of these circuits are readily available (Short, 1987, Hall and Clarke, 1991). When anaesthetizing larger animals (>20 kg body weight), the relatively high gas flows needed when using a Bain’s circuit or T-piece may be uneconomic. In these animals a rebreathing system with a carbon dioxide absorber (see fig 15-2) may be used. Although these systems are economical to use, considerably

![Schematic presentation of an inhalation anaesthesia system (rebreathing system or anaesthetic circuit).](image-url)
more experience is necessary to maintain an adequate and stable depth of anaesthesia. Use of closed circuit anaesthesia is described in Short (1987), and Hall and Clarke (1991).

**Artificial ventilation**

When artificial ventilation is used, fresh gas is forcibly blown into the lungs using either manual pressure or a mechanical ventilator. Animals must be artificially ventilated during thoracotomy or when a muscle relaxant is used. It is also advisable to apply artificial ventilation during prolonged experiments (> 1–2 hours), in order to ensure adequate gas exchange. During artificial ventilation a positive airway pressure exists during inspiration. The expiratory phase is a passive phase caused by the elasticity of the lungs. The increase in intra-thoracic pressure during inspiration reduces cardiac output and, to minimize this effect, the inspiratory phase is kept relatively short, (± 30% of the respiratory cycle).

Although every species has its own ventilatory requirements, as a general rule a tidal volume of 10–15 ml/kg must be administered to ensure adequate respiratory function. The respiratory rate required varies in different species, ranging from 10–15 breaths per minute in dogs, sheep and pigs, to 60–150 breaths per minute in rabbits and small rodents (Table 15-7). Ventilators designed for use in man can usually deliver tidal volumes ranging from 50–1500 ml at rates of 8–60 breaths per minute. Human infant ventilators are capable of supplying smaller tidal volumes, but it is preferable to use a purpose-designed laboratory animal ventilator (e.g. the Harvard Rodent Ventilator) for small rodents. These ventilators should be capable of delivering tidal volumes as low as 0.2 ml at frequencies of up to 150 per minute.

During mechanical artificial ventilation, attention must be paid to the airway pressure to prevent overinflation and barotrauma. Inflation pressures should generally not exceed 10 cm of water in small animals. To attain good artificial ventilation, it is advisable to use a capnograph, to measure the car-

<table>
<thead>
<tr>
<th>Table 15-7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Artificial ventilation</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Species</th>
<th>Frequency/ min</th>
<th>Tidal volume (ml)</th>
<th>Inspiration/ expiration time (%)</th>
<th>Pressure (cm H₂O)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>100–130</td>
<td>0.5–1</td>
<td>35/65</td>
<td>5–15</td>
</tr>
<tr>
<td>Rat</td>
<td>50–80</td>
<td>3–10</td>
<td>35/65</td>
<td>5–15</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>30–50</td>
<td>8–20</td>
<td>35/65</td>
<td>5–15</td>
</tr>
<tr>
<td>Rabbit</td>
<td>30–50</td>
<td>40–60</td>
<td>35/65</td>
<td>5–15</td>
</tr>
<tr>
<td>Bird</td>
<td>6–12</td>
<td>depends on size</td>
<td>35/65</td>
<td>5–15</td>
</tr>
</tbody>
</table>
bon dioxide content in the expired gas, and to maintain this within the normal range of 4–5%. Unfortunately, many capnographs are incapable of recording accurately the expired carbon dioxide concentration from small (<500 g) animals, because the volume of expired gas is too small.

Fluid administration: Whenever possible an indwelling intravenous catheter must be placed for fluid administration at a basic rate of 10–15 ml/kg/hr. With blood loss a higher infusion rate is necessary to compensate for this loss.

Neuromuscular blocking agents

In order to facilitate artificial ventilation and, under specific circumstances, the endotracheal intubation, and in order to obtain relaxation of the skeletal muscles, neuromuscular blocking agents (muscle relaxants) are sometimes administered. When using these drugs, one should remember that the animal is completely immobilized and will not move in response to painful stimuli, even if consciousness has returned. Muscle relaxants should therefore only be used under strictly defined conditions, and only under the supervision of an experienced animal anaesthetist. When using muscle relaxants, the heart rate and blood pressure should be monitored continuously. An increase in either of these parameters in response to surgical stimuli should be assumed to indicate an inadequate depth of anaesthesia, and additional anaesthetic should be administered. Dosages of neuromuscular blocking agents are listed in Table 15-4.

Monitoring of vital functions

Throughout the period of anaesthesia, it is important to assess the adequacy of the animal’s vital functions, and to ensure these are maintained within acceptable limits. The extent and complexity of this monitoring will depend upon the nature and duration of the experimental procedure. A basic assessment of the animal can be undertaken by simple observation, for example the colour of the mucous membranes, the pattern and rate of respiration and the heart rate and pulse can be assessed easily in most species. Repeated observation of these parameters is often difficult during an experimental procedure, and so it is often useful to employ electronic monitoring devices. Monitoring equipment also enables accurate assessment of parameters such as blood oxygen and carbon dioxide concentrations and body temperature, which cannot be obtained by simple observation. Electronic monitoring may include:

– electrocardiogram and heart rate
– cardiac output
– arterial, pulmonary artery and central venous pressure
Anaesthesia, analgesia and euthanasia

- the capnogram (expiratory CO₂ concentration)
- arterial blood gas and acid base status (PaO₂, PaCO₂, pH, base excess, bicarbonate)
- arterial oxygen saturation using a pulse oximeter
- respiratory volume and frequency
- airway pressure
- body temperature
- electroencephalogram (brain activity).

It is also advisable to monitor the proper function of the anaesthetic equipment, particularly during prolonged periods of anaesthesia – for example the concentration of inhalational agents and inspiratory oxygen concentration. When considering purchase of monitoring equipment, ensure that the device will function successfully in small animals. For example, many heart rate monitors are incapable of recording heart rates in excess of 250 beats per minute and the normal resting heart rate of rodents and rabbits will often exceed this rate.

During anaesthesia of small (<10 kg) animals, special attention must be paid to the maintenance of the body temperature. Small animals cool rapidly when anaesthetized, and this is a significant cause of increased mortality. Heating blankets, heating lamps and other devices should be used to maintain body temperature. Body temperature should be monitored continuously, using an electronic thermometer, to ensure that both hypothermia and hyperthermia are avoided.

It may be necessary to provide intravenous fluid to replace that lost from the respiratory tract and by haemorrhage following surgery. Detailed descriptions of fluid therapy are given by Michell et al. (1989). As a basic guide, approximately 10–15 ml/kg/hour of Lactated Ringers solution should be infused intravenously. In small mammals, 10–15 ml/kg of normal (0.9%) saline can be administered subcutaneously or intraperitoneally, although this will be absorbed slowly and will be ineffective for the treatment of acute fluid deficits. All fluids should be warmed to body temperature before administration in order to prevent excessive cooling of the animal.

If the animal’s eyes remain open during anaesthesia, ophthalmic ointment or artificial tears should be applied to prevent drying of the cornea or, alternatively, the eyelids should be taped closed.

Complications of anaesthesia

Most anaesthetic agents have several (unwanted) pharmacological side effects. Occasionally, serious complications may occur because of the depression of vital organ function which, in an extreme situation, can result in an autonomous fatal process, leading to shock and death (fig. 15-3).
Complications of anaesthesia may result in an autonomous process of disregulation of vital functions, leading to shock and death.
Depression of spontaneous ventilation can occur when the concentration of injectable or inhalational anaesthetic agents is too high. This will result in hypoxia and hypercapnia. Eventually ventilation will stop and cardiac arrest will occur. The mucous membranes turn purple-blue, because of the increased concentration of non-oxygenated haemoglobin in the blood (cyanosis). If inhalation anaesthetics are being administered, the vaporizer must be switched off and the anaesthetic circuit flushed with oxygen. If injectable anaesthetics are being administered by continuous infusion, the infusion pump must be switched off. If oxygen is being supplied, the fresh gas flow rate must be increased to approximately three times minute volume and ventilation assisted by manually depressing the reservoir bag or occluding the outflow of a T-piece or Bain’s circuit in patients which are intubated. In small animals which are not intubated, oxygen must be administered by facemask and ventilation assisted by manually compressing the chest. If respiratory arrest occurs during anaesthesia, then artificial ventilation has to be continued until detoxification (metabolism or excretion) of the anaesthetic has advanced to an acceptable level. When the CO\(_2\) concentration decreases due to artificial ventilation, the driving force for the respiration is abolished. When the CO\(_2\), is allowed to increase to normal values then spontaneous ventilation will resume. Respiratory function can sometimes be stimulated by administration of doxapram (5–10 mg/kg by any convenient route). When the respiratory depression is the result of the use of opioids, naloxon or another opioid antagonist may be employed to counteract this depression. When animals are positioned in an unphysiological position, interference with pulmonary perfusion and ventilation may occur. This may lead to ventilation-perfusion disturbances resulting in a decreased arterial oxygenation and increased arterial CO\(_2\) tension.

Cardiac arrhythmias and cardiac arrest

Unless cardiac activity is being monitored with an electrocardiogram, arrhythmias are difficult to diagnose. Arrhythmias can cause serious disturbances in circulatory function, and may require therapy. Detailed information on this topic can be found in Short (1987). During anaesthesia, deep plane of anaesthesia, hypothermia and vagal stimulation may induce bradycardia. Pain, hypoxaemia, hypercapnia, and hypovolemia result in tachycardia. If cardiac arrest occurs, it can be treated by external cardiac massage: the thorax must be compressed at a rate of 70–80 per minute (in the dog) and this should be coupled with the measures described above for correcting respiratory failure. As an emergency measure, adrenaline (0.1–0.2 mg/kg) must be administered either by deep intratracheal administration or by intracardiac injection.
Complications of anaesthesia

Cardiac failure and hypotension

Hypotension can arise because of vasodilation or depression of myocardial contractility caused by the anaesthetic agent, or as a result of blood loss or sequestration of blood into the tissues during surgical manipulations. Decreased capillary refill, low arterial blood pressure, tachycardia, and pale colour of the skin and mucous membranes are some of the signs of hypotension. Intravenous administration of fluids is usually required to correct this problem. It is preferable to use whole blood to treat haemorrhage. Blood must be collected from a donor animal of the same species in acid citrate dextrose (1 part ACD to 3.5 parts of blood). The incidence of adverse transfusion reactions is low following a single transfusion and, in any event, cross-matching is rarely practicable in animals. Blood should be replaced at a rate of 10% of the animal’s circulating volume every 30 minutes but, if rapid haemorrhage has occurred, then replacement must also be as rapid as possible. If blood is not available, plasma volume expanders such as Haemaccel (Hoechst), gelofusin or starch solutions should be used or, if these are unavailable, lactated Ringer’s solution or normal saline should be administered.

Regurgitation

During anaesthesia, dogs, cats, and primates may vomit, and this material may be inhaled into the lungs. As mentioned earlier, withdrawal of food for 12–16 hours reduces the risk of vomiting. Ruminants will regurgitate during anaesthesia, and intubation with a cuffed endotracheal tube is essential in these species. Withholding food for 16–24 hours in these species may be beneficial in reducing the tendency for gas accumulation in the rumen. When aspiration of stomach contents takes place, a clinical pneumonitis will result with an impaired gas exchange leading to hypoxaemia and even death as a consequence. The volume and pH of the aspirate is an important factor in determining lethality.

Hypothermia

Hypothermia can occur during anaesthesia. Not only is the thermoregulation disturbed, but the loss of body heat is also increased due to artificial ventilation, fluid administration and vasodilation. As a consequence the temperature must be monitored at all times, and heating must be supplied when indicated.
Long-term anaesthesia

In some experiments, particularly those concerned with monitoring physiological processes, a stable plane of anaesthesia lasting several hours is essential. This can be achieved either by the use of long-acting anaesthetics, by continuous infusion of short-acting drugs, or by continuous administration of volatile anaesthetics. Further details can be found in Flecknell (1996).

Post-operative care

Monitoring procedures may need to be continued in the immediate post-operative period to ensure that the animal is recovering satisfactorily. It is particularly important to maintain body temperature, and an incubator should be provided for small animals. This must be maintained at 25–35°C for adult animals, and 35–37°C for neonates. Large animals should be provided with heating pads or heating lamps. Body temperature should be monitored regularly to check the adequacy of the measures used. The bedding material provided must be both comfortable and should also provide effective insulation. Sawdust and woodshavings are not suitable, as the animal may inhale particles of bedding and dust may adhere to the animal’s eyes, mouth and nose. Purpose-made bedding material such as “Vetbed” is preferable for all species, alternatively towels or blankets can be used. The animal should be housed individually in quiet surroundings and should be observed frequently until normal activity has resumed. Endotracheal tubes must be removed as soon as the swallowing reflex has returned, but continued attention should be paid to respiratory function until recovery from anaesthesia is complete.

Post-operative pain relief is required after all surgical procedures. In order to provide an appropriate analgesic for an appropriate time period, it is important to attempt to assess the degree of pain that is present. Pain assessment in animals is difficult, and considerable experience of the normal behaviour of the species is necessary. Significant signs of pain may include abnormal behaviour, altered posture, and reduction of food and water intake and weight loss (see chapter 14). Since small rodents are relatively inactive during the day, it may be necessary to observe animals during the dark phase of their photoperiod in order to make an accurate assessment of their well being.

The administration of opiates (e.g. morphine, buprenorphine, nalbuphine), non-steroidal anti-inflammatory drugs (NSAID’s, such as flunixin, carprofen), or local anaesthetics can alleviate post-operative pain. The use of these agents has been reviewed by a number of authors (Flecknell, 1984; Liles and Flecknell, 1992, Flecknell and Waterman-Pearson, 2000; Hellegers, 2000). In general, opioids are required to control post-operative pain.
and, of the drugs available, buprenorphine has a prolonged duration of action in most species (6–12 hours) and can be used safely to provide effective pain relief. NSAID’s are generally less effective analgesics, but flunixin and carprofen and other more recently available agents appear to have a potency approaching that of some opioids (Table 15-8). In many instances, effective pain relief can be provided by administering opioids during the first 24 hours post-operatively, followed by the use of a NSAID for a further 24 hours. Post-operative pain rarely seems to persist for longer than 72 hours.

Analgesics have side effects that may interfere with particular experimental protocols. Opiates can cause respiratory depression, hypotension and constipation, but these effects are rarely of clinical significance in animals. NSAID’s reduce the synthesis of prostaglandins and can have negative effects on the wound-healing process, although this seems to be of little clinical significance. They can disturb blood coagulation, and some may affect renal function. Careful assessment of the pharmacology of the different analgesics available should enable an appropriate analgesic regimen to be developed. If systemic analgesics are contraindicated, surgical wounds may be infiltrated with bupivacaine, a long-acting local anaesthetic, to provide a short (4–6 h) period of analgesia.

In the post-experimental period, the animal must be observed several times a day. Attention must be paid to the surgical wound and the animal must be prevented from mutilating the affected body area, or from disturbing implanted instrumentation (catheters, transducers etc.) by biting, licking or scratching. In carnivores and primates, neck collars are sometimes needed to prevent licking of the wound. In chronic experiments, it may be advisable to implant catheters and other instrumentation beneath the skin.

Euthanasia

The EC Directive (see chapter 2) states that an animal should not be kept alive after an experiment if pain and distress are likely to be experienced, and the keeping alive of the animal is not necessary in order to achieve the aims of the experiment. Laboratory animals are also killed in order to obtain organs or tissues for further experimentation, or to examine the morphological effects of the experiment. Other reasons for euthanasia include the presence of an incurable disease state or (under special conditions) a surplus of animals.

There are some specific requirements to be considered when animals are euthanized. The most important is that the method must be humane. When organs or tissues are harvested for further experimentation, the method should have no influence on the studies that will be undertaken on these organs and tissues. Furthermore the method must be reliable and effective, economical,
Table 15-8
Doses of analgesics for post-operative pain relief (drugs listed in alphabetical order)

A. Mice and rats

<table>
<thead>
<tr>
<th>Drug</th>
<th>Mouse</th>
<th>Rat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspirin</td>
<td>120 mg/kg per os 4 hourly</td>
<td>100 mg/kg per os 4 hourly</td>
</tr>
<tr>
<td>Buprenorphine</td>
<td>0.05–0.1 mg/kg s.c. 12 hourly</td>
<td>0.01–0.05 mg/kg s.c., i.v. 8–12 hourly</td>
</tr>
<tr>
<td>Butorphanol</td>
<td>1–5 mg/kg s.c. 4 hourly</td>
<td>2 mg/kg s.c. 4 hourly</td>
</tr>
<tr>
<td>Carprofen</td>
<td>–</td>
<td>5 mg/kg s.c. 12–24 hourly</td>
</tr>
<tr>
<td>Codeine</td>
<td>60–90 mg/kg per os 20 mg/kg s.c. 4 hourly</td>
<td>60 mg/kg s.c. 4 hourly</td>
</tr>
<tr>
<td>Flunixin</td>
<td>2.5 mg/kg s.c., i.m. 12 hourly</td>
<td>2.5 mg/kg s.c., i.m. 12 hourly</td>
</tr>
<tr>
<td>Morphine</td>
<td>2.5 mg/kg s.c. 2–4 hourly</td>
<td>2.5 mg/kg s.c. 2–4 hourly</td>
</tr>
<tr>
<td>Nalbuphine</td>
<td>4–8 mg/kg i.m. 4 hourly</td>
<td>1–2 mg/kg i.m. 3 hourly</td>
</tr>
<tr>
<td>Paracetamol</td>
<td>300 mg/kg per os 4 hourly</td>
<td>100–300 mg/kg per os 4 hourly</td>
</tr>
<tr>
<td>Pentazocine</td>
<td>10 mg/kg s.c. 3–4 hourly</td>
<td>10 mg/kg s.c. 4 hourly</td>
</tr>
<tr>
<td>Phenacetin</td>
<td>200 mg/kg per os 4 hourly</td>
<td>100 mg/kg per os 4 hourly</td>
</tr>
<tr>
<td>Pethidine</td>
<td>10–20 mg/kg s.c. or i.m. 2–3 hourly</td>
<td>10–20 mg/kg s.c. or i.m. 2–3 hourly</td>
</tr>
</tbody>
</table>

B. Guinea pigs, rabbits, dogs and cats

<table>
<thead>
<tr>
<th>Drug</th>
<th>Guinea pig</th>
<th>Rabbit</th>
<th>Dog</th>
<th>Cat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspirin</td>
<td>85 mg/kg per os 4 hourly</td>
<td>100 mg/kg per os 4 hourly</td>
<td>10 mg/kg per os 6 hourly</td>
<td>!</td>
</tr>
<tr>
<td>Buprenorphine</td>
<td>0.05 mg/kg s.c. 8–12 hourly</td>
<td>0.01–0.05 mg/kg s.c. or i.v. 8–12 hourly</td>
<td>0.01–0.02 mg/kg s.c. or i.v. 8–12 hourly</td>
<td>0.005–0.01 mg/kg s.c. or i.v. 8–12 hourly</td>
</tr>
<tr>
<td>Butorphanol</td>
<td>–</td>
<td>0.1–0.5 mg/kg i.v. 4 hourly</td>
<td>0.4 mg/kg s.c. or i.m. 3–4 hourly</td>
<td>0.4 mg/kg s.c. 3–4 hourly</td>
</tr>
<tr>
<td>Codeine</td>
<td>–</td>
<td>–</td>
<td>0.25–0.5 mg/kg per os with paracetamol 6 hourly</td>
<td>–</td>
</tr>
<tr>
<td>Flunixin</td>
<td>–</td>
<td>1.1 mg/kg s.c., i.m. 12 hourly</td>
<td>1 mg/kg per os daily</td>
<td>1 mg/kg s.c., daily for up to 5 days</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>10 mg/kg i.m. 4 hourly</td>
<td>10 mg/kg i.v. 4 hourly</td>
<td>5–10 mg/kg per os 24–48 hourly</td>
<td>–</td>
</tr>
</tbody>
</table>
### Table 15-8 (continued)

<table>
<thead>
<tr>
<th>Drug</th>
<th>Guinea pig</th>
<th>Rabbit</th>
<th>Dog</th>
<th>Cat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphine</td>
<td>2–5 mg/kg s.c. or i.m. 4 hourly</td>
<td>2–5 mg/kg s.c. or i.m. 2–4 hourly</td>
<td>0.5–5 mg/kg s.c. or i.m. 4 hourly</td>
<td>0.1 mg/kg s.c. 4 hourly</td>
</tr>
<tr>
<td>Nalbuphine</td>
<td>1–2 mg/kg i.v. 4–5 hourly</td>
<td>0.5–2.0 mg/kg s.c., i.m. 3–8 hourly</td>
<td>1.5–3.0 mg/kg i.v. 3 hourly</td>
<td></td>
</tr>
<tr>
<td>Paracetamol</td>
<td>–</td>
<td>–</td>
<td>10–20 mg/kg per os plus codeine 6 hourly</td>
<td></td>
</tr>
<tr>
<td>Pentazocine</td>
<td>–</td>
<td>5 mg/kg i.v. 2–4 hourly</td>
<td>2 mg/kg i.m. 4 hourly</td>
<td>8 mg/kg i.p. 4–6 hourly</td>
</tr>
<tr>
<td>Pethidine</td>
<td>10–20 mg/kg s.c. or i.m. 2–3 hourly</td>
<td>10 mg/kg i.m. daily</td>
<td>10 mg/kg s.c. daily</td>
<td></td>
</tr>
</tbody>
</table>

**C. Primates, pigs, sheep and goats**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Primates</th>
<th>Pigs</th>
<th>Sheep and goats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspirin</td>
<td>20 mg/kg per os 6–8 hourly</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Buprenorphine</td>
<td>0.01 mg/kg i.m. or i.v. 8–12 hourly</td>
<td>0.01–0.05 mg/kg i.m. 8–12 hourly</td>
<td>0.005–0.01 mg/kg i.m. 4–6 hourly</td>
</tr>
<tr>
<td>Flunixin</td>
<td>2.5–10.0 mg/kg i.m. daily</td>
<td>1 mg/kg s.c. daily</td>
<td>1 mg/kg s.c. daily</td>
</tr>
<tr>
<td>Morphine</td>
<td>1–2 mg/kg s.c. 4 hourly</td>
<td>up to 20 mg total dose i.m. 4 hourly</td>
<td>10 mg total dose i.m. s.c. 4 hourly</td>
</tr>
<tr>
<td>Pentazocine</td>
<td>2–5 mg/kg i.m. 4 hourly</td>
<td>2 mg/kg i.m. 4 hourly</td>
<td>–</td>
</tr>
<tr>
<td>Pethidine</td>
<td>2–4 mg/kg i.m. 3–4 hourly</td>
<td>2 mg/kg i.m. 4 hourly</td>
<td>200 mg i.m. total dose 4 hourly</td>
</tr>
</tbody>
</table>

Dose rates based on published data reviewed by Flecknell, 1996 and Flecknell and Liles, 1992, and clinical experience at the Clinical Research Centre, Harrow and the Comparative Biology Centre.

easy to perform, and must be safe for the laboratory personnel. Before attempting any method of euthanasia, it is essential that the personnel involved have undergone appropriate training. In addition to selecting a method of euthanasia that is humane, it is important that animals are handled carefully to minimize any distress. Vocalizations and the release of pheromones from frightened animals may cause anxiety and distress in other animals. For this reason, whenever practicable, animals should not be killed in the presence of others.
There are several methods by which animals can be killed and these can be classified into two broad groups: the pharmaco-chemical methods and the mechanical-physical methods (Table 15-9).

Pharmaco-chemical methods: A drug or other chemical compound is administered that leads to the death of the animal. The most frequently used method is the administration of an overdose of a general anaesthetic, leading to cardiac and respiratory arrest and the death of the animal. Intravenous or intraperitoneal injection of an overdose of pentobarbitone (100–150 mg/kg) is frequently used because it is relatively rapidly acting, easy to administer and inexpensive. Barbiturates may cause pooling of blood in organs due to vasodilation, which may affect histological studies.

Euthanasia, using inhalational anaesthetics, can be carried out with ether, halothane, enflurane or isoflurane. Frequently, carbon dioxide is used as an inhalational agent for euthanasia. Exposure to 100% CO₂ can induce severe dyspnoea in several animal species and appears to cause distress in conscious animals. It is therefore preferable to use a combination of CO₂/O₂ (6:4) and a humidifier. After the animal has lost consciousness, the concentration of CO₂ is raised to 100%. Animals must remain in 100% CO₂ for at least 10 minutes to ensure that they are dead. Neonatal animals are relatively resistant to the effects of CO₂, and exposure may need to be continued for 30–60 minutes, so other methods are preferable. Carbon dioxide and inhalational agents can induce pulmonary oedema, and this may interfere with subsequent post-mortem investigations.

Mechanical-physical methods

Because most pharmaco-chemical methods have the potential to interfere with certain experiments, mechanical-physical methods may be preferable e.g. for harvesting organs and tissues destined for biochemical or histological post-mortem examination. Whenever possible, a sedative or anaesthetic drug should be administered before employing this method. The use of all mechanical-physical methods may be distressing to the personnel involved. This aspect should be taken into consideration when selecting a method for euthanizing the animals. Physical methods include decapitation and dislocation of the cervical vertebrae. Decapitation can be performed by using scissors or a guillotine. Dislocation of the cervical vertebrae is carried out by stretching the animal and rotating the neck. The spinal cord is disrupted and nerve impulses to the vital organs such as the respiratory system and the heart are no longer transmitted. Dislocation of the cervical vertebrae is an acceptable method in mice, rats, hamsters, gerbils, puppies, kittens and small birds, but not in larger animals. If it is done quickly and expertly it is a painless method. To confirm death, this method can be followed by exsanguination or destruction of the brain.
### Table 15-9
Methods of euthanasia

<table>
<thead>
<tr>
<th>Drug/method</th>
<th>Species</th>
<th>Site of action</th>
<th>Safety</th>
<th>Application</th>
<th>Induction rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ether</td>
<td>rodents, cats, pups, small birds</td>
<td>direct inactivation of cortex, subcortex, vital centres medulla oblonga</td>
<td>inflammable explosive</td>
<td>easy in perspex box</td>
<td>slow</td>
</tr>
<tr>
<td>Halothane</td>
<td>rodents, cats, pups, small birds</td>
<td>same as ether</td>
<td>chronic inhalation can be dangerous</td>
<td>easy to perform</td>
<td>quick</td>
</tr>
<tr>
<td>Carbon dioxide</td>
<td>rodents, cats, pups, small birds</td>
<td>same as ether, plus myocard depression</td>
<td>danger minimal</td>
<td>closed box</td>
<td>quite fast</td>
</tr>
<tr>
<td>Barbiturates</td>
<td>all animals</td>
<td>same as ether</td>
<td>safe</td>
<td>animal has to be immobilised for i.v. injection</td>
<td>fast induction</td>
</tr>
<tr>
<td>Cervical dislocation</td>
<td>laboratory animals weighing less than 200 g</td>
<td>direct inactivation of brain</td>
<td>safe</td>
<td>experience and skill necessary</td>
<td>quite fast</td>
</tr>
<tr>
<td>Decapitation</td>
<td>rodents and young rabbits</td>
<td>direct inactivation of brain</td>
<td>chance of mechanical injury</td>
<td>easy with minimal skill</td>
<td>medium fast; unconscious after 13 sec</td>
</tr>
<tr>
<td>Microwave</td>
<td>mice, rats and animals of equal weight</td>
<td>special equipment needed</td>
<td>no changes in tissues used for brain research</td>
<td>very effective if animal in correct position</td>
<td>only small animals</td>
</tr>
<tr>
<td>Freezing</td>
<td>small animals under 20 g body weight</td>
<td>total inactivation of enzyme activity</td>
<td>safe</td>
<td>effective liquid nitrogen</td>
<td>acceptably quick</td>
</tr>
<tr>
<td>T61</td>
<td>dogs, cats, laboratory animals, birds</td>
<td>anaesthesia, unconsciousness; muscle relaxation</td>
<td>safe in case animal can be handled easily</td>
<td>if not intravenously not always handled</td>
<td>fast induction; sometimes effective muscle twitching</td>
</tr>
</tbody>
</table>
For neonates and small animals weighing less than 20 grams, euthanasia can also be carried out by instantaneous freezing of the animals by immersing them in liquid nitrogen, although the efficacy of this procedure in producing rapid loss of consciousness has been questioned. An alternative technique is the application of microwaves to the central nervous system, which causes instantaneous death without any change in the biochemistry of the animal. If microwaves are employed, it is essential that only specially constructed apparatus is used.

Larger animals such as pigs, ruminants and horses, can be rendered unconscious by captive bolt pistols, and then killed immediately by exsanguination from a cut through the carotid arteries.

References


16 Experimental procedures

V. Baumans, R. Remie, H. J. Hackbarth and A. Timmerman

Introduction

This chapter contains a brief description of some basic procedures, such as the administration of drugs and the collection of body fluids. Basic principles of surgery together with some surgical procedures will also be discussed. The description of technical procedures is designed to give a general background and is not intended to be used as a direct practical guide. The use of experimental techniques requires specific skills that can be obtained only by intensive and careful training under the supervision of an experienced laboratory animal scientist or animal technician. One should never use a technique that has not been fully explained and demonstrated by an experienced person. Also, practising on a dummy or a non-living or unconscious animal should always precede the use of techniques in conscious or anaesthetised animals.

For a more detailed description of the procedures described in this chapter, see the list of recommended literature.

Administration of drugs or other substances

There are three methods for the administration of drugs to laboratory animals distinguishable by the route of administration. They are enteral, parenteral or (trans)dermal.

(Trans)dermal application

When using this method of application, the drug is applied in solution or ointment form on the (shaven) skin or directly on a mucous membrane. This method is not very accurate, due to the variability of skin penetration and the
risk that the animal starts licking or rubbing the treated area. The applied substance may cause discomfort to the animal, for example, when the drug is irritating the skin or the mucous membrane.

**Enteral administration**

In this method the substance is brought into the gastrointestinal tract via the mouth (orally) or through the anus using a suppository. The latter method is not very practical when working with small laboratory animals. The simplest procedure of administration is through the food or drinking water. This is not possible, however, with substances which are unpalatable. When administered via the drinking water, the substances must be soluble and chemically stable. This method causes difficulties when it comes to measuring accurate individual doses.

It is possible, and also more accurate, to administer substances by means of a stomach tube (external diameter for mice: 0.8 mm; for rats: 1–2 mm; for guinea pigs: 1.5–2 mm; for rabbits/cats: 3–5 mm; for dogs: 5–7 mm). A curved needle with a blunt end is recommended for this purpose when dealing with small laboratory animals (fig. 16-1). The animal needs to be held firmly by the scruff whilst passing the tube along the palate into the oesophagus. When restraining mice and rats by the scruff, the head will be held up, thereby gently introducing the needle into the oesophagus; for larger animals e.g. dogs or pigs a surgical gag may be required (fig. 16-2). In cats, primates and horses a catheter passed through the nose is preferable. This method, however, does require experience as it is important to prevent the tube from entering the trachea. If this does happen, the animal will cough and it is possible to feel the tube touching the cartilage rings of the trachea. Also air can be sucked or blown into the tube, which is not possible when the tube has rightly passed into the oesophagus.

The presence of condensation on a mouth glass or movements of some hair held in front of the tube will also be an indication that the tube has passed into the trachea.

**Fig. 16-1. Curved blunt cannula for oral dosing.**
Parenteral administration refers to any method of administration other than those mentioned above, mainly, therefore, via an injection.

Injection methods. The most frequently used injection methods are:

- intracutaneous (i.c.) or intradermal (i.d.): into the skin;
- subcutaneous (s.c.): under the skin; resorption of the substance is slow;
- intramuscular (i.m.): into the muscles; the muscles of the posterior thigh or back are the most commonly used; resorption is quicker but this method is as a rule more painful;
- intraperitoneal (i.p.): into the peritoneal cavity; resorption is relatively fast through the peritoneum;
- intravenous (i.v.): into the vein, this is the fastest and the most accurate method.

In table 16-1, specifications are given for several injection techniques in mammals. Table 16-2 shows injection sites in birds, reptiles and amphibians.
Table 16-1
Injection techniques: some specifications per species

<table>
<thead>
<tr>
<th></th>
<th>Mouse 20–25 g</th>
<th>Hamster 25–30 g</th>
<th>Rat 250 g</th>
<th>Guinea pig 350 g</th>
<th>Rabbit 2.5 kg</th>
<th>Cat 4 kg</th>
<th>Dog 20 kg</th>
<th>Pig 50 kg</th>
<th>Primate 10 kg</th>
<th>Sheep 60 kg</th>
<th>Horse 500 kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral</td>
<td>blunt cannula; firm restraint vertical posture, pass tube along palate into oesophagus</td>
<td>tube and mouth gag</td>
<td>nasal tube</td>
<td>tube and gag</td>
<td>tube and gag</td>
<td>nasal tube</td>
<td>tube and gag</td>
<td>nasal tube</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Max. vol.</td>
<td>0.5</td>
<td>0.5–1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>7.5</td>
<td>10.0</td>
<td>20.0</td>
<td>100</td>
<td>30</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Diameter (mm)</td>
<td>1.0</td>
<td>1.0</td>
<td>2.0</td>
<td>2.0</td>
<td>5.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Intracutaneous

<table>
<thead>
<tr>
<th></th>
<th>skin of the back/abdomen</th>
<th>0.05–0.1 ml per injection site for all species</th>
</tr>
</thead>
</table>

Needle

<table>
<thead>
<tr>
<th></th>
<th>26G</th>
</tr>
</thead>
</table>

Subcutaneous

<table>
<thead>
<tr>
<th></th>
<th>skin of the neck</th>
<th>skin of the neck</th>
<th>skin overlying neck</th>
<th>skin overlying neck or dorsal chest</th>
<th>skin overlying neck</th>
<th>skin overlying neck or dorsal chest — near the elbow — thoracic wall</th>
</tr>
</thead>
<tbody>
<tr>
<td>Max. vol.</td>
<td>0.5–1.0</td>
<td>0.5–1.0</td>
<td>1.0–5.0</td>
<td>1.0–2.0</td>
<td>1.5–5.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Needle</td>
<td>26G</td>
<td>26G</td>
<td>25G</td>
<td>25G</td>
<td>21G</td>
<td>23G</td>
</tr>
</tbody>
</table>

Experiment procedures
Table 16-1 (Continued)

<table>
<thead>
<tr>
<th></th>
<th>Mouse 20–25 g</th>
<th>Hamster 25–30 g</th>
<th>Rat 250 g</th>
<th>Guinea pig 350 g</th>
<th>Rabbit 2.5 kg</th>
<th>Cat 4 kg</th>
<th>Dog 20 kg</th>
<th>Pig 50 kg</th>
<th>Primate 10 kg</th>
<th>Sheep 60 kg</th>
<th>Horse 500 kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intramuscular</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Max. vol.</td>
<td>0.05</td>
<td>0.05</td>
<td>0.1</td>
<td>0.1</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Intraperitoneal</td>
<td>lateral to the midline next to the umbilicus</td>
<td>n/a</td>
<td>mid-line between umbilicus and pelvic rim</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Max. vol.</td>
<td>1.0</td>
<td>1.0</td>
<td>5.0</td>
<td>10.0</td>
<td>20.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Needle</td>
<td>25G</td>
<td>25G</td>
<td>24G</td>
<td>24G</td>
<td>21G</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intravenous</td>
<td>lateral tail vein (difficult); sublingual vein; jugular vein; sublingual vein; penile vein</td>
<td>tail vein; hind limb vein; jugular vein; sublingual vein; penile vein</td>
<td>front limb, hind limb vein; marginal ear vein</td>
<td>front limb, hind limb vein; front limb, hind limb vein</td>
<td>ear vein; jugular vein</td>
<td>front limb, hind limb vein; jugular vein</td>
<td>front limb, hind limb vein</td>
<td>ear vein; jugular vein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Max. vol.</td>
<td>0.2</td>
<td>0.3</td>
<td>0.5</td>
<td>0.5</td>
<td>1–5</td>
<td>2–5</td>
<td>10–15</td>
<td>16–24G</td>
<td>21–25G</td>
<td>16–19G</td>
<td>16–19G</td>
</tr>
</tbody>
</table>

1Maximum volume in ml.
2Needle size in G (Gauge):
27G = 0.40 mm 23G = 0.60 mm 26G = 0.45 mm 22G = 0.70 mm
25G = 0.50 mm 20G = 0.90 mm 24G = 0.55 mm 19G = 1.00 mm
3Syrian hamster
4Not applicable
Experimental procedures

When applying injection techniques, there are several points that deserve special attention:

- Use clean, sharp, sterile needles.
- Use appropriate needle size. A thin needle causes less pain and prevents the fluid from flowing back. (The required thickness of the needle (gauge) will depend upon the viscosity of the fluid. When using very thin needles, there is a risk of cracking.)
- Never inject more fluid than the recommended maximum volume (table 16-1).
- Avoid air bubbles in the injection fluid (embolism).
- The injection fluid must be brought to room or body temperature prior to use. Injection of cold fluids is painful!
- Care must be taken when giving intraperitoneal injections, as there is a risk of damaging internal organs. To avoid the urinary bladder the injection should be given slightly off the midline. The needle should neither be inserted horizontally (between the skin and the abdominal wall) nor vertically (risk of causing damage to the kidney). The injection should be placed into the lower left quadrant of the abdomen. There is low risk of causing damage to the intestines (mainly due to their mobility).
- To reduce the risk of damage it is important to use a short needle.
- Some injection fluids can cause tissue irritation (for example, if the pH is too high or too low), and should therefore be administered after having been diluted with saline solution or sterile water. These substances should, by preference, be given intravenously, as they quickly become diluted in the blood. When using the intraperitoneal route, dilution also occurs, but there is a risk of peritonitis and/or invagination of the intestines.
- Administration of large amounts of fluid should be carried out intravenously (in large animals) but can also be given intraperitoneally or orally.

<table>
<thead>
<tr>
<th></th>
<th>Birds</th>
<th>Snakes</th>
<th>Tortoises</th>
<th>Frogs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subcutaneous</td>
<td>neck</td>
<td>—</td>
<td>limb</td>
<td>—</td>
</tr>
<tr>
<td>Intramuscular</td>
<td>pectoral muscle; hind limb muscles</td>
<td>dorsal muscles</td>
<td>hind limb muscles</td>
<td>—</td>
</tr>
<tr>
<td>Intraperitoneal</td>
<td>halfway sternum-cloaca</td>
<td>2/3 heart-cloaca</td>
<td>between tail and limbs</td>
<td>midline</td>
</tr>
<tr>
<td>Intravenous</td>
<td>hind limb veins; wing vein</td>
<td>intracardial ventral abdominal vein; jugular vein (anaesthetised)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Dorsal lymph sac</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 16-2
Sites of injection in birds, reptiles and amphibians
depending upon the experiment. Always inject slowly, especially when administering large volumes intravenously, to avoid pain and shock.

From the site of injection, drugs or other substances will be transported by the blood circulation to the target tissues. The rate of this process will depend upon the route of administration; it will be most rapid using intravenous injections and much slower when using oral administration.

**Special injection techniques.** If a compound has to act directly on the brain, the blood–brain barrier can be circumvented by injecting directly into the cerebrospinal fluid. The site for this injection is the lumbosacral area (between the last lumbar vertebra and the sacrum) or the cerebello-medullary cistern (between the skull and the first cervical vertebra). It is also possible to inject compounds directly into the brain (intracerebrally) under anaesthesia.

For the exact administration of drugs into specific sites of the brain, a stereotactic apparatus is necessary. The head of the anaesthetised animal is fixed in such a way that the required sites of the brain can be precisely indicated, using species specific co-ordinates (see page 331).

Compounds can also be administered directly into other specific locations of the body, for example, into a joint or into the trachea.

---

**Collection of body fluids**

**Collection of blood**

Blood samples can be obtained from various sites of the body, using a variety of methods: from the veins, from the arteries, or by puncturing the orbital vessels or cardiac puncture. When repeated blood sampling is required, the implantation of indwelling cannulae should be specifically considered. The choice of the method will depend upon several factors, such as the purpose of blood collection (arterial, venous or a mixture of the two), the duration and frequency of sampling and whether or not it is a terminal experiment. It is important to choose the method of blood collection whilst designing the experiment, because in some species (for example, the hamster), collection of a substantial amount of blood is only possible in the anaesthetised animal.

In small laboratory animal species, blood collection is usually done under anaesthesia, ensuring the immobilisation of the animal. In case blood is collected from a conscious animal, one should take into account that biochemical parameters may deviate as a result of stress induced by the procedure.
Vein puncture. For blood collection, veins are selected which lie close to the skin and which can easily be distended by pressure. The most commonly used vessels are the veins of the neck (the jugular vein), the thigh (the femoral vein), the cephalic vein on the dorsal side of the fore limb, the lateral tail veins (mice, rats) and the saphenous vein on the medial or lateral side of the hind limb (table 16-3). With regard to rabbits, the marginal ear vein is the most commonly chosen blood vessel.

When collecting blood, the hair covering the chosen area should be clipped or shaved, then swabbed with a suitable antiseptic. Having distended the vein, using pressure, the needle should then puncture the skin and be advanced into the vein. The blood can either be allowed to drip from the needle directly into sample tubes, or a syringe or vacuum tube can be used, but the latter only in larger veins. Before removing the needle the pressure on the vessel should be released. Haemostasis is achieved by applying gentle manual pressure.

Puncture of the orbital blood vessels. In small rodents the veins are both small and often difficult to reach. Therefore blood sampling from the orbital blood vessels is sometimes used as an alternative. For this procedure the anaesthetised animal is firmly held by the skin at the nape of the neck. This causes distention of the jugular vein. A fine glass tube or a Pasteur’s pipette is then placed at the inner canthus of the eye and gently advanced alongside the globe into the vessels. The tube ruptures the vessels and blood can be withdrawn by capillary action. Contamination with tissue fluids and porphyrins from the Harderian gland can occur. It is not possible to take sterile blood samples using this method. Also, complications such as haemorrhage, inflammation and blindness may occur, especially when the same eye is used repeatedly. Moreover, this technique may be aesthetically unpleasant for the operator to perform. For these reasons this method is not considered acceptable in some countries.

Cardiac puncture. In this method, blood is collected directly from the ventricle of the heart of the anaesthetised animal. Puncturing the atrium can be dangerous, due to the risk of leakage into the pericardium, resulting in cardiac arrest and death. When the animal has to survive the procedure, these risks have to be taken into account.

Blood collection from arteries. In order to obtain oxygenated blood, arteries can be punctured or cannulated. For this purpose, the femoral artery (thigh) or the carotids (neck) are frequently used. In rabbits, the central ear artery is most suitable.

Collection from the tail. In small rodents, like mouse and rat, small volumes of blood, e.g. for a blood smear, may be obtained by snipping off the tip of the
Table 16-3
Collection of blood: site and maximum volumes

<table>
<thead>
<tr>
<th></th>
<th>Mouse</th>
<th>Hamster</th>
<th>Rat</th>
<th>Guinea pig</th>
<th>Rabbit</th>
<th>Cat</th>
<th>Dog</th>
<th>Primate</th>
<th>Pig</th>
<th>Sheep</th>
<th>Horse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jugular vein</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Vein fore limbs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vein hind limbs</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Femoral vein</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ear vein</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Tail vein</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Orbital puncture</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Cardiac puncture</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tail tip</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Max. vol. (ml)</td>
<td>0.3</td>
<td>0.3</td>
<td>2.0</td>
<td>5.0</td>
<td>15</td>
<td>20</td>
<td>20</td>
<td>100–500</td>
<td>200–500</td>
<td>200–600</td>
<td>500–700</td>
</tr>
</tbody>
</table>

1Depends on body weight (see text).
Experimental procedures

tail. In the rat this method can only be considered acceptable when performed under general anaesthesia. Afterwards, the wound should be cauterised. In situations where anaesthesia is contra-indicated, puncturing of the tail vein can be useful. The tail must first be warmed in order to dilate the vessels. The animal should be placed into a restraining device, after which a “butterfly” needle (25G) is introduced into one of the tail veins and blood can then be dripped directly into a sample tube. If a vein is punctured percutaneously (through the skin), contamination with tissue fluids may occur, which can influence the outcome of the experiments. If the experiment requires sterile blood, then surgical exposure of the vein under anaesthesia may be necessary.

Collection site in other species. In birds, blood samples can be obtained by cardiac puncture or by a small incision in the comb. The wing vein, the limb vein or the jugular vein can also be used. In tortoises and snakes, cardiac puncture or puncture of the jugular vein can be performed under anaesthesia.

Maximum volumes. The total volume of blood in a living animal is rather constant and is about 8% of its body weight. If the sample volume exceeds 10% of the total blood volume, hypovolaemia and cardiovascular failure (“shock”) may occur. As a general rule, a maximum of approximately 8 ml/kg bwt can be removed once a fortnight (table 16-3).

Implantation of indwelling cannulae. For blood collection, the same size needles can be used as for intravenous administration of drugs. In large animals, trocars (large hollow needles containing a sharp metal wire) can be used. Sometimes repeated blood sampling is required. In larger animals (the size of the rabbit or larger), it is possible to perform vein punctures daily. In small rodents, an indwelling cannula can be implanted via the jugular vein into the cranial vena cava or via the femoral vein into the caudal vena cava. The catheter should be looped under the skin of the neck or the back, respectively, exteriorised at the top of the head and secured there with screws and acrylic glue. The catheter’s dead space is usually filled with polyvinylpyrrolidone, in saline with heparin (PVP-solution). It is important to perform these procedures under aseptic conditions.

Exsanguination. To obtain the maximum amount of blood, decapitation can be performed using a guillotine or scissors, or by puncturing the aorta under anaesthesia. In this way about 30 ml/kg bwt or up to 50% of the total blood volume can be obtained. In mice, exsanguination is also possible by removing the eyeball under anaesthesia and collecting blood from the eye artery, followed by dislocation of the cervical vertebrae.
Collection of body fluids

Collection of faeces and urine

Metabolic cages (fig. 16-3). Metabolic cages must be used for the quantitative collection of excreta, such as urine and faeces. The animals are housed on a grid above a funnel in which the urine and/or faeces are collected and separated.

Mice and rats often urinate and/or defecate purely as a result of being picked up, which may provide the opportunity for collecting small samples of urine and faeces.

Catheterization. In several species, it is possible to collect urine by urethral catheterization. A catheter is introduced into the urethra and aseptically ad-
Experimental procedures

vanced up into the bladder. In male animals this procedure is relatively simple
due to the fact that the urethra terminates in the penis; however, it is neces-
sary to use sedation in some species e.g. the cat.

In the majority of female mammals the urethra opens into the vagina and is
not visible. In the mouse, the rat, the hamster and the guinea pig, however, the
urethra and the vaginal orifice are completely separate. The urethral orifice is
located ventrally from the vaginal orifice. If catheterization has to be per-
formed in female animals, very detailed knowledge of the anatomy is re-
quired, together with well developed technical skills.

The outer diameter of the catheter used will depend upon the species (for
example, for a guinea pig: 0.5 mm, for a dog: 3 mm).

Collection of other body fluids

Other body fluids which are sometimes collected are cerebrospinal fluid
(liquor), bile, lymph or ascites fluid.

Liquor. The collection of liquor can be achieved at two different places: by
puncturing the cerebello-medullary cistern between the skull and the first
cervical vertebra (atlas) or from the lumbosacral space between the last lum-
bar vertebra and the sacrum. The dura mater should be punctured between the
vertebrae with a cannulae containing a mandrin. The mandrin should then be
removed and cerebrospinal fluid can be collected using suction. The proce-
dure is generally carried out under sedation or local anaesthesia.

Bile. For the collection of bile, the abdomen of the anaesthetised animal is
opened and a cannulae is introduced into the bile duct (ductus choledochus).
The bile duct runs from the portal area of the liver to the duodenum. This
procedure is generally part of a terminal experiment, as no bile passes to the
intestines after cannulation and therefore digestion of food will be impaired.
For chronic experiments, a loop can be made in the bile ducts or two cannulas
can be inserted into the bile duct, one in the direction of the liver and one
toward the gut. Both cannulas can be connected and tunneled subcutane-
ously to emerge at the crown of the head. In this way the enterohepatic
circulation is restored.

Lymph. For the collection of lymph the thoracic duct, which lies ventral to the
spine between the vertebrae and the aorta, can be cannulated.

Ascites fluid. The implantation of hybridomas into the peritoneal cavity of
mice and rats makes it possible to obtain monoclonal antibodies. The animals
will produce peritoneal fluid (ascites), into which the monoclonal antibodies
are secreted. The ascites can then be collected and the monoclonal antibodies purified. The amount of ascites should not exceed 20% of the bodyweight. The collection of ascites through puncturing should preferably be performed under general anaesthesia. It is not advisable to puncture more than once, as haemorrhages or peritonitis can result. It should be mentioned that this technique may cause severe discomfort to the animal. In vitro techniques are available and must be used whenever possible.

**Surgical procedures**

**Introduction**

Surgical procedures may be carried out on laboratory animals for various reasons, e.g. for the teaching of technical skills, the testing of surgical techniques or materials, or for obtaining a (patho)physiological model (such as a renal artery stenosis for the induction of a hypertensive model, or a partial hepatectomy for the induction of a regeneration model).

In recent years, a distinction is often made between macro- and microsurgery. The term microsurgery refers to surgery which is performed under an operation microscope. This development has facilitated organ transplantations even within small rodents e.g. the rat.

The performance of surgical procedures requires specific skills which can only be gained by training under experienced supervision in a well-equipped laboratory. Specific knowledge with reference to anaesthesiology and anatomy is a prerequisite.

**Hygiene and asepsis**

Every surgical procedure brings with it the risk of infection. Attention must be paid to asepsis (prevention of contamination through working under sterile conditions) and also, where necessary, to antisepsis (prevention of infections by using antibiotics, for example). The operating room, the animal, the surgeon, the instruments and materials/solution are all possible sources of contamination. The risk of infection can be minimized by taking adequate precautions. Work should be carried out in clean rooms. After having removed the hair – not in the operating theatre – the incision area should be prepared with antiseptics such as 70% ethyl alcohol and/or iodine solution. Sterile drapes and instruments should be used along with sterile clothing i.e. gowns, gloves, face masks, caps etc. In practice, these precautions are usually not fully applied when using small rodents, although it has never been substantiated that these animals can resist surgically induced infection better than other ani-
mals. This implies that, for small rodents, it is also advisable to work under aseptic conditions and, if necessary, in combination with antibiotic prophylaxis before surgery.

**Instruments (fig 16-4)**

A great variety of surgical instruments is available. A basic set of instruments would consist of: a scalpel, straight or curved anatomical forceps, straight or curved surgical forceps, ring-handled preparation scissors (sharp/sharp and sharp/blunt), towel clips, artery forceps, a needle holder, needles, skin clamps and a skin clamp applicator or suture material. Some procedures will require specific instruments in addition to those mentioned, for example, a wound holder.

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**Fig. 16-4.** Overview of a basic set of surgical instruments. 1 = ring handled scissors (straight and curved, blunt/blunt, blunt/sharp, sharp/sharp); 2 = preparation scissors; 3 = skin clamp applicator and remover; 4 = wound holder; 5 = towel clip; 6 = scalpel, holder and blade; 7 = serrated tip forceps (Pean); 8 = needles; 9 = needle holder; 10 = anatomical forceps (curved and straight); 11 = surgical forceps; 12 = bulldog haemostatic clamp; 13 = rat-toothed tip forceps (Kocher).
Specific knowledge of the anatomy of the blood vessels is a prerequisite for successful surgery. Some bleeding during surgery is inevitable, but severe loss of blood can be prevented by blunt dissection or by avoiding sanguinary tissues. For example, less bleeding will occur when using the linea alba instead of an incision through the abdominal muscles when performing abdominal surgery. When employing blunt dissection, the closed scissors are brought into the tissues and then opened, thereby tearing the tissue from the blood vessels. Haemostasis can be aided by electrocautery, or by ligating the blood vessel with thread, or with clamps (liga-clamps).

Suturing

The closing of the incision at the end of a surgical procedure generally requires the restoration of the original anatomical situation. The various tissue layers which were separated have to be sutured. For example, in the case of an incision into the abdominal wall, the peritoneum, the abdominal muscles, the subcutaneous tissue and the skin must be sutured (fig. 16-5). Sometimes two layers are sutured simultaneously e.g. the peritoneum with the abdominal muscles and the skin with the subcutaneous tissue. It is vital that no subcutaneous cavity develops, in which germs can multiply, thus causing inflammations.

The most basic stitches are shown in fig. 16-6: the interrupted stitch and the continuous stitch. The continuous stitch can be executed quicker than the interrupted stitch but, if the suture breaks, the wound can unravel completely and therefore this method is not recommended for larger wounds. A variant of the interrupted stitch is the U-stitch, which allows both inversion and eversion of the wound edges. Inverted stitches are used when closing the urinary bladder and the uterus; the mucosal layer is not pierced (risk of leakage), only

Fig. 16-5. Section through the skin and the abdominal wall. The incision should preferably be made along the midline (linea alba). 1 = skin; 2 = subcutis; 3 = m. obliquus abdominis ext.; 4 = m. obliquus abdominis int.; 5 = m. transversus abdominis; 6 = peritoneum; 7 = m. rectus abdominis.
Experimental procedures

The muscle layer and the serosa. Everted stitches are used for the skin, as they minimise the risk of contamination of the wound. Stitches should not be applied too tightly, since this causes local tissue damage and necrosis. A certain degree of swelling of the wound can be expected to occur under normal conditions.

Suture material can be either absorbable or non-absorbable. Absorbable material can be made from catgut, vicryl or dexon, which vary in absorption rate. Non-absorbable material can be cotton, silk, mersilene, nylon or stainless steel.

Absorbable material is not as strong as non-absorbable material, and is more expensive.

Non-absorbable suture material, however, has the disadvantage that it can cause fistulas when remaining within the body. Threads can be made by one fibre (monofil) or plaited by several fibres (polyfil). Suture material is available in various diameters, from 5 to 12-0 USP (= U.S. Pharmacopea, where thread diameters are described, 5 being the thickest and 12-0 the thinnest). The chosen diameter will depend upon the tissue and the tension.

Absorbable suture materials should be used, unless the suture is used to fixate a cannula or a specific structure that has to remain in position over a longer period of time.

Needles with eyes have to be threaded manually and the needle eye can cause tissue damage. A system which causes less damage to the tissue is where the needle is bonded to the thread (atraumatic).

Needles can be obtained in a variety of curved or straight shapes and sizes. Their tips are, as a rule, either round or triangular. The latter, having cutting edges, are generally used for piercing through tough tissue such as skin. Round-tipped needles are used for soft tissue, such as the intestines.

Having placed the stitches, the suture has to be knotted (fig. 16-7). One
Surgical procedures

Such possibility is a surgeon’s knot, in which the first half hitch contains an extra twist to prevent slipping, for example, for tissues under tension. A reef knot can also be used, for example, for skin. A granny knot should not be used, because it comes undone easily. Skin wounds can be closed using metal clamps, using application forceps or by a subcutaneous continuous stitch of absorbable material. By suturing in this way, the stitch cannot be removed by biting or scratching.

Wound treatment

The wound requires daily inspection. A drain which has been placed into the wound, can be removed after 2–3 days. Draining the wound may be necessary if the wound has been contaminated, or when a quantity of exudate is expected.

A wound which is closed properly and heals normally, requires no special treatment. Post-operative infections should be treated with antibiotics.

It is advisable to allow an infected wound to heal per secundam by forming scabs, whilst treating them with saline and mild disinfectants (Hibitane®, Betadin®).

Post-operative care

If the animal has lost much blood and/or fluids during an operation, then fluid therapy may be necessary. Small laboratory animals should be treated after an operation with a warm saline solution given subcutaneously to prevent dehydration, depending on the duration of the procedure.

Hypothermia must be prevented. This can be achieved by the use of heating aids, such as aluminium foil or cotton wool placed over the body, or with lamps or heating pads.
Experimental procedures

The wound should be inspected daily. If the animal is trying to remove the stitches, dressings or an Elizabethan collar should be applied. Sutures or skin clamps should be removed after 7–10 days.

Post-operative analgesia. See chapter 15.

Invasive techniques

Ectomies. One of the purposes of surgical procedures is the creation of a specific animal model. An important technique used to achieve this is the ectomy. Ectomy (ectomein: to cut out) means the removal of organs or parts of organs. The reason for carrying out this procedure may be the study of the effect of the removal of certain hormones. The most frequently used ectomies in laboratory animals involve the endocrine and the immune system.

The endocrine system
– hypophysectomy (hypophysis): the central regulation of the endocrine system is removed.
– (para)thyroidectomy ((para)thyroid glands): these glands are located in the neck region lateroventral to the larynx); it is difficult to remove these separately.
– pancreatectomy (pancreas)
– adrenalectomy (adrenals)
– gonadectomy (gonads)

The immune system
– thymectomy (thymus; located centrally in the thoracic cavity): not used very often any more, since the development of genetically thymus-deficient animals, the so-called “nude mouse” or “nude rat”
– lymphadenectomy (lymph nodes)
– splenectomy (spleen).

Other ectomies
– hepatectomy (liver): generally one lobe is removed, a so-called partial hepatectomy. To reach the liver, located in the cranial part of the abdominal cavity, a laparotomy is necessary.
– nephrectomy (kidney)
– hysterectomy (uterus)
– removal of parts of the brain

Fistulas. Fistulas are artificial orifices within the body (stomata), which open into certain parts of the gastrointestinal tract, the gall bladder or the urinary
Surgical procedures

bladder. Fistulas to the gastrointestinal tract can be used in studies of the digestion of nutrients, together with studies of secretory products from the gastrointestinal tract.

Transplantations. Formerly, the most frequently performed transplantation was (partial) skin transplantation for genetic quality monitoring of inbred strains. At present, other methods for controlling the genetic integrity of inbred strains are increasingly being used (chapter 7). Transplantation of lungs, heart, liver, kidney and pancreas can also be performed in laboratory animals. Due to the development of microsurgery, these techniques can be carried out in animals as small as the rat. The purpose of these procedures is to study reject reactions, and to test medications which can prevent the rejection.

Implantations. Implantation means the introduction of material or tissues into an animal. The implantation of tumour material is rather common, especially in the “nude mouse”, into which human tumour material is often brought. This technique is carried out frequently under the skin, but can also be performed in other areas, for example, under the capsule of the kidney or liver, or in the cheek pouch of the hamster.

Shunts. Shunts are connections between blood vessels, which are generally made between arteries and veins e.g. the arterio-venous shunt between the carotid artery and the jugular vein. Shunts between veins can, however, also be made, such as a portocaval shunt between the portal vein and the caudal vena cava. Blood coming from the intestines will not reach the liver, but will pass directly to the caudal vena cava.

Stereotactical procedures

In brain research, stereotactical techniques are commonly employed for the application of unipolar or bipolar needle electrodes into several areas of the brain. Through these electrodes, potentials can be diverted or current pulses can be administered. The same techniques can be used to introduce thin cannulae into the brain, through which small amounts of drugs can be administered which will either stimulate or inhibit the function of some areas of the brain.

For the application of instruments into special areas of the brain, a stereotactic atlas and a stereotactic apparatus are needed. These use a three dimensional classification of the brain, derived from the bony skull. There are three distinguishable planes:
- a horizontal plane which passes through the centre of the bony outer ear
and the rim of the orbit (in the mouse, rat and guinea pig this is the maxillar rim between the incisors)

– a frontal plane which passes through the centre of the bony outer ear and runs perpendicular to the horizontal plane

– a sagittal plane which passes through the median of the skull and runs perpendicular to the horizontal plane.

Using the stereotactic apparatus and the co-ordinates from the atlas, electrodes or cannulas can be implanted into specific defined areas of the brain.

**Perfusions**

Perfusion refers to the act of flushing the body or an organ with fluids. The blood is removed from the body and/or the organ and replaced with perfusion fluid. The animal will not survive such an experiment unless just one organ is perfused (e.g. isolated, perfused kidney). The arteries and the veins of the organ (in the liver also the portal vein) are freed from the surrounding tissue in the anaesthetised animal. A cannula is introduced into the artery and fixed using a ligature. After cutting through the veins and the arteries, the organ is removed from the body and the cannula is connected to the perfusion fluid. The organ can now be studied extracorporally for a while, or cells can be isolated for an *in vitro* cell culture.

**Biotelemetry**

Biotelemetry is a low stress method to measure physiological parameters such as heart rate, electrocardiogram, blood pressure and body temperature in freely moving laboratory animals. An implantable transmitter is placed in the peritoneal cavity of the animal under anaesthesia. The transmitter is equipped with flexible leads for measuring heart rate and electrocardiogram. The device sends a frequency-modulated signal to the receiver underneath the animal cage. Through a data acquisition system, the raw data are converted and stored in the computer.

**Imaging techniques**

Radiological studies are frequently performed upon laboratory animals. This requires knowledge regarding adequate anaesthesia and cannulation techniques. When contrast fluid has been introduced through the cannulae, the blood flow through the organ can be studied.
Imaging techniques

Scanning techniques are used for the study of organ transplants and for tumour growth. For this technique, special radioactively labelled substances are injected which attach themselves to the transplanted tissue or tumour material. The volume of the transplantant or tumour can be estimated by a scanner. In a CT-scan (computer tomography) the absorption of photons is used. With NMR (nuclear magnetic resonance) a superior tissue differentiation can be achieved. NMR is a technique whereby differences in magnetic quality of compounds within different tissues become visible and result in an image of the tissue. The signal is derived from protons of the compounds present in the body.

Literature

Experimental procedures
17 Reduction and replacement concepts in animal experimentation

J. Nab, M. Balls, J. B. F. van der Valk and C. F. M. Hendriksen

Introduction

The concept of alternative methods has already been discussed in chapter 1. To summarize, every method or procedure that results in the replacement of an animal experiment, or the reduction of the number of animals required, or the refinement of procedures so that animal suffering is reduced, is considered to be an alternative method. This definition corresponds to the “Three Rs” of Russell and Burch.

Although widespread acceptance of this concept of alternative methods is relatively recent, several methods are already in existence, which have resulted in the replacement, reduction or refinement of animal use.

The introduction of anaesthetics was very significant for the refinement of animal experiments. Ether was the first anaesthetic to be administered to a human patient, in 1846. Soon afterwards the same substance was also used in animal experiments. In Great Britain, the use of anaesthetics was made compulsory for painful experiments in 1876, on the introduction of the Cruelty to Animals Act.

Tissue culture is another alternative approach that has a long history. In 1885, Wilhelm Roux succeeded in keeping cells from a chicken embryo alive in a warm saline solution. Growth of cells in vitro (literally: in glass), was described for the first time in 1907 by Ross Harrison. The cell culture technique expanded rapidly after the introduction of antibiotics. The addition of antibiotics to the culture medium opened up the possibility of culturing cells under relatively simple conditions. Recent developments in this field are specifically related to the standardization of cell culturing techniques. Synthetic culture fluids are of a major interest, as they can replace serum factors such as hormones, which can vary considerably with regard to quality.
Nowadays alternative methods are being applied in almost every part of biomedical and veterinary research and testing, and in education.

The impetus for the development of alternative methods may, to a large extent be ethical, but other factors also play a role. The use of laboratory animals is expensive, and the performance of animal experiments is time-consuming and often difficult to standardize. Alternative methods which allow the replacement of the whole animal are usually less complex and the experimental conditions are easier to standardize. However, it should be pointed out that the simplicity of an alternative may be its strength, especially when mechanisms at the organ/tissue/cell level are under investigation; but can also be its weakness, due to the fact that the response in a simplified system may differ from the response of the organism as a whole. This could be a drawback, just as species differences limit the application to man of data obtained in animal experiments.

In previous chapters it was shown that the performance of research in a technically and scientifically responsible way implicates refinement of animal experiments and a reduction of animal numbers. Research in a responsible way starts with a literature search. The reliability of the experiment and the number of animals required is also influenced by the microbiological status of the animals, the use of the adequate animal model, anaesthesia, housing and other aspects. In this chapter the alternatives most-commonly in use will be described. Next, there will be an elucidation of the tiered approach, and of alternatives in education.

In addition, attention will be given to the storage, exchange and use of experimental data, and to the effects of alternative methods on animal use statistics.

**In vitro techniques**

*In vitro* methods are, at present, the most important category of alternative methods to animal experimentation. Not every *in vitro* method can be regarded as an alternative model. In some research areas, *in vitro* methods are inextricably bound up with the type of research. In these situations the use of *in vitro* methods does not have an effect on the use of experimental animals and can therefore not be regarded as an alternative to animal experiments.

*In vitro* methods cover research on cell organelles, cells, tissues and organs. The term 'tissue culture' encompasses a broad collection of techniques, which are used to keep cells, tissues, organs or parts of organs alive outside the body in a nutrient culture medium, for at least 24 hours. An environment is created for the living cell or (piece of an) organ, which resembles, as far as possible or needed, the normal physiological conditions found *in vivo*. 
Tissue cultures can be divided into two main categories: organotypic cultures and cell cultures. Organotypic cultures consist of parts of a tissue or organ, or sometimes an intact organ, which have been placed into culture medium. The aim of organotypic culture is to maintain the structural and functional relationships between the cells and tissues of the organ in question.

However, the compact three-dimensional structure of organotypic cultures prevents nutrients from reaching all the cells and the removal of waste products. This means that organotypic cultures usually have only a limited life span. When employing this technique, fresh material from animals or humans is required for each experiment.

Nowadays, modern tissue culturing techniques enable the reconstruction of organs. For instance, reconstructed skin is being applied as a means to extend our knowledge on biological processes in the skin, but also as an alternative to animal experiments in, for instance, skin corrosivity and irritancy screening. After dissociation of the different skin components the cells are cultured to obtain a large number of cells. A three-dimensional skin can be reconstructed by seeding selected cells on a suitable substrate under appropriate conditions.

Cell cultures can be distinguished from organotypic cultures, due to the fact that the connection between the individual cells is disrupted either enzymatically or mechanically. When dispersed cells are placed in culture medium, this is known as a primary cell culture. When the cells are cultured for more than one generation, they are called secondary or serial cell cultures. Depending upon the type of cell and the technique used, cells can be kept as a monolayer or as a cell suspension. The lifetime of primary cell cultures is limited. In some cases, cells can be immortalised by spontaneous or engineered transformation. These so-called cell lines or continuous cell cultures retain the potential to divide ‘for ever’ if treated appropriately. By exposing certain kinds of cells to appropriate physical or chemical stimuli, they are stimulated to differentiate. During differentiation, the cells regain more or less their original properties, or in the case of stem cells, develop the characteristics they would normally develop in vivo.

Cell lines are a homogenous and reliable substrate and their application decreases the reliance on animals as cell donors. The culturing of cells usually results in the loss of morphological and biochemical properties, resulting in a progressive divergence from features of the original cell type. For that reason, cells with a low in vitro passage number are stored in nitrogen for later use. The use of cell lines can be improved by applying molecular biological techniques. Genetically modified cells may carry a specific new feature, such as a human receptor. In this way these cells may become a useful model for receptor-binding studies in the screening of pharmaceuticals.

Cell cultures are frequently used in vaccine production; for example, the poliomyelitis vaccine is commonly produced from kidney cells of the mon-
Reduction and replacement concepts in animal experimentation

(key (*Macaca fascicularis*)). The effect of technical improvements of the *in vitro* production of this vaccine on the number of monkeys required in The Netherlands is shown in table 17-1.

*In vitro* studies offer several advantages. They are generally more sensitive than *in vivo* studies, since the isolated cells and organs receive no input from other body parts. The models can be grown under well-defined conditions and there is no need to apply analgesia or anaesthesia, which in an *in vivo* experiment may also affect the results of the study. Dependent on the type of research, the interpretation of results of *in vitro* studies is usually easier, although the extrapolation to a complete living organism may be more difficult. To decrease the extrapolation step, computer programs evaluating the biokinetic properties of a substance are being developed. Also, hepatocyte cultures to study metabolites are applied. To further improve the extrapolation to the human situation there is a tendency to use human tissues for research.

A point for consideration is the fact that isolated cells do not live in their natural environment and therefore may behave differently. In particular, cell lines, which usually are chromosomally aberrant, may have different properties compared to their chromosomally normal healthy ancestors.

Use of lower species

There are some situations where the use of lower organisms, such as bacteria, fungi, insects, or molluscs, can reduce the required number of vertebrate

Table 17-1

Poliomyelitis vaccine production: the impact of changes in methodology on the number of monkeys used in The Netherlands

<table>
<thead>
<tr>
<th>Year</th>
<th>Technique</th>
<th>Number of monkeys per year</th>
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<tbody>
<tr>
<td>1965</td>
<td>– use of imported monkeys</td>
<td>4570</td>
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<tr>
<td></td>
<td>– dissociation of cells by <em>in vivo</em> trypsinization of kidneys</td>
<td></td>
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<tr>
<td></td>
<td>– culturing of cells in monolayers</td>
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<tr>
<td>1970</td>
<td>– replacement of monolayers by culturing on Sephadex microcarriers</td>
<td>1590</td>
</tr>
<tr>
<td>1975</td>
<td>– replacement of imported monkeys by captive bred monkeys</td>
<td>463</td>
</tr>
<tr>
<td>1980</td>
<td>– replacement of primary cell cultures by tertiary cultures</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>– replacement of <em>in vivo</em> trypsinization by <em>in vitro</em> trypsinization</td>
<td></td>
</tr>
<tr>
<td></td>
<td>– improvement of perfusion technique</td>
<td>20</td>
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<tr>
<td>Near future</td>
<td>– use of cell lines instead of cell cultures</td>
<td>0</td>
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</table>
Use of lower species

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animals. One example of this is the use of bacteria in the Ames test, to screen new compounds for mutagenic properties, which are believed to be one of the causes of carcinogenesis. Micro-organisms such as yeast are used on a large scale as vectors for the expression of specific genes, that code for antibody fragments or even vaccine antigens. By transgenesis, plants can also be made suitable for vaccine production.

Another example of the use of lower organisms as an alternative method is the Limulus amoebocyte lysate (LAL) test, which is used for pyrogenicity testing. Until recently, rabbits were used exclusively for this test, which is requested for all materials that will be administered parenterally. If there is a certain increase of body temperature after the intravenous administration of the test material, it is considered to be pyrogenic. In the LAL test, the amoebocyte lysate is extracted from blood of Limulus polyphemus (the horseshoe crab). This lysate is converted into a gel by endotoxin, which is the most important pyrogenic compound.

Despite the fact that, for a number of products, the LAL test cannot be applied for technical reasons, the introduction of the LAL test has greatly reduced the number of rabbits used for this purpose. Nevertheless, it should be realized that ethical objections, which are voiced against the use of vertebrates may be equally applicable to some classes of invertebrates.

Immunological techniques

Immunological techniques form the basis for a number of in vitro methods. Their application is especially useful in diagnostic testing, vaccine quality control and fundamental immunological research. Well-known techniques in this area include enzyme linked immunosorbent assays (ELISAs), the haemagglutination test and the radioimmuno assays (RIAs). These in vitro assays are very sensitive, but in some cases they lack specificity, i.e. in the ability to distinguish between related antigens or antibodies, so an animal test is still necessary.

In 1975, Köhler and Milstein made a special contribution to the improvement of the specificity of antibodies. They succeeded in producing hybridoma cells by fusing antibody-producing lymphocytes and a certain type of long-lived lymphoid cell-derived, cancer cells, namely myeloma cells. After selection and cloning, each hybridoma cell clone is able to produce an antigen-specific antibody (a monoclonal antibody).

Until recently, many animals, particularly mice, were used for the production of monoclonal antibodies. These animals were injected intraperitoneally with hybridoma cells. After 10–14 days, the ascites fluid containing the monoclonal antibodies could be collected. This technique causes substantial
suffering to the animals, due to the fact that the ascites fluid increases pressure on the organs in the abdomen and in the thoracic cavity (fig. 17-1). This \textit{in vivo} production can now be replaced by several \textit{in vitro} techniques, for example by culturing hybridoma cells \textit{in vitro} in fermentation systems and/or hollow fibre systems (fig. 17-2). Through recent improvements of the \textit{in vitro} culture systems, monoclonal antibodies can now be produced at quantities and costs that are competitive with those of the \textit{in vivo}-produced monoclonal antibodies. Therefore a number of European countries (e.g. the Netherlands, Switzerland, United Kingdom) have issued guidelines to restrict the use of animals for production purposes of monoclonal antibodies to only very few exceptional cases.

**Physical-chemical methods**

An overview of alternatives in biomedical research is not complete without considering physical-chemical methods. These methods are used, among others, to unravel the composition of complex chemical mixtures. HPLC (High Pressure Liquid Chromatography) is one of the most prominent examples. A field of testing where this method has resulted in replacement of the use of laboratory animals is the statutory quality control of hormones. Until very recently, the potency testing of products such as insulin, calcitonin and oxytocin, required animal models, and large numbers of animals were used for these purposes. Now, most of the naturally derived hormones are, or will shortly be, produced by rDNA techniques, resulting in better-defined and purer products. This has enabled the introduction of HPLC, which is now the officially required technique for potency testing of some hormone preparations.

**Mathematical models and computer modelling**

It is well known that relationships exist between molecular structure, the physical-chemical properties and the biological activities of compounds. Using this knowledge, it is possible to predict the biological activities of many types of new compounds, including their toxicity, or to improve the effectiveness of drugs, by introducing minor changes into their molecular structures. This method can also be used to select and prioritise when analysing a series of related compounds. An initial screening can be made, ensuring that only a small number of drugs, selected from a much larger number of potential candidate compounds, are tested on animals.

For the development of new drugs, profound and detailed knowledge is
Fig. 17-1. *In vivo* production of monoclonal antibodies by means of ascites in the mouse.

Fig. 17-2. *In vitro* production of monoclonal antibodies in a hollow fibre system.
vital, such as knowledge of the three-dimensional structure of the receptor or about the biological processes that are involved in its effects. Specific groups of atoms, the placing of groups within the molecule, electron charges, etc., may all contribute to the biological activity of the drug and therefore must be understood and taken into account.

Biological activity can only be determined by the interaction of compounds with living biological systems, by (animal) research and human experience when working with known substances, although in vitro studies are making an increasing contribution. The computer can then be used to design new compounds with the required structures and properties. This technique is called computer aided drug design, or rational drug design, and it has been applied in the development of e.g. AIDS medicines.

This phase in drug discovery must always be followed by in vitro tests and animal experiments. However by using computer aided drug design the number of compounds, which need to be tested on animals, can be reduced by a preliminary selection on the basis of desired biological activity.

Many of the processes, which take place in living organisms, can be expressed as mathematical equations, meaning that mathematical models of many physiological, biochemical, pathological and toxicological events can be developed. In most cases, these models are produced and used on computers, so they are known as computer models or computer simulations.

Physiologically based pharmacokinetic (PBPK) modelling can make predictions of the absorption, distribution, metabolism and excretion of a drug in the body, based on the physiological parameters of the organism, the physical-chemical properties of the drug, and its potential for metabolism. In this way a prediction can be made of the possible exposure of body tissues to the drug, and also its likely effectiveness and toxicity.

**Human models**

Most of the results that have been obtained through experimentation on animals will be extrapolated to man. This implies that the human being would be the best model for research and testing. However, there are ethical, legal and practical objections against the use of human models for this purpose. Nevertheless, there are more and more occasions when humans or their tissues can justifiably be used as test subjects.

Human material is increasingly being used in in vitro research. There is, for instance, great interest in human skin and liver models. Organotypic skin models have been developed from human skin tissue and are used for basic research and for testing. Human liver research can contribute to the development and testing of medicines, since great differences exist between
human and animal metabolism. Human blood is being used to screen for pyrogens (and methods currently undergoing validation may soon replace the rabbit test and the LAL test). When a pyrogenic substance is added to human blood, white blood cells start to produce certain types of cytokines. These cytokines can subsequently be measured with ELISA, bioassay or RNA-detection methods.

Unfortunately, human material is not available at all times, nor is it always available in sufficient quantities. The establishment of human tissue banks that could preserve tissues and could mediate between supply and demand, would facilitate the provision of human tissue for scientific research.

Human subjects can only be used as “guinea-pigs” in compliance with strict regulations. The risks involved must not outweigh the desired benefit to be achieved through the treatment. When performing clinical tests on healthy volunteers, only a minimal risk can be tolerated. There must not be any irreversible side effects from the treatment. Current modern techniques such as magnetic resonance imaging (MRI) and spectroscopy (MRS) enable non-invasive and safe studies to be conducted in humans.

Patients and healthy volunteers must be fully informed prior to the commencement of the experiment, and approval from a medical ethical committee must be attained.

When using human volunteers, the problem of inter-species extrapolation is avoided. Extrapolating data from animal experiments to man can cause problems due to the differences between man and animals, with respect to anatomy, physiology, metabolism, biokinetics, and pharmacological and toxicological responses. It is also possible that some effects, which may occur in humans, such as dizziness, changes in mood, etc., are either not detectable in the animal, or may occur at such a low frequency as not to be detected. By using human volunteers, these problems are to a great extent removed; the results only have to be extrapolated within the same species, but inter-individual human variation must still be taken into account.

**Telemetry**

Telemetry permits the continuous measurement of several parameters from freely moving animals. Animals are internally equipped with miniaturised measuring devices connected to a transmitter enabling a wireless readout of data. Devices have been developed to measure body temperature, blood pressure, heart rate, electrocardiogram, etc. Telemetry permits continuous measurement, enabling longitudinal studies in one animal without disturbing it, thereby avoiding stress artefacts. In addition to a reduction of animal numbers and suffering, telemetry allows for better research.
**Tiered approach**

It is not always possible to completely replace an animal test by only one new procedure. The alternative is generally a simplification of reality and cannot always be used for the whole spectrum of applications. For that reason, there are many alternatives being applied in stepwise testing schemes. On the basis of the results of these studies, it is decided whether and how studies will be continued. Further studies may be based on animal procedures or other alternative models. This approach leads to the appropriate use of one or more pragmatic steps. A simplified example for evaluation of the skin corrosive properties of chemicals is given in fig. 17.3.

At first, computer models are used to evaluate the physical-chemical properties of a substance. With a positive result, it can be decided to label the

![Diagram](image-url)
Tiered approach

A tiered approach for testing a substance as being corrosive. With a negative result, the pH value can be measured. With a pH above 11.5 or below 2.0, the substance is regarded as corrosive. If the pH is between 2.0 and 11.5, the substance is tested with a validated, non-animal alternative model. If the result is negative again, it is verified by testing in one animal. Next, the skin irritation properties of the substance will be tested, which can need to be followed by an eye irritation test. A stepwise approach can also be applied for these two tests.

This simplified example of a tiered approach uses three pre-screens before it might be necessary to perform an animal test, only to confirm its non-corrosiveness. As a result, both animal use and animal suffering can be reduced. Within the pharmaceutical industry, the application of this kind of approach has resulted in a reduction of 50% in the number of experimental animals.

Other alternative methods

In some cases, such as with demonstrations for teaching purposes, the use of laboratory animals can be replaced by using organs obtained from the slaughterhouse. Another example of this is the use of bovine eyes from the slaughterhouse to provide corneas for an in vitro eye irritation test, to replace the Draize eye test, one of the routine toxicity tests, which is performed on rabbits. Some parameters of the rabbit eye irritation test can also be simulated very well using isolated eyes of chickens from the slaughterhouse. Total replacement of the eye irritation test on rabbits will only be possible if the other aspects of the test (e.g. inflammation and recovery from damage) can also be modelled. In order to achieve this, the non-animal test will need to be extended by using other methods, such as the chorio-allantoic membrane (CAM) of embryonated chicken eggs and cell culture systems. Another application of slaughterhouse material is the use of pig ears in tests for skin irritation and skin permeability.

Storage, exchange and use of research data

In many cases, the decision whether or not to perform an animal experiment is based upon the results of previous animal experiments, as the hypothesis to be tested is deduced from earlier results. This implies that, as in other branches of science, easy access to research data is a prerequisite for progress. The unnecessary repetition of research that was performed earlier has little scientific value. If such unnecessary research is carried out on laboratory animals, this results in the needless use of animals. Thus, for the scientist, as well as for the animal, it is very important that relevant data are kept up to date and are readily available.
Scientific journals are the most important source of information, as these cover the latest results of research. Journals are a primary source of information. More and more of these journals become accessible in digital format. Information from congresses also belongs to this primary information category. Secondary source information can be obtained from other publications, such as books, symposia, review articles, reports, and on the Internet. Reference manuals, citation indexes and data books also belong to this category.

Apart from these two categories, there is also the “grey” literature, which consists of reports that are not formally published (lectures, international reports, government papers, etc.). Publications which are “in preparation” also belong to this group.

Digital information is becoming increasingly important. Rapid developments in computer technology have improved the storage, exchange and retrieval of such information. By using the Internet it is possible to have at one’s disposal the most current information, and to consult large databases very easily. Special databases have been set up in which all aspects of animal experiments and alternatives can be retrieved. One example is PREX, an online information service at Utrecht University, which contains databases for veterinary sciences and laboratory animal science. NORINA is an online database on alternatives in education. It is important that various types of information can be found at one single source. ALTWEB is such an example. On this Internet site, information can be retrieved on alternatives for animal experimentation, there are links to other information sources, and it contains digitised versions of workshop reports and journals on alternatives.

**Alternative methods in education**

Animal experiments for educational purposes can often be replaced by alternative methods. The processes of learning and development of skills are the ultimate goals of animal experiments in education, rather than the verification of scientific hypotheses. Therefore, animal experiments in education are very often repetitions of earlier experiments. The protocol for such an experiment is an exact repetition of an earlier experiment; and the result of the experiment is also the same, subject to some biological variation.

For that very reason, these experiments can be replaced relatively easily. The animal is here the learning tool. The way in which the animal experiment should be simulated depends upon the learning goals of the experiment: either illustration of theories or training of skills. Some specific alternatives are available for use in education, for example physical/chemical or three-dimensional models; preserved preparations; participation in research; audio-
Alternative methods in education

Visual materials; education by computer. Non-invasive techniques, that can be safely applied to the students themselves can also be a useful alternative. Audio-visual material is the most widely applied alternative method used for educational purposes, such as the teaching of facts, giving demonstrations and instructions, and for the development of ethical values. New computer technology has permitted the introduction of various alternatives in education, such as interactive learning programmes, simulations, digital video and websites. Many learning goals can be achieved with this technology: knowledge, insight and application. With multimedia, it is possible to demonstrate the results of an experiment, making it even more realistic. In the future, ‘virtual reality” seems to have unlimited prospects. The limitations of computer simulations for research purposes do not apply to education, as the model does not necessarily have to be complete. In education, the goal is to teach the basic principles of science, and a properly designed teaching programme based upon well-known facts can achieve this.

Animals are also used for the training of manual skills. For this purpose alternatives are not always available. Slaughterhouse material can be used for the training of the technique of laparoscopy, in combination with a device that simulates the blood flow.

An artificial rat has been developed for the training of microsurgical techniques.

Validation

During the last 20 years, particular emphasis has been given to the development of alternatives to animal testing. This has resulted in various kinds of procedures that, in principle, have the potential to replace, reduce and/or refine the use of animals. Whether an alternative method indeed is accepted and implemented depends to a large extend on the results of a formal validation study or evaluation. Validation is defined as the scientific establishment of the relevance and reliability of the procedure for a specific purpose. Relevance refers to the credibility of the model; that is its ability to correctly predict the test parameter of interest. So, an in vitro mutagenicity test should be able to discriminate between mutagenic and non-mutagenic chemicals. For verification of the relevance, results from the alternative procedure are often compared with those from the classical test, often called the “gold standard”. Reliability on the basis of such comparisons is usually characterised in terms of sensitivity, specificity, repeatability, precision and robustness. Reliability has to be assessed by applying the new procedure under precisely defined conditions in intra- and inter-laboratory studies.

Several international bodies, such as the WHO and the OECD, have issued
guidance on the validation of tests to be used for regulatory purposes. The guidelines of the European Centre for the Validation of Alternative Methods (ECVAM) of the European Commission are widely accepted. According to these guidelines, a validation study should be divided into several successive phases. The prevalidation phase aims to optimise the alternative model, to produce standard operating procedures to optimise the prediction model used, to convert the test results into a statement about toxicity in vivo, to facilitate the transferability of the method to other laboratories, and to evaluate the performance of the test with reference standard chemicals.

The next phase in the validation process is the formal validation study. In this phase, coded compounds are tested in a multi-laboratory study, and the results are independently received and analysed. The last step is an evaluation of the acceptability of the method and the drafting of a test guideline. A proposal is then made to regulatory authorities to consider the use of the alternative test for their purposes.

Results of pre-screening tests are not used for regulatory purposes (e.g. batch release by the National Control Authorities) but, for instance, for in-process control only. Therefore, an absolute confirmation of test relevance is less important. Contrary, in depth validation is a pre-requisite when the alternative procedure will be used for regulatory purposes, such as mandatory required toxicity tests or vaccine batch release tests. Generally, validation of these procedures is based on international collaborative studies, requiring the participation of many laboratories. Emphasis in these studies is given to intra- and inter-laboratory variation and to the test relevance.

It will be obvious that these collaborative studies are time consuming, extremely expensive, and logistically very complex. Problems related to validation studies are one of the main reasons why only few alternative procedures have been accepted so far by the regulatory bodies. Other reasons for the lack of acceptance are related to the animal model (the “golden standard”) against which the alternative procedure is validated. These animal models use different test parameters or are characterised by large variation, thereby complicating the demonstration of a good correlation.

Therefore, a sense of reality, both with regard to the conditions for a validation study and to the limitations of the “golden standard”, is a pre-requisite for the acceptance of alternative methods.

The effect of alternative methods on the use of laboratory animals

Since the beginning of the 1980s, the use of animals in experiments has been declining in most European countries. This can be put down to the synergistic effects of several factors: to improved legislation on the control and registra-
The effect of alternative methods on the use of laboratory animals

...tion of animal use, to increased efficiency, to the improved quality of laboratory animals, to economic factors, and to the greater emphasis placed on the ethical aspects of animal use, e.g. by the activities of the animal ethical committees. The use of alternative methods is one of the factors. A good example is the use of animals in education. In The Netherlands, a reduction of about 50% has been achieved, mainly because of the introduction of alternative methods.

A factor that is limiting the acceptance of some replacement alternatives might be their incompleteness, compared with the use of an intact organism. Physiological processes, such as absorption, biotransformation and excretion, can only be simulated to a certain degree in tissue cultures, and computer simulations are a simplification of reality. For these reasons, the possibility of extrapolating the results obtained from these systems, and applying them to humans, might be limited. When extrapolating information from laboratory animals to human beings, species-specific differences must be taken into account. However, when computer simulations, lower species or in vitro systems are used, the distance between the model and man is even greater. This means that an animal experiment must sometimes still be performed, in order to confirm the results obtained in the alternative tests. Considering the implementation of alternatives, a distinction should be made between fundamental and applied research. Generally, the implementation of three Rs methods in fundamental research can be relatively easy achieved as researchers are free to use the methods they prefer. Acceptance is usually based on in-house validation and publication in peer-reviewed journals. Applied research, and in particular regulatory testing, is based on sets of requirements and guidelines. Alternative methods are only used when accepted by the responsible international or supranational bodies, such as the Organisation for Economic Co-operation and Development (OECD). Acceptance is based on the results of extensive multi-laboratory validation studies. As a consequence, the implementation of three Rs methods in regulatory testing is a tedious and time-consuming process. Even after successful validation, the widespread implementation can be hindered by lack of harmonisation of test guidelines between the regulatory bodies. However, if the alternative method is accepted, reductions in the use of animals can be substantial as these tests are performed in a routine way.

What the effect of the increased use of transgenic animals in research will be on the total numbers of animals is still to be seen. The construction of a new transgenic strain generally requires large numbers of animals. These transgenic strains are used for an increasing number of purposes. As a consequence, total numbers of animals used in biomedical research tend to increase again. On the other hand, however, it might be possible by the use of transgenic animals to optimise scientific research, ultimately leading to a reduction in...
numbers of animals. In addition, transgenic techniques might allow the breeding of humanised animal models and thereby reduce the extrapolation-gap between the animal model and the human situation.

The future of alternative methods

Developments in tissue culture, computer technology, immunological techniques and physical-chemical methods as alternatives to animal experiments make fast progress. By application of DNA-chip technology (micro-array technique) in human tissue, insight can be gained in the involvement of genes in specific diseases. In this field the use of animals can partly be avoided. Ethical considerations are not the only reason for these developments, but could be the stimulus to exploit the use of alternatives in an optimal way.

It is expected that in the future animal experiments will still be necessary. However, the goals of the animal experiments may shift from primary research towards the verification of data from in vitro experiments. Besides that, verification research must always be performed on humans in the last phase of biomedical research.

The effect of alternative methods on the use of laboratory animals in the future is difficult to predict. Fewer animals will be needed for the solving of scientific issues, but on the other side alternatives may generate new questions that may have to be solved by means of animal experimentation.

Literature


Internet (relevant websites)

FRAME: http://www.frame-uk.demon.co.uk
Norina: http://oslovet.veths.no/norina/
PREX: http://prex.las.vet.uu.nl/
NCA: http://prex.las.vet.uu.nl/nca
ALTWEB: http://altweb.jhsph.edu/

Centres for alternatives to animal experimentation

Germany
Zentralstelle zur Erfassung und Bewertung von Ergänzungs- und Ersatzmethoden zum Tierversuch (ZEBET), Bundesgesundheitsamt, P.O. Box 33-00-13, 1000 Berlin 33, Germany.

Great Britain
Fund for the Replacement of Animals in Medical Experiments (FRAME), Eastgate House, 34 Stoney Street, Russel and Burch House, 96–98 North Sherwood Street, Nottingham NG1 4EE, Great Britain.
Reduction and replacement concepts in animal experimentation

Italy
European Centre for the Validation of Alternative Methods (ECVAM), Institute for Health and Consumer Protection, Joint Research Centre, European Commission, Ispra Site, T.P. 020, 21020 Ispra, Italy.

The Netherlands
National Centre for Alternatives to Animal Experiments (NCAD), Utrecht University, Yalelaan 17, De Uithof, 3584 CL Utrecht, The Netherlands.

Switzerland
Schweizerisches Institut für Alternativen zu Tierversuchen (SIAT), ETH-Zürich, Turnerstrasse 1, 8006 Zürich, Switzerland.

U.S.A.
The Johns Hopkins Center for Alternatives to Animal Testing (CAAT), Johns Hopkins School of Public Health, 615N Wolfe Street, Room 1604, Baltimore, MD 21205-2179, U.S.A.

Institute for In Vitro Sciences, Inc., 21 Firstfield Road, Suite 220, Gaithersburg, MD 20878, U.S.A.
18 Ethical aspects of animal experimentation

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Introduction

Since the publication of Peter Singer’s book “Animal Liberation” in 1975, there has been a continuous flow of books and essays concerning ethical aspects of animal experimentation. Singer’s main point is that the difference in the treatment of sentient non-human (higher) vertebrates compared with the treatment of (certain groups of) human beings, is something that cannot be consistently argued for. Accepting the proposition that animals can be used for experiments which are not admissible in human beings is, according to Singer, a matter of unjustifiable discrimination. He labelled this type of discrimination “speciesism” as an analogy to racism and sexism, which also discriminate on morally irrelevant grounds. Singer’s views have met substantial opposition from traditional ethicists who argue that direct obligations can only exist towards persons capable of being aware of the duties and obligations involved, and who are therefore capable of reciprocation. All human beings are considered to be potentially similar and thus should be treated in the same way. In contrast, animals are not morally conscious beings and do not, therefore, need to be considered on a level of equality in our moral deliberations.

This kind of criticism has stimulated Tom Regan to write his book “The Case for Animal Rights” (1984). He defends the position that we have a direct obligation towards higher vertebrates not to harm their wellbeing, in the same way that we are under an obligation towards the mentally retarded, the elderly, and children. They all have a value of their own which, to Regan, is the primary moral argument for the equal treatment of both human beings and (higher) non-human vertebrates.

In this chapter we have abstained from discussing the merits or shortcomings of moral defences or moral rejections of animal research, but instead
have focused upon the problems and procedures caused as a result of the prevailing concept of the morally restricted use of animals in scientific research. Every researcher working with live animals needs to evaluate his experiments in ethical terms. How such an evaluation may be achieved, and the possible pitfalls which may be encountered during the process, are discussed in this chapter.

The moral relevance of animals

Animals are often viewed as merely a means to the solution of a problem. Animals are not regarded as ends in themselves. In scientific publications, animals are generally assigned to sections dealing with “Materials and Methods”, and in the research laboratory animals are no more than experimental objects: they are considered as live instruments, their only value being their capacity to maximize the reliability and validity of scientific experiments.

In the field of ethics, the approach whereby the value of animals is dependent upon human objectives has been labelled *anthropocentric*. The animal here appears to be reduced to its instrumental value. In the course of the last decade, however, the anthropocentric approach has met with an increasing amount of criticism. In literature specialized in professional ethics, a fundamental discussion has arisen concerning the moral status of animals in relation to the moral status of human beings. According to the prevailing public opinion in western civilization, only human beings are considered to be morally relevant, the argument being that only human beings are capable of self-consciousness, of being responsible for their actions, of justifying their decisions and of making promises. Counter-argument here would be that, although these characteristics may be typical for moral actors, ethics has to concern itself with a wider domain of subjects. This wider domain includes subjects which may not be moral actors, but which are nevertheless considered to be equally in need of our moral concern. An example here would be people who possess the characteristics at issue to a lesser degree, for example, the mentally retarded, who are of course nevertheless regarded as morally relevant. When it comes to experimenting on human beings, it is precisely because of their vulnerability that those groups of people, who may not be (fully) capable of asserting themselves, are supposed to receive special forms of protection not provided to those fully equipped to reach an independent, conscious decision to participate as experimental subjects in scientific research. Therefore, experiments carried out on patients and elderly people, who may find themselves in positions of dependency similar to those of the mentally retarded, also meet with heavier criticism and scrutiny than would normally be the case.
Any serious consideration of the moral position of animals requires a detailed analysis of which similarities and differences between animals and human beings are considered to be morally relevant ones. For example, when adopting the thesis that animals are morally irrelevant because they lack the characteristics typical of the moral actor, it is also necessary to justify the moral distinction in the treatment of a class of people which lacks, in a similar way, the key characteristics associated with moral actorship. This theoretical distinction does have consequences for the welfare and health of the animals.

The problem of the moral status of animals can also be illustrated by referring to the declaration of human rights. Some of the most fundamental human rights apply to every human being, irrespective of differences in race, sex, culture, etc. In other words, as far as these fundamental human rights are concerned it is not possible to provide a moral justification for the unequal treatment of men and women, or of coloured and white people. These are differences which are morally irrelevant with respect to the basic rights of humanity.

Similarly, in the debate concerning the moral status of animals, the question is central as to whether the external differences between humans and animals does incorporate the morally relevant aspects which may be used to justify unequal treatment. A question closely related to this is whether or not there are certain general rights which may apply to both humans and animals, and which may serve as grounds for saying that, with respect to these particular rights, equal treatment is justifiable in principle. The “in principle” clause here implies that equal treatment is justified in the absence of a more fundamental ethical requirement, which may overrule these shared rights. Rights are usually conferred on entities which are said to possess intrinsic value (German: Eigenwert). The acknowledgement of the intrinsic value of animals is viewed by many as a logically necessary prelude to the discussion of the “rights” of animals. To others, the acceptance of the “intrinsic value” of animals raises no problems, whereas they may object to the idea of extending “rights” to animals. They make the assumption that, for the sake of animal welfare, the acceptance of such rights is not necessary; they consider it to be sufficient that we accept to have certain duties to the animals.

The biocentric approach emphasizes that all living creatures possess intrinsic value. In the zoocentric approach the intrinsic value of animals in particular is acknowledged. It implies that animals should not be used exclusively as a means, and that they ought to be protected for their own good. When justifying this idea, reference is made to the fact that what we are concerned with here are organisms with a considerable degree of autonomy. They are subjects with species-specific interests, to whom it matters whether these interests are accommodated or not. These species-specific interests
Ethical aspects of animal experimentation are particularly susceptible to damage caused by human beings. The intrinsic value of animals is, like all values, “conferred” by man. In contrast to instrumental value, intrinsic value, however, is not derived from human interests and objectives. Many people prefer, therefore, to speak of the “acknowledgment” of the intrinsic value of animals to emphasize its special character. It is said that the intrinsic value of animals is a necessary antecedent to our valuation of animals, and it would be a fallacy to reverse matters by representing the intrinsic value as its consequence.

The acknowledgement of the intrinsic value of animals implies that we have direct moral obligations towards animals, as opposed to the traditional indirect obligations (e.g. because the animal happens to be the property of another person, or because other people might be offended by cruel behaviour with respect to animals). This acknowledgement that animals have a value of their own is being incorporated into the value systems of an increasing number of people. This process is being reflected by reforms of, and amendments to, various animal protection laws.

Acknowledgement of the intrinsic value of animals has consequences for the researcher which have, as yet, not been exhaustively reflected upon. A few of these consequences are listed below:

- The scientific quality of an experiment is a necessary condition to be satisfied prior to any ethical evaluation taking place: i.e. experimental procedures, which are found to be methodologically unsound, are to be viewed as ethically unacceptable;
- When alternative methods are available, which do not require the use of animals, performance of the animal experiment should not be allowed, even if the alternative method is more expensive;
- If there is no alternative available, and there is a conflict between human and animal interests, due weight must be given to all interests under consideration. It must be understood that violation of the intrinsic value of animals cannot be justified; It may, however, be tolerable if the consequences of not performing the experiment are graver than the adverse effects imposed on the animals;
- In cases where the experimental use of animals is considered to be tolerable, it is essential that the animals involved are allowed to pursue their species-specific behaviour as far as possible before, during, and after the performance of the experiment.
- Researchers engaged in animal experiments have the moral obligation to search for alternative methods in order to reach their scientific aims. Acknowledgement of the intrinsic value of animals is interpreted by some ethicists as a principle of moral justice. This means that animal protection must not be dependent upon human compassion or sympathy, but should be regarded as a direct moral obligation to treat animals respectfully due to their
intrinsic value. Justice in relation to animals is often misunderstood as a demand for animals to be treated like people. Although some animals have similar characteristics and requirements as human beings, they are also in many ways very different. Justice would dictate that they receive similar treatment to humans in similar circumstances (as animals are capable of feeling pain and suffering in the same or a similar way to ourselves), but that they also receive different treatment in different circumstances.

For a long time, the presumption has been that animals are receiving greater justice the more they are treated like human beings. That is, however, not true as animals do not “want” a life style worthy to that of men, but one worthy of their own species. Every animal is, after its kind, a perfect being in itself and not, as is often assumed, merely an intermediate form of life between unicellular organisms and human beings. To judge animals in terms of their man-like qualities, and to ascribe to them inherent values appropriate to man, would merely constitute a new form of anthropocentric thinking.

Advocates of “animal rights” would make the same point by saying that animals “have a right to be treated as such”. Their position would protect animals against exclusively utilitarian deliberations based on the animals’ usefulness to human aims, in the same way that the conferral of rights on human beings provides individuals with a safeguard against considerations based upon what is commonly called the “general interest”. This would imply that certain actions may be considered inadmissible, irrespective of the importance of the expected benefits for human beings.

Acknowledgement of the intrinsic value of animals may also lead to acceptance of the idea that humans and animals should be viewed as equals, at least in some relevant moral aspects. A consequence of taking this position is that questions may be raised concerning medical ethics which require experiments on human subjects to be preceded by animal experiments. It may also be argued that criteria applied to animal experiments should, to a certain extent, be similar to the criteria applied to experiments performed on human subjects; for example the scientific merit of the experiment, the selection of subjects, and a cost-benefit analysis. It should, however, be recognized that in animal experiments ‘informed consent’ of course cannot be obtained from the experimental subject. An ‘animal counsel’ might be appointed in order to ensure that the animals’ best interests are taken fully into consideration.

**Moral problems in ethical dialogue**

The acceptance of the intrinsic value of animals as a point of departure implies that researchers will have to make allowances for the animals’ interests. Should a conflict of interests arise between human beings and animals, the
moral actors (those who are capable of making a moral decision) are the ones who are responsible for an ethically justifiable deliberation. When faced with the question as to whether or not a decision is ethically justified, two aspects should be examined in detail. First, the procedures followed to arrive at the judgement (who should be involved in the decision-making process), and secondly, the content of the judgement (i.e. the validity of the arguments used). This section focuses upon the first aspect; the content of the judgement will be looked into in the next section.

The starting point in normative ethics (in contrast to descriptive ethics) is the individual person, the moral actor, who is capable of making a free choice between alternative courses of action. The choice should be based upon a rational consideration of all the relevant facts, values, and normative principles, and this should be done with an attitude of impartiality and disinterestedness. What this implies is that it is not sufficient to say “this is my choice”; on the contrary, the assumption is that any other moral actor in the same or similar circumstances would arrive at the very same judgement. This is why normative ethics are sometimes said to show a tendency towards universalization (i.e. general applicability), when pronouncing prescriptive statements.

If ethical deliberation and choice are considered valid not only for the individual himself but also for anyone else in the same circumstances, it would follow that out of respect for the autonomy of other persons, arguments used in a decision-making process should be submitted to the scrutiny of other moral actors. It is in this context that we usually speak of a person having to “justify” or “account for” his actions. This ethical accountability does not entail a duty to answer to higher placed persons, authorities, or institutions, which would be the case if the accountability at issue was a legal or public one arising out of one’s position in the social structure (positional duty). Ethical accountability is based on the acceptance of the fundamental equality of all other moral actors irrespective of their social position. As far as ethical issues are concerned, any moral actor should have the fundamental freedom to participate in discussions and decision-making. The ethicist should also not be allowed to take up a privileged position in the decision-making process on the grounds of having attained a certain expertise with respect to substantial ethical argumentation.

Although the concept of the ethically autonomous person is an important starting point, it is also obvious that its definition and place in normative ethics implies a strong social element. Therefore, on one hand, deciding an ethical issue may be an extremely personal and individual process in which one’s conscience, philosophy of life, and moral qualities (“virtues”) play an important role. On the other hand, however, its tendency towards universalization will turn normative ethics into a social process in which the rational components of individual deliberations are called into question in a dialogue.
It follows, therefore, that the establishment of a dialogue concerning ethical questions will constitute an essential element of normative ethics. Since research involving animal experiments raises ethical questions, researchers cannot afford to dispense with this kind of dialogue. In so far as researchers have not been trained to apply ethical reasoning to their own research, participation in this dialogue will require a process of readjustment. The objective bias of scientific research can easily lead to the rejection or evasion of ethical problems. In the natural sciences, this bias is apparent in the tendency which exists to reduce animated nature to a material “object”, apparent in an emphasis on the non-moral, instrumental value of animals, and in a view of ethics which more often than not is emotivistic. The latter term indicates a view in which scientific statements are represented as objective, rational and universal, whereas moral statements are represented as their opposites: subjective, irrational, and contingent. In other words, moral statements are suggested to be no more than the expression of someone’s emotions. When taking this view of ethics, science and ethics are bound to be conceived of as two separate enterprises diametrically opposed to, and therefore hard to combine with, each other. Where ethics are, however, based upon reasoning, the idea of consensus is considered as a realistic possibility and that therefore a dialogue backed up by arguments may very well be expedient. What needs to be understood is that the exclusion of ethics on the part of researchers may result in a “moralizing” attitude on the part of the public, leading to an undifferentiated rejection of animal experiments and an indisposition to listen to arguments. What may then develop instead of a dialogue is a process of antagonism in which considerations of strategy will replace those of ethics.

Negotiations based on strategy may be accompanied by the use of rhetoric language, or fallacious reasoning, aimed at getting the most out of a bartering process. The conclusion of this process will be a compromise leaving both parties dissatisfied, as their differences of opinion will remain unaltered. The party who has had to yield will persevere in its desire to achieve its frustrated aims. Should it actually have the power to enforce its aims, it would certainly do so. If, on the other hand, both parties wish to solve their conflict, it will for all practical purposes become a necessity to adopt measures other than strategic ones, and to enter into a discussion concerning the content of the conflict, the presuppositions of their differing points of view, and the merits of their diverging opinions. This is a procedure which requires both parties to try to reach a consensus about what is to be considered morally right and wrong, without resorting to instigations and threats.

One way of arriving at an ethical dialogue between researchers on the one hand and parties outside the field of scientific research on the other, is to establish an ethics committee such as the “Animal Experimentation Committees (AECs) or the “Animal Care and Use Committees” (ACUCs). Members
of such committees need to have some experience in conducting an ethical
dialogue. It is of the utmost importance that local committees exchange sug-
gestions with respect to procedures to be followed and ethical criteria to be
applied, in order to be able to learn from each other and to achieve some form
of consistency. It goes without saying, due to the nature of an ethical dia-
logue, that its results should be accessible to the public.

A model for ethical reasoning

Ethical dialogue is primarily concerned with arguments. If a dialogue is to be
conducted regarding the admissibility of an animal experiment, an attempt
should be made to keep to the kind of arguments that are pertinent to the
subject. This has to do with the nature of the question giving rise to the dia-
logue. It also provides a method of solving conflicts when the debating par-
ties differ in their opinions.

The dialogue at issue here is the question as to whether a proposed animal
experiment is admissible or inadmissible from an ethical point of view. This
question involves more than the mere approval or disapproval of a random
piece of conduct. It involves an assessment of carefully planned actions which
are always performed intentionally. A person acting intentionally has reasons
for his actions. These reasons may be represented as something “behind” a
researcher pushing him on as it were, or as something “in front of” him which
he is aiming for. In the former case, the researcher’s reasons may be called
his “motives”; in the latter case, they may be called his “objectives”. In a
dialogue, these reasons should be made explicit, and should be discussed as
to whether or not they are important enough to justify performing the pro-
posed animal experiment. This type of deliberation is what is meant by using
arguments pertinent to the subject.

Take, for example, the case where researchers on the one hand, and mem-
bers of an ethical committee on the other, have a disagreement with regard to
a proposed animal experiment. The researchers are inclined to consider it
justified, whereas some committee-members have serious objections. In the
last section we have discussed that strategic negotiations aimed at enforcing
and imposing a particular opinion on others is not satisfactory from an ethical
point of view. What is missing in such cases is a genuine attempt to elucidate
and possibly to solve a substantial disagreement. When researchers and a
committee become involved in a struggle for supremacy, the basis for ethical
dialogue will be undermined in the process.

Why should the reasons submitted by researchers, whether or not they are
sufficient to justify performance of a proposed animal experiment, be the
object of an inquiry, and why should any attempts be made to resolve dis-
agreements by means of arguments? These questions can be answered by the fact that there is an intention to arrive at an accountable collective decision. The decision at issue, i.e. “admissible” or “inadmissible”, is a moral judgement regarding the propriety of actions. The term often used in this context is “normative direction for conduct”. Being able to account for such a decision implies the ability to state reasons in support of it, reasons that are sufficient to justify the decision. There may be good reasons for deciding in favour of admissibility, but there may also be grave reasons for deciding against it, for example, the experiment in question will cause the laboratory animals to suffer severe discomfort and/or the experiment’s scientific value is only of minor or moderate importance. In a dialogue, such points of criticism should not be simply discarded. The reasons in favour of the experiment should be specified and shown to be greater than the reasons for deciding against the experiment, or – more strongly – why the reasons in favour invalidate the reasons against. To summarize, the purpose of a dialogue is to arrive at justifiable normative directions for conduct. Arguments that ultimately carry weight are the ones providing good reasons, i.e. reasons which are sufficient to justify normative directions for conduct.

Ethical reasoning may also be the object of assessment with respect to its quality. Good reasoning should be valid, and arguments supporting such reasoning and conclusions should be both tenable and relevant. Validity has a bearing upon relationships of logical entailment between arguments and conclusion. Prominent ethicists disagree on the question of how strict a logically valid entailment should be, but it is beyond dispute that valid reasoning should be consistent and provide sufficient reasons for making its conclusion acceptable. Tenable arguments are those which correspond with the issue, i.e. they correspond with the facts of the case and the norms and values subscribed to. Those arguments are called relevant which have something to add to the authority of a judgement that a certain piece of conduct is morally right.

In order to arrive at good reasons for normative directions for conduct, it is advisable to take the following points into consideration:

– Has it been established that the proposed animal experiment satisfies required standards of scientific quality?

– Are the animals involved in the experiment expected to suffer any adverse effects? Assessment of adverse effects should account for their severity, duration and frequency.

– Are there any possibilities for replacement, reduction or refinement of the animal experiment?

– What degree of importance should be attached to the animal experiment? Is the experiment of primary or only of marginal importance; and of actual or only of potential importance?
Ethical aspects of animal experimentation

— Will the adverse effects suffered by the animals be compensated for by the importance of the animal experiment?

The first question on the list needs to be examined in more detail i.e. the process of deliberation in which one interest is balanced against another. First of all, it is necessary clearly to articulate which intuitive moral judgments there are with respect to the proposed experiment at issue. Subsequently, it will be necessary to search for those ethical principles which we are committed to and which we think may be pertinent to the case we are concerned with. Finally, it is necessary we may try to establish a correlation between these ethical principles on the one hand, and intuitive moral judgments on the other, in order to derive from this correlation good reasons for a conclusive decision. The procedure described can be illustrated by means of the following example as to whether an acute toxicity test is ethically admissible or not. The substance to be tested is an insecticide. It is estimated that the test will cause the animals involved to suffer considerable discomfort. The test has been requested by a private company, since the insecticide has to meet certain legal requirements if the company in question is to be allowed to sell it on the market. The test would need to be conducted on rats.

It is necessary to articulate clearly which intuitive moral judgments we have concerning this particular case, for instance, that it is right to test a substance with respect to its toxicity prior to its being used by consumers. These intuitions present themselves as self-evident and almost as unquestionable; often they are rooted in upbringing and experience, and they deserve serious consideration with regards to ethical reasoning. However, intuitive moral judgments alone form insufficient ground for a justified decision. They may be misplaced or distorted, as a result of the fact that we may only perceive what we wish to perceive, and intuitive moral judgments may also be incompatible. Along with the intuition that testing a substance for toxicity is the right thing to do, we may, for example, also have an intuition that it is wrong to cause rats to suffer. In such case, intuitive judgments are not sufficient when determining which decision should be taken.

It may be necessary, then, to examine critically our intuitive moral judgments. In a dialogue conducted by a committee, these intuitive moral judgments and lines of reasoning can be examined for compatibility with those of others. It is also possible to try to find ethical principles that are pertinent to the case at issue. Examples here would be the principle that we ought to promote other people’s health, the principle that we ought to protect animals from suffering or that we ought to respect their autonomy, the principle of non-maleficence, the principle of beneficence, or the principle of justice. These ethical principles may be used critically to examine intuitive moral judgments, by means of establishing a correlation between them. This process of reasoning proceeds in three successive stages. First of all, intuitive moral judgments
A model for ethical reasoning

may be critically assessed, both by viewing them from the perspective of certain ethical principles and by confronting them with morally relevant facts. For example, the intuition that it is wrong to make rats suffer may be critically assessed as to whether or not it should be considered misplaced. If it is possible to produce good reasons for protecting rats from suffering pain, then the intuition does not seem to be misplaced.

Conversely, in the second stage, ethical principles may be critically examined in the light of intuitive moral judgments and whatever information is relevant to the problem. Ethical principles should not be isolated from the intuitions and experience from which they originate. One subject for discussion may surround, for example, whether the principle of justice should be taken to imply that animals and human beings ought to be treated equally, or whether it should be understood as implying no more than that rats ought to be taken into consideration in so far as that they have the ability to experience suffering consciously. In the latter case, a generalized requirement to treat animals and humans equally would not represent a correct interpretation of the principle of justice.

In the third stage, the process of establishing a correlation between ethical principles and intuitive moral judgements may continue until a reflective equilibrium has been reached. Intuitive moral judgements are critically assessed and ethical principles are reciprocally examined until a certain degree of correspondence has been reached between acceptable intuitive moral judgements on the one hand, and accepted ethical principles on the other. When that stage is reached, intuitive moral judgments and judgements examined for their compatibility with certain ethical principles are said to be consistent. In the example used above, the reflective equilibrium under concern may be approached in this way. The validity of the intuitive moral judgement would acknowledge that it is wrong to cause rats to suffer. It would, however, also be acknowledged that it would be unacceptable to consider this judgement so important as to prevent us from alleviating potential human suffering. Therefore, we would acknowledge the validity of the principle that we ought to promote other people’s health, but not to the extent that obligations derived from this principle should always override the obligation to protect rats from suffering. Is the conclusion, therefore, that we are obliged to allow the toxicity test to be performed? This would depend upon how important the result of the test is expected to be for promoting people’s health. Refrained from performing the test may only delay official approval of the insecticide by a few months, i.e. until other information would become available by which an approval is possible as well. In that event, the intuitive moral judgement may be that, until that time, it would be better to carry on without the insecticide. Acceptable intuitive moral judgements, together with authoritative ethical principles, might then constitute sufficient reasons to reach a negative decision:
meaning that under the circumstances, the toxicity test is to be viewed as ethically inadmissible. In reality, the situation presenting itself is generally much more complex. The insecticide might, for example, be considerably superior to similar products, or its being brought onto the market might be of eminent importance to the economic survival of the company producing it. If so, the ethical principle of beneficence may also be at issue, which may give rise to a reconsideration of the intuitive moral judgement which was initially acknowledged.

Pitfalls in ethical argumentation

The previous section illustrated a possible line of reasoning which may be useful to follow when trying to find good reasons for a particular ethical decision. Sound reasoning is an important piece of equipment in such an approach. In this section, some of the pitfalls and fallacies which may frustrate a constructive ethical dialogue will be discussed.

One of the major pitfalls constituting an impediment to constructive ethical discourse may be in the form of an irritated response when positions believed to be sacrosanct come into question (“why engage in animal experiments?”). Viewed from the perspective of ethical discourse, a response of evasion (“there aren’t any alternatives”) or of escapism (“lay-people with a lack of scientific expertise in a committee are only going to cause problems”) are inappropriate. History has shown several examples of research methods, which were presumed to be unassailable by a small subculture of experts engaged in their practice, which will no longer be tolerated in most civilized countries.

Another pitfall in ethical argumentation is related to an inability to allow for lines of reasoning beyond the scope of one’s own conceptual framework. Every social group has its own particular set of moral precepts, which is also the case for a group of researchers. These norms and values, taken to be self-evident by the members of a particular social group, will not be doubted until an alternative presents itself, or until outsiders (members of their families, anti-vivisectionists, or their students) draw attention to and call into question the underlying moment of choice with respect to their conduct. If participants in an ethical dialogue show an indisposition to have their own preferences or presuppositions genuinely called into question, controversies will develop which will prevent a constructive ethical dialogue.

It is vitally important to establish a correct balance between the research method proposed and any alternative methods which may deserve consideration. Here the pitfall would be to overlook the fact that a choice between two alternative methods accounts for four aspects to be balanced against each
other, i.e. the arguments for and against both methods. Often, arguments in favour of one alternative are balanced against arguments disfavouring the other, e.g. “advantage of an animal experiment is the possibility to examine the entire physiological system; disadvantage of a cell culture is that in the course of time characteristic functions of cells will disappear”. A particular position has, in effect, been taken disregarding the fact that there are still two other aspects which need to be brought into question: “disadvantage of an animal experiment is that the animals involved may suffer adverse effects; advantage of a cell culture is its speed, its reproducibility, and the large number of tests which can be performed within a short space of time”.

Situations of choice may give rise to quandaries. To find oneself in such situation is to acknowledge the existence of good arguments both for and against alternative experimental procedures, while neither the arguments for nor the arguments against have the cogency to invalidate the other. For instance, a situation may arise in which one procedure will involve the use of twenty rats, while the other is expected to achieve similar results of equal validity with the use of “no more” than five chimpanzees. In this example, the differences of number and emotional distance to the animals are at issue, which are aspects which are difficult to relate to each other. The quandary faced here is, in point of fact, a choice between two evils. A quandary which often occurs is the question whether the researcher uses a few animals several times (e.g. 2, 10 or 20 times) or whether he will use more animals only once. The quantitative reduction of the number of animals must be weighed up as an alternative against the reduction in suffering of the individual animal. When taking into account that no laboratory animals live out their expected life span, the reduction in suffering has to take preference over the quantitative decrease. In the previous section, we have seen that quandaries may be solved by resorting to principles of a more fundamental nature. The pitfall with respect to the type of choices between alternatives is that they are conceived of as subjects of dispute. This often results with the parties to the alleged dispute becoming engaged in a trench warfare, instead of attempting to elucidate the structure and background of the quandary and turning it into the subject of discussion.

It would be very helpful to an ethical dialogue to be able to have a reliable assessment of the severity of discomfort to be expected for the animals involved, and of the relevancy attached to the objectives expected to result from an animal experiment. Here the pitfall presents itself in the form of an unqualified acceptance of arguments assigned by an authority, particularly when the authority in question happens to be the expert who is himself engaged in the animal research. This does not mean that it is wrong to respect the expertise of competent and accomplished men and women; it does mean, however, that to accept an argument without qualification on the sole ground
that it has been advanced by someone with authority will reduce any further
discussion to a superfluous exercise. It is precisely this discussion about pre-
supposed norms and values which constitutes the foundation of ethics. To cat-
egorize adverse effects of research procedures on the basis of a pre-established
index of severity may result in a subsequent neglect of the real problems under
consideration, which will again jeopardize a serious ethical discussion.

When attempting to arrive at a process of deliberation, whereby the bal-
ance to be achieved will be based on related variables, the choice often made
is for costs and benefits to be balanced out against each other. The pitfall here
is one of "reduction"; instead of deliberating on ethical grounds, attention is
focused exclusively on economic aspects. Costs incurred, for example, by
the acquisition, housing and care of laboratory animals may be balanced out
against expenditures expected to be saved on health care facilities as a result
of introducing, for example, a new therapy into the system. Although this is
an important and necessary deliberation within the framework of effective
health care management, it is not an ethical one. It may turn into an ethical
deliberation, but only when the motives (values) behind these economic ef-
forts are brought into question ("why should any efforts be directed at achieving
a minimum in expenditures on laboratory animals and a maximum in savings
on medical techniques?"). By asking questions such as these, the economic
discussion is abandoned and the ethical discussion is entered into. One con-
ceivable answer to this question might refer to the axioms of utilitarianism
requiring the minimization of suffering and the maximization of happiness of
the greatest number (of morally relevant beings: subjects capable of suffer-
ing or of having interests).

An important category of pitfalls, which frequently recurs in ethical rea-
soning, is constituted by what are commonly referred to as fallacies. Falla-
cies are specious arguments: persuasive on the surface, but incorrect on
closer inspection. Their most powerful, and at the same time most alarm-
ing, feature is that they often (irrelevantly) capitalize on human sentiment.
Consequently, they may defeat any attempt of conducting a dialogue based
on rational arguments. Classical fallacies are the spurious counter-argu-
ments derived from the art of rhetoric. The common denominator of these
arguments is that they do not respond to arguments advanced by the oppo-
nent, but that they elaborately expound on another (irrelevant) aspect. Ex-
amples here would be:

— appeal to the public: "If AIDS research centres are not granted exemption
from restrictions on import trade in chimpanzees, this country will not
produce the solution to the AIDS epidemic." In addition to making the
questionable assumption that chimpanzees are indeed necessary to AIDS
research, this argument implicitly addresses national sentiment in order to
persuade the audience.
Pitfalls in ethical argumentation

- *argumentum ad hominem (personal attack):* “Is this man being paid by some animal welfare organization?” Argument suggesting that the speaker in question is to be regarded as suspect, in order to be at liberty to dispense with the effort to refute whatever has been proposed by him.

- *unwarranted appeal to compassion:* “Such a poor little puppy ....” Argument designed to emphasize natural feelings of sympathy that young animals in particular tend to evoke, in order to evade substantial discussion of the moral principles and facts at issue.

- *argument from authority:* “The (British) Home Office Guidance on the operation of the Animals (Scientific Procedures) Act 1986 (or the (US) Animal Welfare Act) requires. . . .” Or alternatively, the authority of a personality such as Albert Schweitzer may be used as a means of intimidating opposing partners in a dispute. What should count, and therefore should be discussed, are the arguments used by these authorities instead of their names.

- *appeal to involvement:* “In your family too, there may be someone suffering from a heart condition. . . .” Argument persuading the audience to form their opinion on the basis of their potentially personal involvement rather than a rational assessment of the moral problem under review.

A second group of fallacies is not specious in the sense that they distract from relevant arguments by irrelevantly focusing on human sentiment, but rather in the sense that they draw logically false inferences between premises. These inferences may in themselves be relevant enough, but the result is that the conclusions reached are of a somewhat dubious nature. Examples of this group are:

- *argument from ignorance:* “Animals do not feel pain, because it has never been verified that they do feel pain.” The lack of verification of one thing does not lead to the conclusion that the reverse must be true.

- *circular argument (begging the question):* “Animal research will always be necessary. After all, testing drugs on animals will warrant their safety for human consumption. In those cases where animal research turns out to be inconclusive, or even to yield false prognoses, we should actually increase the number of animal tests.” The matter in dispute is assumed to be true. Logically false presupposition is that there are no alternatives at all to the one in question.

- *argument from correspondence:* “With animal research it will be the same as with the first steam engine; after a while no one is going to have a problem with it anymore.” Logically false assumption here is that analogies will show a correspondence in every aspect, whereas they may only show correspondence in some (minor) aspects.

- *unwarranted generalization:* “Animal experiments do not always lend themselves to reliable extrapolation to human beings; therefore it is better not
to engage in animal research at all.” What has been observed to be true in some cases is inferred to be true in all cases.

- **a priori reasoning (accident):** “Benefits to people are a good thing. Gene therapy may be a benefit to many people. Therefore gene therapy is a good thing.” What may very well be true as a vague, general rule, is inferred to be true in a particular and very specific case.

- **post hoc ergo propter hoc (after this, therefore on account of this):** “If animal research had not been tolerated in the past, science would never have progressed.” Sequence is confused with consequence. The inference is logically false, since it cannot possibly be falsified, as is the case with all historical arguments.

- **naturalistic (is/ought) fallacy:** “The natural order of things is such that plants, animals, and humans can only survive at the expense of other forms of life. The use of animals for the benefit of humanity thus fits into the natural order of things.” From the way things are it is inferred that this is the way they ought to be: values are inferred from facts. The argument is typically conservative in that it rejects out of hand any possibility of change in the future.

- **on not being consequent:** “Being against animal experimentation and still accepting medicines for your sick child is not consequent.” Although not consequent it might very well be consistent (‘consequent’ referring to acts, as opposed to ‘consistent’ referring to principles). The opponent suggests that you are betraying your own ethical principles. In other words that you are not ‘strong’ enough to participate in a serious discussion, which is of course an ‘argumentum ad hominem’. The point is, however, that the principle of equal treatment under equal conditions is misused because it has been overlooked that different conditions allow for (or even require) a different treatment. The difference in this example is that the direct moral obligation towards your own child overrules (not erases!) the general moral principle to care for animals.

- **equivocation:** “Researchers have the obligation to minimize adverse effects of their research procedures on the animals involved.” Ambiguous terms and concepts are used to conceal a lack of consensus. “Adverse effects” is open to different interpretations made by researchers on the one hand, and representatives of animal welfare organizations or activist groups on the other. For example, to the latter, laboratory confinement will in itself constitute an unacceptable hardship, while to the former confinement presents no problem so long as legally required standards on this point are satisfied. What exactly is implied here by the obligation to “minimize”? Will it still apply when its discharge may become economically inexpedient?
Biotechnology raises new moral problems

As outlined in chapter 7, transgenesis and gene targeting are two biotechnological procedures that have a great impact on developments in biomedical research. However, the introduction of these technologies is challenging the ‘R’ of reduction. The number of animals needed for the production of 3–4 founders is substantial and can exceed 100–150 individuals. Also, since the phenotype of the transgenic founder animals may differ, often more than one transgenic line has to be maintained. As a consequence, housing facilities for transgenic animals have doubled in recent years.

The production of transgenic animals is also raising new ethical questions. Concern is focused on the transformation of a ‘natural’ animal (wild-type mouse, or classical inbred line) into a new creature (the transgenic mouse). Unlike previous debate on animal experimentation, the primary focus here is not on the balance between animal welfare and scientific aims, but on the very act of making the transgenic animal.

Applying the three R’s, and in particular the balancing of suffering against research goals, falls short in the case of technologies such as genetic engineering:

– **Reduction** is still a relevant issue, but it scores negatively when all the animals needed to establish a successful founder animal are taken into account.

– **Replacement** by in vitro cultures is inappropriate when applied to a technology that already takes place in a cell culture of oocytes or blastocysts. The moral issue of the genetic modification is an act at the cellular level. Of course, replacement (in its original meaning) remains an option after establishment of the new transgenic line in the “traditional” follow-up animal experiments (i.e. toxicology, sera research, bioassays for diagnostics, basic research).

– **Refinement** is irrelevant because one manipulates the oocyte, which has no capacity to suffer. Researchers using biotechnology claim that the techniques do constitute a refinement, when compared to the less directed and rather crude classical mutagenetic technologies (such as induction of cancer by radiation and chemicals). Opponents question that biotechnologies are a refinement, referring to the low success rate of microinjection and the low predictiveness of ‘knock-out’ experiments. Biotechnologists define refinement as the use of a ‘micro tool’ whereas its critics define refinement as assuring a high level of ‘controllability/predictiveness’. Both definitions are valid but a muddled discussion results when they are not clearly distinguished.

– The **principle of non-maleficence** (‘do no harm’) presupposes a subject which can be hurt (for some philosophers, this is already problematic in
the case of animals), and/or a subject who performs the act causing the hurt. Clearly, whether the introduction of genes into the genome of the mouse is a case of non-maleficence, is not easy answerable by applying the traditional definition of this principle.

– The principle of beneficence (‘do good’) comes down to carrying out euthanasia where the level of suffering has become unacceptably high. The fundamental question of whether transgenesis can be considered an act of beneficence is a priori to any discussion about the expected profits (for the human beings) of the animal experiments for which transgenic animals are used.

In other words, the question of whether the act of changing the genetic makeup of the animal is morally right, has to be answered in relation to the cultural norms of society; not simply within the scientific community.

In several European countries the intrinsic value of animals is already acknowledged within debate on animal use. A consequence of acknowledging intrinsic value is that every change to the essence of an animal (i.e. to the ‘mousiness’ of a mouse) has to be justified in a moral sense. The introduction of genes from another species into the genome of a mouse, is such a change.

Once genetically engineered animals are created, their use in experiments must follow the same ethical evaluation as for all other animal experiments. The new moral issue is linked to the technology itself, to the very creation of the animals in the first place, not to their use in experiments. Dealing with these complex issues requires a wide-ranging and open communication in society at large. It is through such dialogue that we will develop the new ethical framework needed for evaluating the admissibility of the application of these biotechnological procedures.

Conclusions

When performing research involving animals, it is important for researchers to be well aware of the fact that animal experimentation has become a fundamental part of scientific procedures in the study of living nature. From the perspective of animal experimentation, the instrumental value of animals is central to the success of the experiment. With the acknowledgement that at least all vertebrates should be considered to possess a value of their own (Eigenwert), animal experimentation is no longer regarded as self-evidently justified from an ethical point of view. In addition to having at least to make an attempt at reduction, refinement, and replacement of animal experiments, researchers will also have to embark upon the question of whether the methods used will be compensated for by the results of their research. Solving this question will require an ability to engage in ethical reasoning and to examine
ethical arguments for their compatibility to those of others. This “ethical dialogue” should be conducted by ethical committees. Such ethical reasoning may be required to meet certain standards, both with respect to form and to content, in order for researchers to be able to maintain that there are “good reasons” in the ethical sense to pursue their lines of research. Awareness of fallacies (logical pitfalls in ethical argumentation) is an important element in the process of finding good reasons. By promoting, as far as possible, the public nature of ethical deliberation, a collective process of learning may develop, resulting in the interests of humans and animals being balanced against each other with increasing care. Public accessibility provides, at the same time, a safeguard for society against neglect of the acknowledgement that animals possess a value of their own. To the researcher engaged in animal experimentation, it may be a comfort to know that a positive judgement by an ethical committee to perform an animal experiment is supported by people who lack any direct interest in the research in question.

Literature

Ethical aspects of animal experimentation
on the approximation of laws, regulations and administrative provisions of
the Member States regarding the protection of animals used for
experimental and other scientific purposes

(86/609/EEC)

THE COUNCIL OF THE EUROPEAN COMMUNITIES,

Having regard to the Treaty establishing the European Economic Community, and in particular
Article 100 thereof,

Having regard to the proposal from the Commission¹,

Having regard to the opinion of the European Parliament²,

Having regard to the opinion of the Economic and Social Committee³,

Whereas there exist between the national laws at present in force for the protection of animals
used for certain experimental purposes disparities which may affect the functioning of the common
market;

Whereas, in order to eliminate these disparities, the laws of the Member States should be
harmonized; whereas such harmonization should ensure that the number of animals used for
experimental or other scientific purposes is reduced to a minimum, that such animals are
adequately cared for, that no pain, suffering, distress or lasting harm are inflicted unnecessarily
and ensure that, where unavoidable, these shall be kept to the minimum;

Whereas, in particular, unnecessary duplication of experiments should be avoided,

³ OJ No C 207, 18.8.1986, p. 3.
HAS ADOPTED THIS DIRECTIVE:

Article 1

The aim of this Directive is to ensure that where animals are used for experimental or other scientific purposes the provisions laid down by law, regulation or administrative provisions in the Member States for their protection are approximated so as to avoid affecting the establishment and functioning of the common market, in particular by distortions of competition or barriers to trade.

Article 2

For the purposes of this Directive the following definitions shall apply:

(a) ‘animal’ unless otherwise qualified, means any live non-human vertebrate, including free-living larval and/or reproducing larval forms, but excluding foetal or embryonic forms;

(b) ‘experimental animals’ means animals used or to be used in experiments;

(c) ‘bred animals’ means animals specially bred for use in experiments in facilities approved by, or registered with, the authority;

(d) ‘experiment’ means any use of animal for experimental or other scientific purposes which may cause it pain, suffering, distress or lasting harm, including any course of action intended, or liable, to result in the birth of an animal in any such condition, but excluding the least painful methods accepted in modern practice (i.e. ‘humane’ methods) of killing or marking an animal; an experiment starts when an animal is first prepared for use and ends when no further observations are to be made for that experiment; the elimination of pain, suffering, distress or lasting harm by the successful use of anaesthesia or analgesia or other methods does not place the use of an animal outside the scope of this definition. Non experimental, agricultural or clinical veterinary practices are excluded.

(e) ‘authority’ means the authority of authorities designated by each Member State as being responsible for supervising the experiments within the meaning of this Directive;

(f) ‘competent person’ means any person who is considered by a Member State to be competent to perform the relevant function described in this Directive;

(g) ‘establishment’ means any installation, building, group of buildings or other premises and may include a place which is not wholly enclosed or covered and mobile facilities;

(h) ‘breeding establishment’ means any establishment where animals are bred with a view to their use in experiments;

(i) ‘supplying establishment’ means any establishment, other than a breeding establishment, from which animals are supplied with a view to their use in experiments;

(j) ‘user establishment’ means any establishment where animals are used for experiments;

(k) ‘properly anaesthetized’ means deprived of sensation by methods of anaesthesia (whether local or general) as effective as those used in good veterinary practice;

(l) ‘humane method of killing’ means the killing of an animal with a minimum of physical and mental suffering, depending on the species.
Article 3

This Directive applies to the use of animals in experiments which are undertaken for one of the following purposes:

(a) the development, manufacture, quality, effectiveness and safety testing of drugs, foodstuffs and other substances or products:
   (i) for the avoidance, prevention, diagnosis or treatment of disease, ill-health or other abnormality of their effects in man, animals or plants;
   (ii) for the assessment, detection, regulation or modification of physiological conditions in man, animals or plants;

(b) the protection of the natural environment in the interests of the health or welfare of man or animal.

Article 4

Each Member State shall ensure that experiments using animals considered as endangered under Appendix 1 of the Convention on International Trade in Endangered Species of Fauna and Flora and Annex C.I. of Regulation (EEC) No 3626/82¹ are prohibited unless they are in conformity with the above Regulation and the objects of the experiment are:

– research aimed at preservation of the species in question, or
– essential biomedical purposes where the species in question exceptionally proves to be the only one suitable for those purposes.

Article 5

Member States shall ensure that, as far as the general care and accommodation of animals is concerned:

(a) all experimental animals shall be provided with housing, an environment, at least some freedom of movement, food, water and care which are appropriate to their health and well-being;

(b) any restriction on the extent to which an experimental animal can satisfy its physiological and ethological needs shall be limited to the absolute minimum;

(c) the environmental conditions in which experimental animals are bred, kept or used must be checked daily;

(d) the well-being and state of health of experimental animals shall be observed by a competent person to prevent pain or avoidable suffering, distress or lasting harm;

(e) arrangements are made to ensure that any defect or suffering discovered is eliminated as quickly as possible.

For the implementation of the provisions of paragraphs (a) and (b), Member States shall pay regard to the guidelines set out in Annex 11.

Article 6

1. Each Member State shall designate the authority or authorities responsible for verifying that the provisions of this Directive are properly carried out.

2. In the framework of the implementation of this Directive, Member States shall adopt the necessary measures in order that the designated authority mentioned in paragraph 1 above may have the advice of experts competent for the matters in question.

Article 7

1. Experiments shall be performed solely by competent authorized persons, or under the direct responsibility of such a person, or if the experimental or other scientific project is authorized in accordance with the provisions of national legislation.

2. An experiment shall not be performed if another scientifically satisfactory method of obtaining the result sought, not entailing the use of an animal, is reasonably and practicably available.

3. When an experiment has to be performed, the choice of species shall be carefully considered and, where necessary, explained to the authority. In a choice between experiments, those which use the minimum number of animals, involve animals with the lowest degree of neurophysiological sensitivity, cause the least pain, suffering, distress or lasting harm and which are most likely to provide satisfactory results shall be selected.

Experiments on animals taken from the wild may not be carried out unless experiments on other animals would not suffice for the aims of the experiment.

4. All experiments shall be designed to avoid distress and unnecessary pain and suffering to the experimental animals. They shall be subject to the provisions laid down in Article 8. The measures set out in Article 9 shall be taken in all cases.

Article 8

1. All experiments shall be carried out under general or local anaesthesia.

2. Paragraph 1 above does not apply when:

(a) anaesthesia is judged to be more traumatic to the animal than the experiment itself;

(b) anaesthesia is incompatible with the object of the experiment. In such cases appropriate legislative and/or administrative measures shall be taken to ensure that no such experiment is carried out unnecessarily.

Anaesthesia should be used in the case of serious injuries which may cause severe pain.

3. If anaesthesia is not possible, analgesics or other appropriate methods should be used in order to ensure as far as possible that pain, suffering, distress or harm are limited and that in any event the animal is not subject to severe pain, distress or suffering.

4. Provided such action is compatible with the object of the experiment, an anaesthetized animal, which suffers considerable pain once anaesthesia has worn off, shall be treated in good time with pain-relieving means or, if this is not possible, shall be immediately killed by a humane method.
Article 9

1. At the end of any experiment, it shall be decided whether the animal shall be kept alive or killed by a human method, subject to the condition that it shall not be kept alive if, even though it has been restored to normal health in all other respects, it is likely to remain in lasting pain or distress.

2. The decisions referred to in paragraph 1 shall be taken by a competent person, preferably a veterinarian.

3. Where, at the end of an experiment:
   (a) an animal is to be kept alive, it shall receive the care appropriate to its state of health, be placed under the supervision of a veterinarian or other competent person and shall be kept under conditions conforming to the requirements of Article 5. The conditions laid down in this subparagraph may, however, be waived where, in the opinion of a veterinarian, the animal would not suffer as a consequence of such exemption;
   (b) an animal is not to be kept alive or cannot benefit from the provisions of Article 5 concerning its well-being, it shall be killed by a humane method as soon as possible.

Article 10

Member States shall ensure that any re-use of animals in experiments shall be compatible with the provisions of this Directive.

In particular, an animal shall not be used more than once in experiments entailing severe pain, distress or equivalent suffering.

Article 11

Notwithstanding the other provisions of this Directive, where it is necessary for the legitimate purposes of the experiment, the authority may allow the animal concerned to be set free, provided that it is satisfied that the maximum possible care has been taken to safeguard the animal’s well-being, as long as its state of health allows this to be done and there is no danger for public health and the environment.

Article 12

1. Member States shall establish procedures whereby experiments themselves or the details of persons conducting such experiments shall be notified in advance to the authority.

2. Where it is planned to subject an animal to an experiment in which it will, or may, experience severe pain which is likely to be prolonged, that experiment must be specifically declared and justified to, or specifically authorized by, the authority. The authority shall take appropriate judicial or administrative action if it is not satisfied that the experiment is of sufficient importance for meeting the essential needs of man or animal.

Article 13

1. On the basis of requests for authorization and notifications received, and on the basis of the reports made, the authority in each Member State shall collect, and as far as possible periodically
make publicly available, the statistical information on the use of animals in experiments in respect of:

(a) the number and kinds of animals used in experiments;
(b) the number of animals, in selected categories, used in the experiments referred to in Article 3;
(c) the number of animals, in selected categories, used in experiments required by legislation.

2. Member States shall take all necessary steps to ensure that the confidentiality of commercially sensitive information communicated pursuant to this Directive is protected.

Article 14

Persons who carry out experiments or take part in them and persons who take care of animals used for experiments, including duties of a supervisory nature, shall have appropriate education and training.

In particular, persons carrying out or supervising the conduct of experiments shall have received instruction in a scientific discipline relevant to the experimental work being undertaken and be capable of handling and taking care of laboratory animals; they shall also have satisfied the authority that they have attained a level of training sufficient for carrying out their tasks.

Article 15

Breeding and supplying establishments shall be approved by or registered with, the authority and comply with the requirements of Articles 5 and 14 unless an exemption is granted under Article 19 (4) or Article 21. A supplying establishment shall obtain animals only from a breeding or other supplying establishment unless the animal has been lawfully imported and is not a feral or stray animal. General or special exemption from this last provision may be granted to a supplying establishment under arrangements determined by the authority.

Article 16

The approval or the registration provided for in Article 15 shall specify the competent person responsible for the establishment entrusted with the task of administering, or arranging for the administration of, appropriate care to the animals bred or kept in the establishment and of ensuring compliance with the requirements of Articles 5 and 14.

Article 17

1. Breeding and supplying establishments shall record the number and the species of animals sold or supplied, the dates on which they are sold or supplied, the name and address of the recipient and the number and species of animals dying while in the breeding or supplying establishment in question.

2. Each authority shall prescribe the records which are to be kept and made available to it by the person responsible for the establishments mentioned in paragraph 1; such records shall be kept for a minimum of three years from the date of the last entry and shall undergo periodic inspection by officers of the authority.
Article 18

1. Each dog, cat or non-human primate in any breeding, supplying or user establishment shall, before it is weaned, be provided with an individual identification mark in the least painful manner possible except in the cases referred to in paragraph 3.

2. Where an unmarked dog, cat or non-human primate is taken into an establishment for the first time after it has been weaned it shall be marked as soon as possible.

3. Where a dog, cat or non-human primate is transferred from one establishment as referred to in paragraph 1 to another before it is weaned, and it is not practicable to mark it beforehand, a full documentary record, specifying in particular its mother, must be maintained by the receiving establishment until it can be so marked.

4. Particulars of the identity and origin of each dog, cat or non-human primate shall be entered in the records of each establishment.

Article 19

1. User establishments shall be registered with, or approved by, the authority. Arrangements shall be made for user establishments to have installations and equipment suited to the species of animals used and the performance of the experiments conducted there; their design, construction and method of functioning shall be such as to ensure that the experiments are performed as effectively as possible, with the object of obtaining consistent results with the minimum number of animals and the minimum degree of pain, suffering, distress or lasting harm.

2. In each user establishment:

(a) the person or persons who are administratively responsible for the care of the animals and the functioning of the equipment shall be identified;

(b) sufficient trained staff shall be provided;

(c) adequate arrangements shall be made for the provision of veterinary advice and treatment;

(d) a veterinarian or other competent person should be charged with advisory duties in relation to the well-being of the animals.

3. Experiments may, where authorized by the authority, be conducted outside user establishments.

4. In user establishments, only animals from breeding or supplying establishments shall be used unless a general or special exemption has been obtained under arrangements determined by the authority. Bred animals shall be used whenever possible. Stray animals of domestic species shall not be used in experiments. A general exemption made under the conditions of this paragraph may not extend to stray dogs and cats.

5. User establishments shall keep records of all animals used and produce them whenever required to do so by the authority. In particular, these records shall show the number and species of all animals acquired, from whom they were acquired and the date of their arrival. Such records shall be kept for a minimum of three years and shall be submitted to the authority which asks for them. User establishments shall be subject to periodic inspection by representatives of the authority.
Article 20

When user establishments breed animals for use in experiments on their own premises, only one registration or approval is needed for the purposes of Article 15 and 19. However, the establishments shall comply with the relevant provisions of this Directive concerning breeding and user establishments.

Article 21

Animals belonging to the species listed in Annex I which are to be used in experiments shall be bred animals unless a general or special exemption has been obtained under arrangements determined by the authority.

Article 22

1. In order to avoid unnecessary duplication of experiments for the purposes of satisfying national or Community health and safety legislation, Member States shall as far as possible recognize the validity of data generated by experiments carried out in the territory of another Member State unless further testing is necessary in order to protect public health and safety.

2. To that end, Member States shall, where practicable and without prejudice to the requirements of existing Community Directives, furnish information to the Commission on their legislation and administrative practice relating to animal experiments, including requirements to be satisfied prior to the marketing of products; they shall also supply factual information on experiments carried out in their territory and on authorizations or any other administrative particulars pertaining to these experiments.

3. The Commission shall establish a permanent consultative committee within which the Member States would be represented, which will assist the Commission in organizing the exchange of appropriate information, while respecting the requirements of confidentiality, and which will also assist the Commission in the other questions raised by the application of this Directive.

Article 23

1. The Commission and Member States should encourage research into the development and validation of alternative techniques which could provide the same level of information as that obtained in experiments using animals but which involve fewer animals or which entail less painful procedures, and shall take such other steps as they consider appropriate to encourage research in this field. The Commission and Member States shall monitor trends in experimental methods.

2. The Commission shall report before the end of 1987 on the possibility of modifying tests and guidelines laid down in existing Community legislation taking into account the objectives referred to in paragraph 1.

Article 24

This Directive shall not restrict the right of the Member States to apply or adopt stricter measures for the protection of animals used in experiments or for the control and restriction of the use of animals for experiments. In particular, Member States may require a prior authorization for experiments or programmes of work notified in accordance with the provisions of Article 12(1).
Article 25

1. Member States shall take the measures necessary to comply with this Directive by 24 November 1989. They shall forthwith inform the Commission thereof.

2. Member States shall communicate to the Commission the provisions of national law which they adopt in the field covered by this Directive.

Article 26

At regular intervals not exceeding three years, and for the first time five years following notification of this Directive, Member States shall inform the Commission of the measures taken in this area and provide a suitable summary of the information collected under the provisions of Article 13. The Commission shall prepare a report for the Council and the European Parliament.

Article 27

This Directive is addressed to the Member States.

Done at Brussels, 24 November 1986.

For the Council

The President

W. WALDEGRAVE
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