An introduction to

Biomedical Laboratory Research

By

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PREFACE

Together, we have worked for more than half a century within the field of cell physiology. As university researchers we have also taught physiology, cell biology, and educational techniques to medical and other students and to postgraduates.

The motivation to write this book had various roots. Firstly, we had each worked quite independently, without much supervision, on our own doctoral thesis projects and came to the early realization that there is an upper limit to the utility of the "trial-and-error" approach. We felt that some basic information concerning the craftsmanship of biomedical laboratory research would profit the novice. Secondly, since we enjoy teaching, we arranged in 1983 a basic course, containing most of the subjects of this text, aimed primarily at doctoral students from the medical and biological research professions. As the course developed, we read and searched more systematically to compile teaching material for the course, which steadily improved during the following years. Thirdly, courses like the one we had initiated - with a defined syllabus - became obligatory in Norway (as well as in other countries) for students preparing themselves for doctorates in medicine and science. Furthermore, stricter time limits were imposed for the completion of a thesis.

Gradually our samplings from the world scientific literature and our own writings evolved into a compendium. The reader will find that we lean heavily and perhaps shamelessly on our more distinguished national and international colleagues and scientific heroes (partly in the form of expository "boxes", tables, and appendices), but we have nevertheless increasingly supplemented and complemented earlier editions of the compendium with our own notes, as a response to feedback from course participants. We have also referred to alternative, as well as more advanced and penetrating literature to help the reader who might feel that the text is too introductory or superficial, or both. To increase the practical utility of the book, we have concluded each chapter with one or more summary checklists and with exercises for self-study or small group work. Now we feel that the text should prove valuable to masters and doctoral students engaged in biomedical laboratory research.

Several good colleagues and friends - in addition to our own former students - have helped us to improve the text during the last 15 years. They are too many to be mentioned individually. We thank them all. Special credit, however, is due Joel Glover, Ph. D, and journalist Anne Berit Morrow, M.A., for providing numerous well-informed suggestions concerning the style and logic of the presentations. The

short-comings that remain are of course the sole responsibility of the authors. In particular, the reader will often find that we have pursued certain idiosyncrasies. For example, we prefer medians to arithmetic means and nonparametric to parametric statistics. You are welcome to disagree with us, but be prepared to defend your views with informed arguments!

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Haakon B. Benestad Jens-Gustav Iversen

Chapter 1: Theory of science

A practical approach.

A popular, albeit misleading, view of science is that it seeks *the truth* about nature. Both the reasoning of philosophers and the experience of scientists have taught us that the final and absolute truth is confined to theology. A more modest goal may be to give the best possible description of nature, preferably as general theories or natural laws. Since such generalisations may be modified or altered in the course of time, a scientific "truth" must always be regarded as preliminary.

Scientific truth is provisional

Consequently, science proceeds by

1) development of new theories (or improvement of old ones) that may explain all observations and obtained data and

2) communication of these theories in a way that convinces the scientific community that they really are the best possible description of nature.

How do we generate reliable theories?

Impressed by the logic of Euclid's geometry, antique and medieval philosophers were satisfied that they could reach true conclusions also in natural sciences by mere deduction from self-evident axioms. Whether or not such conclusions were in agreement with factual observations, could apparently be trivial to them. Medieval scholastics may exemplify a degenerate extreme of this tradition. As a reaction against scholastic speculation, renaissance philosophers (e.g. Francis Bacon, 1561-1626) emphasised the importance of factual observations (empiricism). Bacon assumed that theories or generalisations could be made on the basis of observations only by "induction". In the centuries following it became increasingly obvious that Bacon's concept was also too simple. Nonetheless, "naive inductionism" may still appear in some texts. (Box 1.1).

Firstly, many theories could explain the same set of observations, and it was not possible to distinguish between them by means of induction. Secondly, whereas deduction was based on rigorous logic, generalisation is not logically secure in the same way. As David Hume pointed out: If B follows A in every case you have observed till now, there is no logical necessity to expect that B will follow A the next time. Consequently, Bacon's empirism was revised during the following centuries to develop into what is now called the hypothetico-deductive method.

Box 1.1 Naive inductionism.

The idea that in scientific inquiry inductive inference from antecedently collected data leads to appropriate general principles, is clearly embodied in the following account of how a scientist would ideally proceed:

> If we try to imagine how a mind of superhuman power and reach, but normal so far as the logical processes of its thought are concerned, ...would use the scientific method, the process would be as follows: First, all facts would be observed and recorded, *without selection* or *a priori* guess as to their relative importance. Secondly, the observed and recorded facts would be analysed, compared, and classified, *without hypothesis or postulates* other than those necessarily involved in the logic of thought. Third, from this analysis of the facts generalisations would be inductively drawn as to the relations, classificatory or causal, between them. Fourth, further research would be deductive as well as inductive, employing inferences from previously established generalisations.*

This passage distinguishes four stages in an ideal scientific inquiry: (I) observation and recording of all facts, (2) analysis and classification of these facts, (3) inductive derivation of generalisations from them, and (4) further testing of the generalisations. The first two of these stages are specifically assumed not to make use of any guesses or hypotheses as to how the observed facts might be interconnected; this restriction seems to have been imposed in the belief that such preconceived ideas would introduce a bias and would jeopardise the scientific objectivity of the investigation.
But the view expressed in the quoted passage - I will call it *the narrow inductivist conception of scientific inquiry* - is untenable, for several reasons.

* A.B. Wolfe, "Functional Economics," in the Trend of Economics, ed. R.G. Tugwell (New York. Alfred A. Kuope, Inc., 1924) p. 450 (italics are quoted).

From: "Philosophy of Natural Science" by Carl G. Hempel

In this century this method has been most sharply articulated by Sir Karl Popper.

Briefly, Popper's scheme is the following:

Deduction

Axiom(s)

⇓ Logic

Derived statements

Induction

Theory (generalisations)

↑

Observations (data)

Hypothetico-deductive scheme

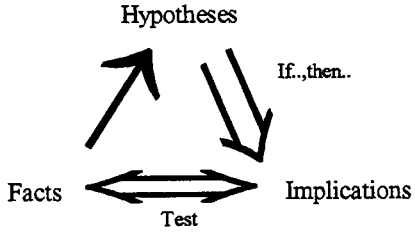

Fig. 1.1

1. You start with a hypothesis that you think may explain certain factual observations. How this hypothesis emerges is not important. Since hypotheses cannot be constructed by logic, you must certainly be able to combine imagination and creativity with scientific knowledge.

2. From this hypothesis you may deduce certain implications by logical reasoning, often in the form "If.. , then.. ".

3. You should then check these implications against reality, i.e. observed data. If the data are not already available (e.g. in the literature), you should seek to find them, in natural sciences often by performing experimental tests. Implications that in no way can be checked, are of no interest in science, as are hypotheses that infer only non-testable implications.

Hypotheses are never verified, just falsified

4. Observations may either agree with your implications or not. If they don't, your hypothesis has been falsified and should be rejected or at least modified. On the other hand, if they do agree, the hypothesis is not verified, but only supported or strengthened. This "verification problem" is an important point.

Even if all the observed data may be "explained" by your hypothesis, other, quite different, hypotheses might offer equally plausible explanations. You can (and should) then strengthen your hypothesis further in two ways. Firstly, you may test other implications, and the more threatening the test is to your hypothesis, the better (Karl Popper's urge, see Box 1.2). Secondly, you should also test the implications of alternative hypotheses. By falsifying them and thereby gradually excluding other explanations of your findings, you will as a consequence gain growing confidence in your own hypothesis.

In this way a hypothesis may be increasingly likely , and you may call it a theory or even a "natural law". But still it can never be "proven". Indeed, even generally

accepted theories and natural laws must be regarded as preliminary generalisations

Box 1.2 The Success of refutation.

Refutations have often been regarded as establishing the failure of a scientist, or at least of his theory. It should be stressed that this is an inductivist error. Every refutation should be regarded as a great success; not merely a success of the scientist who refuted the theory, but also of the scientist who created the refuted theory and who thus in the first instance suggested, if only indirectly, the refuting experiment.
Even if a new theory (such as the theory of Bohr, Kramers, and Slater) should meet an early death, it should not be forgotten; rather its beauty should be remembered, and history should record our gratitude to it - for bequeathing to us new and perhaps still unexplained experimental facts and, with them, new problems; and for the services it has thus rendered to the progress of science during its successful but short life.

From: "Conjectures and Refutations. The Growth of Scientific Knowledge" by Karl R. Popper.

Science advances by the testing of hypotheses

which may be revised in the future, like Einstein's revision of Newton's mechanics (Box 1.3).

According to the hypothetico-deductive scheme, scientific progress requires

1) hypothesis-makers (with imagination, creativity and knowledge),

2) theorists (e.g. mathematicians),

3) experimenters (with practical intelligence and experimental skill).

Since one person rarely fulfils all these requirements equally well, the scientific community benefits from a great variety of talents engaged in the scientific process.

Box 1.3 Scientific Truth.

Scientific truth in the sense I shall describe and explain it below is often thought of as the goal of a scientist's work; though "asymptote" would be the better word, for there can be no apodictic certainty in science, no finally conclusive certainty beyond the reach of criticism. There is no substantive goal; there is a direction only, that which leads toward ultima Thule, the asymptote of the scientist's endeavours, the "truth."
The scientist is in bondage to that conception of truth which has brought it about that aircraft fly and physicians sometimes cure their patients.
The most heinous offence a scientist as a scientist can commit is to declare to be true that which is not so; if a scientist cannot interpret the phenomenon he is studying, it is a binding obligation upon him to make it possible for another to do so. If a scientist is suspected of falsifying or inventing evidence to promote his material interests or to corroborate a pet hypothesis, he is relegated to a kind of half-world separated from real life by a curtain of disbelief; for as with other human affairs, science can only proceed on a basis of confidence, so that scientists do not suspect each other of dishonesty or sharp practice, and believe each other unless there is very good reason to do otherwise.

From: "The Limits of Science" by Peter Medawar

Requirements of a sound hypothesis

As mentioned, to be of scientific interest hypotheses should generate *testable implications*. Science is "the art of the soluble" (Box 1.4). The key word is "testable". The lack of appropriate methods often impedes scientific progress. An apparent scientific revolution in a field is often preceded by the introduction of a new and powerful method. In our opinion too little attention has been given to development of new methods and too little prestige attached to this important task.

Hypotheses must be testable

Box 1.4 The Art of the Soluble.

Following the lead of Bismarck and Cavour, who described the art of politics as "the art of the possible," I have described the art of research as "the art of the soluble."
By some people this was almost wilfully misunderstood to mean that I advocated the study of easy problems yielding quick solutions - unlike my critics, who were studying problems of which the main attraction (to them) was that they could not be solved. What I meant of course was that the art of research is that of making a problem soluble by finding out ways of getting at it - soft underbellies and the like. Very often a solution turns on devising some means of quantifying phenomena or states that have hitherto been assessed in terms of "rather more," '-rather less," or A-a lot of," or - sturdiest workhorse of scientific literature - "marked" ("The injection elicited a marked reaction"). Quantification as such has no merit except insofar as it helps to solve problems. To quantify is not to be a scientist, but goodness, it does help.

From: "Advice to a Young Scientist" by Peter Medawar

Design of hypotheses is closely related to development of methods and design of experiments. Many hypotheses are too ambiguous to be ruled out, and thus all too easily supported. Ideally, you should construct two alternative, mutually exclusive, hypotheses, and then design the "crucial" experiment that distinguish between the two. This procedure is certainly very efficient, because you do not waste time and energy on inconclusive experiments (Box 1.5).

Box 1.5 Strong Inference.

Certain systematic methods of scientific thinking may produce much more rapid progress than others.

Strong inference consists of applying the following steps to every problem in science, formally and explicitly and regularly:

1) Devising alternative hypotheses.
2) Devising a crucial experiment (or several of them), with alternative possible outcomes, each of which will, as nearly as possible, exclude one or more of the hypotheses.
3) Carrying out the experiment so as to get a clean result.
4) Recycling the procedure, making subhypotheses or sequential hypotheses to refine the possibilities that remain; and so on.

It is like climbing a tree. At the first fork, we choose - or, in this case, "nature" or the experimental outcome chooses - to go to the right branch or the left; at the next fork, to go left or right; and so on.
It is clear why this makes for rapid and powerful progress. For exploring the unknown, there is no faster method; this is the minimum sequence of steps. Any conclusion that is not an exclusion is insecure and must be rechecked. Any delay in recycling to the next set of hypotheses is only a delay.

John R. Platt

A hypothesis should have *explanatory power*. It should thus be connected to the generally accepted set of theories in the field. In physiology and derived disciplines this often means reductionism; the scientific programme is to explain biological processes by means of physical and chemical laws. A biological explanation may for example refer to evolutionary theory in which biological phenomena should appear functional (improve the animal's "reproductive success" i.e. its potential to get surviving offsprings).

Sometimes you have to choose between two (or more) hypotheses, and you are not able to exclude any of them with tests. The common rule is to choose the simplest (or least complicated) hypothesis ("Ockham's razor", Box 1.6). One problem nevertheless remains : There is no generally accepted rule for deciding what is simple and what is complicated.

Box 1.6 Ockham's razor.

The principle of parsimony, whose frequent use by Ockham gained it the name of "Ockham's razor", was employed as a methodological principle of economy in explanation. He invoked it most frequently under such forms as "Plurality is not to be assumed without necessity" and "What can be done with fewer [assumptions] is done in vain with more"; he seems not to have used the formulation "Entities are not to be multiplied without necessity." The principal use made by Ockham of the principle of parsimony was in the elimination of pseudo-explanatory entities, according to a criterion he expresses in the statement that nothing is to be assumed as necessary, in accounting for any fact, unless it is established by evident experience or evident reasoning, or is required by the articles of faith.

WILLIAM OF OCKHAM (c. 1285-1349), the most influential philosopher of the fourteenth century.

Ockham's razor

Defenders of declining theories tend to rescue their ideas from falsifying observations by proposing *ad hoc* hypotheses. As mentioned above it is quite normal and commendable to modify a hypothesis when new facts make it necessary. In fact this is fundamental for the scientific progress. An *ad hoc* hypothesis, however, diverges from modified hypotheses in being more complicated and less testable and thus hampers rather than facilitates scientific development.

Scientific revolutions

Popper's scheme for scientific progress seems logical and realistic, and should be a guideline for working scientists. The problem is that if you are looking back over the history of science, you will find that science in fact did not always proceed according to this framework.

Thomas S. Kuhn has introduced the word *paradigm* to describe a sort of conceptual consensus among scientists in a certain field. Science is rather trivial in periods when *one* paradigm is dominating, he claims. However, one paradigm replaces another by "scientific revolutions", during which a new way of thinking is introduced, and after a period many scientists are convinced that the new paradigm is superior to the old one. Established scientists, though, will often tend to stick to the old way of thinking. The new paradigm may therefore not become the dominating one before these people have left the scientific arena.

To a biomedical scientist Kuhn's description appears a bit exaggerated, and it is not easy to recognize his important elements in one's own experiences. Nevertheless, some of his points make sense to us. Scientists, as other human beings, are inclined to habitual thinking, and will hardly believe in theories that ruin or undermine basic pillars of their established conceptual basilica. They tend to be more sceptical of hypotheses that do not fit into current scientific "knowledge", than of hypotheses that confirm their own ideas. If we look back over the history of science, we will find that people with revolutionary thoughts had to work hard (or wait long) to get their ideas generally accepted, even though the experimental evidence favoured them (e.g. Semmelweiss, Mendel, Rous, McClintock). The sceptical establishment had to expire before the new ideas were generally accepted.

It is certainly not easy to recognise one single ruling paradigm in contemporary biomedical science. Apparently progress takes place more by *evolution* - where old theories are more or less continuously replaced by new (and better) ones-, than by *revolution*, where the old paradigm is suddenly replaced by a completely new (but not necessarily better) one. One reason for this may be the lack of new unifying concepts or all-covering theories in this field at the moment. Progress is frequently characterized by mapping new land or finding small bits in a large puzzle.

New paradigms don't overcome before the supporters of the old ones have passed away

Limits to (biomedical) science

Biomedical science, as all natural science, strives to give the best possible description of reality. The philosophical problem: *What is reality?,* is set aside. We who do research in this field, take for granted that phenomena which we observe with our senses or indirectly with instruments are factual. Phenomena that are not generally accepted as factual or real, may not be attacked by the scientific method dealt with in this chapter. Nevertheless, such phenomena may be essential to the individual, to his way of looking at his life and at his world. Science cannot help us to solve religious or metaphysical problems.

Explanations in natural sciences are of a "causal" nature. We explain the reaction chain of the observed mechanisms by general "natural laws". A and B gives C, and if D is present , E will occur etc. Whether the causal relationship is deterministic or stochastic, such descriptions in any case leave *no role to the human personal "soul"* and its possibly free will.

In biology causal analysis may be supplemented or replaced by *functional* explanations. Teleology (Greek *telos*: end, purpose) gives a bad taste to many experimental biologists, but it should not. The operation of natural selection, as phrased by Darwin, means that only organisms that are functionally able to grow up and get progeny, will survive. Therefore, structural traits or physiological mechanisms found in various species to day should in principle be - or have been in the course of evolution - advantageous for the organisms' ability to produce surviving offspring. Biologists could thus profitably look for both cause and functional significance.

In the social sciences (as also in the humanities), on the other hand, causal and functional explanations are often felt to be insufficient. Human behaviour can appear "irrational" to an unfamiliar observer, and yet become meaningful when the cultural context of the participant(s) is taken into consideration. Therefore behavioural sciences may use *hermeneutic* methods to interpret, give meaning to, social transactions and human conduct. You want to *understand* rather than *explain* a phenomenon.

If you observe a person raising his right hand in a special manner, you may *explain* the movements physiologically (causally) on the basis of nerve impulses and a complex pattern of muscle contractions. You could also *explain* them functionally as being intended to, for example, prevent sunshine from blinding the eyes. But you may also *understand* the movements as a sort of salutation.

Such understanding must in some way be founded on general principles ("covering-laws"), which in the end are empirically based. Thus, hermeneutic explanations may also be analysed within the hypothetico-deductive scheme: *If* a certain interpretation is "true", *then* you should expect to find a certain behaviour in a certain setting. If the movement of the right hand denotes a salutation, you should expect to recognise the same gesture in comparable situations. If you never find this, your interpretation has been falsified. In practice, however, it may be intricate with such methods to choose between alternative explanations. Falsification in Popper's sense may be impracticable. In fact, Popper did argue against the scientific value of for example psychoanalysis (and Marxism) for this reason. Consequently, although medical practice has to rely on social sciences as well as on natural sciences, the former may constitute an uncertain base for medical routines.

A practical approach

Below you will find extracts from an article written by James L. Gowans (J.Physiol.(1959) 146, 54-69), dealing with the following observation and problem:
Blood lymphocytes are replaced at least 11 times a day by cell entry from the two large lymph ducts. From where do these cells come, and where do they go?
Gowans forwarded a *hypothesis* that may explain these observations: *"Lymphocytes recirculate between lymph and blood"*. We shall go through the experiments presented with two questions in mind:

1. How did he support this hypothesis?
2. He also referred to two alternative hypotheses. How did he try to falsify them?

THE RECIRCULATION OF LYMPHOCYTES FROM BLOOD TO LYMPH IN THE RAT

In all the mammalian species which have so far been examined, large numbers of lymphocytes enter the blood each day from the main lymphatic vessels in the neck (reviewed by Yoffey & Courtice, 1956).

Whaler & Widdicombe (1936) estimated that the average time spent by lymphocytes in the blood of the rat was probably less than one hour. Many theories have been advanced to explain this rapid turnover of lymphocytes in the blood but none of them has good experimental support (Yoffey & Courtice. 1956: Florey & Gowans, 1958; Trowell, 1958).

In a previous paper (Gowans, 1957 a) it was shown that in order to maintain the output of lymphocytes from a thoracic duct fistula in an unanaesthetized rat it was necessary to re-infuse continuously into the blood all the lymphocytes which issued from the fistula. When either cell-free lymph or lymph which contained killed lymphocytes was re-infused a profound fall in the output of cells from the thoracic duct eventually occurred similar to that described by Mann & Higgins, (1950) in rats which received no intravenous replacement of either cells or fluid. The aim of the present investigation was to discover the mechanism by which the intravenous infusion of living lymphocytes influenced the output of lymphocytes from the thoracic duct. Essentially, this aim was achieved by determining the fate of thoracic duct lymphocytes after their transfusion into the blood.

METHODS

In this section J.L. Gowans describes how he is able to drain lymph continuously from the thoracic duct of the animal, and at the same time infuse intravenously cell suspensions or solutions.

RESULTS

Lymphocyte transfusion on the fifth day after thoracic duct cannulation

The earlier experiments (Gowans. 1957 a) suggested that the output of lymphocytes from the thoracic duct was in some way determined by the number of lymphocytes which entered the blood. If this were so, a suitable transfusion of lymphocytes would be expected to restore to a high level the low output of cells which results from the prolonged drainage of lymph from the thoracic duct. The experiments illustrated in Fig. 2 confirmed this expectation.

A continuous intravenous transfusion of lymphocytes from a series of donor rats was started at the end of the 4th day after cannulation of the recipient rat. All the lymph from the thoracic duct of the recipient rat was collected for cell counts and then rejected. When the transfusion was between litter mates of the same inbred strain, the output of cells from the recipient rat rose progressively and levelled off at a point below the initial output, but within the range of normal values for the output in rats of this age and strain. In contrast. a transfusion from hooded into albino rats resulted in a slower rise in output which terminated abruptly on the 4th day. Despite the continued transfusion of lymphocytes, the output fell progressively to a level below that at the start of the transfusion (Fig. 2). Thus, the fall in the lymphocyte output described by Mann & Higgins (1950) can be reversed by transfusing lymphocytes from one rat into another, providing the genetic disparity between the donor and the recipient animals is not too great.

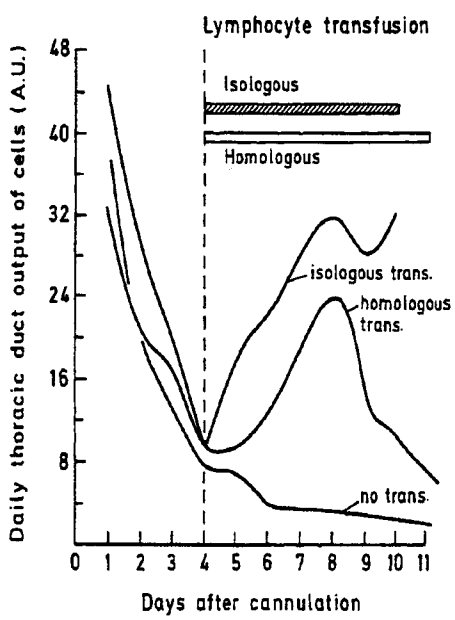

Fig. 2

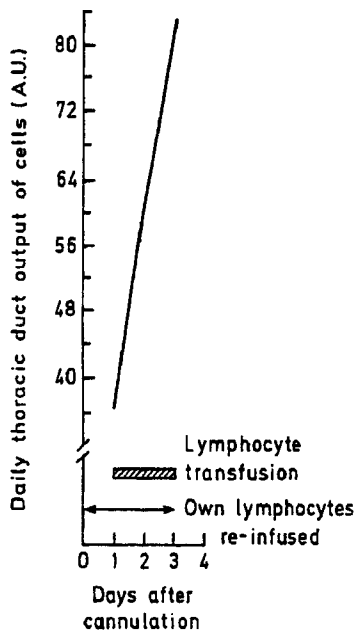

Fig. 3

Fig. 2. Output of lymphocytes from the thoracic duct of three albino rats.
Fig.3. Output of lymphocytes from the thoracic duct of a rat which received an intravenous transfusion of lymphocytes from two successive donor rats.

In this section Gowans formulated - quite explicitly - a hypothesis to explain earlier experimental findings: Output of lymphocytes from the thoracic duct (t.d.o.) is dependent

on the amount of lymphocytes infused into the blood (b.i.); *t.d.o= f (b.i.)*. He then deduced an implication ("If this were so, ..") which was subsequently tested (Fig. 2). The observations supported his hypothesis, but it had to be modified : *t.d.o. = f (b.i.), provided the genetic disparity .... is not too great.*

He then forwarded another modification of his hypothesis : *t.d.o. = f (b.i.); but not beyond a "physiological level" of output.*

The implications were tested (Fig. 3), and this modification of the hypothesis was falsified:

> *Lymphocyte transfusion on the second day after thoracic duct cannulation*
>
> Since the first day's high output of lymphocytes can be maintained or restored by the intravenous infusion of lymphocytes, it might be supposed that there is a more or less constant physiological level to which the output always adjusts itself, given a sufficient input. Fig. 3 shows that this is not so. A vigorous transfusion of lymphocytes, together with the continuous reinfusion of all the lymphocytes from the recipient's own thoracic duct, increased the output to a value more than double that of the first day.

The previous hypotheses that were tested, remained on an experimental level. Now Gowans wanted to generalise to a physiological level. He presented three (mutually exclusive) hypotheses to explain his findings. Two of them had a common implication: After reinfusion of lymphocytes to blood, newly formed cells should appear in the thoracic duct. This was subsequently tested:

> *Mechanism by which the input of lymphocytes into the blood increases their output from the thoracic duct*
>
> The transfused cells could cause an increase in the output from the thoracic duct by three possible mechanisms: (1) Chemical substances derived from the transfused lymphocytes might be essential for the formation of new lymphocytes in the lymph nodes of the recipient animal. (2) The transfused cells might repopulate the lymph nodes of the recipient animal and, by successive cell divisions, give rise to a progeny of new lymphocytes. Or (3), the transfused lymphocytes might recirculate from the blood back into the thoracic duct lymph, i.e. the transfused cells and the extra cells emerging from the thoracic duct are identical. Mechanisms (1) and (2) would both operate by initiating a burst of new lymphocyte production in the recipient animal.
>
> *Formation of new lymphocytes.* The possibility that the intravenous transfusion of lymphocytes had initiated the production of new lymphocytes in the recipient animal was tested by infusing tritium-labelled thymidine into one femoral vein of a rat while lymphocytes were transfused simultaneously into the other. Autoradiographs were prepared from successive 4 hr samples of lymphocytes collected from the thoracic duct of the recipient. If the extra lymphocytes which emerged from the thoracic duct had been formed after the start of the transfusion, their DNA would have become labelled. The results of the experiment are shown in Fig. 5 and Table 2. During the rising phase of the output more than 90 % of the cells in the lymph were small lymphocytes; 2% or less of these contained labelled DNA. This very low proportion of labelled small

lymphocytes was not due to an inadequate level of tritium-labelled thymidine in the animal, since, during the same period, more than 90 % of the larger lymphocytes, which are known to divide in the thoracic duct lymph, became labelled. It was concluded that almost all the small lymphocytes from the thoracic duct of the recipient rat were 'old' cells, in the sense that their DNA had been formed before the start of the transfusion. This experiment rules out mechanisms (1) and (2) unless it is held that 'new' lymphocytes can be produced by some process that does not involve the synthesis of new DNA at any stage.

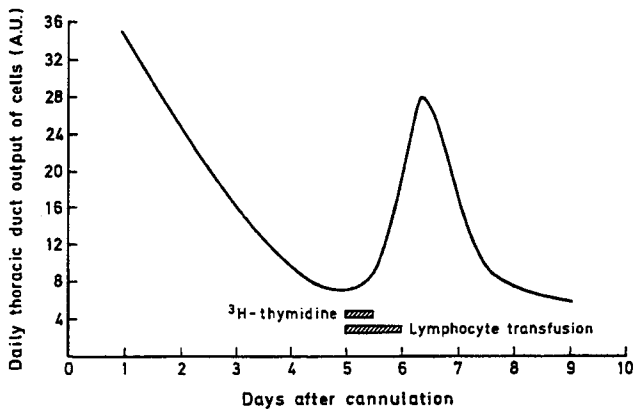

Fig. 5

Fig. 5. Output of lymphocytes from the thoracic duct of a rat which received an intravenous transfusion of lymphocytes during the 6th day after cannulation. Tritium-labelled thymidine was infused intravenously during the first 12 hr of the transfusion.

TABLE 2. Proportion of small lymphocytes and of tritium-labelled lymphocytes in autoradiographs prepared from the collections of lymphocytes in Fig.5

Collection no.	Small lymphocytes (%)	Labelled lymphocytes	
		Small (%)	Medium and large (%)
1	88.8	0	40
2	88.8	0.1	66
3	92.4	0.4	88
4	93.0	2.0	93
5	94.3	0.9	97
6	96.7	1.2	97
7	94.9	1.6	93
8	97.0	0.4	91
9	95.6	1.1	91
10	94.3	1.0	77

After having falsified the two competing hypotheses, Gowans proceeded to gain support for his own "recirculation" hypothesis. If lymphocytes recirculate, then the same cells that are infused into the blood stream, should reappear in the thoracic duct. This was tested with radiolabelled cells, and seemed to be the case (Fig. 6). This observation might,

however, have a trivial explanation, and this "alternative hypothesis" was tested in a control experiment (Fig. 7) and falsified.

Recirculation of lymphocytes. The exclusion of mechanisms (1) and (2) strongly suggested that the lymphocytes which were transfused into the blood found their way back into the thoracic duct lymph. This hypothesis was tested directly by transfusing ^{32}P-labelled lymphocytes into a rat and following the appearance of radioactivity in the cells emerging from the thoracic duct.

The transfusions were begun at the end of either the first or the 5th day after cannulation of the recipient rat (Fig. 6). In each case a continuous rise in the radioactivity in the cells issuing from the thoracic duct started during the first 4 hr. The radioactivity curves had a time course which was similar to that of the cell output curves illustrated in Fig.5.

It could be argued that the radioactivity in the thoracic duct lymphocytes was due to leakage of ^{32}P from the transfused cells and its uptake by the recipient rat's own lymphocytes. However, when the amount of radioactivity which had been transfused into a rat as ^{32}P-labelled lymphocytes (Fig. 6), was infused into another rat as inorganic phosphate (Fig. 7), the cells from the thoracic duct contained a negligible amount of radioactivity.

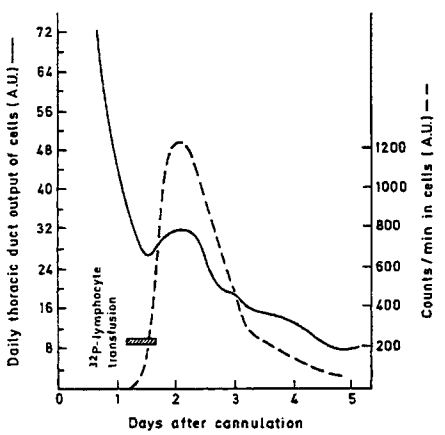

Fig. 6

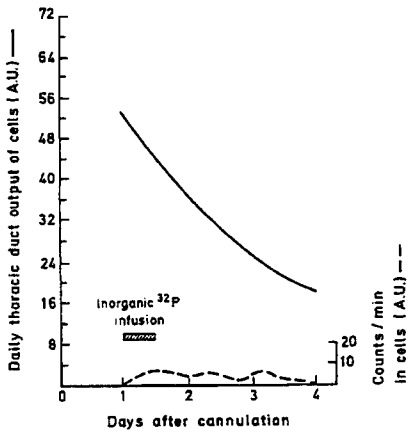

Fig. 7

Fig. 6. Output and radioactivity of lymphocytes from the thoracic duct of a rat which received an intravenous transfusion of ^{32}P-labelled lymphocytes. In this experiment the number of cells transfused was insufficient to cause a very noticeable change in the output of lymphocytes from the thoracic duct, for the recipient's own output was falling rapidly. When the transfusion was begun later the radioactivity curves and the cell-output curves followed each other closely in the same animal.

Fig. 7. Output and radioactivity of lymphocytes from the thoracic duct of a rat which received an intravenous infusion of ^{32}P as inorganic phosphate. Total counts/min in infused phosphate was the same as the cell-bound radioactivity of fig. 6.

The proportion of newly formed lymphocytes normally present in thoracic duct lymph

The idea that lymphocytes continuously recirculate from the blood to the lymph would provide a satisfactory explanation of the apparently high turnover of

lymphocytes in the blood of the normal animal. This explanation would become even more plausible if it could be shown that the number of new lymphocytes formed each day was only a small fraction of the total number which normally issues from the thoracic duct.

Mann & Higgins (1950) showed that the output of lymphocytes from the intestinal lymph duct in the rat was almost equal to that from the thoracic duct. Thoracic duct lymphocytes in the rat, therefore, are gathered from the lymphatic bed drained by the intestinal duct.

It seemed inconceivable that the observed output of lymphocytes from the thoracic duct could be produced each day by the relatively small amount of lymphoid tissues in the intestinal lymphatic bed. Accordingly, an estimate was made of the proportion of newly formed lymphocytes in the thoracic duct of a freshly cannulated rat. Tritium-labelled thymidine was infused intravenously into a rat for the first 12 hr after the cannulation of its thoracic duct. Successive 24 hr samples of lymph were collected for a total period of 4 days. The rat received no transfusion or re-infusion of lymphocytes. Autoradiographs were prepared of the cells in each sample of lymph and of histological sections of the mesenteric and caecal lymph nodes at the end of the experiment. The results are shown in Table 4. During the first 2 days, when sufficient tritiated thymidine was present to label over 90 % of the large and medium lymphocytes, only about 1% of the small lymphocytes contained labelled DNA.

TABLE 4. Proportion of tritium-labelled lymphocytes in autoradiographs prepared from successive 24 hr collections of lymphocytes from thoracic duct. Tritiated thymidine was infused intravenously at 20 Fc/hr during the first 12 hr after cannulation

Day after thoracic duct cannulation	Labelled lymphocytes	
	Small (%)	Medium and large (%)
1	0.8	94
2	1.3	92
3	1.5	79
4	0.6	22

The very small proportion of labelled small lymphocytes detected in this experiment would still be consistent with a high rate of formation of new lymphocytes if the newly formed cells remained in the lymph nodes for some days before their release into the lymph. However, the autoradiographs of the lymph nodes taken from the rat at the end of the experiment again showed extremely few labelled small lymphocytes.

In this last Results section Gowans tested the three hypotheses ("mechanisms") against each other. The two alternative hypotheses implied the presence of a high proportion of newly formed lymphocytes in thoracic duct, whereas his own "recirculation" hypothesis implied the opposite. The observations obviously favoured the latter. (This experimental design may serve as an example of "strong inference", see Box 1.5).

DISCUSSION

The route taken by lymphocytes in passing from the blood into the lymph is not known. Sj`vall (1936) argued from circumstantial evidence that

> lymphocytes were normally formed at a low rate in the lymph nodes and that, in order to maintain the output of lymphocytes from the lymph ducts, they must recirculate from the blood to the lymph. He proposed that lymphocytes entered the tissue spaces and returned to the blood via the peripheral lymphatics. A slow recirculation of this kind probably does take place through most of the tissues of the body, but it is not large enough to account for more than a fraction of the thoracic duct output. Thus Yoffey & Drinker (1939) found only a few lymphocytes in the peripheral lymph from the hind limbs of dogs and cats, and Baker (1933) noted small numbers in the peripheral lymphatics of the cat intestine. Since large numbers of lymphocytes are added to lymph as it passes through a lymph node (Baker, 1933), and new lymphocytes are formed at a low rate in the nodes, it is possible that the main channel of recirculation is through the lymph nodes.

In the omitted part of the Discussion Gowans found support for his hypothesis in the literature.

The observation that peripheral lymph contains only a small number of lymphocytes, urged him to extend his hypothesis: Recirculation of lymphocytes from blood to lymph takes place mainly in the lymph nodes. Testing of this hypothesis would then be his next goal.

Summing up

How Gowans tested his own hypothesis:

If lymphocytes recirculate, *then* he expected that lymph drainage should lead to a fall in lymphocyte output from the drain. Reinfusion intravenously of drained cells should re-establish the lymphocyte output. Experiments showed that this was the case (Figs. 2 and 3). Testing of another implication is presented in Fig. 6, and a trivial explanation of this observation was excluded by a control experiment (Fig. 7).

How Gowans falsified alternative hypotheses:

If reinfusion could lead to proliferation of lymphocytes (and thereby explain the increased output from the thoracic duct), *then* one should expect an increased fraction of postmitotic cells in the lymph drained after cell reinfusion. The experiments did not show this (Figs. 6 & 7, Table 4.)

This article represented, in fact, *an example of a "scientific revolution" in a small scale*: Professor Yoffey, to whom Gowans referred in the text, was an established haematologist in 1958. He was a strong defender of one of the alternative hypotheses mentioned, and an equally strong opponent of Gowans' theories. He was, in fact, so influential that Gowans had problems getting this article published. By hindsight, Gowans' arguments are convincing, but obviously not compatible with the haematological paradigm of Yoffey.

Checklist 1.1: Theory of science.

1. What is your main working hypothesis, and how do you plan to test it? Can you design mutually exclusive hypotheses?

2. Could your hypothesis have other testable implications?

3. Can you envision that new methods, procedures or experimental designs might change apparently non-testable implications to testable ones? Do you consider it worth while to engage in such developmental or methodological work?

4. Could your findings possibly be explained by alternative hypotheses? How can they be tested (falsified)? Or: Which control experiments have to be done to exclude that your results have a trivial explanation?

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Exercise:

Reread the extracts from the article by James L. Gowans.

1) Try to fit each of Gowan's experiments into a hypothetical scheme as outlined in this chapter. How did Gowans strengthen his hypothesis?
2) Gowans experienced difficulties when trying to publish this paper (and others related to this one). Influential journal editors rejected them. Can you find any clues in the text as to the reason for this?

Chapter 2: The beginning

About yourself

Why do you want to do laboratory research work? Because you need scientific merits to realise your career aspirations? Because you are genuinely interested in how things in nature function? Presumably two main pillars of scientific motivation are ambition and curiosity; both are legitimate, but we think a scientific career will be more satisfying for you if curiosity is the stronger one. Certainly many successful scientists cite curiosity as a major driving force in their work, and we know from personal experience that the satisfaction of curiosity is a rewarding experience. Are there other personal qualifications needed to be a good scientist? We believe that a firm will to find the true answer to important problems is the only real prerequisite. You should also be willing to work hard - for a long time!

Curiosity should be stronger than ambition

Competent scientists who have contemplated this question of personal qualifications agree that intelligence is not a limiting factor, provided it is above a certain threshold. A particular kind of professional training may not be essential either. Joshua Lederberg started his work on genetic recombination in bacteria - which later won him the Nobel Prize - while a medical student; and we know top scientists who began their careers as laboratory technicians. Perhaps you will end up learning what you need to know only when the need arises? (see Appendix).

A strong motivation is needed

Whether you start working at a university, a research institute, a hospital laboratory, or in industry is also probably unimportant. However, the kind of mentor or supervisor you get, may be decisive. He or she should of course be fully competent, but also have the spare time and inclination to instruct you and discuss with you. If you set out solving your own specific scientific problem, it may not be easy to find optimal working conditions. Sometimes it is better to start as a collaborator in a project group that has a good leader, who can stimulate everyone to yield their best.

A competent and motivated supervisor is one of the keys to success

The kind of workplace you have chosen may entail duty work – such as teaching - which might distract from your "real work". Teaching and related activities, however, can also serve as a life saving device when you feel utterly unsuccessful due to lack of scientific progress. Sooner or later the scientist's disease, Acedia, will probably hit you (Granit, 1941). Acedia means "bitter distaste" and arises when enthusiasm succumbs. Your working pace slows down. The experiments - previously exciting - have been converted to soulless routines,

The scientist's disease, Acedia

and everything of importance seems to have been done by others. You are reluctant to communicate your research findings, and you may even feel physically ill. There are two main causes of Acedia. You may have concentrated too heavily and exclusively on your scientific work. Then the disease is akin to the burnt-out syndrome, and the treatment is to live a more normal life. The other cause of Acedia is too much ambition. The cure in this case is to consider work a goal in itself and not just a means to serve your ambitions.

A compromise between egocentricity and sociability

You should be aware that a balance must be struck between egocentricity and sociability - between scientific work and self-fulfilment, on the one hand, and a social and family life on the other. If you want to be a top scientist, you must engage very intensively in your scientific pursuits - as an elite athlete or artist has to practise her talents. If you are not careful, this may jeopardise your relations with spouse, family, and friends.

Your goals

Discoveries, improvements, confirmations and refutations

Every scientist wants to make great discoveries. Alfred Nobel's great awards in science are awarded for definable discoveries. However, in chemistry, of which Nobel had first-hand experience as an inventor of smokeless powder and dynamite, he did allow that a prize could also be given for improvements. Indeed, his own major contribution was an improvement: He made the use of dynamite nearly foolproof by adding kieselguhr to the original "blasting oil" (nitroglycerol). Consequently, you may be satisfied by making improvements! You should probably also feel contented if you succeed in delivering experimental evidence for a view that is probable, but not yet established. An example of this most common type of discovery, namely the refutation of all but one among a number of alternative hypotheses, is the following: The theory of chemical transmission at synapses had been suggested by T.R. Elliot in 1905, but it was not proved until very much later, by Loewi and Dale (Granit, 1972).

There are other kinds of scientific work that do not qualify as the making of discoveries, but which are nevertheless as valuable as discoveries for the progress of scientific knowledge. The mathematical elaboration of hypotheses and the construction or adaptation of apparatus or methods to test them, may rely mostly on craftsmanship. Moreover, the entrepreneurial qualifications demanded by team works and by "big science" are also something different. The making of a chance discovery (Fleming's observation of the bacteriocidal properties of the

Penicillium notatum mold) is a very different process than analysing the significance of a discovery and pursuing its application (the industrial production of penicillin). The scientific enterprise includes a multitude of different goals, and a variety of human personalities and qualifications is required to pursue them (see Appendix).

Irrespective of your kind of scientific work, it will help if you have the alertness to see the unexpected and to draw fruitful analogies between various areas of research. Undoubtedly, the generation of certain kinds of hypotheses and their testing does require imagination (Root-Bernstein, 1985) and intuition. But Pasteur did remark that chance only favours the prepared mind. So, it may be necessary to prepare your mind by hard work! Austin (1978), who has retold the adventure of the three princes of Serendip and elaborated on the concept of serendipity, also proposed a new name for the variety of chance observation that springs from a firm basis of knowledge. He called this propensity altamirage and distinguished it from serendipity (Box 2.1).

Serendipity and altamirage

Box 2.1 Various aspects and kinds of good luck

	Elements involved	*Personality traits*
Chance I	**"Blind" luck.** Chance happens, and nothing about it is directly attributable to you, the recipient.	None. («Serendipity»)
Chance II	**The Kettering Principle.** Chance favours those in motion. Events are brought together to form «happy accidents» when you diffusely apply your energies in motions that are typically non-specific.	Curiosity about many things, persistence, willingness to experiment and to explore. («Serendipity»)
Chance III	**The Pasteur principle.** Chance favours the prepared mind. Some special receptivity born from past experience permits you to discern a new fact or to perceive ideas in a new relationship.	A background of knowledge , based on your abilities to observe, remember, and quickly form significant new associations. («Serendipity»)
Chance IV	**The Disraeli principle.** Chance favours the individualised action. Fortuitous events occur when you behave in ways that are highly distinctive of you as a person.	Distinctive hobbies, personal life styles, and activities peculiar to you as an individual, especially when they operate in domains seemingly far removed from the area of the discovery. («Altamirage»)

Modified from Austin (1978)

The scientific problem and how to approach it

First of all, your scientific problem should be an *important* one. Its solution should make a difference. It will probably take a lot of time and effort, so avoid trivialities.

Sir Peter B. Medawar, nobelist and philosopher of science, has claimed that science is the art of the soluble. That means, your hypotheses or solutions to your scientific problems should be *testable.* Really testable, in a decisive way, so that you don't end up with "a so-what-paper". It is important that you test as directly as possible the essence of your hypothesis and not some more or less logical consequences or implications elaborated from it.

Occam's razor

Try to use the principle of Occam's (Ockham's) razor when you formulate your working hypothesis; i.e. choose the simplest hypothesis that can explain all the facts that are the premises for your own work. Then go ahead - learn the necessary experimental techniques and read the literature to be sure that your problems are really unsolved ones.

Importance, testability, congruence, and reproducibility

It is of prime importance that your experiment or intervention be compared with appropriate controls (control phases, control subjects, etc.), so that *only one difference* - i.e. the intervention or substance you want to examine, remains between the test and the control. Equally important: The results should be reproducible! This requires careful planning and documentation. There are therefore four guidelines for your scientific work: importance, testability, congruence between test and control (except for one factor), and reproducibility.

Premature problems, bandwagonry, and postmature problems

A few more words about the choice of scientific problems. Scientific problems are sometimes classified as pre- or post-mature, depending on whether the tools necessary to solve them are available. Some premature problems ripen very quickly when a technological innovation suddenly makes possible a new line of attack. It is often thought that technology is founded upon science. But a more realistic view is that there is an interplay and mutuality between the two, so that technological breakthroughs may advance science, as well as vice versa (Barnes, 1985). New apparatus and techniques may lead to a reorientation of scientific research, and this is legitimate when it helps to solve problems of long standing. However, technique oriented research - to the exclusion of problem-oriented research - may easily lead to bandwagonry and trendy, opportunistic, and unoriginal work. The ultimate challenge for the alert and penetrating researcher is to discover – and solve - important postmature problems.

Reading the literature

Read selectively!

The scientific literature is enormous. It easily overwhelms you ! Yet you have to be knowledgeable to a certain degree. To make the job easier, some division of labour concerning reading could be arranged within your peer group, e.g. with regular literature seminars. Otherwise, it is probably good advice to read thoroughly a couple of general science journals with good and authoritative reviews and overviews (like Nature and Science), and then perhaps five high-prestige journals in your field of research. Additionally, you should arrange for some continuous or periodic computer-based literature retrieval, so that you don't overlook the information that might be valuable when you are going to write your report or article.

Literature storage

You also need a good archiving or filing system. The minimum requirement if you use the old-fashioned index card system, is to have one archive arranged by main themes and one arranged by first author. A much more versatile system, which we recommend (see Chapter 8), is computer based filing. You can then rapidly search for all authors and all kinds of index words, and easily find your reprint or article copy, since they can be given an identification number before storage.

Selection of methods and techniques

It is very important to choose optimal methods and experimental subjects. Perhaps the general approaches are so firmly established in the laboratory you have joined, however, that a newcomer has difficulty changing them. It takes courage to switch to an unconventional methodology. Not only can it require more work than is easily anticipated to establish new techniques; but there may also be resistance to change because lots of basic or background information has already been accumulated on the standard preparation. It is, however, important, and often elegant as well, to secure your main points with the results of diverse approaches. So do not be afraid to change if the problem dictates change!

It is hard to "leave an winning team" - even if it is not winning any longer

One way to apply a new preparation to your problem is to collaborate with another laboratory, where the relevant techniques are routine. If you have to do it yourself, don't waste your time trying again and again to repeat the specialists' procedures as described in the Materials and Methods sections of their articles.

Collaborate with competent peers!

Rather you should try to raise the money necessary to go to their laboratory and see and learn the procedures yourself. That way, you may also be able to obtain samples of critical reagents that are known to be effective, thus facilitating the localisation of possible faults in your own procedures.

Can your problem best be solved with human volunteers, or should unanaesthetised or anaesthetised animals be used? Or perhaps an organ preparation, a tissue slice, a cell culture, or a subcellular system will be the appropriate choice? Remember that the higher level you choose, the higher the probability that your results are both physiologically meaningful and misleading, in the sense that you cannot control the influence on your measured variables as closely as you would wish. For example, if you inject substance A to an experimental animal, it may cause secretion of hormone B. which effects C, the response recorded. Thus, you are - at least in a way, wrong in concluding that A caused C. Furthermore, C may hardly be detectable - or not at all - because homeostatic mechanisms of the animal have been evoked to conserve the steady state. Finally, if A was a monoclonal antibody intended to bind to B and thereby offset the physiological effects or roles of B, no inhibition may be detectable if parallel regulatory mechanisms can take over B's normal roles. There are many examples of such potential redundancy among physiological mechanisms (Box 2.2), one being the numerous chemotactic factors generated in inflamed tissues to attract leukocytes. It is often necessary to start out at the subcellular or cellular level, in vitro, and only much later advance to the organ or intact animal levels, to perform meaningful physiological experiments.

The choice of appropriate level of complexity - from subcellular system to the intact organism

Needless to say, for ethical, technical, and economical reasons experiments should be done - or at least initiated - on animals rather than man, provided the animal model is relevant, so that results obtained can be applied to the human case. We shall have more to say about experimental animals in Chapter 3.

Scott and Waterhouse (1986) have pointed out that sometimes man has to be the experimental subject, for example when you want to evaluate new drugs or medical treatments. The same applies of course to epidemiological research. The relative virtues, drawbacks, and pitfalls of transverse population studies, as well as retrospective and prospective longitudinal studies, are beyond the scope of this book.

Box 2.2 When expected results are not found

As further examples, if a single carotid body is rendered functionless, three other peripheral chemoreceptor areas and the central chemoreceptors are still present to monitor and so control ventilation and gas tensions in the blood and cerebrospinal fluid. Similarly, the source of 'ergoreceptors' - neural inputs from an exercising limb has often been investigated by cutting nerves from the muscle, tendon, ligaments, cartilage or surrounding skin, etc. If, as is intuitively likely, the CNS makes use of all ascending information, removal of one small part is unlikely to make much difference except possibly transiently .

There is a second and related reason why findings that disagree with predictions do not necessarily disprove a hypothesis. When attempts are made to produce a change in some physiological system, homeostatic mechanisms will 'correct' any imposed 'anomalies' by negative feedback mechanisms. Thus, if as a result of stimulating or cutting an afferent neural pathway from an exercising limb to the responses to exercise *were* modified, then chemoreceptors would be likely to correct any change in ventilation fairly soon.....

As a further example, the effect upon heart rate and vascular smooth muscle of noradrenaline is far less marked and shorter-lived when injected intravenously rather than added to an isolated heart or artery preparation. It would be incorrect to infer from this that noradrenaline acted through different mechanisms *in vivo* from *in vitro;* in the former case, metabolic removal is more rapid and homeostatic mechanisms are present to oppose any changes resulting from direct effects upon the heart and blood vessels.....

However, it would be wrong to infer that no such correcting mechanisms existed in *in vitro* preparations, rather they exist at a more local level. Thus, if an isolated organ is being perfused by a medium containing a vasoconstrictor drug, there seems to be no reason to believe that, should tissue ischaemia begin to arise, local changes in arteriolar tone will not occur reflexly to combat the abnormal partial pressures of C02 and 02- Further, if isolated cells or tissue slices are bathed in a medium to which some substance is added, it is likely that the preparation will respond by metabolising or sequestrating the added substance. That is, the size of the stimulus will change.

In summary, therefore, the effect of multiplicity of homeostatic mechanisms is that many stimuli appear to be ineffective or only transiently effective.

Two major approaches have been used in attempts to overcome these difficulties. The first is to control all pathways or mechanisms except the one under consideration so that the others continue to pass on ' no change' or ' normal' information.....

The second way in which attempts have been made to overcome the problems that arise due to the compensatory activities of homeostatic mechanisms is to deliver a stimulus that is so large that it 'overloads' the homeostatic system.

Scott and Waterhouse (1986)

Extrapolations to man

Even though you want your final conclusions to pertain to humans, sometimes you are forced for technical reasons to choose an experimental animal. An example is the squid giant axon used to illuminate properties of excitable membranes in general - also those of the much thinner axons of human neurons. In this case it is usually important to choose a species that allows extrapolation to man. If you are studying vagal influence on the heart, you should prefer dogs (with high resting vagal "tone", like man) rather than cats (where the sympathetic nervous system dominates at rest). Similarly, corticosteroids affect the immune apparatus and may kill lymphocytes - animal species vary markedly in their sensitivity concerning these effects.

Establishing the techniques

Define the precision of your techniques

Technical proficiency is essential if your results are to be reliable and reproducible. Like anything else, proficiency in experimental technique requires training. It is essential to familiarise oneself with all chosen techniques and methods at an early stage of work and to practise them diligently. You

Box 2.3 Precision of an instrument

The precision of an instrument is a measure of the variability or random error associated with repeated measurements of a sample, that is, the reproducibility of the measurement.....
When the precision of an instrument is quoted by a manufacturer then, naturally, the optimum value is quoted. This value is often obtained under a particular set of conditions. For example, it is likely to be the precision with which measurements can be made over a particular section, often the mid point, of the range of values that the instrument is capable of measuring Measurements made at either end of the range can often be rather less precise than those in the middle of the range. Temperature may also influence the precision of an instrument as well as the substance being measured. For example, many blood gas analysers will measure a partial pressure of gas in air with a greater precision than the same value for the partial pressure of the same gas in blood. Sample size may also be important. In some cases, the development of instruments designed to measure a small sample (and necessary to measure samples obtained by micropuncture of kidney tubules, for instance) may result in a loss of precision of the instrument.
Thus, the experimenter needs to do rather more than merely look up the precision of the instrument in the manufacturer's specifications. What he must determine is the precision of the machine under the conditions of his particular experiments.

Scott and Waterhouse (1986)

may need to reproduce some earlier findings obtained with the techniques, to convince yourself and others that your work is reliable. Moreover, you should always perform a number of replicate analyses, with each technique, and examine the spread of the data. This spread, dispersion or variation determines the *precision* of the technique (Box 2.3).

Perform a proper number of replicate analyses

Knowing the precision of your methods, you can make a rational choice concerning the number of replicate measurements required to obtain a convincing result. The mathematical evaluation of precision is discussed in detail in Chapters S and 6.

Establish dose-response and time course relationships. Know the response characteristics of your instruments and procedures

For multistate variables you have to establish "dose-response" or strength of influence relationships. Moreover, you should determine the time course of the processes and effects you have set out to study. In this connection, you should be aware of the response time of your apparatus or measuring devices. You must know whether the inertia or lagtime of some mechanical component precludes the exact registration of rapid biological processes. You should also know whether the measurements are stable, and how to recalibrate or compensate for a drift in either direction during the course of the experiment.

It is important to avoid *systematic errors* of measurement; in scientific jargon this means that the *accuracy* should be high (more about this in Chapter 5). First, you may have to calibrate your apparatus or system of analysis against known standards, which may or may not be commercially available. You may also have to perform re-calibrations during the course of an experiment. Use your imagination and try to conceive of possible "false results", i.e. so-called artefacts, and how to avoid or compensate for them! For example, movements of recording devices (due to respiratory movements, animal walking, etc.) may give rise to artefacts. Another common source is sampling error. The temptation to record certain object populations (perhaps largest neurons, most accessible blood vessels, densest cell colonies in culture) at the regular cost of others could lead to such error. Scott and Waterhouse (1986, pp. 55-56) give examples of such artefacts and claim that they can often be disclosed by a careful *inspection of the raw data.* Certainly, such an inspection is essential in any case, and in particular when a correlation between an independent and a dependent variable is possible or sought for.

Avoid systematic errors

Close inspection of the raw data!

A quality control of your procedures includes more than the calibration of apparatus and avoidance of artefacts. Try to show that your drug, "factor", or intervention really performed as anticipated. That means, you need a kind of

"positive control". If you can't find any influence of your intervention on the process you are studying, there is more than one possible interpretation! You may have (i) falsified your working hypothesis, (ii) been mislead by compensatory reactions taking place in your object of study, which have abolished a real influence (possibly detectable only during a short initial stage of the experiment !), or (iii) not really intervened in the way you had intended. For example, if we find that the immunological graft-versus-host (GvH) reaction in the rat does not affect the formation of blood cells, we must show that we indeed induced an unequivocal GvH reaction, for example by demonstrating the enormous growth of the spleen.

Positive controls, sensitivity control, and specificity controls

Similarly, you also have to demonstrate that you can detect what you purport to detect (sensitivity control). In other words, not only should the intervention be substantiated, but its strength and effect should be known and preferably standardised. If the stimulus strength required to induce an effect is outside the physiological range, then caution must be exercised with regard to the interpretation. Supra-physiological strengths of stimuli or concentrations of "factors" can give rise to artefacts in a number of ways: (l) In the intact animal non-specific stress or alerting responses may have given rise to the effects you observe. (2) High doses of drugs or "factors" may have non-specific actions. (3) Sensitive cells and tissues - more or less roughly manipulated in vitro - may yield quite aberrant responses (Box 2.4). If you are conducting in vitro studies, you should

Box 2.4 On the possibility of in vitro artefacts

We all too often forget that we might be asking too much from the cells we are studying. We perforate their host; we pull them out from their natural environment; we separate them from their friends and relatives; we put them in a merry-go-round that would make any one of us dizzy for the rest of the day; we keep changing the climate around them between nice and warm and freezing cold; we make them swim around in all sorts of specially prepared cocktails and confront them with nasty chemicals, and then - alas - we plead: "Behave naturally!"

Lappegård (1987)

also bear in mind that responses of neoplastic cell lines do not necessarily mirror the behaviour of their normal in vivo counterparts.

Both (1) and (2) should remind you how important it is to include *specificity controls (negative controls)* in your experiments as well. You must show, for example, that an agent claimed to stimulate cells to secrete does not

work by damaging cell membranes so that secretory granules, as well as cytoplasmic constituents (e.g. lactic dehydrogenase) leak out. You should demonstrate that the effects evoked by a putative transmitter or hormone agonist are via the receptor (neutralise the effects with specific receptor blocking drugs!) - rather than by non-specific actions. Be aware, however, that for "anatomical reasons" specificity could be present in vivo, even though apparent non-specificity was found in an in vitro system. For example, rapid degradation or dilution in vivo might limit the effect of a high concentration of some agent.

Control and monitor the experimental conditions

Finally, you have to *control* your *experimental conditions.* Human subjects should be rested, at steady state, and adequately informed of the circumstances of the experiment. Animals should of course be healthy and have had an adequate opportunity to adapt to the animal house and the experimental paradigm (see Chapter 3). Diurnal variations of physiological parameters should be taken into account; as a general rule all experiments should be performed at the same time of day. Anaesthesia, respiration, circulation, electrolyte and fluid balance, hormone concentrations, and the like must be controlled and standardised as much as possible. The same applies to physicochemical parameters of in vitro organ or tissue preparations, cell cultures, and subcellular systems. Temperature, pH, osmolality, etc. should be recorded. Particularly with in vitro preparations - but also in vivo - it is essential to demonstrate that the organism, organ, tissue, cell, or reaction mixture displays a reasonably normal functional activity.

Pilot experiments

Now, you may want to do some pilot experiments. Try to design them as completely as possible, so that the results may be added to those of the main experiments - if everything works well. If everything doesn't work well you should reconsider all the points mentioned above, including the "level" of your investigation (man, animal, organ, tissue, cell, subcellular fraction).

The experimental design

The experimental protocol: rules of craftmanship - and the advantage of a "paired design"

Some considerations are of prime importance. You are going to perform *important and decisive experiments,* very often attempting to falsify a hypothesis (see Chapter 1). Your *random sample* of human subjects, animals, cells, etc. must be *representative* for the total population to which you want to apply your final conclusions. *Test and control experiments* should either be run simultaneously, or their sequence should be varied. A *paired design is* better than an unpaired one, because each subject, animal, etc. can serve as its own control, so that the

interindividual variations do not add to the spread of your data. For illustration, if you want to compare the effectiveness of a new sun tan lotion with the old, established lotion, two alternatives spring to mind. You could apply the new lotion to one side and the old lotion to the other side of the body of the experimental subjects and then record the effects. Or you might give the new lotion to one group of people and the old lotion to another group that matches as far as possible the first one. The first alternative provides the better comparison.

If your intervention has a reversible effect (like drug effects on patients or on an organ preparation), a good approach is to start with a control phase. Measure your variables and confirm steady state (baseline situation), then introduce your intervention (experimental phase), measure again, and finally revert to control conditions. If the two control phases give very much the same recordings, these can be averaged and a paired comparison performed with the experimental results.

Remember that only one "factor" should be changed in the experimental group, or phase. This will necessitate sham operations, solvent injections, and other sham procedures applied to the controls.

Sometimes several control groups or phases must be included, for example to exclude certain interpretations of your main experimental data. It may be a matter of taste whether this approach to sharpen the conclusions should be called controls or tests.

The experimental protocol

The considerations mentioned above are essential for the writing of a good experimental protocol. We advice you strongly to engage in the self-disciplinary exercise of writing a good protocol! Time spent on careful planning may easily pay off as much more time saved by avoiding inconclusive or improperly controlled experiments. Some people shun this kind of mental exertion, but nevertheless make great discoveries (see Szent-Györgyi in Appendix). Please, don't rely too much on this for your own part, unless you are a specially gifted investigator.

Thinking before action - write a detailed protocol and arrange group seminar

To write a good protocol, you have to ask yourself several questions (see below and Checklists 2.1 and 2.2) - but you should ask the advice of others, too. We strongly advocate holding a group seminar on your experimental plans. At these meetings, be sure to involve the technicians. Medawar (1979) had the following to say about this: " ... technicians are colleagues in collaborative research: they must be kept fully in the picture about what an experiment is

intended to evaluate and about the way in which the procedures decided upon by mutual consultation might 'conduce to the sum of the business' (Bacon) ... ". Try to promote reciprocal respect and interest for the various projects that are planned or implemented in your group and at your institution. In general, everybody performs as good as she or he can, and most of your peers are decent people....

Questions (summarised in Checklist 2.2) to be answered by the protocol

You may start your protocol by describing your *scientific problem,* or the *goal of the investigation.* Make clear *why it is important to* find an answer. Find out *what has been done before;* include some central *references.* Try to analyse the *consequences* of the various possible outcomes of your investigation.

Describe in detail the *experimental design, the materials and the methods* (see Chapters 6 and 7). How can you be sure about *validity* (whether you measure what you want to measure) and *reliability* (i.e. reproducibility) of your findings? *How much* experimental material do you need? Here you must weigh the ideal against the limitations set by time, money, and other resources. If you think that you will need the help of a *statistician,* contact him now! He may evaluate probabilities of statistical type II errors (i.e. your material is too small to reveal the existence of real differences between test and control - see Chapter 6) and give you indispensable comments on your experimental design as well.

Write down how you are going to avoid *systematic errors.* In addition to the points mentioned earlier, consider the possibility of changes in the values of *essential variables,* due to the mere passage of time (growth, ageing, seasonal variation, wear and tear of instruments, deterioration of chemicals and biological material, effects of repeated registrations). Experimenter or *observer bias* must also be avoided. Subjective evaluations, such as differential counting of cells and similar types of scoring should be done "blindly", i.e. without the identity of the preparation known to the observer. In experiments with human subjects (such as the sun tan lotion investigation mentioned above) the design should if possible be "double blind", so that neither the subject nor the observer knows which part of the body has been exposed to the experimental intervention and which to the control procedure.

Make clear how you are going to *treat your data.* For example, very special statistical tests are required if you want to check for statistical significance and then collect more data, if significance was not attained. (For statistics, see Chapter 6; for data storage and treatment with a computer, see Chapter 8).

Design one or more *registration forms* (Fig. 2.1) to help you remember to

Fig. 2.1 Example of registration form

Exper.No	Date	**Purpose:**

Exper. animals: Species/strain: Sex: No: Age: Weight:

Cells retrieved for culture:

Methods: *[Reference to detailed descriptions in a book on/collection of standard methods. Point out possible modifications here]*

Exper. started, Hr.: terminated, Hr.:

Cell No. inoculated per dish:

Culture harvest, date: Hr.:

Culture (No.)	Suspension volume (ml)	Cell conc. (10^6c/ml)	Cells harvested (% of inoculate)	^{3}H-thymidine (incorp. per 10^6 cells)

Comments (continued overleaf):

Conclusions:

write down routine data concerning experimental materials, conditions of the experiments, references to methods detailed in the protocol, and the like. Take care that the forms do not prepare the grounds for systematic errors, for example by invariably putting test results (and therefore perform tests!) before the controls. We are very much in favour of registration forms that force us to write down both the purpose of the experiments and the tentative conclusions that we can draw from the results.

The names of your *collaborators* must be written down, and it must be made clear what are the responsibilities of the various participants, where the different parts of the project should be undertaken, and preferably also the deadlines for accomplishing the tasks.

Some frustrations may be avoided by making clear at an early stage who are probably going to be the authors of the finished article, and who will be the first *author (see* Chapter 9). Your work may have to comply with certain *rules,* e.g. for animal experimentation, and be sanctioned by e.g. a *research ethical committee.* Make a written account of these matters. Everybody involved should have a say on the elaboration of the experimental protocol, and obtain a copy of its final version. In addition to the protocol and the collection of registration forms (with data sheets, etc.), it is often claimed that it is a good idea always to *keep a notebook* for writing down good ideas that come to your mind, suggestions made by colleagues at meetings, and the like.

The production phase

Now you are doing laboratory experiments and accumulating data. Try to establish *routines* that promote rational working procedures, cleanliness, and a pleasant atmosphere in the laboratory. Laws, regulations, and common sense dictate laboratory procedures which involve drugs, toxic substances, radioactive isotopes, or dangerous microbes. Take care to ensure that such *materials* are *stored and handled* in an optimal way. This is important; clever experiments are done in vain if you use labile chemicals or biological materials that have deteriorated because they have not been properly treated and stored. (Room temperature, refrigerator, -20°C, -50°C, -80°C deep-freezer, liquid nitrogen? Use of a desiccator? Half-life under best possible conditions?) For example, if you remove the packaging of deep-frozen materials before they have been warmed to room temperature,

Establish good laboratory routines

condensation of water vapour may have nasty effects. As a general rule, if there are any doubts whether a sample of material is indeed intact, buy a new one !

Cleaning can be a critical factor. Aluminium coating or left-over detergents on glassware can have disturbing effects. Some plastic or polymer materials adsorb proteins and peptides, so you may have to check that abnormal losses do not occur during washing, concentration, or isolation procedures.

Cleaning, sterilisation, adsorption, and contamination

Biologically active substances like mycoplasma and bacterial endotoxins must be excluded from several kinds of experiments; endotoxins can be detected even in some commercial tissue culture media!

What if you have to *sterilise* your materials? You have to know whether they can stand autoclaving, boiling, or dry heat - or whether sterile filtration, irradiation, or treatment with ethylene oxide gas should be the method of choice.

Instrument controls and calibrations

The *responsibility for routine controls* and calibration of laboratory equipment and apparatus should be shared by the members of your group. Even electronic balances need recalibrations (- use for example a properly sized old-fashioned balance weight -) perhaps twice a year.

Finally, be alert to possibilities for changing your working conditions so that they can be even better and more enjoyable for yourself and your colleagues. Be a bit lazy - as recommended by a nobelist - in the sense that you *try to simplify* your experimental designs with the aim of obtaining as reliable answers as before in an easier, less laborious, more direct, and more elegant way!

Data storage, processing, and interpretation

Documentation - and its storage

Take into account any possible *future requirements for documentation!* That means that you have to store your raw data, together with the written procedural details, for a long time. Our own experience is that it is tempting to make too few or too short notes - we think we will remember, but time goes more quickly than we would like to think, and important details may be forgotten when the final article is written. You must be able to reconstruct exactly what happened - at a much later time than anticipated.

When you have assembled your filled-in registration forms (see above) in a book (- some research administrators advise against loose-leaf systems, to diminish the risk of fraud), they should be proceeded by one or more *contents* pages, referring to the experiment or page numbers. Consider also the safety precaution of keeping duplicate copies of key results outside your laboratory.

Have a close look at your raw data before you process them. *Visualisation* of the data can be very useful in this connection. For example, a scatter diagram, or dot plot, can be a rewarding way of doing this (see Fig. 5.6 b). Then you may discover for example peculiar clustering of the data, "outliers" (i.e. freak or extreme values), or correlations that were unexpected.

Before data processing: look once more carefully at the raw data!

The data processing, perhaps performed with a computer (Chapter 8), should not be too intricate! Nevertheless, e.g. logarithmic transformations may be required if you are studying processes running an exponential course, like cell proliferation or the decay of a radioactive specimen. Likewise, you may want to work with logarithms to allow your data to fit a normal (Gaussian) distribution (see Chapter 5).

Drawing conclusions

You can start drawing *conclusions* when you have done a series of experiments, adhering to at least the most essential of the principles outlined above (see Checklists 2.1 and 2.2). You should feel confident that the recorded effects were specific and caused by the stimulus or intervention applied by you. Then, after having processed the data, you perform the statistical analyses (Chapter 6). Calculate also the *relative size of the experimental changes,* not just the statistical significance of differences between test and control. The former indicates whether the experimental outcome is *biologically (as well as statistically) significant,* the latter may be high even though the difference in fact is a minor one (when your data are numerous). According to your scientific problem and with the use of common sense, you should decide whether results should be expressed per organ, per 100 g of tissue, per weight unit of DNA, as a percentage of the (median or mean) control value, etc. Your background knowledge of biology should be solid enough so that you can decide whether your results and preliminary interpretations are biologically reasonable. Of course, the opinions of your supervisor and colleagues can be helpful in this regard.

Beware of correlations, and don't equate them with causality

Be cautious about accepting apparent correlations between two variables, and never equate a correlation with causation! If two variables include a common "factor", there must be a correlation between the two, but it may be biologically meaningless. There is a correlation between shivering and goosebumps (piloerection), sharing the factor "temperature", but neither causes the other.

It is not easy to establish *causality* beyond reasonable doubt. A close correlation between A and B *could* mean that A causes B (but also that C causes both A and B or that both A and B increase or decrease just as a function of time). *The time aspect* may give you a lead; does A always precede B with a constant

period of time? Often you will have to perform new kinds of intervention or experiment to settle the question. Remember then *Koch's postulates* for microbial causation of disease (with a bit of imagination you can adapt them to other situations): (1) The organism must always be found in a given disease. (2) The organism must not be found in other diseases or in health. (3) The organism must be cultivated artificially and reproduce the given disease after the inoculation of a pure culture into a susceptible animal. (4) The organism must be recoverable from the animal so inoculated. To these a more modern criterion can be added: It should be possible to nullify or abrogate the effect of the causative organism – or else "factor", drug, biological agent, hormone - your choice! This could be done by removal, by neutralisation (with for instance anti-sense RNA, ribozymes or (monoclonal) antibody), or by competition (with enzymatic inhibitors or receptor antagonists).

Koch's postulates

Furthermore, transgenic animals, site-directed mutagenesis, and gene knockout by homologous recombination also represent powerful analytical techniques.

What about unexpected results?

Generally, *three types of results may prove problematic for interpretation:* (1) There is - contrary to expectation - no difference between test and control. (2) There is a difference, but not the expected one. (3) The results are equivocal. The first case has been touched on above (redundancy of mechanisms and homeostatic regulation). Counter measures to these possibilities are given in Box 2.2. Case (2) is the really exciting one - you may be on the track of a brand new mechanism or hypothesis - or you may have discovered the solution to another problem! If you have to face possibility (3), the inconclusive results, our advice is that in general, it is prudent to leave it and start anew with another experimental design, approach, or problem. Perhaps, at a later date, you may revert to your former problem with sharper weapons.

From qualitative results to mathematical models

As alluded toearlier, there is often a *developmental line* from the simpler objects of research to the more complex ones and finally to the integrated physiology of intact man. Similarly, there is a line from the qualitative results of research work via the quantitative ones to the investigation of regulatory mechanisms and

physical and mathematical models of integrated control systems. If your field of research is a fairly advanced one, it may be a valuable exercise to participate in such model building. Often it clarifies where quantitative information is still insufficient (-or that lack of reliable data may be more widespread than you had realised). You may learn more about which important data you should try to assemble. The mathematical models may also generate testable hypotheses, which you would not have thought of otherwise. Similarly, it may point out unproved premises for some taken for granted mechanism - and you will have to examine these premises experimentally.

APPENDIX

(- for those specially interested)

Personal qualifications of competent scientists

The scheme of thought I have outlined in this third lecture explains the balance of faculties that should be cultivated in scientific research. Imaginative scientists are by no means the most effective; at their worst, uncensored, they are cranks. Nor are the most critically minded. The man notorious for his dismissive criticisms, strenuous in the pursuit of error, is often unproductive, as if he had scared himself out of his own wits - unless indeed his critical cast of mind was the consequence rather than the cause of his infertility.

Medawar (1969)

.... personality is at least as important as intelligence in determining an individual's creativeness. This conclusion was based on an analysis of eminent persons by Cox (1926), Roe (1952) and MacKinnon (1962). Cox subjected to analysis the biographical data of 300 eminent persons, eliminating those born before 1450 and those whose achievement could not be justified on grounds of ability, for example, those of aristocratic lineage. All her subjects proved to be of high intelligence; but personality was equally important. She remarked: ".... high but not the highest intelligence, combined with the greatest degree of persistence, will achieve greater eminence than the highest degree of intelligence with somewhat less persistence." Roe tested and interviewed sixty-four of the most eminent scientists in America. She agreed with Cox, and remarked: "The one thing that all these sixty-four scientists have in common is their driving absorption in their work." MacKinnon, studying eminent architects, concluded: "Our data suggest, rather, that if a person has the minimum of intelligence required for mastery of a field of knowledge, whether he performs creatively or banally in that field will be crucially determined by non-intellective factors."

Pickering (1974)

An anxiety that may trouble some novices - perhaps particularly women, who are victims still of the socially engendered habit of self-depreciation - is whether they

have brains enough to do well in science. It is an anxiety they could well spare themselves, for one does not have to be terrifically brainy to be a good scientist. An intellectual antipathy or a total indifference to the life of the mind and an impatience with abstract ideas can surely be taken as contraindications, but there is nothing in experimental science that calls for great feats of ratiocination or preternatural gifts for deductive reasoning.

Medawar (1979)

There is a substantial gap between the image of the medical scientist that most people carry in their mind and the actuality of his personality, his hopes and his interactions with colleagues and society.
.... worth stating that the central prerequisite for major progress in science is a judicious mixture of imagination and discipline. The former is at least as important as in the arts or the humanities, and the latter even more so, because good science requires a great degree of repetition of effort.....
Few fields of decision-making are as complex and as uncertain as the prospective assessment of human creativity. After fifteen intensive years in medical research, I still make big mistakes in choosing new staff. The three qualities which appear most desirable are analytical ability, originality and drive.

Nossal (1975)

In science creativity demands flexibility: a creative scientist should have a high degree of emotional and psychological freedom coupled with an equal degree of organised precision.

Kohn (1986)

But after half a century of scientific activity I have had the opportunity to observe the development of many contemporary as well as younger colleagues.....
It seems then that some of those who as young men did not show much promise of originality, although quite capable of the necessary intellectual effort, later have given original contributions to our science.....
I think I have said enough in defence of my thesis that acquired originality exists. Such acquisition requires intense work - ....

Granit (1972)

After dinner the winners were cheered four times, and replied with short speeches. Perutz said, in part, "I was deeply moved by the Royal Swedish Academy's

decision to elevate me, a man of modest gifts, to the Olympian heights of a Nobel Laureate. On hearing the news, a friend who knows me only too well sent me this laconic message: "Blood, toil, sweat and tears always were a good mixture." .... "Despite the twenty-five years for which I have been at it, the task which I have set myself has only just begun."

Judson (1979)

The personality of some prominent scientists

There is no question of Pauling's charisma or ability. "Pauling is arguably the greatest scientist alive today," science writer Graham Chedd has said. Pauling's contribution to the understanding of chemical bonds won him the Nobel Prize for chemistry in 1954. Some say his discovery of the cause of sickle cell anemia, part of his development of the concept of molecular disease, deserves another Nobel Prize. He has also made major contributions in the areas of antibody structure and function, protein structure, ...
He is, by the estimates of friends and enemies, a genius. And he had the satisfaction of receiving the Nobel Peace Prize in 1963 for his political activities opposing war, particularly his work promoting a nuclear weapons test ban.....

Pauling does not take it lightly that his non-scientific activities have cost him a great deal of time. He economises on time wherever he can. Students and reporters have noticed, for example, that an interview with Pauling lasts as long as he feels is warranted, and no more. There is a minimum of small talk, such as winding-down and leave-taking at the end of a conversation. When you talk with Pauling on a matter in chemistry, one graduate assistant observed, as soon as Pauling feels you have covered the ground, you may suddenly find yourself staring at each other for a couple of minutes, and you take your leave; his mind has taken off on a tangent he now pursues on his own. Or, as you are walking along chatting with him, he may suddenly stop, take out the pad and pencil he always carries with him, and jot down an idea. He never engages in chitchat on trivial topics, according to the assistant. "At dinner at his house, you might pick up and read an encyclopaedia." The conversation would be, perhaps, about the animals in the ocean, "just intellectual stuff - never about how his aunt and uncle were feeling. His mind is too busy with other things." This is one of his problems getting along with university administrations, the

assistant feels. "He is not a politician - I could never see him having tea with the dean, having tea with anyone."

Goodell (1977)

.... Outside of science, Monod was spectacularly a public man. He spurned the rule of public reticence beyond the circle that most scientists observe, that all enforce upon each other, that none violates without hazarding his reputation. Monod had will, assurance, vanity, an ideological edge, an appetite for polemic, an actor's need for public attention. "There were two personalities in Jacques; or anyway at the simplest level there were two, one of which was repressed for a long time," Jacob said - repressed as the reading out of a gene is repressed in the theory they made together. Monod was the summation of an extraordinary series of oppositions, paradox raised to a principle. His style was as quintessentially French as Linus Pauling's was American. He was a multiple outsider.....

Monod was a product of the French academic system; he was dissatisfied with it from the time he first came to the Sorbonne in 1928 to the days of riot and near revolution in Paris in May of 1968, when he publicly crossed the barricades to be on the students' side. Monod knew by his teens that he would be a biologist. His great work began only when he was nearly forty. He did that work at the Institute Pasteur, itself founded in deliberate opposition to the universities; at the Pasteur, he was a member of the smallest, liveliest, least typical unit; he rose to be director of the institute - .....

Judson (1979)

The significance of this matter is not that it throws any sinister light on Fleming but that it reveals what was his greatest weakness: his quite extraordinary inability to communicate. Whatever may have been the factors which later held him back from pursuing penicillin more aggressively, he would certainly fail to communicate his original interest and excitement, the interest which his genuine results should have aroused, to others.....

"Fleming was a man of few words - and ideas did not greatly interest him. Women on meeting him for the first time were often nonplussed by the unemotional, "almost basilisk" stare, which some of them mistook for rudeness, while others were fascinated. But there was much friendliness and kindness behind that apparently cold exterior, as many can testify." ....

Fleming was a man very much loved by the many who knew him well, by those who passed beyond the barriers of his silences. There is no doubt too that the concealed man had a fine character, just, honest, generous and loving.....
Strangely enough Fleming was not shy of broadcasting or meeting journalists and writers.....
He was often teased about his disorderly habits.

Wilson (1976)

.... In an interview recorded in 1967, Florey said: "People sometimes think that I and the others worked on penicillin because we were interested in suffering humanity - I don't think it ever crossed our minds about suffering humanity; this was an interesting scientific exercise .... It might have been in the back of our minds .... but that's not the mainspring" (Macfarlane 1979. p. 285). But this statement is difficult to reconcile with the tremendous efforts they made to achieve a practical result. Several of Florey's other investigations also had a clear-cut practical objective: ....
But in all cases his approach was .... scientific as opposed to empirical: he aimed at an understanding of the processes that were at work. He chose problems in which he could see a way forward, and devised methods that gave direct and decisive answers to straightforward questions.
Florey acknowledged an element of luck in his research. According to Abraham (1971, p. 288) he "remarked with a characteristic lack of vanity that he was not by temperament, training or ability one who could deal easily with abstract ideas and that his scientific activities had been guided by a series of lucky accidents". But this is only a negative aspect of his abilities, and Abraham continues: 'Whatever the truth of these remarks they provide an inadequate explanation of his achievements, for it was his own ability to discern potentially fruitful fields of research in which he could function most effectively that was a major factor in his success ...." Even if Florey was indeed guided by "lucky accidents", none of his scientific achievements seem to have depended on chance observations, except in the sense that his penicillin work depended on that most famous of chance observations, Fleming's noticing a contaminated culture plate many years before.

Huxley (1982)

Imagination and intuition in scientific work

According to this second view, science, in its forward motion is not *logically* propelled. Scientific reasoning is an exploratory dialogue that can always be resolved into two voices or two episodes of thought, imaginative and critical, which alternate and interact. In the imaginative episode we form an opinion, take a view, make an informed guess, which might explain the phenomena under investigation. The generative act is the formation of a hypothesis: "we must entertain some hypothesis," said Peirce, "or else forgo all further knowledge," for hypothetical reasoning "is the only kind of argument which starts a new idea." The process by which we come to formulate a hypothesis is not illogical but non-logical, i.e. outside logic. But once we have formed an opinion we can expose it to criticism, usually by experimentation; this episode lies within and makes use of logic, for it is an empirical testing of the logical consequences of our beliefs. "If our hypothesis is sound," we say, "if we have taken the right view, then it follows that ...." - and we then take steps to find out whether what follows logically is indeed the case. If our predictions are borne out (logical, not temporal predictions) then we are justified in "extending a certain confidence to the hypothesis" (Peirce again). If not, there must be something wrong, perhaps so wrong as to oblige us to abandon our hypothesis altogether.

Medawar (1969)

One of the most lucid and informative accounts of the creative process is by Helmholtz, the great German physicist, given at a banquet in honour of his seventieth birthday. He said that, after previous investigation of a problem " .... in all directions, ...happy ideas come unexpectedly without effort, like an inspiration. So far as I am concerned, they have never come to me when my mind was fatigued, or when I was at my working table. .... They came particularly readily during the slow ascent of wooded hills on a sunny day." He thought the creative process could be divided into three states. The first is preparation, in which the relevant data are
collected and worked at; the second is "incubation", in which one is not consciously thinking about the problem; the third is that in which the "happy idea" suddenly appears, the state of illumination or inspiration. Then follows the hard work of verification.

Pickering (1974)

Unfortunately, solving problems is not all there is to the scientific endeavour. Even more important than solving problems is *finding relevant* problems - formulating questions that really matter. The world offers us an infinite number of problems to solve, of which we select some and disregard others. Much of the art of doing science is then deciding which problems to concentrate on and which to ignore.

....

.... The ability to solve problems requires logical thinking, and hence a rational mind, whereas the ability to identify consequential problems is only in part based on logic; mostly it is based on instinct, intuition, subconscious perception, a sixth sense, inborn proclivity, talent, irrational impulse or whatever you might want to call it.

There is plenty of evidence from the history of science for an independent assortment of these two traits.

Klein (1985)

About team work in science

.... each also knows that an atmosphere can be created in which one member of the team sparks off the others so that they all build upon and develop one another's ideas. In the outcome nobody is quite sure who thought of what. The main thing is that something is thought of. A young scientist who feels a strong compulsion to say "That was my idea, you know" or "Now that you have all come round to my way of thinking ...." is not cut out for collaborative work. He, and his colleagues as well, would do better if he worked on his own.

A few Polonian precepts can do something to indicate whether a scientist is cut out for collaboration. Unless he likes his colleagues and admires them for their special gifts, he should shun it; collaboration requires some generosity of spirit, and a young

scientist who can recognise in himself an envious temperament and is jealous of his mates should on no account try to work with others.

Medawar (1979)

The importance of important problems

The purpose of scientific enquiry is not to compile an inventory of factual

information, nor to build up a totalitarian world picture of natural Laws in which every event that is not compulsory is forbidden. We should think of it rather as a logically articulated structure of justifiable beliefs about nature. It begins as a story about a Possible World - a story which we invent and criticise and modify as we go along, so that it ends by being, as nearly as we can make it, a story about real life.....

.... The scientific method is a potentiation of common sense, exercised with a specially firm determination not to persist in error if any exertion of hand or mind can deliver us from it. Like other exploratory processes, it can be resolved into a dialogue between fact and fancy, the actual and the possible; between what could be true and what is in fact the case.

Medawar (1969)

It can be said with complete confidence that *any scientist of any age who wants to make important discoveries must study important problems. Dull* or piffling problems yield dull or piffling answers. It is not enough that a problem should be "interesting" - almost any problem is interesting if it is studied in sufficient depth. A problem must be such that it *matters* what the answer is - whether to science generally or to mankind.

Medawar (1979)

On the nature of scientific evidence

.... to establish firmly the logical relationship between the hypothesis and the specific prediction following from the hypothesis. It is no use spending a great deal of time, effort and money for the purposes of designing and executing a series of experiments to answer a particular question if, at the end of all this, answering that question is not a decisive test of the hypothesis. For example, suppose the hypothesis is that a high-fat diet can result in premature death. Suppose also that it is known that diets rich in fat can cause obesity. To show that obese subjects have a decreased life expectancy in comparison with non-obese controls does not necessarily support the original hypothesis. Obese individuals might differ from controls not only in the size of their fat stores but also in the intake of some other foodstuffs. A decisive test of the hypothesis would require the two groups of

individuals to be identical in all factors (including degree of obesity and dietary habits) except for fat intake.....

Scott and Waterhouse (1986)

Here is an extract from the *New Yorker* magazine's "Letter from London" of January 31, 1953.

The Ministry of Health's recently published figures showing that in the week of the great fog the death rate for Greater London jumped by twenty-eight hundred were a shock to the public, which is used to regarding Britain's unpleasant climatic effects as nuisances rather than as killers. .... The extraordinary lethal properties of this winter's prize visitation ....

But how lethal *was* the visitation? Was it exceptional for the death rate to be that much higher than usual in a week? All such things do vary. And what about ensuing weeks? Did the death rate drop below average, indicating that if the fog killed people they were largely those who would have died shortly anyway? The figure sounds impressive, but the absence of other figures takes away most of its meaning.

Huff (1973)

Never confuse correlation with causation

.... the relationship between age and some physical characteristics of women, begin by measuring the angle of the feet in walking. You will find that the angle tends to be greater among older women. You might first consider whether this indicates that women grow older because they toe out, and you can see immediately that this is ridiculous. So it appears that age increases the angle between the feet, and most women must come to toe out more as they grow older.

Any such conclusion is probably false and certainly unwarranted. You could only reach it legitimately by studying the same women or possibly equivalent groups - over a period of time. That would eliminate the factor responsible here. Which is that the older women grew up at a time when a young lady was taught to toe out in walking, while the members of the younger group were learning posture in a day when that was discouraged.

When you find somebody - usually an interested party - making a fuss about a correlation, look first of all to see if it is not one of this type, produced by the stream of events, the trend of the times. In our time it is easy to show a positive

correlation between any pair of things like these: number of students in college, number of inmates in mental institutions, consumption of cigarettes, incidence of heart disease, use of X-ray machines, production of false teeth, salaries of California school teachers, profits of Nevada gambling halls. To call some one of these the cause of some other is manifestly silly. But it is done every day.

Huff (1973)

How and what to read

.... The great incentive to learning a new skill or supporting discipline is an urgent need to use it. For this reason many scientists (I certainly among them) do not learn new skills or master new disciplines until the pressure is upon them to do so; whereupon they can be mastered relatively quickly. It is the lack of this pressure on those who are forever "equipping themselves" and who show an ominous tendency to become "night-class habitués" that sometimes makes them tired and despondent in spite of all their diplomas and certificates of proficiency.
Similar considerations apply to a novice's inclination to spend weeks or months "mastering the literature." Too much book learning may crab and confine the imagination, and endless poring over the research of others can sometimes be a research-substitute, much as reading romantic fiction may be a substitute for real-life romance.
.... The beginner must read, but intently and choosy and not too much.
It is psychologically important *to get results,* no matter if they are not original.

Medawar (1979)

I have said (pp. 251, 258) that although Niedergerke and I came across the changes of band pattern by chance, as a by-product of experiments planned for investigating different phenomena, we might well have planned our experiments to look for those band changes if we had been familiar with the late 19th-century literature and with Jordan's paper of 1934 (see A. F. Huxley & Niedergerke 1958). Likewise, we would have got more quickly to the final answers about inward spread if we had known of the suggestion by Retzius (1881, only partially correct; see A. F. Huxley 1980, p. 11) that the reticulum stained by gold chloride might be involved in the inward spread of activation, or of Veratti's now famous paper of 1902 which showed by means of Golgi staining the different arrangements of the reticulum in different animals, or of Nyström's paper (1897) showing that (in heart

muscle) the reticulum consisted of tubules capable of admitting particles of Indian ink and therefore open to the extracellular space.

How many more suggestions comparable to these are still sitting unknown in the massive literature not only of the late 19th century but of the first half of the 20th? Ought some proportion of our scientific effort to be devoted to searching the old books and journals for hints such as these? It would be a daunting task; most of this old work has been superseded by more recent experimentation, and it is not easy to know which of the unfamiliar observations to take seriously.

The other side of this coin is the advantage of coming fresh to a subject, without knowing the existing literature so well as to be inhibited from having ideas outside the currently accepted framework. I benefited in this way through moving from nerve to muscle, and thus not being over-familiar with the biochemical story of continuous actomyosin filaments that was generally believed around 1950. Before H. E. Huxley's 1953 paper electron microscopists claimed to have confirmed the existence of continuous filaments (see A. F. Huxley 1980, p. 34), and I doubt whether this claim would have been made if the micrographs in question had been examined without a preconceived idea about the structure within a myofibril.

Huxley (1982)

How one reads depends upon immediate goals. Are you perusing a current journal to find out what's going on? This sort of reading is much like looking at the morning newspaper; a general blur may be all that you want. Are you trying to teach yourself about a subject? This will take more care, but can often be done with minimal effort through the use of reviews. Or are you digging in to examine some problem with the thought of pursuing it or extending your own previous research? This may take the fine-toothed comb approach in which every fragment that a particular article provides is gleaned.

Ratnoff (1981)

On the planning of experiments

Dionysians and Apollonians

Wilheim Ostwald (1) divided scientists into the classical and the romantic. One could call them also systematic and intuitive. John R. Platt (2) calls them Apollonian and Dionysian. These classifications reflect extremes of two different

attitudes of the mind that can be found equally in art, painting, sculpture, music, or dance. One could probably discover them in other alleys of life. In science the Apollonian tends to develop established lines to perfection, while the Dionysian rather relies on intuition and is more likely to open new, unexpected alleys for research. Nobody knows what "intuition" really is. My guess is that it is a sort of subconscious reasoning, only the end result of which becomes conscious.

These are not merely academic problems. They have most important corollaries and consequences. The future of mankind depends on the progress of science, and the progress of science depends on the support it can find. Support mostly takes the form of grants, and the present methods of distributing grants unduly favour the Apollonian. Applying for a grant begins with writing a project. The Apollonian clearly sees the future lines of his research and has no difficulty writing a clear project. Not so the Dionysian, who knows only the direction in which he wants to go out into the unknown; he has no idea what he is going to find there and how he is going to find it. Defining the unknown or writing down the subconscious is a contradiction in absurdum. In his work, the Dionysian relies, to a great extent, on accidental observation. His observations are not completely "accidental," because they involve not merely seeing things but also grasping their possible meaning. A great deal of conscious or subconscious thinking must precede a Dionysian's observations. There is an old saying that a discovery is an accident finding a prepared mind. The Dionysian is often not only unable to tell what he is going to find, he may even be at a loss to tell how he made his discovery.

Being myself Dionysian, writing projects was always an agony for me, as I described not long ago in *Perspectives of Biology and Medicine (3).* I always tried to live up to Leo Szilard's (4) commandment, "don't lie if you don't have to." I had to. I filled up pages with words and plans I knew I would not follow. When I go home from my laboratory in the late afternoon, I often do not know what I am going to do the next day. I expect to think that up during the night. How could I tell then, what I would do a year hence? It is only lately that I can see somewhat ahead (which may be a sign of senescence) and write a realistic proposal, but the queer fact is that, while earlier all my fake projects were always accepted, since I can write down honestly what I think I will do my applications have been invariably rejected. This seems quite logical to me; sitting in an easy chair I can cook up any time a project which must seem quite attractive, clear, and logical. But if I go out into nature, into the unknown, to the fringes of knowledge, everything seems mixed up and contradictory, illogical, and incoherent. This is what research does;

it smoothes out contradiction and makes things simple, logical, and coherent. So when I bring reality into my projects, they become hazy and are rejected. The reviewer, feeling responsible "for the taxpayer's money," justly hesitates to give money for research, the lines of which are not clear to the applicant himself.
A discovery must be, by definition, at variance with existing knowledge. During my lifetime, I made two. Both were rejected offhand by the popes of the field. Had I predicted these discoveries in my applications, and had these authorities been my judges, it is evident what their decisions would have been.
These difficulties could perhaps be solved to some extent, by taking into account the applicant's earlier work. Or, if the applicant is young and has had no chance to prove himself, the vouching of an elder researcher acquainted with the applicant's ability may be considered. The problem is a most important one, especially now, as science grapples with one of nature's mysteries, cancer, which may demand entirely new approaches.

Szent-Györgyi (1972)

Beware of experimenter effects (bias)

The experimenter effect is particularly prominent in behavioural research, where people exchange signals unintentionally, without speaking. These signals may be transmitted by gestures, by auditory or visual channels, by touch or even by smell. In every experimental situation the experimenter may thus convey to the subjects his (or her) feelings without even knowing that he has done so.....
Rosenthal and his collaborators [21,22] carried out experiments designed to detect the experimenter effect on rats. .... They had to train rats in seven different tasks. The experimenters were deliberately biased by having been provided with false information that some of the rats were "bright" and the others were "dull" while in fact all the rats were from the same colony, were of the same age and sex and had performed similarly. The "intelligence" of the rats was said to have been determined in previous maze running experiments. Eight teams were given rats described as "bright", and six teams were told their rats were "dull". At the end of the experiment, the experimenters had to rate themselves, as well as the rats. It turned out that the experimenters believing their subjects to be generally "bright" observed better performance on the part of the rats and rated themselves as more "enthusiastic, friendly, encouraging, pleasant and interested" in connection with the performance of their rats, than the experimenters working with "dull" rats. The

differences between the two groups were statistically significant. The explanation given to the experimenter effect in rats was that the rats defined as "bright" and supposedly performing better, were liked better by their experimenters and were therefore touched more. Indeed, Bernstein showed in 195723 that rats learned better when they were handled more by the experimenters. If mere physical contact could affect the learning behaviour of rats surely more dramatic effects may be expected in human experimentation.

...

A significant number of cases attributable to experimenter bias have been reported in the field of astronomy. Re-examination of certain astronomers' published data indicated that the reported observations could not have been possible. John Flamsteed, an English astronomer of the seventeenth century, was the proponent of an (incorrect) theory predicting that the Pole Star would be closer to the North Pole in winter than in summer. He published his own astronomical observations to confirm his hypothesis; in other words, he found what he expected to find. When his colleagues later showed him that his observations had been erroneous, Flamsteed admitted the error and blamed it on the instruments, although he never succeeded in identifying the source of the error!

Kohn (1986)

Some possible consequences of failures and unexpected findings

I will argue that science involves a special mode of thought and is unnatural for two main reasons, ... Firstly, the world just is not constructed on a common-sensical basis. This means that 'natural' thinking – ordinary, day-to-day common sense – will never give an understanding about the nature of science. Scientific ideas are, with rare exceptions, counter-intuitive: they cannot be acquired by simple inspection of phenomena and are often outside everyday experience. Secondly, doing science requires a conscious awareness of the pitfalls of 'natural' thinking. For common sense is prone to error when applied to problems requiring rigorous and quantitative thinking; lay theories are highly unreliable.

....

The physics of motion provides one of the clearest examples of the counter-intuitive and unexpected nature of science. ... Imagine being in the centre of a very large flat field. If one bullet is dropped from your hand and another is fired horisontally from a gun at exactly the same time, which will hit the ground first?

They will, in fact, hit the ground at the same time, because the bullet's rate of fall is quite independent of its horizontal motion.

...

I would almost contend that if something fits in with common sense it almost certainly isn't science.

...

In fact one of the strongest arguments for the distance between common sense and science is that the whole of science is totally irrelevant to most people's day-to-day lives. One can live very well without knowledge of Newtonian mechanics, cell theory and DNA, and other sciences. On the other hand, science can enormously enrich one's life, and in modern society knowledge is essential for innumerable policy decisions that affect our lives (...).

Wolpert (1993)

Methodologists who have no personal experience of scientific research have been gravely handicapped by their failure to realise that nearly all scientific research leads nowhere - or, if it does lead somewhere, then not in the direction it started off with. In retrospect we tend to forget the errors, so that "The Scientific Method" appears very much more powerful than it really is, particularly when it is presented to the public in the terminology of breakthroughs, and to fellow scientists with the studied hypocrisy expected of a contribution to a learned journal. I reckon that for all the use it has been to science about four-fifths of my time has been wasted, and I believe this to be the common lot of people who are not merely playing follow-my-leader in research.

Why do scientists hold or come to formulate erroneous opinions?

Medawar (1969)

If, in spite of the most anxious precautions, a scientist makes a mistake about a matter of fact; if the results were caused by an impurity in a supposedly pure enzyme preparation or if hybrid mice were used in error for mice of an inbred strain, then the mistake must be admitted with the least possible delay. Human nature is such that the scientist may even gain credit from such a declaration and will not lose face except perhaps in the bathroom mirror.....

Though faulty hypotheses are excusable on the ground that they will be superseded in due course by acceptable ones, they can do grave harm to those who hold them because scientists who fall deeply in love with their hypotheses are proportionately

unwilling to take no as an experimental answer. Sometimes, instead of exposing a hypothesis to a cruelly critical test, they caper around it, testing only subsidiary implications, or else follow up sidelines that have an indirect bearing on the hypothesis without exposing it to possible refutation.
I cannot give any scientist of any age better advice than this: *The intensity of the conviction that a hypothesis is true has no bearing on whether it is true.* The importance of the strength of our conviction is only to provide a proportionately strong incentive to find out if the hypothesis will stand up to critical evaluation.

Medawar (1979)

In basic research, everything is just the opposite. What you need at the outset is a high degree of uncertainty; otherwise it isn't likely to be an important problem. You start with an incomplete roster of facts, characterised by their ambiguity; often the problem consists of discovering the connections between unrelated pieces of information. You must plan experiments on the basis of probability, even bare possibility, rather than certainty. If an experiment turns out precisely as predicted, this can be very nice, but it is only a great event if at the same time it is a surprise. You can measure the quality of the work by the intensity of astonishment. The surprise can be because it did turn out as predicted (in some lines of research, 1 per cent is accepted as a high yield), or it can be confoundment because the prediction was wrong and something totally unexpected turned up, changing the look of the problem and requiring a new kind of protocol. Either way, you win.

Thomas (1974)

Among the experiments I did while in Australia was one in which I infused blood into a sheep on two occasions. The first time was without incident, but the second time a small quantity of blood (40 ml) caused a profound reaction (figure 7). It was my first experience with a change in microvascular leakiness to liquid and protein. I wasn't looking for it. 1 wasn't even thinking about it. It just happened.....
We made radioactive liposomes and showed a high percentage uptake in the lungs of sheep, goat, pig, and cow, but essentially no uptake in rabbit, rat, and dog. We used fluorescent liposomes to localise the site of uptake and found by quantitative histology that more than 90 % of the liposomes were associated with large mononuclear cells in the pulmonary capillaries, which by ultrastructure we subsequently found to be macrophagelike cells.

We set about to remove the macrophages from the lung and have obtained large numbers ....

When these cells are placed in primary culture, they behave as macrophages should. They actively secrete lysozyme .... They make a large quantity of thromboxane under baseline conditions and even more when stimulated by calcium ionophore.....

.... results are dramatic. As little as 0.01 ml of rabbit blood injected intravenously or intra-arterially into the goat causes pulmonary hypertension (figure 10), whereas injection of relatively large quantities of goat blood into the rabbit has no effect. We hypothesise that the intravascular macrophages are responsible for the reaction to the foreign red blood cells.

To sum up: I waited 18 yr. between my observation of a transfusion reaction in a sheep and our discovery for a reasonable explanation. It is often the unexpected experimental result that leads to the most interesting new concepts. The take-home message of this story is not to forget about *unexplained* results of experiments, even though it isn't possible to follow the trail immediately. .... In this paper, I have described the way I and my colleagues made some of our discoveries, not in the neat logical manner that journals and study sections prefer but in the chaotic, free-wheeling, unplanned, and unprogrammed environment of the real research laboratory.

Staub (1987)

An old view of the experimental sciences - still valid?

But they are to know,
that in so large, and so various
an **art** as this of **experiments**
there are many degrees of
usefulness:
some may serve for real and plain
benefit, without much **delight,**
some for teaching
without apparent **profit;**
some for light now,
and for use hereafter;
some only for **ornament** and
curiosity.
If they will persist
in condemning all **experiments,**
except those which bring with them
immediate **gain**
and a present **harvest;**
they may as well cavil at
the Providence of God,
that He has not made
all seasons of the year,
to be times of **mowing, reaping,** and **vintage.**

Thomas Sprat (ca. 300 years ago).

Research Project, Checklist 2.1.

The problem.

1. Is it possible to obtain *important and reliable answers?* ("The art of the soluble")
2. Is its solution important enough to make you willing to spend *much more (x3?) time* than you think the project will require? ("What's worth doing, is worth doing well")

The method

1. What is the *precision?* (SD, CV=SD/mean * 100%, quartile interval - see Chapters 5 and 6). How many replicate measurements are needed to establish statistical significance?
2. Have *dose-response* relationships been established?
3. Are *time-courses* of reactions etc. known?
4. What is the *accuracy?* Are controls with "known answers" (methodological quality tests) included?
5. Are *positive controls* and sensitivity controls included?
6. Are *negative controls* (specificity controls) included?
7. Are *physicochemical and biological parameters* controlled, like pH, temperature, osmolality, conditions of rest or depth of unconsciousness, blood pressure, arterial blood gases?
8. Is your experimental *preparation*/target *functioning adequately?*

The experimental design

1. *Test = control, except for one variable?*
2. Is the design appropriately *"blind"* (or "double blind") and *randomised?*
3. The *validity* of the study? Do you measure what you intend to measure? Have you controlled every step in the logic between observation and interpretation? Have you designed the study such that these steps are few? Can the results mean anything else? Can the interpretation be *secured by alternative approaches?*

4. Are your results *reproducible?*

The interpretations

1. Are results and interpretations *biologically sound* and reasonable?
2. Are the differences between tests and controls both *statistically and biologically significant?*

Experimental protocol, Checklist 2.2

1. Project title.
2. The purpose and problem. What do you want to know? Why?
3. The background. What is already known?
4. Experimental design. How are you going to do it?
5. Materials. What kind of preparation will you choose? (animals, cells, etc.)?
6. Variables. Which data will you record? How and when?
7. Analysis. How are you going to process the data?
8. Presentation of results. Scientific article? Popular press? Co-authors and first author?
9. Funding and administration. Who is going to do what, and where, and when? Who is covering the costs?
10. Time schedule. When shall the project be finished?

When this checklist is used to outline a **grant application,** it should be supplemented with:

11. Possible impact. What is the importance of the intended project?
12. Management of techniques. Are all methods needed in current use, or must some of them be introduced?

Exercises

1. Analyse your own motivation for embarking on research work, and your working environment. Can you find any ideas for improving your situation in the sub-chapter "About yourself"? Discuss these and the following matters with your supervisor/peers/class mates.
2. Try to analyse your own personality profile, to find out whether you emotionally would be best satisfied with for example the repetitive, accurate, biochemistry-type experiment or the more dramatic, surgical whole-animal approach. If you are given the opportunity to choose your field and way of study, whether you are practical or theoretical, patient or impatient, systematic or speculative, specialistic or generalistic, an "accumulator" or "guesser" should definitely influence your choice!
3. Can you defend your choice of scientific problem after having read this chapter? Do you disagree with the authors on any important matter?
4. How can you cope with the scientific literature in a rational and economical way?
5. How are you going to process your data?
6. Evaluate your chosen methods, techniques, and approaches. Discuss with your supervisor/peers/class mates how these could be supplemented.
7. Design an experimental protocol and evaluate it in discussions with your supervisor/peers/class mates.
8. Should any of your laboratory routines be improved?
9. Look up the following pages in Nature (London): (1988) 333:816-818; 334:287-290. Which of the rules and suggestions offered in this chapter have been violated?

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Chapter 3: Animal experiments

Animal experimentation in general

Animal experiments are indispensable to biomedical research, even though they should be reduced to a minimum,. You have to work on living animals to study for example the **integrated regulation** of body function, **vaccination** against human or animal infectious diseases, or **experimental therapy** of model diseases established in animals. The experimental approach with **intervention** on experimental animals has been of crucial importance for the understanding of a large number of diseases and conditions. Some human disorders and conditions, unfortunately, are not easy to reproduce in good animal models. It will often be the case that such conditions remain poorly understood; psycho-social disorders, fibromyalgia, bone marrow aplasia, Alzheimer's disease, leg cramps, and tickling are examples that come to mind.

But before one embarks on in vivo studies, a solid base of data obtained from work with isolated organs, tissue slices, cells, or subcellular systems is usually required. The whole organism is so complex that you **need knowledge** about component mechanisms **from in vitro work** in order to interpret in a confident way the results of your interventions during experimental animal work. For example, if a bacterial component (endotoxin) is injected i.v. into a rabbit and a febrile response is observed, this does not mean (as was for long the widely held view) that endotoxin directly affects the temperature regulating centres of the brain. (But it stimulates macrophages to secrete cytokines that do have this effect.) Noradrenaline injected to an experimental animal may slow the heart rate, but when applied to an isolated heart in vitro will speed up the rate. Here, in vivo contraction of small arteries raised blood pressure, which evoked a baroreceptor response and a nervous reflex that slowed the heart rate in order to lower the blood pressure, overriding the direct effect of noradrenaline on the heart. Thus, the first example shows how you can be misled by relying solely on animal experiments, whereas the latter shows that animal experiments may ultimately be required to settle what are the in vivo effects and side effects of an agent or a procedure.

Find out whether animal experiments represent the best approach to your problem

The valuable criticism of and assistance with this chapter, offered by two head veterinarians, Stian Erichsen and Dag Sørensen, of the Norwegian State Institute for Public Health (SIFF), and the National Hospital, Oslo, are gratefully acknowledged.

The animal rights "credo" is that animals should be treated on equal terms with humans and experiments postponed until they could be performed safely and painlessly on volunteers. Certainly, much human suffering that has now been relieved by vaccination programmes, modern drug treatment, etc., would still have persisted if animal experiments had not been conducted in the past.

Animal experiments are necessary to investigate the integrated function of organisms

Box 3.1. Animals rights and Animal Wrongs

At a recent demonstration against a new animal care building at the University of California, animal rightists put up a sign that said, "These animals are to be used by torturers, imprisoners, and murderers." Torturers? Some procedures that these animals undergo, such as removal of blood samples and surgery under anesthesia, are no different from procedures performed on humans at any hospital. Imprisoners ? The cages in a research laboratory are no different from the cages in a humane society or a veterinary hospital. Murderers? A strange term to apply to animal experimenters when a conservative estimate is that pounds and shelters kill a hundred stray cats and dogs for every such animal sacrificed in research.

Kosland (1989)

The three R's

Russell and Burch introduced the «Three R concept» (Replacement, reduction and refinement) as a main guideline for responsible use of animals in experiments, in their book "The principles of humane experimental technique" (Methuen, London, 1959). *Replacement* refers to the use of in vitro and other methods instead of living animals. Examples are cell and organ cultures, computerized models and video tapes. *Reduction* refers to a decrease in the number of animals required for a given experiment. This can be achieved by choosing suitable experimental procedures, by controlling environmental factors, by standardisation of the animal population, and by statistical power analyses. *Refinement* refers to any decrease in the incidence or severity of painful or distressing procedures applied to animals in research. Legislation mandating the incorporation of the three Rs into animal research and testing has been passed in the United States and Europe.

Thus, always consider carefully whether your scientific project has really advanced to the stage where in vivo experiments are justified or indispensable; in particular, **in vitro culture** of cells, organs, or micro-organisms (see Chapter 4) and **computer simulations** may represent good alternative or supplementary approaches. Moreover, in vivo bioassays and toxicological tests can increasingly be replaced by **physico-chemical, biochemical, and immunological methods** of analysis, as well as by the fascinating techniques developed by **molecular biologists.**

Remember also that animal experiments can mean very different things (Fig. 3.1). Complex biological phenomena may best be analysed in **simpler organisms**, like insects or leeches (Box 3.3), at least at an early stage. But it is well to keep in mind that the results of such analyses cannot necessarily be extrapolated to hold true for all animals, let alone human beings.

Remember alternatives to experiments on warm-blooded animals !

Often a certain animal species is used by **tradition** in a field of research, so that a solid experimental data-base for comparison is available. It may therefore be preferable to choose a warm-blooded animal, like the mouse, for your experiments. In any case, treat your animals like your **precious instruments** in regard to control and calibration! Inherent in animal experimentation is a diversity of variables (Fig. 3.2). As far as possible the variables should be known and controlled (see Checklist 3).

"Calibrate" and check your animals

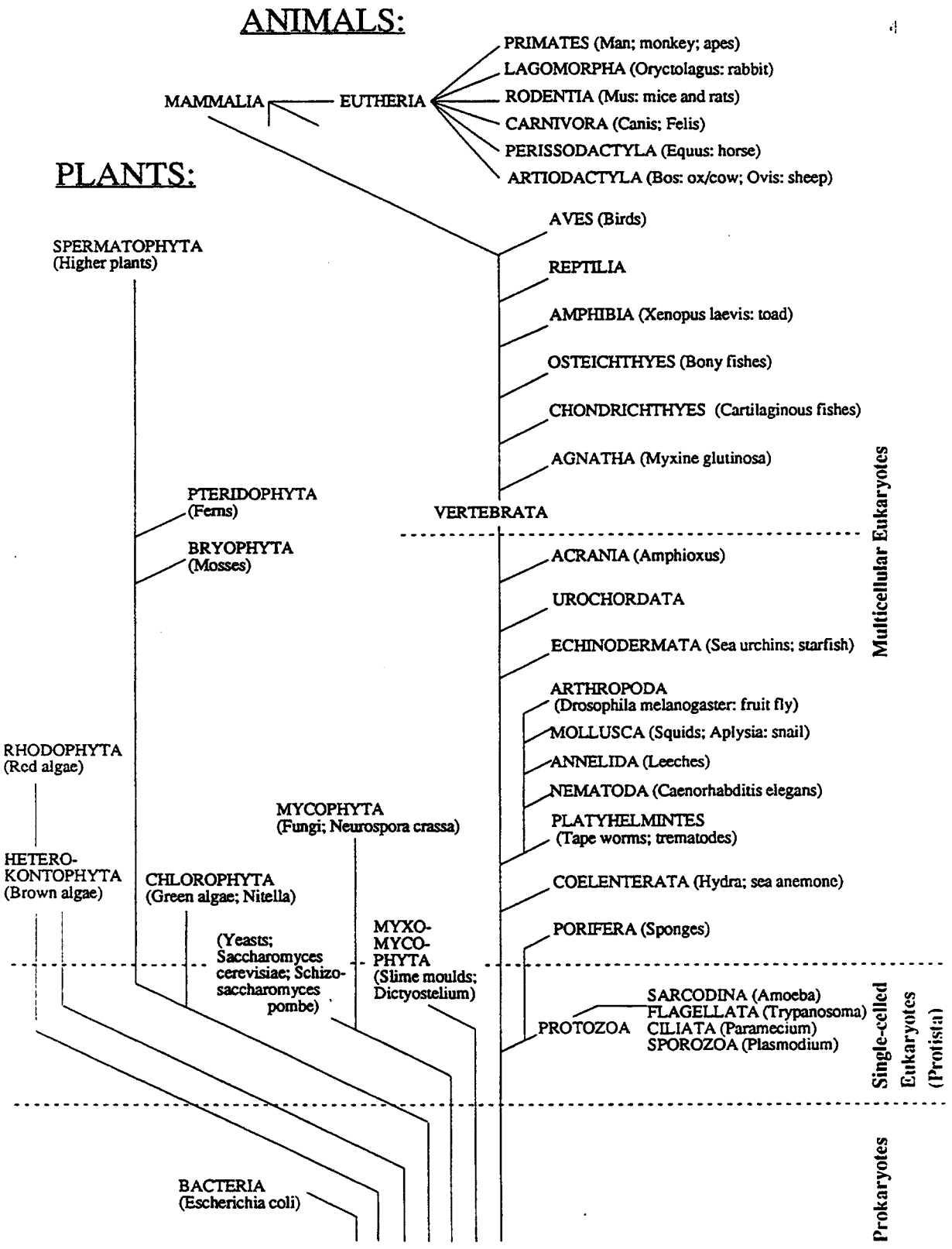

Fig. 3.1. Rough and schematic evolutionary relationships among some commonly used experimental organisms. The diagram does not depict evolutionary time periods, and shows a non-comprehensive selection of phyla, classes, sub-classes, orders, genera, and species, with examples and English names in parenthesis.

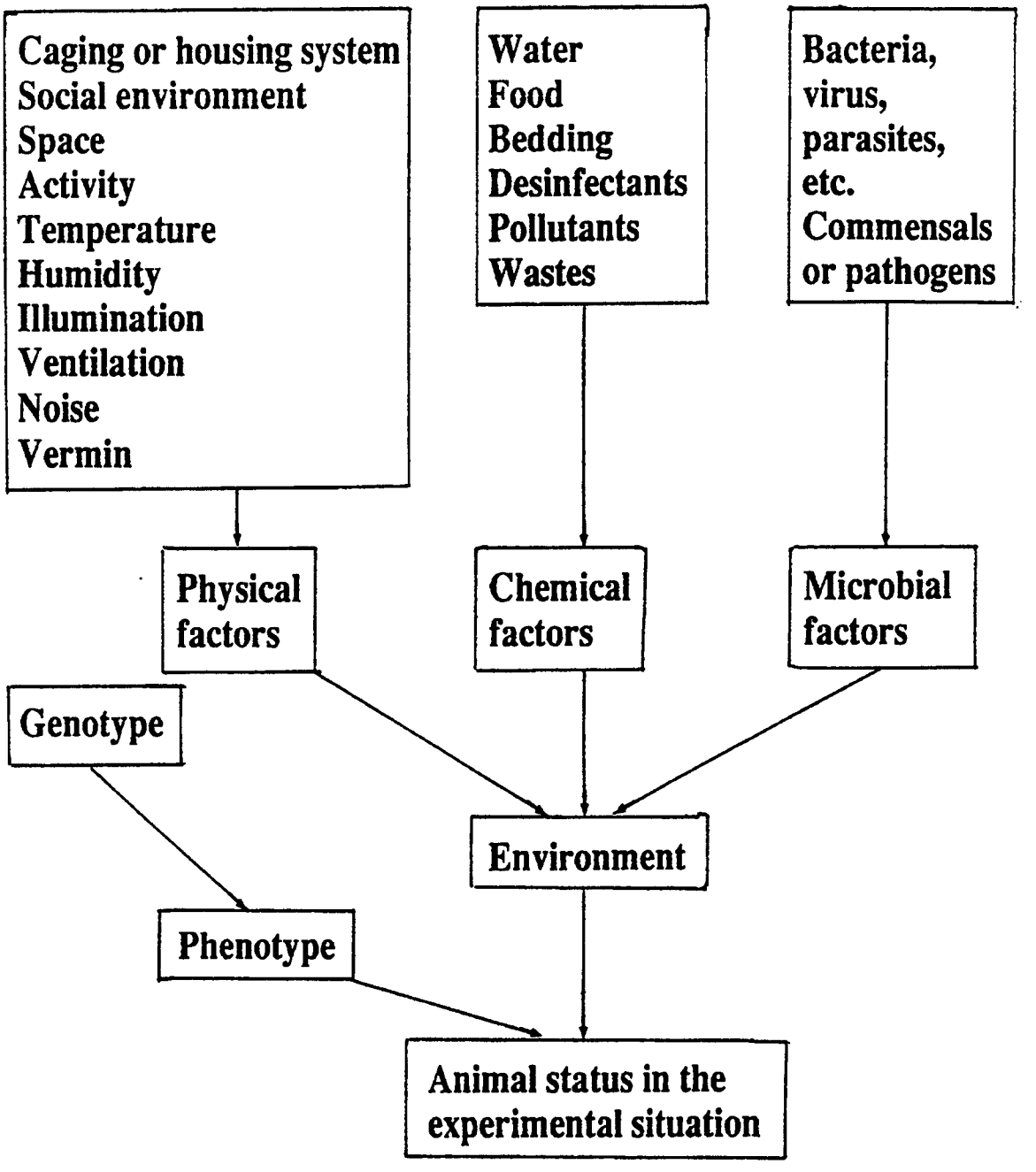

Fig. 3.2. The actual properties of the animals in the experimental situation should be as uniform as possible.

Animal models

The significance of results obtained in animal experiments is dependent upon the selection of a suitable animal model. The extent to which the results can be «extrapolated» to man depends upon the type of animal and the nature of the research. There are no general rules for the choice of a proper animal model, nor are there any rules for the extrapolation procedure.

Animal models used to increase our knowledge of human reactions and human disease can be divided into four groups: Induced models, spontaneous models, negative models, and orphan models. The induced and spontaneous models are the most important ones.

In *induced animal models* a disease, disorder or other change is induced experimentally, - by surgery, by the transfer of genetic material, or by the administration of other kinds of biologically active substances. Thereby, an intended «likeness» is obtained, e.g. with regard to disease manifestations or etiology between experimental animal and man. By interfering with the environmental, dietary, endocrine, immunological, infectious, or genetic state of the animal, models have been created for a great number of diseases and malfunctions. A promising, as well as controversial, group of induced animal models is established in transgenic animals (Box 3.2). To date, mice are the preferred species for research purposes, although other species - including fish - are receiving considerable attention. The production of transgenic animals containing foreign genes may result in hitherto unrecognised welfare problems, and researchers must be alert to signs of distress and pain in these animals. *Spontaneous animal models* of human disease are those which have arisen by natural genetic variation. Hundreds of strains/stocks of animals have been analysed and categorised as displaying spontaneous diseases, which resemble those in man.

Negative models occur in 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 a specific effect in other species or strains. The underlying mechanisms of insensitivity can, however, be studied, to provide further insight.

Orphan animal models refer to models where a disease or condition is initially recognised and studied in an animal species, with the assumption that a human counterpart might be identified at a later stage. Papilloma viruses in malignant

Box 3.2. Transgenic animals - a link between molecular biology and whole animal biology

Techniques based on the manipulations of DNA, today referred to as molecular biology, initially had little impact on fields dealing with more complex systems such as clinical medicine and even basic physiology. However, this has changed over the last 10-20 years. One of the major contributions that now link molecular biology to the study of whole animals, is techniques that allow the insertion of recombinant DNA into the genome of whole animals in such a way that the artificial gene is propagated through the germ line. Such animals are referred to as *transgenic animals,* and the inserted piece of DNA as the *transgene*. The first successful transgenic mice were reported in 1980 (Gordon, J.W., Scangos, D.J., Plotkin, D.J., Barbosa, J.A. & Ruddle, F.H. Proc. Natl. Acad. Sci. 77:7380-7384). Today transgenic animals have been created also in other species such as rats and pigs and other farm animals.

There are two types of transgenic approaches. In one type DNA is injected into one of the two nuclei of a newly fertilised egg. In a reasonable fraction of the embryos the injected DNA is subsequently incorporated at a random spot on the genome. In a different approach DNA is targeted to a specific point in the genome where the sequence of nucleotides is known. The goal is to get the artificial DNA to displace the targeted DNA and take its place in the genome. Since this displacement is a rare event, one has to transfect the DNA into a high number of embryonic stem cells. The transgene is designed in such a way that a successful targeted displacement confers the resistance against cytostatic drugs. By applying these drugs to the transfected embryonic stem cells in tissue culture dishes, the cells where the desired displacement has taken place can be highly enriched. The transgene containing cells are subsequently injected into an early developing embryo (blastocyst) and will form part of the adult animal. If one is lucky, the transgene will enter the germ line.

Random insertion techniques can be used to overexpress a biologically active protein by coupling the coding sequence of its gene to a very active promoter. Furthermore, by selecting a tissue specific promoter, the expression can to some extent be targeted to certain cells. Random insertion techniques have also been used to study gene promoters themselves. The promoter is then coupled to a biologically inactive *reporter gene*, a gene which gives a visible or measurable product. Thus, cells where the promoter is activated, can be detected.

Targeted displacement is most widely used to disrupt a known gene to give a so called *gene knock-out*. More recently, mutations more subtle than such null mutations have been used, and endogenous genes have been replaced by a copy of itself with less dramatic alterations.

While all the techniques pertaining to transgenic animals are available in textbook format (e.g. Hogan, B., Costantini, F. & Lacy, E.: Manipulating the Mouse Embryo, a laboratory manual. Cold Spring Harbor Laboratory, 1986), practical use requires considerable skills when it comes to recombinant DNA technology, handling of embryos, and animal husbandry. In addition, the procedures are extremely time consuming and most researchers discover that a smoothly running transgenic facility requires more dedication and money than most scientific projects. Even so, transgenic techniques are increasingly popular, and have yielded spectacular results within several fields of biology.

In spite of its successfulness, there are several limitations and intrinsic problems that often make results obtained with transgenic animals difficult to interpret. One problem is life itself, which encompasses so many back-up mechanisms, and so much redundancy that manipulation of one gene often has disappointingly small effects. Another major problem is connected to the fact that the transgene is present in all cells throughout development. This often leads to early death or to undesired effects during animal development. Nonetheless, one can expect that in the time to come many of these problems will be overcome by using gene regulatory sequences with more selectivity in directing overexpression, knock-outs, or mutations to the desired time and place within the intact animal.

By courtesy of Kristian Gundersen, Department of Neurophysiology, University of Oslo, Jan. 1996

epithelial tumours and Marek's disease virus as a lymphoproliferative agent, are two examples of orphan animal models.

Only rarely do animal models fully mirror the human condition in health or disease. Very often, the selection of an animal model is based on a similarity between man and animal in regard to only one aspect of the phenomenon under study. Preferably, a variety of spontaneous and induced models of a given human condition should be employed.

Allergy to laboratory animals

Allergy to laboratory animals (ALA) is a well-known occupational disease in persons who engage in animal work. Allergic or hypersensitivity reactions can be divided into four types; types I, II, III, and IV. Type I reactions (immediate reactions, atopic allergy) are the most common. This type of reaction occurs most frequently in individuals who are hereditarily predisposed to develop increased immunoglobulin E (IgE) antibody responses to antigenic stimuli. Common examples are hay fever, urticaria, and bronchial asthma. When the antigen (allergen) binds to IgE on tissue mast cells, these cells release inflammatoy mediators like histamine and various cytokines which trigger the hypersensitivity reaction. Moreover, atopic persons tend to have exaggerated responses to many different irritants, for example cigarette smoke. Employees in biomedical research laboratories come into contact with organic materials from the animals in many ways. Injections, testing, training, operations, killing, feeding, or blood sampling are examples of tasks which expose a person to allergens.

Animal allergens have traditionally been thought of as fur, hairs, or dander. But skin-prick tests show that they are present in hair, skin, feces, urine and other animal materials. With modern immunological techniques it has been possible to characterise the allergens further. In mice, the main allergen is apparently a urine molecule, possibly prealbumin. Serum albumin is also an important suspect. In rats, serum albumin, alpha-2-globulin and prealbumin seem to be the predominant culprits. Somehow the blood-borne allergens must be able to contaminate the animals' fur, etc., possibly via the urine or feces. There are no significant cross-reactions between species, but cross-reactivity between strains seems likely, since the structure of the protein allergens are almost identical. Air in the animal house and in laboratories where animals have been kept, will show a significant loading of allergens. In conclusion, allergens are widespread wherever

animals are kept, and it is not possible to work with animals without being exposed to allergenic material. If an atopic disease is the result, the wearing of a gas mask (or similar protective measure) or a change of work may be the only options.

Legislation

Be aware of laws and regulations !

As a research worker you must know the relevant **laws and regulations** pertaining to animal experimentation in your country or state, as well as the policies chosen by your own institution. Even though you may not be personally responsible for the laboratory animal husbandry you should know the elementary rules of animal welfare (see later; Table 3.2; and the suggestions for further reading).

The use of live animals for experimental purposes in Europe is regulated both at the international and the national levels, involving both European intergovernmental organisations and national governments. The international bodies include both the Council of Europe (CE) and the European Union (EU), both of which have adopted supranational regulations on the use of live animals for experimental and other scientific purposes in member states. The aims of these initiatives have been to assist member countries in their efforts to modernise legislation in this particular field, and not the least, to harmonise such legislation. The enforcement of legislation on experimental animals is the responsibility of each independent country.

Before you perform animal anaesthesia, surgery, or other experimental manipulations, you should be **qualified** through training or experience under competent guidance. Some training may be obtained using animal cadavers. Proper qualification aims to ensure that tasks be accomplished in a manner which is both humane and scientifically acceptable.

A sentence stating that the present work has been carried out in accordance with the national laws and regulations, should be a natural part of the Materials and Methods section of articles dealing with animal experiments.

Some notes on the use of laboratory animals

Since a **uniform experimental situation** (Fig. 3.2) during an experimental period is an important goal, catching wild animals is rarely the method of choice for the supply of laboratory animals. An **accredited supplier** (see Table 3.1 for definitions of terminology), running a high-quality breeding station, who can specify and document the properties of his animals, should in general be chosen (unless your own institution can guarantee the same kind of quality product).

Use quality tested animals with product specification

If animal uniformity is essential and you intend to work with mice or rats, **inbred animals** should be chosen. They are more expensive and often less robust than **outbred animals** (Table 3.2), but for all practical purposes they are isogenic, like identical twins. Also isogenic is the hybrid animal that is a product of a cross between members of two inbred strains; it is a heterozygous animal and often more robust than its parents. Special animal types, such as the **mutant mice** - of which there are more than a hundred different types simulating human diseases - and rats which differ genetically only at one histocompatibility chromosome locus, have offered important scientific advantages. However, the development of various genetic engineering techniques has expanded this field and the scientific and technological possibilities enormously. In particular, **transgenic animals,** produced by introducing a certain foreign gene into the fertilised egg, offer exciting possibilities (Box 3.2).

SPF, inbred animals, or genetically modified animals?

The laboratory mouse has retained part of the temperament of the wild type animals. Therefore, for some purposes at least, it may be prudent to choose **females**, lest the characteristic fighting of males lead to wounds, infections, etc. during establishment and maintenance of a cage hierarchy. If males are to be used, it is important to establish the experimental groups before sexual maturity is reached. This will reduce their inclination to fight.

Concerning the **microbiology** of laboratory animals, you have four choices: conventional animals, specified pathogen-free (SPF) eggs or animals (Caesarean section or hysterectomy-derived), and two types of gnotobiotic animals (see Table 3.1) ((i) germfree or axenic and (ii) gnotobiotic animals associated with one or more defined type of microorganism). The gnotobiotic animals must of course be reared and maintained in isolators, and strict regimes of handling, sanitation, food preparation etc. must be adhered to, which requires specialist veterinary supervision. In comparison with SPF and conventional animals the gnotobionts have a less developed immune system, slower passage of intestinal

contents, lower turn-over of intestinal epithelium, and at least in rats a lower basal metabolic rate and cardiac output.

There are elaborate procedures for the breeding of **SPF animals**, similar to those used for the gnotobionts, including strict rules for the personal hygiene of the investigator (see "Guide for the Care and Use of Laboratory Animals" in the References section). Compared with **conventional animals**, the SPF varieties are better standardised, more uniform, have a lower morbidity and mortality and a higher resistance against some agents (endotoxin), drugs (e.g. cortisone), and physical insults (e.g. ionising radiation). During infection they may produce less pus than conventional animals. They also have a steeper growth curve and longer life span. On the other hand, their breeding and transport is definitely more complicated. Taken together, however, SPF animals should be preferred to conventional ones. Even if you have to maintain them under conventional conditions in your animal house, the SPF status may remain for weeks or months. At least you know what you have received, and the risk of introducing **epizootic** is minimized.

Some common laboratory animals

Mice are economical and well characterised laboratory animals

About 90% of laboratory animals are rodents, and the majority of these are **mice**. Mice are convenient experimental objects in immunology, experimental haematology, pharmacology, embryology, neurobiology, and oncology. They are cheaper and require less space than other mammals. Moreover, they are better genetically characterised and tailored to various applications than most other species, because of the existence of so many inbred, congenic, and transgenic strains.

The rat: an unpopular, but convenient and intelligent laboratory animal

Laboratory **rat** stocks and strains derive from the brown rat, or Norway rat (*Rattus norvegicus*; less used synonyms are *Mus norvegicus*, the Hanovarian rat, or in German "Wanderatte"). A more proper name would have been Rattus asiaticus, since it migrated into Europe from the east about a quarter of a millennium ago, and is now ubiquitous, like the mouse. The rat is one of man's close companions, but unpopular and even feared. Rats destroy one fifth of the world's crops each year, can carry microbes that are pathogenic to humans and may even kill small children by direct attack. Nevertheless, among laboratory workers who have learnt to know and appreciate the domesticated laboratory rat, the attitude is very

different: Treated with calm patience these animals are more peaceful than mice and easy to manipulate. Their intelligence is high enough to make them suitable for behavioural research; and the many inbred, congenic, and recombinant strains that have been developed and genetically characterised in recent years, have made the rat a major experimental animal in pathology, immunology, genetics, oncology, pharmacology, and physiology (e.g. endocrinology and nutrition research) (Gill et al. 1989). The rat may represent a good compromise between economic considerations (size; space) and size considerations (possibility of surgical procedures or multiple blood or organ sampling).

The rat: A compromise between economy and size

As a laboratory animal the **rabbit** has been used for production of immune sera, diagnosis of infections, tests for pyrogens and toxicity of agents and drugs, but also for various kinds of physiological studies.

The hare animals are no longer considered belonging to the rodents (order *Rodentia*), but to their own order, *Lagomorpha* (Fig. 3.1). As the rodents, they have very long, continuously growing incisor teeth (but in contrast to the rodents four of them in the upper jaw), an almost exclusively vegetarian diet, and a large coecum.

Even though some strains of rabbit may show anger and aggressive behaviour, rabbits in general are silent and timid creatures. If scared, the rabbit may let out heart-rending yells, often quite out of proportion to the damage inflicted. Worse, if frightened while restrained or sitting on a smooth support, violent contraction of the muscles in its back may break its weak lumbar spine. A quiet and careful approach to rabbits is therefore strongly recommended.

Don't let the rabbit break its back.

The **cat** (*Felis catus*) and the **dog** (*Canis familiaris*) were domesticated during prehistoric times. There exist numerous strains or breeds, but the variability is less marked for the cat than for the dog. Dogs have been used as experimental animals in physiology, pharmacology, and experimental surgery. Their blood pressures are 100-180 mm Hg systolic and 60-120 mmHg diastolic; other physiologic variables are given in Table 3.2. The dog is a very expensive laboratory animal, due to slow reproduction and growth, big size, and special requirements concerning husbandry. There are strong objections to their use in research among both scientists and non-scientists. If they are indispensable for a given research project, they should be provided by an accredited vendor or breeder. The beagle is often a convenient choice, being small (adult weight 10-17 kg, shoulder height 33-38 cm), smooth-haired, fertile, and friendly. If this is too small, the Labrador retriever has many of the pleasant properties of the beagle,

Anatomical and physiological characteristics

with weight of 25-35 kg and shoulder height of 55-60 cm. The cat is physiologically a quite labile animal. It has been used in anatomy, physiology, pharmacology, and toxicology, and a solid data-base has been accumulated. However, to obtain them in a safe and reliable way has been as problematic as for the dog. The mini-pig (see later) may represent a good alternative to these larger experimental animals.

In earlier times biologists were engaged in research on the taxonomy and diversity of the species. Similarities among different species were noted, however, and it was increasingly realised that results obtained from biological investigation of a certain kind of animal - or even bacterium or plant - could have relevance to other species, including man. So the large ova of the toad (*Xenopus laevis*) became a favoured object of study for developmental biologists because of accessibility and size; the giant axon of the squid became a favourite of neurobiologists for the same reason. Bacteria (*Escherichia coli*) and their viruses (phages) - and more lately yeasts (*Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*) as well - have been chosen by molecular biologists. Simple metazoa like the hermaphrodite worm *Caenorhabditis elegans* have been studied by neurobiologists, development biologists, and cell biologists, since all cells of these organisms are visible under the light microscope, and their genetics and development can be precisely defined. Modern experimental biomedicine abounds with similar examples. A fuller account of most of these examples - as well as the use of the fruit fly (*Drosophila melanogaster*), plants, primates, and man as experimental systems - is given in a special issue of Science (1988; vol. 240, NO. 4858). Another example is provided by Box 3.3.

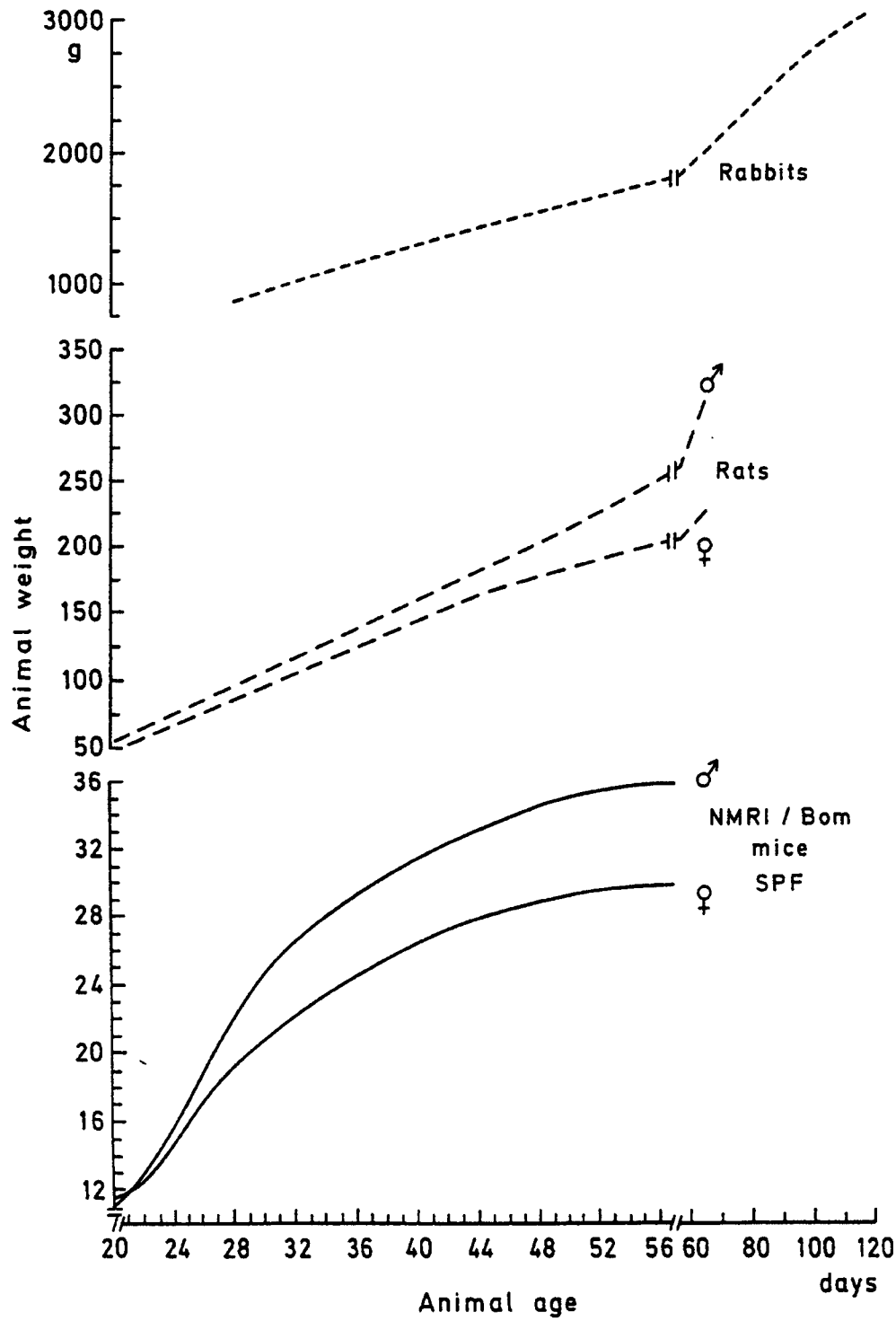

Fig. 3.3. Examples of growth curves for common laboratory animals. You should establish the normal or optimal curves for your own strain(s), so that you can tell whether your experimental procedures affect the weight gain of your animals.

APPENDIX
(- for those specially interested)

Laboratory animals husbandry - some points for consideration

Make sure that rules for good animal husbandry are followed

Good husbandry minimizes variations that can modify an animal's response to experimentation. **The caging or housing system** should facilitate animal well-being and meet research requirements. It should provide space which is both adequate and comfortable, permitting freedom of motion. There should be a resting-place appropriate to the animal species. The enclosure should be escape-proof, and the animals should have easy access to food and water, be provided with adequate ventilation, be able to easily maintain body temperature, urinate, defecate - and if appropriate, reproduce - and still keep dry, clean and comfortable. Inspection of cage occupants should be possible without disturbing them.

Several physiological variables are sensitive to manipulations and suboptimal care of the animals

The animal's **social environment** may be important. Attention should be given to whether the animals are naturally territorial or communal; accordingly, they should be housed singly or in groups. A rat who is caged alone may develop a nervous disorder. On the other hand, too many female mice (> about 30) caged together may lead to infertility. Population density can also affect metabolism, immune responses, and behaviour. Group composition should be kept as stable as possible, particularly for dogs, primates, and other highly social animals. After transfer from the commercial breeder to your animal house, the animals should be allowed some days (for mice about half a week, at least) to adapt, before being used for experimentation. Be aware that when selecting mice from a big cage for your experiments, you easily introduce a bias towards the more sluggish mice! So, a truly random allocation to test and control groups involves optimally two to five mice caged together, with at least one day allowed for new mouse hierarchies to be established in the cages. As a general rule, only compatible animals should be kept together, while newly grouped animals must be closely inspected to detect injuries due to fighting.

Box 3.3 Use of a cold-blooded animal as experimental object

THE LEECH - useful experimental preparation the study of the nervous system.

Many advances in the biomedical sciences have been made through the prudent choice of experimental preparations that simplify the analysis of molecules, genes, and cells. The squid giant axon yielded up the mechanism of electrical impulse activity in neurons, bacteria and bacterial phages facilitated the first attempts at unravelling the complexities of gene regulation, the fruit fly *Drosophila* and the nematode *Caenorhabditis* have proved invaluable in fathoming the mysteries of embryonic development. One organism that has contributed significantly to our understanding of how the nervous system functions and develops is the leech.

The leech's blood-sucking nature, considered repugnant by some, also provided it with an important position in the medical armamentarium of earlier centuries. Although indiscriminate blood-letting is no longer a popular medical practice, the isolation of powerful anticoagulant and anti-tumour activities from leech saliva has once again placed the leech in the medical spotlight. Indeed, surgeons have discovered that leeches provide the best means for drainage of post-operative occlusions following the reattachment of amputated digits.

The leech's behavioural repertoire is dominated by the activities involved in finding and feeding from its prey. It is aroused by and orients toward water movements, swimming to their source with a graceful and effective undulation. Once attached to the prey, it uses its front and rear suckers to crawl to the most delicate regions of skin, where the blood supply is nearest the surface. Although hardly of the same order of complexity, the behaviour of the leech thus embodies many of the fundamental features of the behaviour of vertebrates: rhythmic locomotion, sensory discrimination in several sensory modalities, sensory feedback, choice, and even learning, to name a few.

Neurobiologists find the leech an attractive preparation because of the small number of neurons in the leech's nervous system: about 15 thousand, in contrast to the billions of nerve cells in mammalian brains. Moreover, since the leech is an annelid worm, its nervous system is segmented, the neurons being clustered into nearly identical units called ganglia. The 350-400 neurons in each ganglion are relatively large and can be individually identified on the basis of morphological, biochemical, and electrical properties. Most of them are present as bilaterally symmetrical pairs, so the actual number of different neuron types is about 200. It is therefore a straightforward (although time-consuming) task to determine the synaptic connections between neurons. By using semi-intact preparations it is then possible to correlate activity in different neuronal networks with the actual behaviour of the animal. This has generated tremendously useful information about the neural basis of behaviour, ranging from the mechanisms of single synapses to the properties of complex networks, and including the role of hormones in behavioural regulation. Many of the principles discovered in the leech and other invertebrate organisms appear to apply to the vertebrate nervous system as well.

In addition to appealing to the neurobiologists, the leech has attracted embryologists interested in how the nervous system develops. Some leech species have very large eggs which are amenable to lineage analysis and experimental manipulation. In these species it has been possible to determine which blastomeres give rise to different parts of the nervous system, and what sorts of signals instruct the blastomeres to express different fates. The neurons can be identified at early stages after they are generated, facilitating the study of differentiation: how different neurons acquire their unique sets of phenotypic characters. Synapses can be studied as they develop, and it should be possible to correlate the construction of neural networks with the emergence of behaviours.

The leech typifies the "simple system", in essence a smaller scale version of more complex organisms, with similar operating principles but more amenable to analysis. In the elucidation of the genetic and environmental influences that shape the nervous system, it may provide a paramount preparation for bridging the gap between zygote and brain.

Glover, J. (1990), personal communication

Consider the possibility that the animals need to be **exercised.** This judgement must be based on an understanding of species or breed temperament, nature of the research, expected duration of laboratory residence, etc. Use of a treadmill or exercise wheel, walk on a leash, access to a run, or release from cage to an animal room are some of the possibilities. Animals with specialised locomotor patterns should be allowed to express these patterns. Without possibilities for exercise caged laboratory animals lose their fine appetite regulation and get fat when offered pellet foods ad libitum (freely). **Obesity** may restrict their life span and increase their cancer incidence.

Rats and mice are highly versatile and readily adapt to laboratory caging systems; other animals may have specific **needs concerning** their **environment.** **Temperature** and **humidity** are probably the two most important factors, potentially affecting metabolism and behaviour. Recommended temperatures generally lie below the animal's thermoneutral zones; these and the best humidity values are given in Table 3.2. **Ventilation** should not only be adequate to supply oxygen and remove carbon dioxide, but thermal loads and gaseous and particulate contaminants should also be removed from the cages. A rough rule is that 10-15 room air changes per hour is sufficient. Nevertheless, absorbent and non-toxic bedding should be used and changed frequently, to reduce formation of **ammonia** from urea in the animal's urine. For mice and rats two or more changes per week is enough. Experimental exposure of rodents to elevated temperature, humidity, or ammonia can increase their susceptibility to infectious, toxic, and other harmful agents. Ammonia (> 25 ppm) may paralyse respiratory epithelial cilia.

A time-controlled **lighting system** should be used - and checked periodically - to provide a regular diurnal lighting cycle (e.g. 12-hour lightness and 12-hour darkness). Since **albino** laboratory animals are popular, lower light intensities than previously used are now recommended ("Guide for the Care and Use of Laboratory Animals") and considered sufficient to maintain cleanliness and otherwise good house-keeping practices. Light levels of 320 lx approximately 1 m above the floor appear to be enough and safe, so that retinal damage in albino mice and rats can be avoided.

Noise control is important, especially concerning high-frequency sounds. Human and animal areas should be separated and bells and telephones kept outside the animal rooms. Noisy activities like cage washing and refuse disposal should be carried out away from the animals. Continuous exposure to sounds above 85 dB have caused stress indicators like eosinopenia (i.e. low number of eosinophil granulocytes in blood) and increased adrenal weights, as well as reduced fertility in rodents. Increased blood pressure has been observed in non-human primates.

Animals should be fed **palatable, uncontaminated, and nutritionally adequate food** daily, according to particular requirements dictated by e.g. species (vitamin C to guinea pigs), category (gnotobionts), age, and physiological state (newborns, reproduction, or lactation), or experimental protocol.

Animal foods may be a source of experimental failures

The food supplier should be able to document that recommended nutritional allowances for the various animal species, as issued e.g. by the National Academy of Sciences - National Research Council in USA, have been adhered to, and that periodic quality checks have shown freedom from chemical and microbial contaminants. The formulation and preparation of the food can also be important; for example, pellets may be too solid to be eaten by very young animals. Some contaminants, in concentrations too low to cause clinical toxicity, can induce synthesis of hepatic enzymes that can alter an animal's response to various drugs. Moreover, diets can contain mycotoxins such as aflatoxin B1(a potent hepatic carcinogen), pesticide residues, heavy metals like lead and cadmium, the oestrogenic agent diethyl stilboestrol, etc.. Allergies and toxic manifestations may therefore occur. Even if the producer states that "the food contains all what the animal needs - as a well-balanced diet", it may contain ingredients that block or prevent the biological process you are going to study. For example, a too copious amount of iodine will block the uptake by the thyroid gland of radioactive iodine that you have administered to your rats.

Don't forget the storage of the food either. The date of manufacture and other factors affecting shelf life of food should be known. Refrigeration preserves nutritional quality and lengthens shelf life; nevertheless, food storage time - like drugs' and other agents' storage time! - should be reduced to the lowest practical minimum and the recommendations of the manufacturer followed. Autoclavable diets may require adjustments in nutrient concentrations, kinds of ingredients, methods of preparation, as well as supplementation with ingredients (like vitamin C) that have to be sterilised by other methods.

Animals should have continuous access to fresh, uncontaminated **drinking water**. Automatic watering systems should be inspected daily for leaks and be flushed weekly to ensure proper functioning. Drinking tubes and bottles should be autoclaved once a week. Bacterial growth can be prevented by acidification of the water (pH 2 with HCl).

Cleanliness is essential in an animal facility; this applies to the animal rooms, corridors, and storage spaces as well as to cages, etc.. Solid-bottom rodent cages and accessories should be washed once or twice a week and rinsed in hot water (>80^{o}C) for a period long enough to kill vegetative pathogenic organisms. Cage racks should be washed at least monthly. Wire-bottom rodent cages and cages for all other animals should be washed at least every 2 weeks. Automatic washing machines are highly recommended. UV lamps should be dusted as often as judged necessary, possibly every day.

Surgery, except on rodents, should be conducted only in facilities intended for that purpose, performed or directly supervised in all cases by **trained, experienced personnel. Aseptic techniques** must be used on most animals, including rabbits, that undergo major survival surgery, i.e. intervention that

penetrates a body cavity or has the potential for producing a permanent handicap in an animal that is expected to recover. Asepsis means wearing of sterile surgical gloves, gowns, caps, and face-masks; use of sterile instruments; and aseptic preparation of the surgical field. Some of these precautions may be omitted when you are going to perform survival surgery on rodents; however, sterile instruments, surgical gloves, and aseptic procedures should still be used.

In general, surgery requires tranquillising drugs, anaesthesia, analgesics, and aseptic technique

Most animal species - the mouse being an exception - should be given **sedation** (tranquillising drugs) before major surgery (see Table 3.2); all laboratory animals should be properly **anaesthetised** and given **post-operative analgesics** (Table 3.2). Carstensen (1978) has pointed out that **anaesthetics and drugs** used in connection with surgery may have various **untoward effects**, e.g. on nervous system function, brain blood flow, cardiovascular variables, liver and kidney function, temperature regulation and fluid balance. For example, barbiturates may increase blood glucose concentration, ether anaesthesia may raise prolactin concentration in blood six-fold and thyroid stimulating hormone two-fold. Glucocorticoids that have proven useful in veterinary practice, may spoil your experiment by influencing a number of metabolic processes. Similarly, some antibiotics can adversely influence the intestinal flora of rabbits and small rodents and should not be used. Acquaint yourself with such side effects of your relevant drugs ! Furthermore, **injections and infusions** should always be properly described : **where** (e.g. s.c., i.p., orally, by stomach tube (gavage) - to give an agent mixed with food or by gavage may not be equivalent !), **when** (see below about natural rhythms), and **what** (drugs designated by their chemical as well as registered or pharmacopaeial names).

Beware any possible side effects of drugs and agents!

Appropriate facilities and equipment should be available for **post-surgical care**, and the animal house personnel thoroughly informed and instructed about the operation, administration of analgesics, supportive fluids, heating pads, etc. and about the care for surgical incisions and importance of appropriate medical records.

If the animal has to be killed, the **termination of life** must never be brutal, but quick, without struggle, discomfort, or pain (Table 3.2).

In animal experiments, **time scheduling** is important, because of rhythmic alterations in various physiological variables, i.e. the so-called bio-rhythms (Wollnik, 1989). This applies to the timing of surgery and other interventions, as well as to sampling of blood, urine, cells, or (pieces of) organs. Body temperature, aspects of metabolism, blood pressure, concentration of blood cells, enzyme

Consider possible biorhythms!

activities in blood and organs, blood mineral concentrations and hormone levels may all display **a circadian rhythm.** For example, corticosterone levels in rat plasma may vary four-fold; similarly marked variations have been noted for mitotic indices in mouse liver and endotoxin sensitivity of mice, just to mention some very different types of variables.

Other variations may bear relationship to **food intake, physical activity, or sexual cycle** (Table 3.2). **Circannual rhythms** and **life phase variations** have also been observed, for example a higher incidence of cleft palate in pregnant mice given cortisone during the winter than summer months. A note of caution should be introduced here, however; don't take all postulated bio-rhythms at face value! Special statistical tests should be used to validate claims for e.g. circadian cycles, and more numerous data than in most other research fields are often needed to establish unequivocally that there are rhythmic and not just erratic variations of the variable studied.

Consistency in physiological measurements can only be assured by including methodological control experiments. Otherwise, unexplained variability may arise. For example, when **blood samples** drawn from various parts of the cardiovascular system are analysed and data compared, unanticipated, and often marked differences have been observed: Tail blood from rats gives consistently higher haemoglobin concentrations than cardiac blood, and repeated sampling of mouse venous blood shows that the leukocyte concentration is not stable, but decreases markedly from the first to the sixth sample, approaching the concentration in the aorta. Another source of variability may arise simply from the disturbance introduced by the investigator entering the animal room and moving the cages ! This can within minutes increase (rat) heart rate, haemoglobin concentration, prolactin and thyroid stimulating hormone concentrations in blood - the hormone concentrations in fact several-fold. "Normal" values are therefore actually base levels for a specific stress situation, hopefully common to all the animals.

Importance of methodological control experiments

All test **substances, drugs, and chemicals** used should be well defined and specified according to purity and kind and amounts of possible impurities and additives. Their shelf-lives should be known, as well as their stability in solution, particularly at different pH. Many solutions have to be freshly prepared just before use. The **solvent** can also be important and have substantial effects of its own (check with proper controls!). Benzyl alcohol, when used as a preservative, can have an immobilising and hyperglycaemic effect on mice. Di-methyl-

Consider solvent and volume effects on your injected animals

sulphoxide (DMSO) may cause marked permeability changes in cell membranes and thereby facilitate resorption of other substances. Isotonic 5.5% glucose for infusion, when stabilised by hydrochloric acid to pH 3, may inactivate a freeze-dried enzyme if used to dissolve it. Pellets, tablets, and sustained release preparations, or special diets, may reduce the bioavailability of your experimental substance, for example because bentonite, being a filler in some tablets, may bind the active component. Again, positive controls (see Chapter 2) are important.

Consider also **the volume of solutions** given to laboratory animals (Table 3.2). A constant dose of 50 mg/kg pentobarbital delivered by gavage to rats in the form of a 0.1% solution will reportedly induce sleep after 4.9 min, whereas a 1.0% solution takes 7.1 min. The increased effects of a low concentration seems related to (i) rapid stomach emptying, and (ii) a larger absorptive surface in the gut for a larger volume. By the way, when rats have been fasted overnight (16 h), but with free access to water - which has been a usual preparation for gavage - body weight decreased significantly, and blood haemoglobin and leukocyte concentrations, as well as serum glucose concentration, increased. Therefore, the fasting period should probably not be longer than necessary to empty the stomach.

Finally, take care to establish fool-proof methods of **animal identification.** Cage cards might include name (and local telephone number) of the investigator, experiment and experimental group designations, information on the kind of animals and interventions performed, as well as pertinent dates.

Selected aspects of laboratory animal biology

Bewildering animal nomenclature

The **nomenclature** of laboratory animals is a complicated matter. In short, traditional names like the Wistar rat, the Sprague-Dawley rat, Long-Evans rat, Swiss mouse, and New Zealand White rabbit are obsolete and misleading, since the potential genetic variability hiding behind these names is enormous. For example, the theoretical number of generations between the original and present-day Swiss mice (200) corresponds to the distance from us to the Neanderthal man! An internationally established naming system has now been adopted, at least for inbred mice. An inbred strain shall be given a name consisting of two to four capital letters followed by a dash and a short-hand designation for the producer (site), in maximum three letters, the first being a capital. Catalogues giving strain and producer designations exist, and some examples are given below - including exceptions due to usage established before the introduction of the present rules:

AKR, BALB/c, CBA, C3H, C57B, DBA, NZB. Examples of institutional names are:
J Jackson Laboratories (USA)
Tif Tierfarm AG (Switzerland)
He Heston (American investigator)
Orl. C.S.E.A.L. (French breeding station)
Han Zentralinstitut für Versuchstiere (Hannover, Germany)
Bom Gamle Bomholtgård (Danish breeding station).

Complete names: AKR/J, C3H/Tir, DBA/Bom, etc. In the Materials and Methods section of your manuscripts it should not be necessary to give the addresses of these producers, which are accredited and registered. You may have to expand the designation, however, if an animals substrain (Table 3.1) has been used: C57B/Tif Bom means that the C57B strain was transferred to Gamle Bomholtgård from Tierfarm AG; and DBA/He J Bom that the DBA mice came to Bomholtgård from Jackson laboratories, to which the strain had been given by Dr. Heston.

Animal colours

The **genetics of fur colour** is another interesting, but complicated matter. There are several chromosomal loci for multiple allelic genes that influence colour and colour patterns. Among these loci, A-, B- and C-locus are particularly important in rodents. The **A-locus**, named after a South-American rodent, the **agouti**, contains the A-genes that determine the so-called wild animal colour, or distribution of coloured bands or zones in the animal's hairs. With a capital-A-gene the animal can have a dark patterned back, with mixtures of black, grey, brown, yellow, and red, whereas the abdomen is lighter grey-white or white. This colour pattern, probably serving to camouflage the animals living in their natural habitat, is common among wild-type rodents and other animals, such as the wild rabbit. Homozygocity for the a-gene, however, will cancel the agouti pattern, so that the fur may be homogeneously black or brown.

B in **B-locus** stands for **black**. Presence of a B-gene is a necessary condition for the manifestation of an A-gene. Thus, AA . BB gives a typical agouti fur, whereas aa . BB codes for a unicoloured black coat and aa . bb - as well as AA.bb - for a unicoloured brown coat.

C in **C-locus** means "**full colour**", so that a C-gene is necessary for the appearance of both A- and B-gene effects. Consequently, AA . BB . cc is an **albino** animal, lacking the tyrosinase enzyme. It is unable to synthesise the black pigment melanin and has got a white coat, red eyes and light red skin.

The mouse

Several different ways of life are found among the rodents; some live in underground caves, some in or close to water, some on the ground, and some in trees. Rodents can be either diurnal or nocturnal - day or night animals. The house mouse, *Mus musculus*, is a social, nocturnal creature. The mice in a cage tend to congregate and sleep during day-time and swarm actively during night-time.

The origin of the mouse probably was in Asia; migration made it ubiquitous. The wild-type animal is very temperamental and excitable. It will immediately try to escape or bite you if you try to catch it by its tail. The domesticated varieties may on rare occasions expose similar activities, but mostly the laboratory mice are docile and easy to handle. Furthermore, they are lively, clean, and tidy small animals. Remember, however, that they can easily climb a rough-surfaced wall!

The mouse tail: a heat regulatory organ convenient for iv. injections

Except for a sparse supply on the legs, the mouse lacks sweat glands and regulates its body temperature by varying the blood flow to its tail. Temperature regulating centres in the hypothalamus control the blood flow - which may be large - through the one artery and three veins of the tail, like skin blood flow is regulated in other mammals. Under extreme heating conditions mice will lick

themselves and their neighbours, to cool down by evaporating the spit deposited on their coats.

All rodents have four very long and curved cutting (incisor) teeth, two in the upper and two in the lower jaw. They are root-less, which means that the enamel organ persists, producing continuous growth throughout life at a speed of about 2-3 mm a week. If constant length is not maintained by normal gnawing and thereby wear and tear of upper and lower incisors against each other, the teeth can be painlessly trimmed with a pair of nippers.

Particulars about the rodent gastro - intestinal tract

The rodent menu is mainly vegetarian, with a variable meat component. The most conspicuous feature of the rodent digestive tract is the huge coecum. This is a storage, fermentation and sorting site; during night some of its contents are sorted out, passed on through the large bowel and eaten from the anus (i.e. coprophagy). Thus, by this process - called coecotrophy - food is passed twice through parts of the digestive tract, presumably because some essential nutrients produced by microbial activities in the coecum can not be absorbed by the large bowel (B-vitamins, vitamin K, essential fatty acids).

The sex life of the mouse

Rodents are very fertile, with large litter sizes. This is probably an adaptation to naturally high mortality, due to numerous predators and few effective defence mechanisms in their natural habitat. Mouse generation times and oestrous cycle times are both relatively short (Table 3.2), which is also an advantage for laboratory animal husbandry. Mating takes place during the night. A recommended procedure is to bring one male to a cage with e.g. four females. The female mouse is in oestrous approximately 13 hours of the 4-5 day oestrous cycle. A rubber-like, yellow-white vaginal plug can be detected the next morning if mating has taken place. This plug is formed by the secretions of the coagulation gland of the male, situated next to the seminal vesicles.

An examination of the female's oestrous phase can also be used to predict the time of mating: microscopy of a stained vaginal smear shows (i) numerous leukocytes, some small polygonal and nucleated epithelial cells, and mucous during di-oestrous, (ii) large polygonal cells, but few or no leukocytes and no mucous during pro-oestrous, (iii) large polygonal cells only - now cornified and without nuclei - during oestrous, and (iv) some cornified cells, some nucleated polygonal cells, and lots of leukocytes during post-(met-)oestrous. However, not all matings lead to impregnation, and diagnosing pregnancy before day 16 is very difficult. Without fertilisation, mating results in an approximately 12-day period of pseudo-pregnancy.

Useful genetic variants

Several hundred mouse mutants have been generated. These may serve as valuable models of human diseases or special physiological conditions, for example the diabetic mouse, the athymic (and "nude") mouse, the muscular dystrophic mouse, the obese mouse, various types of anaemic mice, etc. An increasing number of useful transgenic mice are also produced (see Box 3.2).

Easily manipulable, except for major surgery and repeated sampling

A mouse can be lifted and manipulated by its tail. Subcutaneous injection can be performed through the skin of its back. The easiest method is to place the mouse on the cage lid and restrain it by holding its tail; the mouse will then naturally try to pull itself free from your grasp. Otherwise you may stretch the animal between your left thumb and forefinger (grasping its neck skin) and your little finger (pressing its tail root against your palm). Now the mouse should be sufficiently immobilised so that you can inject your agent intraperitoneally (go through the lower part of the abdominal wall) or by gavage (rodents lack a

vomiting reflex). Mark the necessary distance (from mouth to stomach) on the blunt gavage tube beforehand. Finish by letting the mouse play out its aggressiveness by allowing it to bite e.g. the syringe afterwards. Intravenous injections or bleeding (Table 3.2) requires restraint in a perforated cylindrical holder or anaesthesia/analgesia or both. Intravenous injections are best performed into a dilated tail vein of a heated animal. A heat lamp over the animal or the tail, or submersion of the tail in hot water (not burning, <43°C!), may be sufficient. Don't use xylol treatment of the tail skin to dilate the veins; after repeated use the skin becomes parched. Bleeding is best performed from a metatarsal vein; heparinised haematocrit tubes being filled from a puncture wound. The leg is shaved, silicon grease applied to the skin over the vein, and stasis obtained by stretching the leg skin across the vein. By rubbing the stab wound within a few hours you may re-start the bleeding and get a new blood sample. Bleeding from the retroorbital sinuses should not be performed, for ethical and aesthetic reasons.

Infectious diseases of mice

Be aware of the possibility that your animals may be or become infected, and that you should yourself probably be vaccinated against tetanus.

Beware infected animals

Mice may be infested with mites or ring worms in their skin and tape-worms or pin-worms in their guts. More seriously - and usually with no clinical signs - rodents may have viral infections that can change the function of the immune or other organ systems. Examples are provided by mouse hepatitis virus, minute virus of mice, and lactic dehydrogenase virus.

Since the beginning of the 1960s the microbiological status of rodents, especially mice and rats, has been highly improved. However, this does not mean that mice and rats today never suffer from infectious diseases, and therefore that the influence of micro-organisms on research is a historical event - even though the animals have been delivered by commercial breeders providing long lists of micro-organisms claimed to be excluded. Microorganisms can in fact influence the experimental results, although disease symptoms are not observed.

As health monitoring techniques have been improved, more and more micro-organisms are found to be latently present in laboratory animals. For example, mouse hepatitis virus (MHV) is a worldwide infection in laboratory mice, caused by a single-stranded RNA virus belonging to the Coronaviridae family. MHV is extremely contagious. The pathogenesis is influenced by factors like virus strain

and mouse strains; however, current evidence suggests that the infection usually runs its course in 2-3 weeks, and there is no carrier state. Transmission is by direct contact, fomites, and airborne particles. Furthermore, MHV is a frequent contaminant of transplantable tumours and cell lines.
MHV has been reported to alter many experimental results. Examples are perturbed immune function (like inhibition of lymphocyte proliferative responses in mixed lymphocyte cultures, alteration of phagocytic and tumouricidal activity, increased susceptibility to other indigenous pathogens, activation of natural killer cells, and production of interferon), delayed increase in plasma lactic dehydrogenase activity following infection with lactic dehydrogenase virus, and occurrence of anaemia, leucopenia and thrombocytopenia. In atymic (nu/nu) mice, the virus can also cause spontaneous differentiation of lymphocytes bearing T-cell markers, alter IgM and IgG responses to sheep erythrocytes, enhance phagocytic activity of macrophages, cause rejection of xenograft tumours, impair liver regeneration after partial hepatectomy, and stimulate hepatosplenic myelopoiesis.

Subclinical infections can be exacerbated by thymectomy, whole-body irradiation, reticuloendothelial blockade by iron salts, and administration of cortisone, cyclophosphamide, antilymphocyte serum, chemotherapeutic agents, or halothane anaestesia.

Health monitoring programmes are gaining high priorities among biomedical research workers, since infections in laboratory animals, whether producing clinical disease or not, can influence the outcome of many kinds of experiments. Research data obtained from defined, health monitored animals are in general reliable and reproducible. An additional benefit is the reduced risk of zoonotic infection of personnel handling laboratory animals or products derived from such animals. It should be emphasised, however, that in many kinds of experiments certain infections are of secondary importance and do not necessarily disqualify results derived from the use of such infected animals. The main purpose of health monitoring is to achieve «calibrated», defined animals, thereby avoiding spurious experimental results.

As mentioned above, there is also a possibility that your animal colony can be infected by **microorganisms carried in cell lines**. In principle, cell lines can contain the same kinds of intracellular microorganisms that are present in live animals. Most of them are viral, but also mycyplasmas and intracellular bacteria

have been contaminants. The presence of microorganisms in biological materials may be detected by serological analysis of animals previously injected with the material (e.g. with the Mouse Antibody Production (MAP) test). Contamination from other sources than the inoculated cells must be excluded by performing the antibody production test while the animals are kept in isolators.

The rat

Much of what is written above about laboratory animals in general and about rodents and mice in particular applies to the rat. For example, rats are nocturnal and social animals; they have continuous growth of incisor teeth, large coecum and coecotrophy (but the rat lacks a gallbladder, in contrast to the mouse), high fertility with short oestrous cycles, characteristic phase-dependent vaginal cytology, vaginal plug formation after mating, and 12-14-day pseudopregnancy.

Handling techniques

The rat should not be lifted by its tail, but taken with a careful grasp around its body. Seek instruction in animal handling from a competent scientist, veterinarian, or animal house worker! Gavage and intraperitoneal, subcutaneous, and intravenous injection can be performed as for mice. With male rats the dorsal vein of the penis can also be used for injections; a tongue or hind leg vein has also been used. The rat is big enough to permit introduction of permanent (i.e. up to a few days) catheters for example into the aorta (via a carotid artery) or into the thoracic lymph duct. The latter is also feasible in the mouse, but more difficult. Bleeding the rat is as with the mouse, from a metatarsal vein. But since larger volumes can safely be sampled, a larger blood flow can be obtained from the puncture wound by artificially increasing the hydrostatic pressure drop from vein to atmosphere. This is done by applying a plastic cylinder – which serves as a blood receptacle - with a tight-fitting (rubber) collar to the leg, just after the vein has been punctured. A side tube to the cylinder, connected to a suction pump, lowers the pressure inside the cylinder, for as long as the bleeding lasts.

Rat infections

Rats are in general resistant to infections, but may contract murine virus pneumonia, which has been asymptomatic in the mouse hosts. This pneumonia can be transmitted to the offspring during the first post-natal day and shorten the rats' life span to less than one year. Rats are also susceptible to mycoplasma otitis and Bartonella infections of the red blood cells. The latter, latent infections may lead to a lethal anaemia after splenectomy.

The rabbit

The European rabbit (*Oryctolagus cuniculus*) probably originated in the west Mediterranean countries; Romans who came to Spain thought that these animals looked like small dogs and therefore coined the name *cuniculus*. Since then, man has exported the rabbit to all parts of the world, partly as a domestic animal and partly as wild game. Wild animals rest in burrows during the day and seek the feeding fields to eat grass and juicy plants during night. The rabbits are very social animals and tend to gather in colonies. Rabbits are fertile too, so where natural predators are absent and living conditions good, they have caused great damage both to the ground (burrowing) and the vegetation (Australia, New Zealand, UK). Rabbits engage in coecotrophy during the night.

Rabbit habits

The rabbit's well developed fur tends to conceal that the animal has a long cylindrical body form. The long ears are not very hairy, however, and function in thermoregulation in much the same way as the tails of mice and rats.

The ears are richly vascularized, with large marginal veins and a large central artery; these vessels can be cannulated. The smaller and most peripheral veins can be used for blood sampling. By lowering the pressure with a suction device similar to that described for the rat, large blood samples can be obtained. The cylinder, accommodating the whole ear, then rests against the rabbit's cranium. During such manoeuvres the rabbit may be immobilised by wrapping it in a towel or putting it in a box. It can be lifted by the neck skin, but should be supported from the ventral side. (Risk of columna fracture!).

Ears as thermo-regulators

The ovarian cycle of the rabbit lasts about 15-16 days, but ovulation has to be induced - by mating, artificial stimuli, or reciprocal excitement of two females. Conception may take place from day 2 - 14 of the ordinary cycle. Pseudo-pregnancy (nest-building, mammary gland growth, sterility) lasts 16-19 days, pregnancy 28-36 days. Trial and error is the way to find out whether a female is receptive: the male rabbit must be brought to her, and not vice versa.

The sex life of the rabbit

Many female rabbits are rather careless towards their naked, blind, and helpless new-born offspring, neglecting to place them in a nest made from vegetable materials and hair. To avoid their freezing or starving to death, rabbits should be looked after during parturition.

Laboratory rabbits are bred in numerous, often small units around the world, probably implying a high degree of genetic variability. Inbred rabbits have to our knowledge not yet been produced, and it would take at least a human generation (30 years) to accomplish the minimum number of 20 brother x sister matings required to make an inbred animal strain.

Rabbits frequently harbour *Pasteurella multocida* and *Bordetella bronchiseptica*, which are potentially pathogenic to other species, like guinea pigs (who are especially susceptible to *B. bronchiseptica*). Keep such species in separate animal rooms if possible.

Outbreeding and infections

The dog and the cat

These pet animals are in fact beasts of prey, or carnivorous, belonging to the order *Carnivora*. Their teeth and jaws are constructed accordingly. The muscles of mastication are strong, the skull solid, and the brain therefore well protected. Nevertheless, these animals are not exclusively carnivorous; we have seen a dog tricked into eating carrots! Food is not well chewed, only so much as to allow the pieces to be swallowed. The

stomach is a simple reservoir, the bowel relatively short and almost without a coecum. These animals are much less fertile than the rodents (Table 3.2). There are both diurnal and nocturnal species. Most live on the ground, but good climbers or swimmers also exist. Some predators live and chase singly, others in pairs or larger groups. Some attack the prey rapidly and by surprise, others hunt it until it is exhausted. The olfactory sense is extremely well developed.

The domestication of the dog took place so long ago that it is uncertain whether the dog has ever existed as a wild-living species, separate from other species of the genus *Canis*, such as the precursors of the wolves or jackals. Random or planned crossings and selections produced at first a domesticated dog fit for hunting, later for guarding herds and residences, and finally also for social purposes. Consequently, a large variety of dog strains or breeds with very different properties exists.

The cat is definitely nocturnal and may eat almost whatever animal it can manage to chase, including birds and fishes. Cats are of necessity genetically heterogeneous; furthermore, they often present considerable hygienic problems, harbouring parasites and the very contagious microbes causing cat plague and respiratory infections. The emotional objections to their use are the same as for the dog.

Other animals

The Syrian or golden **HAMSTER** (*(Meso)cricetus auratus*). Adult animal weight < 140 g, length ~ 15 cm, plus 1-1.5 cm tail. Nocturnal, mostly territorial animal. Easily manipulated. Omnivorous. Truly inbred strains of hamsters do exist, but are rare.

Hibernates <9°C (with 6-8 heart contractions per min). Low degree of histocompatibility gene polymorphism. Cheek pouches: tube-like extensions of the mucous membrane of the mouth, forming one big subcutaneous sack on either side, reaching as far as the shoulder girdle (~ 1 cm wide, 3-5 cm long when empty - can be markedly dilated when filled). Food or offspring may be carried in the pouches. The pouches can easily be turned inside out and used to study blood circulation, tumour growth etc. in vivo. In addition, they are so-called immunologically privileged sites. Gut microbial flora sensitive to antibiotics, which may therefore kill the animal. Low water demand; kidneys able to produce highly concentrated urine.

Best animal house conditions: Temperature 22 +/- °C, relative humidity 50 +/- 5%, light on at least 12-14 h/d (- could be during night, to synchronise activity period of animal and research worker). Hamster fights should be stopped; the female may kill the male even after being mated repeatedly during an hour's time! Mothers may also exhibit cannibalism against their offspring.

Body temperature: 36.1 - 38.9°C; heart rate: 100 - 300 min^{-1}, respiratory rate: ~ 75 min^{-1}. Life span probably ~ 3 yr.

The **GERBIL**, the Mongolian desert rat, or sand rat (*Meriones unguiculatus*) is a peaceful, hardy, frightless, adaptable, and extraordinarily teachable animal that has gained increased popularity. It can do with metabolic water and water present in its food, so except for inspection and social contact with the animal house personnel, the caging is very simple (very low urine production; faeces almost completely dry). Activity day and night, with a maximum after midnight. Predisposed to epileptic fits provoked by manipulation etc. Adult body weight: females 70-80 g, males 90->100 g; length 11-13 cm, plus tail 9-11 cm. Maximal life span possibly 5 yr. Normal body temperature 35.8-39.0°C.

Relative moisture in animal house should possibly be below 50%. Females may be so strongly monogamous that they refuse to accept a new male instead of their old mate; the males take part in rearing the young.

The gerbil is used for example by neurobiologists, and carries potential for very wide usage (physiology, pharmacology, neurology, psychology, oncology, infection pathology).

The **GUINEA PIG** (German: "Meerschweinchen" = 'Little ocean pig'; French: 'Cobaye') (Cavia porcellus) stems from South America. Social (communal) and nocturnal animals. Probable food supply for the Inkas, but also pet animals, since they are easily domesticable and confident creatures. Like monkey and man, the guinea pig can not synthesise vitamin C (ascorbic acid), and its daily demand per gram body weight (0.02 mg) is huge. Its cellulose hydrolysing gut (Gram-positive) microbial flora is as sensitive to antibiotics (including penicillin) as that of the hamster (Gram-negative bacteria replacing the usual flora easily lead to a lethal 'intoxication'). Adult body weight, at an age of 5-6 months, is 700-800 g, body length 20-25 cm, no tail (and lack of unhaired parts of body surface may make temperature regulation difficult; keep room temperature below 26°C!). Cannot climb; kept in detention by ~ 30 cm high box walls.

Body temperature: 37.4-40°C; heart rate: 130-180 min^{-1}; respiratory rate: 90-150 min^{-1}. Life span ~ 8 yr.

The guinea pig is used in tuberculosis research, in allergology and immunology, in microbiology and toxicology.

The **PIG** (family: *Suidae*) may provide good models of human diseases, except those affecting the digestive tract. When smaller animals like the dog and the cat are difficult to use in physiological experiments and transplantations requiring major surgical intervention, the pig, **SHEEP** (genus: *Ovis*), or **GOAT** (genus: *Capra*) may be preferable.

The **MINI-PIG**. The potential value to biomedical research of physiologically and anatomically normal pigs of small adult size led to planned development of the miniature swine, based on the availability of adequate genetic resources and breeding methodology.

There is considerable variation in the growth rate and size at maturity of the various types of swine. Commercial breeds of pigs typically have a birth weight of 1-2 kg and achieve a weight at sexual maturity greater than 100 kg. They continue to grow at a lesser rate for several more years and may achieve a size of 200 kg or more during their commercial life-time. This heavy weight makes them undesirable for long-term studies, unless massive growth is particularly desirable. In response to this problem, several breeds of miniature swine and a Micropig® have been developed through genetic selection for small size at maturity. In the United States, these include Yucatan Micropigs® (Charles River Laboratories, Inc., Wilmington, MA) and miniature pigs of several varieties: Yucatan, Hanford, Sinclair, Pitman-Moore and Hormel. Typically, miniature pigs have a birth weight of 600-1000 g and a weight at sexual maturity of 20-40 kg. Micropigs have a birth weight of 600-700 g and a weight at sexual maturity of 14-20 kg.

In general, it is easy to provide a uniform and predictable pig size, which is dependent on breed selection. Cost varies widely for commercial swine. Commercial breeds of SPF swine are more expensive than non-SPF swine, but generally cost less than healthy purpose-bred dogs. Miniature pigs and Micropigs® are generally comparable in cost and health status to purpose-bred conditioned dogs.

BIRDS (*Aves*) are very different from the mammals mentioned above. In addition to differences well known by everyone, they have colour vision, which laboratory mammals lack. Digestive tract without teeth, with several proximal (digestive) reservoirs ('stomachs'), and most often two blind guts (coeci). Air sacs that penetrate even into their skeleton are used to ventilate the lungs. Cloaca as receptacle for contents of digestive and genito-urinary tracts. Bursa Fabricii in the wall of the cloaca serves as maturing organ for virgin B-lymphocytes; birds lack well-structured lymph nodes. Only left ovary active; if the right anlage is developed in old birds, it may produce androgenic hormones ("The hen that became a cock"). Elongated and nucleated red blood cells. End product of protein catabolism is uric acid, not urea as in mammals. Body temperature ~41°C, - sometimes even normally exceeding 44°C!

Common laboratory animals are the hen (*Gallus domesticus*) and the quail (*Coturnix coturnix*). Hens (adult body weight 1.5-3 kg) have been used for diagnostic purposes and for physiological, biochemical, and nutritional experiments; the eggs for virus diagnosis and vaccine production, as well as for embryology. The quail is extraordinarily adaptable to a life in custody, even though it is the only migratory bird among the gallinaceous birds.

Adult female body weight: ~ 130 g. Easier husbandry than for the hens, and similar areas of application.

Table 3.1. A very short dictionary

Species.	All the animals of the same kind that can (actually or potentially) mate together and produce fertile offspring.
Stock.	A collection of animals being grown or maintained for breeding or for experimental use.
Family.	A breeding group generally descended from a single pair of parents.
Strain.	A group of animals of known ancestry maintained by a deliberate mating system; generally with some distinguishing characteristics.
Substrain.	Any strain separated after *eight to nineteen* generations of brother x sister inbreeding and maintained thereafter in the same laboratory without intercrossing for a further *twelve or more* generations shall be regarded as a substrain. It shall also be considered that substrains have been constituted (a) if pairs from the parent strain (or substrain) are transferred to another investigator, or (b) if detectable genetic differences become established.
Inbred strain.	A strain shall be regarded as inbred when it has been mated brother x sister (hereafter called b x s) for *twenty or more* consecutive generations. Parent x offspring mating may be substituted for b x s matings, provided that in the case of consecutive parent x offspring matings the mating in each case is to the younger of the two parents.
Line.	Part of a family of animals separated from other parts by one or more generations of independent ancestry.
Subline.	A division of a line.
Random breeding.	Mating of animals by chance, without regard to relationship, to genotypic or phenotypic resemblance.
Outbreeding.	Mating system in which the relationship between mated pairs is less than the average relationship of contemporary individuals, i.e. the deliberate avoidance of inbreeding even to the extent of introducing animals from outside.
Inbreeding.	Mating of closely related animals.
Hybrid.	The immediate product of a) an inter-specific cross, or b) a cross between two inbred strains.
Back cross.	The cross of an F_1 hybrid to either of its parents: in mice, to animals of either parental strain.
Selection.	Causing or permitting some kinds of individuals to produce more offspring than others.

Homozygous. Having identical alleles at a given locus.

Heterozygous. Having different alleles at a given locus.

Phenotype The appearance or properties of an organism.

Genotype. The genetic composition of an organism.

Isogenic. Having identical genotypes.

Coisogenic. Having in theory identical genotypes except for a designated difference.

Congenic. Having similar genotypes.

Performance tested. Refers to an animal population (usually a strain or one of its subdivisions) shown to posses a continued ability to exhibit certain responses or characteristics for which the population is primarily maintained.

Certified grade. Refers to animals endorsed by competent authority as conforming to certain defined and accepted minimum requirements.

Commercial grade. Refers to animals of undetermined quality that do not necessarily conform to accepted minimum requirements.

Accredited supplier. A supplier who raises stocks of laboratory animals which conform to defined and accepted minimum requirements.

Enzootic. A disease within an animal group which remains over a considerable period of time within the group.

Epizootic. A disease which affects many animals at one time.

Specific pathogen free (SPF). Animals that are free of specified microorganisms and parasites, but not necessarily free of the others not specified

Germ free. Animals that are free of all demonstrable microorganisms and parasites, resulting from use of closed system sterile technique.

Gnotobiote. A specially reared organism, the microfauna and microflora of which are specifically known in their entirety.

Gnotobiotics. The science of rearing organisms, the microfauna and microflora of which are specifically known in their entirety.

Transgenic organisms.	Produced by introduction of extra or mutant genes into a fertilised egg cell (or a totipotent plant cell), so that this DNA becomes a. permanent part of the genome. The transgenic organisms express the new gene(s) and pass it on to their progeny.
Knock-out mice.	Transgenic mice in which both copies of a gene have been eliminated by homologous recombination.

Based on Sabourdy (1964)

Table 3 .2. Useful data on some commonly used experimental animals

	Mouse	Rat	Rabbit	Dog	Cat
Biological variables :					
Typical body weights (b.w.) of exp. animals	20-30 g	200-400g	2.5-4 kg	10-35 kg	2.5-4 kg
Life span	C3H: 390-420d Outbred: 700-750d	2-3 yr.	7-8 yr.	13-17 yr.	13-20 yr.
Body temperature (°C)	35.5-39.5	35.5-39.5	37.5-39.5	37.0-39.0	38.5-39.5
Heart rate (min^{-1}, minimum average & range)	470 (300-650)	350 (260-450)	260 (205-308)	110 (77-138)	150 (110-226)
Respiratory rate (----- " ------)	138 (90-180)	92 (80-150)	40 (35-56)	19 (14-28)	26 (20-30)
Blood volume [1] (ml/100 g b.w., average)	8	5	7	9	7.5
Water required daily (ml)	3-7	20-45	80-100/kg	25-35/kg	100-200
Urine excreted -"- (")	1-3	10-15	50-90/kg	65-400 [2]	50-120
Food required -"- (g)	3-6	10-20	75-100	250-1200 [2]	110-225
Digestible protein (%)	12	12	14	20	30
Haemoglobin concentration (g/L)	140	145	120	150	120
Erythrocyte life span (d)	20-40	60-70	50-70	100-120	70-80
Leukocyte concentration (WBC count) (10^9 cells/L blood)	10	15	9	11	18
Differential count, neutrophil granulocytes (%)	10	20	40	60	60
Breeding, reproduction, and husbandry:					
Sexual maturity	6-8 wk	7-11 wk	4-5 mo	9-15 mo	5-12 mo
Sexual cycle length (d) and type [3]	4-5, P	4-5, P	14-16, induced P	21, M (2 times/yr)	14-24, SP
Breeding behaviour [4] and season	H(1m to 4f) all year	H(1m to 6f) all year	Ph(f to m) all year	Ph(f to m) biannual	Ph(f to m) Jan.-Sept.
Duration of sexual receptivity	10-20 h	10-20 h	Ovulation variable	4-8 d	3-8 d, irregular
Optimal reproductive span [5]	6-8 mo	8-10 mo	3 yr	6-7 yr	6-7 yr
Light hours per day for optimal functioning	14	12-14	12-14	10-12	12-14
Gestation period (d, average & range)	20 (19-21)	21 (20-23)	31 (28-36)	63 (56-70)	62 (58-66)
Litter size (range)	6-12	6-14	1-16	3-10 breed dependent	1-8
Birth weight (g)	1-1.5	4-6	<100 g	- " -	90-140
Litters per year	4-5	5-6	5-6	1.5-2	2.5

Age at weaning	19-21 d	20-22 d	6-8 wk	6-8 wk	4-8 wk
Weaning weight (g)	8-14	40-50	breed de-pendent	breed de-pendent	~ 600
Thermoneutral zone (°C) [6]	30-32	28-31	16-20	-25 -+32 (includ.polar dog)	
Animal house temperature, during breeding	18-26	18-26	16-21	18-29	18-29
Animal house temperature, during experimental period	21-23	21-23	16-21	18-29 (27-29 post-ope-ratively)	18-29
Animal house relative moisture during breeding (%)	40-80	40-80	40-60	30-70	30-70
-----" ----- relative moisture, experimental period	50-60	50-60	40-60	30-70	30-70
Minimum space recommendations:					
Type of housing	cage	cage	cage	pen/run/ cage	cage
Floor area per animal (cm^2)	95 (>25g b.w.)	190 (200-300g)	2 800 (2-4 kg)	11 200 (15-30kg)	2 800 (< 4kg)
Height, from the resting floor to the cage top (cm)	13	18	36	(90)	60
Blood volume available (ml):					
From adults, routine sampling	0.1	0.25	1.0	2.0	1.0
--- " --- , maximum	0.007/g	0.005/g	7/kg	9/kg	7/kg
--- " --- , after killing	0.025/g [7]	0.02/g [7]	35/kg	45/kg	35/kg
Maximum injection volumes (ml):					
Intravenously, one rapid inject.	0.5	1.0	10		
Orally, fasting state	0.5 (1)	2 (5)	5		
Subcutaneously, several sites	1	3	10		
Intramuscularly, per site [8]	not done	0.1	0.5		
Intraperitoneally	1	4	10		
Sedation :[9]					
Propionylpromazine (Combelen vet.R, Bayer, FRG)			0.3-0.5 mg/kg s.c. i.m.(or i. v.).Dura-tion 2-4 h		
Xylazine (Rompun vet.R, Bayer) [9]					3-5 mg/kg s.c., after 12 h of fasting
Fentanyl/Fluanisone (HypnormR, Janssen, Belgium,0.2 mg. fen.+ 10 mg flu./ml)		0.05 ml/ 100 g	0.05-0.1ml/ kg s.c. or i.m.		

Anaesthesia : [9, 10, 11]

Midazolam (Dormicum R, Roche, Switzerland) + Hypnorm R (see above)	0.05-0.075 ml dilution/10g s.c. or i.p. (30-60 min duration) [12]	0.15-0.2 ml dilution/ 100g s.c. or i.p. (20-90 min)	2 mg/kg i.p.,then after 5 min 0.3 ml/kg i.m. or 0.1 ml/kg i.v. [13] (40-90 min)		
				See e.g. Flecknell (1987)	
Analgesia:					
Buprenorphine Hydrochloride (Temgesic R, Reckitt & Colman, Hull, England)	0.1-0.3 mg/ kg s.c. (6-8 h duration)	0.1-0.3 mg/kg s.c. (8-12 h)	0.02-0.05 mg/ kg s.c. (8-12 h)	0.01 mg/ kg s.c. (8-12 h)	0.01-0.02 mg/ kg s.c. (8-12 h)
Euthanasia:[14]					
Cervical dislocation	Acceptable (A.)	Not acceptable (N.a.) for rats >50 g	N.a.	N.a	N.a.
Carbon dioxide (100% or better: 70% + 30% O_2) in an uncrowded chamber	A. for adult mice	A. for adult rats			
Halothane (Halothan R, Hoechst, FRG), moistening cotton wool in an uncrowded chamber, placed in ventilated cabinet. Removal of dead animals >10 min after respiratory arrest.	A. for newborn and young mice	A. for newborn and young rats			
Pentobarbital (pentobarbitone)	25 mg i.p.	100 mg/kg i.p.	100 mg/kg i.v.		100 mg/kg i.v.
Thiopentone Sodium (Pentothal R, Abbott labs, England)			30-40 mg/ kg i.v.+ exsanguination		
Decapitation or head blow + exsanguination, on special occasions and by trained personnel only	A.	A.	A. <300 g b.w.		

Footnotes

1 : Cage life tends to make animals fat, if they are fed ad libitum. Then the blood volume given as a percentage of the body weight will be reduced.

2 : Breed dependent

3 : P = polyoestrus, SP = seasonal polyoestrus, M = mono-oestrus

4 : H = Harem mating (1 male (m) to number of females (f)). Ph = polygamous, but usually hand mated, take (male to female)or (female to male).

5 : Refers to that period of the female's life during which fertility and litter size are maximal and reproductive complications minimal. Breeding stock will usually be replaced at the end of this period.

6 : Temperature range where extra metabolic energy is not needed to produce or dissipate heat.

7 : Our own experience is that these estimates taken from the literature are too low, e.g. it is usual to obtain 1.0 ml blood from a mouse.

8 : Whole or part of intramuscular injection to small animals - even to rabbits - is often deposited intermuscularly, so that the agent is resorbed as if it had been injected s.c.

9 : The animals should be well adapted to the situation and in a quiet environment for optimal effect of the drugs.

10 : Beware that an anaesthetised animal is not just an unconscious creature with extinguished nerve reflexes; it is affected by drug(s) that may have side effects, which could ruin the experiment. Remember also that during anaesthesia small animals in particular may lose body heat, which may be disastrous.

11 : Ether should not be used; it is explosive and may lead to haemolysis and disturbed antigen/antibody reactions in the animal. Furthermore, when applied on "open mask", the very cold inhalation gas may cause a dangerous fall in body temperature.

12 : Dormicum and Hypnorm are diluted separately, equal volumes of drug and sterile water. The final mixture contains equal volumes of the two dilutions and must not be refrigerated. Shelf life about 8 weeks.

13 : The effect of fentanyl can be cancelled by Naloxone Hydrochloride (Narcanti ^{R}, Du Pont Ltd.,England)

14 : Acceptable methods should (i) depress the central nervous system to ensure insensitivity to noxious stimuli, (ii) be rapid, (iii) be carried out by trained personnel, and (iv) not interfere with planned post mortem evaluation. A

follow-up examination should confirm the absence of a heartbeat. Sometimes (rabbits, cats, dogs) sedative drugs should be used before the killing, which should as far as possible be aesthetically acceptable as well. Consult the relevant laws and regulations!

APPENDIX: TRANSGENIC OR KNOCK-OUT ANIMALS

Some web sites:

Internet Resources for Transgenic and Targeted Mutation Research:

http://www.bis.med.jhmi.edu/Dan/tbase/docs/databases.html

NIH directory of Knockout Mice & KO Help Desk:

http://www.ncifcrf.gov/VETPATH/nihtg.html

INDUCED MUTANT RESOURCE (IMR). Transgenic and Targeted Mutant Mice. The Jackson Laboratory:

http://www.jax.org/resources/documents/imr/

Animal experiments, checklist 3

1. Animals. Species, strain, stock etc. Category (SPF, germ-free, etc.)
Sex; age; weight (development); sexual cycle
Source; date of delivery
Interventions and conditioning
Compliance with regulations

2. Housing. Temperature range
Relative humidity (range)
Illumination
Caging (mode of selection of animals; no. of animals per cage)
Feed (producer; type; batch no. and date of production; anti microbial precautions; ad libitum or quantity per animal) and water (tap water, additions of HCl or vit. C, or otherwise)
Hygienic state (handling routines, barrier system, filter cage tops, etc.)

3. The experiment. Test substances, apparatus, equipment: Chemical or technical name/description; producer; catalogue no.; batch no.; amount; time, route, and rate of administration: orally, by gavage, by injection : s.c., i.p., i.m., i.v., i.a. Additives; solvents.
Sedation, anaesthesia, analgesia: Drug description, dose, time of administration, duration.
Other experimental interventions; blood or urine sampling

4. General condition. Level of consciousness and activity, behaviour; coat; respiration; hydration; pain.

5. Euthanasia. Method; dose; time.

6. Necropsy and post mortem examinations. Procedure; results of inspection, histology, chemical and other analyses.

(All these points may not be strictly relevant for your experimental protocol - and even less so for the Materials and Methods section of your article - but you should be prepared to argue why!)

Exercises

1. Present pros and cons for your choice of experimental animal.
2. Could your protocol be improved, concerning your use of experimental animals (e.g. distribution of animals to test and control groups or choice of anaesthesia, analgesia, food supply, method of killing)?
3. Can you find alternatives to your use of laboratory animals?
4. Look up or construct a growth curve for optimally fed animals of the strain that you use. If your animals deviate from a standard curve, what is the reason?

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Chapter 4: Cell and tissue culture

Introduction

Let us start with a simple metaphor. If you wanted to learn how an old-fashioned, mechanical alarm clock works, you could carefully remove its back cover and scrutinise its interior. The result would probably be a general understanding of the clock's mode of behaviour. An alternative is to take the clock entirely apart and make detailed studies of a few of its parts at a time, to analyse in depth how these components interact, and then try to synthesise a coherent scheme of the clock in action. At some stage, you may want to go back to the whole, working assembly again to test the appropriateness and completeness of your scheme. (By then you would probably need a new clock.)

Holistic vs. simplistic approaches

It's the same thing with biological sciences. According to your research problem, you may choose to work with intact or reasonably intact organisms (see Chapter 3), or you may have to isolate organs, tissues, cells, or subcellular components for investigation. Then, at some stage, you may want to revert or advance to the integrated organism - to clarify the meaning of the now well characterised, isolated parts.

The time perspective

Time scales differ. Whole organisms can be studied during their entire life spans; organs removed and perfused with blood or a blood substitute for hours to days; tissue slices incubated in artificial extracellular fluids (culture media) perhaps for hours, - and isolated cells for hours to months, depending on their nature and the circumstances of the culture conditions. Subcellular components, like enzymes, their substrates, hormones, RNA, or DNA may be stored for long periods of time - freeze-dried (lyophilised) or frozen (at minus 20-196^{o} C; the latter being the boiling point of liquid nitrogen). Some cell types can also be stored at low temperatures.

Organs, pieces of tissue, cells, or subcellular components can be studied for hours to months in vitro

A mature mammalian organism is made up of cells that can be roughly classified in one of three categories: 1. Cells that cannot divide (like nerve and striated muscle cells). 2. Cells that constitutively divide (i.e. cells of 'renewal tissues', e.g. bone marrow cells and epithelial cells lining our inner and outer surfaces). 3. Cells that conditionally divide rapidly (to repair a cell loss - like

glandular cells in liver, kidneys, and salivary glands; as well as many types of connective tissue - stromal - cells). With current techniques, cultures of cells of all three categories have characteristic and limited life spans; and some cells survive such a short time even under the best culture conditions that we may speak about incubation rather than culture. Nerve, muscle, surface epithelial, and glandular cells can often be grown for days only; bone marrow and connective tissue cells for hours to months.

Static and (conditionally) renewal tissues; stem and transit cells

This last statement requires an explanation. Renewal tissues such as bone marrow cells consist of stem cells on the one hand and maturing and mature transit cells on the other. The definition of a stem cell is a cell that by division can give rise to daughter cells that 'have two options': either remain a stem cell or differentiate, and thereby in such renewal tissues start a suicidal course, maturing to a functional end cell with a limited life span (i.e. a transit cell). Now, if you isolate functional end cells like neutrophilic granulocytes from blood and culture them, you can perform meaningful experiments with your cultures for only a few hours. This is because these cells are 'front line soldiers' in the combat against infections, apparently genetically programmed to die (that is, to die apoptotically) shortly after having performed 'their duty' (of devouring invading bacteria). If you culture the other type of 'professional eater cell' (phagocyte), the macrophage, your cultures can be extended to weeks, because these cells may have long natural life spans (and may even function as their own stem cells).

A fragmentary overview of some technical developments

When tissue culture was first successfully attempted, at the beginning of the 20th century, small pieces of tissue were explanted to the culture vessel, either from a cold- or warm-blooded animal. Cells sometimes migrated out from the explant, and cell division was only seen among those cells. About 1950 a rapid expansion of the field took place (Box 4.1). Culturing of dispersed cells (single-celled suspensions) was introduced; refined culture media and culture technology appeared (antibiotics, disposable plastic vessels, clean air equipment etc.); and by and by it was discovered that most cells need specific polypeptide or glycoprotein growth factors for optimal survival and growth in vitro (-literally meaning 'in glass', therefore slightly inappropriate now) (Box 4.1-3).

The art of cell culture developing into a science

For example, the history of haematopoietic cell culturing is illuminating. Until the mid-1960's, attempts at extended culturing of bone marrow cells had been unsuccessful. The art of cell culturing was by then not much more than 50 years old; we call it an art, because no one really understood what was going on in the cultures. The exercise - at least as it started - resembled alchemy more than science, and in the hands of Carrel, one of the pioneers, it even approached or perhaps exceeded the limit of fraudulence (Kohn, 1986).

Box 4.1. The history: Some Landmarks in the Development of Tissue and Cell Culture

1885 **Roux** showed that embryonic chick cells could be maintained alive in a saline solution outside the animal body.

1907 **Harrison** cultivated amphibian spinal cord in a lymph clot, thereby demonstrating that axons are produced as extensions of single nerve cells.

1913 **Carrel showed** that cells could grow for long periods in culture provided they were fed regularly under aseptic conditions.

1948 **Earle** and colleagues isolated single cells of the L cell line and showed that they formed clones of cells in tissue culture.

1952 **Gey** and colleagues established a continuous line of cells derived from human cervical carcinoma, which later became the well-known HeLa cell line.

1954 **Levi-Montalcini** and associates showed that nerve growth factor (NGF) stimulated the growth of axons in tissue culture.

1955 **Eagle** made the first systematic investigation of the essential nutritional requirements of cells in culture and found that animal cells could propagate in a defined mixture of small molecules supplemented with a small proportion of serum proteins.

1956 **Puck** and associates selected mutants with altered growth requirements from cultures of HeLa cells.

1958 **Temin and Rubin** developed a quantitative assay for the infection of chick cells in culture by purified Rous sarcoma virus. In the following decade, the characteristics of this and other types of viral transformation were established by **Stoker, Dulbecco, Green,** and other virologists.

1961 **Hayflick and Moorhead** showed that human fibroblasts die after a finite number of divisions in culture.

1964 **Littlefield** introduced HAT medium for the selective growth of somatic cell hybrids. Together with the technique of cell fusion, this made somatic-cell genetics accessible.

Kato and Takeuchi obtained a complete carrot plant from a single carrot root cell in tissue culture.

1965 **Ham** introduced a defined, serum-free medium able to support the clonal growth of certain mammalian cells.

Harris and Watkins produced the first heterokaryons of mammalian cells by the virus-induced fusion of human and mouse cells.

1968 **Augusti - Tocco and Sato** adapted a mouse nerve cell tumour (neuroblastoma) to tissue culture and isolated clones that were electrically excitable and that extended nerve processes. A number of other differentiated cell lines were isolated at about this time, including skeletal muscle and liver cell lines.

1975 **Köhler and Milstein** produced the first monoclonal antibody-secreting hybridoma cell lines.

1976 **Sato** and associates published the first of a series of papers showing thatdifferent cell lines require different mixtures of hormones and growth factors to grow in serum-free medium.

1977 **Wigler and Axel** and their associates developed an efficient method for introducing single-copy mammalian genes into cultured cells, adapting an earlier method developed by **Graham and van der Eb.**

Modified from Alberts et al. (1994)

Box 4.2 The surprising story of a balanced salt solution for cell culture

From «**This week's citation classic**», Current Contents, No. 5, Jan. 30, 1984:

Hanks J H & Wallace R E: Relation of oxygen and temperature in the preservation of tissues by refrigeration. *Proc. Soc. Exp. Biol. Med.* **71**:196-200, 1949.

John H. Hanks, October 17, 1983:

«This story illustrates the art of producing a quoted paper by being an unremitting failure in one's primary purpose. The steps were: (a) stubborn investigation of a problem that has not yet been solved after 110 years of effort by scores of investigators, (b) making sense by making an autoclavable balanced salt solution (BSS), and (c) helping John Enders *et al.* to develop cell cultures for the propagation of polio virus.

These accidents happened because in 1939 I had been induced by the Leonard Wood Memorial (American Leprosy Foundation) to investigate the microbiology of leprosy at the Philippine Culion Leprosarium.....I was given free rein to search for the central problems in bacteriology or immunology and to investigate the problem I thought most important. It became evident that without cultivating the causative agent, one cannot gain the knowledge required to cope with a stubborn infectious disease.

After about one year I had shot my bolt of brilliant ideas for inducing *M. leprae* to grow in bacteriologic media. The glowing report of Timofejewsky.....suckered me into the belief that, if only I could learn to maintain human cells from susceptible patients in tissue culture, a useful (though more costly) tool could be made available. The trials and errors doubled sweat production in that sticky environment. The recommended filter sterilisation of high-bicarbonate BSS opened a can of worms. Dissolved CO_2 (and changes in pH) were modified by pressures, temperature, and CO_2 concentrations. I terminated this foolishness.....by adjusting the ratio of phosphate buffers to pH 6.8 to limit the hydrolysis of glucose, and by including phenol red colour indicator so that pH could be seen across the room at any time and by autoclaving. Stimulation of cell respiration was ensured by one-tenth the usual bicarbonate or by the bicarbonate in serum. If interested in tissue cultivation, hundreds of biochemists, or country boys might have done the same.

The third step arose from a long-standing friendship with Enders at Harvard University. He, Tom Weller, and/or Frank Robbins began coming to my lab to discuss uncertainties or problems in their efforts to obtain standard replication of the polio virus in an optimal cell culture system.....Their investigation resulted in replacing •30 monkeys with •12 cell cultures (and a Nobel prize). One of their rare mistakes was to recommend that Hanks BSS was the way to go.

The paper including BSS probably owes its popularity to the innate wisdom and laziness of humanity. It is useful to autoclave all possible reagents employed in cell cultivation. If contamination occurs, one has a specific list of components which need not interfere with sleeping at night.»

(Barret, 1986)

Box 4.3. Some growth factors and their actions

Factor	Composition	Representative activities
Platelet-derived growth factor (PDGF)	AA, AB, or BB A chain=125 aa B " =160 aa	Stimulates migration and proliferation of fibroblasts, smooth muscle cells, and neuroglial cells
Epidermal growth factor (EGF)	53 aa	Stimulates proliferation of many cell types
Insulinlike growth factor II	73 aa	Collaborates with PDGF and EGF, stimulates proliferation of fat cells and fibroblasts
Transforming growth factor β	Two chains, each 112 aa	Potentates or inhibits response of many cell types to other growth factors. Affects differentiation of some cell types
Fibroblast growth factor	Acidic: 140 aa Basic: 146 aa	Stimulates proliferation of many cell types, including fibroblasts and endothelial cells. Induces mesoderm in Xenopus embryo
Interleukin - 2	153 aa	Stimulates proliferation of T lymphocytes
Nerve growth factor	Two chains, each 118 aa	Promotes axon growth and survival of sympathetic and some sensory and CNS neurons
Granulocyte/ macrophage colony-stimulating factor (GM-CSF)	25 000 d glycoprotein	Stimulates proliferation of G/M-precursors, enhances survival of immature and mature cells in vitro, affects G vs. M differention commitment, stimulates mature G and M functionally

It was of course known that when evolution produced multicellular organisms, the unicellular forerunners brought a portion of their surrounding sea with them into the new assembly, to constitute the extracellular fluid of man, animals, and plants. Normal life functions depend upon the homeostasis - equilibrium - of this extracellular fluid; and this is the task of several of our organ systems - lungs, heart, blood, bowels, kidneys, etc.. Osmolality, alkalinity (pH), ionic composition, gas pressure (pO_2 and pCO_2), nutrient, vitamin, and hormone concentrations, as well as temperature must be kept within strict limits. It is therefore only natural that the first culture media were various tissue extracts. By and by refined methods of analysis and new materials and products paved the road for the advent of standardised cell and tissue culture media of various types and known composition. Even then, and until the most recent decades, some extra material of unknown, but definite significance - present e.g. in sera or tissue extracts - had to be added.

Foetal calf serum - very expensive - has often been used, and still is, even though cheaper substitutes sometimes exist. We now know that foetal calf serum is devoid of potentially harmful antibodies and contains adhesive proteins, important as cell anchors to the cultured cells' plastic or connective tissue support. Most decisive, however, is the serum's content of relevant growth factors for the cells to be cultured; most of them being local hormones or paracrine agents. In haemopoietic cell cultures they are called colony stimulating factors, interleukines, or cytokines. Such factors control not only cell division in culture, but also cell maturation and cell survival. Conditioned media, which may replace or supplement the source of serum, are culture media harvested from cultures of cells that are usually of a different kind than those of the subsequent main culture. The success of bone marrow culturing, for example, has depended on the production of media conditioned by lymphoid, stromal, or other cell populations; and on the later purification, characterisation, amino acid sequencing, and gene cloning of the growth factors these cell populations produce (Box 4.3 and 4.4). Adhesive proteins (free in solution or integral parts of cell membranes), as well as growth factors, are often involved when cells need 'feeder cells' to grow in culture, or otherwise when cell-to-cell interactions are observed.

A recently achieved stage: chemically defined culture media, including artificially synthesised growth factors

Another important advent - which was particularly relevant to bone marrow culturing - was the production of viscous, semi-solid media, by addition of the polysaccharides agar or methylcellulose to the culture media. This

Clonal cultures in viscous media

prevented daughter cells from leaving their birth-place, so that cells stemming from a single progenitor (or stem) cell (i.e. a cellular clone) would stay together in a colony. In a week, mouse bone marrow cells would generate colonies visible to the naked eye (minimum cell generation times about 8 hours); human cells would need about twice this time (minimum generation times about 24 hours). A special property of blood cells is that they can grow like this in colonies, without a solid support. Other normal cells cannot; - they need a glass, plastic, or other solid surface to adhere to. Cancer cells, on the other hand, can often grow in suspensions and form colonies, like the blood cells. Finally, it should be mentioned that the most immature bone marrow stem cells apparently do not thrive in the presently available semi-solid media - nor do they thrive on plastic surfaces. To behave reasonably physiologically, they need cell-to-cell contact with certain bone marrow stromal cells (Box 4.4). It should be borne in mind that other cells as well - even if they survive and grow on a plastic surface - may perform quite differently and more naturally when present in a cellular niche that is similar to their natural one, thus containing certain connective tissue cells, as well as certain types of collagen fibres, proteoglycans, etc., making up a defined three dimensional scaffold.

Solidly supported *vs.* suspended cell growth

In this way haemopoietic stem cells, with current technology, can produce haemic cells for weeks in culture. Ultimately, however, this haemopoiesis ceases. Even cultures of fibroblasts - which are among the easiest ones to culture - tend to die out. Here, it seems that a kind of cellular senescence is programmed into the cells, so that fibroblasts from embryos will divide longer than fibroblasts from young animals - which in their turn will divide longer than fibroblasts from aged animals (The 'Hayflick limit'; see Box 4.1). The biological basis of the 'Hayflick limit' seems to be the loss of the chromosomes' telomeres. Telomeres are stretches of repeated polydeoxynucleotides forming the extremities of chromosomes. The standard DNA replicatory mechanism cannot duplicate telomere DNA; it takes special RNA primers and the telomerase enzyme to do the job. Therefore, in the absence of telomerase a segment of each chromosome's DNA will be lost in each cell division. When a critical telomere reduction has occurred, the cell can no longer divide. Significantly, the telomerase enzyme is active in cancer cells, and possibly also in true stem cells.

The "Hayflick limit" - an upper limit to the number of mitoses in culture and in vivo

Box 4.4.Uses of the long-term bone marrow culture technique

Long-Term Bone Marrow Cultures and their uses

The long-term culture of bone marrow cells is a technique in which primitive, developing and mature haemopoietic cells can be maintained and produced *in vitro* for more than 1 year (in the case of mouse cells) and several months (for human bone marrow). The cultures are established from whole bone marrow, in the absence of added growth factors. During the first 2-3 weeks of culture an adherent multi-layer of heterogeneous cell types form, which is derived from the marrow stromal cells. Haemopoietic cells present in the initial marrow inoculum attach to and migrate within the adherent layer of cells, and establish close interactions with the stromal cells. The cultures are fed weekly by replacing some of the culture medium with fresh medium, and the proliferation, self-renewal and differentiation of haemopoietic cells in the cultures is sustained for long periods. The most primitive cells tend to be retained in association with the adherent layer of cells and the more mature cells tend to be released into the growth medium, so that these maturing and mature cells are harvested during feeding of the cultures and can be assayed by various techniques to monitor the haemopoietic activity in the LTBMC.

The key characteristic of this technique is that the cellular environment for haemopoiesis, which exists in the bone marrow and which plays an important role in controlling haemopoietic cell development, is reproduced in the cultures. Extensive investigation and comparison of the cell types comprising the cellular environment *in vivo* and in LTBMC support the view that the cultures reproduce *in vitro* many of the cellular interactions between haemopoietic cells and their stroma, and that the haemopoietic cells produced have the same developmental capacity and functional characteristics as those produced in vivo. Even now, LTBMC is the only *in vitro* technique which supports the self-renewal of haemopoietic cells.

The major advantage that LTBMC offers over working *in vivo* is the simplification that results from isolating the haemopoietic process, in itself a complex mechanism involving multiple cell types and regulatory molecules, from the rest of the organism. Secondly, haemopoiesis in the cultures is accessible. The LTC have been successfully used in experiments to further our understanding of:

1 chemical and viral leukaemogenesis;
2 interactions between haemopoietic cells, stromal cells and regulatory molecules produced by the stromal cells;
3 growth requirements and growth characteristics of normal versus leukaemic cells.

LTC of human bone marrow has also been of value in "purging" malignant cells from marrow for patients with acute myeloid leukaemia. An aliquot of patients' marrow is grown for 10 days in LTC. During this time the patient receives intensive chemotherapy to ablate the leukaemic cells in their remaining marrow. Throughout the 10-day culture period the normal haemopoietic progenitor cells persist while the leukaemic cells are depleted. The cultured marrow is then re-infused to the patient and re-establishes haemopoieis. This type of autologous bone marrow transplant has been performed over the last 10 years and shows as good a success rate as any other transplant procedure in use. A newer and potentially very exciting application of LTBMC that we have been developing in the last 3 years is their use for the growth of haemopoietic stem cells as targets for gene transfer for therapeutic purposes i.e. gene therapy. Preclinical experiments to establish a protocol for the treatment of the lysosomal storage disorder, Hurler's syndrome, by gene transfer into haemopoietic stem cells are now complete and a clinical trial is anticipated in the near future. The longer term aim of using LTBMC in gene therapy is to develop new strategies for the treatment of malignant disease.

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By courtesy of Elaine Spooncer (1996).

Applications of cell cultures

The generation of large, homogeneous cell populations

The possibilities offered by cell culturing to obtain homogeneous cell suspensions, to increase by cell multiplication the amount of cellular material available for analysis, and to set up simple, experimentally manipulable cell inter-action systems in the culture dish (Box 4.5) have been a necessary condition for the great advances made within cell biology during the last decades. The challenges posed by 'the riddle of cancer' (which in fact may be many riddles - not just one!) and the desire to understand, diagnose, prevent, and treat virus diseases have meant that mostly cells from warm-blooded animals and man have been used. However, cells from lower species have increasingly been introduced, to solve problems e.g. within the fields of developmental biology, insect control in agriculture, and infections in fish farms.

A *primary culture* is started from cells taken directly from an organism; when transferred to a new culture vessel ('subcultured'), the cells become a '*cell line*'. Cultured normal cells may spontaneously or by experimental manipulations (chemicals, irradiation, or virus) become immortal (Fig. 4.1). They then form a *continuous* cell line, and we say that the normal cells have been *transformed.* Transformed cells may be *oncogenic*, i.e. able to produce tumours upon inoculation into animals, or not. Oncogenic cell lines may give rise to *benign or malignant* (i.e. invading and metastatic) growth (see Definitions below). Transformation seems to be a genetic change in the cells, leading to a short-cutting or extinction of regulatory feedback loops, so that the cells are no longer dependent on addition of extracellular factors that are normally important to cell growth or survival.

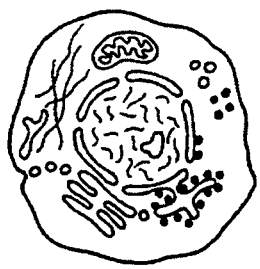
Primary cultures *vs.* various kinds of cell lines

An experimental system consisting of a single cultured cell type can now be reached by various approaches. A reasonably homogeneous primary culture (see Definitions below) can be made by dispersion of the cell in a tissue fragment, followed by cell isolation/separation procedures. Cells can be dispersed mechanically (e.g. by pipetting or whorlmixing), enzymatically (e.g. by trypsin digestion of intercellular substance), chemically (e.g. by ethylenediaminetetraacetic acid (EDTA) complexing Ca^{2+} and Mg^{2+} ions, which are often instrumental to cell adhesion), or by various combinations of these three methods. Cell isolation may be based on cell size (e.g. cell sedimentation or centrifugal elutriation), cell density (e.g. density gradient centrifugation), surface properties (e.g. cell adherence to plastic or to antibody-coated vessel surfaces or

Cell isolation techniques

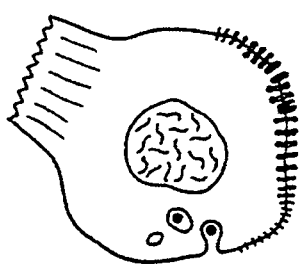
beads), or on combinations thereof (e.g. fluorescence activated cell sorting, where cells above or below a certain size threshold, with or without attached fluorescent antibody against a cell surface antigen, can be isolated).

Box 4.5. USAGE OF CELL CULTURES



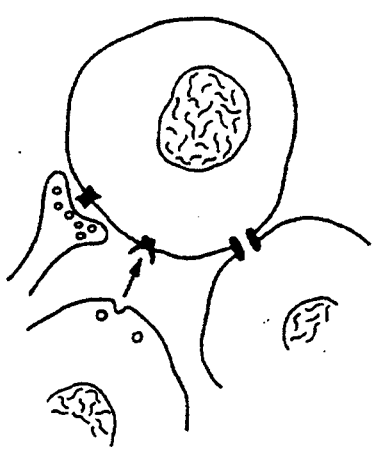
Cellular biochemistry and molecular biology

(Intermediary metabolism; DNA/RNA synthesis; cell transfection and gene therapy; production of recombinant proteins; anti-sense RNA; S/N/W blotting; polymerase chain reactions; gene knock-out; site-directed mutagenesis; screening for genetic diseases; etc.)



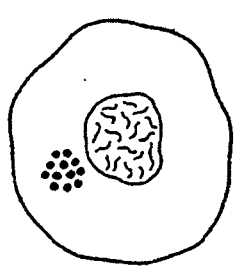
Cell biology

(Movement; secretion, endocytosis; cell adhesion; cell division, diferentiation and maturation; carcinogenesis; membrane fluxes and transport; membrane potentials; etc.)



Cell communication

(Hormones; paracrine agents; neurocrine transmitters; cell-to-cell (metabolic) cooperation and signalling; cell-to-matrix interactions; receptors; etc.)



Miscellaneous

(Cell multiplication; cell fusion (with e.g. hybridoma formation); virus culture and other infections; drug actions; toxicity studies; development of vaccines; etc.)

Alternatively, homogeneous cultures can be obtained by serial subculturing, starting with a heterogeneous primary culture, from which a certain cell type may outgrow the other cell types. An even simpler solution is to use a continuous cell line (see Definitions below). One great advantage of continuous cell lines is that you can exactly reproduce experiments performed elsewhere in the world (Table 4.1).

Manipulation of cultured cells

Cell culturing has many applications

Somatic cell genetics received a strong impetus from the discovery that mitosis could be induced in normal blood lymphocytes in culture by the plant lectin phytohaemagglutinin (that had already been used for decades to aggregate and thereby sediment red blood cells). Immunology benefited as well, in that proliferating and differentiating lymphocytes could be studied. By and by improved culture techniques facilitated reproducible studies of cell interactions in culture, such as between T and B lymphocytes and between lymphocytes and antigen presenting cells, to mimic in vivo interactions during antibody production.

A further major advance was the technique of cell fusion (Harris, 1970). With one of the fused cells enucleated, important information on the interplay between cytoplasm and nucleus was gained. A cell with two nuclei is called a heterokaryon; after mitosis a hybrid cell with one large nucleus is formed. Hybrids between human and mouse cells sometimes loose chromosomes, and it is the human chromosomes that are lost. By correlating the changes in cell phenotype to the loss of chromosomes - that is random - during mitosis, it has been possible to assign certain genes to specific chromosomes; for example, the gene coding for human insulin is on chromosome number 11.

Hybridomas and monoclonal antibodies

Of immense practical importance was the discovery that hybrid cells formed by an immune cell (determined to produce a specific antibody) and a cell from a plasmacytoma cell line (i.e. a transformed, antibody-forming cell line) may form a continuous cell line - originating from the hybrid cell and therefore a clone - that will secrete the specific antibody to the culture medium. This antibody is a so-called monoclonal antibody, and such antibodies have become an indispensable tool in cellular and molecular biology, as well as in diagnosis and therapy of human and animals diseases (see Box 4.6).

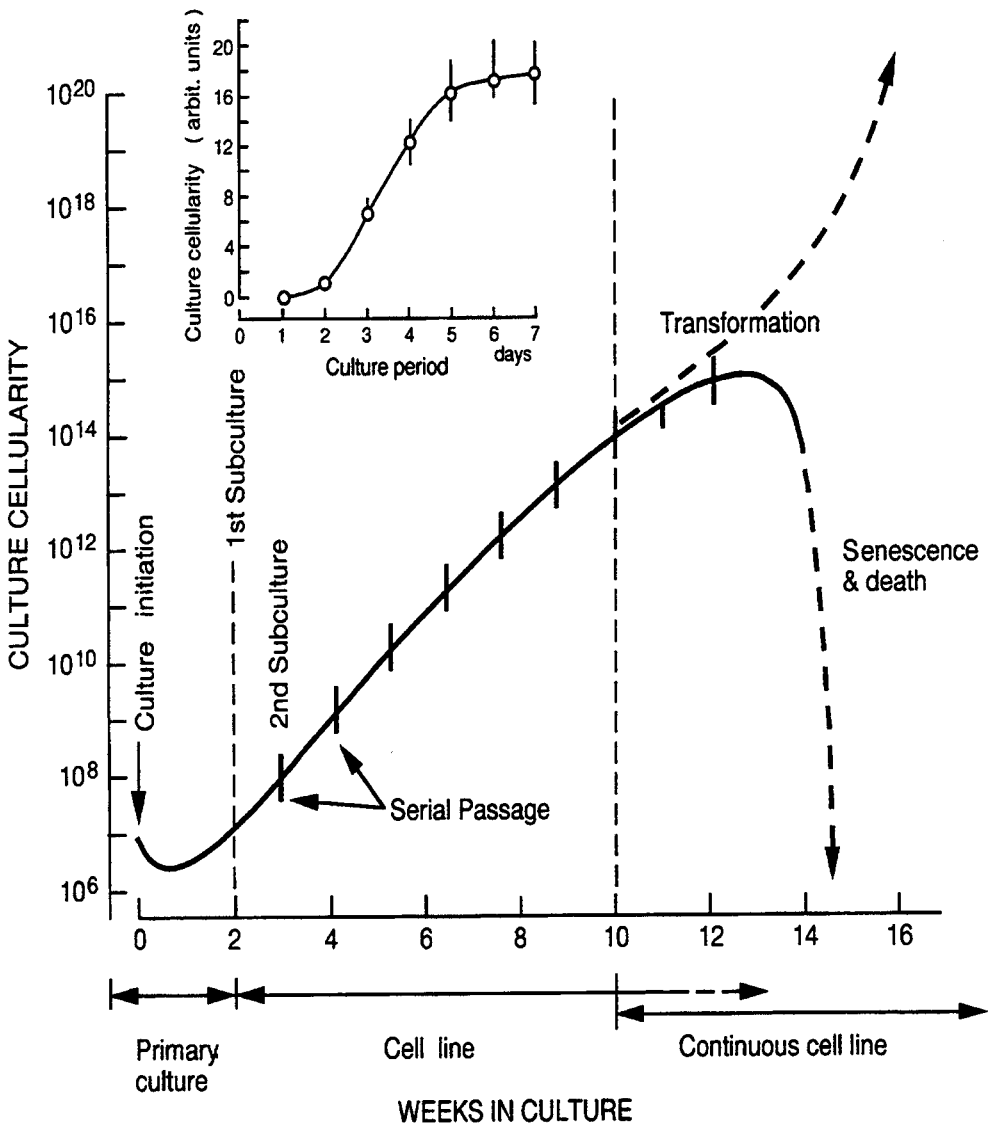


Fig. 4.1. Various fates of cultured cells. Evolution of a hypothetical cell culture. The Y axis shows the accumulated cell yield, assuming no cell loss at passage, on a log scale. A continuous cell line can arise at any time, not necessarily after 10 weeks, as depicted here. Likewise, senescence may occur at any time - here after about 12 weeks - but for human diploid fibroblasts most likely after 30-60 cell doublings (or 10-20 weeks, depending on the doubling time). *Inset:* Growth of mouse bone marrow cell colonies in primary culture (in viscous, methylcellulose containing medium. Supravital staining based on mitochondrial dehydrogenase activity. Quantification with digital image analysis of video pictures of the culture dishes.)

(Based on Freshney (1987) and own results)

TABLE 4.1 Examples of cultured cell lines and strains that express differentiated properties *in vitro*

Origin	Cell line	Species	Marker
Finite cell lines			
Pigmented retina	Retina	Chick	Pigmentation
Calvaria	Cartilage	Chick	Cartilage synthesis
Epidermis	Keratinocytes	Human	Cornification
Skeletal muscles		Chick	Myogenesis
Hypothalamus	C7	Mouse	Neurophysin Vasopressin
Continuous cell lines			
Spleen	Friend	Mouse	Haemoglobin
Hepatoma	H-4-11-E-C3	Rat	Tyrosine aminotransferase
Myeloid leukaemia	K562	Human	Phagocytosis, formation of superoxide
Glioma	MOG-CCM	Human	Glial fibrillary acidic protein
Pituitary tumour	GH2, GH3	Rat	Growth hormone
Melanoma	B16	Mouse	Melanin
Neuroblastoma	C1300	Rat	Neurites
Kidney	MDCK	Dog	Transport mechanisms
Myeloma	Various	Mouse	Immunoglobulins
Pulmonary artery	CPAE	Cow	Factor VIII, angiotensin converting enzyme
Foreskin	Melanocytes	Human	Melanin
Spleen	CT11-2, HT-1	Mouse	Interleukin 2

American Type Culture Collection is a unique private, non-profit resource dedicated *inter alia* to the distribution of living cell lines. Address:10801 University Blvd., Manassas, VA USA. Tel.: (+1) 703-365-2700.Internet address: http://www.atcc.org/

Box 4.6. Monoclonal antibodies

When challenged with foreign macromolecules, either as parts of invading microorganisms or as experimentally injected isolated molecules, most vertebrates respond by producing antibodies. Because they elicit an immune response, the foreign molecules are referred to as antigens. At the amino-terminal end the antibodies possess a small groove, the antigen-binding site. The surface of this groove is complementary in shape and distribution of electric charges to a corresponding area on the surface of the antigen. The antigen-binding sites of antibodies bind with relatively high affinity to these areas, called the antigenic determinants or epitopes, via multiple weak intermolecular interactions, just like hormones bind to their receptors or substrates bind to enzymes. As a rule the epitopes are unique to each antigen. The antibodies can therefore distinguish with exquisite specificity between an amazingly large number of different antigens, a property which has made antibodies a versatile tool in biomedical research and in clinical medicine.

Previously the research laboratories had no option but to use polyclonal antibodies. These are obtained by simply injecting the antigen into an animal (e.g. rabbit, sheep or horse), usually by repeated injections spaced several weeks apart, and then bleeding the animal to get serum. The serum always contains a blend of different antibodies to the antigen, each binding to or "seeing" different epitopes on the same antigen, or seeing the same epitope in different ways, with dissimilar affinities. Because different antibodies are produced by different antibody-forming cells, the blend is referred to as polyclonal antibodies. A major drawback with this method is that unless the injected antigen is completely purified the serum contains antibodies directed against the impurities. This is particularly a problem if the antigen is a membrane protein, let us say the CD4 molecule on T4-lymphocytes, which is not readily isolated from the whole cell. In theory, this problem could be solved by immunising with T4-cells and then absorbing out antibodies produced against other antigens on the cells. In practice, this approach is difficult because complete absorption of unwanted antibodies requires the availability of related cells that exhibit all the antigens that the T4-cells do except the CD4-molecule. Another drawback with the traditional method of producing antibodies is that each individual animal responds in a unique way to an antigen, producing different sets of antibodies with dissimilar titers and also with variation between different bleedings from the same animal. This means that it is often difficult to compare results from similar tests made with antibodies from different batches of antisera.

These shortcomings would be overcome if single cells producing antibody to a given epitope could be expanded in culture to a large population in which all the members continued to produce the same antibody. Unfortunately, normal antibody-producing cells do not grow in culture, but die within a few days. This obstacle was circumvented when Köhler and Milstein got the idea in 1975 of fusing antibody-producing cells isolated from the spleen of an immunised mouse with malignant mouse cells. The hybrid cells retained the ability to produce antibodies from the normal, antibody-producing fusion partner and the power to survive and divide infinitely in culture from the malignant fusion partner. The latter fusion partner was selected with care. It was a myeloma cell, originally derived from a normal antibody-producing cell, but transformed to a cancer cell. Furthermore, it was deficient in an enzyme so that it could not survive in a selective medium, called HAT-medium. This meant that after fusing antibody-producing cells with myeloma cells and culturing the cells in HAT-medium, only hybrids of normal cells and myeloma cells survived. The potency of this technique was rapidly realised when other scientists subsequently showed that they could grow single hybridoma cells and test their ability to produce antibody of a certain specificity.

To give an example, a mouse is immunised repeatedly with whole T-4 cells and the spleen taken out four days after the last immunisation. Then the leukocytes are teased out. Some of these are antigen-stimulated B-lymphocytes that will mature to antibody-forming cells. Hybridoma cells are made by cell fusion techniques and then cultured in HAT-medium on 96-well tissue culture plates under conditions so that on

average only one hybrid cell per well grow up into a colony. The supernatants from each well containing a single colony is then tested for production of antibody against a panel of positive and negative control cells. Clones producing antibody only against the positive controls are then selected for expansion and further testing and selection. Ultimately cultures of hybridoma cells are obtained, all derived from a single fused cell, hence their designation monoclonal. All these cells produce identical antibodies. These cells can now be propagated infinitely and the secreted antibodies harvested from cell culture supernatants or from the ascites fluid of mice injected intraperitoneally with the hybridoma cells. The different properties of the antibody, such as specificity, affinity, class and stability, are thoroughly investigated. Thereafter the clones, supernatants or ascites fluid can be distributed to all interested laboratories throughout the world. As all involved use the same, well characterised reagent, the variability and uncertainty inherent in the traditional method are eliminated.

Another advantage with this technique is that the order of events can be completely reversed, i.e. the antibody can be produced first and the antigen detected and characterised afterwards, with the antibody as a tool. In fact, the first monoclonal antibody produced using the described selection procedure was raised against rat T cells and found to split rat T cells into two subpopulations. It was subsequently shown that the population which bound the antibody consisted of helper T cells, and still later that the antigen recognised was a molecule now known as CD4.

Monoclonal antibodies have numerous other applications in clinical medicine and biomedical research. A well-known example is modern pregnancy tests, sold as self-to-do kits in pharmacies. As early as two weeks after fertilisation the urine of the pregnant woman contains traces of the hormone human choriogonadotropin (hCG), which can be detected by using a monoclonal antibody against hCG and latex particles coated with the hormone. If hCG is absent from the urine, the antibodies will crosslink the particles to easily visible clusters, whereas free hCG in the urine will compete for the binding sites and thereby inhibit cluster formation. The cited example is based on competition for antigen. Variants of this test are based on direct detection of hCG. Similar principles, whether competitive or direct, lies behind a large array of other tests, such as radioimmunoassay (RIA) based on scintillation counting of radiolabeled ligands, and enzyme-linked immunosorbent assay (ELISA) based on photometric measurements of converted substrates catalysed by enzymes coupled to antibodies. Note that these tests are also performed with polyclonal antibodies, but the monoclonal antibody technique has greatly extended their use.

Antibodies, in particular monoclonal antibodies, can also be used as staining agents to visualise the presence and distribution of molecules on cells (immunocytochemistry) or in tissues (immunohistochemistry). The antibodies must then be tagged with a tracer that can be made visible, such as a fluorochrome, which can be detected by fluorescence microscopy, a radioactive marker, which can be visualised by autoradiography or an enzyme that catalyses the deposition of an insoluble, coloured end product (see also fig. 5.5).

There are also interesting applications of monoclonal antibodies in clinical medicine, in what is referred to as immuno-targeting. The antibodies are then used as vectors to carry attached groups - like cytotoxic agents or isotopes - to selected cells in the body, e.g. cancer cells.

New methods of making monoclonal antibodies are explored, based on genetic engineering. In these techniques cDNA libraries from antigen-stimulated spleen cells are ligated into expression vectors and used to transform bacteria. It is therefore likely that in the future bacteria will replace hybridomas for production of monoclonal antibodies.

The monoclonal antibody technique has developed into a large, world-wide industry that has greatly facilitated biomedical research and radically transformed laboratory medicine. Moreover, it has interesting potential applications in clinical medicine. (Fossum, S. (1996), personal communication)

The latest developments in cell culture technique are based molecular biology. Genes can be introduced into cells to transform them, to manipulate them in specific ways, or to make them produce protein factors or hormones, like human interferon, insulin, or erythropoietin. Addition of an anti-sense RNA species can disclose whether a certain protein (coded by the relevant 'sense' mRNA) plays a hypothesised functional role. The polymerase chain reaction can be used to detect an actively transcribed gene in a single cell! It is easy to comprehend what enormous developmental potential such techniques convey to both basic biological research and areas of applied research, from medicine to agriculture. Sometimes, the post-translational modification of proteins, like glycosylation, is not necessary for biological function. Then, prokaryotic organisms that are easier to culture, can be used to host the producer gene, rather than eukaryotic cells (yeast cells or e.g. mammalian cells).

Cell culture and genetic engineering

The further and varied uses of cell cultures, for example to perform chromosome analysis on foetal cells obtained by amniocentesis, to diagnose virus infections and make viral vaccines, to assess toxicity of pharmaceutical compounds and environmental pollutants, and perhaps form tissues suitable for transplantation purposes or reconstructive surgery lie outside the scope of this chapter. So do technicalities, such as techniques of asepsis, preparation and sterilisation of culture media, cell separation and quantification, control of mycoplasma and other infections, and also control and assessment of endotoxin contamination and the functioning of incubators. Even so, the appendix to this chapter gives an example of the formulation of a cell culture medium. Suffice it to say that cell culturing without expert help is not something to be undertaken lightly, for "just a few experiments". In few areas is Murphy's law more apt: "What can go wrong, goes wrong!" Luckily, however, the potential gains are major ones, as shown in Table 4.2.

Avoid tricky pitfalls and adhere to stringent procedures

Tissue cultures and organ preparations

Cultured tissues fragments and perfused, isolated organs hold an intermediate position between the cell cultures and the whole, integrated organism. Experiments with such preparations may therefore yield data which might be more physiologically relevant, but also more difficult to interpret, than those obtained with cell culture. Two examples are presented in Box 4.7 and 4.8.

Box 4.7. A useful tissue preparation: Brain slices

For many biological problems a simplification of the complex *in vivo* structure has aided analysis of the underlying basic mechanisms. This is particularly important in the nervous system with its inherent complexity. While elements of the peripheral nervous system can sustain isolation and handling in an external tissue bath for several hours, the central nervous tissue is more sensitive. Initial attempts (Andersen 1956) to prepare an isolated preparation of the hippocampal formation made use of the hibernating hedgehog, relying upon the fact that this animal survives reduced temperatures down to about 2°C and then has a smaller demand for oxygen. However, these early attempts failed. In the 1960s, the British biochemist James McIlwain tested the viability of slices taken from brain tissue, maintained in a modified Warburg flask. With different slices of the cerebral cortex he was able to record membrane and action potentials of cortical neurons. However, these experiments were critized because the values recorded were lower than those obtained in acute experiments. Hence, it was inferred that the tissue had been damaged. Better signals were obtained when McIlwain used sections of the olfactory tract and the underlying pyriform cortex cut parallel to the surface. Potentials could then be recorded from the olfactory tract fibres, and a subsequent field potential suggested the presence of a certain synaptic activity as well.

In 1971 Andersen, Bliss and Skrede found that the major hippocampal neurons and their interconnecting axons are oriented in a single plane. This allowed a slice of hippocampus nearly normal to the longitudinal axis of the structure to be cut out; this so-called laminar arrangement for cutting slices proved successful.

Importantly, the dissection should be performed at a low temperature (~ 0-4°C) to reduce the damage caused by the reduced oxygen availability. Slices between 250 and 700 µm thick may retain good responses for 10-20 hours. They can be maintained in two ways, either in the interface between the culture fluid (similar to cerebrospinal fluid but without protein) and the (95%) oxygen/(5%) carbon dioxide containing gas phase or in a fully submerged position. The first technique is called the interface technique. Here, the slices are carried by a nylon net covered by tissue paper to make a smooth surface. The upper 30µm usually contains damaged tissue due to the mechanical disruption. However, below this level cells which show remarkably good physiological responses, can be found, down to a depth of about 250 µm.

The better performance of cells in the upper than in the lower part of the slices is taken as evidence that the oxygen supply is more efficient from the gas phase than from the water phase. The viability of the slices is critically dependent upon the film of fluid on top of the slices. Therefore, the surface must be even and strictly horizontally placed to avoid drainage of the fluid, with consequent damage through drying-out. With submerged slices, there is of course no danger of drying-out. On the other hand, the cell viability is slightly lower here. Therefore, the physiological responses are somewhat muted, and individual cells are less easily identified than in the interface slice. However, this method is suitable for pharmacological experimentation.

In properly treated slices the cells regularly show all the basic properties seen under physiological conditions *in vivo*. Fiber volleys, synaptic potentials, action potential, and after-hyperpolarization are readily demonstrated. Both extracellular and intracellular recordings are possible, and patch clamping of the excised patches or whole cell patch recording is readily attained. The latter is most easily performed on slices taken from young animals, but patching may be successfully applied even with 40 days old rats. In addition, optical techniques (e.g. differential contrast microscopy) are easily performed, and metabolic studies can be correlated with the physiologically recorded responses.

The advantages of the slice preparation are manifold: First, the slice allows precise positioning of both stimulation and recording electrodes. Furthermore, the cell structures may be identified with dyes. Most importantly, the blood brain barrier is functionally absent, allowing experimental control with interstitial fluid variables like Ca^{2+} concentration and pH. In addition, micro-surgery can be performed, dividing the slice into smaller elements and in fact partly dissociate its cells. Thus, a strand of the alveus, containing mostly CA1 axons, can be dissected free and used as an artificial nerve for both stimulation and recording. Finally, by appropriate colouring of tissue components it is possible to use optical methods to compare physiological, anatomical and biochemical features of identified parts of the hippocampal structures.

Andersen, P and Langmoen, IA (1980) Quart.Rev.Biophys. 13: 1-18.
Andersen, P (1981) Trends in Neurosciences 4: 53-56.

Andersen, P.O. (1994), personal communication.

Box 4.8 An isolated, perfused rat lung preparation

Isolated, perfused lungs were first used in 1967, in a study on trans-vascular fluid exchange and hypoxic pulmonary vasoconstriction. Since then, this whole organ preparation has been used extensively as a research tool, *e.g.* for studies on vasoactive substances, microvascular permeability, endothelins and metabolic functions of the lung. Rats are convenient research animals, inexpensive and in no danger of becoming extinct. The lung preparation makes it possible to control, and alter with precision, inflow- and outflow pressures and blood flow. When constant flow perfusion is used, vasoconstriction or vasodilation is detected as changes in inflow pressure. Pulmonary microvascular pressure can be determined with the so-called double occlusion technique. Also the ventilation pattern, *e.g.* tidal volume, ventilation frequency, inflation pressure and end-expiratory pressure, is easily controlled and altered.

The fluid filtration rate of the pulmonary vascular bed can be determined by monitoring the weight of the lungs during standardised elevations of the pulmonary venous pressure. Lungs perfused with homologous blood can be maintained without spontaneous edema formation for at least 6 hours. This allows quantitative studies on the importance of the various plasma components and blood cells in maintaining microvascular integrity. Also the importance of blood flow pulsatility and ventilation pattern on gas exchange and the ventilation/perfusion ratio has been studied with this preparation.

In the lungs the blood is exposed to a very large endothelial area. The isolated lung preparation is therefore useful for studies on endothelial functions, such as activation and deactivation of hormones and vasoactive substances. Lung endothelial cells display many membrane receptors with specific functions, *e.g.* inactivation of bradykinin and transformation of angiotensin I to angiotensin II. By using the rat lung preparation it has recently been found that endothelin-1 increases microvascular permeability, but only in the presence of white blood cells and certain plasma protein fractions.

It is tricky and it usually takes considerable time to establish this preparation and to become comfortable with it, but the rewards may be great for a dedicated investigator.

Hauge, A. ACTA PHYSIOLOGICA SCANDINAVICA (1967) 72: 33-41
Helset, E, Kjæve, J, and Hauge, A. CIRCULATORY SHOCK (1993) 39: 15-20

Hauge, A (1996), personal communication

Table 4.2. Advantages and disadvantages of cell culture

Pro	Contra
1. Control of cell environment (Gas pressures, humidity, temperature, pH, osmolarity)	1. Expertise needed. (For example, since contaminating microorganisms tend to outgrow and destroy cultured eukaryotic cells.)
2. Reproducibly homogeneous cell sample. (For example, clonal cell lines may be stored in liquid N_2.)	2. Artefactual or unphysiological findings. (Cells from multicellular organisms normally interact with other cells and intercellular material, which are often absent from the culture. In vitro, cell metabolism tend to be glycolytic rather than oxidative; and if cell differentiation occurs, the more mature cell forms may be underrepresented or missing, compared to the *in situ* (tissue *in vivo*) situation. Finally, nerves and blood vessels, carrying regulatory signals, are absent.)
3. Economy. (Doses of test agents much lower than for *in vivo* use.)	3. A low ratio of harvested tissue (in weight units) to resources (time and money) invested.
4. Ethics. (The normal questions of patient and animal experimentation are avoided.)	4. Instability of continuous cell lines. (A major problem with the aneuploid chromosomal constitution. Even with genetic stability the growth rate may vary from one passage to the next.)

Definitions

Anchorage-dependent cells or cultures. Cells, or cultures derived from them, which will grow, survive, or maintain function only when attached to an inert surface such as glass or plastic.

Aneuploid cell. The nucleus does not contain an exact multiple of the haploid number of chromosomes.

Cell culture. The growing of cells *in vitro*, including the culture of single cells. In cell cultures, the cells are no longer organised into tissues.

Cell generation time. The interval between consecutive divisions of a cell. This interval can be determined, for example, with video filming through a phase contrast microscope. *This term is not synonymous with "population doubling time*".

Cell hybridisation. The fusion of two or more dissimilar cells leading to the formation of a synkaryon.

Cell line. A cell line arises from primary culture at the time of the first subculture (Fig. 4.1). The term *finite* or *continuous* is used as prefix if the status of the culture is known. If not, the term *line* will suffice. *The term "continuous line" replaces the term "established line"*. In any published description of a culture, one should make every attempt to publish the characterisation or history of the culture. If such has already been published, a reference to the original publication can be made. In obtaining a culture from another laboratory, the proper designation of the culture, *as originally named and described*, must be maintained and any deviations in cultivation from the original should be reported in any publication.

Cell strain. A cell strain is derived either from a primary culture or a cell line by the selection or cloning of cells having specific properties or markers. The properties or markers must persist during subsequent cultivation. In describing a

cell strain, its specific features must be defined. The terms *finite or continuous* are to be used as prefixes if the status of the culture is known.

Chemically defined medium. A nutritive solution for culturing cells in which each component is of known chemical structure.

Clone. A population of cells derived from a single cell by mitoses. A clone is not necessarily homogeneous and, therefore, the terms *clone* and *cloned* must not be used to indicate homogeneity in a cell population, genetic or otherwise.

Contact inhibition of locomotion. A phenomenon in which two cells meet, locomotory activity diminishes and the forward motion of one cell over the surface of the other is stopped.

Density dependent inhibition of growth. Mitotic inhibition correlated with the increased cell density.

Diploid. The state of the cell in which all chromosomes, except sex chromosomes, are two in number and are structurally identical with those of the species from which the culture was derived.

Epithelial-like (Epithelioid). Resembling or characteristic of, having the form or appearance of epithelial cells. In order to define a cell as an epithelial cell, there must be definitive characteristics present typical of epithelial cells. Until such time as this is possible it would be most correct to use the terms *epithelial-like or epithelioid.*

Euploid. The situation which exists when the nucleus of a cell contains exact multiples of the haploid number of chromosomes.

Explant. Tissue taken from its original site and transferred to an artificial medium for growth.

Explant culture. The maintenance or growth of an explant in culture.

Fibroblast-like (Fibroblastic). See epithelial-like (epithelioid) above.

Haploid. Possessing half the diploid or normal number of chromosomes found in somatic or body cells. Such is the case of the germ cells, ova or sperms, following the reduction divisions in gametogenesis.

Heterokaryon. Genetically different nuclei, irrespective of their number, in a common cytoplasm, usually derived as a result of cell to cell fusion.

Heteroploid. The term given to a cell culture when the cells comprising the culture possess nuclei containing chromosome numbers other than the diploid number. This is a term used only to describe a culture and is not used to describe individual cells. Thus, a heteroploid culture would be one which contains *aneuploid cells.*

Histiotypic. Resembling a tissue *in vivo* in form or function or both. For example, a suspension of fibroblast-like cells may secrete a glycosaminoglycan-collagen matrix, and the result is a structure resembling fibrous connective tissues, which is, therefore, histiotypic. This term is not meant to be used along with the word "culture". Thus, a tissue culture system demonstrating form and function typical of the cells *in vivo* should be said to be *histiotypic.*

Homokaryon. Genetically identical nuclei, irrespective of their number, in a common cytoplasm, usually derived as a result of cell to cell fusion.

Hybrid cell. The term used to describe the mononucleate cell which results from the fusion of two different cells, leading to the formation of a synkaryon.

In vitro neoplastic transformation. The acquisition, by cultured cells, of the property to form neoplasms, benign or malignant, when inoculated into animals. Many transformed cell populations which arise *in vitro* intrinsically or through deliberate manipulation by the investigator, produce only benign tumours, that is, tumours which show no local invasion or metastasis following animal inoculation. If there is supporting evidence, the term "*In vitro malignant transformation*" can be used to indicate that a cell line does, indeed, invade or metastasise.

In vitro transformation. A heritable change, occurring in cells in culture, intrinsically or resulting, for example, from treatment with chemical carcinogens, oncogenic viruses, irradiation, etc., leading to the acquisition of altered properties such as morphological, antigenic, neoplastic, proliferative, etc. This expression is distinguished from "*in vitro* neoplastic transformation" in that the alterations occurring in the cell populations may not always include the ability of the cells to produce tumours in appropriate hosts. The type of transformation should always be specified in any description.

Organ culture. The maintenance or growth of organ primordia or the whole or parts of an organ *in vitro* in a way that may allow differentiation and preservation of the architecture and/or function.

Organotypic. Resembling an organ *in vivo* in three dimensional form or function or both. For example, a rudimentary organ in culture may differentiate in an *organotypic* manner, or a population of dispersed cells may become rearranged into an *organotypic* structure, and may also function in an *organotypic* manner. This term is not meant to be used along with the word "culture" but is meant to be used as a descriptive term. Neither should the word "growth" be used along with this term since the tissue need not actually be increased in size or number of cells in order to be *organotypic*.

Passage. The transfer or transplantation of cells from one culture vessel to another. This term is synonymous with the term "subculture".

Passage number. The number of times the cells in the culture have been subcultured. In descriptions of this process, the ratio or dilution of the cells should be stated so that the relative cultural "age" can be ascertained.

Plating efficiency. The percentage of inoculated cells which give rise to colonies when seeded into culture vessels. The total number of cells in the inoculum, type of culture vessel and the environmental conditions (medium, temperature, closed or open system, etc.) must always be stated. This term is often expressed as the percentage of individual cells in the vessel which give rise to colonies. If one is

certain that each of the colonies arose from single cells, then one may properly apply the term "cloning efficiency".

Population density. The number of cells per unit area or volume of a culture vessel (see also Saturation density).

Population doubling level. The total number of population doublings of a cell line or strain since its initiation in vitro.

Population doubling time. The interval, calculated at the logarithmic phase of growth, in which, for example, $1.0x10^6$ cells increase to $2.0x10^6$ cells. This term is not synonymous with "cell generation time".

Primary culture. A culture started from cells, tissues or organs taken directly from organisms. A primary culture may be regarded as such until it is subcultured for the first time. It then becomes a "cell line".

Pseudodiploid. This describes the condition where the chromosome number of the cell is the diploid one of the organism but, as a result of chromosomal rearrangements, the karyotype is abnormal and linkage relationships may be disrupted.

Saturation density. The maximum cell number attainable under specified culture conditions in a culture vessel. This term is usually expressed as the number of cells per square centimetre in an anchorage dependent culture or the number of cells per cubic centimetre in a suspension culture.

Seeding efficiency. The percentage of the inoculum which attaches to the surface of the culture vessel within a given period of time. The conditions under which such a determination is made should always be reported. This term is synonymous with the term "attachment efficiency."

Subculture. See "passage."

Substrain. A substrain can be derived from a strain by isolating a single cell or groups of cells having properties or markers not shared by all cells of the strain.

Surface or substrate dependent cells or cultures. See "anchorage dependent cells."

Suspension culture. A type of culture in which the cells multiply while suspended in medium.

Synkaryon. A hybrid cell which results from the fusion of the nuclei it carries.

Tissue culture. The maintenance or growth of tissues, *in vitro*, in a way that may allow differentiation and preservation of the architecture and/or function.

(Modified from Shaeffer, W.I. (1979))

APPENDIX : A cell culture medium:
ISCOVE'S MODIFIED DULBECCO'S MEDIUM (IMDM)

Guilbert and Iscove demonstrated that precursor cells of erythrocytes and macrophages could be cultured in a totally defined serum-free medium when supplemented with albumin, transferrin, lecithin, and selenium. This medium is a modification of Dulbecco's Modified Eagle's Medium (DME) containing selenium, additional amino acids and vitamins, sodium pyruvate, HEPES buffer, and potassium nitrate in place of ferric nitrate. Further studies demonstrated that Iscove's Medium would support murine B lymphocytes, haemopoietic tissue from bone marrow, B cells stimulated with lipopolysaccharide, T-lymphocytes, and a variety of hybrid cells.

A FORMULA

COMPONENT	g/L
INORGANIC SALTS :	
Calcium Chloride • $2H_2O$	0.219
Magnesium Sulfate (anhydrous)	0.09767
Potassium Chloride	0.330
Potassium Nitrate	0.000076
Sodium Bicarbonate	3.024
Sodium Chloride	4.505
Sodium Phosphate Monobasic (anhydrous)	0.109
Sodium Selenite	0.0000173
AMINO ACIDS :	
L-Alanine	0.025
L-Arginine • HCl	0.084
L-Asparagine • H_2O	0.0284
L-Aspartic Acid	0.030
L-Cystine • 2HCl	0.09124
L-Glutamic Acid	0.075
L-Glutamine	0.584
Glycine	0.030
L-Histidine • HCl • H_2O	0.042
L-Isoleucine	0.105
L-Leucine	0.105
L-Lysine • HCl	0.146
L-Methionine	0.030
L-Phenylalanine	0.066
L-Proline	0.040
L-Serine	0.042
L-Threonine	0.095
L-Tryptophan	0.016
L-Tyrosine • 2Na • $2H_2O$	0.10379
L-Valine	0.094
VITAMINS :	
D-Biotin	0.000013
Choline Chloride	0.004
Folic Acid	0.004
myo-Inositol	*0.0072*

Niacinamide	0.004
D-Pantothenic Acid (hemicalcium)	0.004
Pyridoxal • HCl	0.004
Riboflavin	0.0004
Thiamine • HCl	0.004
Vitamin B-12	0.000013
OTHER :	
D-Glucose	4.500
HEPES	5.958
Phenol Red •Na	0.016
Pyruvic Acid •Na	0.110
SPECIFICATIONS :	
pH at 25° C	7.0 ±0.3
Osmolality (mOsm/kg H_2O)	276±5%
Grams of powder required to prepare 1 L	17.7

Cell culture, checklist 4

1. **Cells.**

- Designation (see Table 4.1 and Definitions).
- Information on origin, vendor, or supplier of cell lines, number of "passes" (subculturing) of cell lines, etc.

2. **Culture conditions.**

- Culture medium (designation, producer, sometimes even catalogue, lot. or batch no.).
- Additives: serum (designation, producer, sometimes even catalogue lot or batch no.); antibiotics; antimycotic agents; "feeder cells"; extra buffers (e.g. hydroxyethylpiperazine-ethanesulfonic acid - HEPES), reducing agent (like mercaptoethanol); hormones; growth factors; conditioned medium; agar or methylcellulose (to make medium viscous); test substances.
- Physicochemical conditions (pH, % CO_2 in air or % CO_2 and O_2 in specially prepared gas mixture; humidity; temperature; osmolarity).
- Culture harvest (use of mechanical devices ("rubber policeman"), EDTA, enzymes, etc.).
- Precautions against contamination (e.g. mycoplasma screening; endotoxin analyses of culture supernatants).

3. **The experiment.**

- Apparatus (producer's name and address).
- Analyses (references!).
- Methodological tests (e. g. of reproducibility, precision, and accuracy; dose-response and time-course experiments).
- Scoring the cultures: coding of experimental and control plates/ dishes; culture time(s) (sometimes even time of the day of cell inoculation/plating and harvesting should be stated in protocol and scientific article).

(All these points may not be strictly relevant for your experimental protocol - and even less so for the Materials and Methods section of your article - but you should be prepared to argue why!)

Exercises

1.How is (or could possibly be....) cell culturing useful for the solution of you scientific problem(s)?

2.Can you make fruitful use of the cell culture technology, e.g. the commercially available monoclonal antibodies? How?

3. Which are the necessary precautions to bear in mind when you interpret results obtained with cell, tissue, or organ cultures (and extrapolate to human physiology or pathology)?

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Chapter 5: The Data

Measurements

Biological data representing measurements performed on a sample of individuals, will vary. The data will be distributed in either a discrete (for categorised variables) or a continuous way. Neither will repeated measurements on a single individual - nor replicate, almost simultaneous measurements, generally give exactly the same results. The data distributions may be symmetrical (Fig. 5.1) or asymmetrical (Box 5.1, Table 5.1) around an arithmetical mean (average) value. (You will learn more about data distributions from the next chapter.)

The distribution of the measurements (data) is important

A special case of the symmetrical distributions is the bell shaped normal or Gaussian distribution (after the German mathematician Johann Carl Friedrich Gauss, 1777-1855). Here, ~ 68% of the values lie within the interval between the inflections of the curve (Fig. 5.1). The distance from the arithmetical mean ($\bar{X}$) to one of the inflections is the standard deviation (SD) of the data. About 95% of large samples of normally distributed data will fall within the interval $\bar{X} \pm 2 * SD$.

Arithmetical mean (average) value:

$\bar{X} = \Sigma_i X_i / n$

(X_i represents each of the measured values;

n = number of X_i's)

Standard deviation:

$SD = \sqrt{\Sigma_i (X_i - \bar{X})^2 / (n-1)}$

Standard error (of the mean):

$SE(M) = SD / \sqrt{n}$

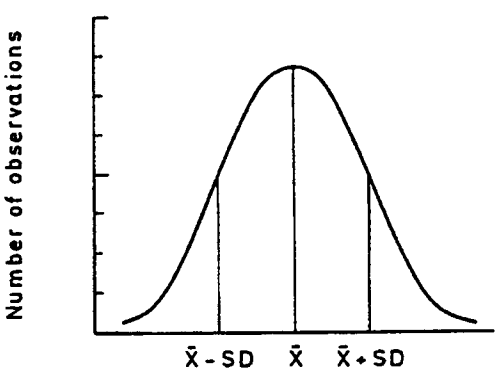


Fig. 5.1 *The normal distribution of data and some descriptive statistics.*

Asymmetrical distributions may be skewed so that there is a long "tail" towards the higher values. Then, a distribution close to the normal one can sometimes

be obtained if you use mathematically transformed data (logarithms or square roots of your raw data).

Finally, there may be "outliers" - very deviant values caused by e.g. crude measurement errors - so that the distribution does not conform to any general pattern. Irrespective of the type of data distribution, it can be represented by a localisation parameter and a measure of uncertainty for this localisation. The localisation parameter may be the arithmetical mean ($\bar{X}$, Fig. 5.1, Table 5.1) or the median ($\tilde{X}$, Table 5.1). The median is found by ranking the values from the lowest to the highest and picking out the centre value (for an odd number of data; the mean (average) of the two centre values for even numbers of data). Obviously, the median value will be much less affected by a few "outliers" among a restricted number of replicate data than will the mean value (Box 5.1).

Table 5.1 Replicate measurements of blood haemoglobin concentration (mmol/L), used to exemplify estimates of localisation parameters and variability

Measurements in the order obtained		Measurements ranked	Calculations		
10.15		8.97	No. of observations	:	47
9.80		8.97	Mean ($\bar{X}$)	:	9.94
9.93		9.34	Standard deviation (SD)	:	0.40
11.14		9.34	25% percentile	:	9.74
10.49		9.46	Empirical median ($\tilde{X}$)	:	10.02
10.15		9.46	75% percentile	:	10.10
11.04		9.59	95% Confidence		
10.10		9.59	interval of the median,		
8.97		9.71	non-parametric method	:	
9.77		9.72	Lower limit (LL)	:	9.80
9.90		9.72	Upper limit (UL)	:	10.05
9.46		9.74			
9 46	~ 25% ->				
9.80		9.77	95% Confidence interval of the		
9.72		9.80	mean, parametric method:		
10.20		9.80			
9.59		9.80	$\bar{X}$ +2.01 * SD/ √47		
9.59	LL*->	9.80	LL: 9.82		
10.10		9.80	UL: 10.06		
9.34		9.90			
9.80		9.93	(The coefficient 2.01 has been taken from		
9. 74		9. 93	a t-distribution; some other examples of		
10. 05		10 02	coefficients for conversion of SE to 95 % c.i.:		
10 02	$\tilde{X}$ ->	10.02	n	coeff.	
10.05		10.02	5	2.78	
10.02		10.02	10	2.26	
10.09		10.02	20	2.09	
10.02		10.05	30	2.04	
9.72		10.05	40	2.02	
10.02	UL*->	10.05	60	2.00	
9.93		10.09	∞	1.96)	
10.23		10.10			
10.02		10.10			
8.97		10.10			
10.10	75%->				
10.05		10.10			
10.10		10.10			
10.18		10.15			
10.10		10.15			
10.49		10.18			
9.80		10.20			
10.02		10.20			
9-93		10.23			
9.80		10.49			
9.71		10.49			
		11.04			
		11.14			

* See Table 5.2

The distribution can be represented by its localisation and variability parameters

Sometimes, when measurements are grouped in size categories, the mode is used. The mode is the most frequently occurring size, or the size category with most entries, as when measurements are categorised with a multichannel analyser. For example, the data of Table 5.1 can be grouped, so that each compartment width is 0.5 mmol/L haemoglobin. The mode is then 10.25 mmol/L, representing the interval 10.00-10.50 mmol/L, which has the highest number of measurements (23). With a symmetrical distribution, such as the normal distribution, the mean, median and mode will be identical values. Not so with a skewed distribution (Box 5.1)!

Box 5.1 A skewed distribution of survival times: mean, median, and mode

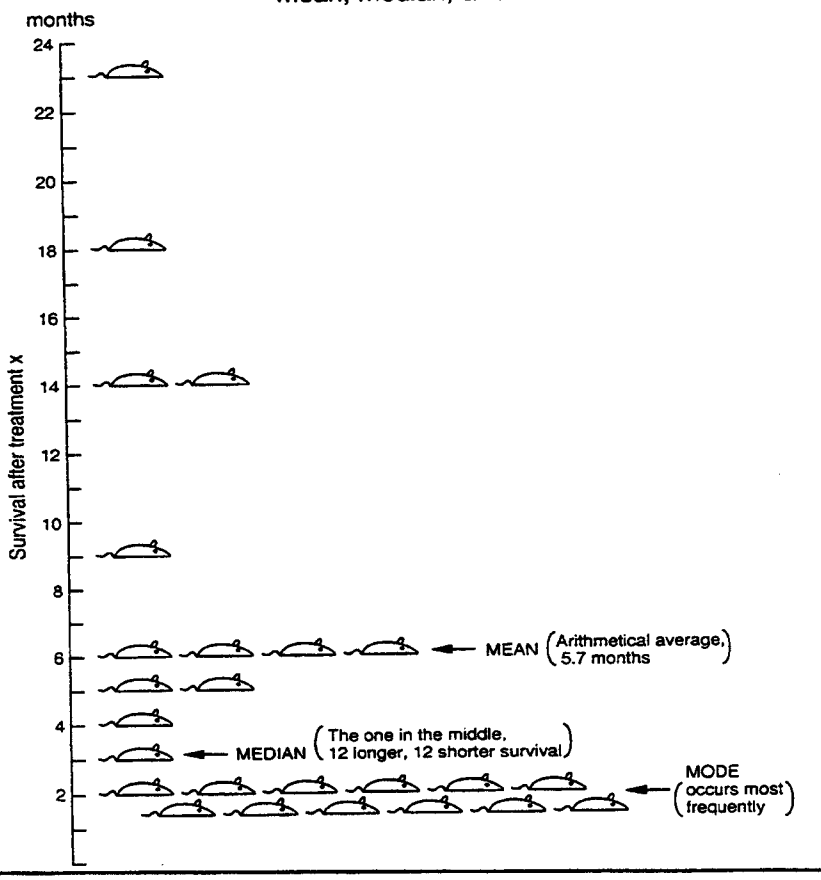


Table 5.2. Estimation of confidence intervals of medians

						$\gamma \approx$ 0.95	
n	d	γ	n	d	γ	n	d
6	1	.969	17	5	.951	26	8
	2	.781		6	.857	27	9
7	1	.984	18	5	.969	28	9
	2	.875		6	.904	29	9
8	1	.992	19	5	.981	30	10
	2	.930		6	.936	31	10
9	2	.961	20	6	.959	32	11
	3	.820		7	.885	33	11
10	2	.979	21	6	.973	34	12
	3	.891		7	.922	35	12
11	2	.988	22	6	.983	36	12
	3	.935		7	.948	37	13
12	3	.961	23	7	.965	38	13
	4	.854		8	.907	39	14
13	3	.978	24	7	.977	40	14
	4	.908		8	.936	41	14
14	3	.987	25	8	.957	42	15
	4	.943		9	.892	43	15
15	4	.965				44	16
	5	.882				45	16
16	4	.979				46	17
	5	.923				47	17
						48	17
						49	18
						50	18

γ = width of confidence interval (0.95 = 95%)
n = number of observations
d = rank position of confidence interval limits; i.e., when the observations have been ranked, the interval limits can be found as the d-th lowest and highest values (exemplified in Table 5.1)

Traditionally, the uncertainty of the mean has been indicated by the *standard error of the mean* (SE or SEM); $SE = SD\sqrt{n}$. We recommend the use of the *confidence interval,* because this interval not only tells us the probable location of the true, universal mean or median, but it can also be used to assess both the magnitude and the statistical significance of differences or ratios between groups of data. The confidence interval is that interval of the data distribution which includes the true, total population (or universal) localisation parameter (in contrast to the sample localisation parameter), in a given (usually high) proportion of replicate experiments. A confidence interval of 0.95, for example, will include the true localisation parameter in 95% of these experiments.

Confidence interval (or SE) as indicator of the uncertainty of a localisation parameter

You have obtained in an experiment a number of independent replicate data - with a certain intra-experimental variation. If the data are normally distributed, the approximated 95% confidence interval is given by $\bar{X} + 2 * SE$ (Table 5.1). Alternatively, you can find the median value and its 95% ($\gamma = 0.95$) confidence interval from Table 5.2 - small numbers of data requiring interpolation - or more easily with a computer (see Chapter 8). Now, if you repeat this kind of experiment numerous times, the interval limits will vary, but in 95% of the experiments the intervals calculated will include the true mean or median value.

It is clear that the relative width of the confidence interval will decrease with increasing number of replicate values (see formula for SE and inspect Table 5.2). The intuitive explanation is that we obtain a better estimate of the localisation parameter in this manner.

Confidence interval of a difference: a valuable parameter

Sometimes the mean or median with confidence interval is most appropriately given for the *difference* between a test and a control group of data. If the experimental design is a paired comparison (see Chapter 2), this may be particularly recommendable. In this method each experimental subject can serve as its own control, eliminating inter-subject variability (Table 5.3). The estimate of a difference between test and control results can be informative in other instances as well. If its 95% confidence interval does not include the zero value, the difference is statistically significant at the 5% level (see Table 5.3 and Chapter 6). Similarly, if the mean or median of a control group is not included in the 95% confidence interval of the test group - and vice versa - then the two groups of data are also significantly different ($P<0.05$, see Chapter 6). To find the confidence interval of a ratio - with variability in

both numerator and denominator - may be more complicated, but reasonably simple and reliable procedures do exist (Liestøl and Benestad, 1986).

Sometimes, we want to indicate the *dispersion or variability of the data* rather than the statistical reliability of the localisation parameter. Then the SE or the confidence interval is not the proper choice, but rather the SD, the coefficient of variation (CV), fractiles, or percentiles. The SD gives an estimate of the width of a Gaussian curve of distribution, from the mean to the inflection (Fig. 5.1), irrespective of the number of measurements used to construct the curve. The relative width, i.e. the SD related to the mean value, is the CV (CV = SD/ $\bar{X}$*(100%)).

Variability parameters of a distribution: SD, CV, fractiles, percentiles

With a non-Gaussian or unknown type of distribution of the data, fractiles or percentiles may be preferable to SD or CV. Fractiles and percentiles are equivalent concepts. If you rank your data from the lowest to the highest value, then the point of division between the lowest 25% or quarter of the data and the highest 75% or three quarters of the data represents the 25 percentile or 1/4 fractile. The median represents the 50 percentile; the point of division between the lower 90% and the upper 10% of the data is the 90 percentile, etc. Now, as a measure of data dispersion you may use the quartile interval, i.e. the interval between the 25 and the 75 percentiles (between fractiles 1/4 and 3/4). This interval encompasses half the data and is therefore quite resistant (robust) towards the influence of "outliers". With a normal data distribution, an estimate of the SD can be obtained by multiplying the quartile interval by 0.74. (Try with data of Table 5.1, which are not normally distributed!)

Table 5.3. ***Paired comparisons: Changes in blood coagulation and bleeding times with hard exercise***

Test procedure	Before (B.) of after (A) cycling *	Mean (min)	Median (min)	95% confidence interval of the median	95% c.i. of the median B-A difference
Blood coagulation time	B	3.1	3.2	2.4-3.3	0.001-0.609
	A	2.8	2.6	2.3-3.1	
Bleeding time	B	5.8	5.3	4.5-7.0	0.13-1.84
	A	4.8	5.0	4.3-5.5	

* 17 medical students working for 5 min at near maximal aerobic capacity on a bicycle ergometer.
Note the widely overlapping confidence intervals of the group medians, which do not indicate statistically significant differences between A and B. Significant differences exist, however, as demonstrated with paired comparisons, A versus B for every subject (last column). There is a lot of inter-individual variability which is removed by looking only at the differences between individual B-A pairs.

The fractile values can be determined by simple, linear interpolation or by a slightly more complicated interpolation procedure that is easily performed with a statistical computer program (Chapter 7).

Use of the 95% range: reference (or "normal") values

The 2.5-97.5 interpercentile range for a variable, called the 95% range, measured in a presumably healthy population, is often used to indicate the "normal values" or "reference range" for that variable. It should be noted that 5% of normal individuals will have values falling outside this range. If a large number of different laboratory tests are performed on e.g. a blood sample drawn from a completely healthy person, the probability is high that at least one of the test results will fall outside the reference range. In fact, if 12 tests are performed, the chance of obtaining at least one "pathological" result is almost 50%. If the common sense or clinical acumen of the person in charge is not too well developed, the poor subject of investigation may be thrown into a series of new, more elaborate and worrying examinations ("the Ulysses syndrome"). This possibility of obtaining falsely positive results (see Chapter 6) should be kept in mind by laboratory research workers as well.

Errors

Systematic and random errors; accuracy and precision; validity and reliability

Two different types of error exist, i.e. *systematic* and *random* errors, which can affect our localisation parameters (e.g. medians), our measures of uncertainty (e.g. 95% confidence intervals), and our measures of variability (dispersion) (e.g. quartile intervals). A *systematic error,* or bias, leads to untrue medians, even with large numbers of observations. There is a deviation from the true value. The *accuracy* of the method is bad. To exemplify, if you shoot with a rifle at a target and the sight is not properly adjusted, your shots may be closely clustered (so the precision is high and the SD, CV and quartile intervals have low values). But you hit off the target (so the accuracy is low and the localisation estimate, $\tilde{X}$ or $\bar{X}$, is bad). A *random error,* on the other hand, would occur if you were shooting with a well adjusted sight, but with low *precision*. This time your shots are spread over a large area, and the SD, CV and quartile intervals have high values. The centre of the dispersed hits could, however, still be the "bull's eye". There is no systematic error, only large random error(s), and the localisation estimate may incidentally be good.

Concepts connected with the two kinds of error are *validity* and *reliability* (see also Chapter 2). An investigation is valid if its results tell what they are meant to tell, and it is reliable if the results are trustworthy, in the sense that a repeat investigation will give similar results as the original one. Thus, research work may be without systematic errors, accurate, and valid - or it may be without marked random errors, precise, and reliable; these are related but not synonymous concepts.

Examples of *systematic errors* are: faulty procedures (when you use 0.025 rather than 0.020 ml pipettes), faulty or no calibration (e.g. of modern, electronic weights, which should be checked at intervals), and some types of psychological bias (when subjective assessment or evaluation is affected by the investigator's expectations).

Some types of systematic errors can be avoided by adherence to a strict *standard procedure* (Box 5.2). Other types are more troublesome. For instance, poor reproducibility from one experiment to the next may depend on biological variations between groups of experimental animals or instability of reactants. It may be difficult to reproduce exactly the optimal conditions for every repeat of your experiment. It may also be difficult to hit a growing cancer in an experimental animal at exactly the

Box 5.2. Experimental errors.

In order to measure temperature, ... we may not estimate the tenths properly; our eyes may be misaligned, introducing parallax error; or we may simply make a mistake in recording the scale reading. If only the bulb is immersed in a fluid appreciably different from the air surrounding the shaft, another error is introduced, since the thermal expansion of the mercury in the glass column is not the same as that in the immersed bulb. If the bulb is not allowed to come to complete thermal equilibrium with the medium to be measured, another error will result from this source.

Finally, when the thermometer was manufactured, it had to be *calibrated* This means that the temperature registered by the thermometer had to be compared to that measured by some other instrument, presumably of known accuracy. The subject of primary and secondary temperature standards is actually quite complex; ... result is that a manufacturer usually guarantees that the reading from a mercury thermometer will fall within specified error limits of the true *temperature. For ordinary thermometers the range is usually + 1. For thermometers of this type, it is clearly of little value to try to read them to the nearest 0.1°C, unless one is interested in temperature differences, and not in the absolute value of the temperature ....

Complete elimination of experimental (measurement) error is never possible. Even apparently insignificant details may contribute to experimental precision. For the temperature example, a good procedure for maximum precision in measuring differences might be to always set the thermometer in the same place when not in use, to insert it into the measured solution in exactly the same fashion each time, to allow it to come to equilibrium for exactly the same length of time in each solution, to insert the thermometer to exactly the same depth each time, and to make the reading facing the thermometer at the same angle and height each time. Above all, an evaluation of error magnitude should be carried out for critical measurements, for example by repeated alternate measurements of the same two solutions, and comparison of the replicate measurements.

(From: Cameron, 1986)

same developmental stage in each experiment, just to mention a few examples. Therefore, it is very important to run the tests and the controls as parallel as possible, as stressed in Chapter 2 and 3. Arrange replicate experiments at the same time of day, to avoid possible diurnal variations. Moreover, let the same person perform all the experiments, and in the same replicate experiments at the same time of day, to avoid possible diurnal way every time (Box 5.2.). Finally, whenever possible quality control should be introduced - not only in routine laboratories, but also in experimental research - so that standard (or reference) samples, with known properties or

composition, be analysed together with the unknown test and control samples, at regular intervals.

To control and minimize errors: standardise procedures, run test and control in parallel, increase number of replicate analyses, include reference samples, count more subjects or incidents

Fortunately, we are often interested in the differences between the test and the control. Provided that you use strictly identical designs for the test and the control analyses, running tests and controls in parallel and "blindly" if necessary (see above and Chapter 2), the differences may not be affected by systematic errors. The systematic errors can then - after a careful consideration! - be neglected.

The only certain about *random errors* is that they always will occur. They may be due to limited exactness of analytical equipment, imprecise weighing and pipetting, and apparently random processes. Random processes may take place in biological specimens (say the growth of cancer cells in an animal or in a culture dish) or otherwise (e.g. the decay of a radioactive nuclide or the number of particles or cells present in a small sample of the total). Errors are unavoidable in circumstances which are governed by chance: A given atom in a sample of radioactive material may disintegrate or not within a certain second; and a specific cell will or will not be included in a 20 µL blood sample for counting.

When you count white blood cells in dilution in a haemocytometer, the counts might be 17,20,23,23,24,26,27,29,30,35 in ten 0.1 µL compartments, when the cellular concentration in undiluted blood is 5000 /µL. Under certain conditions (see Chapter 6) your counts fit a certain asymmetric distribution, called the Poisson distribution (after the French mathematician Simon Denis Poisson, 1781-1840). Given one of these counts, it is then possible to calculate its 95% confidence interval (see Box 5.3, which gives a specialised example of such intervals). With high counts (= x) the Poisson distribution approaches a particular Gaussian (normal) distribution. In this case the SD is $\sqrt{x}$. Then, the 95% confidence interval is approximately given by $x \pm 2 * \sqrt{x}$ (see above). In other words, if your cell count is 1000, its 95% confidence interval is 937-1063. Note that these intervals must be calculated from the raw data, for example the 26 cells in one compartment of the haemocytometer, and not from the derived result, 5200 cells/µL or $5.2 \cdot 10^9$ cells/L.

Again, you can see that by increasing x, the *relative* width of the interval will decline. It follows that you can almost eliminate this kind of random error by counting a sufficiently high number of cells, radioactive disintegrations, or whatever. To increase

the counting time of a radioactive specimen beyond that giving a total count of 10 000 (with a 95% confidence interval of ~4% and CV of 1%) may not be worthwhile, however, if *other* sources of random error, contributing much more to the total error, still persist. Here - as always in scientific endeavours - common sense is indispensable.

Box 5.3 Random errors, exemplified with variability of differential counts of white blood cells

95% confidence intervals for the percentage of cells with a particular characteristic, given that **a** % of cells with this characteristic are found in a study of **n** cells

	n			
a	100	200	500	1000
0	0 - 4	0 - 2	0 - 1	0 - 1
1	0 - 6	0 - 4	0 - 3	0 - 2
2	0 - 8	0 - 6	0 - 4	1 - 4
3	0 - 9	1 - 7	1 - 5	2 - 5
4	1 - 10	1 - 8	2 - 7	2 - 6
5	1 - 12	2 - 10	3 - 8	3 - 7
6	2 - 13	3 - 11	4 - 9	4 - 8
7	2 - 14	3 - 12	4 - 10	5 - 9
8	3 - 16	4 - 13	5 - 11	6 - 10
9	4 - 17	5 - 15	6 - 12	7 - 11
10	4 - 18	6 -16	7 - 14	8 - 13
15	8 - 24	10 - 21	12 - 19	12 - 18
20	12 - 30	14 - 27	16 - 24	17 - 23
25	16 - 35	19 - 32	21 - 30	22 - 28
30	21 - 40	23 - 37	26 - 35	27 - 33
35	25 - 46	28 - 43	30 - 40	32 - 39
40	30 - 51	33 - 48	35 - 45	36 - 44
45	35 - 56	38 - 53	40 - 50	41 - 49
50	39 - 61	42 - 58	45 - 55	46 - 54

For **n** = 100 the confidence limits were calculated exactly; for **n** > 100 with **an** > 2000, the normal approximation was applied; and for **n** > 100 with **an** < 2000 Poisson's approximation was used.

(From: Rümke, 1960)

Analyse all sources of experimental error and minimise them - if necessary!

All sources of experimental error should be analysed carefully and pains taken to increase the analytical precision if the purpose of the investigation makes this desirable. Provision of better equipment, performance of higher numbers of replicate measurements, and simply more technical training are all points to consider in this regard. But remember also the principle of the blunt axe: you need not sophisticate your methods if they already work with a precision sufficiently high to solve your

problem. In any case, however, you should know their performance by making a number of replicate measurements to find the precision (given as CV or quartile interval) of your various analytical procedures (Chapter 2 and Box 5.2).

Data presentation

A satisfactory characterisation of a group of measurements must in addition to the median (or mean) include a confidence interval (or SEM) or alternatively an interpercentile interval (or SD) (see above). Furthermore, replicate number (N.) and the unit of measurement should be given. Whenever possible, the SI (Système internationale) units should be used, and powers of ten replaced by the SI abbreviations (Table 5.4).

Give information on the localisation parameters, their uncertainty (or the dispersion of the measurements), SI-units of the measurements, the number of replicates and experiments

One of the cornerstones of reliable scientific research is reproducibility; the number of times a given experimental result has been obtained should not be omitted, as is so often done in contemporary scientific literature.

For example, a cell count may be given as 1.12 (1.00 - 1.25) * 10^9 cells/L (median, 95% confidence interval, N = 20). An alternative would be 1.12 (1.00,1.25) nL^{-1}. More important than the choice between these options is that the width of the confidence (or interpercentile) interval should determine the number of "effective" or "significant" digits to be presented in both the interval and the median value. For example, the following would give a false impression of precision: 1.1224 (1.0015-1.2468) *10^9 cells/L. Our opinion is that the *interval* should not have more than 2 "effective" or "significant" digits (i.e. you don't count the first zero(s), if such occur). Consequently, rather than as 0.2453 (.2453) the interval should be given as 0.25 (.25) and the other values adjusted according to this: 1.12 (1.00-1.25). Another example, this time of high-precision measurements: 1.1224 (1.1215-1.1268). Some authorities go further than this, however, and claim that when several data are listed for comparisons, as in tables, two "effective" digits only should be given (Ehrenberg, 1977).

Table 5.4. Système International d'Unités (SI units): some examples

Names and symbols for basic SI units

Physical quantity	Name of SI unit	Symbol for SI unit *
length	metre	m
mass	kilogram	kg
time	second	s
electric current	ampere	A
thermodynamic temperature	Kelvin	K
luminous intensity	candela	cd
amount of substance	mole	mol

Special names and symbols for some derived SI units

Physical quantity	Name of SI unit	Symbol for, SI unit	Definition of SI unit
energy	joule	J	$kg\ m^2\ s^{-2}$
force	Newton	N	$kg\ m\ s^{-2} = Jm^{-1}$
power	watt	W	$kg\ m^2\ s^{-3} = Js^{-1}$
pressure	Pascal	Pa	$kg\ m^{-1}\ s^{-2} = Nm^{-2}$
electric charge	coulomb	C	A s
electric potential difference	volt	V	$kg\ m^2\ s^{-3}\ A^{-1} = JA^{-1}\ s^{-1}$
electric resistance	ohm	Q	kg m2 s-3 A-2= VA-1
frequency	hertz	Hz	s^{-1}
concentration	mole per litre		$mol\ L^{-1}$ ($mol\ dm^{-3}$)
(radio)activity**	bequerel	Bq	s^{-1}
radiation dose	gray	Gy	Jkg^{-1} (lGy = lOO rad)

Prefixes for SI units

The following prefixes may be used to indicate decimal fractions or multiples of the basic or derived SI units.

Fraction	Prefix	Symbol	Multiple	Prefix	Symbol
10^{-1}	deci	d	10	deca	da
10^{-2}	centi	c	10^2	hecto	h
10^{-3}	milli	m	10^3	kilo	k
10^{-6}	micro	u	10^6	mega	M
10^{-9}	nano	n	10^9	giga	G
10^{-12}	pico	P	10^{12}	tera	T
10^{-15}	femto	f			
10^{-18}	atto	a			

*Symbols for units do not take a plural form and should not be followed by a period; e.g. 5 cm, but not 5 cms or 5 cm. (except at the end of a sentence). ** 1 μCi = 37 kBq

*Note that expressions like mg/kg/d and mmol/mL/s are not correctly written; the designations should be mg/kg * d (or $mg\ kg^{-1}\ d^{-1}$) and mmol/mL*s (or $mmol\ mL^{-1}\ s^{-1}$).

Tables and illustrations - general advice

First you have to decide which points you have to make (your aims and goals), and to whom you want to communicate (see Chapter 7). Ask yourself whether the data must be presented at all, or whether a qualitative statement in the text of your manuscript will suffice. Then you should consider whether the data or a summary of them may be given in the text. If data are numerous and important, you choose a table or a figure (graph). A general rule is: a table if exact values are important or comparisons with data obtained by others are essential; a figure if a relationship between an independent (x) and a dependent (y) variable is a main point (such as a time course). Since many readers will peruse only the title, abstract and illustrations of your paper, this may influence your choice of illustration material (line drawings, photographs of equipment or recordings, etc.) and also underscores the rule that tables and illustrations with their titles, legends and footnotes should be self-explanatory in principle.

Consider the precise function of your data presentation! Data in text, table or figure - and how?

Next, you must ask yourself what information the typical reader or listener already has, and construct titles, designations, axis labels, column headings etc. with this in mind. Furthermore, the design of tables and illustrations must be adapted to meet the requirements set by your chosen journal (full page or column width, etc.). Start construction of tables and figures at an early stage of your work; this endeavour may direct your attention to weak points needing supplementary or control experimentation. When your main data have been summarised in this way, the order of presentation is ordinarily an easy choice and helps you get started writing the manuscript.

Use your imagination and draft several versions - if possible - of your tables and illustrations. A maximum amount of information should be given in a minimum of space - without overloading, ambiguity, or an aesthetically poor impression! Sometimes tables or figures should be split to make two or more simpler ones. In other cases a composite figure would be appropriate, e.g. two figures in a panel, with a common x axis (Fig. 5.2). It is a pity if your results are not properly communicated, considering the time, efforts and resources needed for their acquisition. Remember also that figures for a written article often are unsuitable for

Prepare alternative drafts of tables and figures when the data are at hand

oral presentation, while tables almost always are (see Chapter 7). You may therefore, in any case, need two versions of each table and figure.

The general design of a table is as follows:

Table X. *Title*

Design of a table

Boxhead for stub	Boxhead (= column headings) with units
Stub (= reading column)	Field (with data) ← (Row)
Table footnotes	↑ (column)

General information about the contents of a table - in addition to that provided by the title - can be given in the legend (footnotes) below the table. Sometimes footnotes are used to provide information on parts of the table or single entries.

Careful construction of titles and legends

Figures have a legend (caption), and its first sentence should be equivalent to the title of a table. We feel the title should often be formulated as a succinct statement of the conclusion to be drawn from the table or figure, and wish to discourage the dull "The effect of ...". In any case, the title should be short.

Since tables and figures must be self-explanatory, a compromise must be found between the requirements for brevity and clarity. Greater size tables and figures can allow for longer legends. Methodological details should in general be given in the Materials and Methods section. However, you may have to include some technical information of a general nature in the legend, and certainly abbreviations used (sparingly, if possible!) in tables and symbols used in figures must be explained. Moreover, your choice of localisation parameter, measure of its uncertainty (or variability), number of replicate analyses in each experiment and total number of independent experiments should be stated. If normalised data are presented, the raw data corresponding to the 100% control values should also be given. Having provided this information in the legend of the first table or figure in a series, you may later on

just refer to it ("See legend of Fig. 1 for further information"). If you have not done it before, you should now read carefully the "Instructions for authors" of the journal to which you plan to submit your article.

When you have drafted all your tables and figures, examine them for uniformity! Have you used identical axis labels in all figures presenting the same kind of data? The same column headings in different tables - when appropriate? You should also scrutinise your illustration material, and even more so your tables, for superfluous information. Could some words, parts of pictures, non-essential data be deleted? And, by all means, arrange a "rehearsal" by (a) well-informed, critical, non-specialist colleague(s) at the draft stage.

Check your tables and figures for appropriately uniform design and consistency, and search for redundancy

When you have finished drafting your manuscript (Chapter 7), it is time to mark in its margin the approximate position of tables and figures. They should appear, of course, in their correct, ascending order, as also used during the writing. Check carefully for consistency, both within the total illustrative and tabulated material and between this and the text. Inconsistencies are confusing and depressing. In a doctoral thesis a photograph showed a section of a urinary bladder 2 hours after injection of a cytotoxic agent, with cell loss, focal necrosis and partly denuded basement membrane (pointed out in the figure legend). In the text, the author claimed, under the heading "Histologic alterations", that initial changes appeared 4-6 hours after the injection! See also Table 5.5, where one main deficiency is the inconsistency between the experiments reported.

More about tables

Data should be arranged so that the same kind of data are grouped in columns. Order the columns to facilitate the communication of your main point(s); data to be compared should stand side by side. Control or normal values are often appropriately placed near the start of the table, i.e. up or to the left in the field. The usual reader will process your information from left to right, and from top to bottom; construct the table with this in mind, and your points may be taken more readily.

Data arrangement to facilitate communication of main points

Consider deletion or transfer to the table legend or to the text of (i) data that can be easily calculated and (ii) monotonous data (especially multiple entries of 0,

100, +, or -)(Table 5.6). Be uniform concerning the number of digits given and avoid conveying an impression of falsely high precision (see above and Tables 5.5 and 5.6).

A few notes on craftsmanship

The decimal mark should be a point (period), although in some countries a comma is used. In a column, the marks should be on a vertical line. The same applies to the ± signs before SE or SD or the dashes of confidence or quartile intervals.

The problem of the proper way of presenting the units of measurement, in the column headings, is the same as for the axis labels of figures (see Fig. 5.2 and below). Horizontal rules should be used sparingly, vertical ones not at all. Sometimes, spacing can be used to group rows of data together. The space between columns should be as small as aesthetically and practically acceptable, however.

Table 5.5. Poorly designed table

Table Y: *Labelling indices (%) of cultured spleen cells from leukemic* mice

Culture period (days)	Experiment 1		Experiment 2	
	Unseparated cells	G_0G_1 fraction	Unseparated cells	G_0G_1 fraction
6	16.0	43.5	4.8	33.2
9	24.0	27.6	5	22
13	11.6	26.6	4.5	12
20	-	-	4	6.5

First of all, the experimental material is too meagre; the difference between the two experiments is too great. No measure of variability or number of replicates is given. Taken at face value, it is not justified to give decimals (and this is done inconsistently). The dash sign is not explained.

Table 5.6. Another poorly designed table

Table Z: *Cellularity and differential counts of peritoneal exudate cells, accumulated one hour after injection of chemoattractant FMLP*

	Dose of chemoattrac-tant, µg	Treatment time, h	Total cellularity (x 10^6)	Differential counts(%) Macro-phages	Lympho-cytes	Neutro-phils	Eosino phils
Salinecontrol	0	1	5.8±3.2	76.6±8.6	16.5±8.6	6.1±4.7	0.7±1.3
Treatment with FMLP	25	1	14.0±5.6	63.4± 8.6	26.8±8.8	9.1±5.2	0.7±0.8
FMLP	50	1	21.1±7	63.9+11.7	23.0±9.5	12.1±7	1±0.8
FMLP	100	1	25.5±5.4	64.5±10.1	16.8±7.7	17.6±8.8	1.2±1.3

Redundancies in the left part of the table should be removed; for example, column 1 (reading column) be deleted, col. 2 be headed: dose of FMLP (preferably given in µmol or nmol, since MW of FMLP, which is a microbial peptide, is known), col. 3 be deleted. The variability (SEM are given) of replicate analyses (the number of which should be given in the legend or in the field) indicates that the data be presented without decimals (as has in fact been done inconsistently - for part of three entries), according to the "significant digits" convention. The data furthermore demonstrate the inappropriateness of parametric methods (i.e. calculation of SEM) applied to data that are not normally distributed: Taken at face value, cell counts can apparently have negative values here!

More about figures

A goal: rapid communication of scientific findings and ideas

The Russian author Ivan S. Turgenyev (1818-1883) said that "a picture may instantly present what a book could set forth only in a hundred pages". Rarely, scientific illustrations can attain this goal, but we should try hard - without overloading the graph. "Graphical excellence is that which gives to the viewer the greatest number of ideas in the shortest time with the least ink in the smallest space" (Tufte, 1983). Examples of good illustrations, obtained by courtesy of respected colleagues, are given as Figs. 5.3-5.5.

The function of various types of figures (graphs)

First you have to decide which amount of data you may present in each figure and the type of figure that is appropriate. Line graphs (Fig. 5.2) are often best to visualise dynamic comparisons, or the relationships between an independent variable (x, say time or concentration of a chemical) and a dependent variable (y). Column (Boxes 5.4 and 5.5) or bar (Fig. 5.6) graphs may be chosen for discontinuous variables and for ratios. They may also be useful when static comparisons are to be made, and when a quicker and more striking impression of sizes and differences is wanted than can easily be given by a table. The scatter diagram (scatter plot) - with or without a

regression line (see Chapter 6) included - is often the best way of illustrating the relationship between x and y values when neither of them can easily be predetermined in the experiment (Fig. 5.6).

Beware of more complicated geometric figures as means of depicting size variations; two drawn cows, the one covering twice the area of the other, may well represent a 100% increase of milk production to you, but the reader who makes comparisons on a linear basis, may be misled.

The virtue of *photographs* (Fig. 5.3, 5.5) is authentication. However, line drawings may be superior concerning clarity and emphasis of the essentials. Therefore, a combination of the two should sometimes be chosen. Alternatively, photographs of gels, blots, et cetera may be retouched (inform how, in the figure legend or Materials and Methods section) and equipped with inscriptions, letters, or (various kinds of) arrows pointing at important detail, to be explained in the legend. Take care that such markers are clearly visible: use white letters or arrows if the background is dark, or insert a white area if black symbols have to be used.

Microphotographs should have a scale on them, giving an appropriate dimension (Fig. 5.5). Photos may haphazardly be enlarged or diminished during the printing process; therefore a statement of a x800 magnification, given in the legend, is not clear - additionally, does this mean linear or area magnification? The legend should also inform about staining method for tissue sections.

Don't overload!

Don't overload your figures; excessive detail tends to reduce their effectiveness. In a line graph, three or four curves are a maximum, the higher number only permissible when the curves are not crowded together or cross each other repeatedly, or both.

Beware of depicting different variables (like body temperatures and erythrocyte sedimentation rates) on the same y axis (which then has to be given a scale without designation of the unit of measurement). Should the material be presented in two or more panels of the same figure? Two or more figures? Can some of the numbers on the axes (Fig. 5.7) be deleted? Can you simplify the axis labels, without obscuring the points you want to make, and rather give the specific explanation in the legend?

Labels on the curves or explanation of the symbols in the figure space, can accelerate comprehension and is nice for slides. The same applies to a short title in the

figure space, but these additions may lead to overloading and should then be relegated to the figure legend.

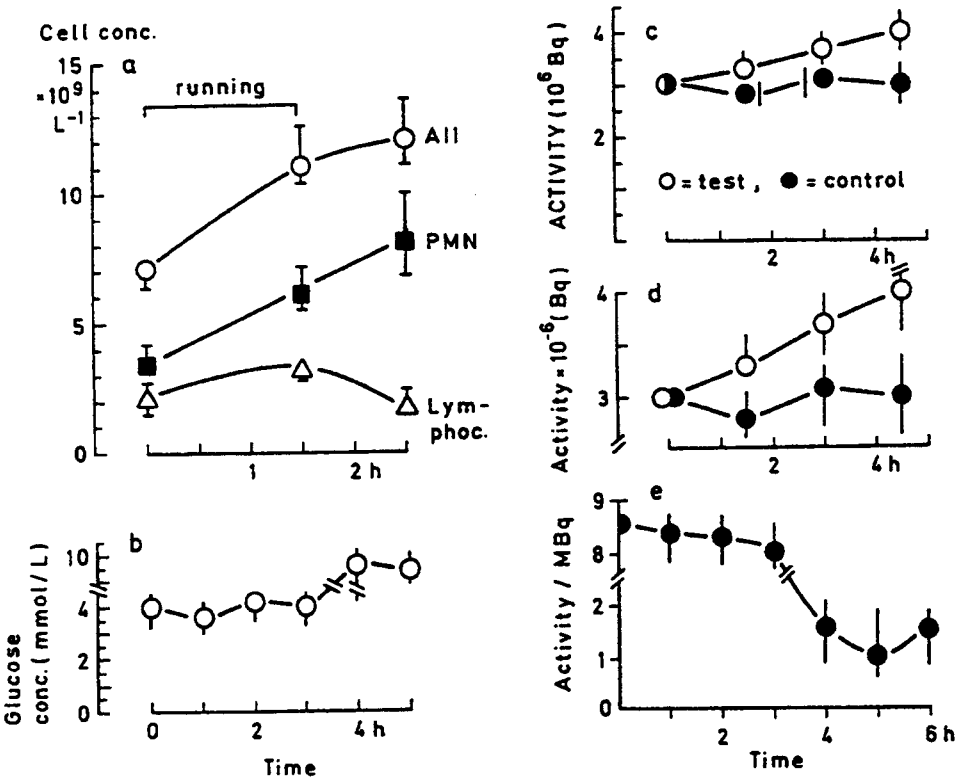


Fig. 5.2. Five panels illustrating various ways of constructing line graphs, some of them not recommendable. Note (i) the various ways of labelling the axes, (ii) the misleading impressions that can be created by manipulation with the scales (panel b and c vs. d), (iii) the acceptability of axis displacements, (iv) the marking of discontinuities, (v) the explanation of the symbols in the figure space (panel a and c), (vi) the displacement of symbols (panel d) or variability bars (panel c) to avoid overlap, (vii) the utility of combining panels (e.g. panel a and b), (viii) the usage of the SI system and avoidance of powers of 10 (panel e), (ix) the better readability of words written in lower case letters than in equal-height upper case letters (on y axis of panel c), and (x) the aesthetic appeal of the spacing between the curve lines and the data symbols, as well as the dispensability of the horizontal finials of the variability bars (shown in panel a).

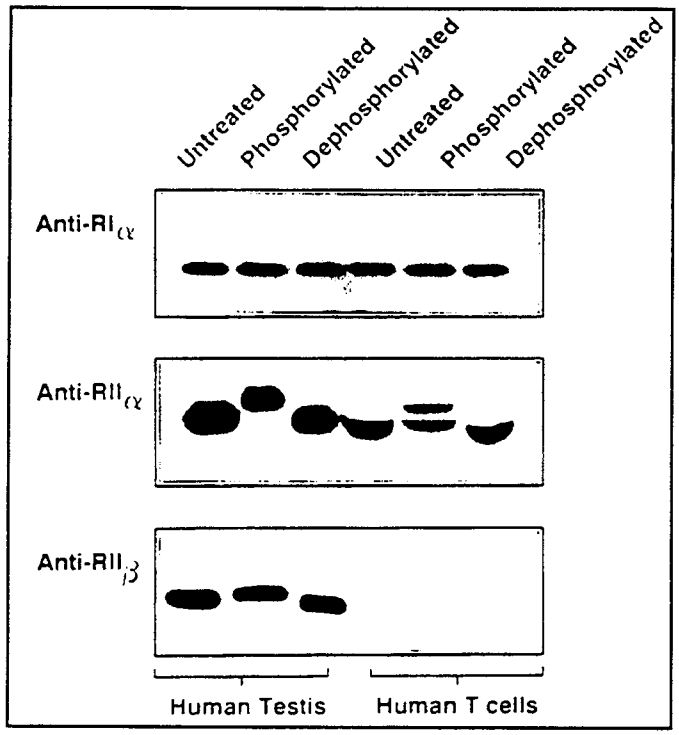
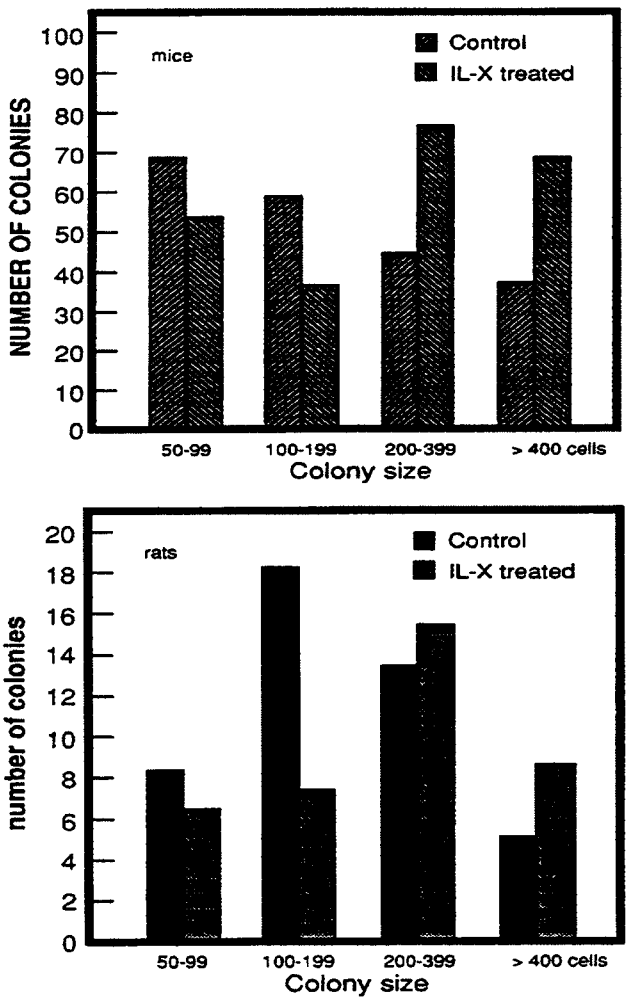


Fig. 5.3. Demonstration by immunoblotting (Western blots) of T lymphocyte extracts that three R subunit proteins with apparent molecular masses of 49, 51, and 54 kDa correspond to only two regulatory subunits (Rla and Rll«) of a cyclic AMP-dependent protein kinase.
T. Lymphocyte extract and - as a positive control - purified testis RI and RII were phosphorylated and dephosphorylated. All samples were resolved with sodium dodecyl sulphate-polyacrylamide gel electrophoresis, transferred to nitrocellulose filters, and reacted with subunit-specific antisera (anti-RI_α , -RII_α , or - RII_β). Immunoreactive proteins were visualised with ^{125}I-labelled protein A and radioautography. [During the same investigation, mRNAs for only two of the R substances, i.e. RI_α and RII_α had been detected with Northern blots in extracts from highly purified human blood T lymphocytes]. (By courtesy of Dr. Bjørn Skålhegg, Dept. Med. Biochemistry, Inst. of Basic Medical Sciences, University of Oslo, 1996).

Pictures of gels, blots, etc. require that great attention is given to technical details, photographic technique and labelling of the pictures (see instruction to authors given by Proc. Nat. Acad. Sci. (USA)). What may be adequate descriptions and labelling for specialists (above), may not be so for the general reader.

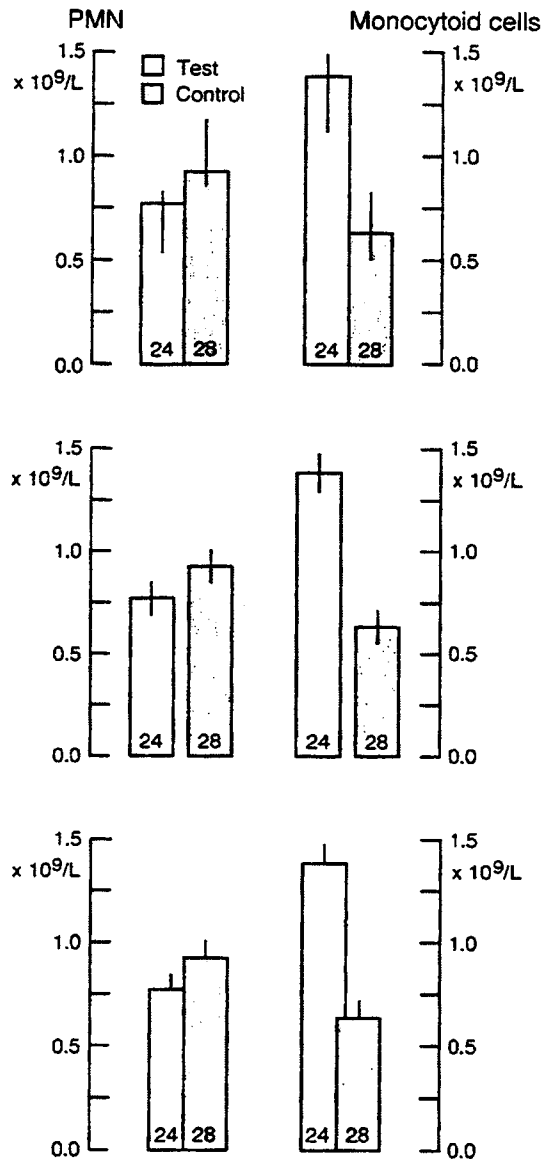
Box 5.4. Poor construction of column graph



Common faults of craftsmanship:

- Y axis scales different in the two panels to be compared
- Insufficient contrast between columns for controls and IL-X treated animals
- Unneccesary to distinguish more than two column types (test and control)
- Inconsistent use of type size (and "mice" and "rats" written with too small letters)
- Inconsistent use of upper and lower case
- Excessive numbering on Y axis
- Heavy black frames

Box 5.5. Various ways of designing column graphs



Upper panel: A conventional column chart. You must be sure that the left- and right-hand scales unequivocally refer to the left- and right-hand columns, respectively. Median values are shown, with their 95% confidence intervals (estimated with a non-parametric method and therefore asymmetric around the medians) and number of replicate values. Alternatively, the Y axis designations may run vertically upwards, along the axes.

Middle panel: Variant design, with space between the columns (not having the same width as the columns!). SE(M)'s are here shown as measures of uncertainty of mean values.

Lower panel: A third variant, with slightly overlapping columns and only 1 SE(M) drawn for each column.

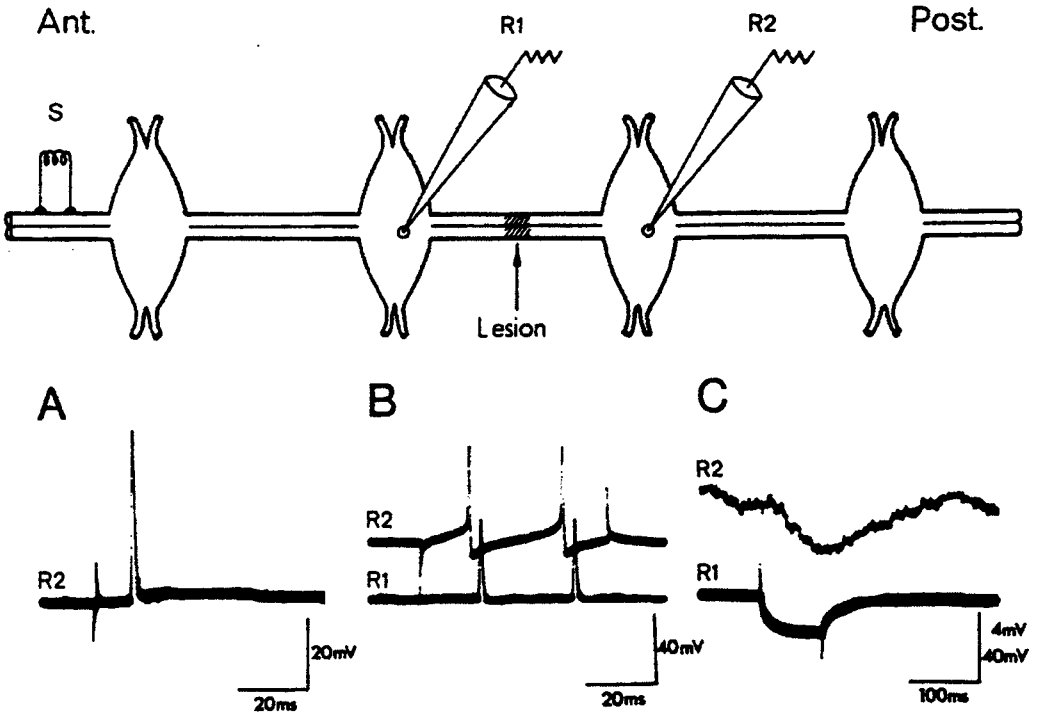


Fig. 5.4 *Demonstration of repair of a severed nerve tract in the central nervous system of the leech.* The top part gives a schematic representation of the preparation and the experimental arrangement. Four segmental ganglia with their connecting nerve cord are included. The nerve cord had been surgically interrupted several weeks before the final experiment at site marked with arrow. Stimulation (S) site at anterior end of nerve cord. Recording electrodes (R1, R2) are intracellularly in ganglion cells in 2nd and 3rd ganglion.
Records in lower part all show membrane potential as a function of time. A. Nerve impulse in cell R2 after stimulation at S. B. Activation of cell R2 (two impulses) is followed by activation of cell recorded by R 1. C. An induced shift in membrane potential in cell R1 is spread passively to cell R2. Collectively, the three sets of records demonstrate that the normal interconnections have been re-established between the cells in the second and third ganglion. (By courtesy of Professor Jan K.S. Jansen, Institute of Physiology, University of Oslo).

A long text would have been needed to fully match the information contents and precision of this kind of illustration.

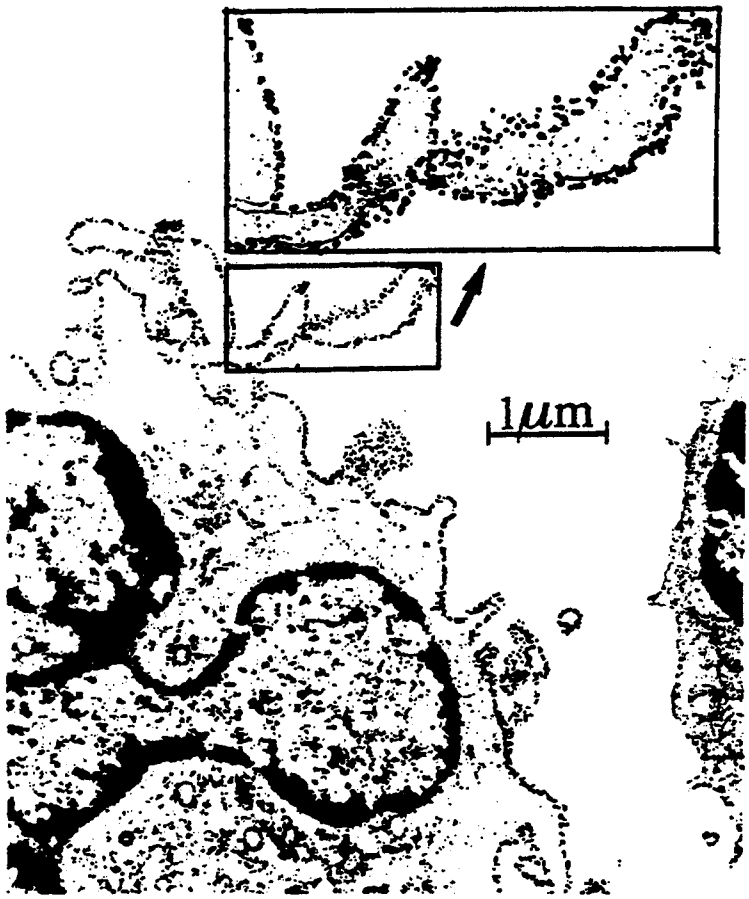


Fig. 5.5 *Electron micrograph of a veiled cell sampled from rat lymph, showing the highly irregular surface of the cell, thrown into large folds or "veils".* The cells have been reacted with antibody-coated gold particles (black dots on cell surface), embedded in plastic (Araldite) and sectioned (50 nm thick slices). Antibodies against Major Histocompatibility Complex class II antigens were used, binding of gold particles thus depicting the distribution of this antigen with even distribution on one cell, the other one is negative. (By courtesy of Dr. Sigbjørn Fossum, Dept. Anatomy, Inst. of Basic Medical Sciences, University of Oslo).

With such immunocytochemical methods one can map the localisation, density, and distribution of particular molecules in cells and tissues.

Don't waste space!

On the other hand, don't be extravagant; you may be rudely punished by the reviewers of your article! Can two or more figures be combined to one? Can your photographs be cropped; is exuberant empty space present in the figures? Can the scales of the axes be more "economical"? The axis labels should not be placed so that they increase unnecessarily the space occupied by the figure in the journal or book. Axes should not extend further than needed to include your data (Fig. 5.7).

Clarity is essential, concerning not only the general design, but also the details that are presented. Bear in mind the reduction in size that occurs during printing. The "rule of four" may give a rough guide: Use for your artwork a sheet of paper not larger than A4 (21 x 30 cm); the height of capitals (uppercase letters) should be at least 4 mm; the letter and curve lines should be at least 0.4 mm thick; the range of line thickness should be less than 4 "units" (i.e. the range from the thinnest to the thickest line should be no more than e.g. 0.4-0.8 mm). This means that in the final print the height of capitals should be at least 2 mm and line thickness at least 0.1 mm.

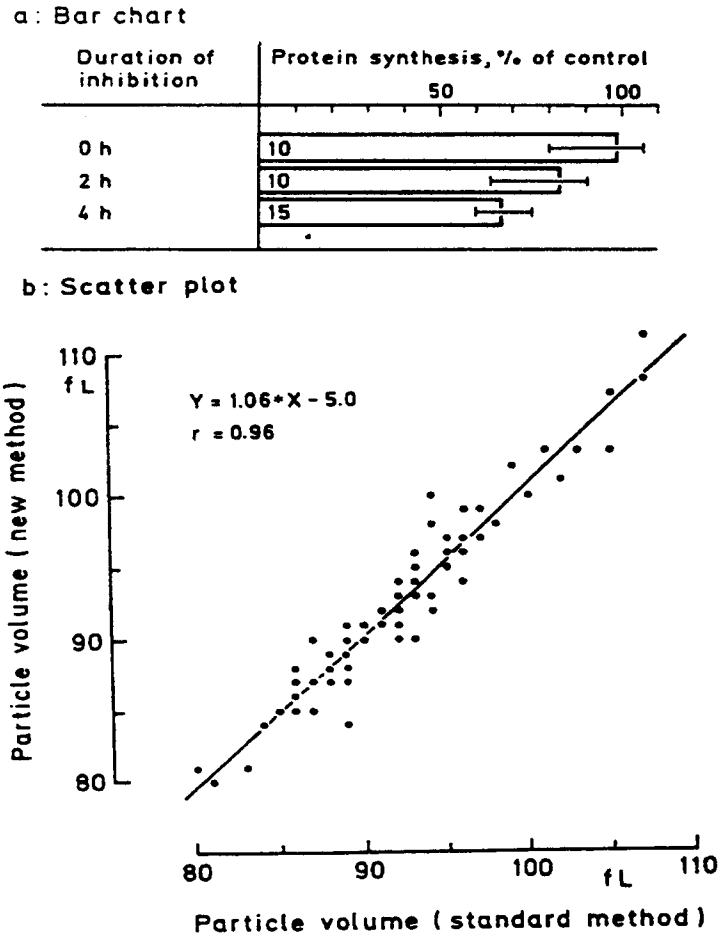


Fig. 5.6 *Examples of bar chart and scatter plot.* Numbers in the bars give the number of replicate analyses. The regression line (here very close to the line of identity, X = Y.), its formula, and the correlation coefficient (r) are indicated (see Chapter 6). Y = particle volume recorded with new method; X = particle volume recorded with standard method. Sometimes the line should be omitted and the formula and r-value given in the text or the figure legend. Here, information on the 95% confidence intervals of the coefficients may also be presented (slope or declination: 1.00-1.13; intercept: -11 - +1).

Usually, the lines of bars, columns, and curves can be drawn thicker than the x and y axes, which again should be thicker than column or bar cross hatchings and lines indicating confidence intervals or SE. All lines should be broken at sites of discontinuity or change of scale (Fig. 5.2).

When scales do not start at zero (logarithmic scales at 1), this should be easily seen. Even so, it may be possible to mislead the reader by your choice of axis scales (Fig. 5.2). Marker lines (calibrations) on the axes may be numerous, but if so do not number all the marks.

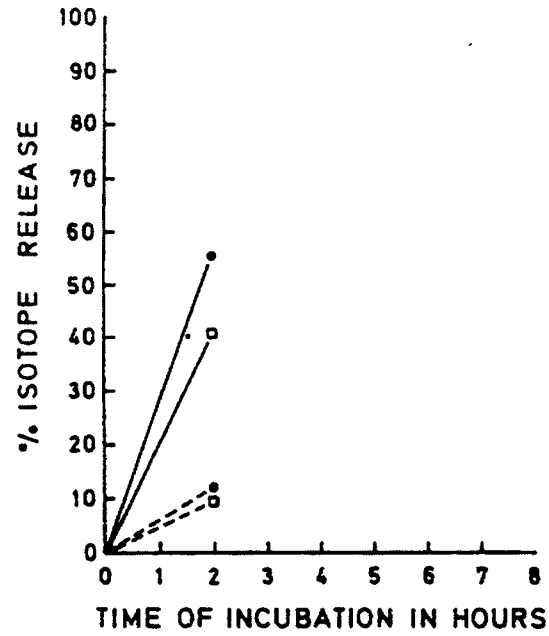


Fig. 5.7 *Chromium-51 release from labelled cells in the presence (—) or absence (---) of killer cells.*

- Osmolarity of medium raised from 290-320 mosM with sodium chloride;
, Osmolarity of medium raised from 290-320 mosM with sucrose.

All data are *average values* from *a* minimum of four determinations at each point.

Poor figure design: Axes extending too far beyond data points, which have no indications of variability. Axes excessively labelled with numbers. Curves inappropriately extrapolated to origo, where there are no data points. Double zeros at origo is unnecessary. Readability of upper case words is inferior to that of lower case words, which vary both in form and height. Terminal finials on x and y axes are lacking. The whole figure could probably have been replaced by a small table or a sentence in the text.

Curves should be broken also when they cross other curves, and we suggest that they terminate before touching the symbols for the data points (Fig. 5.2). These symbols should stand out clearly and therefore should not be drawn too small. (A compromise may be necessary in the case of dot plots with many dots, see Fig. 5.6.)

Use standard symbols, i.e. circles, triangles, and squares - open or closed or both. Curves can be drawn solid or in various ways broken (dotted or dashed). It is often unnecessary to vary both type of symbol and type of curve. If you have to, and if you have to use more than the standard types of symbol, your graph is probably overloaded. Beware of extrapolation of your curves outside the range of your data!

Authorities certainly disagree when it comes to details of figure construction. We prefer the mode presented in panel a of Fig. 5.2 to the others, but sometimes the instruction to authors or the options of your computer program force you to follow a different style. We think that marker lines may as well be drawn on the inside as on the outside of the axes, as long as they do not interfere with bars, columns or data points. Such interference may be prevented if you make a short displacement of one of the axes (Fig. 5.2). The origo need not be marked with two zeros (often you can skip both!). The x and y axes should stop at a marker line; the confidence intervals or SE, however, need no short horizontal finial. Columns and bars should be wider (often preferable) or narrower than the interval between them, and the ground line of column graphs may well be omitted (Box 5.5). You can get more advice of this kind from Boxes 5.4 and 5.5.

The upshot of following all or most of these rules is that computer-drawn figures will often be better suited as a draft for the artist than directly for printed work. Luckily, however, modern computer programs often give aesthetically satisfactory graphs.

Different routes of communication need differently designed data presentation!

Finally, we want to emphasise that different ways of communication require differently designed data presentation. Most of what has been written in this chapter (measures of variability, number of replicates, footnotes to tables, et cetera) applies to the written article, in need for complete documentation. On the other hand, the illustrative material of a talk must set forth your main points in a way that makes them easy to grasp; - and then you have to neglect the rest and all the "hedging" (see Chapter 7). In a poster presentation an intermediary or combined approach may be aimed at: Variability of letter size, colour, et cetera, as well as the possibility of giving a "handout" to specially interested viewers, provide for both emphasis on main points and penetration into experimental details (see Chapter 7).

Checklist 5.1

1. Are your data appropriately presented - in text, tables (exact values important for comparisons), or figures (data most important to communicate; relationships between variables)?

2. Are your data fully characterised (localisation parameter, measure of uncertainty or variability, number of replicate analyses/experiments, units of measurement)?

3. Are your tables and figures self-explanatory, clear, uniform, and consistent – both in isolation and in conjunction with the text?

4. Are the table headings and the first sentences of the figure legends concise, informative and easily understandable ?

5. Are any of your tables or illustrations overloaded? Can anything be removed (monotonous or easily calculable data in tables, words and numbers in graphs, parts of photographs)?

6. Is there too much open space in tables or illustrations?

7. In tables, have you arranged uniform data in columns and placed the columns in an order facilitating rapid understanding and relevant comparisons?

8. Are your illustrations aesthetically pleasing? (Repeat rules suggested in this chapter if necessary). Will they tolerate the necessary reduction in size? Are axes and curves appropriately broken at discontinuities? Are axes scaled properly? Have you avoided potentially deceptive presentation?

9. Have you arranged a "rehearsal" with (a) critical colleague(s)?

Exercises

1. Use your own experimental data to construct (i) a table, and (ii) an illustration. Analyse the results carefully: Which of the suggestions and rules given in this chapter have you followed, and which have you neglected (and why)?
2. Illustrate the data presented in Tables 5.3 and 5.6.
3. Which are systematic and which are random errors among those mentioned in Box 5.2?

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Chapter 6 : A first-aid assistance in statistics

Introduction

In the preceding chapter you have learned about acquisition and presentation of data. Sometimes you can draw unambiguous conclusions from the data directly, but very often you need backing by some sort of statistical treatment. Biomedical statistics is, however, an extensive subject that is mastered by professionals only. On the other hand, every scientist needs some basic comprehension of statistical concepts to be able to handle his/her problems with a few, well defined, statistical methods.

Our advice to the scientist-to-be:

1. Join an elementary course in statistics as soon as possible.
2. Learn a few simple methods that you can utilise on your own data (methods not used, are soon forgotten). Be particularly aware of the restrictions or assumptions of these methods.
3. Gradually enlarge your repertoire of statistical methods as the needs arise.
4. Be cautious and conscious! Stick to those methods you manage. When in doubt, ask a statistician - preferably at an early stage of your work.

The aim of this chapter is *not* to teach you statistical methods, but rather to give some examples of the structure of statistical methods, mainly tests. Our hope is that even the most ignorant should get an intuitive comprehension of the reasoning behind statistical testing of hypotheses. We have also emphasised some important topics that are too often ignored (e.g. Type II error).

As you have seen in the preceding chapters, all the data we collect are burdened with uncertainty due to methodological, statistical, or biological variation.

Observed data are always uncertain

The task of the statistical test is to cope with this uncertainty. Your conclusions can never be 100% certain, all you can do is to specify a degree of uncertainty, i.e. the probability of being wrong.

Statisticians often distinguish between the "observed" and the "true". If you could make an infinite number of observations, you would get true information, but since you are only able to make a limited number of observations, you have to generalise and assume that the observed data are representative for the whole set of

possible observations. Your observations then constitute a randomly selected sample of all possible observations. Another set of observations would constitute another sample, which may differ from the first, and so on.

When in the previous chapter you estimated a median with its 95% confidence interval from a limited number of observations, you thereby implied that there is a "true" median , which has a probability of 95% of being included in your confidence interval.

Testing of significance.

In a typical experiment you make a set of control observations which are compared with the corresponding observations in a "treatment" or "test" situation in which (preferably) only one variable differs. You will always observe a difference between the two sets of observations. The statistical problem is whether this difference is "real", or if it as well might be explained by coincidence, as a result of the "inherent" variability of the biological preparation or experimental procedure. This problem might be phrased: *"What is the probability of getting a difference like this by chance?"* Often, however, the statisticians prefer another type of formulation (for reasons which, we hope, will be more clear later in this chapter).

Statistically significant difference: The null hypothesis is unlikely

At first, statisticians begin with an assumption that the two sets of observations in reality have been sampled from the same population, which would mean that there is no real difference between them. This is called a *null hypothesis*. Then, if this statement turns out to be too unlikely, the null hypothesis is rejected.

In theory you should start the experiments by formulating the null hypothesis and the rejection level, i.e. the lowest probability you would tolerate without rejecting the null hypothesis. This probability level is often called "α" or "the significance level". Accordingly, we often state that the difference between the two sets of observations is statistically significant at the appropriate (5%, 1% or .1%) level. (The actual probability calculated from the data is designated P, whereas the rejection probability, as mentioned, is called α. Thus, the null hypothesis may be rejected if $P \leq \alpha$.)

In practice, research workers tend to do the statistical work after they have collected the data. This may be forgivable if you have outlined a more or less definite hypothesis in advance. But if you just go through your data afterwards,

looking for statistically significant differences, you may be deceived.

A rejection level of 5% means that in one of twenty investigations where there are no real differences, the data will by coincidence nevertheless give statistical significance.

Equally important as the statistical significance is the **biological** significance. A tiny dissimilarity between two groups of data may be real and statistically significant, but of no practical concern. Furthermore, a statistical test does not guarantee against possible experimental faults. You should accordingly always recheck an unexpected and interesting finding, using another type of experiment or a different experimental approach, if at all possible.

"A difference is a difference only if it makes a difference"

The sampling problem

When you make a statement on the basis of your observed data, you are always implying that your data are "representative" of some more general or underlying phenomena. If you have measured e.g. haemoglobin (Hgb) concentration in a blood sample from a young, healthy man, you may assume that the measured value reflects a normal Hgb level in young, healthy men - or at least the Hgb concentration in the blood of this particular man. Obviously, such implications require unbiased sampling. The young, healthy man may have his permanent residence at high altitude, or he may have just finished a hard bicycle ride to your laboratory. In these cases physiological polycythaemia or hemoconcentration may impair your conclusions.

All statistical treatment is based on proper sampling. Since mathematical skill is not required for this part of the task, there is no excuse for the non-statistician to neglect this. You should therefore always ask whether the data you have collected, are representative of the underlying phenomena ("true" population) that you want to study. When you compare two groups of individuals, it is of particular importance to be cautious. The groups should be carefully matched for all properties that might influence the results, except the one property you want to investigate.

Statistical tests.

You want to find out whether a certain treatment affects the patients' Hgb value.

Your measurements (mmol/L) are:

Control group (C): 10.5 11.2 10.4 10.5 9.6 10.4

Treatment group (T): 6.5 10.4 8.1 9.4 ,

which can be visualised as in Fig. 6.1 .

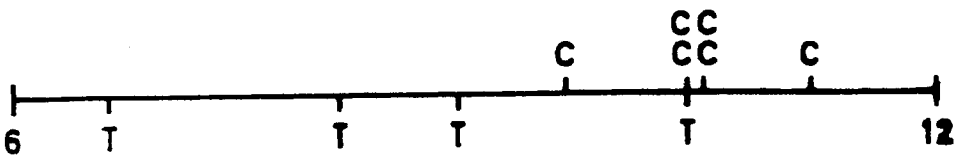


Fig. 6.1 Haemoglobin measurements of control (C) and treated (T) patients displayed on a linear scale.

Intuitively, you get the impression that the treatment has lowered the Hgb values, but you want to convince yourself that this apparent effect is not due to pure chance. Your null hypothesis is then that there is no real difference between the two groups. You recognise accordingly the need for a statistical treatment of the data, to find the probability that you may reject the null hypothesis even though it in fact is true! This is, in a way, the probability of being misled to believe in a treatment that was really worthless.

Ranking leads to simpler statistical treatment

If you look at the line in Fig. 6.1, you will find three of the four T-patients at the left end, and this may be significant. The observation can be formalised by ranking the data on the line from left to right. The T-group will then get rank numbers 1, 2, 3, 6 and the control group 4, 6, 6, 8, 9, 10 (data with the same value being given average rank value, here: (5+6+7)/3 = 6).

If you add all rank numbers in each group together, the rank sums obtained will reflect to which extent the values in the two groups differ. A low rank sum in one group (and a correspondingly high in the other) is not likely to occur by chance. We shall see that this likelihood can be estimated precisely for a certain rank sum when the sizes of the data groups are known.

What is the probability of getting a certain rank sum by chance?

In this case the rank sum in the T-group is (1+2+3+6) = 12. If you at random select four numbers among the rank numbers one to ten, it can be shown that you have $(10 \cdot 9 \cdot 8 \cdot 7)/(1 \cdot 2 \cdot 3 \cdot 4) = 210$ different, equally likely, possible combinations. In general: If you sample s numbers of a total of N, you have

$\binom{N}{S} = \frac{N \bullet (N-1) \bullet (N-2) \bullet \ldots \bullet (N-s+1)}{1 \bullet 2 \bullet 3 \bullet \ldots \bullet s}$ different possibilities.

(You may grasp this intuitively if you imagine that you in the example above have 10 possibilities when you pick the first number, 9 left when you pick the second one and so one. This way you will have 10·9·8·7 different possibilities to chose 4 different numbers in a certain order. For our purpose the order in which the four numbers are selected, has no relevance. The 4 numbers can be ordered in 4·3·2·1 different ways (try yourself!). We must therefore reduce the number of possibilities by this factor, the result being the expression above (by convention written $\binom{1}{4}^{0}$)).

Box 6.1 Ordered or unordered sampling?

To illustrate the difference between ordered and unordered sampling, consider a population of 4 marbles labelled A,B,C,D. An ordered sample of three marbles is drawn.
You have then 4·3·2=24 different possibilities as shown below (4 possibilities when picking the first marble, 3 when picking the second and 2 when picking the third).
However, since a sample of three may be ordered in 1·2·3=6 (i.e.3!) different ways, you will get only 4·3·2/3!=4 different possibilities of unordered samples.

ordered samples	ABC	ABD	ACD	BCD
	ACB	ADB	ADC	BDC
	BAC	BAD	CAD	CBD
	BCA	BDA	CDA	CDB
	CAB	DAB	DAC	DBC
	CBA	DBA	DCA	DCB
corresponding unordered samples	ABC	ABD	ACD	BCD

In this case we have 10 ranked values. If the 4 T-values are scattered randomly among these, we have 210 equally likely sets of rank numbers for the T-group. We can find out by trial that only two such sets (viz. 1+2+3+6 (as here) and 1+2+4+5) give the rank sum 12. The probability of getting this rank sum is thus 2/210 = .0095. You are not satisfied as yet, however. If the rank sum had been lower than 12, you would still believe that the treatment had a definite effect. More precisely, you would ask: what is the probability of getting a rank sum of 12 or lower by chance? Now you will find two more possibilities (1+2+3+4 = 10 and 1+2+3+5 = 11). Consequently, the chance is 4 to 210 of getting a rank sum of 12 or lower, i.e. the probability is 4/210 = .019 (or 1.9%) that you are wrong when you reject your hypothesis that the treatment does not lower the Hgb values.

But you are still not completely satisfied! If the treatment had appeared to

increase the Hgb values, you would have regarded that as an interesting observation as well. Therefore, you also have to test the probability of getting extremely high rank sums. The distribution of rank sums is symmetrical, so the probability of getting a rank sum equally extreme in the opposite direction is also 4/210. Such a *two-sided test* then gives a probability of .038 (or 3.8%), which in conventional terms means that the difference observed between the two groups is statistically significant at the 5% level. (In real life, computer programs or tables make it much easier to perform the test than shown here.)

You have now rejected the null hypothesis that no difference exists between the two groups (with less than 5% probability of being mistaken). You have used a non-parametric method, Wilcoxon's two sample test (equivalent to the Mann-Whitney test), two sided (or two-tailed).

Non-parametric methods do not use estimated parameters

Such tests are called non-parametric because the data are not assumed to comply to certain distributions that are defined by parameters such as mean and standard deviation. An alternative *parametric* test is Student's t-test. For this test to give reliable results, however, the observed values must be (approximately) normally distributed and the variance (SD^2) in the two groups must be (approximately) equal (the latter requirement is hardly fulfilled in our case). When you are using a non-parametric test like Wilcoxon's, you replace the observed values with rank numbers. Thereby, you lose some information, but on the other hand the rank sums obtained have a well known and invariable distribution, so that you can reliably predict the probability that a certain rank sum can occur by chance. Therefore, you are in general safer when using non-parametric methods. In marginal cases, though, you may miss statistically significant differences, because you are not using all the information inherent in the data.

The choice between two-sided and one-sided tests may seem puzzling, but is easily explained. You are almost always interested in changes in either direction; consequently, you should as a rule use two-sided tests. But sometimes you can *in advance* state that only changes that go in one direction are relevant. You may e.g. want to confirm preliminary experiments, and if they are not confirmed, you would put the data aside. In such cases it may be appropriate to use one-sided tests.

Paired comparisons

When you want to find a possible effect of a certain treatment, as above, the

problem is often to discover the real effect in an ocean of variability. There is biological variation between individuals, temporal (diurnal or day-to-day, etc.) variations, methodological variations and - not the least - your measurements are often not precise enough. You may get rid of some of these variabilities with a paired comparison, to which you have been introduced in the previous chapter. You may e.g. compare the Hgb value before and after treatment of each person, if the interindividual variation is marked, or you can do two parallel experiments on the same day to avoid day to day variations etc. You can then use the difference (or the ratio) between the two measurements for statistical treatment.

Sign test: "head or tail?"

The simplest test for paired comparisons is the *Sign test.* If the changes brought about by an experimental procedure tend to go in the same direction, you feel that this is not likely to occur by chance. It is like flipping a coin. The probabilities of getting heads or tails are equal, so the chance of getting only tails in a series of tosses is low.

Let us look at a case like the one mentioned. You want to check whether a certain treatment affects the Hgb level. This time you treat 8 persons and measure Hgb before and after treatment (Table 6.1).

Table 6.1. Haemoglobin measurements (mmol/L) on 8 persons before and after drug treatment.

Person	A	B	C	D	E	F	G	H
Before	9.9	9.7	10.2	11.5	9.9	9.1	11.3	10.3
After	9.3	9.6	10.0	11.0	9.2	9.4	10.4	9.8
Change	-0.6	-0.1	-0.2	-0.5	-0.7	0.3	-0.9	-0.5

Thus, 7 persons had reduced values after the treatment, whereas one had increased. Using the coin tossing analogy, you may ask: what is the probability of getting one (or no) head when tossing a coin 8 times?

Each time you are tossing, you have two possibilities, head or tail. Thus 8 tosses give 2^8 (= 256) different, but equally possible sets of heads and tails. Only one set contains no heads at all, namely tail for each toss. Consequently, the probability is one in 256 to get no heads by chance. Furthermore, you may get one head in 8 different ways (head only at the first toss, only at the second and so forth). Altogether this gives a probability of (1+8)/256 = .035 to get one or no heads.

In our case this means that the probability is 3.5% that the apparent reduction in Hgb values after treatment is incidental. If you *in advance* announced that you were exclusively interested in reduction (so that if the treatment appeared to increase the Hgb values, you would not use this information), you may stop after this one-sided test. Otherwise you have to test the possibility of getting one or no tails as well, i.e. the other extreme end of the distribution. You would then end up with a probability of (9·2)/256 = .071, which means that there is no statistically significant difference at a 5% level in Hgb after treatment.

The Sign test is evidently a crude test that uses a small amount of the information at hand. Another non-parametric test - also named after Wilcoxon (W's test for paired comparison) - uses more of the available information. In this test the differences between the paired observations are ranked according to their absolute values. The rank numbers are then allocated two groups, one for each direction of change. (Pairs with 0 difference (equal values) are excluded.) The two rank sums are then used for the analysis.

In our case we get a minor group containing one number and with rank sum 3, and a major group with the remaining seven numbers and rank sum 33 (1+2+4+5+6+7+8). What then is the probability of getting rank sum three (or lower) by chance when you are ranking 8 differences in this way? You know already (from the Sign test example) that you have 2^8 (= 256) different possibilities of separating 8 numbers in two groups (in the former example with heads and tails, in this example with positive and negative differences). How many of these possibilities leads to a rank sum of 3 or lower?

By trial you will find the following possible samples of rank numbers in the minor group that meet the requirement: 0 (all changes in one direction); 1; 2; 3; 1+2. Thus 5 of 256 possibilities make such low rank sums. If you include the possibility of getting as extreme rank sums in the other direction, you end up with a probability of 10/256 = .039 (or 3.9%) that such a difference (or a larger) has come about by chance. Thus, with this test you can demonstrate a statistically significant difference at the 5% level with the two-sided as well as the one-sided test.

For paired comparisons it is less risky to use a parametric test, such as Student's t-test, than when comparing two groups. With this test you can examine whether the mean difference between the pairs are different from 0 (or the mean ratio is different from 1). In this case you are not obliged to assume that the variance is equal in the two groups. The only assumption you have to make, is that the

differences (or ratios) are normally distributed.

Before you finish the paired comparisons section, you should note that with these methods we were able two detect much smaller effects of treatment than when comparing two groups. If you had analysed the data in table 6.1 as two independent groups, using e.g. the Wilcoxon test demonstrated earlier in this chapter, you would find an almost equal rank sum in the two groups. Correspondingly, comparing paired with independent t-tests, you would get p = .021 (paired) and p = .28 (independent). (See also Table 5.3).

Method of choice: paired comparison!

Covariation

Height and weight

You have observed that some people are fat and some are thin, and you want to investigate this variation in corpulence further. To this end you weigh all students in a class, and not surprisingly you find a considerable inter individual variation. You then cleverly think that a person's weight may be related to his/her stature. So if you in some way could take account of the variation in height, the variation in weight due to various degrees of fatness (or other factors) could appear to be much smaller. Consequently, you measure the height of the students as well. The results are shown in Table 6.2 and Fig. 6.2 .

Table 6.2 Height and weight of twelve students in a class

Height (cm)	Weight (kg)
150	54
154	58
158	62
160	60
161	65
166	64
172	69
174	71
179	74
181	78
184	76
190	80

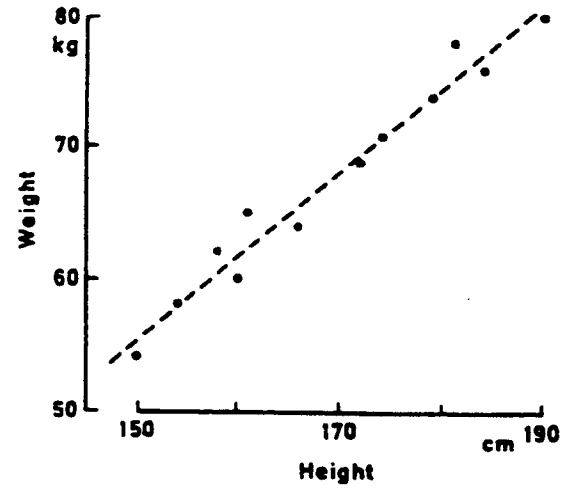


Fig. 6.2. X-Y plot of the data in Table 6.2.
The regression line is stippled.

As expected you can observe a clear relationship between height and weight. In the graph a straight line can be drawn, so that the measurements either lie on this line or rather close to it. Such a *regression line* is constructed so that the sum of the squared (vertical) distance between each dot and the line is minimised (method of least squares). This sum gives an indication of the variation of the weights due to other factors than the students' heights. In other words, the variation of weights in the class can be attributed to 1) a variation due to difference in height (illustrated by the straight line) and 2) a variation due to other reasons (illustrated by the deviation of each point from the straight line). If all the points were lying on the line, all the weight variation could be explained by variation in height. If, on the other hand, it is so difficult to construct a straight line that fits the distribution of the dots that it could as well be drawn parallel to the abscissa, then the deviations from this line is equal to the original variation, and there is no association at all between height and weight.

A mathematical way to express variability is by the sum of squares (s.s.), which is calculated by adding together the squares of the differences between the values of each observation (x) and the mean value $(\overline{x}), \sum(\overline{x}-x)^2$

(The variance (var x) is the average of the squared differences; or more precisely s.s./(n - 1). The standard deviation (SD x, see chapter 5) is the square root of the variance,) $SD(x) = \sqrt{\text{var}(x)}$

If we look at the variation of weights (y) in our example and calculate s.s., we will get 773 ("total s.s."). If we then construct the regression line and calculate s.s. as the sum of the squared deviation of each y value from this line, we get 27 ("residual s.s."). (The line is in reality, as mentioned above, constructed to minimise just this sum.) The difference between total s.s. and residual s.s. (773-27 = 746) is called "s.s. due to regression". We can get a mathematical expression for the association between y and x (weight and height) by calculating the ratio between total s.s. and s.s. due to regression, r^2 = s.s. due to regression/total s.s. = 746/773 = .965.

This mathematical treatment leads in two directions.

First, we can define the linear relationship between x (independent variable) and y (dependent variable) by constructing the regression line or, more precisely, estimating the parameters **a** (intercept) and **b** (slope) in the equation y = a + bx. It is also possible to estimate the standard errors (or confidence intervals) of these parameters. By such a regression analysis we assume 1) a linear relationship between y and x and 2) that if we had made enough observations, we would be able to confirm that for each value of x, the y values would be normally distributed around the regression line, with mean value on the line and a uniform SD (Fig. 6.3).

Regression analysis establishes a linear relationship between x and y; y = a x + b

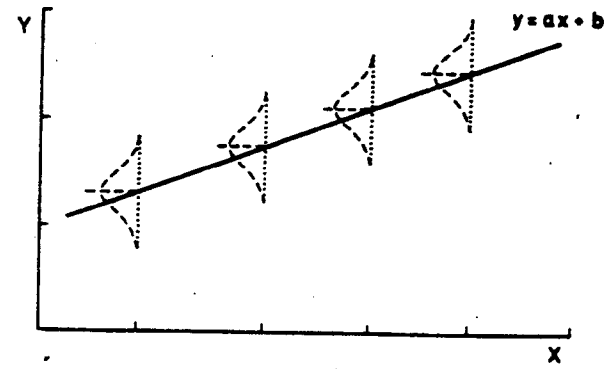


Fig. 6.3 *The y values are (ideally) normally distributed(with constant SD) around the mean values on the regression line y = ax + b, as shown for four randomly chosen x values.*

If the relationship between the variables is not linear, you may transform the data in various ways to obtain linearity, e.g. by means of logarithmic transformations or by using inverse values. In such cases you have to be very careful. For example, inverse values have quite a different distribution than the original data (note 2. assumption above!). To avoid such problems, it is now possible to buy computer programs that may fit untransformed data directly to e.g. Michaelis-Menton graphs with the method of least squares. The use of this method is thus not restricted to straight lines.

Correlation: Relationship between two variables.

Second, we can concentrate on the mutual relationship between the two variables. The correlation coefficient, r, (see r^2 above) is a measure of the degree of linear relationship between the two variables. Thus r may vary from +1 to -1, so that +1 indicates a complete positive correlation, 0 no correlation at all, and -1 complete negative correlation (i.e. all the data points can be placed on a straight declining line, so that when x increases, y decreases). In our example we can calculate,

$r = \sqrt{.965} = .983$ which means that there is a strong association between weight and height.

The requirement of linearity and normal distribution is in principle the same for calculation of r as for the regression analysis. If you are in doubt and for example suspect the presence of a sigmoid relationship, you better use measures for correlation (Spearman, Kendall) that do not require linearity.

Last but not at all least: Before you undertake any calculations, plot your data in a scatterplot! You can then more confidently make up your mind whether, and in case

how, you shall perform your statistical analysis.
It should be unnecessary to remind the reader of the distinction between correlation and causal relationship, but since we may almost every day experience logical short-cuts on this matter, we cannot resist doing it! *You should not accept a causal relationship without correlation, but correlation alone does not imply causal relationship.*

Analysis of variance

The principle of this analysis is to allocate the total variability of a set of observed data to the different sources of variation, as shown above for linear regression.

The simplest form is *one way analysis of variance* (frequently the abbreviation "anova" is used), where grouped data may be analysed to specify the variation within each group and between the groups.

An example: You suspect that the marks given at an oral examination on certain days are depending on the mood of the examiner, and you want to investigate this by comparing the marks given by that examiner on three different days of oral examination. Thus, the null hypothesis to be tested is that there is no significant difference between the examination results on these three days. Obviously, the marks will differ between students, which is reflected in an estimate of the total variation. The total variation, however, may be separated into variation *within* days and variation *between* days. Provided that the null hypothesis is true, all the samples (marks) may be regarded as drawn randomly from the same population, characterised by the estimated total sample mean and total variance. If the variation between days significantly exceeds the variation within days, the two variances are not estimates from the same population, and the null hypothesis should be rejected.

This type of analysis may be extended to relationship between several variables. The observed (dependent) variable will often be influenced by more than one (independent) variable. In these cases it is also possible to analyse the source of the variation. *Two-way analysis of variance* and *multiple regression* may then sort out the contribution to the total variation from each of the different independent variables. If the variable is discontinuous, as for instant a binary variable like sex, you may use a *logistic regression.*

In all these tests there are assumptions of normal distribution. If those are not met, you may perform non-parametric tests such as the *Kruskal-Wallis* (for One

way) and *the Friedman* (for Two way analysis of variance) tests.

Regression and variance analyses have recently been expanded and further developed into a variety of very useful statistical methods. It is beyond the scope of this introductory text to deal with this complicated, but very important field; but we will certainly encourage readers to enlarge their statistical repertoire in this direction.

Distribution

The distribution of your data often determines which statistical method is appropriate to use. It may help you to decide the kind of distribution if you know the way the data have been acquired.

A series of **measurements** is often (approximately) **normally** distributed (see Chapter 5). In such cases you may use a Student's t-test and other tests based on normality. If the normality of the distribution is in doubt, you should rather use one of the non-parametric methods mentioned above.

Fraction estimates are binomially distributed.

The **binomial** distribution is often obtained when you estimate a **fraction**, proportion or percentage. When you want to estimate the fraction of lymphocytes among blood leukocytes, you may score the number of lymphocytes among e.g. 200 leukocytes in a smear. Let us suppose that you score 80 cells as lymphocytes which means a fraction of .40 (or 40%). You can get a better estimate by counting more cells, but knowing that the distribution is binomial, you may be satisfied when you have determined the precision of your estimate. By looking up in tables based on binomial distribution (Box 5.3) you can find confidence intervals for fraction estimates (in the example above the 95% confidence interval is .332 -.472).

In this case you imply that you have a fixed probability of scoring a lymphocyte each time you observe a cell. The cell has then either the morphological property of a lymphocyte or not - consequently the term binomial (Bi nomen = two name). When you toss a coin (with probability .5 of getting a head), you will get the same type of distribution (see Sign test above).

You can also investigate the frequency of a phenomenon using another scheme. You have two groups of cancer patients, 10 patients in each group. One group is given the best standard treatment, the other receives a new promising drug. After 5 years you examine the fate of your patients, and find out that 7 persons in the treatment group, but only 2 in the control group, are alive. Has the drug had any

effect, or may the apparent effect be explained by coincidence? To make the data easier to grasp, it is common to expose them in a so called 2x2 contingency table (Table 6.3).

Table 6.3 Drug treatment of cancer patients: Survival after five years .

	dead	alive	total
control	8	2	10
treated	3	7	10
total	11	9	20

The null hypothesis is that there is no real difference between the two groups. The proportion of persons alive after 5 years is 9 out of 20. If you randomly separate these 20 patients into two groups - 10 in each - what is then the probability of getting 2 living persons (or fewer) in one of the groups? In a more general way: If you have N elements (patients) and r of these have a special property (being alive after 5 years), and if you then randomly draw a sample of s elements (patients in the control group) from these N, what is the probability of getting d with the special property (patients alive in the control group) ?

It can be shown that d is **hypergeometrically** distributed and the probability of d being a certain number can be calculated as $\frac{\binom{r}{d}\binom{N-r}{s-d}}{\binom{N}{s}} = \frac{\binom{9}{2}\binom{11}{8}}{\binom{20}{10}}$

(You can pick s elements out of a total of N in $\binom{N}{s}$ different ways (see Wilcoxon's test above). Of these you have $\binom{r}{d}$ possible ways to chose d special ones and $\binom{N-r}{s-d}$ possibilities to get (s-d) non special ones .)

The appropriate test of the null hypothesis based on a hypergeometrical distribution is called the **Fisher-Irwin** test (or **Fisher's Exact** test). You may look it up in elementary textbooks, and it is included in the most common computer program packages in statistics. (Larger samples may be tested with the Chi square test , which is not discussed here).

In this case a Fisher-Irwin test will show that P = .035 (one-sided) and P = .07 (two-sided) , which means that the treatment group has a statistically significant better survival rate than the control group at the 5% level. As you are interested in a possible adverse effect of the drug as well, the test must be performed two-sided - then the null hypothesis cannot be rejected at the 5% level.

2 x 2 contingency tables: use Fisher-Irwin test!

Another special distribution is the **Poisson** distribution (see also Chapter 5). When you e.g. are assessing radioactivity in a specimen, you will record a certain number of counts per minute. You assume that you have a certain probability of recording one disintegration within a very short time interval - depending on the type and amount of radioactive material. If you examine 1 Bq (=60 DPM) of a radioactive material (and the counting efficiency is 100%) , you will on average measure 1 count per second. But disintegration is a stochastic (random) process, so that in some seconds you may record two or more counts, while in others none at all. The distribution of number of counts in each time period conforms to a Poisson distribution. This you have to take into account when you are estimating confidence intervals or are comparing two measurements. The same distribution is relevant when you are counting a limited number of events within a certain time period or elements within a certain volume (if random in time or space!).

Counted numbers conform to a Poisson distribution.

When you count a large number of events (elements), your estimates become more precise, and at the same time the Poisson distribution becomes more symmetrical and conforms better to a normal distribution. You may then safely use a normal approximation to the Poisson distribution. The best estimate of the variance of the counts is then equal to the number counted (and consequently SD may be estimated as the square root of that number).

In general, all these special distributions (binomial, hypergeometrical and Poisson) may be approximated to normal distributions provided the number of observations are large enough. But when the observations are few, exact estimates of confidence intervals and exact tests are advisable.

Confidence interval of a difference between two groups.

Both parametric and non-parametric methods are now available for such estimates. We will consider a standard parametric method.

In the Hgb case above you can easily calculate that the mean value in the C group is 10.4 and in the T group is 8.6. Thus the difference between the two means

group is 10.4 and in the T group is 8.6. Thus the difference between the two means is 1.8. What then is the confidence interval of this difference?

The formula to calculate this interval may look awfully complicated, but it helps to approach the solution step by step.

The problem is to estimate the standard error of the difference between the means, as the 95% confidence interval is - as stated in the previous chapter - approximately equal to the mean ±2·SE (the factor depends on the number of observations, or strictly the "degrees of freedom", d.f., and may be found in a t-table).

You start by assuming that the variances of the two groups of data (var c and var t) are equal. You can then obtain the best estimate of this common variance (var (c,t)) by calculating a "pooled" average of the two. The square root of this estimated variance divided by the number of data (n_c and n_t) in the two groups then gives you a pooled standard error of the two means:

$$\text{var}(c,t) = \frac{\text{var}(c) \bullet (n_c - 1) + \text{var}(t) \bullet (n_t - 1)}{n_c + n_t - 2}$$

$$SEM_c = \sqrt{\frac{\text{var}(c,t)}{n_c}} \qquad SEM_t = \sqrt{\frac{\text{var}(c,t)}{n_t}}$$

Since the variance of an arithmetic sum (in this case a difference) is the sum of the variances of each element, we can then easily calculate the standard error of the difference between the two means. this is the square root of the sum of each "weighed"standard error squared $SE_d = \sqrt{SEM_c^2 + SEM_t^2}$

When you recall that the variance of n observations of x (with mean x) has the variance: $\text{var}(x) = \frac{\sum(\bar{x} - x)^2}{(n-1)}$ you realise that the entire formula will be

$$SE_d = \sqrt{\frac{\sum(\bar{x}_c - x_c)^2 + \sum(\bar{x}_t + x_t)^2}{(n_c + n_t - 2)n_c} + \frac{\sum(\bar{x}_c - x_c)^2 + \sum(\bar{x}_t - x_t)^2}{(n_c + n_t - 2)n_t}}$$

We can then go back to the comparison between the Hgb values in the two groups. We found that the difference between the two means was equal to 1.8 and may now calculate the standard error of the difference to 0.7 (Try yourself!). You have then to consult a table for t-distribution and will there find that at 6+4-2 = 8 "degrees of freedom" the 95% confidence interval will be the mean value ±2.3 SE_d, which here gives 1.8 ± 2.3·0.7 or 0.2 - 3.4.

Testing of hypotheses

Let us reconsider the results of the Fisher's test applied to the example concerning survival of cancer patients. We found that the probability was .07 that a difference equal to or larger than the observed difference between the two groups might have arisen by chance. In this instance we could not reject the null hypothesis that the new drug treatment had no effect, at the 5% level. But this does not necessarily imply that the drug had no effect. On the contrary, we strongly suspect that the drug may be helpful, and that we have to reinvestigate its effect on more patients.

There are thus in principle two ways in which you may be mistaken when you are testing your null hypothesis.

1. You may reject a true null hypothesis, i.e. you conclude that there is a difference, although the apparent difference is incidental. The significance level, ", specifies the risk you are willing to take to do this (type I) error.

2. You may accept a false null hypothesis, i.e. you are not able to show that a real difference is statistically significant. This eventuality is called type II error, and it is also possible to specify a probability level, 8, analogous to ".

You may also be wrong if you do not reject the null hypothesis.

(To calculate the probability of doing a type II error is for a non mathematician even more difficult than the corresponding calculations for type I error (significance tests). You must in fact first formulate an alternative hypothesis (to the null hypothesis), which specifies the minimal difference you are interested in detecting. In our example the alternative hypothesis might state that the new drug increases the survival rate from e.g. 20% to 50%. It is then possible to calculate the probability of being mistaken if you reject this hypothesis - given the size of the experimental material.)

Just a look at the confidence intervals may give you a good indication of the risk of doing a type II error. In Fig. 6.4 two comparisons (A and B) of two groups of data are displayed as medians with their 95% confidence intervals. In neither A nor B are the two groups statistically different at the 5% level (see Chapter 5). But you can also observe that whereas the two groups of data in A are almost equal (the median values do not differ much), the two groups in B might be quite different.

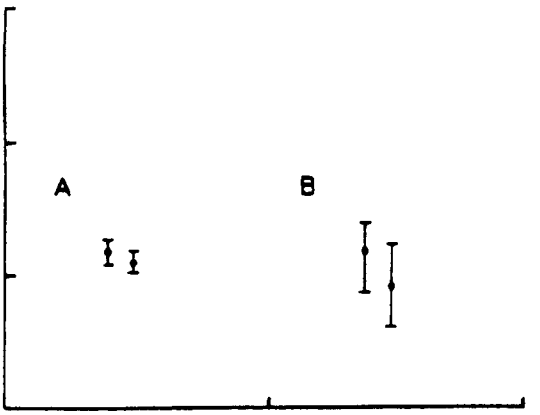


Fig. 6.4 Two comparisons (A and B), each between two groups of data, are displayed as medians with their 95% confidence intervals.

If you conclude that there is no difference between the two groups in B, you may very well commit a type II error. In such cases you should formulate your conclusions carefully. Stating that "The two groups are equal ..." is never acceptable. "It was not possible to demonstrate any statistically significant difference between the two groups" is not misleading in the same way.

Ideally you want to avoid both types of errors. You want the statistical test to tell the "truth": either there is a difference or there is not. The problem is that if you reduce the probability of performing a type I error by lowering ", you will at the same time increase the risk of committing a type II error, and vice versa ("Scylla or Charybdis" problem).

You can diminish the probability of committing a type II error by either (i) refining your methods (reducing the variability of the data or increasing the precision) or assembling more observations (getting more data).

Table 6.4 Minimal number of items (patients etc.) required, in each of two equally large groups, to avoid type I or II errors, according to specified probabilities.

	Improvement of incidence (e.g. survival rate) (%)			
	20 → 50		20 → 30	
ß	$\alpha = 0.05$	$\alpha = 0.01$	$\alpha = 0.05$	$\alpha = 0.01$
0.10	42	63	320	485
0.20	30	49	230	374
0.50	19	34	101	202

Table 6.4 may illustrate the problems. Let us go back to the survival rate of the differently treated cancer patients. You realise that you have too few patients to be able to draw reliable conclusions, so you decide to perform a more extensive investigation. How many patients should be included in each group this time? Obviously this will depend on the rejection levels you prefer, and how marked effect of the drug you want to detect.

If you set the risk of doing a type I error (α) at 5%, of type II error (β) at 20%, and you want to detect an increase in survival rate from 20% to 50% or more, you need 30 patients in each group. However, you may feel that you ought to change to the new treatment even if the new drug offers only a slightly better prognosis than the standard treatment. You also want to be quite sure whether the new treatment really is the better one. On the other hand, you would not miss an opportunity to improve the survival rate of these patients, either. If you on this background choose as the alternative hypothesis an increase in survival rate from 20% to 30%, $\alpha = .01$ and $\beta = .10$, you must have 485 patients in each group.

To avoid both type I and type II errors, your methods should be precise, your observation many or both.

When using this experimental design, you have to stick to the number of patients (or experiments) you have calculated in advance is needed. An advantageous design in such cases is sequential trial. Methods are now available that permit termination of an investigation before a prefixed number of patients are observed (or experiments performed). You are allowed to observe the outcome of each case, and on the basis of the accumulated data decide when you have sufficient number of observations to avoid both Type I and II error with prestated α and β (see

also below).

The statistical method you chose, may influence the possibility of doing a type II error. A comparably simple test like the Sign test does not use much of the information inherent in the data. If you find a statistically significant difference, all is well, but if you are not able to reject the null hypothesis this way, you may easily commit a type II error and miss a genuine difference. A parametric test like Student's t-test uses more of the present information, and is therefore more likely to detect a true inequality; the test is said to have a stronger **power**. (On the other hand, these tests require - as already mentioned - annoying assumptions to be satisfied, they are less **robust**).

How many?

Before you start the experiments, you better consider how many measurements you ought to do, or how many experiments you should include in the series.

In most cases you know in advance something about the precision of your methods and the variability of the experiments. Often the measurements are reasonably precise but you may expect occasional "outliers". The median of **three** parallel measurements will not be much influenced by single outliers, and should accordingly be preferred to duplicate determinations. Of course poor precision must be compensated by correspondingly more replicate measurements.

If you have performed one successful experiment and the results appear clear cut, you should repeat it at least twice. Many unforeseen circumstances (e.g. laboratory demons!) may influence the results, so even the most convincing experiment can turn out not to be reproducible. In fact, this applies also to the "all or none" kind of experiment. In continuous recordings, for instance, you may after some stimulation of cells or an organ observe sudden deviations from a stable baseline. You have to convince sceptics, and then three separate observations should be sufficient; of course, under the assumption that you never observe spontaneous deviations that large from the baseline and that possible stimulus artefacts have been taken into account. In such cases you use the information you have already got about the normal behaviour of the registration and the possible influence of randomness. If you, on the other hand, want to quantify the response or compare different sets of responses, you should let the number of observations be based on statistical judgement (see above).

Repeat an interesting observation at least twice!

Some experiments are unmistakably failures. Already when the experiment is going on, you may reject the results. But sometimes you do not find out until **after** you have seen the results, that the experiments did not fit into the rest of the series. In such cases you have to be cautious, but in our opinion it should be acceptable to look back to find reasonable rejection criteria. Such criteria should definitely provide a reasonable explanation of why this particular experiment went wrong, and the practice should certainly result in rejection of all experiments that satisfy the chosen criteria. If at all possible, however, you should outline the rejection criteria in advance, e.g. on the basis of pilot studies.

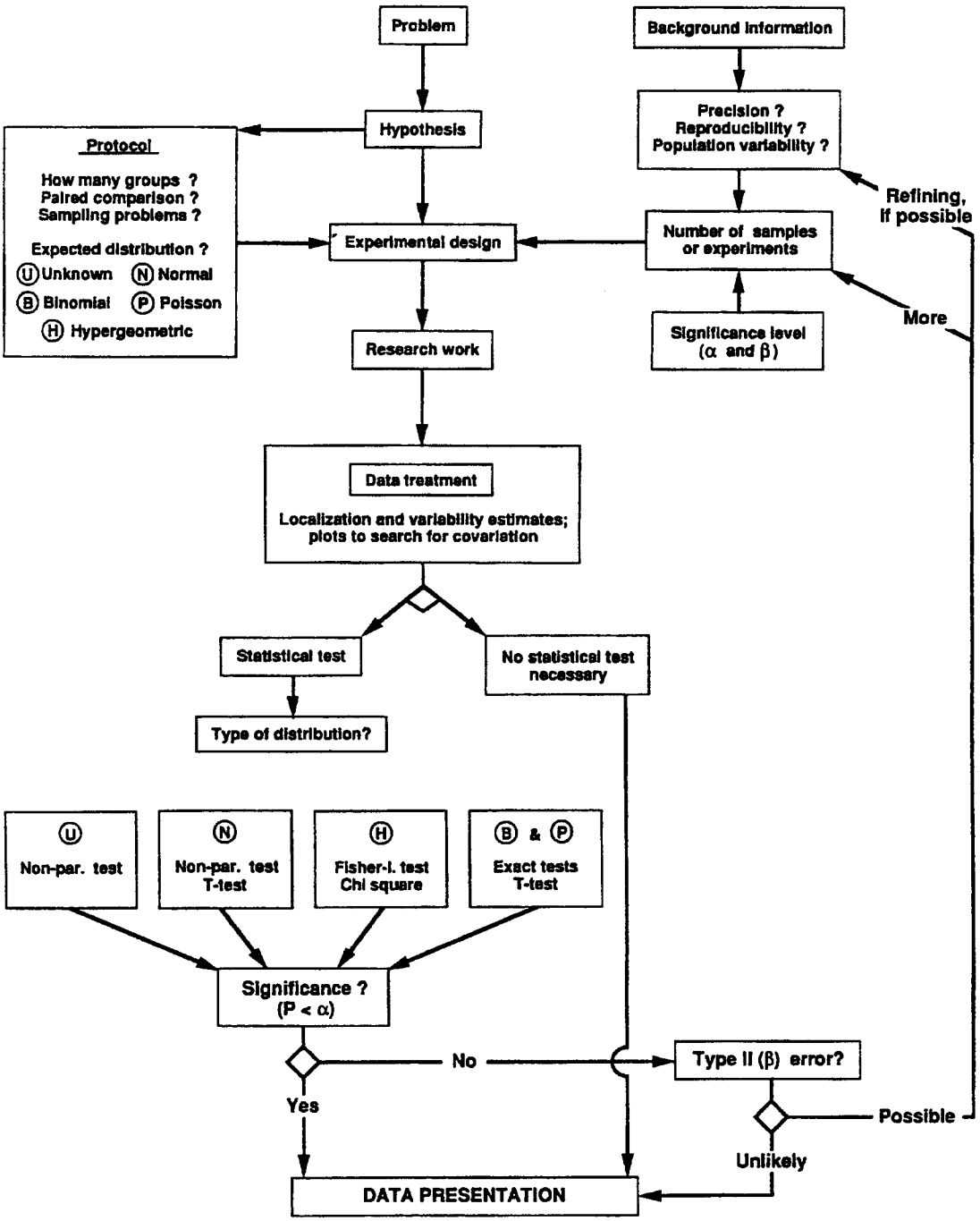
When you are performing a series of experiments, it may be tempting to terminate the series when you have just obtained statistical significance. This is not allowed if you apply the statistical tests outlined in this chapter or in most elementary textbooks in statistics. If you make decisions in the course of an experimental series, it is like deciding whether to stop or continue coin tossing after having got 6 tails with six tosses. When you are gambling, it may be advisable to quit then - before the luck changes. Estimates of probabilities, however, may be biased by such a procedure.

As a rule you should therefore go through the whole series as you had planned at the outset.

In clinical trials it is regarded unethical not to give all patients the best treatment, as soon as this has been established. Thus the trial should be terminated as soon as the statistical significance level is reached. Statisticians have for this purpose worked out special protocols, so called **sequential analysis**, in which the kind of bias mentioned above has been taken into account.

Main points of this chapter are summarised in flow chart 6.1.

Flowchart 6.1.



Exercises.

1. You want to find out whether a certain drug affects the blood pressure of healthy people. You decide to give the drug to one group of volunteers and a sham drug (placebo) to another group and then to compare the systolic blood pressure in the two groups.

   How will you select the groups? What is the rationale for giving placebo in this case?

   After two weeks' treatment you can measure the following values (mm Hg):

Drug treated:	Sham treated:
120	100
130	115
125	125
130	110
135	100
115	105
110	
115	

   Is there a real difference between the two groups?

   Formulate a null hypothesis, choose " (significance level) and an appropriate statistical test. If you set " = 0.02, can you then reject the null hypothesis?

2. Another experimental design might reveal an effect of the drug in a more efficient way. Propose an alternative protocol, including statistical method(s)!

3. After two weeks' treatment you observe that 4 of the 8 drug treated persons - but none of the 6 controls - complain of dizziness.

   Is the apparent difference between the groups statistically significant at the 5% level? (Hint: use a 2 X 2 contingency table!)

4. How many persons do you need in each group to be reasonably certain that the drug either has or has not this side effect? Presumably, 20% of untreated persons complain of dizziness. You want to be able to detect an increase in this frequency to at least 30%.

Suggestions for further reading.

Introductory:

Altman, D.C. (1991)
Practical statistics for medical research.
Chapman and Hall, London.

Bland, M. (1987)
An introduction to medical statistics.
Oxford University Press, Oxford.

Bourke, G.J., L.E. Daly and J. McGilvray (1985)
Interpretation and use of medical statistics.
3. ed. Blackwell, Oxford.

Conover, W.J. (1980)
Practical non-parametric statistics.
2. ed. Wiley.

Gardner, M.J. and Altman, D.G. (eds.) (1989)
Statistics with confidence. Confidence intervals and statistical guidelines.
British Medical Journal, London

Hodges, J.L. Jr. and E.L. Lehmann (1970)
Basic concepts of probability and statistics.
2. ed. Holden-Day, San Francisco.

Noether, G.E. (1976)
Introduction to statistics.
2. ed. Boston.

Rowntree, D. (1981)
Statistics without tears. A primer for non-mathematicians.
Penguin Books, London.

Classical reference books:

Armitage, P. and G. Berry (1987)
Statistical methods in medical research.
2.ed. Blackwell, Oxford.

Snedecor, G.W. and W.G. Cochran (1980)
Statistical methods.
7. ed. Iowa State Univ. Press, Ames.

Snedecor, G.W. and W.G. Cochran (1980)
Statistical methods.
7. ed. Iowa State Univ. Press, Ames.

Chapter 7: How to communicate

General

Appraise the situation and the reader/listener/ viewer; adapt your message accordingly

To communicate means to share a message with someone - not just to deliver it and hope that it will be perceived (lat. communis = common)! As the sender of a message, you must be aware of the kind and *condition of the receiver(s)* of your message (Fig. 7.1). This seems self-evident, but it is nevertheless very often neglected, at least in part. "What do or will they want to know?" is an all-important question. What is *the function* of your communication?

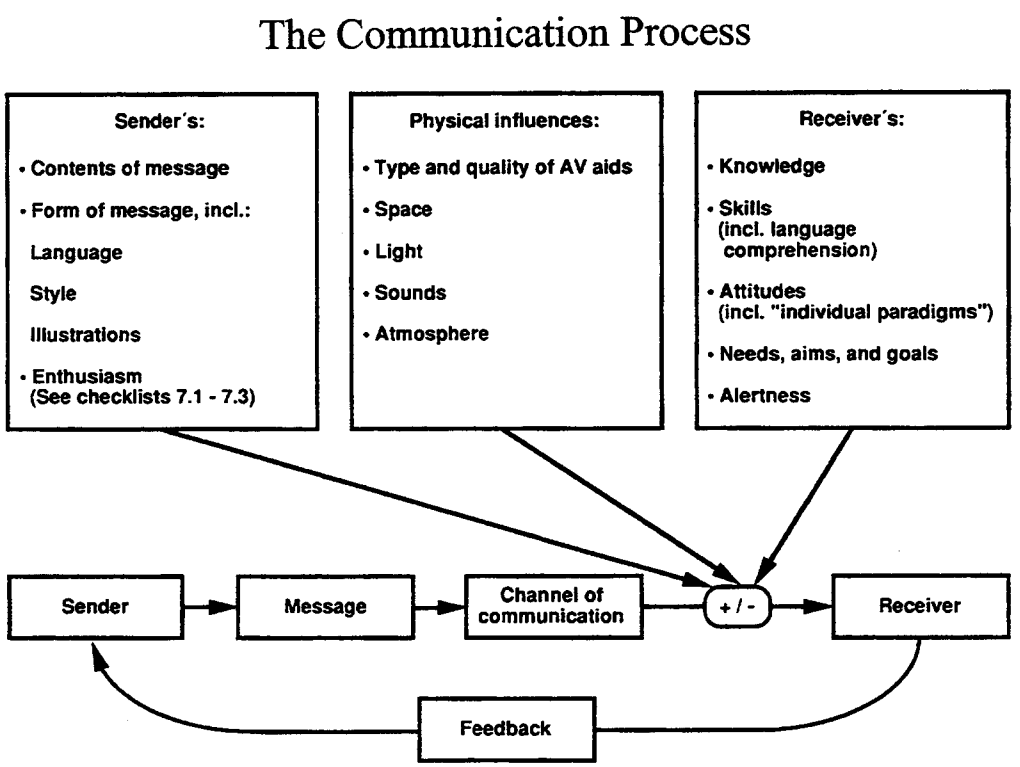


Fig. 7.1 *Improvement (feedback), facilitation (+), and inhibition (-) of the communication process.* The ideal is identity between original meaning of the sender and perceived meaning of the receiver. The feedback may be formal (oral or written) or informal (spontaneous oral or behavioural responses); it may concern the communication process, the result (the learning), or both. AV = audio-visual.

Other important considerations: intention and mode of the presentation

Also essential is the adaptation of your message to the mode or channel of communication. Think of the different ways the novelist, the opera singer and the newspaper journalist send a message! Successful presentations of your scientific work as a written article, an oral presentation at a meeting, or a congress poster may be almost as diversified as these three examples of communication. Check carefully the points made in Checklist 7. 1 and Fig. 7. 1 and be prepared to give reasonable explanations if you choose to ignore any of the suggested considerations !

Box 7.1 Why not say it clearly?

If we want to eliminate a major component of bad writing, namely, the excessive use of to be, we must first become sensitive to the deadly quality of the prose in which this excess occurs ..... :

This small monograph is *an excellent summary of current concepts of neurophysiology. It is profusely illustrated with beautiful pictures and clear diagrams. The text* is *relatively simple and is obviously written for the non expert, for there* ***are*** *very few references cited.*

We may link an adjective directly to the subject and thus eliminate an is. Instead of saying "The text is relatively simple," we need merely say, "The relatively simple text" and then attach the subject, *text,* to some other verb. In the present example we say, "The relatively simple text is obviously written...." One is has disappeared, along with an *and.*
Another common technique converts a noun complement into a verb: "is a summary" becomes *summarizes:* "This small monograph summarizes current concepts...." What, then do we do with the *excellent* that originally modified *summary?* We convert it into an adverb that modifies *summarizes, so* that *excellent summary* becomes *excellently summarizes.* "This small monograph excellently summarizes current concepts...." Another is has disappeared, along with the preposition *of.*

.....

This excellent volume ***is*** *a compilation of papers presented at a symposium*

[↓]

This excellent volume compiles the papers presented at a symposium.

[↓]

This excellent volume brings together the papers read at a symposium.

Another **empty construction** is the all-too-common usage, "The fact is that," "It is clear that," and similar clauses. These we should eliminate ruthlessly. Sometimes, to the resulting shortened version we can profitably add an adverb to render the sentence more euphonious and provide a better transition. Then a sentence such as

The ***fact is that thieves*** *were an important class in the total social structure*

becomes

Indeed, thieves were an important class in the total social structure.

.....

The **overuse of prepositions** is a severe and extremely common fault..... Convert a prepositional phrase into a participle.... Convert a prepositional phrase to an adverb..... Change the passive voice to the active:

*There had been major changes **in** the presentation related **to** the data accumulated as a consequence **of** exhaustive study **of** the results **of** treatment **in** cancers **of** the head and neck, breast, and gynecological tract.*

[↓]

The author changed his presentation after he had exhaustively studied the results of treated cancers of the head and neck, breast and gynecological tract.

.....

Always try to eliminate an **impersonal it:** "It seems to me that" means I *believe; "it is* perfectly obvious that" means *obviously; "it is* possible that" means *perhaps.*

Adapted from King (1978)

The scientific article: General points

Study craftsmanship, practise, and get feedback!

There is only one way of learning to write a good scientific article, and that is to practice: write and rewrite, over and over again! Study carefully the good writing of others! Read an exposition of good English writing and study the available texts on scientific writing (Box 7.1, 7.2; Checklist 7.2; list of recommended reading). But most importantly, try and try again, and let your own private referee(s) give you feedback about your performance. The referee may be your mentor (supervisor), a colleague (who should not be a specialist in your lane of research), or both.

Box 7.2 More suggestions for better writing

Literary courtesy decrees that the author convey a useful message in language that does not exact undue mental effort from the reader.....

Superfluity

Deleting trite, superfluous phrases like *physical examination revealed, suffice it to say, for all intents and purposes, in the matter of,* and *in respect to will* facilitate the reader's comprehension and invigorate your style without jeopardizing your meaning. Equally dispensable are such introductory encumbrances as it *is interesting (important) to note that, it is obvious (clear, well known, readily seen) that, it should be remembered that.....* In the mistaken belief that pomposity is more professional, they will write *effectuate an alteration* for *change, found to be* for *was, give rise to* for *cause, in close proximity to* for *near, in the event that* for *if, in view of the fact that* for *since,* and *produce an inhibitory effect on* for *inhibit. It is the opinion of the author is* also difficult to justify when I *think is* more precise and straightforward.....

Sentence structure

Blanket condemnation of the passive voice is misguided, since the passive is not only appropriate but preferable when the agent or the action is irrelevant or unknown. .... When, however, the agent is named in the sentence, the active voice is more direct and more vigorous. Weak and incontinent: *Breaking [grammatical] rules ... is now employed in medicine by the educated writer to confuse his equally educated reader [8].* Recast in the active: *The educated*

medical writer breaks [grammatical] rules.... to confuse his equally educated reader. (Improving the syntax does not, of course, remedy the inaccuracy of the statement). .....

The lifeless *Dissection of the aneurysm was carried out carefully by the surgeon* takes on vitality when recast as *The surgeon dissected the aneurysm carefully* because *dissected* evokes an image of action.....
Loose, obscure writing is inevitably linked to loose, imprecise thinking. Cluttering your writing with the irrelevant and the superfluous suggests an inability to stay on a straight mental path.

From DeBakey & DeBakey (1982)

A participial phrase at the beginning of a sentence must refer to the grammatical subject.

Walking slowly down the road, he saw a woman accompanied by two children.

The word *walking* refers to the subject of the sentence, not to the woman. If the writer wishes to make it refer to the woman, he must recast the sentence:

He saw a woman, accompanied by two children, walking slowly down the road.

Participial phrases preceded by a conjunction or by a preposition, nouns in apposition, adjectives, and adjective phrases come under the same rule if they begin the sentence. The examples in the left-hand column, below, are wrong; they should be rewritten as shown in the right-hand column.

On arriving in Chicago, his friends met him at the station.	When he arrived (or, On his arrival) in Chicago, his friends met him at the station.
A soldier of proved valor, they entrusted him with the defense of the city.	A soldier of proved valor, he was entrusted with the defense of the city.
Young and inexperienced, the task seemed easy to me.	Young and inexperienced, I thought the task easy.
Without a friend to counsel him, the temptation proved irresistible.	Without a friend to counsel him, he found the temptation irresistible.

Adapted from Strunk and White (1968)

We believe that it is an obligation - and also a means to self-education - to review the manuscripts of colleagues. It is well to be questioned at an early stage on dubious points, omissions, extravagance, faulty or incomprehensible logic, unclear writing or illustrations, inconsistencies, or insufficient evidence, rather than later - and

then often, alas, as harsh criticism - from the journal's referees. Wise, however, is your private reviewer if he or she starts out by pointing to the good or laudable aspects of your attempt, for criticism is often a painful process!

When you have decided who shall be your readers, chosen your highest priority journal accordingly, read its information to contributors carefully, drafted the tables and figures (see Chapters 5 and 6) - it is time for still more brainwork before starting the actual writing. Ideally, you should have time, quiescence, motivation, and you should enjoy the prospect of writing about your work. If not, and if you have to press yourself to get started, delay it - but only if chances are good that your writing conditions will soon improve! Awkward formulations that have been put on paper are often hard to rectify. Start with a written disposition of the article or part of it - or with a clear mental picture of the essentials and their proper place in a logical stream of information. Rarely is the logic too simple and the sentences or the whole paper too short! Before getting started, you should also be conscious about the main questions usually asked by the editors of the journal referees (Box 7.3).

Enjoy writing your story, in a simple, logical way

The scientific article: The organisation

The title should be brief and specific. Try to avoid general words and phrases like "A study of .. ", "The effect of.. ", "Results from .. ". The title should be rich in key words, but assembled in a readable form. You should avoid abbreviations, jargon (if possible), and overloading of words modifying each other, like "tobacco mosaic virus transformed long-passaged cell lines", or " .. growth from peripheral blood human megakaryocyte progenitor cells".

Title: Informative and readable

The *abstract* should state the subject- or perhaps even better: the problem(s)- your general approach, the results, and your main conclusions - in generally understandable terms. The contents must be clear to the reader before and without studying the rest of the paper, and the abstract must be suitable for reproduction in abstract service publications. Remember that most readers will satisfy themselves with your title and abstract!

Abstract: A minipaper, intelligible in itself

Avoid abbreviations if they are not really needed or used repetitively. In ordinary papers and congress abstracts you should be informative, not indicative - as you are when you write for example “The results will be discussed …”.

Box 7.3 Questions posed by journal editors

Is scope of paper suitable for-the journal ?
Is the paper clearly written ?
Is the language acceptable ?
Are methods appropriate ?
Are methods satisfactorily described ?

Are illustrations adequate ?
Are tables adequate ?
Is discussion relevant ?
- too long ?

Other questions asked by editors:

Is the article considered to be of sufficient scientific value to warrant publication?

Is the investigation well planned?

Are the conclusions justified?

Are the references to the literature considered satisfactory?

Are all the figures and tables necessary ? If no, what can be deleted?

Experimental Material:	Control:	Statistical Treatment:
Adequate ?	Sufficient ?	Adequate ?
Inadequate ?	Insufficient ?	

Are any unresolved ethical issues raised by this paper or the work it reports?

If human or animal studies are included, is there an acceptable statement in the text regarding informed consent or permission to perform the experiments, respectively?

Box 7.4 How to interpret a research report

What he says:	What he means:
Introduction.	
"It has long been known that..."	I haven't bothered to look up the original reference.
"This appears to be an unexplored area...."	I've been so busy writing that I have had no time to look into the matter.
".....of great theoretical and practical importance."	.....interesting to me.
"While it has not been possible to provide definite answers to these questions " "While it has not been possible to evaluate conclusively ...."	The experiments didn't work out, but I figured I could at least get a publication out of it.
Material and methods.	
"High purity...." "Very high purity...." "Extremely high purity...." "Super purity...."	Composition unknown except for the exaggerated claim of the suppliers.
"....accidentally strained during mounting"	...dropped on the floor.
".... handled with extreme care throughout the experiments"	... not dropped on the floor.
Results	
"Typical results are shown ..."	The best results are shown.
"Some of the observations were clearly atypical and were omitted from the study"	To include them would ruin the findings.
"It is evident from the table ...."	My conclusions are not contradicted by the table.
"Although some detail has been lost in reproduction, it is clear from the original chart that ...."	It is impossible to tell from the chart that ....
"Presumably at longer times ...."	I didn't take time to find out.

"These results will be reported at a later date ...."	I might possibly get around to this sometime.
"The agreement with the predicted curve is excellent"	Fair.
".... good"	Poor.
".... satisfactory"	Doubtful.
".... fair"	Imaginary.
"As good as could be expected, considering the approximations made in the analysis".	Non-existent.
"Three of the samples were chosen for detailed study"	The results on the others didn't make sense and were ignored.
<u>Discussion</u>	
"It is suggested that ...." "It is believed that ...." "It may be that ...."	I think
"Well known"	(i) I happen to know it; (ii) well known to some of us.
"Obvious", "of course"	(i) I was not the first to think of it; (ii) I also thought of it independently, I think.
"It is generally believed that ...."	A couple of other blokes think so too.
"It might be argued that ...."	I have such a good answers to this objection that I shall now raise it.
"Correct within an order of magnitude"	Wrong.
"It is to be hoped that this work will stimulate further work in this field"	This paper isn't very good, but neither are any of the others on this miserable subject.
"The most reliable values are those of Jones"	He was a student of mine.
"This study seeks to synthesize previous work in this field ...."	I was too lazy to do any original work.
"It is clear that much additional work will be required before a complete understanding ...."	I don't understand it.
"Unfortunately, a quantitative theory to account for these effects has not been formulated ...."	Neither has anybody else.

"Limitations of time and resources prevents me ...."	I'd better stop here before I get all confused.
Acknowledgments	
"Thanks are due to Joe Glotz for assistance with the analysis and to Dave Doc for valuable discussions ...."	Glotz did the work and Doc explained what it meant.
	(Compiled from various known and anonymous sources)

Yet restrain yourself so that you do not exceed the limits (e.g. 250 words or 3% of the total paper length) imposed by the editor. Delete doubtful points from the summary or abstract!

After the abstract you may present *index words,* naming the main methods, experimental animals, cells and tools. Index words should preferably be chosen among those given by Index Medicus and otherwise according to the instructions. The abstract and index words may well be the last paragraphs you draft.

Introduction: What is your problem and why is it important?

The start of the *introduction* seems to be the most common place for platitudes, but they should be deleted wherever they occur (Box 7.4). Once again, consider whom you are aiming at when you construct the few sentences that introduce your topic and place it into its wider context.

A short historical review may then be necessary to put your work in perspective and indicate its relevance - and above all, its importance (see Chapter 2). Reference to review articles allows you to condense this part of the introduction. Be certain that it is quite clear what your references are meant to indicate. Be honest! The last two suggestions apply to the rest of the paper as well.

Finally, indicate the main problem(s), perhaps also your intention(s), and possibly briefly the main procedure(s) and result(s) as well. Avoid redundancy and repetitions, however (Box 7.5).

The *Materials and Methods* section may be written at an early stage. Study the pattern adopted by your chosen journal before you start writing.

Box 7.5 The state of the art: A miserable affair? - Consider this ironical description

First of all, the manuscript must not be a bad one. After all, the oligarchy is not unintelligent and they can tell a bad manuscript when they see one except when it is their own. But the manuscript must not be a *very good* one either. It must not contain any radically new ideas, untested approaches, discoveries that open new vistas. It must conform to the usual standard, it must not tower above this standard. There is a long list of examples supporting this conclusion. The most outstanding of these is the paper by Hans Krebs describing the Krebs cycle, work later honored by a Nobel prize, but rejected by *Nature* where it was submitted originally.

The subject of the paper must *be fashionable.*

Also, you must use *sophisticated* techniques.

Finally, you must write your paper to conform with the established norm. Speculate as little as possible, and if you must speculate, do so along the well-established lines. Repeat the main points at least three or four times so that you drive them into the minds of the reviewers and the readers should there be any. The *Abstract,* the final paragraph of the *Introduction,* the *Results,* and the opening paragraph of the *Discussion* are the places where the reviewer expects to find such repetitions. He feels cheated if you avoid some of this redundancy. A good thing is to give the reviewer an extra bonus by adding a summary at the end of the *Discussion.* The reviewer will appreciate your generosity and will take it into account when making recommendation on the manuscript.
Utmost care is required for putting your results into proper context. It is extremely important what and whom you cite. Here it is absolutely crucial that you stay in the mainstream of current thinking. Any deviation will be looked upon with suspicion, any negligence in acknowledging a popular idea will be regarded as ignorance or, even worse, as conceit.

Klein (1985)

Materials and Methods: the cookbook

You should consider the following points too, which may not be self-evident: Experimental animals: Inbred? Specific pathogen free (SPF)? Particulars about sex, age, strain, feeding, housing, day-night rhythm? Randomisation procedure? Time of the day when experiments were performed, in case of diurnal variation of parameter examined? (See Chapter 3.) Biological materials, if variable: Batch or lot or code number? Name and acknowledgement of the donors of recombinant proteins, monoclonal antibodies et cetera? Patients or human material: Particular, ethnic group? Did you obtain the informed consent of patients et cetera (see Box 7.3 and Chapter 9)?

Consider replacement of r.p.m. (rotations per minute) with G (or g), for the bottom of the centrifuge tube, or for a specified interface. Moreover, 18-20°C is better than "room temperature". Have you remembered to specify all important physico-chemical parameters like those just mentioned; as well as pH, concentrations (preferably in mol/L), gas pressures, volumes of solutions, storage conditions?

It may be possible to condense this section by reference to previous, detailed descriptions, so that just the principles of the procedures are described, plus modifications and the reasons for them. These modifications, as well as new methods, should be presented in detail, however.

A paragraph with the subheading "Experimental design" may be appropriate to indicate how the various materials and methods were employed.
Sometimes a sub-section headed "Methodological considerations" can be used to present results and discussions of methodological investigations that you feel are too trivial or peripheral for the Results and Discussion sections.

The final paragraph should inform about data treatment, data presentation, and statistical methods used. Concerning hypothesis testing, you should specify the significance level and whether one- or two-sided (-tailed) tests have been used (see Chapter 6).

Finally, this section is the part of your manuscript where the passive voice is most appropriately used, since what was done is more important than who did it. Otherwise, try to use the active voice - "I" or "we" are not prohibited! - especially when you interpret or describe what you mean.

Results: What you found, ordered in a logical way

The *Results* section: Here you tell a story, or stories (with subheadings, if permitted), briefly, in the past tense. This section may also be drafted at an early stage, when provisional tables and figures are ready (see Chapter 5). We personally dislike the sloppy "It can be seen from Figure 1 that blood pressure remained constant ...". Imagine that the page charge is US $ 130 (which it may well be!) and write: "Blood pressure remained constant (Fig. 1), ...". More important, a good story does not begin by telling about the uneventful. Place the important points early in the paragraph, and the important words near the beginning of the sentence!

Extensive discussion is not allowed here, but you may have to explain shortly why an experiment was done, what it meant and how it led to the next type of experiment. You may also have to document experimental premises for the main approach.

Don't repeat data in the text, that are already in the tables or figures. Rather, describe main trends and findings, either qualitatively, or in rough quantitative terms (e.g. doublings, a 25% decrease).

An illustration summarising all replicate experiments of a certain type is certainly more convincing than a figure showing a "typical" or "representative" experiment (which is probably the best one). If, however, the summarising figure must be based on normalised data, the second alternative may nevertheless be the better one (see Chapter 5). Then, you may add that four replicate experiments gave virtually (or essentially) similar results. Murphy's law states that what can go wrong, goes wrong; but you need good reasons to exclude atypical results (see Chapter 6). Consider, therefore, describing the typical features of the atypical outcome.

Discussion: the interpretation, in relation to common knowledge, and the importance of the results

The *Discussion* section should also, as a general rule, be written in the past tense. For general conclusions the present tense may be appropriate. You might start by recapitulating what you found, related to the problem(s) stated in the introduction. You should then tell what this means. Next you have to draw upon the common body of scientific knowledge, referring to work done previously by yourself or others.

A possible lack of complete honesty and knowledge will now be evident to your well-informed reader. Are you fully aware of the important previous work done in this area? Be careful to cite explicitly the relevant results of others. Are the points and comparisons you want to make so important that you should refer to those making the original observations, or will a reference to a review paper suffice? You know that your references to others' work will rarely be thoroughly checked during the reviewing process, after you have submitted your paper to publication - does this mislead you to study the literature less than carefully? As you become a well established scientist, will you be able to resist the temptation to restrict the references to work done by your own countrymen and peers, and then to work done by peers in your own laboratory and yourself, and finally only to your own work during the last ten years --?

Sometimes, it is not possible to tell what your observations mean. There are several options. Start with the most plausible interpretation. Allegedly, Hans Selye said: "Our facts must be correct. Our theories need not be, if they help us to discover important new facts".

It may be relevant to point out sufficient and necessary conditions for making the present observations, and also why nobody else found what you found - and what

remains to be found. Above all, pinpoint the news value and importance of your work (but again: be utterly honest)!

Sometimes you have to discuss groups of observations, each one addressing a separate (sub-)problem. Then you either start with the most important, or if that is difficult to decide, you may follow the order of presentation chosen in the Results section. Optionally, you may mark these separate discussions with sub-headings or short italicised introductions.

The final paragraph may contain some concluding remarks, and it may be headed *"Conclusions"* to herald the authors' views on the significance of their work, on prospects for future work, or on other aspects of the investigation, perhaps in a more subjective way than allowed for the rest of the paper.

Common faults of the Discussion are lengthy repetitions of the Introduction and of the Results. Often the Discussion is too long, extending far beyond a reasonable interpretation of the new hard facts. Don't consider every scrap of your work - but don't conceal important negative results or discrepancies with the results of others either! Don't try to document that you have read all the literature in your field of study, and don't exaggerate the generalisation and importance of your work. But don't be too modest either! You may criticise the works of others - preferably in the way of understatement - but never raise personal criticism! There is much subtle steering between Schylla and Charybdis; apply common sense (the most important in science!) and you will come through with a concise, complete, and fair account.

In the short *Acknowledgement* section you should thank all sources of financial and other support. Furthermore, you may say what your helpers did and then thank them for that. To appreciate the expert technical assistance of trustworthy and efficient laboratory personnel is the least you can offer them, we think.

Acknowledgements: financial support, technical assistance, et cetera

Finally, the *control or checking procedures* take time and deserve that time. Most importantly, check that all data - including those in references - are correct. Have a special intention with each review: Should sentences be divided in two or three? Can you find more redundant words, clauses, sentences, or paragraphs to delete? Are there sentences with too many prepositions, nouns that could be converted to verbs, passive voice constructions, warning words like "is", "of", "by", "it", "very", et cetera?

Can the orthography, grammar, and style be further improved? - Are the verbs in correct tense and correct - singular or plural - form? The word processing programs

of modern computers can help you with some of these tasks, but there are no real substitutes for training and hard work.

Are all the references that are listed cited in the text - and vice versa? Now, go back to the Instructions to Authors and check that you have followed them. Then, at last, you may write a letter to the editor to introduce your manuscript, perhaps something like this:

Dear Editor,

I have enclosed a manuscript in quadruplicate, "Membrane ... " by H.B., R.H., and KL. I hope that you may find the paper suitable for publication in Exp. Hematol. The investigation concerns ...., which we feel falls within the scope of your journal. All data presented in the article are original data of the authors. The data have not been published previously. We now submit the article to your journal only. The manuscript has been reviewed and approved by my co-authors.

Yours sincerely

The final repair; be flexible, but not unreasonably so

How you should cope with rejection or conditional acceptance of your paper is dealt with in some of the books recommended for further reading. Here, we shall give you two pieces of advice only, for your reply to the editor, if you have to revise your manuscript. (1) Be polite. Point it out if the reviewers did thorough and competent work. Use careful understatement if you have to chastise the anonymous reviewers for misunderstanding, sloppy work or queer ideas - even if they have been rude and unreasonable (we don't think they often behave like this!). (2) Answer all points raised by the reviewer, briefly and specifically. Indicate which and where changes have been made in the manuscript, or explain why you think changes should not be made.

The oral presentation

There are marked differences between the oral and the written communication

Frequently one is required to present one's work orally - at a seminar, at lab discussions, or at a congress. Checklist 7.3 gives an overview of some main points, which are elaborated upon in the boxes (7.6, 7.7). It is wise provided you are not very experienced - to prepare a manuscript for formal sessions. But remember that it must

be very different from the written article, and preferably do not use it after finishing your own rehearsals in private. The other way round is also true: a good speech is not *easily* transformed to a good article.

Box 7.6 Letter to the Editor: After a Congress and Before the Next One: A Plea for Better Performances

The last ISEH meeting, in Baltimore, was a success! Except for some of the discussions of posters, everything had been organized in an exemplary way. It was, however, especially disheartening to witness, over and over again, the down-right poor oral presentations of scientific papers. Sindermann (1) has prepared a short and incomplete list - in 10 points - of "admonitions and advice on how not to present a scientific paper." In regard to the Baltimore meeting, I would like to cite some of his suggestions: "Read it word for word from single-spaced typed pages .... Never ... run through a practice session, ... Tables should have tiny printing and at least thirty lines of numbers to ensure maximum invisibility of data .... Then leave the slide on the screen for a very short period, to thwart the eager types with excellent vision who may be seated near the front .... When you present the paper, act genuinely disinterested in the data and the audience; never, never give an indication of enthusiasm for your research; . . . keep the room moderately to totally dark during the entire paper, with slides interspersed throughout ...."

Speakers adhering conscientiously to some or most of these rules may tax heavily the patience of innocent congress participants, who may have made financial and other sacrifices to be present. In addition, the higher the travel costs, the more unfamiliar the participants may be with rapidly and indistinctly spoken English language, the less they may understand, and the more dumb and desolate they may feel! I shall not hesitate to characterise as an offence the neglect of foreign visitors' difficulties with spoken English, especially when distracting mannerisms like gapfilling "ahs" or "ers", failure to use the microphones, and "private" discussions in the lecture room are added to the main misery. Those finding it difficult to interpret such discussions that follow the formal presentations, may consult Nolan (2) for advice. Those enjoying participation in discussions that are conceivably unintelligible to part of the audience, might consider the following. Recently, at an international meeting, German was quite unexpectedly used as congress language. How would you have enjoyed that? Or how about reverting to the times of Carl Linné; as a Swede he performed excellently with his school Latin within the academic circles of the Netherlands!

There is no lack of books or articles giving sensible advice on how to speak at scientific meetings (1, 3-7). Apparently, some of the points cannot be made too often. The Nordic Association for Physiology recommends its members first to consider whether the study is at all appropriate for a 10-15 min oral presentation. Then, it should be taken into account that the majority of the audience may not be specialists in the field, so that an ample part of the allotted time be used to introduce and elaborate on the problem(s) that has been approached; whereas sheer data presentation should be kept at a minimum - 4 slides are normally a maximum for a 10-min speech. End the talk with a clear conclusion - preferably on a slide, I would like to add, so that everyone might at least receive that final message. "Under no circumstances whatsoever should a paper be read from a script, " says Sir Peter B. Medawar (7), with emphasis. Less strictly, I would urge the speaker at least to pretend not to read from the manuscript! Authorities unanimously prohibit the use of the same tables for slides as for the printed article. Good and easy readable guides to better slides exist, some of them in fact free of charge (8,9). Let us try to make even the lecture room experience an enjoyable one in London!

Yours sincerely

Haakon B. Benestad, M.D.
Institute of Physiology,--

1. Sindermann CJ. Winning the games scientists play. New York: Plenum Press,1982: 39-54.
2. Nolan SP. On discussional language: a novice's guide to phraseology and translation with suggestions for the moderator. J Irreproduc Res 1981; 27: 10- 1.
3. Calnan J. Barabas A. Speaking at medical meetings - a practical guide. Lond.: Heinemann 1981.
4. Williams PC. Suggestions for speakers and standards for slides. Inst Biol J 1965, May: 1-8. (Institute of Biology, 41, Queen's Gate, London S. W. 7.)
5. Meadow R. Speaking at medical meetings. Lancet 1969; ii: 631-3.
6. Zollir ger RM, Howe CT. The illustration of medical lectures. Med Biol Ill 1964; 14:154-62.
7. Medawar PB. Advice to a young scientist. New York: Harper & Row, 1979: 59-62.
8. Sandøe E, Andersen JD. A guide to better slides for European meetings and congresses of cardiology. C. H. Boehringer Sohn, Ingelheim, Western Germany, 1978.
9. Effective lecture slides. Kodak publication 1974; S-22.

Benestad (1983)

More than ever it is essential that you know your audience, to catch their interest. Connect your theme to general principles. Don't mention details concerning materials and methods, references and acknowledgements!

Stick scrupulously to your allotted time; you may use 40% of it to introduce the subject and your problem(s) (versus perhaps 5-10% in the written article), 40% for the main methods and results (versus 40-60% in the article), and 20% for discussion and conclusions (versus 30-45% in the article).

Remember that in contrast to the written article, some redundancy is needed; you have to repeat important points, phrasing them in a different way, since microsleep is ubiquitous on occasions like these. It is impossible for the receiver of your message to stop or go back, as he can when reading.

Tell a good story that goes home!

Prudently, you start by saying what you are going to say, and you finish with a conclusion - preferably projected on to the screen, to facilitate understanding, since not all scientists can deliver a speech properly and not all scientists can understand oral English (or dialects thereof) sufficiently well.

Supposedly, the maximum speaking speed should be 120 words per minute, but this is too fast. A rule of thumb is that you may speak 80 words per minute, that a slide will roughly take the time of 50 words, that one new idea can be introduced every

3 minutes, and that a 10-minute speech should be illustrated with a maximum of 8 slides (preferably not more than 3 of them containing important data).

Box 7.7 Can a speech make a good journal article?

A speech and a journal article, even if they deal with the same problem and present the same data, are different means of communication, each with its own qualities and rules. One does not succeed readily in the other's form.
...., the transcript of a well delivered speech, based on a few notes, supported by 11 white-on-blue slides and kept as short as possible to allow for a lively discussion, probably would produce a very unpromising manuscript.....

The talk can be enlivened by humorous digression, personal observations and local references; these devices are distractions in a written presentation. The format of the talk can be flexible and the speaker can allow time for immediate feedback; the journal article is precast in a rigid form and feedback is slow and cumbersome. The talk can often only summarize the author's current work and prompt the listener to want to hear more; a good journal article should offer enough detail and "close" some aspects of the topic so that the reader feels that at least one question has been answered .
The differences between these two media are basically due to a simple physiologic fact: the eye is quicker than the ear. We read twice as fast as we talk. But not only can the reader go forward in time faster, he or she can also go back in time - that is, review - *ad libitum.* As a result, the reader is in a position to be more critical; the itinerant eye, directly scanning the text, demands a continuity of thought and a level of supporting detail that the accepting ear, oriented 90° away from the source of its information, does not.
How, then, can a spoken scientific presentation be converted into a good scientific paper? With great difficulty. Details have to be added, statements supported with references, subheadings inserted, digressions pared, and figures and tables condensed, and the line of thought or "critical argument" has to be clarified. ...|

Morgan (1983)

To use uniformly designed *illustrations* is helpful to the audience; it facilitates rapid orientation and understanding. Each slide should illustrate one - or at most two - points. Thus, written material on slides should be restricted to brief summarising statements - the following old-fashioned form may serve as a reminder also for those who prefer computer-made slides:

Some rules of thumb

Use a template this size for writing the original of a text slide, with normal size characters (i.e. height of upper case characters about 2.5 mm).

A combination of upper and lower case letters is easier to read than upper case letters only. Another rough rule of the thumb is that the slide should not have more than 6 words in a title, 6 rows of words or data, 6 columns or bars, 3 columns of data, or 3 curves. Try to label curves, bars, and columns rather than to use symbol codes; otherwise simplify as much as possible (see Chapter 5). You should be able to read the lettering on the slides by the naked eye at normal reading distance against an illuminated background.

Now, prepare thoroughly and rehearse frequently! Total time of your presentation should be 90% of the allotted time, when you have spoken reasonably slowly - slower than otherwise when you come to important points - and have shown all the slides. This gives you 10% leeway for the unexpected.

Suggestions about the performance

If possible, check beforehand that the audio-visual aids really work; and clean your slides. Try to give an impression of friendly and relaxed authority when you present your speech. Tell your story in plain English. Use short sentences and a conversational style. Be clearly seen! Be conscious about the effects of your body language (hand-waving, wandering about, hands in pockets, shy lack of motility, et cetera). Address people at the back of the room - with enthusiasm. If you cannot demonstrate excitement for your subject, nobody else will be excited! Take a deep breath; good breathing allegedly neutralises many faults, such as the lack of communication that results from dropping the ends of sentences. Try to place your voice forwards, in the front of your mouth. Try to make your story as simple, easy to understand, logical and clear-cut as possible; our guess is that the ratio between too complicated and too elementary oral presentations is greater than 10:1.

Remember, that after letting the audience have a quick orientating glance at your slide - in silence - you can explain it!

If necessary, start with the axes. Tell what the picture shows. Laser torches are excellent means of directing the attention of the audience to the essential detail. Don't move the red arrow erratically around; point purposively. If you tremble, use both hands and support your elbow(s) against the lectern. Don't read a text slide to the audience; the listeners read more rapidly than you can speak to them. Plan beforehand when you want to have the main lights on and off.

The discussion is for the whole audience!

During the discussion of your presentation, remain at the rostrum. Repeat each question for the benefit of those who didn't hear them. Aim your reply at the audience, not at the questioner. It is permissible (though seldom done) to take notes, so that you don't forget the first of a series of questions. Thank questioners who make helpful suggestions and try to be polite to the others.

The poster

The use of posters to communicate research results at congresses and meetings has become very common. The poster is a potentially powerful way of catching the interest of the congress participant (Box 7.8, Fig. 7.2) and represents a rational and efficient way of transferring information, comparable to the newspaper. Supplemented with discussions with the viewers and possibly with handouts, the information value of a poster probably largely exceeds that of a newspaper page. And you may make acquaintances and friends for life this way, if you can stand the vulnerable exposition of yourself and your work that lies implicit in the poster mode of communication. It is even better if posters are grouped according to their topics and are formally discussed.

Unluckily, poster sessions are often poorly organised, so they may seem to be a place of relegation for those who want to tell a story that is not too exciting, and for those who have to give some kind of presentation to obtain a travel grant. But it need not and should not be this way!

For planning a poster section, it is important that instructions to the participants are clear, concerning both the production and the exposition of the poster. Its dimensions and orientation - whether "landscape" or "portrait" format - must be

given, as well as the means of fixation to the board. Moreover, information must be explicit concerning the manning of the posters, and the presence of the communicants at the discussion session, if such be arranged. Poster sessions should not be squeezed in between "more important" oral sessions or into inconvenient rooms with too little space and light. Neither should there be insufficient time for viewing.

Poster sessions can also be ruined by the poster authors. If the poster is unattractive, its author shy or not present, or the story trivial, failure will be the result.

As a general rule, when you start planning your poster, use the guidelines for slides for the illustrations and the principles of newspaper lay-out for the text (see above, and Chapter 5).

The Physiological Society of London has issued guidelines for posters, among which some of the following advice can be found. A good poster should not try to make more than one major point; you have a limited amount of space available - yet, all information (text and symbols) should be readable by viewers 2 m away (others say 1 m), meaning that the quantity of information you can present is restricted. Remember that posters need planning; they should not be thrown together at the last minute. Aim for clear and simple presentation of data; posters should not have more than six illustrations, and literary - as opposed to illustrative - content should be kept to a minimum. The illustrative content should cover around 50% of the available area, thus figures and tables should be large. The message to be presented should not require oral explanation. Avoid abbreviations, acronyms, and jargon. Clearly label the order in which your presentation should be read - the spacing may give cues in this regard, as a substitute or supplement to numbering of (sub-)headings, figures, and tables.

Poster planning and construction: newspaper layout for the text and guidelines for slides for the illustrations

Indicators of craftsmanship

The observer probably first looks at the title, or heading, then perhaps at the introduction and the conclusions. If he works in the same field as you, or you have done a good job with the poster, or both, he may go on studying the rest of your message. Consequently, the title should be "catchy", telegram style, with big letters (a minimal capital letter height of 4 cm), to be legible from at least 5 m. (Sub-)headings too have to be short, but meaningful; in short, simple words written with capital letter height of at least 15 mm. The introduction and conclusions also need to be short and to the point, presented in an attractive and easy to read style (capital letter height at least 10 mm). Line lengths of 15-18 cm (or about 40 characters) may be suitable. Appropriate magnification of a text written with a printer, typewriter, or a tape-lettering machine

can be performed with a reflex copying machine (Xerox type) or photography. Drawings and photographic prints in an A4 (21 x 30 cm) format may not need further enlargement. Trim your illustrations to present only the essentials, and they should be clearly visible at a distance. Whenever possible, captions on graphs and diagrams should run horizontally. Colours may enliven your poster, but use them intentionally, consistently, and with some restraint. For example, with felt-tipped markers you can apply the same colour to a certain experimental group, when it is represented repeatedly, by bars, columns or curves in your illustrations. Furthermore, conclusions and (sub-)headings may be colour-underlined to attract the attention of the viewer.

We believe in one exception to the rule that everything should be readable at a distance. You may feel that you have to present some methodological points or specifications that are of no principal importance. For this, you could use a "box of footnotes", written in letters small enough to require genuine interest and closeness to the poster to be read. Again, varying the size of the types is the way newspapers do it.

The line lengths recommended above mean that you present sequence in columns, so the viewers don't need to weave back and forth in front of the poster (a real traffic mess). Put the "footnote box" in one corner, such that those interested and up close don't obstruct the view of others.

We favour a light coloured, unbroken poster background and therefore prefer to construct posters from modular units (the size of which will fit our suitcases) that can be mounted close to each other in rows and columns to make up the whole prescribed poster area. Posters should never be rolled - photographs and applied letters may get damaged, and rolls are inconvenient travelling companions.

Box 7.8 The content of posters

General principles

When studying a poster the reader can linger over it as long as he or she wishes, and in this sense a poster presentation is more like a printed page than a slide. In most cases, however, the reader will be confronted with a large number of posters, and he will be expected to study them while standing up. Information must therefore be presented clearly and concisely so that the essentials of the message are easily grasped. Studies in museums have shown that visitors tire of reading long caption panels very quickly, and while they may begin by systematically reading each one, they soon start to wander at random. After this point they are likely to spend more time on the most visually 'attractive' displays. Posters should stimulate interest rather than present complex details.
In effect, a poster is an advertisement for the author's particular ideas or techniques. He stands in front of his display with something to sell. Good posters use the best techniques of salesmanship. The content should be succinct and to the point, with short pithy subheadings, and the design should be attractive in terms of color, lettering and layout.

The importance of simplicity when presenting introduction and conclusion data on posters is exemplified below.

Introduction

CONGENITAL PARATHYROIDISM is common but not easily recognized

NEW TECHNIQUES of investigation are demonstrated which change the patterns of diagnosis.

Conclusions

- Serial blood tests following injection with 'Isopon A' prove a useful and safe indicator.
- Ultrasound and isotope scanning provide 99% accuracy in diagnosis.
- Therapy with 'Dilactyl' AT THE RIGHT TIME, is shown to be completely effective in all fifty cases investigated.

Adapted from Reynolds and Simmonds (1982)

FUNCTIONAL CAPACITY OF NEUTROPHIL GRANULOCYTES IN DEEP SEA DIVERS

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Løvhaug D (Norwegian Defence Research Establishment)

PROBLEM

Saturation divers work for weeks under high ambient pressures, being exposed to hyperoxic conditions only slightly below the pulmonary danger limit of 50 kPa partial pressure of oxygen. Skin infections and particularly external otitis represent a significant health problem in deep saturation diving. Do divers' granulocytes (PMN) adapt to the hyperoxic conditions in a way that render them less capable of dealing with localized infections?

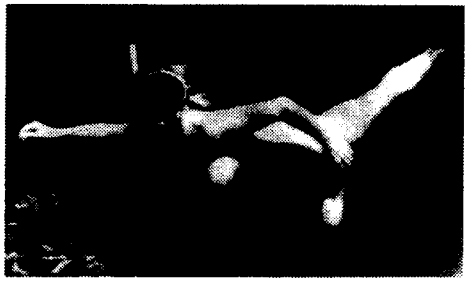

Fig. 1: North Sea diver

RESULTS North Sea divers:

At least until a week after a 11-16-d diving session in the North Sea (50-70 metres of sea water (msw); mean PO_2 slightly below 50 kPa), the divers' PMN could be "primed" to give enhanced respiratory bursts upon stimulation - as recorded in three different assays (Figs.2-4):

(1)

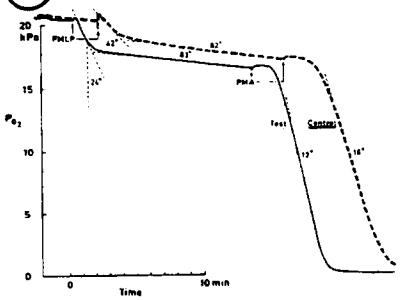

Fig.2: The diver's PMN showed a more rapid drop in oxygen pressure than control PMN, reflecting a higher oxygen consumption rate. The PMN were exposed to two successive stimuli, known to initiate a respiratory burst (increased O_2 consumption), i.e. formyl-methionyl-leucyl-phenylalanine (FMLP) and phorbol myristate acetate (PMA) (at arrows).▲

(2)

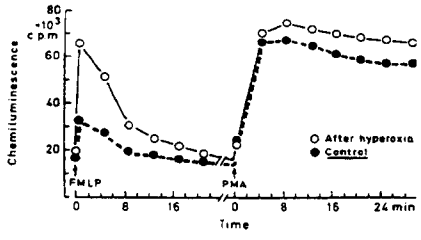

Fig.3: Stronger chemiluminescence responses of diver (5 d after ascent) than of control PMN to FMLP and PMA stimulation.▲

Methods of Respiratory Burst measurement

(Please, take one sample)

NOTES:

▲One more North Sea diver gave similar responses. Both were examined within 7 d of ascent, and in a pilot experiment the responses were largely normalized after 34 d.

■Decompression sickness in mice: Rapid compression to 60 msw in pressure tank; "bottom phase" 45 min; decompression approx. 8 msw/min, slightly less rapid the last 7 msw. Signs of distress: Immediately after ascent many mice were apparently hyperactive and scratching themselves, about a tenth lay flat for a few minutes, seemingly moribund, but none died.

(3)

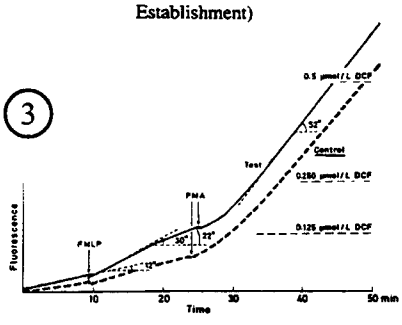

Fig.4: Oxidation of more intracellular non-fluorescent dichloro-fluorescin (DCFH) to fluorescent dichloro-fluorescein (DCF) by diver (test) than by control PMN after FMLP and PMA stimulation. Hydrogen peroxide (H_2O_2) has oxidized the DCFH substrate, catalyzed by PMN peroxidase.▲

Onshore test divers:

During decompression after 25-28-d chamber dives to 360 msw, air micro-bubbles were detected in the divers' blood, and sometimes haemoconcentration and PMN "priming" could be found as well:

(4)

Table 1: Haematological data before the dive and during decompression

Blood Donor (No.)	PCV (% v/v)	Reticulo-cytes (%)	WBC (10^9 / l)	PMN (%)
Before (7)	44 *	0.9 **	6.9	58
During (4)	49	0.3	6.7	50

*: P< 0.05, **: P< 0.01

(5)

Table 2: PMN chemiluminescence before the dive and during decompression

Blood Donor (No.)	FMLP response (% con-trol)	PMA resp. (10^3 c.p.m.)
Before (7)	97 **	33.8 **
During (4)	321	41.1

(6)

Table 3: Hydrogen peroxide availability for intracellular oxidation of dichloro-fluorescin (DCFH) in PMN, before the dive and during decompression

Blood Donor (No.)	Baseline oxidation (%)	FMLP response (% contr.)	PMA response (degrees)
Before (7)	105 *	98 **	56 *
During (4)	207	215	60

Plasma sampled from two North Sea divers 2-6 d (post-dive) and 24-28 d (control) after ascent (from 10-16 d at 68-92 msw) was incubated with PMN from a healthy blood donor, AB0-type 0. DCF fluorescence, in response to stimulation (see Fig.4), was in pilot investigations enhanced by post-dive plasma:

(7)

Table 4: DCF formation by normal PMN incubated in post-dive plasma (% of control)

Stimulus	Incubation time 1 h	3 h
None	129-100	76-103
FMLP	231-147	141-124
PMA	141-116	222-101

Fig. 5. A *real* **saturation** diver

Preliminary results with decompressed mice:

(8)

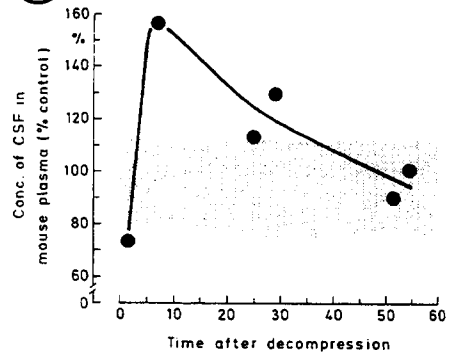

Fig.6: Increased concentration of colony-stimulating factor(s) (CSF) in plasma of rapidly decompressed mice.

CONCLUSIONS

Formation of reactive oxygen intermediates (superoxide, hydrogen peroxide, etc.) by stimulated PMN from the divers was often larger than normal and never detectably reduced. Paradoxically, this "priming" could lead to diminished resistance against infection, if these PMN have lost some ability to localize to sites of infection. A mouse model of decompression sickness may be useful to further illuminate these problems.

B

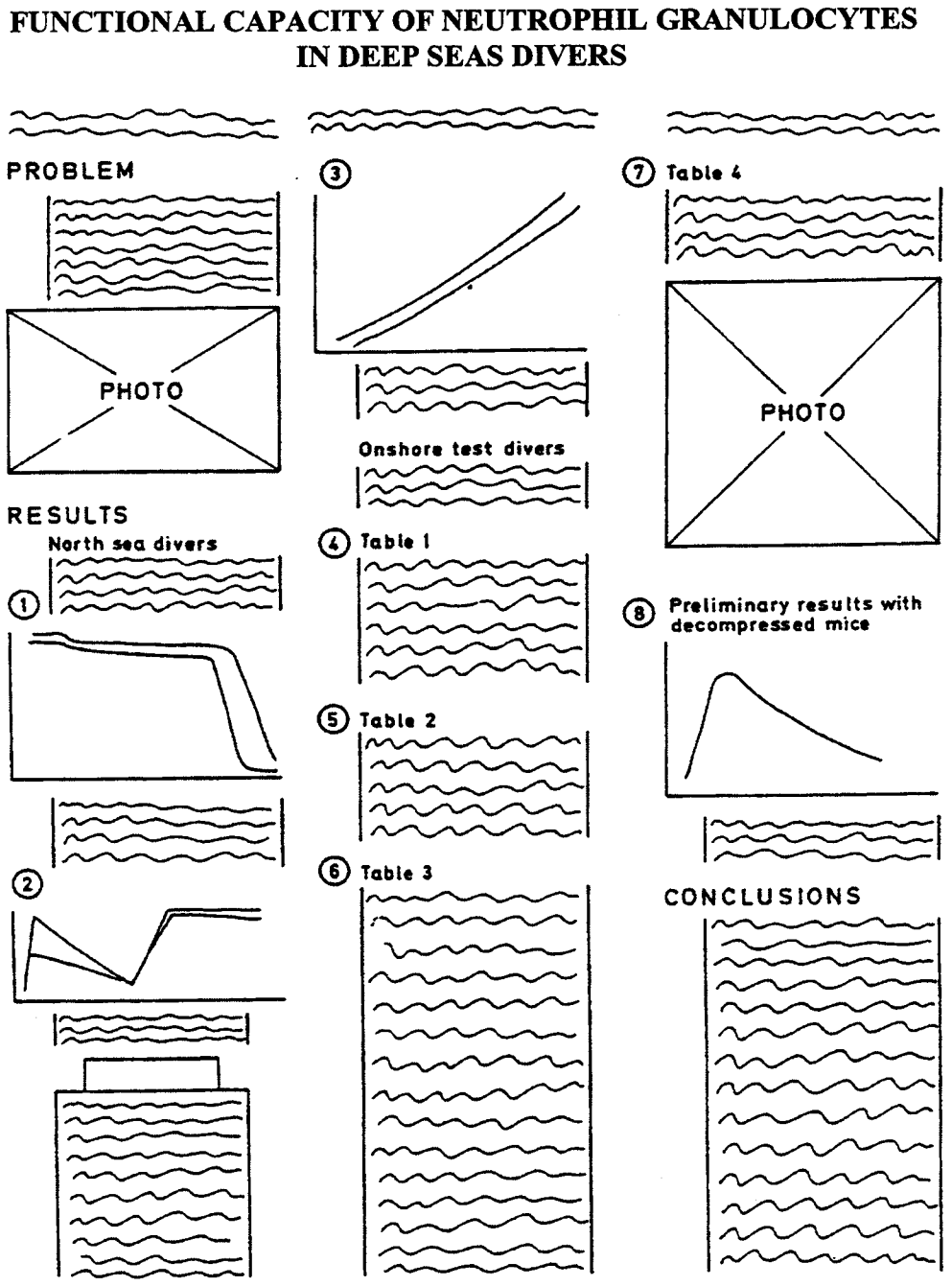

Fig. 7.2. *A poster discussion.* A: as constructed by one of the authors, B.: as tentatively improved on by a professional. She appreciated the balance between pictures, colours and text, but found the whole structure of the poster a bit too 'untidy'. She wanted more orderliness and uniformity (less variation of letter size) and more clear-cut demarcation of margins (with their contents) and columns. The division of the title on two lines is all right, but the authors' names and institutions should span the three columns. Smaller margin on top than at bottom. A newspaper page is constructed correctly!

Checklist 7.1: Communication in general

1) To whom are you communicating?

2) What do they want/need to know?

3) Have you adapted your article, speech, or poster to the answers given to questions 1 and 2 (See Fig. 7.1 and text)?

4) Have you adapted the presentation of your message(s) to your channel of communication (written, oral, pictorial, or combinations thereof)?

5) Are you left with the bare essentials, structured in a simple, logical sequence?

6) Have you pointed out the main problem(s)/intention(s) at the outset, the conclusion(s) at the end, and in between those results that are new and important?

Checklist 7.2: The scientific article

1) Is the *Title* short and rich in information (index) words? Does it indicate the kind of work performed, experimental animal(s) and main method(s)?

2) Does the *Summary/Abstract* answer why, how, and what you did? What did you find, and what does it mean?

3) *Introduction:* What was your problem - and why was it important to solve it?

4) *Materials and Methods:* How did you do it? Will a competent scientist be able to replicate your experiments, given this description?

5) *Results:* What did you find? Are your claims consistent with the data? Can this or other sections be condensed (See Box 7.3)?

6) *Discussion:* Have you briefly summarised what you found and then told what it meant, integrating it into current knowledge? (What next?)

7) *Data presentation* - Tables and Figures: See Checklist 5.1.

8) *References:* Are they comprehensive, but not excessive? Have you allocated priorities appropriately?

9) Have you: *acknowledged* financial, technical, and other kinds of help? *Revised* the final manuscript - particularly all data - carefully? Obtained *permission* to submit it for publication from your co-authors?

Checklist 7.3: The oral presentation

1) Have you got *a good story* - for this audience?

2) Can you indicate the *general aspects and importance* of your theme/work?

3) Have you scheduled an appropriate part of your allotted time (≈40%?) to presentation of *background and main problem(s)?*

4) Will you stick to the recipe: *"Say what you 're going to say - Say it - Say what you've said - Sit down"?*

5) Can you say it in a *simple and logical* way, with short sentences, in an oral and not written form?

6) Have you *avoided overloading* your speech?

7) Is the *illustrative material easily understandable?*

8) Can you deliver your speech *with enthusiasm,* so that those at the back of the room can see, hear, and *understand you easily?*

9) Have you arranged *rehearsal(s)* - early enough to be able to improve visual aids and often enough to achieve excellence?

Exercises

1. Write a part of or a whole article and have it thoroughly criticised by peer(s) and expert(s). (For class courses, the authors of this book may provide you with background material and raw data that can be used as training material in the class; see chapter 10. This way, the participants can compare their contributions and learn from different ways of performing the same tasks).

2. For group exercises in science class courses: Look up the article presented in Nature (London), vol. 226, May 30 1970, page 869. Discuss and criticise the article in a systematic way; according to study design (Chapter 2), data presentation (Chapter 5), statistics (Chapter 6), and form (this chapter).

3. Prepare a 10-minute oral presentation, with due regard to the nature of the target group. Obtain criticism on contents, form, and visual aids from your colleagues/the rest of the class.

4. Construct a poster, based on your own or colleagues' experimental results, or on the group work of exercise 1, for presentation at a (specialised?) scientific congress. Seek feedback from your peers/the rest of the class.

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Chapter 8 : The indispensable PC

Words in italics are explained in the dictionary at the end of the chapter.

This chapter is not written for the experienced computer-fan, but rather for those whose main concern is scientific research, and who accept the P(ersonal) C(omputer) as a valuable tool in this connection, but do not want to spend much time learning to use it. In most laboratories computers are already in industrious use. The problem for the beginner is thus not to decide which hardware or software to acquire, but rather how to get started with the equipment that already is there.

Computers should save time

There are two main reasons to use a computer: 1. It saves time. 2. It treats data accurately and reliably. However, you should be suspicious as to whether you really save time and even as a dedicated PC user always k.eep in mind the following provocative statement:

If the accumulated time-saving effect of using a PC for a certain procedure is A , and the time used to introduce and learn to use the PC (software) for this procedure is B, then always A#B. If unexpectedly A should seem to exceed B, a new and much better program (or a new version of the old one) will certainly just then be introduced. It will be a "must" to learn it, thus causing a rapid increase of B, to once more exceed A.

Two recommendations for the beginner are:

1. Don't start educating yourself by learning to execute PC procedures or programs if you are not sure whether you will actually use your new skills fairly soon.
2. Don't employ equipment or programs without readily available expertise to help you when anything goes wrong (-it always does!).

Rely on available know-how

If you disregard one or both of these rules, you will soon experience that your attention will be diverted from biological to technical problems.

About the equipment (hardware)

Traditionally, information is processed and stored in individual brains or as written text, and communicated by means of written or spoken language, symbols or pictures. The introduction of electronic computers has made us able to store and treat much more information much faster than any human brain. But the computer is no brain. It is rigid and totally dependent on accurate and appropriate input. The information is transformed to *binary digits* - "digitalized" - and treated as such. This is also quite different from how the brain is supposed to work. However, since the introduction of the first computers in the middle of this century, much effort has been spent to produce programs and computer "languages" that conform to the way we think.

Binary digits

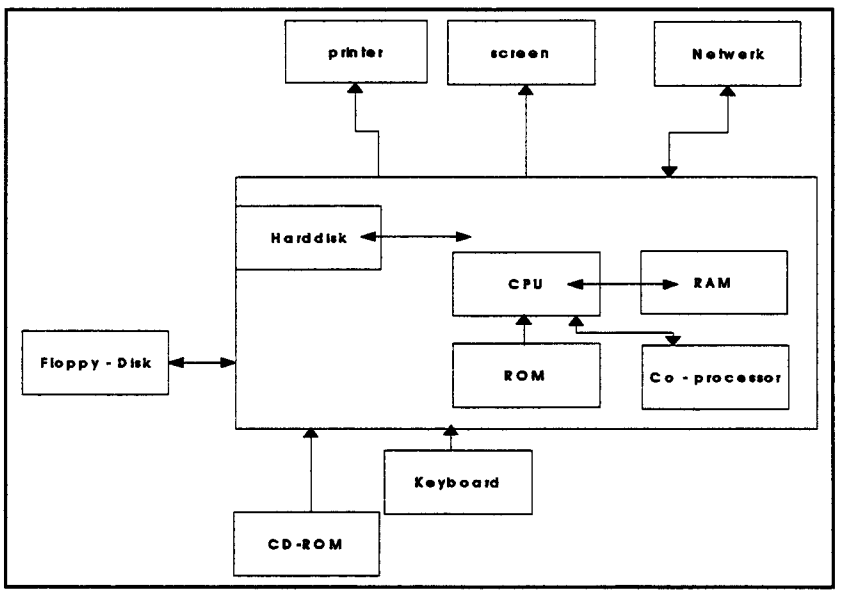

Fig. 8.1 Hardware connections in a PC. Accessory equipment *in italics.*

You settle down at your desk and introduce a 5 V powered electric current into your computer with the ON button. Thereby, you are "*booting*"(see Fig.8.1 and Dictionary at the end of the chapter) the device. Information is then collected either from an external *floppy-disk* - or nowadays most frequently from en internal *hard-disk* - that tells the computer how to work. Such instructions are called the *operating system* and the part of the computer they are collected into and used in is, called the random access memory (*RAM*). When the booting is finished, you can give instructions to the PC from the keyboard or the *mouse*. Usually you want to operate a program that is stored on a disk, e.g. a word processing program. When you give the starting instructions from the keyboard (or the mouse), information is then collected from the disk and stored in the RAM. While you are working, additional information (e.g. about spelling of a word) may be concurrently picked up from the disk. You can at any time observe and control your instructions and the processing of them on the screen. The product (text) may be saved for the future on the disk and the information can be transformed back to a traditional, non-digitalized form by means of a *printer*. In this exercise you have employed the basic components of a PC. In addition, data can be sent between your personal computer and remote computers or data-bases by connection to a *network* or a *modem*.

Recordings from laboratory instruments may also be transferred to the PC. Since such recordings as a rule present information in an analogous form (as temporal alterations of voltage), the signals must first pass through an *analogue-digital converter* (*ADC*), so that the signals are transformed to the binary digits that the PC can treat. Laboratory experiments can also profitably be supervised from the computer. Configuration and calibration parameters may then be set on the basis of simultaneously incoming information (on-line registration).

About the programs (software)

1. Writing ordinary text

In chapter 7 we stated: *"There is only one way of learning to write a good scientific article, and that is to practise: write and rewrite over and over again"*. Writing a scientific article therefore demands drafting, rewriting, correcting, and so on - seemingly almost infinitely and perhaps to your despair - before the product is suited for publication. This task has been substantially simplified after the introduction of computer word processing. The typewriter is definitely out, and there is no way back to the handwritten manuscript. So, if you have got a PC and don't know where to start, start by writing!

The typewriter is out, definitively

Modern word processing programs offer a lot of options concerning fonts, formats etc. You will soon pick up the useful ones. We have personally experienced some especially valuable possibilities:

Macros can profitably be used for standard formats or phrases, e.g. in letter writing. **Search/replace** may be used when a long, "exhausting" term or phrase occurs repeatedly in the text. Then you can substitute e.g. qx for the whole phrase in the first draft, and later replace all qxs with the appropriate wording using the search/replace function.

If English is not your native language, you can benefit greatly from a **dictionary** checking your orthography or a **thesaurus** suggesting the pertinent synonym you may be desperately searching for in your mind.

Desk-top publishing to make posters.

Desk-top publisher, which is an advanced text producer, may be convenient in poster production and for educational purposes, but you should leave its use to people with special talents and enthusiasm until you can cope with plain word processing fairly well. You can manage for a long time with scissors, glue, and a xerox-machine, - especially if you have also access to a laser printer!

Most journals will welcome manuscripts on diskette if one of the major word processing programs has been used.

2. Worksheet

Although designed primarily for business applications such as budget or account work, worksheets (spreadsheets) have proven to be extremely useful for laboratory registrations as well. If your experiments follow a standard protocol, you can design a ready-made worksheet format, in which you can fill in your observations and measurements during or after each experiment. All calculations, and even simple statistical procedures, can be pre-programmed once and for all. You can then store each experiment as a file on your disk. Afterwards you can extract data from all these files for subsequent treatment. Printouts of your files will serve as a written documentation. Figure 8.2 shows an example.

Worksheet as laboratory journal

It is essential to learn to edit and to use formulas for calculations applied to data introduced into the worksheet. Just a little more skill is sufficient to manage statistical treatment of your data as well in the same worksheet.

If your computer is connected to a laboratory instrument, e.g. a balance or a radioactivity counter, the recorded data may be transferred directly into a worksheet.

Additionally, most worksheet software includes some graphics, or may easily be combined with a graphics program (package). After a short training period you should be able to visualise your data as e.g. bars or scatterplots. In our opinion it is worth while to be familiar with some graphics options/programs, but so far we have found few that are well suited to our requirements, since most of them do not offer the possibilities to construct graphs adhering to the standards we have recommended in Chapter 5 (e.g. confidence intervals assymetrically placed relative to the medians, or broken axes and curves).

Intracellular Calcium				
Date:	**19/11-96**	Exp.no: **CG14**		
Cell type:		**GH4C1 cell line**		
Purpose:	**Time course of TRH response**			
Pre-treatment:		**None**		
Viability:		**96.5%**		
Cell conc.:		**2 mill. per ml.**		
Measurements:		F	Ca^{2+} conc (nM)	
F min		**10.00**		
F max		**100.00**		
Base line 1		**22.00**	*17.69*	
Base line 2		**29.00**	*30.77*	
Added (ligand):		**50 nM TRH**		
	1 min	**52.00**	*100.63*	
	2 min	**44.00**	*69.82*	
	3 min	**40.00**	*57.50*	
	5 min	**34.50**	*43.02*	
	n.d.			
	n.d.			
Comments:	**The same time course as exp. CG12**			
Conclusion:		**Max. response 1 min**		
		Half max. 2-3 min		

Fig. 8.2. Example of a worksheet used as laboratory journal.
The standard sheet is collected from the file, and you fill in current information like date etc.) and the measured values (in this case fluorescence intensity, F); this information is here shown in bold print. The calculated values (nM intracellular Ca^{2+}) can then be obtained by a command. In this case they are calculated according to the formula

$$\frac{F - F\,\mathrm{min}}{F\,\mathrm{max} - F} \bullet 115$$

and presented in italics.

Finally, don't forget to fill in possible comments, and always one or more conclusions ("What did you learn?"), shown underlined in the example above.

3.Statistics

Powerful standard packages as BMDP, SAS and SPSS are now available for PCs. For epidemiologists or other scientists that use many different and sophisticated statistical methods, one of these "is a must". Ordinary laboratory scientists may find them laborious and complicated to use. When you select a statistics package, you should not seek greatness of variety of methods offered, but rather simplicity in use, especially concerning input of data. Preferably, you should be able to bring data from your worksheet directly into a program for statistical treatment, without having to execute a number of complicated procedures. As we have stated before, an estimate of the median value of replicate data, with its 95% confidence interval is often sufficient (Chapters 5 and 6). If you in addition can perform the few statistical tests mentioned in Chapter 6, your preliminary statistical repertoire should be satisfactory. In any case, with simple and - if necessary - more sophisticated methods, you should always *learn to understand the underlying principles and premises* of the statistical methods before you learn to execute the program.

Learn the statistical method before performing the test on the PC.

4. Bibliography

You will not have been engaged for a long time in the research business before a mass of copied articles begin to accumulate on your desk. You will soon be forced to group them according to subject or author - or preferably both - but sooner or later you will not be able to cope with the ever growing pile, and you have to begin filing. Our advice is: the sooner the better. Several computerised filing systems are now available to research scientists.

Before you make your choice, be aware of the following minimal requirements:

Input

References may be registered in three ways:

1. Manually from the keyboard.
2. Semi-automatically from a permanent database (e.g. Medline™) either with on-line modem or network connection or from a *CD-ROM* (see below).
3. Semi-automatically from a rapidly and continuously updated, commercially available system (e.g. Current contents™, Reference Update™), which weekly surveys the bibliography of a great number and variety of journals.

Your own reference program should be capable of easily accepting transferred references from such external databases, and you should have the opportunity to add your own notes and key words.

Output

Writing a manuscript, you should also easily be able to transfer a sample of references from your own database to your word processor and to format the reference list according to (almost) any journal style. Moreover, it should also be possible to integrate the references into a manuscript according to the chosen journal style. This option speeds up and facilitates the manuscript production considerably by eliminating previously almost unavoidable printing errors in the reference lists.

Easy to make a reference list

5. Remote connections

You may communicate with the rest of the world through Internet

At many research institutes PCs are connected to a local *network* outfitted with a common *server* computer. Through this network it is possible to communicate with institutions all over the world via the *Internet*. In absence of a network, a *modem* connection to a telephone line may be a satisfactory alternative. Thereby, you can get access to major programs and databases. We have already mentioned Medline, which embraces a major part of the biomedical literature references and spans several decades. With initial help from a librarian, you should be able to pick up literature relevant to your work very easily on your own by on-line searching.

Another option that certainly is useful is electronic post. By this means you can send a letter or transfer a manuscript or other filed information to friends and colleagues all over the world, provided he/she has a PC connected to the network. This possibility of rapid communication may be particularly useful when you co-author a manuscript.

Not only newspapers but also many scientific journals are now available on Internet. All this appears fascinating and you may in this way get new information rapidly, but most scientist will probably still prefer to read books and journals printed on paper.

6. Digital imaging

In many biomedical laboratories where microscopes are routinely used, digital imaging has become a fascinating way to analyse microscopic pictures. The picture is surveyed

by a video camera. The "analogue" video signal is then digitalized by means of a "*framegrabber*". The image is in a way sectioned into small parts, "pixels", each of which is given a numerical value according to darkness and colour. If you use 8 *bits* (i.e. a 256 steps scale) to characterise each pixel, and a picture is divided into 500 x 500 pixels, you need as much as 250 *Kbytes* to store just one picture! This may be compared with ordinary text storage, where 250 000 characters or about 80 pages require a similar magnitude of memory. Thus, the computer needs time and abundant RAM to treat pictures. However, the procedure may be highly rewarding, because objective, quantitative information - which might not easily be gained otherwise - may be obtained.

Digital imaging: objective quantification

7. Graphic presentation

Imagine that you want to use an illustration from a book, modulate or simplify it, add your own text, and make a colour slide of the final product. A few years ago you would need help from at least an illustrator and a photographer to do this. Now you can do it yourself on your PC provided your institute is equipped with the necessary tools.

A scanner transfer a picture (or text) into the computer

First, you need a "scanner" that can copy and digitalize the illustration you want to use, if preferable in colour. (You can also scan a text which initially will be treated as a graph by the computer . By special character recognition programs this text can be converted to *ASCII characters* and used in word processing.)

Then you need a drawing program, which can enable you to make the changes you want, adding text collected from your word processing program and, perhaps, setting a background colour. Finally, you are satisfied with the product as it stands on your screen.

The next step is to "print out" the illustration in a suitable format and design. You may then use a colour printer to obtain a printed picture, optionally as an overhead transparency, or you may use a "slide-maker" to expose a film directly from the PC.

The film may afterwards be processed to a slide in the ordinary way.

This sequence of events illustrates a general trend. We may foresee that even more of the scientific work will take place in front of the computer. Your measurements and observations will go directly into a PC, and will leave it processed as a slide, part of a poster, or of course, as a manuscript on paper or on a disk.

Short computer dictionary

ADC	Analogue-digital converter. Device that transform electrical analogue signals (as temporal alterations of voltage) to a digital form.
ASCII	American Standard Code for Information Interchange. A standard for numerical representation of letters, marks, figures, and numbers (e.g. 65 for the letter A, 48 for the number 0 and 10 for "carriage return"). This standard is used in the communication between computer and printer, and in the internal representation of text in the computer.
Bit	The basic information unit in all computers. It can only have two mutually exclusive values, e.g. true/false, yes/no, 0/1 (binary digits).
Boot	To start a "cold" computer by loading an operating system into the RAM. The term was derived from the sense that the computer intends to get itself started by pulling its bootstraps.
Byte	A group (assembly) of 8 bits, (e.g.: 00111010). One byte may have 256 (2^8) different values and may be used as store for small integers or for letters when they are given a numerical representation (see ASCII).
CD-ROM	Compact Disk- Read Only Memory. Introduced for storing music, but now also available for all sorts of information (dictionaries, etc.). Large capacity, but special equipment and software are required. Application programs are now available on such CDs.
Co-processor	Electronic device designed for fast calculations. May be installed in most PCs, but software that is not conformed to it, is of no use. Speeds up arithmetic operations like multiplication, division, and transcendental functions. Co-processors may be designated e.g. 8087, 80287, 80387.
CPU	Central Processing Unit: the central microprocessor - "brain" - of the computer. Often designated 8086, 80286, or (for Macintosh) 68000, 68020. Software made for one CPU-type may as a rule be used on CPUs with higher - but not necessarily lower - version-number in

the same series.

Floppy-disk — Device for storing data; it is a magnetizable disk (diskette). Its capacity varies between 360 Kbytes and 1.44 Mbytes. At least one floppy-disk driver (diskette station) is required for installation of new soft-ware in the computer (unless a CD-ROM disk drive is connected).

Framegrabber — Device (a specially programmed card) that transforms analogue video signals to digital information. The image is sectioned into small components (e.g. 512 x 512), "pixels", where each pixel is given a numerical value according to darkness or occasionally colour.

Hard-disk — Device for storing data; it is a fixed, magnetizable disk (capacity 20 Mbytes or more). Installed in or attached to the computer.

Interface — Hardware and software components of a connection between two elements of a computer or its peripheral devices. Applications have a "user interface" that notify how the program appears to the user.

Internet — A world-wide network of networks for exchange of information and communication. Electronic mail and world-wide Web are examples of Internet applications.

Kbyte — Measure of data: 1024 (2^{10}) bytes.

Laserprinter — Printer for text and graphics based on xerox technology. High quality print. Resolution: 300 x 300 dots per inch (DPI).

Mbyte — Measure of data: 1048576 (2^{20}) bytes.

MHz — Measure of frequency (10^6 s^{-1}), based on the time the CPU requires to execute one cycle, e.g. retrieve contents of one address from the memory.

Modem — Device for connection between the computer and a telephone. Makes it possible to use external data-bases or to communicate with other computers.

Mouse	Device for moving an indicator on the screen. Is useful e.g. in drawing-programs, some word processing programs and menu operated programs.
MS-DOS	Operating system for IBM-compatible PCs.
Multi-tasking	When some operating systems (e.g. UNIX or OS/2) have the possibility to operate several programs simultaneously.
Network	Two or more computers connected with cable to facilitate common use of printers, hard disks, etc.
Operating system	Basic software in every computer. Takes care of basic operations like storing or finding data on disk, transferring characters from keyboard to screen or printer. In addition, the operating system contains specified commands that are used to start other programs. MS-DOS is an operating system.
Parallel port	Connection to a printer. It operates one way only, i.e. data are transferred only from the computer to the printer .
Pinwriter	Less expensive printer (also called matrix printer).
RAM	Random access memory. The fast working memory of the computer. The program in operation is stored in this memory, as well as transitional data. The content of RAM is lost (made unrecoverable) when the computer is turned off.
ROM	Read-only memory. Memory that is kept unaltered by the programs being executed and also when the computer is turned off. Booting (starting up) procedures are stored in ROM.
Serial port	Connection to printer, modem, net etc. Permits two-way traffic, i.e. data may be transferred both to and from the computer.
Server	Computer connected to network for common use.
Streamer	Device storing data on magnetic tape. Often used for backup of data (and programs) on hard disk.

UNIX	Operating system (developed by Bell Labs) available, but not much in use on PC. Often utilised by servers and work stations. Popular because of its multi-tasking capability.
Windows	A "Macintosh-like" operating system for PC based originally on DOS, but the later version (Windows 95) is a self-sufficient operating system.
Work station	Computer with great calculatory capacity and high quality graphic screen, but with low storage capacity, this being compensated for by connection to a (net-)server.
XY-plotter	Device for drawings (graphs) using a pen and often different colours. Is operated from the computer like a printer. Requires special software.

The indispensable PC. Checklist 8

1. Is your PC sufficiently outfitted (RAM, hard-disk capacity) for the programs you want to use ? Do you need remote connection?
2. Which standard programs (word processing, worksheet, statistics, bibliography etc.) are used by your peers? What do your colleagues recommend, and which practical assistance can you get?
3. Have you explored the **timesaving** options in your programs?
4. Reprints and copies of scientific articles: How to keep order? The most practical and rapid way to find out what is written about an interesting topic?
5. Graphic techniques, poster production, overhead transparencies, and slides: Where is the know-how?

Chapter 9 : How to behave

As stated in chapter 1, you may with scientific methods disclose what is true - or rather (by falsification) what is not true; but you cannot with scientific methods decide what is morally good or evil. In that sense science is "ethically neutral", which does not at all imply that ethical problems are irrelevant to science and scientists.

The philosophical question of how morality is justified may be answered in different ways by different people depending on religious and cultural background. Whether you are a Christian, a Muslim, or an agnostic, you will probably share a system of morals with the rest of the scientific community. Still, in everyday life you will surely encounter a number of smaller or greater conflicts in which ethical reasoning is essential for reaching a solution or a decision on the issue. Ethical reasoning implies that you must relate the arguments used in a controversy to basic, consensual, moral principles, and to clarify how you should best balance conflicting ethical arguments.

In all social contexts, laws, rules, and common judgement steer the behaviour of the participants. In this chapter we will consider some frequently occurring conflicts of interest in the scientific community and some of the formal and informal conventions that you are supposed to follow.

Is science good?

More precisely: Is the science you are involved in beneficial to man or society?
There are good reasons to believe that (even basic) biomedical research is essential to the progress of medical therapy and care. Science in general is a driving force in technological and social development. Knowledge is the foundation of rational behaviour. On the other hand, fundamental criticism has been raised against modern medicine (Illich, 1975), and some side effects of scientific and technological endeavours, like pollution and the seemingly unlimited growth of industrialised societies, are certainly of great concern.

The effects of science may be good or adverse

Thus, the generally accepted favourable effects of science should be weighed against the allegedly evil consequences. Research enterprises directed towards defence (or rather war) objectives (as e.g. nuclear, bacteriological or chemical warfare) are particularly problematic in this respect.

It is of course difficult to foresee the ultimate consequences of your own research, and often scientific knowledge may lead to unexpected virtue as well as evil. In

biotechnology, for example, the ethical aspects of genetical engineering have been the subject of heated debates because the consequences appear so potentially alarming, yet unpredictable. Many scientists tend to leave such ethical problems to professional decision makers (i.e. politicians).

Knowledge oblige

Nevertheless, if you understand that the use of a particular scientific insight might cause harm, you should feel obligated to inform the decision makers (and the public) about the risk or hazard involved. Just as if you knew that a bomb might explode at a certain time and place, you would immediately inform the police and, if possible, the people at that place.

Science versus life

The subject of biomedical research is living material, which may imply experiments on living animals (including humans). The ethical challenge is to balance the value of your research activities against the possible harm you may cause to the living creature involved.

Experiments on animals

Animal experimentation is so essential to biomedical research that you have to accept its value even if you would yourself prefer to abstain from it. (See also chapter 3.)

We human beings have exploited other animals for our own purposes as long as we can recognise, and very few have questioned the morals of this practice.

Box 9.1 GUIDING PRINCIPLES IN THE CARE AND USE OF ANIMALS
Approved by the Council of The American Physiological Society

Animal experiments are to be undertaken only with the purpose of advancing knowledge. Consideration should be given to the appropriateness of experimental procedures, species of animals used, and number of animals required.
Only animals that are lawfully acquired shall be used in the laboratory, and their retention and use shall be in every case in compliance with federal, state and local laws and regulations, and in accordance with the NIH Guide.
Animals in the laboratory must receive every consideration for their comfort; they must be properly housed, fed, and their surroundings kept in a sanitary condition.
Appropriate anesthetics must be used to eliminate sensibility to pain during all surgical procedures. Where recovery from anesthesia is necessary during the study, acceptable technique to minimise pain must be followed. Muscle relaxants or paralytics are not anesthetics and they should not be used alone for surgical restraint. They may be used for surgery in conjunction with drugs known to produce adequate analgesia. Where use of anesthetics would negate the results of the experiment such procedures should be carried out in strict accordance with the NIH Guide. If the study requires the death of the animal, the animal must be killed in a humane manner at the conclusion of the observation.
The postoperative care of animals shall be such as to minimise discomfort and pain, and in any case shall be equivalent to accepted practices in schools of veterinary medicine.
When animals are used by students for their education or the advancement of science, such work shall be under the direct supervision of an experienced teacher or investigator. The rules for the care of such animals must be the same as for animals used for research.

If you agree with breeding and slaughter of livestock animals for meat production, you also ought to accept breeding and painless killing of animals for research purposes. Obviously, it is not always that easy. The most annoying problem is with experiments that may, despite sound precautions, cause pain or severe discomfort to living, unanaesthetized animals. In such cases you must justify, to yourself and others, that the scientific outcome is valid and important and cannot be obtained in other ways.

Most countries have now introduced rigid regulations on animal experimentation, which you certainly should follow conscientiously (see Box 9.1).

In life sciences research on animals is unavoidable

Animal right activists often claim that research on animals is unnecessary and could be replaced by e.g. cell cultures or computer simulations. This is unfortunately far from true. New drugs or therapeutic procedures must always be tested on animals before they are used on man. For example, if the teratogenic drug, thalidomide, had been properly tested on pregnant animals before being introduced as a neuroleptic agent, many seriously crippled children would have been born healthy. In

physiological sciences investigation of regulatory functions (e.g. effects of hormones or the nervous system) is inconceivable without experiments on intact animals.

Box 9.2 RECOMMENDATIONS FROM THE DECLARATION OF HELSINKI

I. Basic Principles

1. Clinical research must conform to the moral and scientific principles that justify medical research and should be based on laboratory and animal experiments or other scientifically established facts.
2. Clinical research should be conducted only by scientifically qualified persons and under the supervision of a qualified medical man.
3. Clinical research cannot legitimately be carried out unless the importance of the objective is in proportion to the inherent risk to the subject.
4. Every clinical research project should be preceded by careful assessment of inherent risks in comparison to foreseeable benefits to the subject or to others.
5. Special caution should be exercised by the doctor in performing clinical research in which the personality of the subject is liable to be altered by drugs or experimental procedure.

II. Clinical Research Combined with Professional Care

1. In the treatment of the sick person, the doctor must be free to use a new therapeutic measure, if in his judgement it offers hope of saving life, re-establishing health, or alleviating suffering.
If at all possible, consistent with patient psychology, the doctor should obtain the patient's freely given consent after the patient has been given a full explanation. In case of legal incapacity, consent should also be procured from the legal guardian; in case of physical incapacity the permission of the legal guardian replaces that of the patient.
2. The doctor can combine clinical research with professional care, the objective being the acquisition of new medical knowledge, only to the extent that clinical research is justified by its therapeutic value for the patient.

III. Non-Therapeutic Clinical Research

1. In the purely scientific application of clinical research carried out on a human being, it is the duty of the doctor to remain the protector of the life and health of that person on whom clinical research is being carried out.
2. The nature, the purpose and the risk of clinical research must be explained to the subject by the doctor.
3a. Clinical research on a human being cannot be undertaken without his free consent after he has been informed; if he is legally incompetent, the consent of the legal guardian should be procured.
3b. The subject of clinical research should be in such a mental, physical and legal state as to be able to exercise fully his power of choice.
3c. Consent should. as a rule, be obtained in writing. However, the responsibility for clinical research always remains with the research worker, it never falls on the subject even after consent is obtained.
4a. The investigator must respect the right of each individual to safeguard his personal integrity, especially if the subject is in a dependent relationship to the investigator.
4b. At any time during the course of clinical research the subject or his guardian should be free to withdraw permission for research to be continued.
The investigator or the investigating team should discontinue the research if in his or their judgement, it may, if continued be harmful to the individual.

Experiments on humans

The key word is *informed consent.* Really informed and real consent! In contrast to animals, man is usually able to understand the purpose and consequences of experiments, and should himself freely decide whether to participate or not.

Informed consent is essential

Experiments on humans are often in the form of clinical trials whose aim is to find out whether a certain (new) treatment of a disease is better than the standard (old) one. The experimental subjects are thus patients that may benefit from the treatment. To avoid bias and "placebo" effects such trials are usually designed as double blind studies, which means that neither the patients nor the experimenters know at the time of treatment, which treatment is being administered in individual cases. It is an unsolved ethical problem how to construct an optimal experimental design in such studies, and at the same time inform the patients appropriately according to the Helsinki Declaration (Box 9.2 and 9.3)

Box 9.3 Consent: How Informed?

The fundamental difficulty, as in so many medical dilemmas, is a conflict of roles and duties. The doctor is committed to the welfare of his patient: anything that impedes or seems to impede his promotion of the patient's care and well-being is a denial of his responsibilities. His duties as an investigator are quite distinct: he has to contribute towards a valid outcome to the trial which may help later generations of patients but which is unlikely to help the patient who is currently being treated. The randomised trial is not easy to reconcile with such a concept of individualised care. Instead of stating or implying that the recommended treatment is the one best suited to the patient's predicament, the doctor has to explain that there is uncertainty about the value of certain forms of treatment. To resolve this in the interests of future sufferers the patient will, if he agrees, be subject to one particular form of treatment: the nature of that treatment will be determined not by his doctor but by a procedure equivalent to the tossing of a coin. There is exceptional difficulty when the randomised treatments have clearly different effects upon the patient, as in mastectomy trials. Is it really compatible with the physicianly role that a patient undergoes a mutilating operation at the toss of a coin? Is it for that matter acceptable that a doctor should fail to offer his patient the preferred treatment because both are participating in a multicentre trial? Not surprisingly, some doctors cope with this acute dilemma by agreeing to a trial in principle and then failing to enrol patients for it, thereby achieving a wholly irrational reconciliation between their duties to individual patients on the one hand and to medical progress on the other. There is, however, one aspect in which consent to a randomised trial differs from consent to routine treatment. The clinician is confronted with the uncongenial task of confessing to both ignorance and impotence. He does not know whether one treatment is better than the other (otherwise he would not be conducting a trial), and he is explicitly abandoning his normal duty of selecting and recommending a particular treatment in favour of chance. This is why many clinicians baulk at the task of enrolling patients in a randomised trial. Is it then necessary to explain the process of randomisation to the patient at all? According to one view there is a moral obligation on the clinician to do this since the fact of randomisation, however distasteful, is of fundamental relevance to the trial and therefore to the patient's decision to participate. It is therefore wrong to withhold such information. Such a view is persuasive. It would not be easy to present a fair picture, complete with a description of alternative forms of treatment, and then omit to mention how one of these is to be chosen. Nevertheless the need to explain randomisation is clearly an important sticking point for doctors if not for patients. Zelen has suggested that this difficulty can be met by a modified trial design. Patients are randomised to two groups: one group receives the best standard treatment and consent is not sought. The other group is allocated to the alternative treatment to be tested, and informed consent to a trial is sought. If such consent is not given patients are reallocated to standard treatment. Faced with poor recruitment the NSABP adopted the Zelen model and the rate of recruitment increased sixfold, but such a change of direction is achieved at a price that is in this case both ethical and scientific. The scientific price is the increased risk of systematic bias. The ethical difficulty is that one group of patients participates in a randomised trial without giving their consent. It might be argued that if the alternative course of not participating was adopted the outcome would be the same for this group of patients. The ethical criticism remains, however, that as a result of the decision to conduct a clinical trial a group of patients received a particular form of treatment by chance and not by choice. On these grounds the Cancer Research Council Working party rejected the Zelen model: it is difficult to refute their conclusion. There is no wholly satisfactory alternative to the randomised controlled trial.

Editorial, The Lancet,
June 30, 1984, p.1445

Another problem is how to inform or get consent or both from e.g. children, insane, mentally retarded, or unconscious patients. Fortunately, most research clinics are supervised by ethical committees from which you may get advice. In any case, you should never initiate a clinical trial if you do not have good reasons to believe that the new treatment is definitely beneficial to the patients.

The patients should benefit

Physiological experiments are often performed on volunteers. Sometimes such experiments involve a slight, but definite, risk of serious complications or even death to the participants. The volunteers should, of course, be appropriately informed about this and appropriate insurance agreements reached, but in addition you should feel confident that they are not in any subtle way forced to volunteer.

Really poor people may, for instance, be tempted by generous pay, or prisoners by prospects of better treatment or a rapid release, to accept an undesirable risk. As a common rule: You should not persuade others to participate in experiments that you would not willingly have participated in yourself!

Internal ethics

The ethical problems mentioned above pertain to the scientist as a member of society. Some moral issues, however, are specific to or more critical to the scientific community than to society at large. Science therefore has, in a way, its own internal moral code.

Fraud.

Dishonesty, though generally condemnable, may in certain circumstances be met by social approval. The different attitudes towards truth exhibited by scientists and lawyers are illustrative (Zuckerman, 1977), and in everyday life "white lies" may even be regarded as respectable. In science, however, fraud is disastrous. Honesty is a *conditio sine qua non* in an enterprise that above all strives to attain the truth. Nevertheless, fraud is seemingly an increasing problem in science.

Fraud is disastrous

The reasons for the apparent increasingly fraudulent behaviour may be many. After a long period with growth of funds and jobs for scientists, resources have lately been severely restricted. In the competition for money and positions, the ambitious scientist must document his or her competence with achieved results and at that almost exclusively in the form of publications. "Publish or perish" is a fact of scientific life. As a

consequence, fabricating results may appear to some a faster and safer way to success than doing experiments.

Erroneous conclusions based on feigned results will not in the long run hamper scientific progress. They will either be ignored because they were of little interest, or they will be falsified by other scientists who cannot reproduce the results. In the short run, though, fraud may load unnecessary work on the deceiver's peers. An even more serious consequence is the decline in respectability that science suffers each time a new incidence of fraud is uncovered.

There is an indisputable distinction between deliberate fraud on the one hand and errors - due to sloppy work or simply human mistakes - on the other. But between these extremes there is a continuous spectrum of more or less conscious manipulation of data ("trimming and cooking" see Box 9.4).

Save your raw data carefully

We will strongly recommend that you adopt a rigid routine in keeping and filing experimental results in the form of laboratory notebooks (journals), registrations etc. (see also chapter 2). All results should be registered; if an experiment or some of the results have to be discarded, the reason should be stated in the notebook. Such procedures, when carried out meticulously, will aid your self control and tend to protect you against the temptation to "adjust" data. A more slovenly practice will certainly make it easier to delude yourself.

Coworkers have common responsibility for their joint conclusions, especially when they co-author a paper. You should therefore welcome, rather than be offended by, a collegial interest in your raw data.

In this connection you should remember that suppression of unexpected findings (which e.g. do not support your favourite hypothesis) is as condemnable as plain forgery, even if this malpractice may be easier to forget or disguise.

Box 9.4 From: Sindermann,C.J. "Winning the games scientists play"

I have developed i series of general and vaguely unsatisfying statements about ethical conduct in science. The list is by no means exhaustive, but it may be useful. Some readily debatable dicta are that scientists will:

- Design and conduct investigations in conformity with accepted scientific methods;
- Report in full, on a timely basis, the results of investigations, basing conclusions solely on objective interpretations of available data;
- Not publish or disclose data provided by others without their expressed permission;
- Not publish or release data anonymously;
- Give proper credit for ideas, data, and conclusions of others;
- Prevent release or publication of preliminary or misleading reports of results obtained;
- Resist temptations to utilise news media as first outlets for significant scientific information, in advance of disclosure to peers through normal publication channels;
- Challenge unethical conduct of other scientists, using scientific journals and scientific meetings as proper forums for debate;
- If in private industry, respect the terms of any agreement concerning proprietary information, but avoid entering into agreements which may lead to prolonged suppression of significant new information;
- Provide legitimate conservative estimates of degree of risk of any activity within their area of expertise, based on the best available evidence, but resist pressures for extrapolation and speculation beyond the logical conclusions derived from that data;
- Resist pressures to support decisions based on social, economic, or political considerations by warping conclusions based on scientific evidence;
- Resist pressures to support publicly an officially declared position by an employer if such a position is clearly not in accord with available scientific evidence;
- Offer scientific advice only in areas in which background or experience provides professional competence;
- Resist temptations to express subjective opinions or views in public forums on scientific matters outside areas of individual competence;
- Discourage, by whatever means are available, the employment of professionals in subprofessional jobs, except as temporary expedients;
- Discourage, by whatever means are available, the employment of subprofessionals in professional scientific positions;
- Encourage, by whatever means are available, payment or adequate compensation to professionals for professional services; and
- Encourage the professional development of scientists for whom he or she has supervisory or management responsibility.

My initial reaction to this list is that it is dreadfully dogmatic and incomplete.

Use and Abuse of Data

Ideas, data, and conclusions are the principal items of commerce for the scientist. As such they can be subject to a spectrum of manipulations which are clearly or marginally unethical. Included are misuses and abuses of data collected by the individual scientist, or those collected by others. I have attempted to categorise and identify both types. Beginning with data collected by the individual, misuses include

- Massaging--performing extensive transformations or other manoeuvres to make inconclusive data appear to be conclusive;
- Extrapolating--developing curves based on too few data points, or predicting future trends based on unsupported assumptions about the degree of variability in factors measured;
- Smoothing--discarding data points too far removed from expected or mean values;
- Slanting--deliberately emphasising and selecting certain trends in the data, ignoring or discarding others which do not fit the desired or preconceived pattern;
- Fudging--creating data points to augment incomplete data sets or observations; and
- Manufacturing--creating entire data sets de novo, without benefit of experimentation or observation.

Ideas, data, and conclusions developed by others may be also misused in a number of ways:

- Premature Disclosure--reporting, discussing, or citing the work of others which is unpublished or in press, without their stated permission;
- Scientific Ectoparasitism--deliberately exploiting or developing ideas or proposals of others, made available in oral or unpublished form for review or comment;
- Mirror Writing--utilising a form of pseudo-plagiarism in which concepts or conclusions developed by others are rephrased or reworded and used without giving adequate credit to sources; and
- Plagiarism--outright lifting of data or text from the published work of others without permission from or credit to original sources.

These categories represent only minor expansions of an attempted codification of abuses made one hundred fifty years ago by the British mathematician Babbage. He, in those long-past simpler days, created three descriptors: "forging" (fabricating and reporting results which were never obtained), "trimming" (manipulating data to improve their appearance and utility), and "cooking" (selecting data which fit a hypothesis and rejecting those which do not). How short is the distance we have travelled in all those years!

Etiquette

Scientist versus science

The scientific community is dependent on free interchange of ideas and data. New and important findings should be published in a concise and comprehensive way as soon as they are sufficiently substantiated. But, each scientist is dependent on salary, grants and recognition and has to compete with his or her fellow scientists to secure these needs.

Scientists compete – secrecy vs. exchange of information

Indicators of scientific competence, such as number of publications (and recently also number of citations), priority of observations, etc. are essential in this competition. A temptation can therefore arise to avoid open exchange of still unpublished ideas and results and to observe exaggerated secrecy. During the years a set of more or less informal conventions (etiquette) has therefore evolved in order to regulate this conflict of interest between science as a whole and that of the individual (ambitious) scientist. Often science journal editors represent the interest of science, and many of the formalised rules have been formulated by them (e.g. Box 9.5).

Box 9.5 Guidelines on authorship

INTERNATIONAL COMMITTEE OF MEDICAL JOURNAL EDITORS

At its last meeting the International Committee of Medical Editors (the Vancouver group) drew up the following guidelines on authorship and on other contributions that should be acknowledged. The committee also expanded the section on information to be given in the covering letter to include details of any conflict of interest and clarify the position of the author responsible for final approval of proofs.

Guidelines on authorship

Each author should have participated sufficiently in the work to take public responsibility for the content. This participation must include: (a) conception or design, or analysis and interpretation of data, or both; (b) drafting the article or revising it for critically important intellectual content; and (c) final approval of the version to be published. Participation solely in the collection of data does not justify authorship.
All elements of an article (a, b, and c above) critical to its main conclusions must be attributable to at least one author.
A paper with corporate (collective) authorship must specify the key persons responsible for the article; others contributing to the work should be recognised separately (see Acknowledgements and other information).
Editors may require authors to justify the assignment of authorship.

Acknowledgements of contributions that fall short of authorship

At an appropriate place in the article (title page, footnote, or appendix to the text; see journal's requirements) one or more statements should specify: (a) contributions that need acknowledging but do not justify authorship, (b) acknowledgements of technical help, (c) acknowledgements of financial and material support, and (d) financial relationships that may constitute a conflict of interest
Persons who have contributed intellectually to the paper but whose contribution does not justify authorship may be named and their contribution described--for example, "advice," "critical review of study proposal," "data collection," "participation in clinical trial." Such persons must have given their permission to be named. Technical help should be acknowledged in a separate paragraph from the contributions above. Financial or material support from any source must be specified. If a paper is accepted it may also be appropriate to include mention of other financial relationships that raise a conflict of interest, but initially these should be outlined in the covering letter.

Information to be included in the covering letter

Manuscripts must be accompanied by a covering letter. This must include: (a) information on prior or duplicate publication or submission elsewhere of any part of the work; (b) a statement of financial or other relationships that might lead to a conflict of interests; (c) a statement that the manuscript has been read and approved by all authors; and (d) the name, address, and telephone number of the corresponding author, who is responsible for communicating with the other authors about revisions and final approval of the proofs. The manuscript must be accompanied by copies of any permissions to reproduce published material, to use illustrations of identifiable persons, or to name persons for their contributions.

Brit. med. J. 291 (1985):722.

Abundant or redundant publication

Scientists nowadays are overwhelmed by an increasing amount of scientific information. The number of articles recorded in the Medline index increases by about 40,000 each year. Double publication has become a problem not only for scientific editors, but for all readers who try to cope with the unmanageable profusion of articles. It is, of course, understandable that you have to prepare for your next grant application, but nevertheless your obligations towards the scientific community should motivate you to publish: (i) only the complete story, if necessary as companion papers, with all control experiments included and (ii) only very important findings as preliminary reports. (See Box 9.6.)

Co-authorship

The same problem occurs again; as long as the number of publications is used as main indicator of scientific competence, the list of authors on each paper will often be long - and unreliable as well. Our recommendations are (see also Box 9.5 and 9.7):

Consider authorship ***in advance***

- Rule-of-thumb: All authors should be responsible for the whole work.
- Consider and come to an agreement on the authorship as soon as possible during the project planning.
- The major contributor(s) first; the primary author should preferably also be the main writer, with the senior researcher at the end. (Except a few journals who want authors ordered alphabetically.)
- Don't offer authorship in return for "visibility" (by including a famous co-author), technical assistance, or supply of materials (Box 9.5).

Box 9.6 Publication: Numbers and Quality

There are many factors contributing to the increase in the bulk of scientific publications. Over the past score of years there has been a great increase in the number of individual workers in many research areas, an increase in laboratories housing such workers, an increase in the number and size of available journals, and an increase in the competitive pressures for available research fund support. There is, in addition, an increased pressure on the individual scientist to publish abundantly and thus to increase his bibliography. Rightly or wrongly he may conclude that his success or failure in attainment of future promotions, outside job offers, procurement of research grant support, memberships in prestigious societies, and medals, prizes, and other rewards are determined largely by the length of his bibliography. This attitude contributes to what, in the minds of some, is excessive and sometimes redundant publication.

I should like to propose a possible action that would tend to offset this frenzy to publish. If those agencies that recommend promotions, appointments, funding of research, memberships in prestigious societies, and the awards of medals and other honours would agree to consider only a very select list of the bibliographic citations, I believe nothing important would have been lost. Let the applicant select, say, one dozen of his bibliographic citations that are most meaningful to him; then the pressure to publish as perceived by the active scientists might be diminished. If the judge in each case is confronted by 12 articles that he may conceivably read rather than by 300 or 500 articles that he will certainly not read, something will have been gained in the process. In this regard it may be pointed out that in nominations to the Nobel Prize only 12 citations are requested. Similarly, nomination to membership in the National Academy of Sciences requires a selective bibliography of no more than 12 publications. Such an action if effective would tend to reduce the present emphasis on the numbers of publications and might restore emphasis where I believe it belongs, namely, on the quality of publications. It would further simplify the task of the awarding committee as well as the chore of that more overworked population, the secretaries who must prepare the nominations.

DeWitt Stetten, Jr.

Citations and priority

Scientific merits are not in general rewarded with high wages and only rarely with prestigious prizes. All the more reason to honour creditable scientists by mentioning their significance to scientific progress. Thus, when describing previous work in your field, you should exercise meticulous care in allotting priorities when you construct your reference list, so that the original and important contributors be displayed. It is recommended that you:

- Try to be accurate when referring to earlier contributions in the field.
- Mention the first (counted from the date when the journal of publication received the manuscript) one or two good original papers and/or appropriate or recent review articles. "Priority" should also be given to the paper that first formulated an important hypothesis. Intricate problems may arise here when you want to distinguish the wheat from the chaff; not only to sort plagiarism from modified hypotheses, but also when a loser in the publication rat race produced the genuine breakthrough.

Concealment

Free exchange of information is important

Scientific progress depends on openness and exchange of ideas, as well as on positive and negative results. Basic research paid by public money should be distributed without restriction to the scientific community, as soon as the conclusions are sufficiently substantiated. On the other hand, you should be careful not to publish premature results. In our opinion you should never leak results to public media, before they are published in the scientific press. Only after scrutiny and (hopefully) acceptance by peers may your findings be worthy of integration in the collective scientific (and public) knowledge.

If you perform applied research, and especially if you are mainly paid by a private firm, economic (or military) concerns may oblige secrecy. This policy must be regarded as an indispensable evil which on the one hand may impede scientific progress, but on the other hand channels funds to useful research enterprises that could not otherwise have taken place. We agree with Sindermann's advice (Box 9.4): "Respect the terms of any agreement concerning proprietary information, but avoid entering into agreements which may lead to prolonged suppression of significant new information".

Box 9.7 Credit and Responsibility in Authorship

Authorship credit on a by-line has come to be the currency of our academic accounting system, taking its values not from the norms of research but from the pressures of the marketplace. In science especially, the journal article is the customary measure in considering candidates for grant awards, society membership, or academic promotion, articles and grants are the currency of a career. Authors need to see their names attached to articles published in the journals considered important by their peers and founders. Researchers pursue their places in the publication marketplace by "authoring" as many articles as they believe are needed for their careers. This is not a concern in itself; but when it is at the expense of substance and integrity, it becomes important to all who share in the enterprise or benefit from it.

There are eloquent descriptions of the pressure to publish and round condemnations of its consequences -"salami science" and the "least publishable unit" and "honorary authorship." If authorship is seen merely as a "money of account," researchers can feel free to give, trade, and buy by-line places. Further, critics will have a hard time justifying why authors should change their entrenched and functional use of by-line space as one more negotiable commodity in the academic marketplace, not essentially different from making arrangements for lab space, assistants, equipment, and the other basics of science research. This concept of by-line authorship as merely one part of the larger research enterprise evolved as science moved from the traditional relatively cloistered academic life, with low salaries, community and accepted scholarly norms, to its present position in big business/big government/big science. Its natural expression came as projects grew so large that many authors were involved; distortion set in when researchers began to publish smaller and smaller bits of their research and yet retained the numerous authors. producing more separate titles for each author to claim. Runaway inflation is no healthier in publishing than in the economy at large.

There is also the problem of fraud. Some have argued that the few known cases of true fraud (as opposed to sloppiness or honest error) in research publishing derive from the pressure to publish. While the basic fault here lies with the individual who succumbs to temptation, the greater fault is in a system that, however unintentionally, fosters irresponsibility. We are obliged to come to terms with the consequences of individual actions that bring the community's integrity into question, and to examine the system that encourages quantity over quality. Further, we need to take seriously the fact that few senior researchers, much less their parent institutions, impress upon junior colleagues the ethical standards upon which the integrity of science and scientific publishing rest. It is not the pressure to publish that should concern us first but rather the scientific community's laxness in making plain and enforcing forthrightly its own standards of work and merit.

The pernicious consequence of using authorship as a currency of exchange is that it dilutes the responsibility for what is reported in the published article. When one person conducts the research and publishes the report, all readers know who is responsible for the completeness, integrity, and correctness of the report. The researcher's results may not stand up to the scrutiny of readers' and colleagues' review, but that is the way normal science works. Should the peers find the work shoddy or the report misleading, they have no doubt about who is responsible. This side of the credit-responsibility coin is important to maintaining integrity in research.

As the number of authors grows, however, the responsibility for the whole article or its parts becomes diffuse. At a minimum, we can expect each author to take responsibility for the article as a whole and for each to defend and confirm the part for which he or she is responsible. But recently editors have seen more and more authors taking the credit but not the responsibility. The major ethical problem here is the uncoupling of responsibility from authorship.

And surprisingly often, these authors show a rather shocking insouciance. Those who believe that they are entitled to credit for work they took little part in, perhaps do not understand, and cannot explain, much less defend, are breaking faith with their peers. This is so because scientific teamwork must be based on trust, and editors' acceptance of the research is most often an act of faith.

The "gentleman's agreement" in science is left over from the days when "gentleman scientists" pursued learning for its own sake and had little reason to lie other than vainglory. Today, when science is big business, the reasons to cheat begin at vanity but quickly go on to encompass the acquisition of grants, tenure, and the rewards of renown. Yet, although science is now a major industry, trustworthiness remains central; it is the weight-bearing member of the scientific edifice. In conducting research, the scientist combines this essential trust of colleagues' integrity with a healthy scepticism about the research being done.

Addeane S. Caelleigh Editor, 1991.

Checklist 9

1. Have you considered the ethical consequences of your research project? May the results be misused in a way you find unacceptable?
2. If you are going to use animals: Have you checked that you are licensed to perform the intended experiments? Have you designed the experiments to minimise distress in your animals, still using enough animals to obtain reliable results?
3. If people are subject to investigation: Have you informed the participants properly and obtained written consent? Has the research protocol been approved by the appropriate ethical committee?
4. Have you let co-workers (blindly) perform the scoring where objective measurement was not possible?
5. Imagine that the head of the institute wants to see your experimental notebook. Are you prepared to show it to her straightaway?
6. Is the "story" complete and reliable, or is publication possibly too early?
7. Have you included in your article **all** facts (methods, results) that may be significant to other workers in the area (even if they are competitors)?
8. Have you checked that you have appropriately cited the **original** contributors to the field?
9. Have you come to an agreement about co-authoring of a planned publication? In which order shall the authors be listed? Who should be acknowledged?

Exercises

1. a) You suspect that one of your companions in the lab have faked data that he is going to publish. How should this suspicion be tackled?

   b) The erroneous results have already been published. Which actions would then be appropriate?
2. You have received financial support from a pharmaceutical firm to test a new drug in your experimental set-up. You find (surprisingly) that the drug may have adverse effects. The firm does not believe in your findings and withdraws the support and the supply of the drug. You are thus not able to continue the experiments, which is essential to obtain publishable results. What shall you do?
3. You have for a long period been working on your own with a research project. Finally, you have collected sufficient data to substantiate your hypothesis. The head of the department where you have been employed, has approved the investigation, but has had no part in the planning and accomplishment of the project. Nevertheless, he disagrees with your interpretation of the data and want to obstruct publication. At this time you move to a new job in another town. Who "owns " the data in this case? Some of your findings may have commercial interest. Who should benefit?

If the head of the department had proposed the original project, would you judge the case otherwise?

4. Outline a double blind clinical trial where the participants are properly informed and have consented (see Box 9.3)!

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Chapter 10: A basic course in biomedical laboratory research

Since 1983 we have arranged a yearly 10-week course in introductory science, mainly for postgraduate M.D.s. There have been 20 or 25 participants in each class. The need for suitable teaching material led us to compile from various sources a small compendium, which was expanded by our own writings. It was rewritten and rearranged as we learned more about the course participants' wishes and requirements, and we changed the course accordingly. Finally, the compendium developed into the present textbook, which we recommend to our students as a basic self-study guide.

The present scheme of our course - which is every year subject to attempted improvements - is given in Table 10.1. There are two major elements in the course organisation: First, student assignments are based on small group work; and second, the core of the assignments has been a set of raw experimental data.

Table 10.1 gives the course outline

We believe that learning depends more on the activity of the student than on the activity of the teacher. Our teacher motto is "Be enthusiastic and lazy!". The time devoted to lecturing has gradually diminished, whereas structured small group work has gained increasing importance.

Main principle: practical work in small groups

A main goal has been to teach the participants to write a scientific article. We have constructed sets of raw data either based on our own or colleagues' unpublished experiments or - probably better- deduced from the elaborated data presented in high-quality articles published in prestigious medical journals. Criteria for the choice of such articles include the general appeal of their main topics and the use of descriptive statistics and the estimation of statistical significance in data presentation. Based on these data and some background information - written in Norwegian - on the purpose and methodology of the experimental work, the students are required as a group to write a complete, scientific manuscript during the course. If one, or preferably two, original articles are used as the data source, their identity are not disclosed until the last day of the course, when the students can compare their work with the original publication(s).

To write a scientific paper

Table 10.1. General course format: A basic course in biomedical laboratory research.

Day	Date	Main theme (lecture)	Invited teacher (Y/N)	Workshop/seminar (Class discussion)	Deadline work task *	Small group work
1	7 Jan.	Theory of science	N	Introduction to scientific writing	-	Statistical exercises
2	14 Jan.	Computer usage	Y	Theory of science	Statistical exercises	Improvement on distributed poor graphs and tables.
3	21 Jan.	Data presentation	N	Statistical exercises Data presentation	Data presentation (group work)	Statistical exercises. Final group discussion (F.g.d.) on own version of 'Materials & Methods'
4	28 Jan.	Epidemiology and clinical research methods	Y	Poster production	Statistical exercises	-
4x	29 Jan.	Experimental animals	Y	Exp. animals : Demo. procedures	-	-
5	4 Feb.	Work in the lab.: Experimental protocol, etc.	Y	'Materials & Methods'	-	F.g.d. 'Results'
5x	5 Feb.	Cell culture & molecular biology	Y	-	-	-
						F.g.d. 'Discussion'
6	25 Feb.	Literature retrieval and use of the scientific library	Y	'Results'	Individual homeworks: Data presentation & Statistical problems	F.g.d. 'Introduction'
7	5 March	Oral presentations	Y	'Discussion'	Two 10-min lectures	F.g.d. 'Abstract'
8	12 March	Statistical highlights (choice of method; type II error)	Y	'Introduction'	Statistical exercise. Evaluation of teaching material	F.g.d. 'Poster'
9	19 March	Ethics, Etiquette	N	'Poster', 'Abstract', General course evaluation	-	Mounting the poster

* In addition, manuscript sections are submitted and distributed to all participants, for small group discussion of the products of the other groups, one week before their plenary discussion.

----.----3 weeks' intermission (with manuscript work, solution of statistical problems, preparation for oral and poster presentations, and reading of recommended literature)

Judgements on the essentials of a paper

It is possible both to complicate and to facilitate the writing of such a manuscript. We make the task harder by exaggerating minor points or trivialities of the total data-base or the methodology. This is to train the students in the art of judgement, emphasising that only important points should appear in print (even if it means that weeks of futile or exploratory laboratory work in real life might never be published). Moreover, we tell the students that the description of methodology and results may be incomplete, so that they are forced to use their creativity to give a full and coherent description.

Group rehearsal before plenary discussion

On the other hand, writing the manuscript is facilitated by our small group approach. We divide the class into four five-member groups. In each group one student writes the first English version of the Materials and Methods section, another student the Results section, etc. The one who is allotted the Abstract also has to construct a title and a list of key (index) words. These first versions are then discussed and improved during group meetings (Table 10.1), and possibly also by e-mail or facsimile letter communications within the group between the meetings.

After four weeks we begin a series of weekly 4-hour plenary sessions devoted in part to discussion of the manuscripts. By this time each student has had the opportunity to read and compare four different versions (one from each of the small groups), based on the same raw materials, and distributed to all participants one week beforehand (see Table 10.1). Only one section of the manuscript is discussed at each of the plenary sessions. Different guest instructors, selected on the basis of their proficiency and specialities, are invited to comment on the different manuscript sections and discuss them with the class. Usually, we also give our private comments in writing to the student authors; these latter comments include orthographic and stylistic details that are not appropriate to the plenary discussion.

Invited teachers comment on manuscript sections

Poster construction task

The database for the manuscript writing is also used to construct a poster presentation. Consequently, four "parallel" posters, one from each group, are presented and discussed on the last day of the course. Moreover, these raw data are also used for individual statistical exercises and visualisation tasks, i.e. the construction of graphs and tables (Table 10.1).

Literature retrieval

Ideally, practice in using literature retrieval systems (e.g. PubMed, Medline or Index Medicus on CD-ROM) should be associated with the manuscript writing. However, we do not want the students to trace the source(s) of their raw data before they finish the manuscript. Thus, we urge them to gain experience by retrieving

literature relevant to their own research projects, and we distribute to them beforehand copies of retrieved abstracts dealing with their manuscript topic.

Overall, the course evaluations have been favourable (otherwise, we would have stopped our self-initiated enterprise long ago). The components that have been most unanimously successful, have in fact been those mentioned above, where the students have to exert themselves the most.

The oral presentation

Activity on the part of the students is also required on course day 7, when two participants each has to prepare a 10-min scientific talk to be discussed by the class. This is preceded by a lecture on the general principles of effective oral presentation. The subject, target group, and language of presentation (Norwegian or, preferably, English) can be chosen by the two performers.

The cafeteria and exemplification principles

It has been a bit problematic to find an appropriate format for the teaching of topics not based on elaboration of the raw data, such as the philosophy or theory of science, the usage of computers, epidemiology and clinical research methods, and work in the laboratory. Here, we are presently trying two different strategies, either alone or in combination, namely (i) the menu principle, with optional participation, or (ii) the exemplification principle, with student tasks or problem solving as starting points for the more theoretical treatment of the topics.

The students are given an option to participate in some parts of the course. For example, the extra (x's in Table 10.1) days, dealing with experimental animals, cell culturing, or molecular biology are for those with special interests. Since the students still have very different backgrounds concerning computer usage, we try to tailor computer training to individual needs - particularly for treatment of raw data, for making graphs, for writing and editing a manuscript, and for literature retrieval and storage. On the day dealing with work in the laboratory, we urge the student groups to prepare answers to a number of specific questions and problems. These concern for example the design of an experimental protocol and laboratory journal/note-book, laboratory safety precautions, collaboration with technicians, sharing responsibilities, laboratory staff seminars, and the storage of laboratory data. Reading of Chapter 2 should facilitate this work.

We have been asked by our students to change our original course format to include some statistics training. We have done so, but the outcome has been less than satisfactory. The heterogeneity of our students' statistical knowledge is possibly even

more marked than their variance in computer proficiency. We firmly believe that all of the students need a thorough understanding of statistics. The simplest solution has been to require a statistics course as prerequisite to our own course. Now, as a prelude to the discussion of statistical problems that are presented to the student groups, we try to teach the bare essentials of combinatorics, descriptive statistics, and hypothesis testing (see Chapters 5 and 6) - perhaps annoying the proficient and frustrating the ignorant.

Statistics preferably needs a separate course

Although we try to lure our students into solving problems and performing work tasks on their own in order to learn the essentials of scientific craftsmanship, we also believe in the value of an inspiring lecture. This is particularly important for such topics as the theory of science, the art of clinical investigation, and the ethics and etiquette of science. However, our teaching of the theory of science also rests heavily on the exercises of Chapter 1. Concerning ethical questions - which close our course - we have tried various alternatives and have chosen to connect the survey of guidelines for ethics and etiquette to a class discussion of problems that the student groups have been presented beforehand as "case studies". The case studies deal with e.g. questions of authorship, duplicate publications, free communication of scientific findings, animal experimentation and handling of animal rights activists, and scientific fraud.

An inspiring lecture is also useful!

Each course day is evaluated by the students, on such points as the relevance of the topic and the quality and tempo of presentations. We have also asked whether some course components should be removed or replaced. Moreover, just prior to termination of the course we have arranged a more extensive questionnaire evaluation of the whole course, enabling us to improve or at least modify the course format in the following year.

The explicit appeal to our students about suggesting new topics of general interest that could be included in the course has not been very fruitful. This is perhaps not surprising, since most of the students have not previously had any experience in the planning and practice of research. On the other hand, the general evaluation of the course always shows that there are differences of opinion concerning the scheduling and structuring of the course. One organisational alternative we have considered is that the course be arranged in two - or three - phases, each lasting two or three full days, with sufficiently long intervals(s) between the phases to allow for the completion of required "homework" and exchange of material between groups.

Never full agreement on optimal course format

Documentation rather than exam

We have stood up against (a light) pressure from the medical faculty to arrange a final exam; as an alternative we have chosen certain kinds of documentation as requirements for the course testimonium. The students must document (i) participation in at least 80% of the course, solution of (ii) individual and (iii) group tasks (see Table 10.1), (iv) writing of a manuscript section, (v) participation in the poster production, and (vi) submission of a written evaluation of the course materials (including the compendium).

The organisation of this course is a process that is continually evolving. We invite our readers to present their suggestions for further improvements!

Exercise

1. Try to design a course format that would be appropriate to the requirements of yourself and your peers.