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# REVIEW

# SOME FUNDAMENTAL THEORETICAL AND PRACTICAL PROBLEMS ASSOCIATED WITH NEUROCHEMICAL TECHNIQUES IN MAMMALIAN STUDIES

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## CRITIQUE

by L. HERTZ

**Abstract**—The assumptions inherent in (i) a pharmacokinetic experiment *in vivo*, (ii) a subcellular fractionation, (iii) a chemical assay in an isolated neuron, are listed as three examples of well known neurochemical techniques. From these lists of assumptions a number of general hiatuses in knowledge of the effect of preparation of tissue on the results of experiment have been identified. The importance and different kinds of control experiments are discussed. Comment is made on how the different parameters to which measurements are referred may affect the result of the experiments. Optimal techniques are preferably non-disruptive and non-invasive. A few new techniques are proposed.

## AIMS OF NEUROCHEMISTRY

One may summarise the following aims of neurochemistry as the study of: firstly, biochemical processes in the normal central nervous system; secondly, the biochemical processes involved in control by the nervous system of other parts of the body and the 'feed-back' from these other parts to the nervous system; thirdly, the biochemical processes and visceral changes triggering neurological and psychological diseases, and the changes in the nervous system resulting from such diseases.

All over the world—especially during the past 35 yr—enormous resources have been devoted to neurobiological research, yet we are still very far from understanding the biochemistry of, for example, thinking, learning, nerve regeneration, manic depression, schizophrenia, multiple sclerosis or the genesis of cancer in the nervous system. This is not to say that pieces of the jig saw have not been found, rather that, so far, not enough of them have been fitted together to make a coherent picture which would indicate, say the biochemistry of thinking, or would lead to a rational treatment for multiple sclerosis.

A number of reasons for this lack of success have been suggested elsewhere (Hillman, 1979). One possible explanation for this is that major shortcomings of techniques currently in use are being overlooked, so it seems opportune to reexamine systematically some of the most current popular techniques, particularly in relation to the assumptions implied by their use and the limitations inherent in the procedures. However, before one does so, it is necessary to state some basic points about the philosophy of research.

## THEORETICAL ASPECTS OF RESEARCH

### *Hierarchy of preparations*

Movement, thinking, learning, active transport and genesis of disease all occur in living animals. Therefore, when one is designing experiments to examine any of these phenomena, the whole living animal must be regarded, *prima facie*, as the best source of information (Hillman, 1976). Tissue to which powerful chemical reagents has been added, or which has been homogenised, centrifuged, frozen, fixed or dehydrated, must yield information of lesser quality than that derived from living animals, when the results of the two

kinds of experiments are incompatible, and when one is attempting to examine properties found only *in vivo*. This generalisation also applies to light vs electron microscopy, in which the former can be used to observe living animal cells, which the latter so far has not been able to do. It also implies that a metabolising tissue, like a brain slice, for example, must be judged to yield better quality information than a histological section, *when the findings arising from the two preparations are incompatible*. It thus becomes evident that one may arrange experiments in a hierarchy (Table 1). At the apex stands the whole living conscious normal human being or animal unaffected in respect of the measurements one is making by the experimental or observational procedure (this particularly applies to experiments on the biochemistry of behaviour). Lower down the hierarchy are the anaesthetised, restrained, dieted and inbred animals. Lower still are the tissue slices and single cells. Further down come histological sections and electron microscopic preparations.

*Findings vs hypotheses*

In addition to an experimental hierarchy, there is an obvious logical hierarchy. A finding which makes no or few assumptions is obviously better than one implying many assumptions, especially if they have not been tested. Findings are more valuable than hypotheses. Extrapolations are guesses involving stretching one's findings beyond the data. From the very nature of such a hierarchy one cannot use a hypotheses as evidence against findings. It is well to note that the following hypotheses are widely regarded as findings: the Davson–Danielli and Singer–Nicholson models of the cell membrane; the presence of a 'packet' of acetylcholine in a synaptic vesicle; the chemiosmotic hypotheses of proton transport in mitochondria. One is not decrying the use of hypotheses to design experiments, but it should be emphasised that they cannot be used to contradict findings, upon which their validity alone depends. A hypothesis is a guess, supposition, extrapolation or fantasy. A junior research worker embarking on a new project naturally expects that his highly respected predecessors have tested all the crucial assumptions, but this may not always be true (please see below). However, the farther the original experiments recede into the past, the more assumptions will have been accumulated, and the more inertia or resistance will be engendered in the scientific community against testing the assumptions, or carrying out the relevant control experiments. Nevertheless, obviously neither the long lineage of predecessors who have failed to carry out such experiments, nor the general inertia of any body of dogma against such a necessity, can validate *in any way*, such partially completed experiments.

Table 1. Hierarchy of preparations

| |
|---|
| Whole conscious human being or animal unaware of being observed. |
| Whole conscious human being or animal observed and behaviour affected significantly by being observed. |
| Human beings or animals on drugs, abnormal diets, restraint, anaesthetised, stressed. |
| Decerebrate preparations. |
| Brain windows. Perfusion *in vivo*. Brain slabs. |
| Isolated brain or head. |
| Cerebral slice or isolated retina. |
| Tissue culture of brain and spinal cords. |
| Isolated sympathetic and dorsal ganglia. |
| Isolated peripheral nerve. |
| Isolated neuron. |
| Homogenate. |
| Histochemical or histological preparation. |
| Electron microscopic preparation. |

The position in the hierarchy is established empirically, and, therefore, may not be universally agreed.

*Generalisations*

If one draws an original conclusion about the chemistry of, say, the frontal cortex of the rat, based on even only four measurements, the following further conclusions are likely to be drawn, unless some well known research worker finds very different values and succeeds in publishing them:

(i) the statistical *validity* based on these few observations in one experimental series is likely to hold in the same preparation in all animals of that species;

(ii) the finding is likely to be true for other areas of the cerebral cortex of a rat and probably other areas of grey matter;

(iii) it is likely to be true for the grey matter of all other mammalian species;

(iv) it is likely to be independent of the breed, diet, clinical state, circadian rhythms and other variables, unless any of these variables has been shown specifically to alter the chemistry of the area under study;

(v) all these expectations are liable to hold for the foreseeable future.

Such generalisations dare not be made lightly. They are recognised less in neurochemistry than in pharmacology and therapeutics, where species variation is a constant consideration.

*Subjective and objective criteria*

Everyone would agree that scientific experiment should be objective, but there are several circumstances in which subjectivity may intervene in experiment or publication. These include:

(a) failure of research workers to carry out control experiments, which they have identified or to which their attention has been drawn;

(b) unreadiness to publish experiments which have been done, but which do not fit in with the hypothesis of the research worker;

(c) refusal to recommend for publication in journals or books findings which contradict the views of the referee, who cannot identify objective criteria for such refusal;

(d) influencing research councils to turn down grants to carry out research which will produce unpopular results or is done by people whom the reviewer does not like;

(e) failure of research workers to cite findings which are incompatible with their own hypotheses.

*Evidence*

It is essential, although not always easy, to distinguish between findings which are compatible with a hypothesis but not crucial to supporting or to denying it, on the one hand—and evidence which bears directly on its validity—on the other hand.

*Vertical approach*

The creation of a hierarchy (Table 1) helps with another problem. If one wishes to find the site of action of a drug, for example, on the brain, the following are a few of the sort of experiments which may be carried out: one may observe its effect on the behaviour of the whole animal; it may be injected and its distribution within the tissues examined; it may be administered to an animal chronically and its effect on the metabolism or the chemistry of the tissues subsequently excised may be studied; it may be placed in an incubating medium containing cerebral slices, in which its effect on biochemistry is studied; its receptors may be examined in subcellular fractions. Of course, the results of all these experimental investigations should be compatible with each other. However, if the drug has a precise effect in a preparation near the apex of the hierarchy, say, it produces effects on behaviour when it is not found in measurable quantities in the brain, one may conclude either that its primary effect is on another organ, or that it is rapidly metabolised in the brain and a metabolite produces the behavioural effects, or it was measured at the wrong time. These conclusions can be elucidated further.

If the drug has an effect on a cerebral slice, and all the preparations higher in the hierarchy, but not on a homogenate, one would then conclude that its effect was on the membranes, which are largely intact in cerebral slices, or that its effect had been destroyed by the medium in which the tissue was homogenised or by the homogenisation itself; in the latter case, one could probably conclude that it was a temperature sensitive mechanism, either located in enzymes or other proteins. Thus, in general, by locating a property high up in the hierarchy, but not lower down, one could define what degree of tissue organisation was necessary for it, and therefore, identify the locus of its mechanism of action. A property present low down in the hierarchy but not higher up is either one which is overridden by mechanisms present in the more complex tissue or is an artefact usually of a technique involving tissue disruption. It seems essential when seeking to detect or measure receptors in homogenates or subcellular fractions to bear in mind that local temperature rises during homogenisation may well alter the measured affinity of the tissue for a transmitter, antigen or ligand (please see below).

*Disruptive techniques*

Any disruptive technique, such as homogenisation, sonication, subjection to powerful chemical reagents or hypotonic solutions, will induce some or all of the following consequences: acidification, autolysis, hydrolysis, proteolysis, diffusion, denaturation, oxidation and micelle formation.

Formally, one may consider disruptive techniques in relation to the Second Law of Thermodynamics. One cannot alter the entropy of a closed system without altering its standard free energy. Free energy determines the equilibria of the majority of enzyme reactions. Homogenisation and centrifugation are clear examples of techniques of changing entropy. Therefore, one can neither ignore the effects of these procedures on enzyme activities, nor deny that they are likely to have any.

*Structure and function*

In recent years it has become fashionable to try to correlate what are called 'structure' and 'function'. This usually means seeking findings which are compatible both with the electron microscopic view of a subcellular organelle, and with the biochemical events believed to be occurring in the organelle. One must question the value of such an endeavour for several reasons. Firstly, the structure one is examining is a

metal deposit on a fixed dehydrated piece of tissue; the metal deposit does *not* metabolise, and has no biochemical properties whatsoever. Secondly, electron and, to a greater extent, light microscopy are used to examine the shapes of structures like mitochondria or membranes. The mitochondria consist of many thousands of molecules, whereas the biochemical properties are essentially studied as reactions of single molecules or chains or cycles involving single molecules. Thirdly, when the electron microscope has been used to elucidate the structure of cell membranes, it has leaned heavily on interpretations derived from the Davson–Danielli hypothesis, and has claimed to demonstrate the 'unit' membrane of Robertson or the Singer Nicholson hypothesis. Evidence has been adduced elsewhere that the 'unit' membrane as seen could have no three dimensional existence (Hillman and Sartory, 1980), and evidence is to be published elsewhere that all three models are incompatible with each other and with generally accepted views about membrane biochemistry. Fourthly, electron microscopy – but more so low angle diffraction – has been useful in demonstrating the shape and dimensions of large extracted and purified molecules like DNA and proteins, but is difficult to interpret in the study of the molecules when mixed or bound with others as they are presumably in living tissues. In these cases, one frequently has to resort to interpretation in the light of the model for which one believes the measurement to be providing the evidence. One may be tempted here to regard the finding plus hypothesis of as good a quality as a pure finding. The reason why this is frequently the case with membrane studies is that all the models which attempt to correlate visible structures with invisible molecules have a large proportion of unknowable supposition, since our methods hover at the border of uncertainty (Hillman, 1972). Most of this uncertainty arises from the vast number of variables in preparation procedures which may affect the result of experiments. There are serious dangers that our studies may be elucidating the vagaries of our methods more than the fundamental properties of the living tissues, which we started out intending to study (please see below).

*Concentrations in biochemical, physiological and pharmacological experiments*

In experiments which are intended to derive information about living cells, it seems highly desirable that in general when studying affinities, enzyme activities or metabolism *in vitro*, one should always attempt to study them in media mimicking serum or cerebrospinal fluid, in preference to media in which they exhibit 'optimal', i.e. maximal activities, which may be totally irrelevant to the state of the tissue *in vivo*. This is particularly true in enzymology. By the same token when one is attempting to elucidate the effect of a drug *in vivo* by studying it *in vitro*, it seems highly desirable to employ concentrations of the drug equivalent to those found in the cerebrospinal fluid or serum when it is being used clinically (Wraae *et al.*, 1976). Sometimes there is a temptation when a drug is found in tissues in concentrations too low to be measured, or it does not have the desired effect *in vitro* at the concentration equivalent to its therapeutic level, to use much higher concentrations in pharmacological experiments. Frequently, such dosages used clinically would be fatal. The employment of high concentrations is usually justified on the grounds that the tissue affected may take up a high concentration of the drug, so that its concentration at the site of action may well be much greater than that in the serum or in the incubating medium. If such a phenomenon is suspected, this should urge the necessity of measuring the concentration in the target organ, and *demonstrate* the high concentration. Nevertheless, if a target organ does accumulate a high concentration in the presence of a low concentration in the serum or in the medium, then it is the low concentration which should be used in experiment: this is especially true if the receptors themselves concentrate the drug or hormone from the serum or medium, from which a much higher concentration still would occur in the receptors. If a clear pharmacological or hormonal action occurs in the tissue in which the drug or hormone concentration cannot be detected, either one should devise a more sensitive method of measurement (probably using radioisotopes), or seek an explanation of the effect of the agent as originating in another organ in which its concentration can be detected by available methods.

*Practical approach to theoretical problems*

Most of the problems in the relationship between the living whole animal and experimental preparations *in vitro* can be avoided or circumvented by pursuing the following stratagem:

(1) Systematic consideration and testing of all important assumptions implied by the use of a particular technique or preparation;

(2) Carrying out appropriate and adequate 'control' experiments;

(3) Careful consideration of what the particular parameter being measured is referred to and methods of calibration.

## ASSUMPTIONS

(In each case 'change' refers to statistically significant change.)

Three examples of quite different techniques are given to illustrate the listing of assumptions and to derive generalisations about the common hiatuses in experimental design.

### *Pharmacokinetics*

A drug is administered intraperitoneally to an animal to study its uptake into the different organs, its excretion and its metabolites. The following assumptions may be identified, that:

(i) the drug is always injected in the same site, whereas it may be subcutaneous, intramuscular, intraperitoneal, into the alimentary tract, or in several of these compartments. The absorption from the different locations into the blood varies with the real site of injection;

(ii) the biochemical changes consequent on the stress to the animal do not alter the distribution in the circulation and the tissues of the drug;

(iii) the changes due to stress do not alter the affinity of the drug for the different tissues;

(iv) the changes due to stress do not alter the excretion by the kidneys, liver, lungs or skin;

(v) the injection does not alter the appetite or thirst of the animal and, therefore, its nutritional status;

(vi) the injection does not alter the animals behaviour in other ways, such as its mobility, its sleeping habits, its resistance to infection or its temperature regulation, all of which could affect drug metabolism;

(vii) repeated injections do not produce accumulation or variation in any of the above effects;

(viii) the injection needle does not wound the animal;

It will be rapidly appreciated that the injection of saline or the vehicle of the drug into control animals makes only a limited impact on these assumptions (v–viii);

(ix) the removal of some animals of a batch does not produce stress to the other ones left in the cage;

(x) the circadian rhythms do not govern any of the previously mentioned effects;

(xi) agonal changes in blood biochemistry do not alter the distribution or metabolites of the drug;

(xii) the measurement of the drug in the tissues is not affected by the presence of the tissue, or that the extraction from each of the tissues is equal. This could only be tested by the use of a recovery method for calibration—please see over;

(xiii) the homogenisation does not affect the affinity of the binding of the drug by the tissue;

(xiv) other reagents besides the extractants do not affect the binding of the drug by the tissue;

(xv) other procedures such as centrifugation, filtration, washing, etc. used in measurement of the drugs do not affect the binding of the drug by the tissue;

(xvi) the post-mortem changes do not affect the binding or any of the measurements;

(xvii) the particular metabolites or their proportions are not affected by the biochemical effects of stress;

(xviii) they are not affected by agonal changes;

(xix) the effect of the metabolites does not change after death;

(xx) the extraction of the drug from each of the homogenates or body fluids is equal or complete;

(xxi) the drug does not itself interfere with the measurement;

(xxii) the calibration of the drug is not different in the presence of tissue as in free solution. The use of standards passed all the way through the whole system warrants this assumption, but not the frequent use of 'internal' standards which are often used only with the final steps of the measurements.

### *Subcellular fractionation*

A second example of assumptions inherent in the particular technique comes from measurement of the subcellular distribution of an enzyme (Hillman, 1972). The main inherent assumptions are that:

(i) the stress of handling and killing has no effect on the result of the experiment;

(ii) the agonal changes have no significant effect;

(iii) post-mortem changes have no significant effect;

(iv) cooling to room temperature, 0, $-25$ or $-196^{\circ}C$ has no significant irreversible effect;

(v) the enzyme activity of a homogenate decreases linearly with dilution;

(vi) the medium in which the tissue is homogenised, containing, for example, sucrose, edta, detergent or bile salts, does not alter the chemical activity significantly and irreversibly;

(vii) the enzyme activity measured finally is not changed significantly by the incomplete replacement of soluble constituents of the tissue which are lost on gross dilution, homogenisation and centrifugation in a quite different chemical environment;

(viii) movement during preparation of known cofactors, such as cations, and co-enzymes, or unknown ones, will not alter substantially the apparent localisation of enzyme activity as measured;

(ix) soluble materials originating from any compartment *in vivo* will not diffuse into the supernatant or become bound to another fraction during preparation, and thus be supposed to have originated in the location where it is found;

(x) no step in the preparation or lytic enzymes will render substances which were slightly soluble *in vivo* more soluble, and thus more diffusible which may change their affinity for the different fractions;

(xi) the heat necessarily generated during homogenisation is so rapidly conducted away that the temperature does not rise sufficiently high to change enzyme activities irreversibly;

(xii) refrigerating the centrifuge diminishes temperature rise at the surface of particles being homogenised;

(xiii) enzyme activities are not irreversibly changed by pressure during homogenisation and centrifugation;

(xiv) the same amount of work is done on each different part of a centrifuge tube;

(xv) the same amount of heat is generated in different layers of the homogenate in media which have different viscosities;

(xvi) the extraction from each of the final fractions is equal and complete;

(xvii) a recovery of enzyme activity in all the subcellular fractions added together of 60–130% of that in the initial crude homogenate implies that the enzyme has not relocated;

(xviii) the enzyme preparation has no significant non-enzymic activity on the added substrate;

(xix) the enzyme activity in the unphysiological substrate mixture is approximately similar to that in the chemical environment *in vivo*;

(xx) the similarity in appearance on electron microscopy of, for example, a mitochondrion in a section to that in a mitochondrial fraction is evidence that its biochemical properties have not changed during fractionation;

(xxi) the microsomal fraction consists mainly of cell membranes and endoplasmic reticulum;

(xxii) the apparently high but unquantifiable incidence of a particular identifiable organelle in a particular fraction as seen by electron microscopy is evidence that the biochemical properties of that fraction are dominated by that organelle;

(xxiii) when the enzyme activity is referred to the protein content of a subcellular fraction, the protein can be measured satisfactorily (please see below);

(xxiv) the calibration method for protein in one subcellular fraction is applicable to other fractions; unless this has been tested for specifically.

*Isolating neurons by dissection*

The third example is the technique of isolation of mammalian neurons by hand dissection, as described by Hyden (1959). The assumptions necessarily implied here are:

(i) the stress of handling and killing has no effect on the result of the experiment;

(ii) the agonal changes have no effect;

(iii) post-mortem changes have no effect;

(iv) cooling or deep freezing has no irreversible effect;

(v) the pressure on the medulla or other tissue while it is being cut does not change the biochemistry or location of the biochemical constituents in the cells;

(vi) constituents of the tissues do not diffuse away in the solutions in which the tissues are placed when the cells are to be taken out;

(vii) the latter solutions do not change the biochemistry of the cells;

(viii) the pressure on the neurons and the breaking of the axons and dendrites on isolating the cells do not change the permeability of the cell membranes or cell biochemistry;

(ix) significant quantities of constituents of the cells do not diffuse away in the fluid which adheres to the cells when they are transferred;

(x) significant quantities of constituents of the cells do not diffuse away in the incubating media;

(xi) the media constituents do not affect the biochemistry of the cells;

(xii) the recovery from the cells is complete;

(xiii) the cell constituents do not affect the calibrations;

(xiv) optical methods of measurement can be calibrated satisfactorily.

One may derive some generalisations about these assumptions. Firstly, the absolute validity of the experiment, as judged by the research worker and anyone in the future who quotes his evidence or uses it as a basis for his own experiments, depends upon the warrantibility of *every single* assumption. Thus, the validity of the result of an experiment depends crucially upon the weakest assumption, and their effect is cumulative. Secondly, examination of these lists reveals a number of important assumptions implied by particular techniques which are common to most procedures *in vitro*. Failure to test the truth of these assumptions represents an important and crucial hiatus in the whole corpus of knowledge. They may either be tested separately and rigorously, or in comprehensive experiments of whole procedures

which cover several of them at once. Each of these control experiments are simple but highly crucial for all cytology. The importance of these assumptions is heightened by the knowledge that several of them have been shown to be untrue experimentally or contrary to the Second Law of Thermodynamics or the Law of Conservation of Energy (Hillman, 1972, pp. 33, 49, 63, 73, 85 and 92). The only circumstances under which these assumptions could fail to have a serious impact on the results of these experiments and the interpretations derived from them would be if their total effects would be too small to be significant in relation to the overall results of the experiments. However, it cannot be stressed to forcibly that unless and until most of these experiments have been carried out satisfactorily, every experiment already in the literature and any of these kind executed in the future must remain unproven. The general proposition here is that many of our most firmly held beliefs are based on inadequate data, and should not yet be accepted.

Thirdly, many of these problems are not relevant if one is examining *mechanisms*. For example, if one has established that the electroencephalograph can be recorded in the isolated brain (White *et al.*, 1963), that electrical excitation may be induced in elements in cerebral slices *in vitro* (Yamamoto and McIlwain, 1966), that isolated neurons take up oxygen (Hertz, 1966), then these preparations can be used for studying the mechanisms of these phenomena and the factors which control them. However, one cannot suppose that the reactions under study occur at the same rate, or in the same locations, or are not grossly modified by other control mechanisms greatly damaged during preparation.

A summary of the general areas in which more comprehensive experiments need to be done urgently to justify currently held beliefs is given (Table 2).

Table 2. Areas in which insufficient comprehensive experiments have been done to test the assumptions inherent in widely used techniques

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| Stress on body biochemistry affecting blood and cerebrospinal fluid composition. |
| Agonal effects on the same systems. |
| Post-mortem changes occurring rapidly in acute animal experiments, but much more profoundly in material from human post-mortems. |
| Homogenisation on chemical activity and localisation. |
| Centrifugation on chemical activity and localisation. |
| Exchange of substances between tissue and media in which it is prepared and incubated. |
| Effects of all chemical agents used, including those which do not naturally occur in tissues, and those which do occur naturally but in different concentrations than are used in the experiments. |
| Extractability of different agents, including drugs from different fractions. |
| Recovery calibrations of added substances, rather than calibration with pure solutions. |
| Satisfactory method for measuring protein when it is used in comparison of the properties of different fractions. |

## CONTROL EXPERIMENTS

An experiment is only as good as the controls and calibrations used in the course of it. Many of the problems of neurochemistry can be obviated or circumvented by doing appropriate controls, but it still seems as if many of these have been done only haphazardly. This is no substitute for a systematic experimental examination of all the steps in each of the procedures just discussed. A few comments are appropriate about particular control systems.

### *Double blind experiments*

Experiments on human beings require double blind measurements in which neither the doctors giving the treatment nor those analysing the results know to which group the particular drug or placebo is being administered. This optimal situation cannot always be achieved, since the effectiveness or side-effects of a drug may well reveal to the patient that he is being given it. Furthermore, in clinical trials, it is often impossible to find enough patients to achieve a statistically significant result; also in common mental diseases like schizophrenia and manic depression, it is difficult to envisage who might serve as control groups for testing. This problem is best obviated by using patients as controls for themselves whenever this is possible.

In drug trials on patients there may sometimes be the ethical problem that a doctor cannot properly withold a treatment he believes to be effective, even before comprehensive clinical trials have been carried out. In the case of testing the efficacy of lithium salts in the treatment of manic depression, Schou and his colleagues devised a trial in which all the patients were put on the lithium salts which they considered likely to be effective. After an adequate course of treatment, half of the group was taken off it in a double blind situation, and given a placebo and the recurrence of acute disease was measured in both groups. The patients who had been taken off the lithium salts had a highly significantly greater relapse rate (Baastrup *et al.*, 1970).

*Previous and parallel controls*

Control experiments can be classified as 'previous' and 'parallel'. The previous control is that which occurs before the agent is applied, and the parallel control is carried out to similar tissues under similar conditions except that the experimental agent is not applied. The necessity for parallel controls is derived from the possibility of oscillations, product inhibitions and circadian rhythms which have been found in enzyme systems and *in vivo*.

*Controls for lesions*

These represent special difficulties. If one uses the tissue adjacent to the lesion, its blood supply, structure and biochemistry, may have been changed by the proximity of the lesion. The tissue farther away in the same organ may have no histological changes, but it may have been affected either by redistribution of the blood supply in the apparently normal tissue, or by changes in the biochemistry of the tumour itself affecting the normal looking tissue. If one uses tissue well away from the lesion, for example on the other side, one is making the assumption—which is probably warranted—that substances from the lesion have not invaded the cerebrospinal fluid, blood or lymphatic system, or that the lesion has not advanced far enough to produce cachexia.

When a lesion is made on one side which causes a 'compensatory' increase of activity on the other side, it is not appropriate to use the overactive side as a control. For example, if one cuts the sciatic nerve on one side, the activity on the other side is increased well above the normal degree. One cannot then simply compare the biochemistry of a limb with little use with one which is being used much more.

In all such experiments one should use other animals, preferably from the same litters as one group of controls, as well as several other kinds of controls indicated above.

*Inhibitors*

The use of a preparation to which a 'specific' inhibitor has been added implies that its specificity has been tested, not only on a few reactions related to the one under examination, but also that it has been tested on virtually every major pathway. For example, although fluoride, cyanide, ouabain and dinitrophenol are considered to be inhibitors of specific enzyme reactions, it is extremely unlikely that such powerful chemical reagents—in the concentrations in which they have been found to be inhibitory—would have no effect on the rate of any other enzyme reaction, or its rate. Until this has been tested under the particular conditions of the experiment in which it is intended to be used as an inhibitor, 'specific' inhibition must be deemed not to have been demonstrated.

*Boiled and fixed tissue*

Since the use of inhibitors is at present somewhat uncertain, one may consider other control systems which may be used. Boiling the control tissues is an old-fashioned method, although it disrupts and denatures tissues. As has been pointed out, these effects are also produced by homogenisation, so this cannot be used as a reason for not using boiled tissue as one of several control systems.

When one is studying reactions of proteins including enzymes *in vitro*, it is useful to have controls which are of similar composition to the experimental tissue, but which do not metabolise. To this end, 'fixed' controls were employed in experiments using cerebral slices (Wraae *et al.*, 1976). These were immersed in isotonic fixative, which was subsequently washed off for a prolonged period of time. The fixed slices were then incubated in parallel with the cerebral slices whose metabolically dependant reactions were being tested. The use of such fixed tissues enables one to find out how much of the reaction or exchange between the tissue and the medium *in vitro* is not dependant upon metabolism or is 'non-specific'; one can then subtract this degree of reaction from that of the metabolishing tissue, to find out how much and what factors determine the metabolic activity relative to the particular reagent under study. Nevertheless, one has also to be aware of the effects of the fixative itself, and the loss of substances from the tissue during the fixation and subsequent prolonged irrigation.

*The rate of enzyme reactions*

One usually measures enzyme activities by measuring the rate of breakdown of substrate present in excess concentration over the enzyme concentration in which the tissue is incubated. One can use a whole homogenate as an enzyme preparation. In this circumstance, one should exclude possible instability of the substrate due to substances in the homogenate (the enzyme preparation) other than the enzyme itself. This can only be done when all enzymes are destroyed by boiling, or the particular enzyme inhibited by fixation, or by an inhibitor demonstrated to be specific for that enzyme, so that the 'non-specific' breakdown of substrate may be measured and a comparison can be made with the test situation, at the same concentration of the substrate suspected of instability.

Of course, *in vivo* the breakdown of substrate is the relevant parameter, as the substrate itself is not 'concerned' whether it is broken down by the enzyme itself, or if it is aided by instability induced by other constituents or conditions in the homogenate *other* than the enzyme itself. In this circumstance the 'blank' for measuring enzyme activity should *not* be an inhibited preparation, but should be the total incubation mixture without any tissue being added at all. Of course, the properties, including the kinetics, of a homogenate used as an enzyme preparation, may well be very different from those found in crystallised, purified enzymes. Furthermore, many tissues evidently have the property of breaking down particular substrates, but the specific enzymes have never been isolated from them. It is unlikely that any enzyme is isolated from a tissue without altering its chemical, including enzymic, activities, possibly quite drastically.

*Controls for axonal transport*

This phenomenon first described by Weiss and Hiscoe (1948) can be seen as the passage of ions, molecules and particles down an axon, whose constriction can stop it. It is generally believed to be a biological process, because (i) it is 'fast' (1.5–400 mm/day) (Ochs, 1977); (ii) it is slowed down or stopped by the application of colchicine; (iii) it travels in both directions.

The following movements can occur in a tube containing an aqueous suspension of fine particles (Bütschli, 1894): Brownian movement, streaming, convection, diffusion in all directions, laminar flow. The rates of these movements depend upon the sizes, charges and chemical natures, of the moving ions, molecules and particles, and the viscosity and chemical nature of the fluid phase. Therefore, Occam's Razor would require that the movement of such a complex material as axoplasm at, say, 37°C would occur without any necessary metabolic, physiological or biochemical mechanism, due to the interplay of each of these physical phenomena. One need only suppose that axonal transport is a different or additional property to the resultant effects of all the movements, if it can be shown to proceed at a significantly slower or faster rate than could be accounted for by all of them acting together. Such a demonstration has not yet been done. It could be difficult to carry out all these necessary control experiments, but one could start by measuring in capillary tubes of about the same diameters as living axons the rate of movements of different constituents either of real axoplasm taken from squid or myxicola, or artificial solutions of similar chemical composition as the living mammalian axon is believed to be.

*Controls of experiments showing biosynthesis and receptors*

One can show *in vitro* that biosynthesis in addition to 'non-specific' uptake into a tissue is occurring by showing that the substance is taken up more in the presence of substrate and glucose, at a higher temperature between 0 and 37°C, and in the absence of inhibitors. However, even if all these control experiments have been done, one still has to demonstrate a biosynthetic pathway within the tissue, and also show that the major uptake can be accounted for by this pathway. When one tries to establish biosynthesis *in vivo*, one has to use a radioactive technique in a whole animal. Unfortunately, attempts to demonstrate biosynthesis by these criteria highlight the difficulty of the problem both *in vitro* and *in vivo*. Any of the latter tests 'damage' the metabolic machinery very seriously, and one may interpret the effect of a poison in stopping any process by virtue of 'killing' the tissue as specific blocking of a particular pathway. Of course, this confusion compliments the belief in the specificity of an inhibitor when it has not been comprehensively demonstrated experimentally. Generally, biosynthesis has been shown unequivocally *in vitro* by the uptake of precursors, and the uptake of the same precursor *in vivo* is assumed to be evidence that the same mechanism occurs in the living animal. The effects of deprivation of substrates and oxygen or the application of inhibitors is often reversible *in vitro* when the same treatment might kill a whole animal.

*Control experiments for 'bound' enzymes*

Much has been written about 'structure-linked' enzymes. This is usually demonstrated by showing in, for example, a mitochondrial or a lysosomal fraction that the addition of detergents, bile salts, freeze-thawing or sonication, increases its enzyme activity. If the increase is considerable this is taken to mean that one of the above agents 'liberates' the enzyme. A much simpler proposition is that the agents themselves activate the enzymes, possibly with the help of activators present in the tissue, or which have relocated during homogenisation, or which have been activated by other agents added during homogenisation. There is a large hiatus here in our knowledge of what these powerful chemical and physical agents can do to enzymes in the presence and absence of homogenates, which is frequently filled with the expression 'structure-linked'.

*An insoluble fraction*

One may consider that any tissue may be divided into its insoluble components, which react with its soluble components. If one wishes to understand the effect of any naturally occurring reagent or a drug on a tissue, it would seem desirable to analyse its effect on each of these components separately and then to see how each of the main elements of the soluble components affect the reaction of the compound under study with the insoluble fraction. In view of the undesirability of the use in extraction of powerful reagents which themselves might further complicate the reactions, it was decided to prepare the insoluble fraction, using only one reagent, water, to which the tissue is normally exposed. An insoluble fraction of brain was prepared by homogenising brain by hand, using a Potter–Elvejhem homogeniser, dialysing it against a very large volume of water and washing it with a large volume of water. This insoluble fraction showed a very different affinity for sodium than for potassium ions when they were added in a range of 'physiological' concentrations (Ahmed and Hillman, 1982).

This insoluble fraction could be used to measure the degree of 'non-specific' uptake of precursors, drugs and antigens in the absence of metabolism. It could also be used in blanks for flame photometry, when substances diffusing out into the perchloric or trichloracetic acid affect the brightness of the flame. It could be used in general as a blank system for homogenates, to see how a natural mixture might affect measurements of nucleotides, proteins and recoveries in general.

## MEASUREMENTS

Every value derived in an experiment depends upon the parameter of the tissue to which it is referred. Employment of any parameters implies certain beliefs about it; some of the important measurements and their implications are listed in Table 3. From this list certain generalisations may be derived. Firstly, virtually all measurements intended to be relevant to tissue *in vitro* imply that there are no changes in weight of tissue during dying and post-mortem. Secondly, it is assumed that there is no exchange—significant in terms of substances being measured in the experiment—between the tissue and any of the pre-incubation and incubation media in which it is immersed. Thirdly, it is supposed that substances measured such as protein, DNA or insulin are recovered completely from the tissue; this has not always been tested. Fourthly, it is assumed that the latter measurements are not interfered with significantly by other substances present in the tissue. Fifthly, there is unfortunately at present no satisfactory method of measuring protein quantitatively. Proteins in tissues exist in very heterogenerous mixtures and the different techniques each measure different chemical properties of the protein. Probably the new partially optical method of Neuhoff *et al.* (1979) will turn out to be the most accurate so far, as it is relatively independent of the nature of the protein which it is measuring. Sixthly, most of the difficulties of reference to which parameters are made are much less relevant if one is doing an experiment only on one tissue, rather than comparing results from different tissues. Seventhly, in a neurochemical investigation, it is probably wise to consider as the relevant parameter its activity or free concentration in the compartment under study if it is possible to establish this. The rates of biochemical reactions are generally dependant upon activities of the constituents, these normally depend upon their concentrations, the affinity of other substances in its immediate milieu, and the rate of the reaction itself. The 'relevant' activities in the undisturbed metabolising tissue are probably the most difficult parameters to ascertain, and this uncertainty dictates the desirability of carrying out experiments *in vivo*, if possible.

## OPTIMAL PROPERTIES OF DIFFERENT PREPARATIONS

There is still a great volume of new findings potentially to be derived from the use of current techniques, despite the large number of uncertainties and untested assumptions associated with many popular neurochemical techniques. The following general attitudes may be appropriate: (i) avoidance, whenever possible, of disruptive techniques; (ii) analysis of the effects of the chemical and physical reagents used on the parameters and their locations being measured; (iii) simulation wherever possible of 'physiological' conditions for biochemical experiments and therapeutic concentrations in pharamacological experiments; (iv) confining the use of a technique of examination of a particular phenomenon to the study of preparations in which it can be demonstrated.

A list of preparations meeting these criteria largely or completely is given (Table 4). This illustrates the wealth of techniques available, with some crucial notes about each of them. Probably the development of quantitative measurements in brain scanning and

Table 3. Parameters of neural tissue to which measurements are frequently referred, and assumptions necessarily implied in reference to a particular parameter

| Parameter to which measurement is referred | Assumptions implied in these measurements |
|---|---|
| 1. Fresh weight | (i) no change in weight during dying or post-mortem |
| 2. 'Fresh' weight after placing tissue in medium | (i) no change in weight during dying or post-mortem<br>(ii) no net uptake or loss of tissue contents including water |
| 3. Dry weight, either after drying at 105°C for 2 h to constant weight, or at 22°C for 16 h | (i) no change in weight during dying or post-mortem<br>(ii) only water is lost—no other volatile components evaporate or become more soluble to be lost on incubation |
| 4. Freeze dried tissue | (i) and (ii) as for dry weight, 3 |
| 5. Tissue water (difference between fresh weight and dry weight) | (i) and (ii) as for dry weight, 3<br>(iii) there is no movement of substances between dry tissue and water during drying |
| 6. Incubated weight | (i) no change in weight during dying or post-mortem<br>(ii) no net uptake or loss of tissue contents including water, after tissue is placed in preparation medium<br>(iii) no loss of tissue on tissue chopper or cutting device, or in glassware<br>(iv) no net uptake or loss of tissue contents including water, after the tissue is placed in the incubation medium, or that the nature of the fluid which adheres to the tissue is known<br>(v) one can distinguish between increase in weight due to swelling of the tissue and adherence of fluid to it |
| 7. Protein | (i) no breakdown of protein during dying or post-mortem<br>(ii) protein is not lost during incubation<br>(iii) the measurement of protein is not affected by the incubating conditions<br>(iv) the recovery of protein is the same from different tissues or fractions compared or the different chemicals used to separate the fractions<br>(v) protein in tissue (as opposed to pure proteins in free solution) can be measured satisfactorily |
| 8. Desoxyribonucleic acid | (i) no change in DNA during dying or post-mortem<br>(ii) equal quantity of DNA in each cell<br>(iii) complete recovery of DNA from each tissue or fraction<br>(iv) DNA measurement in tissue can be measured satisfactorily |
| 9. Potassium ion concentration | (i) no change in potassium ion during dying or post-mortem<br>(ii) most potassium ion is in cells<br>(iii) the extracellular space is small<br>(iv) the extracellular space *in vivo* can be measured<br>(v) the extraction of the potassium ions is virtually complete<br>(vi) other substances in tissue do not affect the measurement of potassium ions |
| 10. Intracellular concentrations by use of inulin, sucrose, thiocyanate, xylose, arabinose to measure extracellular space | (i) these 'markers' remain extracellular<br>(ii) they do not react with tissues<br>(iii) they are not broken down by tissues<br>(iv) exchanges between tissue compartments do not continue during incubation and measurement<br>(v) that their recovery from tissues is complete and rapid<br>(vi) that the tissue does not interfere with their measurement |

phosphate metabolism *in vivo* in conscious human beings are the most promising techniques for the future, especially in experiments attempting to correlate psychological and neurological states with biochemistry and physiology.

## NEW PREPARATIONS

With these general attitudes in mind, I would like to suggest the wider use of some procedures and put forward some further original systems.

Table 4. Some neurobiological preparations, in which the tissue is metabolising, and the cells or parts of cells under study are largely intact

| Technique | Usefulness | Comments |
|---|---|---|
| Nucleide imaging | Cerebral blood flow, metabolism and pharmacology | Ambulant patient |
| Nuclear magnetic resonance | Phosphate metabolism | Ambulant patient. Non-invasive |
| Clinical trial in patients | Metabolism of drugs | Balance studies and urine measurements |
| Desoxyglucose | Regional glucose uptake | In experimental animal |
| Hypothermic cardiac arrest | Reversible cerebral biochemical, and psychological state | Recovery from cerebral ischaemia |
| Metabolism cage | Drug excretion and effect of drugs and diets on behaviour | Animal can be killed and cerebral biochemistry examined |
| Cerebral focal lesions | Experimental epilepsy | Cerebral changes may be due to lesion not epilepsy |
| Injection into ventricles or onto cerebral surface | Effect of transmitters | Ambulant animal |
| Cannulation cerebral vessels | Regional metabolism of brain | Many samples in conscious animal |
| Externalised carotid injections | Effect of drugs on behaviour in conscious animal | Same animal can be used for several observations |
| Iontophoresis of cerebral and spinal cells | Effect of transmitters | Anaesthetised animal |
| Cerebral biopsy | Cerebral biochemistry | Anaesthetised patient |
| Isolated head | Biochemistry of brain, face and scalp | Most cerebral blood flow controlled |
| Isolated brain | Cerebral biochemistry and control of blood flow | Biochemistry of whole brain only |
| Cerebral slice | Cerebral metabolism and electrical activity | Swelling occurs on incubation |
| Isolated retina | Retinal metabolism | Reacts to light |
| Isolated dorsal sympathetic ganglia | Synaptic biochemistry and transmission | Preparation transmits and conducts |
| Tissue culture of brain and retina | Biochemistry of different cell types | Cells dedifferentiate; only embryonic or cancerous tissue |
| Isolated neurons of brain, spinal cord, sympathetic dorsal ganglia | Enzyme activities; transmitters | Firing not yet demonstrated |
| Peripheral nerve | Biochemistry of conduction and 'axonal transport' | Separated from cell bodies |
| Pacinian corpuscle | Biochemical factors controlling pressure receptor | Not much used yet for biochemical investigators |

Please note that although the preparations themselves fulfil these criteria, it is often necessary to disrupt them subsequently to make measurements in them.

(A). Observing the effects under direct microscopical vision of microinjections into living cells *in vivo* or in tissue culture. These techniques were very widely used before disruptive methods became popular in the 1940's. Many distinguished authors practised them (for example, Cowdry, 1924; Gray, 1931; Heilbrunn, 1956; Chambers and Chambers, 1961; McClung-Jones, 1964). They are complementary to iontophoresis used widely with intracellular electrodes in cells in the brain and spinal cord since the 1950's (see Eccles, 1957). One should resist the temptation to fix, freeze or dehydrate the cells, subsequently in the experiment. In addition to microinjection into cells, micropipettes may be used for withdrawing small samples of intracellular fluids for chemical analysis.

(B). Observations under direct microscopic vision of ocular fundi, peripheral nerves, blood vessels, ganglion cells and the surfaces of brain and spinal cord *in situ*.

[Several other under-used techniques have been mentioned elsewhere (Hillman and Sartory, 1980). The following techniques are believed to be original.]

(C). Examining the behaviour of enzymes in their naturally occurring concentrations in blood, cerebrospinal fluid, ventricular fluid, aqueous humor, vitreous humor, saliva, perspiration and milk.

(D). Comparison of the properties of natural mixtures containing, and purified preparations of, proteins, enzymes, transmitters, etc., in media simulating cerebrospinal fluid, serum, cytoplasm, nucleoplasm, to

see how the properties might change when exposed to environments similar to the natural intracellular and extracellular fluids.

(E). The implantation *in vivo* of microscopic chromatography columns in the central nervous system and its extracellular fluids to examine the chemical and immunological properties of the central nervous system in its own chemical environment. One would have to be aware of the effects of any tissue reaction from the column or its contents.

(F). A total system of biochemistry using reagents which the research worker would be prepared to instil into his own conjunctiva, viz, water, normal saline, physiological saline, serum, cerebrospinal fluid, ocular fluids, tears, synovial fluid, lymph and milk. The principle behind this would be that such fluids would not be expected to denature proteins or cause tissue reactions, which might affect the results of one's experiments.

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