



Certainty and Uncertainty in Biochemical Techniques

Harold Hillman 1972

Summary: '*... biochemistry is in a state of uncertainty because elementary control experiments for complex procedures have never been done. I submit that they should and must be done soon.*' - Harold Hillman.

This summary is an overview, by Rae West, of Harold Hillman's 1972 book *Certainty and Uncertainty in Biochemical Techniques*, with my own comments, followed by passages from the book itself. This book (influenced somewhat by Heisenberg) attempts the difficult task of determining the weaknesses of modern techniques used in biochemistry - from about the end of the Second World War to the present day - which tend to be expensive, elaborate, and to involve well-entrenched interest groups. (Hillman doesn't survey the entire range of techniques; for example optical microscopy, LD50 and other empirical tests, ECGs, and EEGs aren't examined here.) Hillman's book shows that much biological work is probably valueless.

Chapters 1-6 deal with six techniques; each chapter lists steps of its technique, followed by considerable detail step by step, [mostly omitted in my scanned-in sections, below] with lastly a consideration of the assumptions inherent in the technique, with suggested control experiments which ought to be carried out to determine the value of the techniques. This layout isn't strictly adhered to, since, for example, an assumption already described needs only a page reference.

Page 105 arranges the techniques in order of undesirability; however the book's order, though putting the three worst first, puts the remainder in no obvious sequence.

Hillman puts considerable stress on heat generation (and states the whole 'study arose out of some thoughts about the relevance of the experiments on friction carried out by Count Rumford in the 1790s..') and this thermodynamic critique gives his criterion of undesirability on page 105.

The first chapter is the longest; the final six chapters are much shorter than the first six.

Chapter 7 briefly summarises, or re-summarises, what needs to be known to find things out - 'the hiatuses in our knowledge.'

Chapter 8 [scanned IN FULL, below] looks at the individual techniques, briefly, but also includes questions and speculations.

Chapter 9, 'The definition of biochemistry', examines the a maze of different attitudes to the word 'biochemistry', including the motivations for measuring biological constituents, the preparative procedures that make in vivo correspondence unlikely, and blurred distinctions which ought to be kept clear. He comments on the lack of success biochemists have 'enjoyed' when using highly energetic techniques on labile things. And says 'One is continually stressing to students the importance of control

experiments..'

Chapter 10, [scanned IN FULL, below] Practical conclusions says things similar to what have been said, plus, however, comments on vocabulary: emotive words and evasiveness; Appendix I [scanned almost IN FULL, below] expands on this point.

Chapters 11 [IN FULL, below] and 12 [IN FULL, below] try to map out proposed future study, bearing uncertainty in mind.

- Rae West.

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CHAPTER 1: SUBCELLULAR FRACTIONATION

Subcellular fractionation consists of the following steps, each of which will be examined separately:

1. Killing the animal.
2. The dead animal cools.
3. The tissue is diluted.
4. The tissue is homogenized.
5. The preparation is added to a sucrose gradient.
6. The homogenate is centrifuged.
7. The substrate mixture is added.
8. The products of the enzymic reaction are extracted.

9. The products of the reaction are measured.

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Assumptions inherent in techniques attempting to examine subcellular localization of enzymes

Many of the assumptions implied in the use of subcellular fractionation have been discussed with respect to particular steps in the process, but a few further remarks are appropriate after listing the main assumptions.

- (a) That killing the animal has no substantial effect on its biochemistry.**
- (b) That cooling does not induce substantial irreversible change in the isolated tissue.
- (c) That the enzyme activity of a homogenate decreases linearly with dilution.
- (d) That the medium in which the tissue is homogenized, e.g. sucrose - or additives which make organelle separation easier, e.g. ethylene diamine tetracetic or bile salts - do not alter the chemical activity significantly and irreversibly.**
- (e) That the enzyme activity measured finally is not significantly changed by the incomplete replacement of soluble constituents, like cations or amino-acids, which are lost on gross dilution, homogenization and centrifugation of tissue in a different environment.
- (f) That movement during preparation of known co-factors, like calcium or magnesium, or unknown ones, will not alter substantially the apparent location of enzyme activity as measured.*
- (g) That soluble materials originating from any part of the cells will not diffuse into the supernatant during preparation, and thus be supposed to have originated in the cytoplasm.*
- (h) That the heat generated during homogenization is so rapidly conducted away that the temperature does not rise sufficiently high to produce significant irreversible change in enzymes.
- (i) That refrigerating the centrifuge prevents temperature change at the particle surface.*
- (j) That enzyme activities are not irreversibly changed by pressure.**
- (k) That the same amount of work is done on the homogenate in different parts of the centrifuge tube.*
- (l) That the same amount of heat will be generated in parts of the homogenate with differing viscosities.*
- (m) That the extraction from each of the final fractions is equal and complete.**
- (n) That the enzyme preparation has no significant non-enzymic effect on the substrate.
- (o) That the similarity of the appearances on electron microscopy of the parent tissue and its subcellular particles shows that its biochemical properties have no been changed by the fractionation.

*Assumption contradicts laws of thermodynamics or physics

**In some experimental systems this has been shown to be untrue.

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Control experiments for the concentration of enzymes in subcellular fractions

Many such experiments have been mentioned in the description of the steps of subcellular fractionation. The only common control experiments at present carried out are the overall recoveries of enzyme activity in subcellular fractions, compared with the enzyme activity in the crude homogenate. Nevertheless, there are several controls which are possible for the whole of the process. These are:

- (i) the use of boiled tissues as parallel controls for heat-labile enzymes, and subtraction of the values of the apparent enzymic activity of these tissues from the substrate breakdown of the unboiled fractions; these would seem to be absolutely urgent and vital;
- (ii) the division of subcellular fractions into two aliquots, one of which should again be subjected to the whole subcellular preparation system, the test of the change in activity of the latter, and the correction for any loss during the preparation;
- (iii) the test of the effect of the preparative procedure on bacteria producing exo-enzymes this would give an opportunity to measure the effect of subcellular fractionation quantitatively;
- (iv) the mixture of boiled bacteria of known and uniform size with different enzymes in order to control such variables as enzyme and product extractability;
- (v) the admixture of purified enzymes with synthetic poly-amino-acids of known composition and size, to try to derive empirical relationships between the sizes and chemical properties of these well-characterized compounds, on the one hand, and the enzyme activity as a result of a particular fractionation procedures, on the other;
- (vi) the subjection of the pure enzymes in dilute solution, but mixed with suspensions of inert *particles* of known size, to the whole preparative procedure, to test the effect of friction of particles of known size; and
- (vii) the subjection of native proteins, like egg albumen and plasma proteins, to the whole procedure, and examining such properties as their viscosity, their absorption, their binding powers, their pH, and their immunological reactions before and after.

Which of these control experiments are relevant or necessary will depend mainly upon the 'enzyme' activities one is attempting to measure (see page 117).

CHAPTER 2: HISTOCHEMISTRY

Histochemical techniques involve the following steps:

1. Killing the animal.
2. The dead animal cools.

3. The tissue is fixed or frozen.
4. It is dehydrated, freeze-dried or subjected to freeze substitution.
5. It is embedded.
6. The tissue block is cut.
7. The section is mounted.
8. The tissue is deparaffinized.
9. The section is incubated with substrate mixture.
10. The reaction is stopped.
11. The tissue is dehydrated.
12. The section is mounted
13. The section is cleared.

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Assumptions implied in use of histochemical techniques

- (a) There is little post-mortem redistribution or loss of enzyme activity.*
- (b) 'Fixatives' stop overall biochemical activity of tissue, but not the enzyme being studied.**
- (c) The effects of freezing, e.g. shrinkage and intracellular crystallization, do not produce irreversible changes in tissue.
- (d) Different parts of the tissue are dehydrated equally.*
- (e) Warming tissue to embed it affects neither the localization nor the activity of the enzyme measured.**
- (f) That cutting embedded tissue does not cause a significant temperature rise.
- (g) That agents such as formalin, alcohol, or propylene glycol, do not affect the enzyme activity.**
- (h) That incubation in aqueous media does not relocate the enzymes or change their apparent activity.*
- (i) That 'non-specific' uptake of substrate, enzyme product, co-enzyme, stain or indicator does not occur.
- (j) That the location or apparent concentration of enzyme is not affected by incubation in unphysiological media.
- (k) That the staining reaction is not affected by the washing or clearing reagent.

* This assumption contradicts laws of thermodynamics or physics.

** In some experimental systems, this has been shown to be untrue.

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Control Experiments

Many of the problems associated with histochemistry could be circumvented by appropriate controls, some of which are already being done. Experiments to examine the effect of each of the steps are mentioned under the respective headings, The

following experiments would test the whole procedure:

- (i) use of boiled tissue as controls especially for known lab enzymes;
 - (ii) use of tissues irradiated with ultraviolet light, if it inhibits that particular enzyme, as a control;
 - (iii) leaving out the substrate as a control;
 - (iv) incubation with inhibitors exhaustively shown to 'specific';
 - (v) preparation of 'dead' organic materials, like wool or wood in the same way as is done for histochemistry. This would not be expected to have enzymic activity but would be relevant to their localization by histochemical preparation.
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CHAPTER 3: ELECTRON MICROSCOPY

When a tissue is prepared for electron microscopy the steps followed are:

1. Killing the animal (page 1).
2. The dead animal cools (page 5).
3. The tissue is 'fixed' (page 41).
4. It is dehydrated (page 45).
5. The cytoplasm is replaced by a non-aqueous solvent.
6. The tissue is embedded (page 45).
7. It is sectioned (page 46).
8. It is stained (page 47).
9. It is mounted (page 46).
10. It is subjected to an electron beam.

Some further considerations in respect of electron microscopy

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Assumptions implied by use of electron microscopic techniques

(a) Assumptions (a) -(f) as mentioned in connection with histochemical techniques (see page 49).

(b) That there are no significant structures which dissolve or diffuse away in the reagents.

(c) That there are no significant structures which are not stained.

(d) That the organelles originally are equally hydrated in vivo, and will shrink proportionately.**

(e) That the heat and irradiation causes no significant change in the size or shape of the organelles.**

(f) That the similarity of electron micrographs from subcellular fractions, fresh tissue, and fixed tissue, means that electron microscopy has no effect on the preparation.

(g) That ability to distinguish organelles clearly on electron micrographs is

evidence of their biochemical viability before fixation.

****** These assumptions have been found experimentally to be untrue.

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Besides the control experiments mentioned in the text, the following experiments for overall examination of the electron microscopic technique would be highly desirable:

(i) the effect of the whole preparation procedure on materials of biological origin, like wool, leather, wood, and pollen, to identify the artefacts;

(ii) examination by light microscopy of the effects of the agents on the relative volumes of tissue components

(iii) identification of the artefacts found on preparation of pure solutions and suspensions of enzymes, proteins, carbohydrates and fats;

(iv) when histochemistry is done with electron microscopy, boiled tissue or tissue with inhibitors should be used as controls;

(v) wherever possible, normal tissue and tissue from an animal subjected to a particular agent should be studied together. Most of the problems of electron microscopy can be avoided using this approach, but it may well be that the effects of preparation are sufficiently large in the experimental and control tissue to mask induced biological change; and

(vi) more general use of light microscopy of unfixed tissue (Hyden, 1961).

CHAPTER 4: RADIOACTIVE MEASUREMENTS

When radioactive measurements are made of rates or metabolic 'significance' of reactions in biological systems, the following steps are taken:

1. Injection or inhalation or addition of the radioactive isotope.
2. Uptake of the isotope by the tissue.
3. Its involvement in a metabolic pathway.
4. The enzymic activity is stopped.
5. The excess radioactivity is washed off.
6. The substances which have incorporated isotope are extracted, or subjected to autoradiography.
7. The compound structure, homogenate or extract containing the isotope is counted.
8. The results of counting are interpreted.

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Assumptions inherent in radioactive isotope rate measurement techniques

(Many of these can be found in Zilversmit, Entenman & Fishler (1943), and Siri (1949). Several of these assumptions are, however, not necessarily relevant to the identification of metabolic pathways.)

- (a) The precursor will mix almost instantaneously with the tissue.
- (b) The reactions involved are actually or formally of the first order.
- (c) The reactions are effectively 'irreversible'.
- (d) There is random participation of precursor molecules in the reaction.
- (e) The newly formed molecules rapidly equilibrate with those previously present.
- (f) There is no 'compartmentalization' of the precursor or product within the tissue.

Other assumptions in the more general use of radioactive isotopes may be added:

- (g) That the 'non-specific' uptake is negligible compared with
- (h) That the chemistry of the radioactive isotope is the same as the non-radioactive one.
- (i) That the radioactivity does not alter the rate of reaction being studied; and
- (j) that the daughter radioactive species or impurities are not present in significant number.

-[No explicit section on control experiments]

-77: 'Several control experiments have already been mentioned. For tissues in vitro, one can use a dead, boiled, or 'inhibited' sample alongside the experimental ones.

In vivo , the following experiments are suggested:

- (i) the examination of the uptake of radioactive tracers into different tissues of a dead animal, using rapid postmortem fixation by perfusion (Brown & Brierley, 1968);
- (ii) the incubation of boiled homogenates and subcellular fractions with the radioactive tracers, and their subsequent complete chemical analysis to define the fate of precursors in the absence of metabolism. The measurements from both these series of experiments should be subtracted from the results of metabolic experiments;
- (iii) as (ii) but with the nonradioactive compounds in physiological concentrations;
- (iv) total recovery experiments for all the radioactive markers used;
- (v) calibration curves with tissue present, not only on pure solutions of the non-radioactive tracer, or the compound in which its incorporation is being studied;
- (vi) analytical experiments to show that the tracer is mainly and most rapidly incorporated into the pathway being studied; there is much metabolic data already available;
- (vii) analytical experiments to show that the incorporation is only occurring at one identifiable site, i.e. not across the blood-brain barrier, the kidney, or the wall of the intestine, which are multi-component systems comprising several anatomical sites and biochemical pathways.

It is clear that control studies for experiments in vivo are much more difficult, but, nevertheless, should be done if we wish to analyse the results of radioactive tracer experiments meaningfully.

CHAPTER 5: ELECTROPHORESIS

Such separations usually consist of the following steps:

1. Killing the animal (see page 1).
2. The tissue is excised (see page 6).
3. The tissue is homogenized (see page 12).
4. The proteins, lipids, or nucleotides, are extracted.
5. The extract is placed on a gel or paper with buffer and solvents.
6. An electric current is passed through the buffer while it is being cooled externally.
7. The protein, lipid, or nucleotide, is separated, eluted, or observed.
8. The concentration of the substrate in the band or eluate is measured chemically or densitometrically.

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Assumptions of electrophoretic techniques

- (a) All the assumptions appropriate to chromatography (see page 92).
- (b) That the heat generated by the electrical or magnetic fields, will not be dissipated in the preparation being separated.*
- (c) That cooling prevents the temperature rising significantly (see page 15).
- (d) That clear separation of peaks implies that the compounds in vivo were distinct, i.e. that bonds have not been broken by the procedure.
- (e) That polymerization or acceleration of reactions between compounds not normally associated does not occur.
- (f) That separation of a biological activity in a peak implies that the biological activity has not been decreased by the separation.

* This assumption contradicts the laws of physics.

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Control experiments for electrophoretic techniques

- (i) Measurement of the loss of biological and chemical activity of temperature-sensitive substances.
- (ii) Recovery experiments for the whole electrophoretic system.
- (iii) Examination of the movements of co-factors in the electrophoretic field, as their migration would affect the enzyme activity detected.

CHAPTER 6: CHROMATOGRAPHY

Chromatography consists of the following stages:

1. Killing the animal (page 1).
2. The dead animal cools (page 5).
3. The tissue is diluted (page 8).
4. The tissue is homogenized (page 12).
5. An extract of the lipids, carbohydrates, or amino-acids is made (page 79).
6. The extract is buffered, and then added to a column, paper or liquid, or put into a gas flow.
7. Solvents are passed in one or two dimensions.
8. Paper is dried.
9. The column is eluted, or the paper is developed.
10. The quantity of the material is measured chemically, densitometrically, isotopically, or by thermal conductivity ionization, or electrical conductivity techniques.

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Assumptions implied in the use of chromatography

(a) All those assumptions appropriate to killing the animal, homogenizing the tissue, and extracting the mixture to be chromatographed.

(b) Adding the extract in a solvent system to the paper or column does not induce significant temperature rise.**

(c) That the same amount of heat would be generated at different points between the origin and the solvent front.*

(d) That 'washing' the column causes no significant loss of the compound or change in its reactivity with the column.**

(e) That drying the spot on the paper does not cause significant irreversible change in its chemistry, e.g. by polymerization, crystallization or dehydration.

(f) That a second solvent system has no effect on the chemistry of the separated compounds.

(g) That desorption or elution of the bands or spots with other solvents does not cause significant temperature rise.

(h) That the measuring system does not degrade the compounds in the bands or spots.

* This assumption contradicts laws of thermodynamics or physics.

** In some experimental systems, this has been shown to be untrue.

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Control Experiments

- (i) All the controls appropriate to the first four steps mentioned previously.
- (ii) Comparison of the activity of a known purified enzyme at each stage of the chromatographic procedure, and overall recovery of the whole process.
- (iii) As (i), but with the addition of excess known purified enzyme to a homogenate.
- (iv) Systematic study of the chemical reactions of paper and column materials together with the substance being separated.
- (v) Examination of the effect of the solvents and the eluting solutions on the enzyme activities.

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CHAPTER 8: THE INDIVIDUAL TECHNIQUES [In Full]

Subcellular fractionation

So many and unlikely are the assumptions involved in subcellular fractionation (page 33) that it is highly unlikely that comparisons of chemical activities between different fractions have any value at all. In the normally accepted procedures, different amounts of work are done in different parts of the centrifuge tubes and different fractions to be compared are centrifuged for different times with added co-factors. The only possible way the experimental results could be justified would be to show that the effects of the procedures were extremely small compared with the parameters which were being measured. These demonstrations have yet to be made.

We should perhaps distinguish between two different kinds of ignorance about subcellular fractions. Firstly, there are the endoplasmic reticula, sarcoplasmic reticula, nuclear pores, etc., which must be artefacts (see page 59). Secondly, there are the nuclei, the mitochondria, and the cell membranes, which can be seen by light microscopy; it would seem quite possible that quantitatively we know very little about their relative biochemical activities. This means that we cannot assess the meaning of any sentence starting, 'mitochondrial fractions' or 'ribosomes' or 'membrane fractions' etc.

It is often said that subcellular fractionation techniques have greatly expanded our knowledge of biochemistry. If all the quantitative information is uncertain, what phenomena which were not previously known in crude homogenates can be regarded as having been discovered by these methods?

Histochemistry

This technique is at its best only descriptive. It cannot be quantitative except in a comparative way because it cannot be calibrated. If one finds an enzyme activity at a particular site one can accept that a certain but unknown proportion of that originally present at that site has survived there or has come from elsewhere; if the enzyme is soluble it may only be a very small proportion. If, on the other hand, one does not detect an expected enzyme, we cannot know whether or not the substrate, co-factors or

indicator have diffused away, or, indeed, have any affinity with the site. The observation that enzymes detected histochemically are usually seen on membranes is not without implications.

Electron microscopy

This is such a drastic procedure that the new information which has been found by it is also very limited. It is a technique examining the leather rather than the skin (see page 103). Because of our ignorance about the complex chemistry and precipitating properties of chemical constituents of living tissue, we cannot know whether any structure seen is an artefact or not, unless it has previously been observed by light microscopy. This is especially true for the cytoplasm which is a suspension in vivo to which we add non-miscible organic solvents.

The relative shrinkage of different parts of cells - which has not been measured - does not permit us to make any measurements of the relative sizes of organelles or the spaces between them. Nor can we know the shapes of particles, since the osmium salts are a shadow and the intense radiation probably makes particles tend to regain their minimum diameters.

With electron microscopy, unlike subcellular fractionation, most of the artefacts and difficulties have been fully described and research workers discuss them publicly. Yet they continue to carry out the techniques as if such limitations had never been demonstrated.

Electron microscopy is a very clear example of an uncertain technique. It is interesting that it was developed for the study of very stable inorganic materials and then taken over by the biologists. In order to examine tissue we must fix, dehydrate and irradiate it. If any part of it is significantly sensitive to any of these steps, we will never see it properly. Yet we have plenty of information about the sensitivity.

The usefulness of the technique is mainly in demonstrating osmiophilic structures without indication of their relative sizes or shapes. It can only give information about labile materials within the system if they have been firmly bound to the tissue before preparation. Even here its usefulness is dependent upon the staining material not affecting the labile activity.

Electron microscopy can also be used to compare normal and abnormal tissue from the same organ but usually the resultant description is only a verbal one. There is pantheon of bodies with particular shapes - Golgi bodies, vesicular bodies - and a family of some of which Galsworthy would have been proud. They have been given living characteristics. Synaptic vesicles have been filled with acetylcholine, although it has been calculated that the amount of acetylcholine liberated during electrical activity would require them to be solid acetylcholine. Lysosomes have been denigrated as 'suicide bags' and theories have been adumbrated to show how they avoid catabolizing themselves,

Electron microscopy was developed for metallurgical purposes. Here the substances studied are inorganic, stable and usually crystalline. Biological tissue is relatively unstable, aqueous and heterogenous.

We may summarize conclusions about subcellular fractionation, histochemistry, and electron microscopy by saying that they may be useful to compare normal and

abnormal tissue. They may also contribute to histology. What little information histochemistry gives us of biochemical relevance is only about the water-insoluble organelles.

Radioactive measurements

These techniques have made important contributions to our knowledge of metabolic pathways. They have a significant advantage in that they are relatively non-energetic and thus do not disturb the tissues which they are being used to examine. However, both in vivo and in vitro, they are not by any means as specific as their protagonists would like them to be, mainly due to the several assumptions which their use implies. In vivo it is often difficult to do the necessary control experiments and, therefore, isotopes are far more useful for detecting metabolic pathways than in measuring the rates of reactions within them.

Many of the alleged quantitative results of experiments have ignored the assumptions listed and understood by the pioneers (see page 74). In vitro, the control experiments are much easier (see page 70) but there are many extra problems related to the unusual environment of the tissue.

The prime advantage of radioactive measurements is that they may avoid the necessity for killing the animal, taking out the tissue and extracting it. They can be used in dynamic studies with several observations on the same animal or acute killing and counting of a whole organ. In many senses, radioactive techniques are the least uncertain. They can sometimes be made quantitative even in vivo.

Electrophoresis

As has been pointed out, this technique is used successfully to separate various biological materials (page 86). The heat generated within the system can be assessed and it is fairly high, although spread over a long period. The cooling and the smallness of the quantity of the extract applied are evidently both effective in preventing excessive temperature rise, otherwise labile biological materials would lose their activity. The measure of recovery of a labile activity is the best criterion for the suitability of a technique for biological studies. Nevertheless, complete recoveries of all isoenzymes, serum proteins, etc., should be done, as there is undoubtedly a considerable heat gradient along the electrophoretic strip.

Electrophoresis is an example of one technique in which the suggested parameters of rate of rise of heat generation and the constant level of heat generation, could easily be indicated.

Chromatography

This is probably the 'mildest' technique involving extraction. It is often used for separating fairly small molecules. Heat is generated during the absorption or desorption but the quantities involved are extremely small and the system approximates very closely to an open one. However, we should recollect that all the other steps in the procedure have already been taken (see page 89); and we are adding a minute extract to the column or paper. It would be more desirable to develop those techniques in which a piece of tissue is placed on the column and the minimum number of solvents extract

and separate it at the same time.

A considerable advantage of chromatography is that it is comparatively easy to use a known quantity of a known substance both as a marker and to measure recovery.

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CHAPTER 9: THE DEFINITION OF BIOCHEMISTRY

105: 'Even without such measurements, we may rank the techniques in view of our separate examination of each, in increasing order of their probable generation of heat and consequent undesirability: radioactive techniques < chromatography < electrophoresis < histochemistry < subcellular fractionation < electron microscopy. This exercise is often not possible as the techniques are not necessarily alternatives. ..'

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CHAPTER 10: PRACTICAL CONCLUSIONS [In Full]

We may put forward a few practical suggestions for the jobbing research biochemist making quantitative measurements intended to be relevant to a situation in vivo (see definition of biochemistry, (d), page 103).

1. Techniques involving homogenization centrifugation, and sonication - all of which generate heat - should be avoided if possible. If unavoidable, they should be done in pre-cooled conditions, minimally, and as slowly as possible.
2. Open systems, with small quantities of materials in thermally well-conducting vessels, are to be preferred to isolated or closed systems, especially with bulk materials
3. Quantitative experiments should never be done without adequate recovery measurements and calibration curves in the presence of tissue.
4. 'Physiological' concentrations, pHs, co-factors, temperatures, and other conditions, should be simulated wherever possible.
5. If the effect of a technique is to change the system randomly more than the difference being detected, that technique produces uncertain results and should be abandoned.
6. Emotive words, like 'contamination', 'optimum', 'specific', 'ageing', 'mild' should be avoided in descriptions of experiments. The actual measurement or operation should be stated (see page 115).
7. Assumptions inherent in any technique whose use is contemplated should be systematically listed. If they have not been tested satisfactorily by earlier workers, the novice should examine them before embarking on the technique. If assumptions have not been examined at all, or cannot be for practical or theoretical reasons, they should nevertheless be recorded in publications, even if distinguished pioneers have omitted to do so.

8. Verbal evasions of questions, citations of similarly behaving authority, and expressions like 'I would think such effects are minimal' are no substitute for properly controlled experiments.

CHAPTER 11: PROPOSED STRATEGY FOR BIOCHEMISTRY [In Full]

The burden of this study is not that all experiments *in vitro* are suspect; rather that an experiment is only as good as its controls. If no controls have been done, which could have been done, the experiment is probably valueless. Biochemistry, which originally studied living tissues, has been carried away by an enthusiasm for physical techniques which may change the nature of the study so that what we discover is more a function of the method we use than the properties we seek to elucidate. In the author's opinion this has largely been the result of the application of high energy to unstable materials. We have learnt techniques from industrial chemists who use them for separating cream, examining ore crystals, or dyeing wool.

Our first task is to find out how far away from the living system we are, and I have suggested that an early step should be to study the biochemical changes that occur during dying and during the excision of tissues. This is fraught with difficulties because of the very rapid agonal changes; but until we can extrapolate back across this chasm of ignorance we will continue to study tissues that are running down - without knowing how far they have run.

Our second series of measures must be serious investigation of the effects of our techniques on the systems we are studying. This is a plea for scientists to use the elementary control experiments which are normally commended to schoolboys. It should not be necessary to make this point so long after Bacon, Harvey, Descartes and Fisher. We are attempting to measure the certainty of our methods upon which the validity of our findings depends otherwise unsupported. Furthermore, if we find that there is too much uncertainty due to a particular technique, we must take our courage in our hands and abandon that technique.

We can assess the uncertainty in a quantitative way. Though it will be tedious we should attempt to measure the rate of heat generation or temperature rise for each technique. We will know beforehand whether it can be used or not by comparing it with the known heat stability of biological materials. It is worth asking the question *en passant*: if a whole mammal cannot tolerate a body temperature of 42°C for long, why should its tissues?

Another aspect of the uncertainty is the degree to which the changes which occur during tissue preparation are reversible. These could be expressed as the ratio of the final to the original activity of the system. In this respect we may ask whether large molecules, other than proteins, 'denature'. The several physical measurements which shelter under this title could well be applied to an examination of whether similar partially reversible changes in the conformation can occur in carbohydrates, lipids, steroids, etc. It seems to be a useful working hypothesis to regard denaturation as a float in the cavalcade of dying. If we could reverse denaturation of tissues completely, we might jam the trigger of death, though this would probably have more unpleasant

and unexpected effects than mankind has ever previously produced.

One way of avoiding uncertainty is to make intensive studies of natural completely unprocessed materials taken from the body before measuring their properties. Such examination could involve urine, saliva, cerebro-spinal fluid, and gastric juice, as well as 'blood, sweat and tears', which all contain extracellular fluids to which we have easy access. Instead of extracting enzymes from them and measuring their maximum activity at an extreme pH, we should be studying properties of naturally occurring enzymes in native mixtures at body pH and temperature. Furthermore in anaesthetized animals, we could study the ocular fluids, the synovial fluids, cartilage, bone, the uterus, the intestine, the kidney, etc., *in situ*.

In vitro there are, of course, many useful experiments that may be done. If a cerebral slice concentrates potassium ions ten times against the medium and requires energy to do so, or if an isolated ganglion transmits impulses, or if a separated perfused heart goes on beating, we are certainly wise to use them to study the mechanisms underlying these phenomena. We cannot, however, rest with the result unless we have explored and demonstrated its relationship to the tissue *in vivo*. With these preparations elicitation of as many of the 'functional' properties as we can that are found *in vivo* will immensely increase their value.

We may indicate other areas of experiment whose more active pursuit might appear advantageous. Firstly, we have the study of single cells, nuclei and giant axons, isolated by hand dissection, especially if they can be shown to retain excitability. Such techniques have been pioneered by Purkinje, Hyden, Pope and Lowry among others. Secondly, we have the artificial membranes made of natural and synthetic extracts by such people as Rudin and Bangham. Thirdly, we can define the properties of well characterized membranes so that we may have a basic physico-chemical knowledge about the behaviour of relatively simple systems; by comparing their properties with those of biological membranes *in vitro* and *in vivo* we can advance our understanding of the latter situation.

A further attitude to experiment which seems valuable is the scrutiny of the non-enzymic properties of biological systems. This is not quite the same as the controls for enzyme experiments. An enzyme is said to accelerate a reaction which would go very slowly indeed without it. It is therefore desirable to collect information about what dead tissue does to substrates of all kinds. This would give us the basic behaviour, deviation from which we can regard as the cunning of biochemistry.

There are two new systems of study which I would like to propose. One of them is the comparison of the behaviour of pure single amino-acids, proteins, carbohydrates, lipids, etc., in three distinct media. These three media are (i) as close a simulation as possible of the extracellular fluid, e.g. cerebrospinal fluid, plasma or Krebs-Ringer solution; (ii) a medium made up with the same constituents as the closest approximation we can design to the intracellular fluid; and (iii) a medium simulating the nucleoplasm as closely as possible. Comparison of the spectra, conductivity and charges of, say, albumen in these three salines at physiological pH, temperatures, and concentrations would throw light on how it might behave in the only conditions to which it is normally subjected. One would then gradually add more complex and esoteric constituents and build up a model of the physico-chemical behaviour of large

biological molecules.

The other system I would like to mention is also new. If present beliefs about subcellular organelles prove misleading we can look for an alternative in Nature. Since we can see the nucleus, the cell membrane and the mitochondria under a light microscope, they cannot be soluble in the cytoplasm. I would, therefore propose a simple separation system based on this fact. Briefly, one would disrupt the tissue by hand homogenization slowly in a large known volume of distilled water which would also act osmotically; this would depend on the absence of significant denaturation. The resultant mixture would then be filtered, if possible. The residue would be slowly evaporated to dryness at a temperature not exceeding 37°C to be reconstituted when needed.

This may be designated the insoluble fraction. The filtrate would be concentrated, also at a temperature not exceeding 37°C, to approximately its original volume and kept in a refrigerator. Slow filtration to separate the nuclei and, or mitochondria might be feasible, but the energy necessary to do so would have to be looked at very closely.

CHAPTER 12: SUMMARY AND CONCLUSIONS [In Full]

A great deal of the modern biochemistry of tissues in vitro is done with unknowing disregard of the laws of thermodynamics and physics. I would conclude that the validation of some of the most popular techniques in world-wide use is grossly overdue. Until and unless this is done, all the findings based on them must be regarded as unproven. If the necessary control experiments should fail to validate the techniques, the techniques should be abandoned. At the moment biochemistry is in a state of uncertainty because elementary control experiments for complex procedures have never been done. I submit that they should and must be done soon.

APPENDIX 1: [scanned almost in full] MISLEADING SYNONYMS

Term	Popular misuse
(a) Activation detected	Increased enzyme activity
(b) Active transport	Concentration different than the medium
(c) Ageing mitochondria	Properties changing with time
(d) Biosynthesis	Uptake of radioactive precursor
(e) Bound substances	Increased extraction by another agent
(f) Contamination	Unwanted enzyme activity
(g) Cytosol	Supernatant
(h) Denaturation	Change of physical properties of protein
(i) Enzyme activity	Breakdown of substrate
(j) Extracellular space	Inulin, sucrose, or raffinose space
(k) Fixation	Arrest of histological change

(l) Inhibition	Decreased enzyme activity detected
(m) Leached	Diffused
(n) Membranes	Subcellular fraction
(o) Mild centrifugation	1000 to 10,000 g
(p) Non-specific uptake	Tissue uptake
(q) Optimum enzyme activity	Maximum enzyme activity
(r) Purification	Increased enzyme activity detected/mg protein
(s) Recovery of activity	Percentage of total activity at end of preparation
(t) Solubilization	Addition of detergents
(u) Transport	Diffusion

The loose use of operational terms often obscures biochemical inexactitudes and assumptions. A few words must be said about some of them.

(a) Activation is often wrongly used to mean that the addition of a particular ion or a number of co-factors to an enzyme preparation increases the quantity of product detected. The apparently increased yield may arise from decreased stability of the substrate, acceleration of the enzyme reaction, increased substrate extraction, or alteration of optical properties of the whole system. The term should really be reserved for substances which have a stoichiometric relationship to the enzyme reaction.

(b) Active transport is not merely the concentration or exclusion of a substance by the tissue. It must have been shown to be against the electrochemical gradient and dependent on the supply of energy. It is not just a partition of a substance in favour of one or other phase of a system.

(c) Ageing of 'mitochondrial' preparations is the change of their properties with time following their isolation. Its existence implies that the effects of the separation procedure outlast the separation itself; the preparations have not reached equilibrium during the earlier measurements following fractionation. Ageing could be a phenomenon similar to dying and its characterization would be useful if one believes in the biochemical significance of 'mitochondrial' preparations.

(d) Biosynthesis is often used as a synonym for the uptake of a known radioactive precursor. However, it should imply much more. The term only has meaning if it has been shown that there is significantly more uptake in the presence of substrate and oxygen than in its absence, that there is significantly more of the precursor in that pathway than 'non-specifically', and that the extractant takes out very little of any product not involved in the pathway postulated. In practice all of these conditions are difficult to satisfy in experiments on living animals.

(e) Bound substances like calcium ions, ribonucleic acid, or proteins, are often so characterized because strong acids, bile salts, detergents, or sonication yield larger quantities from a preparation. This ignores the real possibility that these agents themselves have a special affinity for the 'bound' materials or actually increase the enzyme activity themselves.

(f) Contamination is an emotive term implying that an enzyme activity which is believed to reside exclusively in a fraction in which one is not interested, is found in significant concentration in the fraction to which one has not directed one's attention. The belief behind the concept is that there are enzymes which occur exclusively in one organelle. Since the 'purity' of the fraction is measured by the absence of other enzyme activity, there is no certain way of knowing by these techniques whether there are specific marker-enzymes of particular organelles. Indeed, belief in them makes cellular biochemistry much neater but in view of all the assumptions upon which the belief is based (page 33), it is more in the realm of an aesthetic than an axiom. Here we have uncertainty.

(g) Cytosol is sometimes used as a synonym for the supernatant and such misuse implies that any soluble enzyme present in any cellular organelle in vivo would not diffuse into the supernatant during the preparation (see page 34, (g)).

(h) Denaturation can be used to mean changes in optical, physical or chemical properties (Joly, 1965) - if we may risk such dangerous distinctions. Clearly, in a description of techniques one must specify which particular technique is being used since they each may give different answers.

(i) Enzyme activity is not the same as the ability of the tissue to cause breakdown of substrate. The latter is a function of the total chemical environment of the substrate, including the presence in the preparation of activators, inhibitors, co-enzymes, a source of energy, etc., as well as the pure enzymes (see page 30).

Many substrates, such as cytochrome, or succinate, are very unstable themselves. Lactate and ATP, for example, absorb light. Their instability is increased by warming to 37°C. Any oxygenated mixture containing ions, buffers, and tissue, is liable to break down some substrate. We must draw a clear distinction between two circumstances. Firstly, the activity on a substrate which a tissue in vivo has at a particular moment in the absence of chemical addition of historical accident; this is a single, not necessarily controlled observation and its results cannot be attributed only to enzyme activity; secondly, we have a preparation which we have controlled for all non-enzymic activity by doing relevant preliminary and parallel experiments; in this case, we must subtract the non-enzymic activity from the total activity as measured in the complete preparation.

(j) Extracellular space. Research workers have claimed since the 1940s to measure extracellular space with inulin, sucrose, thiocyanate, raffinose and chloride. There was originally no evidence that they remained extracellular although their tissue concentrations are less than the medium concentrations. It was once supposed, and subsequently believed, that this itself constituted evidence that they did not enter cells. They could be degraded by tissue, react with it: or be repelled by it. In deference to the latter considerations, the original use of the term 'extracellular space' when inulin or sucrose space had been measured has recently given way to the expressions 'inulin' or 'sucrose spaces'; but their proponents still imply that they measure extracellular space, otherwise they would have abandoned using them.

(k) Fixation implies that because one has arrested obvious microscopic change, all biochemical and physico-chemical change within the system has been virtually halted, except in certain histochemical experiments where the fixative obligingly spares the

enzymes we are studying (page 41).

(l) Inhibition has a well understood meaning in classical enzymology which is much more than the decrease of product due to the addition of a small quantity of inhibitor (please see 'activator' above). A specific inhibitor is presumably one that in low concentration decreases the rate of *only one* reaction stoichiometrically. The idea is usually taken to imply that it would not inhibit any other pathway significantly. This belief is often based on an extrapolation from perhaps two or three other reactions of the many thousand of which any viable tissue is capable. Clearly, this is statistically unacceptable. Just because an inhibitor affects a particular reaction in a homogenate furnished to exhibit one enzyme activity at its maximum rate, it does not mean that it would not affect other reactions of the same homogenate incubated under other conditions; nor does it mean *a fortiori* that in a more organized tissue, such as an isolated organ or a whole animal, the inhibition would be at the same site, as specific, or not counteracted by regulatory mechanisms. This latter extrapolation cannot be permitted with the knowledge that aspirin 'uncouples' oxidative phosphorylation extremely effectively.

(m) Leached [omitted]

(n) Membranes. The use of the word 'membranes' in respect of a subcellular fraction in which membrane fragments can be identified histologically should mean that the largest proportion by volume of the material in the fraction originates from the cells' membranes; further, that there is no significant contribution from other material which cannot be identified on electron microscopy or which has similar enzymatic activity. Often, however, it means only that in the particular fraction isolated by a well-defined series of steps, a particular particle or organelle is most commonly identified, not that most of the mass of the fraction is that particular organelle. This may be alright with mitochondria and nuclei, but it is very doubtful with more amorphous fractions containing 'endoplasmic reticulum', or 'microsomes'.

In separations of 'neuron' and 'neuroglial' fractions this distinction between a fraction of a pure cell and the only one in which that kind of cell can be identified, is clarified by the use of the words 'neuroglial fraction' or 'neuroglial-rich' fraction. Whereas the former would be expected to have the biochemical characteristics of neuroglia alone, the latter would not. In respect of the more amorphous subcellular fractions, there is no reason whatsoever to believe that much of the material seen does not originate from the breakage of other organelles. Individual particles are too small and irregular to compare with structures seen in electron-micrographs.

(o) Mild centrifugation, meaning 1,000 to 10,000 g, is to be compared, presumably, with 100,000 g. Among other consequences of the former treatment would be a pressure of 1 to 10 atmospheres (see pages 19-29). The use of the adjective 'mild' implies that it would have little or no effect on the physico-chemical properties of the tissue so treated.

(p) Non-specific uptake of radioactive precursors, for example, is often used as an explanation for an uptake in which the research worker is not interested. It may mean only that the substance being studied is partitioned between the medium and the tissue in favour of the latter in the absence of an energy source.

(q) Optimum enzyme activity [omitted]

(r) Purification of an enzyme may mean either that as a result of a preparative procedure a higher concentration of enzyme activity can be measured than before, or that non-active material has been separated from the enzyme preparation. Another interpretation would be that some steps in the preparative procedure might have increased the activity themselves, for example, by relocating co-factors, by generating heat, by changing the protein conformation, etc. The opposite may be said of loss of enzyme activity during preparation (see page 39).

(s) Recovery of enzyme activity following subcellular fractionation usually means the total enzyme activity of the subcellular fractions as a proportion of the activity of the crude homogenate. When this proportion is low the enzyme activity of a particular fraction is often expressed as a percentage of the sum total of all the activities in the final fractions added together. This has been discussed (see page 39). What the recovery should aim to measure is the total of activity in all the final fractions as a proportion of the tissue before it was homogenized. A method for doing this has been suggested (see page 16).

(t) Solubilization of a fraction often means the addition of a synthetic or natural detergent. When tissue becomes soluble its chemical properties must have changed.

(u) Transport can be defined as movement of particles or molecules, but its use often implies a mechanism for the movement. Diffusion is one possible mechanism; there are many others (see Bayliss, 1959; Wilbrandt & Rosenberg, 1961; Harris, 1960). Diffusion does not require a carrier, a semi-permeable membrane, special affinity of a tissue constituent, or any other external machinery; therefore, Occam's razor encourages us to adduce it as a mechanism for transport before we look for more complicated possibilities. By the same token, unless we have demonstrated unequivocally that it is not the means of transport, we should not attribute it to another.

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