Difficulties with Current Research in Cell Biology and Suggested Solutions

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Introduction

Since the Second World War, there has been a massive expansion in the study of cell biology, but there have been relatively few, if any, critical examinations of the theory and assumptions of its techniques, and such examinations are overdue. Much of biochemistry has been carried out ignoring the laws of thermodynamics, and many of the findings of electron microscopy have not demonstrated the solid geometry of three-dimensional objects. Concerns about these research methods extend not only to the biochemistry of normal cells, but also to fundamental research into the genesis of many cellular diseases, including carcinoma, sarcoma, leukemia, muscular dystrophy, multiple sclerosis and Alzheimer's dementia. Approaches to improve obedience to the laws of thermodynamics and solid geometry are proposed.

Definition

One may define the aim of cell biologists, as to elucidate the structure and chemistry of cells of animals and plants, in such a way that the measurements are not affected significantly by the procedures used to examine them.

A. The Problems

1. The Second Law of Thermodynamics

Living systems are described as open, because materials and energy can exchange freely with their environments. However, it is extremely difficult to make calculations in open systems, because at any particular instant, one can not measure accurately the concentrations of compounds, and the activities and rates of reactions. Most physiological events occur in milliseconds to seconds, while measurements take from minutes to hours.

Unlike living organisms, most experiments in biochemistry are carried out in partially or completely closed systems, in which energy but not materials can be exchanged with their

environments. The systems are almost closed because the tissue, the media, water, reagents, homogenising and centrifuge tubes, all conduct heat poorly. The energy generated by homogenisation, centrifugation, chemical reaction, etc, can not dissipate rapidly, so that the temperature rises. The experiments are, therefore, partially open and partially closed, which makes precise and meaningful calculations extremely difficult.

Homogenising tubes are cooled from the outside by ice; centrifuges are refrigerated, and the air is evacuated from them. The cooling increases the viscosity of all the elements of the system, including the reaction mixtures, and, therefore, increases the energy generated. The cooling of the homogeniser and the centrifuge *increases* the gradient between the reaction mixture and its environment, so that the energy once generated is dissipated more rapidly.

The degree to which biochemical experiments can be considered closed is a measure to the accuracy of calculations made in obedience to the Second Law of Thermodynamics. In the part of the system, which is indeed closed, any significant change in entropy must be accompanied by a change in free energy. Free energy drives all chemical reactions, determining their rates and equilibria. The particular reactions studied are components of the interacting metabolic pathways and cycles, so that any change of entropy or free energy of any part of the system is likely to affect the metabolism widely.

Changes in entropy, (e), and free energy, (f), occur during the following steps of any procedure: dying of the animal, (e); addition of sucrose, edta or deoxycholate (e, f); homogenisation (e); centrifugation (e); rinsing tissue (e); dilution (e); concentration (e); filtration (e); exothermic and endothermic reactions (e, f); boiling (e, f); freezing (e, f); extraction (e); elution (e), precipitation (e); dehydration (e, f); evaporation (e, f); freeze-drying (e, f); solubilisation (e, f); electrophoresis (e, f); chromatography (e). The changes in entropy and free energy occur with respect to the original biochemical state in the living intact animal, and to the previous steps of the whole procedure.

2. **Subcellular fractionation**

Since the 1950's, this procedure has been the very widely used for characterising the chemistry of the organelles, the nuclei, mitochondria and cell membranes, and the location of particular chemical reactions within them. Reactions measured in the subcellular fractions are assumed to be the same reactions at approximately the same rates, as they occur in the cells in the original intact cells in the living animals.

The main steps in the procedure to examine the chemistry, for example, of the nuclei of rat liver, are: the animal is restrained and killed; its abdomen is opened; the liver is

excised; sucrose is added; the liver is homogenised in a cooled tube; the homogenate is centrifuged and separates into layers; the layers are examined under a microscope to determine which contain the most nuclei; the chemical properties of that layer are examined.

The overall assumption of the use of this procedure – rarely stated – is that none of the reagents or manoeuvres alters the rates or equilibria of the reactions being studied. In 1972, I identified 18 different assumptions inherent in the use of this procedure, and by 2008, the list had grown to 23. These included that: homogenisation and centrifugation would have no significant effects on the chemistry; that the same g force is exerted on different parts of the centrifuge tube; that diffusion does not occur during homogenisation and centrifugation; that agents such as sucrose, edta, tris and deoxycholate have no significant effects on the chemistry of the fractions; that the contents of the fraction, other than those by which it is designated, or believed to be enriched, make no significant contribution to the chemistry of the fraction, etc, etc. The trouble is that the validity of the whole procedure depends upon the warrantability of all, including the weakest assumptions, as the strength of a chain depends upon its weakest link.

3. **Electron Microscopy**

The electron microscope was first applied to biological tissues in the 1940's. Since then, it has been widely regarded as superceding the light microscope in providing information about cell structure. Under optimum conditions the electron microscope has a resolution of about 1-10nm, whereas the resolution of the light microscope is 200-250nm. However, the electron instrument has important disadvantages. Living tissue could not survive low pressure, electron bombardment and irradiation, so that the living tissue must be fixed (killed), dehydrated, stained with heavy metal salts, cut into very thin sections, embedded, subjected to low pressure, and bombarded by electrons. The electron micrograph gives an image of those parts of cells upon which the heavy metals has finally been deposited. Structures would *not* be seen: if they did not react with the stain; if they were liquid; if they were broken down by, or soluble in, any of the reagents used in the preparation of the tissue for electron microscopy. In addition to the parts of cells seen, the electron microscopists also sees some of the reagents used, and the products of the reactions of the reagents with each other, and with the cell contents.

Figure 1

If one cuts a section of an orange at its equator, the skin appears to have its minimum thickness. It would impossible to cut random sections of an orange nearer the poles (figure 1) and find each section of the skin appearing to be uniformly thick. Yet if one looks at electron

micrographs of the membranes around the cells, the mitochondria and the nuclei – now renamed the 'unit' or 'trilaminar' membrane – each of the laminae appear to be uniform distance apart. So also do: the cristae of the mitochondria; the lamellae of the Golgi body; the lamellae of the myelinated nerve; and the thylakoids of chloroplasts. When this flagrant disobedience to the laws of solid geometry has been pointed out, electron microscopists have claimed that authors select the clearest micrographs for illustrating their membranes. The sections would be clearest when the sections were cut normal to the microtome. For several years in response to this assertion, I have looked down several electron microscopes and examined hundreds of micrographs in publications. So far, I have not found *one* publication in which a full range of expected orientations was seen in one micrograph.

Figure 2

The 'unit' membrane was first described by J.D.Robertson, in 1959. It is now almost universally accepted as a true model (figure 2), although it has other problems. If the cell membrane is indeed trilaminar and it invaginates to form cisternae, the cisternae should be trilaminar on each side, that is, they should appear on electron microscopy to have four layers (or laminae) with 3 spaces in between; (cisternae are not seen by light microscopy). Even by electron microscopy, cisternae are very rarely seen, indeed, and the regions where they invaginate the cell membranes and the nuclear membranes are even more difficult to find. They are as rare as the region around the mitochondria, which should show similar trilaminar appearances of the invagination of the cell membrane and the mitochondrial membrane, making it also 4 layers with 3 spaces in between.

In lectures at over 200 universities, colleges and institutes in Britain, continental Europe, U.S.A., Canada, Israel, Australia and Thailand, I have asked colleagues to send me any published references or micrographs, showing the expected variations in distance between laminae of 'unit' membranes. After 29 years of failure to elicit such images, I have concluded that the images seen are two-dimensional; they are produced after sections have been cut. That does *not* mean that the membranes around the cells, the mitochondria and the nuclei, are artifacts, but that the trilaminar *appearance* as seen by electron micrographs is.

In addition to the description of the 'unit' membrane, the use of the electron microscope led to the following findings: confirmations of the existence of the Golgi body as a cytoplasmic organelle; the discovery of networks in the cytoplasm, called 'endoplasmic reticulum' and a 'cytoskeleton' (some authors use the latter term to include both); the naming of granules lining the endoplasmic reticulum as 'ribosomes'; the description of the cristae in the mitochondria; the description in the cytoplasm of lysosomes and peroxisomes; the

identification of several filamentous systems, such as microtubules, microfilaments, microtrabeculae, tubulin, actin, spectrin, dynesin and others. Some of these systems have been detected by fluorescence microscopy. They all occupy a substantial proportion of the volume of the cytoplasm. They are only seen in fixed dehydrated tissues. Biochemists have isolated fractions enriched in these particular cytoplasmic structures, and have studied their biochemical properties by subcellular fractionation.

However, if one examines living unicellular organisms or cells in tissue culture by fairly low power light microscopy (x200 to x400), one sees the following movements of relatively large particles in the cytoplasm: Brownian movement; streaming; diffusion; convection; nuclear rotation; phagocytosis; pinocytosis; meiosis; mitosis, secretion and movements of bacteria. Such movements are used, for example, to determine if cells in tissue culture are alive. None of these movements would be possible if the cytoplasm were filled with endoplasmic reticulum, cytoskelecton, Golgi bodies, lysosomes, peroxisomes and mitochondria. The mitochondria are the only one of these structures clearly identifiable in living cells. Before lysosomes and peroxisomes had been described in stained sections, apparent structures in the cytoplasm were usually called ‘Golgi’ bodies.

The viscosity of cytoplasm in living cells has been measured by several different techniques, including, centrifuge microscopy, intracellular injection of fine particles such as ground glass or carbon black; application of magnetic fields and electron spin resonance. They have all shown it to be low. If it were filled with solid networks and filament systems, it would be much higher. When fine particles are injected into cytoplasm, they appear to move freely and not to be obstructed by filamentous systems, or invisible relatively large bodies, such as Golgi bodies, lysosomes or peroxisomes.

It has been suggested that moving particles secrete lytic enzymes which dissolve the cytoplasmic bodies and filamentous systems in front of them, and the latter then re-form by themselves in real time. While this is just possible for the mitochondria and, -if they exist- the lysosomes and peroxisomes, it is extremely unlikely that iron filings or ground glass particles would produce such enzymes, and the mechanisms for secreting them. Paricles in Brownian motion would have a particular problem in ‘deciding’ which part of them would secrete the enzymes after determining fairly rapidly upon which direction they were to take next.

Intracellular movements occur in living cells to which no reagents have been added, while the cytoskeleton is only seen after fixation, addition of heavy metal salts or fluorochomes, sectioning and embedding. Thus conclusions from living cells are more likely

to be true, when the information from the two sources is contradictory. I think that one must conclude that all the structures seen in the cytoplasm by histology or electron microscopy – with the exception of the mitochondria – are artifacts of dehydration and the reagents used to demonstrate them.

Nevertheless, one may still ask what the endoplasmic reticulum, the cytoskeleton and the filament systems are, when one sees them under the electron and fluorescence microscopes. They consist of cytoplasm *minus* some of the contents, soluble in water or any of the reagents used, *plus* some of the reagents used. In the 1960's, and American group led by Abbé Luyet and others, including Rapatz, Tanner, McKenzie, and Meryman, dried out solutions of potassium chloride, alanine, glycine, ethylene glycol and others. They saw all sorts of crystalline patterns, some described as 'spherulites', 'dendrites' and 'spicules'. Living cytoplasm contains water, proteins, lipids, carbohydrates, polypeptides, fatty acids, glycerol, metabolic intermediaries, etc. When the cells are dehydrated, the contents will precipitate, and some crystalline forms will be seen. It is a reasonable questions to ask where all the non-aqueous and suspended contents of the cytoplasm go, when the tissue is dehydrated for histology or electron microscopy.

B. Approaches to Solving These Problems

1. The Second Law of Thermodynamics

The characterisation of living systems as open, while biochemical experiments are carried out in largely closed systems, represents a major challenge which may be impossible to meet ultimately. Biology should be the study of life in *living* intact cells and organisms. It is almost impossible to know the rates and equilibria of living reactions, when they are difficult to study in intact cells, and killing the animals or homogenising the cells changes them significantly. Yet it might be useful to suggest a code of practice designed to approximate as closely as possible to the state of the living animal or plant.

I would suggest the following:

A. Prefer experiments in living intact systems over those in vitro.

B. Employ low energy procedures. Avoid homogenisation, centrifugation, electrophoresis and electron microscopy. (Incidentally, with the exception of homogenisation, the apparatus for carrying out the latter procedures is energy intensive and expensive).

C. Use agents which have minimum effects on normal metabolism in

physiological concentrations;

D. If possible, avoid the use of reagents which do not occur naturally;

E. Test that the reagents used do not affect the normal activities of the cells in the systems one is studying, for example, its oxygen uptake, resting membrane potentials, Brownian movement, streaming, secretion, synthesising proteins, concentrating potassium ions, excluding sodium ions, producing antibodies, etc.

F. Carry out experiments at low, but not freezing temperatures, and slowly, so that energy can be dissipated with minimal rise in temperature.

G. Carry out control experiments to see the effects, whenever one uses unphysiological concentrations of natural substances, or unnatural substances, or manipulations, such as homogenisation, centrifugation, freezing or boiling.

H. Avoid disruptive techniques in favour of micromanipulation.

I. Use light microscopic techniques, bearing in mind that light transmits heat, and can affect metabolism and produce photochemical effects.

J. Avoid histology, histochemistry, freezing procedures and electron microscopy, because of the chemical changes induced in tissue by reagents and agents, used in these procedures.

K. Address fundamental thermodynamic objections to each individual procedure, carry out all relevant control experiments, or abandon that procedure.

L. The quantity of energy needed to analyse a biological system, or the fragility of that system may be so great that the particular procedure – or even all procedures – can not be used for studying that system as a model of the living intact cell.

2. Subcellular fractionation

Elsewhere, I have listed the assumptions inherent in this procedure. Some of them are contrary to physical laws and the Second Law of Thermodynamics.

The following control experiments are suggested:

(i) measure the effects on the system under study of each of the reagents and manoeuvres at each step of the procedure;

(ii) take a well characterised enzyme, such as cytochrome oxidase, succinic dehydrogenase or ATPase, and pass it through the whole fractionation procedure, to measure how much enzyme activity survives the procedure;

(iii) most of the procedures are empirical, so when one has identified the important steps, which affect the enzyme activity, one may be able to modify the procedure to make

it slower, carry it out with weaker reagents, use less co-factors, homogenise the tissue more slowly, or centrifuge it with less force, or for less time;

(iv) mix an enzyme uniformly with a pre-determined mixture of microspheres of know diameters, put it through the whole fractionation procedure and find out the location and recovery of the enzyme;

(v) use boiled enzyme preparations as controls for normal enzymes to find out how much of the breakdown of substrate can be attributed to the enzyme, and how much is due to the instability of the substrate, and the chemical environment, in which the enzyme is studied;

(vi) explore other procedures for localising enzyme and other activities in living cells, preferably with little or no disruption, and under conditions of minimal input of energy. Many of these procedures can be carried out under direct light microscopy. The procedures include: watching unfixed cells, unicellular organisms and cells in tissue culture, by bright field, phase contrast, anopteral, dark ground, differential interference contrast, polarised, Rheinberg, supravital staining, microspectrophotometry, confocal, laser capture, and quantum dot fluorescence microscopy. One may warm or cool cells; one may centrifuge them at low speeds for short times; one may immerse them in fluids of various refractive indices; one may inject fluids or small particles into cytoplasm; one may draw off minute samples from the cytoplasm and the extracellular phase, and analyse them chemically. Thus it can be seen that there are much less energetic, less complicated and cheaper procedures to examine the location of chemical activities within and without cells, than subcellular fractionation.

Even if with all the necessary control experiments, subcellular fractionation could measure the total enzyme activity at each stage of the procedure, it will never be able to pin point with certainty the original location of the enzymes in the intact living cells, because there is no way of knowing whether the enzymes, the substrate, activators, inhibitors or co-factors have relocated during the procedure.

3. **Electron microscopy**

Electron microscopy can not be used for studying living cells. Like histology, it involves dehydration and cutting sections of three-dimensional objects. They both shrink tissues, so that neither procedure can be used for measuring the shapes of asymmetrical organelles, nor their dimensions, incidence, number of processes, direction of movement of particles, or chemistry of cells. Nor can they be used to study the following phenomena;

intracellular or cellular movements; incidence of the particles or subcellular organelles; oedema or atrophy; dying or post mortem changes; hypoxia or ischaemia; the chemistry of organelles.

If one concludes that many of the apparent structures seen by the electron microscope are artifacts, those who regard them as real have a duty to answer the objections on geometrical and logical ground to their alleged existence. The only substantial reason I have heard for the missing three dimensional views of organelles has been that electron microscopists have selected figures for illustrations; this is not satisfactory. Furthermore a *testable* hypothesis of how intracellular movements occur freely in the presence of so much cytoplasmic furniture, should be produced.

The cytoplasm of, say, a fibroblast, contains mitochondria, and an onion skin cell contains chloroplasts. However, cytoplasm also contains a number of amorphous bodies. Authors give names to some of them, and then they prepare fractions, enriched in them. But it is useful to reflect that both by light, and more by electron microscopy, one sees many amorphous small particles, which can only be identified by the history of the preparation or the beliefs of the biologists. There are also many 'particles' or 'bodies', which can not be identified. Among the names given to such appearances include: 'particles in Brownian movement, streaming particles, vesicles, vesicle-like particles, bodies, assembles, axonal particles, inclusions, arrays, ribosomes, glycogen, phagocytosed particles, starch particles, cell debris, neurohumoral particles, secretory particles, broken mitochondria and microbes.' It seems probable that the biochemical properties of all these bodies, may never be known. They may be too small, heterogeneous and variable, ever to know about them with certainty.

Harold Hillman was the Reader in Physiology and Director of the Unity Laboratory of Applied Neurobiology, at the University of Surrey, until his retirement in 1995.

Further reading:

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Legends to figures

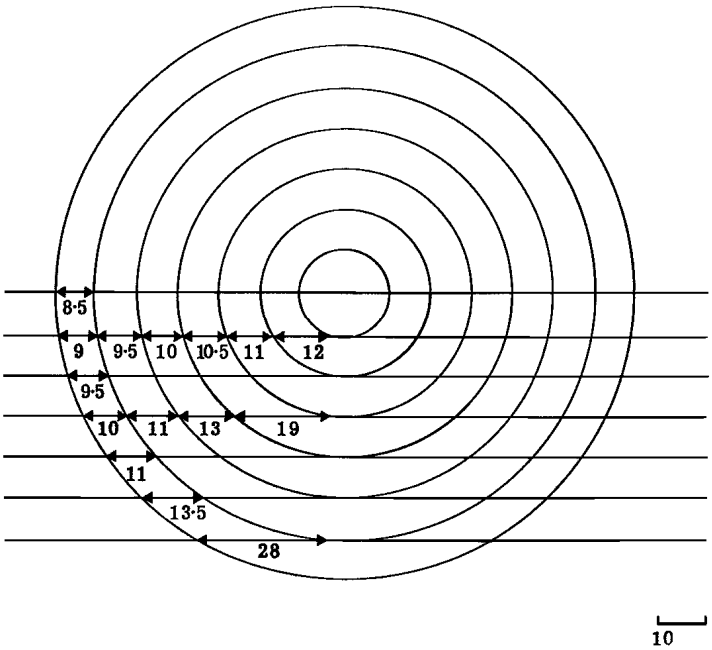

Figure 1. Diagram showing that sections of a membrane, cut at different distances between the poles and the equator, should *not* appear to be equally thick.

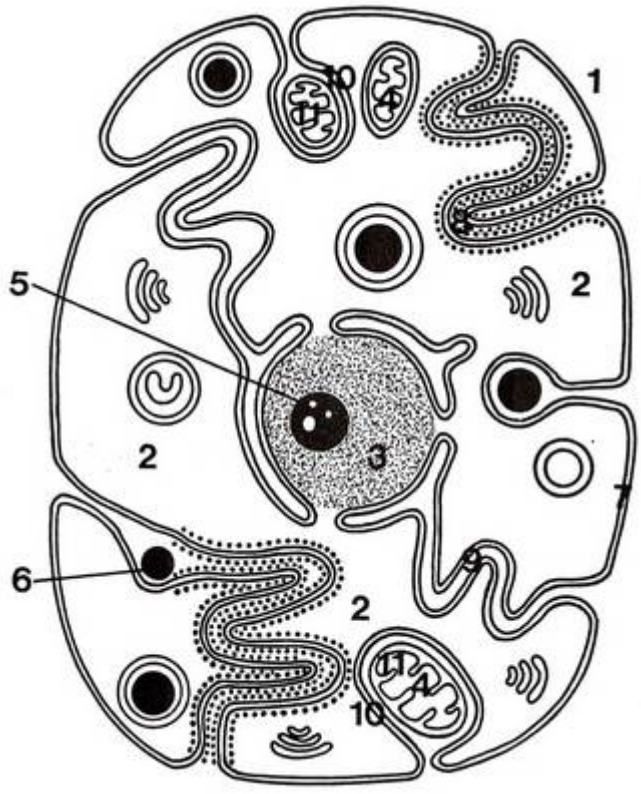

Figure 2. The generalised cell with 'unit membranes'. Different compartments are indicated by numbers. 1 = extracellular compartment; 2 = cytoplasm; 3 = nucleoplasm; 4 = mitochondrial matrix; 5 = nucleolus; 6 = lysosome; 7 = unit membrane; 8 = rough endoplasmic reticulum; 9 = cisternae; 10 = invagination of cell membrane; 11 = mitochondrial membrane.
The problems associated with this model are:
(a) all the membranes appear to have laminae uniformly distant apart;
(b) the mitochondria, the Golgi bodies, the lysosomes, the peroxisomes and unidentifiable particles, could not move around the cytoplasm;
(c) *two* trilaminar membranes should appear around the mitochondria, the lysosomes, the nucleus and the cisternae;
(d) the extranuclear space is continuous with the cisternae;
(e) the section must have been cut through the middle of the membranes around the cell, the mitochondria, the nucleus and the cisternae.