Cell Biology is Currently in Dire Straits

by Harold Hillman

(Unity Laboratory of Applied Neurobiology, 76, Epsom Road, Guildford, Surrey, GU1 2BX, U.K.)

e-mail: harold.hillman@btinternet.com

Summary

During a research career lasting more than 50 years, I have concluded that the following procedures are unsuitable for studying the biology of living cells in Intact animals and plants: subcellular fractionation; histology; histochemistry; electron microscopy; binding studies; use of ligands; immunocytochemistry; tissue slices; disruptive techniques; dehydration; deep freezing; freeze drying; boiling; use of extracellular markers; receptor studies; patch clamp measurements; inadequate calibrations. The main objections to these procedures are: (i) they change the properties of the tissues being studied grossly and significantly; (ii) they ignore the second law of thermodynamics;(iii) they produce artefacts, many of which are two-dimensional; (iv) adequate control procedures have never been published for them. I have described alternative procedures, and suggested that unsatisfactory ones should be abandoned.

I have put forward the hypothesis that the poor quality of cell biology in the 20th century and since is the reason for the failure of medical research to discover the chemical changes *initiating* diseases, so that a rational approach to intervening in the chemistry early on has not resulted.

My background

I am summarising my background to indicate that I have the relevant training and experience in conventional cell biology, neurobiology and physiology, to discuss the subject using normal paradigms. I have London University degrees in medicine, and in physiology, and a doctorate in biochemistry. I was the reader in physiology at the University of Surrey from 1968 until I retired in 1995. I was the Director of the Unity Laboratory of Applied Neurobiology at the University from 1970 until 1995, and I have continued its activities since then. I was the Medical Adviser to the Schizophrenia Association of Great Britain between 1990 and 1993. Since 1960, I have published about 160 full-length research papers and 6 books, on cytology, neurobiology and resuscitation. I was the Founding Editor and Editor- in- Chief of 'Resuscitation' from 1970 to 1985.

Preamble

This open letter to cell biologists concerns very important mistakes made since the early 20th century, but, regrettably, still perpetuated in the 21st century (Hillman, 1972; Hillman and Sartory, 1980; Hillman, 1986; 2008). I have brought evidence that so much research work of poor quality has been published that there is a huge inertia which resists publication of better quality research. Much of the current consensus in the subject is wrong. So this message is mainly addressed to young research workers starting their careers in the early twenty first century, who had not yet carried out and published compromised research. I hope that they will have the strength of purpose to examine the popular consensus critically, and to carry out experiments of better quality.

Aims of cell biology

These have been defined as a description of the structures and chemical compounds of living tissues and their relationships, not affected significantly by the procedures used to examine them (Hillman and Sartory, 1980).

This definition involves the major assumption that evidence from studies of living intact organisms is more valid than that from dead or treated organisms. Such treatments include: killing; fixation; stunning; extraction; dehydration; heating; freezing; embedding; sectioning; staining; chemical treatment; subjection to toxins. This does not mean necessarily that subjection to these agents or manoeuvres themselves renders the procedures valueless, but it does mean that evidence derived without their use is to be preferred to that with their use, when the two are in conflict. It also imposes upon us the duty -- so far largely ignored -- to carry out comprehensive control experiment of the effects of every chemical and every manoeuvre on the results of the experiments.

Schleiden and Schwann (1838) put forward the idea that all tissue consists of cells. Nowadays, this is expressed in modern jargon, that the cell is the 'functional' unit of tissue. This important generalisation applies to unicellular organisms and metazoa. In metazoa, as in colonial organisms, all cells are related to each other. Some of these are separated by thin layers of fluid, the extracellular compartment, but others are so close as to virtually obliterate the compartment between them. The extracellular compartment of metazoa consists of: the interstitial fluid; the plasma; the lymph; the ocular fluids; the serous fluids; and the synovial fluids. All of these are of similar chemical composition.

It is most important to appreciate how much energy is used to separate tissues into fractions enriched by particular kinds of cells, or of particular organelles (Hillman, 1972). It is usual to pretend that no energy is dissipated during the procedures or that it has no effect on the chemistry of the tissues.

Overall conclusions

A. Most procedures used in research in cell biology have ignored the second law of thermodynamics (Atkins, 1994);
B. Control experiments for the effects of reagents and manoeuvres used on the results of experiments have been grossly inadequate;
C. Electron microscopists have ignored the dictates of solid geometry and most of the apparent structures they have detected are artefacts of their preparation procedures (Hillman and Sartory, 1980). They select their illustrations, rather than show typical ones. They prefer the results from metal deposits over those from unfixed living cells.
D. Nuclei, containing nucleoli, and mitochondria, are the only organelles present in cytoplasm (Hillman and Sartory, 1980).
E. Several structures seen in unfixed tissue by light microscopy have been ignored because they are not seen by electron microscopy (Table 4).
F. New high-resolution light microscopic techniques should be used to examine structures seen by electron microscopy (Table 7).

Evidence for these conclusions

A. The second law of thermodynamics

This law states that in a closed system, in which energy, but not matter is exchanged with its environment, any change in entropy must be accompanied by a change in free energy. Living systems are open, that is to say, energy and materials are freely exchanged between the system (animal or plant) and its environment. Ideally and theoretically, we would like to study living organisms. However, their metabolism is changing so much more rapidly than one can measure, the pathways and cycles are so interdependent, and the procedures may well affect the parameters measured. Therefore, nearly all biochemical experiments are carried out in largely closed systems. The energy generated in the system cannot dissipate rapidly because it is surrounded by media which conduct heat poorly, including water, ice, plastic, glass, air and vacuum.

A list of changes in entropy and free energy in chemical procedures is given (Table 1). Of course, added free energy drives chemical reactions to new rates and equilibria away from those in the Intact, living animal or plant. Therefore, in principle, none of these steps should be used to examine the latter, unless and until, their effects on the parameters of the tissue under study have been examined quantitatively. It is rather surprising that jobbing biochemists have not asked themselves forensically what effects routine manoeuvres have on the tissues and parameters they measure. They have also ignored what the second law of thermodynamics dictates about homogenisation and centrifugation. Also, they seem to have forgotten about the crucial necessity of control experiments.

Step	Effect	Change in Entropy (E) or free energy (F)
Killing the organism	diffusion occurs; gradients across membranes and between adjacent tissues diminish	E
Tissue hypoxia	oxidative reactions diminish	E
Post mortem changes	denaturation of proteins; redistribution of solutes	E, F
Tissue cools	rates of reactions diminish	F
Tissue is sliced homogenised, macerated or sonicated	it is subjected to pressure; concentration gradients are diminished; heat is generated; enzymes, substrates and activators diffuse from their sites in vivo to other locations	E, F
Powerful chemically active reagents are added	Tissue is diluted; tissue constituents exchange with reagents and bind to them	E, F
Tissue is 'washed', rinsed, diluted, or eluted	it is diluted; soluble constituents are extracted	E
Tissue is centrifuged or stirred	friction occurs between particles and centrifugation medium; pressure rises; heat is generated	E, F
Enzyme activators and inhibitors added	Change enzyme activities	E
Tissue is filtered, or triturated	it is diluted; may bind to filter paper or column; soluble constituents are extracted	E

Dialysis	small molecules and ions are removed	E
Constituents are concentrated	the affinity for the other constituents is changed	E
Constituents are precipitated or suspended	they change their solubility and reactivity	E
Tissue is dried, dehydrated or blotted	concentrations of all soluble constituents increase; water and volatile substances are removed; proteins are denatured	E, F
Tissue is frozen or heated	reaction rates are changed; dehydration occurs; proteins are denatured	E, F
Crystallization	the solvent is extracted; all bonds with other tissue constituents are broken; the concentration of the substance increases to 100%	E, F
Tissue reacts with added	exothermic or endothermic reactions	E, F
Sedimentation	reagents exceed their solubility products	F
Partition	reagents react with each species and phase	E
Lyophilisation	dehydration and concentration	E
Purification	(see chromatography and electrophoresis)	E, F
Mounting	subjection to xylol or propylene oxide and mountant	F
Staining	organelles coloured, which changes light absorption	E
Antigen – antibody	plus other 'non-specific' reactions	E
Addition of flurochromes	reaction not only with proteins	F
Subjection to x-rays	DNA 'damage'	F, E
Bombardment of electrons	liberation of heat and x-rays	E, F
Addition of non-isotonic reagents	accelerates the movement of soluble constituents	E
Tissues are dissolved in powerful reagents	proteins are denatured, pH changes	E
Substances are extracted	bonds are broken	
Tissue is fixed	enzyme activities are inhibited; proteins are denatured	E, F
Chromatography and electrophoresis	bonds are broken; tissue is heated	E, F
Preincubation and incubation	tissue exchanges with media	E
Contamination	substrates are used up; toxic products are produced	E
Microscopy	tissue is illuminated and absorbs energy	E
Penneabilisation	denatures proteins; opens membrane pores	E

Table 1: Effects of procedures on the entropy and free energy of the chemical reactions within tissues, Ii should be noted that most manipulations change the entropy of the system and may change the free energy.

Subcellular fractionation suffers from two other serious problems. It is recognised that the reaction mixture heats up during both homogenisation and centrifugation. Biochemists seek to prevent this by cooling the homogeniser with ice or refrigeration. This lowers the initial temperature of the mixture. Unfortunately, however, it increases its viscosity, -- as any skier knows. Thus, more heat is generated. It is hoped that cooling increases the rate of heat dissipation, before it can affect the system under study. This hope cannot be tested, but it sounds like an attempt to close the stable door after the horse has bolted.

The other problem with centrifugation is the g force used. The force to which biochemists refer is that applied to the centre of the tube, whereas it varies with the distance between the particular part of the centrifuge tube containing the fraction, and the axis of rotation. It is grossly different in different parts of the tubes. Thus, one is subjecting different parts of the homogenates to quite different quantities of energy. It is not at all surprising that they should exhibit different chemical activities.

B. Subcellular fractionation

One must conclude that hardly any experiments in vitro have been controlled, so that while they may be able to give *qualitative* information about living systems, they cannot tell one quantitative information about the rates or equilibria of, for example, enzyme reactions, pathways or cycles, etc. Diffusion, homogenisation, centrifugation, and heat may cause enzymes, activating ions and substrates to move from their original sites in vivo. They may also change the affinities of drugs.

In subcellular fractionation, the only popular control is to add up all the activities at the end of the procedure, and to compare this total with the enzyme activity of the crude homogenate. If the recovery is between 60% and 120%, the experiment is regarded as satisfactory. However, if it is much lower, recovery in each final fraction is measured as a percentage of the total recovered, rather than the activity of the original homogenates. This is grossly inadequate, especially when trying to locate activity in a final fraction.

Since 1972, I have been insisting that biologists should only be interested in the properties of an organelle in a fraction, if they have demonstrated that these properties reflect those of the same organelle in the living intact organism (Hillman,1979;1991;2008;2009;2010). Nevertheless, no biochemist has ever said that: subcellular fractionation is *not* governed by the second law; control experiment *have* been published; control experiments are *not* necessary; the results of fractionation experiments *do* reflect the properties of the intact tissue in vivo; the latter results do *not* affect these properties; or, the whole question is *trivial*. As Sherlock Holmes said, the curious fact was that the dog did *not* bark in the night.

C. Microscopy

When a tissue is prepared for histology, histochemistry, electron microscopy, or immunocytochemistry, an animal is killed; the tissue is excised; it is fixed or frozen; it is embedded; it is sectioned; it is rehydrated; it is stained; it is mounted; it is radiated by light, or bombarded by electron beams. Living tissue could not survive the dehydration, low pressure, x-irradiation and electron bombardment, which occur in the electron microscope. So, heavy metal salts of osmium, tungsten, manganese, uranium or lead, are deposited on fixed tissue, and these deposits are

examined. When one studies unfixed tissues in physiological media, one is looking at cells, which exchange approximately normally with their environments. In histological sections, one is examining tissue *plus* reagents used in the preparation, *minus* constituents of the tissue (including water), dissolved in or extracted by, the reagents used. The electron microscopists look at heavy metal salts, *plus* other reagents used in the preparation, *minus* substances extracted by the reagents. Virtually nothing is seen if heavy metal salts are not used for staining, as was shown by Weakley in an elegant illustration in her book, ' Beginners Handbook of Electron Microscopy', (1972). In addition, one does not see any cellular structures, which do not react with or dissolve in reagents, including ethanol and acetone.

Electron microscopy has a resolution of up to 25 times that possible by light microscopy, and the following new findings resulted, when the procedure was introduced:

(a) The membranes around the cells, the nuclei and the mitochondria appeared double—dark-light-dark, and J.D.Robertson (1959) gave this 'trilaminar' appearance the name 'unit membrane'. It is very widely illustrated in textbooks;
(b) a network, the 'endoplasmic reticulum' was seen in the cytoplasm;
(c) the existence of the 'Golgi body', described by Golgi in 1898, was 'confirmed' by electron microscopy;
(d) other networks of actin, tubulin, spectrin, vimentin, were seen by fluorescent, as well as by electron microscopy;
(e) the cell membrane was postulated by physiologists to be traversed by 'ion channels'. These were hypothesised to open up to allow the passage of ions, such as K^+, Na^+, Ca^{2+}, Cl^- depending upon the ion species, its voltage, size, shape and charge, sometimes after a delay. No membrane pores have been seen by electron microscopy but one, the sodium acetylcholine channel, has been modelled by Kistler et al (1982). Where are all the tens of channels? Why does the cell membrane appear on electron microscopy as smooth as a baby's cheek?
(f) the myelin sheath was found to consist of lamellae;
(g) two new bodies, originally found in subcellular fractions, were claimed to have been seen in the cytoplasm; these 'lysosomes' and 'peroxisomes' were each believed to contain their own portfolios of enzyme activities;
(h) the nuclear membrane was seen to be penetrated by pores, or pore apparatuses:
(i) shelves seen in the mitochondrial matrix were given the name 'cristae';
(j) the A-bands of muscle were described as being composed of 'thick' filaments, and the I bands of 'thin' filaments. Small 'cross bridges' were seen in the spaces between the thick and thin filaments;
(k) particles seen by electron microscopy either in sections or in subcellular fractions were named 'ribosomes, transmembrane molecules, membrane receptors, molecular motors, synapses and synaptic vesicles'.

With co-authors, I have written extensively on why all these structures (a)-(k) must be artefacts (Hillman and Sartory, 1977a; 1980; Hillman,1986; 2008;), so I will confine myself to

summarising the reasons in respect of each of the particular structures, and I will indicate the likely origins of these artefacts (Tables 2, 3).

Artifacts in cells

Structure	*Why it is an artifact*
1. Unit membrane	Laminae are too uniformly distant apart
2. Ion channels	Only the sodium ach channel has been modelled
3. Golgi body *	Too diverse sizes and shapes by microscopy
4. Endoplasmic reticulum	Not 3-dimensional.Would prevent intracellular movements
5. Cytoskeleton	,, ,,
6. Molecular motors	Not seen by electron microscopy in whole cells
7. Lysosomes	Not seen as a structure in intact living cells
8. Peroxisomes	,, ,,
9. Liposomes	Lipids would be extracted during e.m. preparation
10 .Myelin lamellae	Do not appear in 3 dimensions
11. Membrane receptors	Not seen by light or electron microscopy
12. Transmembrane molecules	,, ,,
13. Membrane carriers	,, ,,
14. Synapses *	Rarely seen by light microscopy
15. Pre-synaptic fibres	,, ,,
16. Synaptic vesicles	Too uniform in diameter. No stalk by electron by microscopy
17. Mitochondrial, inner and outer membranes	One membrane would show up as two lines
18. Mitochondrial cristae	Do not appear in 3 dimensions
19. Nuclear pores	Would connect cytoplasm and nucleoplasm
20. Thick muscle filaments	Do not conform to 3-dimensional geometry
21. Thin muscle filaments	,, ,,
22. Cross bridges	Are not orientated when muscle contracts
23. T tubules *	Not seen in unfixed, unstained muscle
24. Sarcoplasmic reticulum	Would obstruct intracellular movements
25. Caveolae	,, ,, ; also, not seen in unfixed muscle
26. Cisternae	Not seen in unfixed unstained cells
27. Thylakoid membranes	Not seen in 3 dimensions

Table 2. Most of these structures are believed to require the resolution of electron microscopy to see, but a few can be seen by light.*

Likely origins of artefacts

Number Origins

1. It must have arisen after sections were cut. It is 2-dimensional.
2. It is a hypothesis that small currents originate from ion channels.
3. The shapes and dimensions of Golgi bodies are too variable both by light and by electron microscopy.

4. They are precipitates of staining reagents and cytoplasm, which is dehydrated. It is not seen in 3-dimensions in equal frequency.
5. As 4.
6. Arises from a search for the causes of intracellular movements, ignoring well-established phenomena of diffusion, Brownian movement, streaming and convection.
7. Was conceived after a subcellular fraction 'enriched' with hydrolytic enzymes was separated.
8. Similar origin in a fraction enriched in about 30 enzymes including peroxidases and catalases.
9. Ribosomes originated from separation of a fraction containing RNA. They are difficult to identify in whole cells, but can be seen in some fractions.
10. Precipitates of cytoplasm.
11. Myelin in life is a viscous fluid, which precipitates as lamellae, when it is dehydrated for electron microscopy.
12. Arises from the pharmacological idea that drugs and transmitters can rarely act unless they bind to receptors.
13. Arise from the belief that some ions and amino acids are 'transported ' across membranes by macromolecules.
14. Similar origin to latter.
15. A precipitate of silver or heavy metal salts on or near nerve cell bodies and dendrites.
16. A belief that dendrites connect synapses which are located on cell bodies and other dendrites.
17. A fraction of spheres and ovals that can be separated from the central nervous system.
18. Dehydration of the mitochondrial matrix.
19. Precipitate of fluid mitochondrial matrix.
20. Cracks in the nuclear membrane during preparation for microscopy.
21. In life, muscle is a gel, which precipitates, when dehydrated for electron microscopy.
22. Similar explanation to latter.
23.Trapping of particles of stain and myoplasm as a consequence of preparation for electron microscopy. Similar looking structures are seen in nerves.
24. Shrinkage artifacts, not seen in fresh, intact muscles.
25. Precipitate of myoplasm and staining reagents during preparation for electron microscopy.
26. Vacuoles occurring as a consequence of preparation for electron microscopy.
27. Consequences of precipitation, vacuum, electron bombardment and irradiation of cytoplasm.

Table 3. Other explanations for these phenomena are possible. The numbers refer to the structures listed in Tabble2.

D. Cytoplasm

The nuclei, containing the nucleoli, and the mitochondria, are the only structures seen by light microscopy in the cytoplasm of living cells. They can be seen moving in tissue cultures. All the other structures claimed to be present are only seen in electron micrographs of dead tissue, or in fixed stained histological sections. If they existed, the cytoplasm would be almost solid with endoplasmic reticulum, cytoskeleton, Golgi bodies, lysosomes, peroxisomes, contractile proteins and stress fibres. This is hardly compatible with either the low viscosity of cytoplasm, or with the rich variety of intracellular movements seen in living cells-- Brownian movements, streaming, convection, secretion, phagocytosis, pinocytosis, vacuolation, nuclear rotation, meiosis, mitosis, and muscle contraction. Additionally, many of the structures only seen by electron microscopy (Table 2), are not seen in a random selection of orientations. There are two-dimensional (Hillman and Sartory,

1980). Therefore, they must have appeared after the sections were cut. My explanations for the origin of these artifacts are given (Table 3).

E **Neglected structures**

Since the electron microscope was introduced to biology in the 1940s, several structures, clearly visible by light microscopy in unfixed and living tissues have been ignored in the literature (Table 4). Of these, the fine granular material is probably the most important, since it comprises most of the volume of the mammalian central nervous system (Hillman, 1986; Hillman and Jarman, 1991). The existence of this material was originally described briefly by Hodgkin and Lister (1829), but modern neuroanatomists have ignored it completely. They regard it as 'debris' or an artifact. Many hypotheses imply microscopic movements, which, however, have not been observed closely (Table 5).

Other anatomical features which should be examined by high power light microscopy

1. The location of DNA in resting nuclei and in mitochondria.
2. The structure of the nucleolus and the nucleolonema.
3. The structure of the nucleololus.
4. The existence of a nucleolar membrane in neurons.
5. The presence of fine granular material in the central nervous system.
6. The presence of droplets and droplet fibres in the central nervous system.
7. The detailed changes in meiosis and mitosis.
8. The examination of neuroglial nuclei.
9. The origin of axonal inclusions in myelinated nerve fibres.
10. Observations of axonal flow.
11. Remak fibres.
12. The nucleolonema.
13. Intra-cellular movements in axons and myelin sheaths.
14. Schwann cells.
15. Rods and cones.
16. Examination of pre-cancerous cells.
17. Examination of teased cells from tissues affected by many diseases.
18. Dying.
19. Necrosis.
20. Diapedesis.

Table 4. These have not received sufficient attention.

Phenomena involving particulate and macromolecular movements

Davson-Danielli model of cell membrane
Singer- Nicholson hypothesis
Ions crossing channels in cell membranes

Receptor theory
Role of g-proteins in signalling
Pre-existence of all possible antigens in cell membranes
Theory of chemical transmission
Geren model of myelination
Sliding filament hypothesis of muscle contraction
Chemi-osmotic hypothesis
Meiosis
Location of calcium ions in sarcoplasmic reticulum
Mitosis
Presence of cytoskeieton in cell
Contraction of contractile proteins
Apoptosis as a cellular phenomenon
Opening and closing of nuclear pores
Passage of RNA across nuclear pores
Blood-brain barrier
Axonal transport
Molecular motors in action
Pinocytosis
Phagocytosis
Cellular secretion
Vacuolation
Diapedesis

Table 5. Most of these phenomena have not yet been examined by modern techniques of high power light microscopy.

Hypotheses which could be tested by observation

1. Ions cross membranes at channels.
2. The role of g- proteins in signalling.
3. Opening and closing of nuclear pores.
4, Messenger RNA crossing nuclear pores.
5. Pinocytosis.
6. Contraction of contractile proteins in cells.
7. Location of calcium ions in sarcoplasmic reticulum.
8.Phagocytosis.
9. Chemical hypothesis of transmission.
10. Sliding filament hypothesis of muscle contraction.
11. Geren model of myelination.

Table 6. Most of the above should be visible in living cells

Electron microscopists often assert that there are no alternatives to their instrument. I have already pointed out that, in my view, observations on living or unfixed issue yield more accurate information than that from metal deposits. A wide variety of such techniques, both ancient and modern, is available. Some of these are indicated in Table 7.

Light microscopy techniques available

Earlier	*Later*
Bright field	Confocal
Oil immersion	Video-enhaced
Phase contrast	Polychromatic illumination
Anopteral	Optical tweezers
Rheinberg	Laser capture
Polarising	Quantum dot fluorescence
Vertical illumination	Lenseless
Differential interference contrast	Atomic force
Supra-vital staining	
Critical microscopy	
Inverted	
Centrifuge	
Microspectrometry	
X-ray	
Modulation contrast	

Table 7. Most of these techniques can be used in living tissues

I would suggest that, instead of apparently indiscriminate and pragmatic use of powerful and toxic reagents in biological experiments, tissues should be examined in more optimal conditions, which respect the second law of her thermodynamics, and change the biochemistry minimally (Table 8). Of course, it has not been shown that more natural environments for tissues will make experiments more accurate, but it is a reasonable assumption.

Optimal conditions for examining tissues by light microscopy

1. Cells should be living, unfixed, not frozen, manipulated or disrupted, and in situ, if possible.
2. They may be cooled to 273^0K, but not below the eutectic point of the tissue.
3. They should not be disrupted, compressed, centrifuged, penetrated or rendered anoxic.
4. If it is necessary to remove the tissue from the whole animals, it should be examined in plasma, serum, cerebrospinal fluid, lymph or ocular fluids (preferably from the same animals as the tissue), in growth media, or normal saline. These fluids should be isotonic.
5. Tissue should not be dehydrated or subject to low pressure.
6. Tissue should not be subjected to powerful electromagnetic radiation.

7. Tissue should not be subjected to high energy, during examination.

Table 8. Light to illuminate the tissue is probably the only high energy radiation, which cannot be avoided. Minimum light should be used, although the images may be enhanced electronically.

Scientific responses to my criticism

Three papers have taken issue with my views, (Horne and Harris, 1981; Michell, Finean and Coleman ,1982; Hawes,2010. We were not allowed to respond at length to the first two papers, which added up to 23 pages, but the editor did allow us a 2000 word letter in reply. In 2010, the Editor of 'The Biologist' published a paper of mine doubting the validity of the use of subcellular fractionation and electron microscopy (Hillman, 2010). As a consequence, the Editor was rebuked by the Chairman of the Society of Biology, and I believe he was replaced. Apparently, the officers of the Society of Biology, and the officers of the Royal Microscopical Society authorised Professor Christopher Hawes of Oxford Brookes University to 'refute' my views (Hawes, 2010). I consider it highly improper and extraordinary that in this day and age, two learned societies should give their official support to one side in a scientific disagreement. I did not feel that Professor Hawes had addressed the points I had made, but 'The Biologist' did not give me the right of reply. It said correctly that I had written the first paper and Professor Hawes had replied to mine. If I had replied to the latter, it would have to give him space to reply to that.

In questions after my lectures, and in published responses to my views cited above, the following points were made. Firstly, I had not taken into account the massive biochemical and biophysical evidence which had created the current consensus. Those who made this criticism were evidently unaware that a few years before in 1972, I had written 'Certainty and Uncertainty in Biochemical Techniques,' dealing specifically with these aspects of the problem. Secondly, they seemed to believe that if an apparent structure did not conform to the laws of solid geometry, evidence from other totally different studies could prove its existence.

Thirdly, it was asserted that the structures which I had characterised as artefacts (Table 2) had been demonstrated by several different microscopical procedures, which they believed represented different independent lines of evidence. My response was that the alleged structures were not seen in fresh unfixed tissue, but in dehydrated, shrunken, stained preparations, which was the source of all the artifacts.

Fourthly, it was said that the structures were not artefacts, because there were so repeatable. This is extremely poor reasoning. When one looks at the road ahead, it always seems to narrow, but one's car is not usually crushed by it. When one looks at a light through a pinhole, one sees rays all around it, but they do not represent structures. They are highly repeatable.

Fifthly, I asserted that so many structures, such as the 'unit membranes', the myelin lamellae and thylakoids of the chloroplasts, appear only two-dimensional on electron micrographs. This point was answered by listing different publications (Hawes, 2010) in which they were seen in a variety of orientations. Unfortunately, this is not good enough. For example, even if one sees cristae

or thylakoids in a variety of orientations in *different* publications, one should also see them in a variety of orientations if one could see several of them *in the same field*, provided that the magnification was not too great. This is a simple requirement of solid geometry.

Sixthly, electron microscopists have sometimes asserted that the reasons for which the 'unit membranes' are nearly always seen in micrographs in transverse section is that they themselves select for publication in those membranes which appear most clearly. Obviously, there are the membranes which happened to be cut normal to the plain of section. However, if that were so, it would mean that, in the Robertson model, at any particular moment, all 'unit membranes' around the cell, the nucleus, the mitochondria and the chloroplasts would have to be orientated usually and simultaneously normal to the plane of section, whenever the microscopist chose to cut the sections. I believe that Professor Karl Deutsch (1962) was the first person to recognise this geometrical anomaly.

Non-scientific reactions to the publication of my unpopular views

I should like to draw attention to the fact that I regard my views as unpopular, rather than heretical, as I do not believe that scientists should talk in terms of dogma and heresy. In the best of possible worlds, good scientists who hear challenges to their beliefs, assumptions, hypotheses, procedures or conclusions, should examine such criticism with due attention. They should respond by entering into civilised dialogue with their critics. They should be prepared to admit mistakes, if necessary, and change their views. Such reactions have not occurred.

Instead, there have been a large number of reactions. A summary of which I will recount together with my responses to each. (Remarks made by others about me are indicated in the third person).

(i)"The structures he has concluded to be artefacts are described in the first chapters of most of the textbooks of life sciences used by students. We feel certain that the eminent academics and members of learned societies have addressed the problems which Hillman has raised, but we are not sufficiently expert to answer them ourselves".

My answer. Despite the fact that I have been making these points since 1972, and I have had difficulties in publishing them, there has been little response to my criticisms and little willingness to enter into dialogue, other than the publications cited. In 1978, Mr.Peter Sartory and I in the columns of the London 'Observer ' challenged the Royal Microscopical Society to a public debate on these questions anywhere in the world (Hillman and Sartory, 1977b). Only two such debates have ever occurred, one in May, 1980, at Brunel University, and a second in May, 1995, at Sydney University.

(ii) "Hillman is not an electron microscopist, so he cannot comment on electron microscopy."

My answer. For over 30 years I have used this instrument. Even if this assertion were true, that would be irrelevant to the scientific points I have made, because I quote the findings of respected electron microscopists.

(iii) "He seeks controversy."

My answer. This is irrelevant and untrue. Even if it were, it would not affect the validity of the scientific points made.

(iv} "Textbooks would have to be rewritten if he were right"

My answer. Authors of textbooks should be watching and recording new developments and controversies, to keep themselves up to date.

(v) At the Physiological Society, the Royal Microscopical Society, the Anatomical Society and the Biochemical Society, several members objected to my quoting textbooks on the grounds that "You should not believe what you read in textbooks"

My answer. This is either a statement of the obvious fact that writers of textbooks take time to record the latest advances, or it is an expression of extreme cynicism. We wrote a letter to 'Nature' which published (1977b) was asking anyone who expressed such sentiments to justify them. There were no answers.

(vi) "Truth will out" was another reply. That is to say, "these differences will be settled sometime in the future, perhaps as a result of new findings".

My answer. This implies that there is not enough evidence at present to settle the matter. I regard this sentiment as an unwillingness to face contradictions already identified in their own views.

(vii)"No other cell biologists agree with Hillman, or are prepared to say so openly."'

My answer. After most of the 250 lectures on this subject that I have given in Britain, Continental Europe, North America, Israel, Thailand and Australia, members of the audience have come up to me to say they agreed with me. I asked each of them if they were prepared to express the same views in public, not necessarily mentioning my name. Their replies included, "No, no, I am afraid that I cannot do so as I'm completing my Ph.D." "I am applying for a lectureship". "I am seeking funds for my research". "I am applying for a chair". Two skilled electron microscopy technicians, who prepared illustrations for one my books, responded to my request to acknowledge their contributions by saying "No, No. It would be more than our careers would be worth."

In addition to these reactions, there were other personal consequences.

(viii) I was prevented from presenting my views at the International Society for Neurochemistry, the European Society for Neurochemistry, the German Physiological Society and the (British) Physiological Society, most recently, in June,2011. I have been a member of the Society for more than 40 years. No reasons were given for the refusal to allow me to present a paper. Sometimes, I was offered the possibility of presenting my views on a poster. I felt that these topics were too important for a fruit market presentation. When one shows a poster, if one engages in dialogue with one interested person, the others file by. In my view, posters are the tools of the intellectual proletariat, which allow it to demonstrate attendance at a meeting of a learning society, while the high priests proclaim their views.

(ix) In 1964, before I presented my first unpopular paper at the Physiological Society, the Chairman of the meeting at Mill Hill, Professor William Feldberg, told me that he had heard that my

paper would be attacked by a senior member of the Society, whom he was not prepared to name. I believed that I had anticipated the likely awkward questions, and I said that I would withdraw my paper temporarily, if my opponent were to undertake to repeat my experiments. He refused to do so, so I persisted in giving my presentation. For some reason, the physiologist who had agreed to introduce my paper did not turn up. After my presentation, Professor Augustus Born led a strong attack on me. I was asked five questions three of which I had already answered in my presentation. About 200 physiologists, not all of them members of the Society, were present. About 15 voted against, and about 4 voted for publication. The rest abstained, or did not have the right to vote. The abstract was not published. The next Monday, the distinguished biophysicist, Prof J. A. V. Butler, telephoned me to say that -- in his view -- the rejection of my communication by the Physiological Society had ruined my chances of ever being appointed to a permanent academic post in Britain.

Of course, the politics of this situation do not matter. My paper in 1964.reported the effects of light, sound, subjection to an electric field, centrifugation and different concentrations of sodium and potassium ions, on the stability of adenosine triphosphate, creatine phosphate at 37^{o}C and arginine phosphate solutions at 24^{0}C. Most of the material was not published, except in the International Information Exchange 1 on Phosphates, No 190, 3rd July, 1964. However, the effect of light on ATP was the only part which did appear (Hillman, 1966). Professor Albert Amat of the University of Rovera I Virgili in Spain repeated some of my experiments, and I believe that his results were similar to mine. I never found out the reason for the hostility of some biochemists to my findings. It is a shame that the experiments have not been duplicated exactly, and the results were not confirmed or shown to be wrong.

(x) Since 1972, when my book, 'Certainty and Uncertainty in Biochemical Techniques' was published, I have not received a single penny from public funds to support my research in neurobiology, cytology, or resuscitation, other than my salary as reader in physiology at the University of Surrey. My work in resuscitation is entirely without controversy. I was extremely fortunate that the work of the laboratory was supported by the Handicapped Children's Aid Committee of London, and by the late Professor David Horrobin, Managing Director of Scotia Pharmaceuticals.

(xi) In 1958, when the University of Surrey was in serious financial difficulties, it's then Vice-Chancellor, Dr. Anthony Kelly, and the Council of the University attempted to close the Unity Laboratory of Applied Neurobiology. At the time, I was an elected senator. I wrote to my friends in Britain and abroad about the intended closure and several letters in my support were sent to the University of Surrey. The Vice Chancellor did not report them to the Council or to the Senate. The University gave as grounds for the proposed closure: firstly, my research was expensive; secondly, my scientific views were unpopular; thirdly, I had been unable to attract external funds. I circulated documents, showing that my laboratory was the cheapest in the faculty. Then a question was asked in Parliament about whether the cut in funding would limit the expression of unpopular views. I also showed that like me, 70% of the academic staff had not received any outside finance support. The Association of University teachers backed me. Nevertheless, the Council and the Senate decided to close my laboratory, and to take away my tenure as reader in physiology. Despite this decision, I kept on working full-time until I retired aged 65, because of the support indicated above. I believe that at the time I was the only British academic whose tenure was taken away due to the unpopularity of his/her scientific views.

(xii) I have had the greatest difficulty in publishing my books. I have had to submit them to lesser-known publishers, who have not always had adequate marketing departments. Consequently, the books were not reviewed by prestigious journals, and so did not sell well. Similarly, my manuscripts of papers have been rapidly returned. I have been told by the 'European Journal of Neuroscience' that synapses are not central to the interests of its readers, and by the 'Journal of Neurosciences' that the existence of synapses has no implications for the biology of cells.

(xiii) After I had been the Reader in physiology at the University of Surrey for 22 years, I applied for a Chair. At the time, I had published 4 books, and about 80 full-length papers. My application was turned down. The Vice-Chancellor told me that the reason was that a Fellow of the Royal Society had told him that my views were considered controversial among biologists. I did not receive any documents, assessing the quality of my research. The fact that I was the Chairman of the Association of University Teachers at the University at the time may also be relevant, although the University denied that this was so.

(xiv) Whenever I have had substantial queries about consensus views in cell biology , I contacted the pioneers of these views to try to meet them face to face. A month ahead, I sent them two-page summaries of what I wished to discuss. Among those who were not prepared to meet me were: Professors: Augustus Born, Christian de Duve, Hugh Huxley, Sir Bernard Katz; Keith Porter. Those who were prepared to meet me included: Professors, Paul Glees, Sir Ernest Chain, Britton Chance, James Danielli, Hugh Davson, Sir Andrew Huxley, Sir Hans Krebs, and J David Robertson. Most of those to whom I wrote did not reply, but those who did gave the following reasons for refusing: they were busy; they had already heard my views; refutation of my ideas had already appeared in the literature; " more heat than light would be generated if we met". I have always maintained that any author, who has written a book or a paper, or who has appeared on the radio or television, obligates himself or herself, to enter into dialogue with all serious and interested parties.

Courses of action if the criticisms are correct

The following would seem appropriate:

(i) experiments in vivo should be preferred over those in vitro, because the former involve less changes of entropy;

(ii) experiments in vitro should be carried out in physiological media (Table 8);

(iii) research workers should list all the assumptions inherent in their procedures, interpretations and conclusions. They should test or consider the warrantability of each of them;

(iv) they should carry our comprehensive control experiments to examine the effects on their system of all reagents used in the relevant concentrations, and of all physical manoeuvres. If any of the latter is found to have significant effects on results, the experiment should be redesigned to minimise the effects until they are below significance. If the procedures cannot be so modified, they should be abandoned, and different procedures should be designed;

(v) experiments using low energy procedures should be preferred to those using high energy;

(vi) in addition to control experiments on individual steps of procedures, control experiments should also be carried out comprehensively for the whole procedures of subcellular fractionation, electron microscopy, histology, histochemistry, chromatography and electrophoresis;

(vii) electron micrographs rather than diagrams, of whole tissues should be produced, showing, membrane receptors, transmembrane molecules, lysosomes, peroxisomes and molecular motors. The consequences of being unable to do so should be considered;

(viii) students should be encouraged to ask, and insist on answers to, fundamental questions;

(ix) journal referees and members or grant giving bodies should be prepared to enter into dialogue with the authors of manuscripts and applicants for research grants;

(x) ombudsmen should adjudicate the fairness of editorial decisions and applications for research funds;

(xi) research workers should always analyse their own experiments critically.

Modern medical research

It is widely believed that medical research since the Second World War has been very successful. This research work may be divided into three kinds:(a) some important findings have been serendipitous or accidental, for example: discovery of penicillin; use of lithium for bipolar disorders ; neuroleptics for schizophrenia; steroids for skin diseases; (b) many apparent advances have resulted from applications of technology. These include: mass vaccination; intensive care units; cardiac surgery; war surgery; keyhole surgery; tissue transplantation; (c) there is a third kind of medical research into the *genesis* of diseases, such as carcinoma, sarcoma, leukaemia, multiple sclerosis, schizophrenia, Alzheimers' dementia, motor neuron disease, muscular dystrophy, etc. It is absolutely remarkable how *unsuccessful* this sort of research has been. If one knew the basic mechanisms, whose disarray induced disease, one could then design logical interventions to prevent them developing. This is the approach of geneticists who hope to locate the genes causing hereditary and gene-linked diseases, and to intervene early to prevent their development. It remains to be seen whether this experimental approach will be successful.

If one believed that accidental findings or application of technology had produced so many important findings that we should depend on them to advance medical and biological research, one might just as well abandon all basic research, especially on the genesis of disease and the action of drugs. Large cancer research organisations and pharmaceutical companies do not believe that we should just wait for advances to result from accident or good luck. They believe in the power of well designed experiments. Otherwise they might just as well abandon fundamental research, and wait for Godot

My view that the latter research has been so unsuccessful, because it has ignored natural laws is only a hypothesis. However, it is true that the cost of failure so far has been high. The most paradoxical aspect of scientific research is that it is widely believed to be objective, but that

intellectual integrity, which is its highest resolve, is subjective. I do wish it on all my colleagues and successors.

References

Here follows a list of my publications and unpublished manuscripts in date order, plus three written publications, which have taken issue with the views of Mr. Peter Sartory and myself.

Atkins, P.(1994). *The Second Law of Thermodynamics*. Freeman, New York.

Deutsch , A (1962). Artifacts, the great problem of electron microscopy. *Wiss. Zeitschr. De Moritz Arnt Univ., Greifswald. Xi Math. Natur Reihe*,**2.**

Golgi C. (1898). Sur la structure des cellules nerveuses des ganglions spinaux. *Arch. Ital. Biol.,* **30,** 278-286.

Hawes, C.(2010). No problems in cell biology. *The Biologist*,**57,**

Hillman, H. (1966). The effect of visible light on ATP solution. *Life Sci*.,**5**,589-605.

Hillman, H. (1972). *Certainty and Uncertainty in Biochemical Techniques*.Surrey University Press, Henley on Thames.

Hillman, H. (1977a). The unit membrane, the endoplasmic reticulum, and the nuclear pores, are artifacts. *Perception,* **6,** 667-673.

Hillman, H. (1977b). The value of textbooks. *Nature*, **267**,102

Hillman, H. and Sartory, P. (1978). Letter. *Observer,* 1st January, London.

Hillman, H. (1979). Control experiments for biochemical techniques. *Biochem. Soc. Bull***., 1**,3.11.

Hillman, H. and Sartory, P. (1980). *The Living Cell.* Packard Publishing Company, Chichester.

Hillman, H. (1986). *The Cellular Structure of the Mammalian Nervous System.* MTP Press, Lancaster.

Hillman, H. (1991). *The Case for New Paradigms in Cell Biology and in Neurobiology*. Mellem Press, Lancaster.

Hillman, H. and Jarman, D. (1991). *Atlas of the Cellular Structure of the Human Nervous System*.Academic Press, London.

Hillman, H. (2008). *Evidence-based Cell Biology, with some Implications for Clinical Research*. Shaker Publishing, Maastricht.

Hillman. H. (2009). Procedures used in cell biology ignore natural laws. *J. Biol. Phys. and Chem*., **9**,130-135.

Hillman, H. (2010). Two problems with cell biology and what should be done about them. *The Biologist***, 57,** 42-46.

Hodgkin, T. and Lister, H. (1827). Notes on some microscopic observations of the blood and some animal tissues. *Phil. Mag*.**ii,** 130-138.

Horne R.W. and Harris, J.R. (1981). The electron microscope in biology. *School Sci. Rev*., **63,** 53-68.

Kistler, J. et al. (1982). Structure and function of an acetylcholine receptor. *Biophys. J.* **37**, 371-383.

Michell, R. H., Finean, J.B. and Coleman, R. (1982). Widely accepted modern views of cell structure are fundamentally correct. A reply to Hillman and Sartory. *School Sci. Rev***., 63**,434-441.

Robertson J.D. (1959). The ultrastructure of cell membranes and their derivatives, in *The Structure and Function of Subcellular Components.* Cambridge University Press, Cambridge.

Schleiden, M.J. and Schwann T, (1838). *Contributions to Phytogenesis*. Sydenham Society, London.

Weakley, B. (1972). *Beginners Handbook in Biological Electron Microscopy.* Churchill-Livingstone, London.

(Latest version, 29.6.2011)