A Serious Indictment of Modern Cell Biology and Neurobiology

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Since 1970, I have examined large areas of cell biology and of neurobiology, and I have concluded that the cell biologists have made substantial errors in their experiments, mainly because they have not carried out adequate control experiments, have disregarded the laws of geometry, thermodynamics, chemistry and physics, and have been unwilling to recognise the artefacts produced by the procedures used in histology and electron microscopy. Here, I have summarised my publications since 1972. The responses to the points I have made are indicated in *italics*, and my conclusions in **bold** type. I have already published a list of unanswered questions in biology and neurobiology, (Hillman, 2003), most of which still remain unanswered in 2013.

Molecular biology	The chemistry of living intact biological systems. *Many biologists consider that molecular biology, biochemistry and the function of tissues, can be studied by examining dead, homogenised, stained, or tissues which have been grossly changed from their states in vivo.* **Biologists have shown little interest in the effects that the procedures they use, have on the structure and chemistry, of the tissues they are studying. This has lead them into the study of many artifacts, and distortions of the chemistry of the living systems.**
Open or closed systems	Living systems are open, and it is extremely difficult to carry out calculations on them, because they often change more rapidly than they can be measured, and the parameters are often unknown or unknowable. Unfortunately, nearly all biochemical experiments in vitro are carried out in partially closed systems, because the glass, the fluids, the plastic containers and the vacua, in which they are carried out, conduct heat poorly and at different rates. This means essentially that one cannot measure rates of reactions in vitro, which are intended to reflect those in the original living organism. *If one were to adopt this attitude, one could not do any experiments in vitro.* **Therefore, the most useful experiments would be those carried out on living animals and plants, in which the investigation procedures were designed not to change the entropy of the systems significantly.**
Structure	The anatomy of living organisms. This should not include fixing the tissues, dehydrating them, sectioning, or staining them, all of which change them grossly (Chughtai, Hillman and Jarman, 1987, Hillman, 2000). *Most modern biologists consider that they are examining the structure of living tissues by histology, histochemistry, or electron microscopy.* **Examining living or unfixed cells by light microscopy is the best technique, because it avoids using a number of strong chemical reagents and physical manoeuvres.**

Function	The chemistry of intact living biological systems (please note that this is the same as the definition of molecular biology).
The second law of thermodynamics	This states that in a closed system, any change in entropy, such as homogenisation, centrifugation, dilution, filtration, or elution, must cause a change in free energy. Free energy drives the rates and the equilibria of all chemical reactions. Thus, it is not possible to change the entropy of a system without changing its chemistry. *Those who homogenise tissues, or carry out subcellular fractionation, have simply ignored this law, although they have not denied that it is relevant. It has also been said that this is not a useful criticism, because it would make experiments in vitro virtually impossible.* **I have published a list of experiments, mostly in vivo, which can be done with minimal disobedience to the second law of thermodynamics**. (Hillman, 2008, page 430).
Light microscopy and histology	Histology and histochemistry involve fixation, dehydration, sectioning, and staining, tissues, which shrink and distort them, and they are subject to a battery of mostly unnatural chemicals, so that these techniques are not acceptable to find out the structure or the biochemistry of living cells. *It is true that histology, histochemistry, and electron microscopy, require many chemical reagents and mechanical manipulations, but most biologists believe that these procedures give real information about the structure of cells.* **Unfortunately, we have shown the distortions produced by histology and histochemistry, and the artefacts produced by electron microscopy (Hillman and Sartory, 1980, pages 35-78). Therefore, we have concluded that examination only of unfixed and unstained tissues gives reliable information.**
Electron microscopy	Electron microscopy involves depositing heavy metal stains on tissues, fixed by chemical fixatives or deep freezing, and looking at the stains plus tissues, without knowing the contributions of each. The assumption of both histologists and electron microscopists is that the reagents do not change the structure, or alter chemistry of the tissues significantly. *The explanation, which has been offered about our assertion that the procedure for electron microscopy produces many artefacts, is that electron microscopists select the micrographs which they use to illustrate the particular features they wish to show.* **This does not explain why the membranes round the cell, the nucleus, and the mitochondria which appear remarkably uniformly distant apart in virtually every electron micrograph, which will have been cut from different directions. We have concluded that the electron microscope is not a suitable instrument to examine biological tissues, because heavy metal salts are deposited on the fixed tissues, and the procedure produces many artefacts, (Hillman and Sartory,1980).**

The extracellular fluids	These include the serum, the lymph, the interstitial fluids, the synovial fluids, the aqueous and vitreous humours, and the cerebrospinal fluid, all of which are fairly similar in their chemical composition. However, the volume of interstitial fluid can not be assessed accurately by electrical measurements or by electron microscopy. In living brains, it is difficult to know what the resistance of the tissue measures, and shrinkage as a result of preparation for both histology and electron microscopy, prevents one measuring the extracellular space by these techniques. *Much information has been obtained by micro-chemistry of small samples of extracellular fluid, and also by using macro-chemistry on the more abundant fluids.* **The chemistry of most of the extracellular fluids can be measured fairly accurately, but the interstitial fluid is difficult to measure or to know its chemistry.**
The shapes of cells	These can only be studied by light microscopy of living or unfixed cells, as histological, histochemical, immunocytochemical and electron microscopical, preparation procedures, distort and shrink cells in tissues. It makes them more square or cubical, as they compress each other. Most histologists, histochemists and electron microscopists do not pay much attention to these distortions. *They believe that both histology and electron microscopy give more accurate views of the structure and even the chemistry of the tissues, than does the examination of unfixed issue by light microscopy, or observations in vivo.* **Some biologists may not have read the literature on the effects of their procedures on the shapes of cells.**
Three dimensional models	It is impossible to make a three dimensional model of a cell, in which normal intracellular movements of relatively large structures are occurring, if a cytoskeleton is present. The diagrams in classical textbooks of biology for undergraduates, such as 'Gray's Anatomy', show drawings of cells which are a mixture of two and three dimensions, and they are almost as impossible to model as Escher's famous drawings *No one has responded to this assertion, which I have made in books, publications and lectures, in several countries.* **I hope that biologists will be able to do so in the future, because it is very important that illustrations represent genuinely three-dimensional models of cells. In my opinion, they have a duty to abandon these models, unless they can do so.**

The chemistry of the cell membrane	The chemistry of the cell membrane was originally examined by low angle diffraction. (Schmitt, Bear and Clark, 1935). It was also calculated by reference to the experiments of Gorter and Grendel (1925), and the Davson-Danielli (1936) lipid-protein hypothesis. More recently, it has been measured in membrane fractions. *Subcellular fractionation has been used to derive values for the concentrations of lipids and proteins in cell membrane fractions***.** **One cannot study its chemistry by electron microscopy either. Therefore, its chemistry cannot be known despite the confident statements in the literature about it. Various authors have proposed a large number of different orientations of the molecules within it, precisely because there is no way of deciding for certain what their orientations are in life, (see Hillman, 2008, pages 109-110). All standard textbooks of biology show the orientation of the lipids and proteins in the cell membrane, but, unfortunately, these models are only hypotheses. It may be concluded that it is impossible to know the chemistry of the cell membrane, its width, its structure, and the orientation of molecules within it.**
The structure of the cell membrane	This cannot be known (a) by light microscopy, because it is too thin to be resolved; (b) by electron microscopy, because heavy metal salts are deposited on either side of the dehydrated membrane, and the spacing of the deposits are measured; (c) by subcellular fractionation, because the procedure is likely to change the chemistry, (Hillman, 1972). It is believed to consist of lipids and proteins in the proportions stated in the literature, but the values derived by subcellular fractionation cannot be depended upon. When one looks at a cell membrane by phase contrast microscopy, one sees that its refractive index is different from that of the extracellular fluid, and of the cytoplasm. The light microscope can see that the membrane is present, but can not assess its thickness, structure or chemistry. However, the chemistry of the salts used in electron microscopy and in histology is clearly different from the chemistry of the membrane. The cell membrane, which has been renamed, the 'unit membrane' by J.D. Robertson, (1959), is not trilaminar, but it is composed of only one layer. The currently accepted model of the structure of the membrane, (Singer and Nicholson, 1972) is unsatisfactory, because it supposes that, in life, the membrane is fluid, but this can neither be proved nor disproved. It is not proved by the finding that molecules move across its surface. The hypothesis also suggests that protein molecules protrude from surface of the membrane, which they cannot be seen to do on electron microscopy. *Membranologists have never addressed these arguments*. **The trilaminar appearance arises from the fact that the heavy metal salts deposit on *both* sides of any single membrane, so that they all appear trilaminar. As Chairman Mao said," You can not clap with one hand."**

Subcellular fractionation	Subcellular fractionation started in the 1930s and some of its pioneers included Claude, de Duve, Beaufay, and others, (see Claude, 1946, and De Duve and Beaufay, 1981). Biochemists use this procedure widely, because they do not realise that it ignores the second law of thermodynamics, which states that one cannot change the entropy of a system, without changing its free energy. During subcellular fractionation, water, soluble compounds, substrates, enzymes and cofactors, diffuse from the parts of the cells in which they originated to other sites. It is virtually impossible to prevent diffusion, except by binding or precipitation. Control experiments have never been published for the effects of homogenisation and centrifugation on the systems being studied, presumably because it has been assumed that they have no effect at all (Hillman, 1972). *All biologists accept the findings from subcellular fractionation, and they appear in all textbooks to date*. **One of the consequences of the second law is that measurements of enzyme activities, breakdown of unstable substances, and rates and equilibria of reactions, should only be carried out in vivo.**
Compartmentation	When one looks at a metabolic map, one sees hundreds of enzyme reactions, 10 s of cycles and pathways, and so it becomes clear that it simply would not be possible for each reaction to have its own compartment. Even more to the point is the fact that unicellular organisms, including yeasts and plants, can carry out virtually all biochemical reactions in only one apparent compartment. *The underlying belief of those who carry out subcellular fractionation is that the main biochemical activities are located in particular compartments of the cell. For example, they point out that the mitochondrial fraction has been found to be the site of oxidative phosphorylation, while the membrane fraction houses ATPases.* In life, there is likely to be compartmentation of different reactions in different parts of cells, but their location cannot be discovered by using subcellular fractionation. In mammalian cells, modern cell biologists list the following compartments: the extracellular fluid, the cytoplasm, the cytoskeleton, the mitochondrion, the Golgi body, the lysosome, the peroxisome, the nucleus and the nucleolus. It is clear that many chemical reactions must share compartments. **However, the concept of compartmentation cannot justify subcellular fractionation, and the amount of energy used to separate the different fractions makes it very unlikely that one can find out the location and activity of a particular chemical such as an enzyme, by disruptive techniques.**

Ion channels	An electron micrograph of only one ion channel has ever been described, (Toyoshima and Unwin, 1988), whereas there are believed to be 10s of them across the membranes of every nerve cell. The minute patch clamp currents, which physiologists detect, probably come from chemical junctions, and electronic components in the recording systems used (Hillman, 2008, pages 92-93). Patch clamp electrodes have been shown to be blocked up by tissue *There is plenty of physiological evidence for the existence of these channels***.** **It is only a hypothesis that the small currents (pA) originate from the structures postulated to be ion channels. It is a proper question to ask, why all cells do not appear to be pitted by ion channels. Even those who believe that ion channels exist, agree that only one of the large number of different channels postulated, has been detected.**
Active transport	*It is widely believed that the higher concentration of sodium ions outside the cell membranes, and of potassium ions in the cytoplasm are caused by a sodium ion activated ATPase enzyme located within the walls of the cell membrane, and this pump requires energy to work. The distribution of the sodium ions against the electrochemical gradient is taken to mean that they have been pumped.* The following considerations should be borne in mind: (a) the sodium activated ATPase molecule has a larger diameter than the width of the cell membrane, so that it should be seen protruding from the cell membrane on electron micrographs, but it cannot be seen; (b) the finding that the sodium and potassium ion gradients fall when the tissue is deprived of oxygen, substrates, or when it dies, does not itself provide sufficient proof that the energy is needed for a pump. In all the latter circumstances, the tissue is so changed chemically that the redistribution of sodium and potassium ions could be due to a completely different mechanism. One cannot accept the reasoning of 'post-hoc, propter hoc' in this case; (c) the ATPase is isolated by subcellular fractionation; (d) Ling (1962), has put forward an alternative mechanism in which the sodium ion has an affinity for the substances in the extracellular fluid, and potassium ion for substances in the cytoplasm. The concept of a pump is rather nebulous, and it is difficult to prove or disprove**.** **It seems to me that Ling's hypothesis is more sound physicochemically and less mysterious, than the concept of ion pumps.**

Transporters and carriers	These can also not be seen by electron microscopy. Neither the biochemists nor the electron microscopists have offered any explanation for their inability to detect them. Even if the transporters existed in life, it may be impossible to isolate them and to characterise them chemically. *The biochemists drew up by criteria for the concept of membrane transporters and carriers, (Wilbrand and Rosenderg, 1961).* **It may be concluded that they are unlikely to exist as structures in living membranes**.
Receptors	Many receptors have been identified, and they have been sequenced. Their diameters have been calculated, and they are up to 3 times the widths of the cell membranes. Nevertheless, they simply cannot be seen on electron micrographs, where the membranes appear as smooth as angels' cheeks. The main evidence for belief in them is that transmitters and drugs act at extremely low concentrations. *The only explanation for their apparent absence in electron micrographs that has been offered is that they must have moved during the preparation for electron microscopy. It is normally assumed that if a transmitter or a drug acts, it nearly always binds to a receptor, the majority of which are found to be located in the microsomal fraction. This is believed to contain the cell membranes and the endoplasmic reticula. By the way, has anyone seen a microsome?* Another explanation is that the receptors move from their original sites in the cell membrane to other parts of the cell, during the preparation for electron microscopy. If this explanation were accepted, it would mean that electron microscopy was unsuitable for detecting the locations in life of any small structures in tissues**.** **It must be concluded that receptors as structures and sites, simply do not exist in living cells. Elsewhere, I have put forward a different simpler hypothesis for the action of transmitters and drugs (Hillman, 1991).**

Ligands	Sometimes, ligands are employed, when receptors for transmitters, drugs, hormones, antigens, G proteins, agglutinogens, and oestrogens, are being sought, instead of these particular substances. The ligands are believed to occupy the same sites as the latter substances, and are frequently used to locate and isolate them. Sometimes a transmitter or a drug has a completely different chemistry than that of the ligand, which is used to replace it. Also, of course, it may have effects, other than, or in addition to, those of the transmitter or drug itself. Sometimes, ligands are chosen, because they compete with the transmitter or drug, but, often because they are more stable. Also, a ligand , like an activator or inhibitor, may partially activate or inhibit, the original transmitter or drug. This is difficult to interpret. Physiologists, pharmacologists, and biochemists, have never recognised this problem, so that there has been no reaction to it being pointed out. However, for the reasons I have given, it seems to me that the best substance to look for receptors or binding sites of, for example, acetyl choline is acetylcholine itself, rather than nicotine or muscarine. The fact that acetylcholine is more unstable than the latter two substances, seems to be good evidence that the latter substances should not be used as ligands, because this instability is an important chemical differences. *Nowadays,the use of ligands is very widespread, especially in pharmacology, that it has not been questioned, but probably should be.* **I would conclude that their use is not desirable. However, I am not denying that drugs and transmitters do not compete, and may activate or inhibit these particular substances, whether or not their chemistry is similar. It does not seem at all surprising that substances having similar chemistry should activate or inhibit each other**.
Glycolipids	Diagrams often show these as tubes attached to the outsides of cell membranes, but they cannot be seen by light or electron microscopy. *Belief in their existence arises from experiments involving subcellular fractionation.* **This is a totally unsuitable procedure for attempting to understand the chemistry of cell membranes, and less disruptive procedures should be used.**

Cytoplasm	This is a translucent fluid, in which mitochondria can be seen moving in living cells. It has a low viscosity. It contains no Golgi apparatus, endoplasmic reticulum, cytoskeleton, tubulin, actin, spectrin, vimentin, contractile proteins, or any other filaments. The following intracellular movements can be seen by light microscopy in living tissues: Brownian movement, streaming, nuclear rotation, convection, meiosis and mitosis. These movements would not be possible, if the cytoplasm were full of the former structures. The movements can only be seen in living cells, whereas the structures are only seen in dead fluorescent tissues or in electron micrographs. *One explanation, which has been offered by the electron microscopists is that the particles could move through the cytoskeleton. Another is that the moving particles contain enzymes which would dissolve the cytoskeleton in real time, and it would reform after they had passed through.* **The first explanation does not take into account that the particles have diameters several times more than that of the weave of the cytoskeleton (Hillman and Sartory, 1980, page 52). In respect of the second explanation, Brownian movement can be seen in colpoids containing finely ground glass, pollen, and other non-biological materials, which contain no enzymes. I prefer evidence from living tissue to what can be seen in heavy metal deposits on dead tissue. Therefore, one is forced to conclude that the whole cytoskeleton is an artefact of dehydration and staining. I believe that this point is irrefutable.**
Endoplasmic reticulum	This is an artefact, because, in life, (a) the above quoted intracellular movements occur; (b) the cytoplasmic viscosity is low; (c) the reticula do not appear in random orientations. This can be seen by electron microscopy, and by fluorescence microscopy. It is not useful to consider the properties of the subcellular fraction, called the microsomal fraction. *The literature is fairly divided on whether it considers that the reticulum is a network, or is a series of lamellae. Neither of these is seen in all orientations in electron micrographs (Hillman and Sartory, 1980, pages 46-50).* **The endoplasmic reticulum is an artefact of dehydration and staining.**

Golgi body	Golgi, (1898), described the appearance of this body, as a consequence of staining neurons of brown owls with silver salts, and it was subsequently seen in most other stained and unstained cells. It was later also identified in electron micrographs of cells, but it was not appreciated that the appearances, shapes and dimensions, seen by light microscopy, were completely different from those seen by electron microscopy (Hillman, 1978). In the early 20th century, any structure or particle seen in the cytoplasm of stained cells, was called a 'Golgi' body. Other than ourselves, no author has commented on the discrepancies between the light and electron microscopy. *In the early literature, there are many drawings of Golgi bodies by dark ground illumination and in stained tissue*. **I have seen no modern micrographs of living cells, for example, in culture, showing these structures. I believe that the failure to see the Golgi bodies in living cells means that they do not exist in life.**
Lysosomes	These cannot be seen in unfixed cells or in cells in tissue culture. The movements of particles injected into the cytoplasm do not appear to be obstructed by such bodies, which are believed to be about the same size as nuclei. Their existence originated from experiments on subcellular fractionation, in which a certain fraction was found to be rich in acid hydrolytic enzymes. The latter finding does not bear on the question of whether the structures exist in the living intact cells. *Lysosomes sometimes appear as vacuoles adjacent to injected substances, and, sometimes, as granules. It is an open question whether the two different kinds of lysosomes represent the same structure.* **I do not think that one that can conclude that lysosomes exist, until and unless, they can be shown in living or unfixed cells.**
Peroxisomes	The same remarks apply to peroxisomes as to lysosomes. *The fraction of peroxisomes contains a different selection of enzyme activities than do the lysosomes.* **Although it is a widespread practice, one cannot describe the 'function' of a fraction, and believe that that function which one has attributed to it, justifies its existence as a structure, because of the complex chemical procedure which must be used to separate it. The peroxisomes are also artefacts, for the same reasons as the lysosomes are**.
Mitochondria	Mitochondria are filiform structures, which can be seen in continuous movement in the cytoplasm of living cells. By light and electron microscopy, they can be seen in many orientations from circles, to sausage shapes, to worm shapes. *They are widely believed to be the site of oxidative phosphorylation, and of mitochondrial DNA.* **In view of the uncertainty about the preparation of mitochondrial fractions, neither of these localisations can be known with certainty, but there is no doubt that mitochondria exist in the cytoplasm of living cells.**

Mitochondrial membranes	According to the electron microscopists, each mitochondrion is surrounded by a 'unit' trilaminar membrane, the inner layer of which invaginates to form the cristae. *According to J.D. Robertson's (1959) model, the mitochondrion itself should be surrounded by another trilaminar membrane. One should see three laminae around each mitochondrion. This is not seen by electron microscopy. The inner and outer mitochondrial membranes have also been separated by subcellular fractionation, and the fractions contain different enzymes*. **This localisation can not be accepted. It is likely that the mitochondrion is surrounded by only one membrane, and the fractionation yields uncertain information about the localisation of the enzymes.**
Mitochondrial cristae	These appear nearly always as shelves normal to the plane of section, and are rarely, if ever, seen in any other orientation. Fortunately, nature has given us controls, in which the whole mitochondria themselves do appear in three dimensions, both by light and by electron microscopy. *Mitochondriacs have never responded to this observation***.** **It means that the appearance by electron microscopy of cristae must be two-dimensional, and they must be artefacts, due to drying out of the mitochondrioplasm, which must have appeared after the sections have been cut.**
Mitochondrioplasm	This is probably the liquid phase of the mitochondria, whose chemistry can probably not be known, because the subcellar fractionation procedure puts too much energy into the mitochondrial fraction. *Electron microscopy to show the contents of the mitochondria involves dehydration***.** **It is concluded that the chemistry in life of the membranes or the mitochondrial contents, cannot be known, and may never be.**

Cytoskeleton	This is the name given by modern cell biologists and electron microscopists to all the structures, other than the mitochondria, which they see in the cytoplasm. If the cytoplasm were so full of cytoskeleton, it would certainly not permit the intracellular movements which are characteristic of living cells, and can be seen in unicellular organisms, in tissue cultures, and in plants. Peter Sartory and I, (1980, pages 35-78) showed that they are all artefacts. Furthermore, each of the elements of the cytoskeleton should appear in a random range of orientations, which they do not. *Having identified the particular elements making up the cytoskeleton, modern biochemists think that it is necessary to define the 'function' of each of these elements*. **The hindrance which the cytoskeleton would offer to intracellular movements seen in living cells, plus its failure to be seen in three-dimensions, makes it almost certain that the whole cytoskeleton originates from precipitation of this cytoplasm during dehydration, and from the histological and electron microscopic reagents used to stain the tissue. All the elements, of which the cytoskeleton is believed to consist, are artefacts.**
Nuclear membrane	The light microscopists see the nuclear membrane consisting of only one imperforate layer. However, the electron microscopists claim that it is composed of two trilaminar membranes, each with a pale layer between. One of these comes from the cisternae in the cytoplasm, and the other from the nucleus itself. Therefore, it should consist of three or four laminae, but, unfortunately, electron microscopy does not show this. *Diagrams of the cell in the modern textbooks simply do not show the relationship between the cell membranes and the cisternae, and the cisternae and the nuclear membranes.* As stated above, any real layer will stain on both sides with a heavy metal deposit, and appear to consist of two layers. **The simplest hypothesis is that the nuclear membrane consists of one imperforate layer whose chemistry is unknown and probably unknowable.**
Nucleus	This body can be seen floating in the cytoplasm in tissue cultures, whether it is seen to undergo rotation and changes in shape. *It contains DNA. This is proved by spectroscopy, and by nuclear transplantation during fertilisation in vitro.* In addition to the nuclei, which are found normally in cells, many syncytia contain several nuclei surrounded by cytoplasm, without individual membranes surrounding the cytoplasm belonging to a single nucleus. Syncytia are rarely recognised in histology textbooks. These include the naked nuclei of the brain, the Schwann cells, the granular cells of the cerebellum, and syncytia are also found in and many other organs (Hillman 2008, pages 51-52). Once again, the nuclear fraction has been examined by subcellular fractionation, but one cannot depend on the results of these experiments. **The nucleus undoubtedly exists in living cells**

Nuclear pores	Electron microscopists show the nuclei to be perforated by a large number of circular pores, through which they believe messenger RNA travels from the DNA in the nucleus to the ribosomes on the rough endoplasmic reticulum. This journey has never been shown by autoradiography, in tissue cultures, or by fluorescence microscopy. If the pores were circular depressions on golf balls, as one's eye passed from the centre to the periphery, they should appear from circular to oval, to spindle, to slit shaped. *When one looks at electron micrographs, one only sees circles, and occasional ovals. In transverse sections, they appear as cracks in the nuclear membranes*. They are not seen in a range of orientations. The pores are almost certainly due to release of the gases in tissue at the time of electron bombardment, very similar in appearance to the craters on the surface of the moon. They may also be due to cracks in the salt deposited on the nuclear membrane, due to the bombardment of the quite different chemical components in the heavy metal salts, the nucleoplasm, the cell membranes and the cytoplasm. Furthermore, if the pores comprise 10% to 20% of the area of the nuclear membranes, (Feldherr, 1972), it would require an enormous amount of energy to stop the two compartments mixing. Also, they would short-circuit the potential differences which have been recorded across the nuclear membranes, (Lowenstein and Kanno, 1963a, b). The nuclear pores are only seen by the electron microscope, but should be picked up in living cells by modern methods of high power light microscopy, if, indeed, they are present. **The so-called 'nuclear pore apparatuses' represent an exercise in graphic artistry, which does not correspond to the electron micrographic images of these alleged structures. The simplest hypothesis is that pores are bubbles in the nuclear membrane, resulting from the energy released by the electron bombardment. Pores do not exist in the nuclear membranes in f living cells.**
Nucleolus	There are sometimes multiple nucleoli in primitive species, but in mammals there is usually only one. Virtually nothing is known about the chemistry of the nucleolus, because it is normally examined by subcellular fractionation. I have put forward the hypothesis that DNA from chromosomes retreats to the nucleolus during the resting stage between division, (Hillman, 2008, page 197). The nucleolus absorbs ultraviolet light (Caspersson, 1950). This body can be seen by light microscopy in living cells and in cells in culture, where it is seen to move continuously. It is also seen by electron microscopy, where it only appears as a blob. **There is no reasonable doubt about its existence in life.**

Nucleolonema	In Uruguay, Estable and Sotelo, (19 51), found a skein of fibres within the nucleoli of unfixed cells, and we saw the same structure in our isolated unfixed neuron cell bodies, (Sartory, Fasham and Hillman, 1971). However, as soon as one fixes the cells for light or electron microscopy, the whole nucleolus becomes opaque, and the nucleolonema can no longer be seen. *Therefore, its existence has been ignored by modern cell biologists, histologists and electron microscopists.* **This structure certainly exists in the nucleoli of many cells, and needs to be looked at by modern high power light microscopic techniques.**
Nucleolar membrane	My colleagues and I separated cell bodies of neurons in saline or in Krebs-Ringer solution rather than in sucrose, which Hyden had used (Hillman, 1986). We examined the nucleoli of human beings, rabbits, rats, guinea pigs and frogs. We found that the nucleoli became much more translucent in these media, and they appeared to be surrounded by membranes. *This observation has been completely ignored by histologists and electron microscopists*. Neither 'Nature' nor 'Gray's Anatomy,' would publish this finding. However, we have shown micrographs of them, (Hussain, Hillman and Sartory, 1974; Hillman and Jarman, 1991, pages 24, 28, 117). **This membrane undoubtedly exists around the nucleoli of neurons, but we do not know whether they can be seen in other tissues.**

Muscle filaments	*Thick and thin muscle filaments, and cross bridges between them, are the structural components, which form the basis of the sliding filament hypothesis of muscle contraction*.(Huxley and Hanson, 1959). It is an absolutely beautiful hypothesis, but there are some problems: (a) the filaments are too uniformly distant apart in sections. They should appear in a range of distances apart depending upon the angle of section; (b) it is extremely difficult to find oblique sections of muscle in electron micrographs; one usually sees either perfect transverse or perfect longitudinal sections. This would seem to be rather strange, as it is so difficult to align a muscle before it is stained and sectioned; (c) the muscle should contract with the maximal force when it begins to contract, because the cross bridges should be maximally stretched at the beginning. When the muscle has contracted maximally, the force exerted by the transverse component should have reached its maximum, and, therefore, the muscle fibres should narrow their waists. A contracted muscle should look thinner not fatter. The usual explanation given for this is that muscles are isovolaemic, so that a longitudinal contraction must cause a transverse expansion. Unfortunately, this failure of the muscle to contract in its middle is seen not only in the whole muscle, but also when single muscle fibres are dissected out**.** **It must be concluded that the myoplasm in life is a viscous fluid, which, when dehydrated, forms thick and thin filaments. A new alternative theory to the sliding filament hypothesis requires to be formulated.**
Cross bridges	*These are granules seen by electron microscopy between thick and thin filaments in muscle, which are believed to pull the filaments longitudinally, so that the whole muscle contracts.* They are almost certainly artefacts, because, firstly, they are amorphous, and do not appear to be orientated in such a direction as would pull one filament along the other. Secondly, particles with a similar appearance can be seen between sciatic nerve fibres, (Hirokawa, 1982), which, of course, do not contract. Thirdly, when a muscle is stretched beyond the overlap of thick and thin filaments, it should not be able to contract. It does. The cross bridges are most almost certainly formed when particles of the deposit stain used to show up the tissue gets trapped between the thick and thin filaments**.** **The cross bridges are artefacts of preparation for electron microscopy.**

Neuromuscular junctions	*The neuromuscular junction appears as a flat plate arising from the motor nerve sitting on the distal muscle..It has been seen clearly by histologists, and has been studied extensively by electron microscopists. Unfortunately, unfixed and unstained neuromuscular junctions have received little attention from modern high power light microscopists*. In the current literature, the anatomy and physiology of neuromuscular junctions is assumed to be similar to the same properties in synapses. However, there are several differences between the two (Hillman 2008, page 246). **The neuromuscular junction undoubtedly exists, but it is structure has not been studied in living, fresh and unfixed, nerve-muscle preparations.**
Neuroglia	*Virchow, (1846), originally looked at unfixed brain and saw neurons. He called the 'ground substance' between the neurons, 'neuroglia,' meaning nerve glue. Histologists such as Ramon y Cajal, del Rio Hortega, Penfield, and others, used several different staining procedures and concluded that each different staining procedure was specific for a different kind of neuroglial cell; they were named, 'astrocytes', oligodendrocytes' and 'microglia' (Penfield, 1932). The neurohistologists believed that the central nervous system consisted of neurons, their dendrites, and the three different kinds of neuroglial* ***cells****. The electron microscopists also believed that the central nervous system was solid with the four kinds of cell.* **I have brought abundant evidence that there are only two kinds of cells in the central nervous system, these are neurons and naked nuclei. (Hillman, 1985, pages 82-241). The rest is a syncytium packed with mitochondria (Hillman, Deutsch, Allen, and Sartory, 1977). The structure of unfixed microglia has so far only been studied by ourselves. Neuroglia is not comprised of astrocytes , oligodendrocytes, and microglial cells, but consists of a mass of mitochondria.**
Astrocytes	*Astrocytes are star shaped cells which are believed to be one of the three kinds of neuroglial cells*. In extensive examinations of the literature, I have shown that the dimensions, morphology, tissue culture characteristics, staining, appearances by electron microscopy, and other characteristics of these allegedly different kinds of neuroglial cells overlap so much with the same characteristics in neurons, (Hillman, 1985, pages 52-81). **I have concluded that any cell which has processes is a neuron, and that the only other cells in the brain are naked nuclei .**

Oligodendrocytes	*Oligodendrocytes are generally described as cells with rather few processess.* Unfortunately, one cannot count the number of processes in single sections, because one does not know from what direction the microtome knife has come in a particular section. If one cut a neuron from one direction, for example, near the apex, it would appear to have very few processes, but if one cut the cell nearer its base, there would appear to be many more. **As indicated above, any cell with a number of processes is a neuron.**
Microglia	*These cells in the central nervous system can be seen in any histological section. They are surrounded by neuroglia, and are not seen to be enclosed by their own membranes, by light or electron microscopy*. I have called them 'naked nuclei,' In the literature, they are described as neuroglial nuclei, reactive astrocytes, or satellite cells, and some histologists deny their existence completely. However, we have shown many micrographs of them in unfixed brain and spinal cords, (Hillman and Jarman, 1991, pages 37, 50, 81, 104). **There is no doubt about the existence of the naked nuclei, and they can be seen in any histological section or in unfixed central nervous tissue. The absence of cellular membranes around them has not been previously observed, although it is very evident.**
Ependymal cells	These columnar cells lining the ventricles can be easily seen in the sections, and can be dissected out from the lining of the ventricles. **There is no doubt about the existence of ependymal cells.**
Molecular motors	*These are believed to be fine processes attached to intracellular bodies, such as mitochondria, which cause them to move.* Unfortunately, they are **not** seen by electron microscopy attached to the structures, which they are believed to move. Those biologists who believe that they do, indeed, cause these structures to move, do not seem to have heard of intracellular movements, such as Brownian movement and streaming, which can easily be seen in colpoids**.** **No explanation has been offered as to why they cannot be seen, and the most plausible one is that they simply do not exist.**
Axonal flow	*Precursors, proteins and markers, travel down axons at different rates, unless they bind to any other constituent within the axon, or the axon is obstructed. The rates of movement can be measured and classified. However, any substance which is dissolved, or an very small particle, will move up or down the axon, depending upon the local chemistry and temperature*. They will also move as a consequence of Brownian movement and streaming, even without any energy arising from metabolism in the axoplasm. **One has to ask the question, 'What is the point of measuring the rate of flow, as the answer will be a composite of a number of different forces acting on a particular solute or particle?'**

Transmembrane macromolecules	*These are believed to extend across cell membranes.* Inexplicably, they cannot be seen clearly by electron microscopy. **The simplest explanation for this is that they do not exist.**
Axons	An axon is a single nerve fibre connecting the cell body at one end to nerve fibres, and, at the other end, to a neuromuscular junction. It contains a fluid axoplasm. Myelinated fibres are surrounded by myelin sheaths, which are indented at intervals by 'nodes of Ranvier'. **In life, the axoplasm is translucent, and contains a few particles in continuous motion. It does not contain any cytoskeleton. Sometimes, the axonal membrane invaginates into the axoplasm and bits break off, and can be seen moving freely within it**
Saltatory conduction	*In Japan,Tasaki, (1939), and in Switzerland, Stämpfli, (1951), described the jumping of action potentials from one node of Ranvier to the next, as 'saltatory conduction'. They both believed that this explained why the amplitude of the action potentials did not decrease, as they were conducted down the nerve.* However, according to Kerhhoff's Law, there is some difficulty, because the action potential would tend to pass straight down the axoplasm, which has a low conductivity, rather than escape across the axonal membrane at the node of Ranvier**,** where the resistance of the membrane would be higher than that of the axoplasm. **It seems to me that the concept of saltatory conduction is rather unlikely.**
Axoplasm	In life, the viscosity of this fluid has been found to be low, (Hillman and Sartory, 1980, page 57). *Intracellular movements can be seen, particularly by time-lapse photography***.** **It could contain no cytoskeleton**. **Its chemistry has been determined, (Waxman,1978).**

Myelin sheaths and lamellae	*In a myelinated fibre, this is a thick translucent sheath surrounding the axon, indented at the nodes of Ranvier. According to the Geren, (1954), it is composed of the membranes of collapsed Schwann cells, which are believed to wrap themselves around the axon, during the first few days of life.* However, the refractive index of the axoplasm in living or unfixed axons is lower than that of the myelin sheath, whereas if the latter were composed of collapsed cell membranes, it would have a higher refractive index. Furthermore, Brownian movement has been detected within the sheath. When we looked at myelination in unstained sciatic nerve fibres of rats up to 10 days old, (Hillman and Jarman, 1989), it appeared nothing like the Geren model**.** **Furthermore, in the literature, the lamellae are seen to be the same distance apart in oblique sections of sheaths, as they are in transverse sections. The spacing of the lamellae defies geometry. (So far, I have experienced great difficulty in publishing this finding**). **One can only conclude that whereas the myelin sheath exists around living axons, the lamellae are artefacts of dehydration or staining for electron microscopy**.
Schwann cell	Nuclei can be seen nestling along the length of myelinated fibres. It is widely believed that there is one Schwann cell to every antinode, but when one looks closely, one sees that they occur much less frequently**.** **The Schwann cell nuclei appear to be part of a syncytium. At the moment, no one really knows what the Schwann cell nuclei do.**
Microtrabeculae	Wolosewick and Porter, (1979), described a network in the cytoplasm of cultured human cells, which was even finer than the cytoskeleton**.** At an International Conference on Cell Biology in Berlin **i**n the early 1980s, I asked Porter how movements would be possible in the presence of such a fine network. He did not answer the question. If they existed in life,the microtrabeculae would be even a finer hindrance to intracellular movements of light microscopically visible particles, than the endoplasmic reticulum would be. **Microtrabeculae have since disappeared from the literature. One may conclude that they resulted from staining for electron microscopy.**

Synapses	*In 1897, Sherrington, (1897), gave the name, 'synapses', to the junctions he postulated to be present between two different nerves, when a reflex occurred. Held, (1897), and Auerbach, (1898),saw particles of silver salts on nerve cell bodies, and these were regarded as the sites of Sherrington's proposed connections. Later, excitatory and inhibitory potentials were recorded in the region of synapses.(Eccles, 1953, pages 99-115).* It has not been appreciated that it was only an assumption that the intracellular pipettes were recording from the anatomical synapses, that is, the region where the histological granules were seen. It is extremely difficult to know the exact location of the minute tip of a micropipette. At the time, it did not occur to histologists that these granules could be seen not only on the cell bodies and dendrites, but also away from these sites. It was soon believed that all granules seen on nervous tissues, stained by silver or osmium salts, were synapses. This view still prevails today, but it is contradicted by the following considerations: (a) no granules can be seen on the surfaces of unfixed unstained neurons or dendrites; (b) no dendrites from one neuron are seen to be connected by presynaptic fibre to synapses on the surfaces of other neuron cell bodies or dendrites, (Copestake and Hillman, 2013, submitted for publication); (c) the facts that the synapses appear to be far larger by light microscopy and their numbers much smaller, than they appear by electron microscopy, (Hillman, 1985), mean that the two kinds of microscopists have been looking at different structures; (d) the presynaptic and postynaptic thickenings seen on electron microscopy are equally spaced apart in virtually all micrographs; they are hardly ever seen overlapping, as geometry would require. This observation has also been ignored by neurobiologists; (e) it is generally assumed that all granules seen on stained cell bodies and dendrites are synapses, but the vast majority of them sit on single fibres and are not connected to other fibres, so that they are like old-fashioned telephones, which have no connections with the exchanges. It is a proper question to ask whether there are any anatomical criteria which define synapses by light microscopy, other than the presence of a granule in stained nervous tissue. Would it not be reasonable to expect that silver or osmium staining would deposit granules on tissues, such as liver, kidney, spleen, etc, where no synapses are expected to be present; (f) it is only an assumption that any particle, granule, deposit, oval or sphere, is a synapse; (g) in modern textbooks, on the Internet, and even in papers in learned journals, neurobiologists and editors are prepared to accept diagrams, drawings, models or computer reconstructions, of synapses, even although microscopy and microphotography have reached extraordinarily high standards in recent years. It should not be so difficult to find light micrographs of synapses on living or unfixed neurons. *These findings have been overlooked by neurobiologists.* **It must be concluded that synapses are artifacts.**

Synaptic vesicles	These can only be seen by electron microscopy, when they appear as oval or spherical bodies in the pre-synaptic swellings. For a reason that has never been explained, they appear remarkably uniform in diameter and shapes. Even if the vesicles all have the same dimensions, in a thin section for electron microscopy, they should always appear in a range of diameters. They do not. It is also believed that they each contain a 'quantum' of transmitter or inhibitor, which can not be proved. *Until now, there have only been seen by electron microscopy, but it may be that with the higher resolution offered by modern techniques, such as confocal, lensless, and quantum dot, microscopy, their existence could be confirmed.* To me, they appear like bubbles of dissolved gases, which appear in the saucepan, when one boils an egg. The vesicles are too small to carry out chemistry on them, except by subcellular fractionation. **Their uniformity of diameter makes them likely to be artefacts.**
Pre-synaptic fibres	Dendrites of one neuron joining the synapses on another neuron or other dendrites, are simply not illustrated in the literature, either by light or by electron microscopy. *Occasionally, in silver stained sections, one sees a single knob on the surface of one neuron with a small stalk coming from it, but one does not see that stalk attached to the dendrite of a proximal neuron, for example, (Wyckoff and Young, 1956; De Robertis, 1959).* **David Copestake and I have specifically looked at this question, but we have failed to find such a connection in sections of brains and spinal-cords.**
Specific inhibitors	A 'specific' inhibitor means that the particular chemical acts only at one site or on one reaction. In order to be able to say this, one would have to examine whether it had any other major chemical effects. **It is very unlikely that any powerful chemical has only one action.**
Extracellular markers	Substances such as inulin, sucrose, thiocyanate, xylose and arabinose, have been used as markers to indicate the volume of the extracellular space in tissues. Their use implies that the marker: (i) does not bind to the tissue; (ii) is not broken down by it; (iii) is completely recoverable; (iv) does not have any osmotic effects; (v) has no effects itself on the chemistry of the tissue; (vi) does not cross into the cells; (vii) all extracellular markers should indicate approximately the same volume of the extracellular space as each other. **None of these assumptions has been shown to be true, and so, there is no justification for using these markers**

Intracellular pipettes	Pipettes, with tip diameters of less than 1 µm, penetrate the cell membranes, and are used to measure the potential differences between the extracellular fluid and the cytoplasm. Unfortunately, they should only be used when the chemistry of the fluids on either side of the membranes are similar, but differ only in their concentrations. **Regrettably, this is not true for extracellular fluid and cytoplasm, and so measurements with them must be regarded as approximations.**
Ribosomes	*Ribosomes are separated as a subcellular fraction. This fraction contains a relatively high concentration of RNA. The particles are too small and amorphous to be characterised either by electron or by light microscopy*. Ribosomes can not be seen in some cells, such as muscle, and are usually believed to be the granules on the 'rough' endoplasmic reticulum. All cells are believed to contain RNA, and their presence is assumed even when they cannot be seen, presumably because they are too small, or too diffuse. **If the cells have the same chemical properties whether or not the ribosomes can be observed, how can one be certain of their chemical properties when they are seen?**
Necrosis	This is a change in appearance of issues observed in histological or electron microscopical sections. *It is regarded as showing that the tissue is dead. It is usually examined in sections of fixed tissue so that its chemical evolution is not known. It is seen at the sites of bacterial infection, tumour growth, or tissue destruction.* **It deserves serious chemical investigation.**
Apoptosis	This was described by Kerr, Wyllie and Currie, (1972), as being a different histopathological process from necrosis. Apoptosis was considered to be an active form of the dying of cells based on the density of cells and organelles in tissue sections, and several other characteristics differentiating apoptosis and necrosis have since been described. *The concept of apoptosis has spawned a huge literature*. **The differences between the two phenomena are vague, qualitative, and have not been compared statistically, (Hillman, 2008, pages 404-405).** **The authors of the concept do not seem to have realised that in single sections, one can not measure the relative packing of cells, because differently orientated sections of the same tissue, and the shrinkage during staining, would show different spacing between the cells. Virtually nothing has been published about changes in the chemistry and structure of tissue, which occur, when it is stained. The chemistry of necrosis is not known, and the differences between it and apoptosis are quite unconvincing. It has not been shown in unfixed tissues.**

Signalling	Signalling is a modern portmanteau term used to describe the fact that a change of the chemistry or physiology of one part of a tissue causes changes in other parts of the tissue. **If the concept of signalling is no more than an indication that a cause produces an effect, it is rather surprising that such a banal concept has achieved currency among biologists.**
Tissue slices	These are normally cut from whole brains, livers, kidneys, etc, and incubated in specialised media, imitating extracellular fluid. They take up oxygen linearly, but they have several problems. Firstly, the organ must be compressed, when it is sliced, and this must affect its biochemistry, anatomy and integrity. As soon as the slice comes into contact with the fluid moistening the knife, it swells. When it is placed in incubation, it swells again presumably from components coming from the incubating fluid, which gradually becomes cloudy. The slice swells throughout incubation. After incubation, before one weighs it, one must remove incubating fluid, which adheres to it; it is a subjective judgement to know when one has completed this process. Normally, the tissue is homogenised, later, in order to measure its chemical constituents, and losses also occur here. *Much useful biochemical information has come from their use.* **The fact that their weights change during the experiment, means that one cannot know the concentration of any chemical constituent in it, because it must be referred to weight. Therefore, it is highly doubtful if the biochemistry of tissue slices can tell one much about the quantitative chemistry of the living organ from which they came.**
Enzymes	These chemical catalysts act on the rates and dynamic equilibria of the reactions in the living intact tissue of animals and plants. **Breakdown of substrate has often been regarded as synonymous with enzyme activity, ignoring the possibity that the substrate is unstable in the particular chemical environment. After the systems have been subjected to large changes of entropy, these parameters can not be known, so that one should really only measure enzyme activities, in vivo, using non-disruptive procedures.**
Colpoids	Colpoids, (Herrera, 1928), are mixtures of water, salts, oils, and fine particles, in which many sorts of movements can be seen. These include Brownian movement, streaming, diffusion, and convection. No biological tissue is present, but studying colpoids gives one an idea of the behaviour of fine particles in fluids in the absence of metabolism. One can then know if biological systems are demonstrating properties, which do not require metabolism and life. **This area of physical chemistry has received extremely little attention from biologists, and it could yield them much new information.**

What is probably known about the structure biology of most cells in the body, and of neurons.

Tissues are composed of cells and their excretions, and syncytia. One can distinguish an extracellular compartment, cytoplasm, nucleoplasm, and nucleoli. The cell, the mitochondria, the nuclei, and the nucleoli in neurons, are all surrounded by semi-permeable membranes, and the composition all the fluids within each natural compartment is dependent upon those of the other compartments. Each of the membranes is anatomically imperforate. Each is probably composed of a single structural membrane, whose thickness and chemistry is not known, and, possibly, is unknowable. The structure and chemistry of the cell membrane can not be elucidated by depositing heavy metal salts on its surface, and examining it by electron microscopy. Any deposit stain must result in a single thin layer appearing as two lines. The composition of each compartment depends upon the chemicals within it at any particular time, the permeabilities of the membranes between it and other compartments, the affinities of the chemicals on each side of it for each other, the metabolism in that compartment, and the pressures from other tissues; these are due to pressures from adjacent tissues, the blood pressure, gravity, the ambient temperature, muscle contraction, and movements of the whole body.

The extracellular compartment covers the following fluids: blood, lymphatic fluid, interstitial fluid, cerebrospinal fluid, pleural fluid, peritoneal fluid, pericardial fluid, synovial fluids, and ocular fluids; their chemical compositions are very similar, and they also contain oxygen, carbon dioxide, proteins, hormones, steroids, catecholamines , amino-acids, ions, fatty acids, and metabolic intermediaries, which influence the cell membranes and metabolism in different parts of cells. It is unlikely that receptors, pores, carriers, Ion transporters and antibodies, are located in the cell membranes in living cells, because they are believed to be macromolecules, but cannot be seen by electron microscopy.

The idea that the cell membrane contains receptors for: transmitters, drugs, antigens, toxins, hormones, etc., is an unnecessary one, because any of these substances could react with any chemical species, protein, enzyme, cycle, or pathway, within or without the cells. Although it is widely believed that these receptors are largely located in the cell membranes, there is little sound evidence for this view. Evidence from subcellular fractionation can not be adduced, because the procedure ignores the second law of thermodynamics. Intracellular movements, such as Brownian movement, streaming, nuclear rotation, convection, diffusion, meiosis and mitosis, occur in living cells. The nucleus houses DNA, but one does not know how the properties of this macromolecule are affected by the isolation procedures. The mechanism and chemistry of meiosis and mitosis and the changes during cell division, are initiated by the nucleus. In living tissues, there are no cytoskeletons, Golgi apparatuses, lysosomes, peroxisomes, or filaments of actin, tubulin, spectrin, vimentin, or contractile proteins (other than in muscle). There are no molecular motors.

In the central nervous system, neurons can be seen connected by axons, and dendrites form large networks around them. However, much of the volume of the system is occupied by neuroglia. This does not consist of astrocytic, oligodendrocytic, and microglial cells. The neurons are surrounded by a mass of mitochondria in a syncytium. This syncytium is composed of mitochondria and mobile naked nuclei. The existence of neurons and the three kinds of neuroglial *cells*, was based on the belief that there were specific staining procedures for these types of cells. However, when one compares the literature on neurons, astrocytes, and oligodendrocytes, their descriptions overlap almost completely. The fact that most of the central nervous system is a syncytium means that this syncytium is the extracellular fluid of the neurons. It is highly conductive, and permits the naked nuclei to move around in the living tissue. They can congregate rapidly around infective foci and tumours in the living central nervous system. It is rather surprising that authors, such as Hyden, Pigon,

Hamberger, Hertz, Epstein, Hansson, Ronnbäck, and others, who have studied neuroglial clumps, have not examined their structure in the unfixed state by high-power light microscopy. We have concluded that in the central nervous system, any cell with processes or dendrites is a neuron, and the only other cells there are naked nuclei. These nuclei are called neuroglial nuclei, oligodendrocytes, reactive astrocytes, and satellite cells. Examination of any light or electron micrographs shows that the naked nuclei are surrounded by cytoplasm containing mitochondria, but do not each have their own cell membranes -- that is, they are in a syncytium.

In the the literature, silver deposits indicating synapses are found not only on the surface of neuron cell bodies, but also away from dendrites. We have also been unable to find presynaptic fibres going from one cell body to synapses on distal cell bodies, (Copestake and Hillman, 2013). Furthermore, we have shown that the number and dimensions of synapses seen by light and electron microscopy, are completely different, so that the two kinds of microscopes are probably looking at different objects (Hillman, 1985). In addition,pre-and post-synapses thickenings seen by electron microscopy do not appear in a range of orientations in micrographs.Also, the very short fibres attached to synapses in histological sections are only about the same length as the diameters of the cell bodies, and do not stretch across the fields, even in the plane of the sections. Finally, nearly all the illustrations on the Internet, in textbooks, and in published papers, are diagrams or illustrations, rather than micrographs. This is an invitation to anyone, who would gainsay this, to send us references to light microscopic publications showing synapses clearly, or to micrographs of them on neurons or dendrites in living cells, observed by high-power light microscopy. The denial of the existence of synapses and synaptic vesicles, induced me to propose an alternative theory to the chemical basis of transmission, for which Professor Katz was awarded the Nobel prize in 1970.

We have also seen nucleolar membranes around the nucleoli of all unfixed mammalian neurons, which we have examined. This membrane has been illustrated, (Hussain, Hillman and Sartory, 1974; Hillman and Jarman, 1991), but no one has repeated our observations, or denied, or confirmed, them.

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Unknowns and uncertainties about cell biology and neurobiology

These may be listed:

1. In biochemical experiments in vitro, one does not usually know to what extent the system is open or closed, but sadly it is usually partly open and partly closed. This makes it very difficult to relate the experiments in vitro to the living animals and plants, which are all open systems. It also makes it difficult to make real calculations about the rates and equilibria of reactions, which are intended to throw light on the chemistry of the living animals and plants. It seems to me highly likely that the only solution to the problem is to avoid doing experiments in vitro, or in which the entropy of the systems are changed significantly by the experimental conditions.

2. The shapes and dimensions of cells and organelles can not be determined by histological, electron microscopical, histochemical, or immunocytochemical, techniques, since all of them involve dehydration of the tissues, which causes them to shrink, because the tissues themselves contain 60% to 90% water. Some biologists feel that the reagents used in the procedures restore the cell volumes to their states in the living tissues, but the evidence is against this.. The only solution to this problem is to look at unfixed and unstained tissues by light microscopy, or to work on functioning isolated organs.

3. The thicknesses, the chemical composition, and the orientations of the molecules, can not be determined in respect of the membranes around the cells, the mitochondria and the nuclei. The electron microscopists deposit heavy metal salts on both sides of a membrane,

and then examine those deposits. It is generally believed that the membranes contain water according to the Singer Nicholson hypothesis (1972), so there are already shrunk by dehydration to an extent which is not known, after which the distance between the deposits on both sides of a membrane are measured.

4. Results from experiments using homogenisation, centrifugation, and subcellular fractionation, can not be depended upon, because they change the entropy of the systems drastically. Controls could be done on the effects of each of the steps of the procedures on the results of experiments, but, so far, they have not been published.

5. In experiments in vitro intending to measure the same parameters in the living intact animal, the concentration of enzymes, the rates of the reactions and their equilibria, can not be determined, because of the changes of entropy during the procedures.

6. Many apparent structures seen in stained tissues by light or electron microscopy, are small and amorphous, so that their shapes and dimensions cannot be known for certain. Therefore, a number of vague graphic terms are used to describe them; these include: deposits, vesicles granules, secretions, specialisations, Golgi apparatuses, lysosomes, peroxisomes, ribosomes, synapses, and synaptic vesicles.

7. The effects of the chemicals used to extract DNA and RNA on their chemistry need to be studied, because the extraction could influence their properties.

8. The chemistry of necrosis is not known.

9. The chemistry of histological and electron microscopical sections has not been compared with that of these original tissue, so that one does not know what chemicals have been extracted , and what have been added to the tissue, by the staining procedures.

10. The reason for which astrocytes and oligodendrocytes are extremely difficult to find in sections of healthy nervous tissue have not been explained.

11. The mechanisms of phagocytosis and pinocytosis are not known.

12. The usefulness of tissue culture needs careful revaluation, in respect of what information about the original parent tissue one seeks to elucidate.

13. The mechanism of muscle contraction needs to be re-examined, because the thick and thin filaments do not appear in sufficient range of orientations. These filaments and the cross bridges are only seen by electron microscopy, and there are considerable physiological problems connected with this process.

14. If receptors do not exist, those who previously believed that they do, should put forward a new hypothesis for the actions of transmitters, drugs, hormones, enzymes and toxins.

15. A new theory of transmission of signals from one part of the nervous system to another needs to be proferred, in view of the problems with the chemical theory. I have proposed a new theory (Hillman,1991).

Procedures, which should not be used on biological tissues, mainly because they change the entropy of the systems significantly

1. Homogenisation	13. Fluorescence microscopy
2. Centrifugation	14. Electrophoresis
3. Subcellular fractionation	15. Freezing
4. Tissue slicing	16. Freeze-drying
5. Dehydration	17. Heating
6. Fixation	18. Elution
7. Sectioning	19. Extraction
8. Staining	20. Measurement of tissue by its dry weight

9. Histology	21. Dilution
10. Histochemistry	22. Filtration
11. Immunocytochemistry	23. Chromatography
12. Electron microscopy	24. Non-recovery calibrations

More useful techniques

1. Experiments in vivo, involving natural reagents in physiological concentrations.
2. Epidemiology.
3. Photography.
4. Telemetry.
5. Observation by the naked eye.
6. Separated organs which function similarly to those in vivo, such as heart, kidney, limbs and intestine.
7. Micro-dissection.
8. Use of adequate control experiments.
9. Exclusive use of recovery calibrations.
10. Use of radioactive isotopes in whole animals and plants.
11. Use of statistics, especially in histology, histochemistry and electron microscopy.
12. Double and triple blind experiments in all biological sciences.
13. Use of colpoids.
14. Light microscopy of living or unfixed tissues by, bright field, dark ground, phase contrast, anopteral, differential interference, vertical illumination, polarised light, confocal, quantum dot, and lensless, microscopy. Such light microscopy should also be used by pathologists to examine lesions.

List of artefacts in biological tissues

(a) The trilaminar appearances of membranes around the nuclei, mitochondria, and nucleus, but there is no doubt about the existence of the membranes themselves;
(b) Ion channels in cell membranes;
(c) Trans-membrane molecules;
(d) The cytoskeleton, including the endoplasmic reticulum, contractile proteins, tubulin, actin, vimentin and spectrin;
(e) Golgi bodies;
(f) lysosomes;
(g) peroxisomes;
(h) the nuclear pores and the nuclear pore apparatuses;
(i) the lamellae of the Golgi body as seen by electron microscopy;
(j) receptors on membranes, sometimes seen in subcellular fractions, but not by electron microscopy of whole tissues;
(k) molecular motors;
(l) mitochondrial cristae;
(m) thylakoid membranes in chloroplasts;
(n) synapses;
(o) pre-synaptic fibres;
(p) synaptic vesicles;
(q) myelin lamellae, but not myelin sheaths;
(r) microtrabeculae.

This list may not be comprehensive. Detailed consideration of these can be found in Hillman, (2008).

I invite anyone who disagrees or agrees with the views expressed here, or who teaches the current biological consensus, or writes textbooks about it, to communicate with me about any of the statements in this paper. I undertake to respond to all serious, politely couched, viewpoints, as long as I am physically capable of doing so.

Some serious questions addressed to cell biologists

In Hillman (2003), I listed a number of crucial questions not answered by the cell biologists and the neurobiologists. Hardly any of them have been answered. I have now refined some of the earlier questions and added some new ones. The questions are written in normal type, the answers of the cell biologists and the neurobiologists are in *italics*, and my replies to the questions are in **bold** case. I will now list these:

1. What is biology and how does it differ from molecular biology and from the chemistry of living tissues?

Molecular biology is another name for the chemistry of the living tissues. Most biologists and biochemists regard the chemistry of dead, fixed, dehydrated, frozen, homogenised, centrifuged, or extracted tissue, as yielding valuable information about the chemistry of life.

I would put a different emphasis on the problem. It seems to me that our aim must be to study the processes in the living animal, because the more we subject it to reagents and manipulations, the more distant it becomes from living processes. I believe that most biologists are not as concerned as they should be about what their procedures do to living animals and plants, and that many of their findings are artefacts resulting from their own manipulations.

2. Do they (the cell biologists and the neurobiologists) believe that the second law of thermodynamics applies to biochemical experiments?

This question has been ignored.

3. What are the consequences of living organisms being open systems, while the experiments are carried out in partially closed systems?

This question has also been ignored.

It seems to me that most experiments in vitro involve large changes of entropy relative to the state in the living animal, and, therefore such experiments should not be used for measuring the rates and equilibria of reactions in living animals and plants.

4. Why do most biologists believe that electron microscopy gives a more accurate image of the structures of the cells than does light microscopy?

The electron microscope has a higher magnification than the light microscope and they believe that a heavy metal deposit on a dead tissue, gives more accurate information than observing living cells directly by light microscopy.

This seems very unlikely in view of the fact that electron microscopists are looking at deposits of salts of heavy metals on dead tissue. The preparation for electron microscopy includes fixing the tissue, dehydrating it, and subjecting it to a number of toxic and unnatural reagents.

5. How relevant is the chemistry of dead, fixed, or disrupted, tissues to our knowledge of living systems?

One can learn a great deal about the biology of systems using histology, histochemistry, electron microscopy, immunocytochemistry and fluorescence microscopy.

Not about the structure and chemistry of living cells.

6.Why do the laminae of all the trilaminar, ('unit membranes') around the cell, the nucleus, and the mitochondria appear nearly always in electron micrographs to be equally spaced, when sectioning them from random directions should cause them to appear in a range of

spacing?

Some electron microscopists have asserted that the reason for the high frequency of appearance of sections normal to the cell membranes, is because they select the best images of these membranes for illustrations, which are those that are at right angles to the plane of section. Some electron microscopists have denied my assertion, but have never shown any electron micrographs showing that it is not so. In general, this assertion has simply been ignored.

If it is true, it proves that the appearances of these cell membranes are two-dimensional, and must have occurred after the sections have been cut, and been bombarded by electrons in the electron microscope.

7**.** Why are lysosomes and peroxisomes not seen in unfixed and unstained cells?

This question does not seem to have been raised before.

Because they are artefacts of staining.

8. Why has there been so much resistance to the study of the nuclei and the nucleoli of unfixed tissues by light microscopy?

Electron microscopy gives one greater magnification.

The tissue has to be treated with so many powerful chemicals during the staining, and these can cause artefacts.

9. Why has only one ionic channel been shown in cell membranes, when there are believed to be tens of them in membranes?

No explanation has been offered for this.

The small patch clamp currents attributed to these channels could be chemical and electronic artefacts, arising from the chemical connections and electronic circuitry, used to record them, (Hillman, 2008, pages 92-93).

10. Why are not receptors, transporters, carriers, and transport enzymes, which are believed to be macro-molecules, not seen by electron microscopy in the cell membranes?

No explanation has been offered for this.

The simplest explanation is that none of these macromolecules exist within, or on the surface of, cell membranes.

11. Is it possible to make a three dimensional model of a cell containing a cytoskeleton, which permits movements of relatively large light microscopically visible particles?

The usual explanation of this is that the strands of the cytoskeleton open up to allow the particles to pass through, and reform after the particles have passed.

This completely ignores the fact that the particles are much wider than the weave of the cytoskeleton. It seems to me that the concept of a cytoskeleton is incompatible with the intracellular movements seen in living cells.

12. Why are there are very few publications on the effects of the chemicals use for staining in histology, electron microscopy and histochemistry, as well as on the effects of such physical manoeuvres as cutting sections, or embedding on the chemistry of the tissues?

No explanations have been given for this lack of necessary control observations.

If one continues to use these procedures, the effects of the reagents and of the manoeuvres should be examined urgently.

13. Why have biochemists, who carry out subcellular fractionation, not studied the effects of homogenisation, centrifugation and the reagents they use, on the results of their experiments?

There has been no answer to this question.

Such control experiments are needed before one accepts the results arising from any procedures using subcellular fractionation.

14. Do those who carry out the latter technique believe that enzymes, soluble materials, co-factors and substrates, do not diffuse from their original sites during homogenisation, centrifugation and mixing?

I am not aware that this question has been raised before.

It is extremely difficult to stop diffusion. However, this consideration alone makes subcellular fractionation a questionable procedure.

15. Why are not all calibrations carried out with the calibrating solutions added at the beginning of the whole blochemical experiment, rather than is the current practice, when pure solutions of the salts, proteins, or DNA, are used to calibrate the instruments at the end of the experiments?

The latter is the common practice nowadays. This suggestion does not seem to have been made before, so that biochemists have not reacted to it.

Nevertheless, it seems to me to be the obvious and correct method of calibration.

16. Most cell biologists believe that protein and glycolipid molecules protrude from the surfaces of cell membranes, so why are they not seen on electron microscopy?

This question does not seem to have been raised before.

The simplest explanation for this is that they are not present in these locations. The cell membrane by electron microscopy always appears to be very smooth.

17. Do electron microscopists believe that they can measure the width of a cell membrane which has been dehydrated during preparation for electron microscopy?

This question does not seem to have been raised.

Not if they believe that the membrane contains significant amounts of water.

18. Why have the neuroglial cells which modern neurochemists identify by markers not been shown to be the same cells as those stained by classical neuroglial stains?

This question has not been raised before, although the markers are usually found in tissue cultures, and the stains are used for whole nervous tissue.

It is possible that these types of experiments have been carried out, but have not been published. Astrocytes and oligodendrocytes do not exist in life.

19.Why is it so difficult to find astrocytes or oligodendrocytes in unfixed central nervous tissue or in stained sections of healthy tissue?

This question does not seem to have been raised before.

These two types of neuroglial 'cells' simply do not exist in the living central nervous system. The neuroglia is a syncytium mainly of mitochondria and naked nuclei, as originally envisaged by Virchoff, (1846). In the literature, these types of cells are usually represented by drawings or diagrams.

20. Why are no membranes seen by light or electron microscopy around the cytoplasm surrounding the naked neuroglial nuclei or the granular cells of the cerebellum?

This observation and question does not seem to be raised before.

I invite colleagues to look at micrographs of the nuclei, and then answer the question. It seems to me to be undoubted that such membranes simply do not appear.

21. Why have no histologists, other than ourselves, stained neurons from cranial nuclei, anterior horn cells, and cerebellar Purkinje cells with classical neuroglial stains, and they would find as we did, that the stains were not specific for the three different kinds of neuroglial cell, but also stained neurons, (Hillman and Deutsch, 1979)?

Most histologists believe that particular stains are specific for each of the kinds of neuroglial cells, and that they do not stain neurons.

I do not know why these experiments have not been repeated.

22. Why are the dimensions and numbers of synapses very different by light and by electron microscopy*?*

This point has been ignored.

I would recommend histologists and electron microscopists to have a look at this problem.

23. Why are presynaptic fibres, which are widely illustrated in neurobiology books and

papers not been seen in histological sections, (Copestake and Hillman, 2013)?

Physiologists, histologists, and neurobiologists believe them to be present.

They are not.

24. Why do myelin lamellae appear in electron micrographs to be equally spaced in cross sections as in oblique sections?

This finding has not been noted previously.

This finding has been very difficult to publish, (Hillman, 2013)

25. Why do thick and thin filaments of muscle fibres appear equidistant on electron micrographs?

This question does not seem to have been raised previously.

I would request electron microscopists to have another look at their sections.

26. Can modern techniques of light microscopy, such as confocal, quantum dot, or lensless microscopy, show the presence of cross bridges in unfixed and unstained muscle fibres?

They have not yet been examined by such techniques.

It would be very convincing if they were shown in living or unstained tissues.

27. Why do cross bridges not orientate themselves, so that they appear longer when the muscle is relaxed, and shorter when it is contracted?

No explanation has been offered for this, perhaps because it has not been commented on before.

They are artefacts of staining. Probably the myoplasm is a viscous fluid in life.

28. How valid is the use of ligands in biochemistry and pharmacology?

The general view is that they are most useful and most biologists see no objections to them.

I believe that a full international conference on receptor and ligands should be convened to discuss their use, because the use of both of these has been accepted without sufficient discussion and debate.

References

(Most of these are to the publications from my own laboratory, in which I have dealt particular points in more detail, or are to important references by the pioneers of these subjects).

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