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ARTEFACTS IN ELECTRON MICROSCOPY AND THE CONSEQUENCES FOR BIOLOGICAL AND MEDICAL RESEARCH

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ABSTRACT

It has been previously shown that the endoplasmic reticulum, the Golgi apparatus, the lysosomes, the nuclear pores, and the cristae of the mitochondria could not exist in living cells. Therefore, study of them in all life sciences, or any apparent correlation between their 'structure' and 'function' should be abandoned. The consequences to biological and medical research of such conclusions are considered. Semantic and logical analyses of experiments should always be done, and all the assumptions inherent in them should be identified and tested. Research should be more directed to studies on whole living animals, rather than to metal deposits, precipitation patterns, homogenates, or tissue fixed or subjected to other powerful physical and chemical agents known to change them drastically.

Key Words: Cytology. Electron microscopy. Artefacts. Endoplasmic reticulum. Golgi apparatus. Nuclear pores. Membranes.

INTRODUCTION

During an examination lasting several years of unfixed isolated neurons, we compared their structure with electron micrographs in the literature of many neurons and other cells. It became evident that the electron micrographs showed two-dimensional representations of several structures which could not exist in three dimensions. The incidence of the orientation of most membranes seen by electron microscopy was far too frequently normal to the section, instead of being randomly orientated with respect to it, as solid geometry would require. We then investigated with entirely fresh eyes the structure of living cells.

We came to the conclusions (1) that the following structures could not exist at all in living cells: endoplasmic reticulum; lysosomes; nuclear pores; cristae of mitochondria. The Golgi apparatus (which was discovered by light microscopy)was also an artefact. In addition, the 'trilaminar' appearance of the membranes around the following structures was also artefactual, although the structures themselves exist: the cells; the nuclei; the mitochondria; the synapses. We have given detailed consideration to the points which have been proffered against our conclusions, and have proposed how these artefacts arose during the preparation of tissue for electron microscopy (2,3). Although our conclusions have been the subject of much debate, so far there has been little challenge to the evidence which we have adduced.

Briefly, it is as follows:
(i) the apparent remarkably uniform spacing of the two lines of all the membranes seen by electron microscopy is artefactual, because if one cuts any real structure composed of equally spaced lamellae through any plane except its equator, the lamellae must appear to differ in spacing. They do not. The 'trilaminar' appearance seems to be normal to the section in the vast majority, (perhaps 90%), of all the occasions upon which it can be seen clearly.

An attempt has been made to explain this by asserting that the only condition under which the membranes can be seen clearly is when they are at right angles to the section. If this were so, it would mean that 60-80% of all structures seen under the electron microscope (especially in secreting cells) were orientated in the same way, and were not random with respect to the plane of the section.

We have suggested that the trilaminar appearance arises simply due to the fact that the heavy metal stains used in electron microscopy must deposit on both sides of a membrane or cytoplasmic precipitate, and therefore every apparent membrane must appear as two lines; the stains do not dissolve the membranes or replace them, which could be conditions in which they would appear as one line. We have also given detailed reasons why the 'inner' and 'outer' mitochondrial or erythrocyte membranes apparently have different biochemical properties - one is studying them both in different chemical conditions (4);

(ii) the endoplasmic reticulum, the Golgi body, the dictyosomes and the lysosomes are artefacts, because cells which can be observed in life, - protozoa, plant cells, cells in tissue culture - show a large repertoire of intracellular movements: Brownian movement; streaming; free access to all parts of cells of injected particles; vacuoles moving around the cytoplasm; mitochondrial movements; nuclear rotation; axonal flow; pinocytosis; phagocytosis. All these movements have been detected during the last two centuries by light microscopy using as low as two hundred times magnification. They would not permit the existence of a net or flattened vesicles connecting the cell membrane to the nuclear membrane, which was so fine that ten thousand magnification and more was required to see it. It has been suggested that the reticulum is flexible, and we have discussed why this is not acceptable as an explanation (3).

The most cursory observation of living cells leads to the conclusion that the cytoplasm is an aqueous suspension in life. Its viscosity has been measured by a large variety of different methods over the last half century and has always been found lower than that of olive oil or glycerol; a reticulum would have a much higher viscosity, In our view, the dehydration by organic solvents or freezing of tissue, which is done during preparation for electron microscopy, precipitates the cytoplasmic solutes in uniformly spaced rows like crystals;

(iii) the nuclear pores always appear circular or slit-like. They are hardly ever seen in section as any shape intermediate between these two, as geometry would require. We believe that their appearance on plan view of the nuclear membrane is due to bubbles, and on transverse section is due to cracks.

The present paper examines the consequences to biological, biochemical, and medical research of an acceptance of these views. It is complimentary to an analysis of the implications for biological education of our conclusions (to be submitted for publication).

IMMEDIATE CONSEQUENCES

1. For electron microscopy

A research worker accepting our views might be tempted to suggest that it might be worthwhile to do experiments to try to disprove or prove our thesis. As far as we know, no one has denied one of our main pieces of evidence - that intracellular movements occur - or alleged that the structure of cells should not have to be geometrically possible. Nor has anyone so far gainsaid our categorical assertion that it would not be possible to make a three-dimensional model of any complete cell from any electron micrograph of a whole cell, or any diagram based on electron microscopy (1). There would be little point in doing experiments to prove again generally accepted biological phenomena or geometrical considerations. However, there is still some advantage to be derived from examining the appearances which may be obtained when salt solutions, protein solutions, lipids, etc., are subjected to all the steps of electron microscopy. These studies would yield valuable information about the possible crystalline deposits, precipitation patterns and heavy metal appearances, which now appear as structures.

However, an acceptance of our views would unavoidably lead to the abandonment of the study in all the life sciences by electron microscopy, low-angle diffraction, electron histochemistry or morphometry of the following structures: the endoplasmic and sarcoplasmic reticula, the Golgi apparatus, the lysosomes, the mitochondrial cristae, nuclear pores, neurofilaments and neurofibrils. Furthermore, the separation and chemical characterisation by subcellular fractionation of such structures would be regarded as an exercise with no profit to science. This does not mean that all subcellular fractionation is of no value, although it is dangerously at risk. Up to now sufficient satisfactory control experiments have never been published for electron microscopy or subcellular fractionation. The most optimistic judgement we can make of the vast majority of their findings is that they are 'not proven'.

2. Control experiments

There is a very substantial and urgent case for doing many control experiments on the effect of these two techniques on the properties of the biological tissues being studied (4, p.114), especially of microscopic measurements of dimensional changes during tissue preparation.

These control experiments are crucial for the cytologist who desires to understand the biology of living cells. They are of much less significance to the histopathologist. He knows very well that in living healthy persons nuclei are not violet, nor cytoplasm pink, but he recognises the 'appearance' of tissue from a healthy person or animal stained with haematoxylin and eosin, and he also identifies deviations from that appearance which have previously been established by observation to originate from unhealthy organs. He is using artefacts legitimately. In our view there is considerable danger to the whole biological profession from the building of a vast superstructure on such shaky foundations. Biochemical cytology, pharmacology, and toxicology, stand prey to the first serious-minded student who plans to carry out the most obvious, relevant and necessary control experiments. Alternatively, these disciplines will acquire a theological

dogmatism in respect of which such experiments will be discouraged or ignored, or their authors ostracised. This will not advance biological science.

The dimensions of different tissues and different subcellular elements change to different extents with different staining procedures for light and electron microscopy, and there are relatively few studies in which attempts have been made to quantitate these dimensional changes (5, 6, 7, 8, 9, 10). Even when such dimensional changes have been measured, to our knowledge, they have hardly ever been used to correct dimensions such as those of the cell membrane, the nucleus, or the synaptic clefts, etc. We would urge more frequent corrections of measurements using data already acquired.

If the major findings on cell structure which have been made by the use of the electron microscope over a period of 40 years are artefacts, one may ask if this expensive instrument has an application to biology at all. Certainly, it can be used to recognise viruses, especially when clinical, microbiological and immunological findings can be correlated empirically with their identification under the electron microscope. This cannot be interpreted to mean that we know the shape of the living native virus. A second application of the electron microscope is in the study of the structure and classification of diatoms (11, 12). These have exoskeletons composed of siliceous materials resistant to dehydration, heat and electron bombardment.

The electron microscope is widely used to characterise structures in subcellular fractions. This has its dangers. One can undoubtedly detect the same appearance of mitochondria and nuclei in tissue and subcellular fractions, but the correlation between the appearance of 'ribosomes', microsomes, and nuclear pore fractions, with their parent subcellular organelles, is by no means so good. Furthermore, the assumption underlying many of these correlations is that the viewing by electron microscopy of the image of mitochondria in a particular fraction is itself evidence that the biochemical activity of those particular structures has not been grossly changed by the fractionation (4, p.34).

The correlations are particularly poor when attempting to compare membranes seen in electron micrographs with microsomal fractions, lysosomes with their subcellular offspring, and ribosomes with the fraction of the same name. The membranes appear trilaminar and their fractions single layered rings or ovals. The electron microscopist and the biochemist agree that they are the same structure. One looks naively and asks, "How can flattened vesicles become circles?" and one is told, "They round off during the preparation of the subcellular fraction". If this were so it would require all of the following steps: (a) the two layers of each vesicle would join at their peripheries; (b) these joins would be sufficiently well sealed that they would not burst open when the two layers were inflated; (c) after inflation the joins would disappear; (d) every single circular or oval membrane now one layer thick would orientate itself in such a way that it was normal to the section - as according to the electron microscopists it would not otherwise be seen. It should be noted that microsomes are hardly ever seen except in perfect transverse section.

The ribosomes and lysosomes are seen on electron micrographs of whole tissue as granules. Granules are also seen in the fractions, and although the electron microscopists agree with the biochemists as to the identity of the two lots of granules, their evidence is embodied merely in such a statement. We have been unable to find in the literature any comparison which has been validated statistically of the dimensions of these bodies in the two situations.

We would assert that only in the cases of the whole mitochondria, of the whole nuclei and of the whole nucleoli can one identify unequivocally the same structure in the tissue sections as in the subcellular fractions. Since every research workers sees a different group of electron micrographs, we would invite every individual electron microscopist and every biochemist working in subcellular fractionation to make this comparison themselves afresh. We would be pleased to be shown to be wrong in this viewpoint, or if our comments were to result in research workers embarking on such a project under suitable control conditions.

Much effort is used to 'purify' particular subcellular fractions. This means to produce a fraction which appears to be composed of one component alone on electron microscopy. Usually the purity of the fraction is also tested by the activity of enzymes like succinic dehydrogenase or acid phosphatase which are believed to be confined to that particular fraction. However, when the 'marker' enzymes are found in other fractions, they are insulted with the emotive term 'contaminants', which implies unequivocally that one has other independent techniques for showing where the markers were in the living cells. It should be noted that the idea that particular enzymes were confined to particular subcellular compartments was and still is only an assumption. It is then a circular argument to describe the presence of the enzyme elsewhere as 'contamination' or to continue 'washing' the fraction with powerful detergents until it is purified. Furthermore, closer examination of the literature indicates that nearly every enzyme which has been regarded as a marker, for example acid phosphatases for lysosomes, Na-K ATPase for synapses, monoamine oxidase for mitochondrial membranes, has been found with substantial activities in other fractions.

An assertion that a fraction is homogeneous should imply that at least, say 90% of that fraction by weight and volume is composed of the particular subcellular organelle. However, often it means that the one discussed is the only fraction in which that particular organelle can be identified, sometimes only as a small percentage of the material present. Frequently, in fractions there is a considerable quantity of unidentified or unidentifiable material.

3. Correlation with biochemical findings

It is commonly asserted that biochemical findings provide independent evidence for structure as derived from electron microscopic studies. Unfortunately, the biochemical studies of subcellular fractionation are interpreted by reference to the electron microscopy, and, therefore, cannot be regarded as evidence for or against that structure; it is a circular argument to attempt to do so. If the endoplasmic reticulum, the nuclear pores, the Golgi apparatus, and the cristae, do not exist in living cells, no exercise of logic can make study of an apparent subcellular fraction of them of any value whatsoever.

Even if, theoretically, the biochemical findings could be regarded as independent evidence, at present much of that evidence has never properly been controlled (4, p.39). When one prepares a subcellular fraction, one kills the animal; its biochemistry changes. One mixes it with sucrose; its enzymes are inhibited. One homogenises it; this causes considerable local rise of temperature. One centrifuges it; there is a different pressure, a different centrifugal force, a different viscosity and a different heat conductivity between the top and bottom of the tube. One takes off one supernatant and centrifuges it again; this compounds the differences of treatment already given in the last step. One measures an enzyme activity in different phases; this implies that other constituents in quite dissimilar chemical fractions would not affect the enzyme activity or its measurement. We have identified fifteen separate assumptions implied usually unknowingly to the biochemist, by the use of subcellular fractionation. Several of them are contrary to laws of thermodynamics or physics, or have been shown to be untrue by experiment (4, p.33). We have also listed the minimum number of control experiments which would have to be done to validate any major finding about the subcellular localisation of an enzyme. Regrettably, this technique has become very popular and routine without sufficient satisfactory control experiments ever having been done to justify its use.

Unfortunately, again, no exercise of intellect or irritation can absolve us of the responsibility to do the relevant control experiments, if we wish to localise biochemical activity. In view of the energy we often have to inject into our systems to separate their components, and the very sensitive enzyme-substrate systems we are studying, it is probable that preparative procedures which destroy cellular integrity are not suitable for studying unstable materials or active metabolism intended to inform us about events and their rates in vivo.

COMMENTS ON ELECTRON MICROSCOPY

1. Effects on biological tissues

A few further comments can be made about electron microscopy of biological tissues. Research workers who study the endoplasmic reticulum or the cristae of the mitochondria are examining precipitation patterns of the complex and changing mixture of which the cytoplasm and mitochondrioplasm are composed. They have often maintained that even if the electron micrographic images do indeed represent precipitates, changes in their patterns in different physiological and pathological conditions would themselves yield useful information, which might some day be interpretable or correlated with the 'function' in health or disease.

We believe this not to be the case, firstly, because the precipitation, dehydration, high temperature and irradiation would, in our view, impose such drastic alterations in the tissue as to mask the real biochemical changes which homeostatic mechanisms in vivo confine within narrow limits. Secondly, it is an unavoidable if regrettable fact of electron microscopy that - irrespective of the technique used - one is only looking at heavy metals (13). These have been deposited on membranes, but are not the biological structures themselves. To attempt to correlate the 'structure' of mitochondria as seen under the electron microscope, with the 'function' of the fraction which takes up oxygen, is similar to trying to relate a death mask of a face with the

physiology of the brain. They are each quite different structures and we are not even comparing like properties of them.

2. Rapid freezing

Thirdly, it is commonly believed that the use of rapid freezing techniques permit one to cool tissue to -100°C or -196°C without ice crystals being formed and the tissue being dehydrated (14). Crystallographers know that more rapid freezing produces smaller crystals, but the idea that ice will not separate is unfortunately not the case; measurements of conductivity have indicated that freezing of different tissues causes a sudden decrease of conductivity between -50°C and -20°C, and this change has always been interpreted as due to separation of ice crystals (15); to the author's knowledge, it has not been shown using a cryomicroscope, that ice crystals are not formed, and tissue has been shown to shrink considerably on freezing (16).

Fourthly, from the point of view of the cytologist, it is not a useful question to pose - "If the main findings of the electron microscope in tissues are artefacts, for what then should we use the instruments?" Just because they are expensive, we cannot allow the desirability of embarking on any particular series of experiments to be dictated by the instruments that have been purchased; the logical conclusion of such an attitude is science fictional.

Fifthly, we would maintain that continued study of these artefacts is not merely a waste of the research worker's time, but an historical abuse of his skills. This follows, if we are right, irrespective of whether or not we are heeded. In a further sense, the accumulation of data and literature on these artefacts in research in all branches of the life sciences, must also obscure true findings by adding noise to the 'signal to noise ratio' of the collective scientific semaphore of signals.

3. Achievements for biochemical cytology of electron microscopy and subcellular fractionation to date

We have concluded that electron microscopic study of those apparent structures which we regard as not existing in living cells has yielded no information whatsoever relevant to the understanding of disease, or the treatment of patients; this is so despite their use over a period of four decades, and despite the fact that much of the work in these areas is financed by the Medical Research Council and the Agricultural Research Council. We state this categorically not only because we believe it to be true, but also as a sincere invitation to anyone knowing of evidence that this assertion is wrong to step forward and demonstrate it to be untrue. We also issue the same request in respect of subcellular techniques. If this conclusion is accepted, it is no reflection on the technical ability or the sophistication of those who carry out the experiments.

Research workers rarely discuss the generalised lack of success in clinical or applied life sciences because, either they have not noticed it, or they would feel it impolite or dangerous to comment on their colleagues' work. We have discussed this problem elsewhere (17). We regard the failure even to recognise our inefficiency as a substantial contribution to the problem itself.

USEFUL NEGLECTED TECHNIQUES

There is a temptation to regard our views as nihilist in that we suggest the abandonment of some widely used techniques without proposing alternative methods of study. Of course, it is not nihilist to persuade a group of highly intelligent, educated and well paid persons not to waste time, which could be devoted to other more rewarding experiments. Among these are:

(i) light microscopy and photography by bright field, dark-ground, polarising, phase contrast, anopteral, incident illumination and interference techniques of living or unfixed tissue. Obviously the less reagents and agents to which the tissue has been subjected, the more precisely it will reflect the properties of the original living tissue.

(ii) Microdissection of tissue, in which mechanical separation of cells can easily be done (18, 19). Wherever possible, fixation, freezing, dehydration, addition of fluorochromes, embedding and staining, should be avoided.

(iii) Experiments in vivo using radioactive tracers yield very valuable biochemical information, which should not be complicated - unless it is unavoidable - by subsequent heavy processing of tissue.

CONCLUSIONS AND PRACTICAL SUGGESTIONS

We have concluded that most of the findings by electron microscopy of the structure of the cell must be artefacts, and that in subcellular fractionation grossly inadequate control observations have been carried out to date. In both techniques, the results of the relevant control experiments - when they have been done - have been regarded as appendices to the history of biology, and have not, therefore, affected its course. We will now summarise practical suggestions aimed at improving the current parlous state of medical and biological research. These will be mentioned in chronological order in respect of the education of a research worker in life sciences and its practice as a career:

A. Courses in all life sciences should include substantial sections on light microscopy, especially of living tissue. Semantics and logic should also be included in the curricula.

B. Undergraduates, postgraduates and professional research workers should carry out semantic and logical analyses of all papers which are crucial to their projects. In this analysis, all major assumptions should be identified not only of the immediate papers being read, but also all those preceding the immediate papers when they contain assumptions carried over to, and crucial to the ones under consideration. The validity of every assumption should be tested, because the strength of an experiment depends entirely on the warrantableness of every assumption made, including the weakest (4, p.107).

C. Far more attention should be given to the design and publication of control experiments. Indeed, substantive experiments should not be proceeded with until the control experiments have been done. In consonance with this attitude, research workers should be much more ready to abandon,

or admit the error of their experiments. In our view, this would probably require a deliberate and conscious education of undergraduate and postgraduate students about the desirability and necessity of intellectual honesty. Journal editors should be more willing to publish experiments with negative results, control experiments, corrected data and critical comments on the lines of "Matters arising" in 'Nature'.

D. The assessment of the relative validity of experiments in vivo and in vitro, when their results are not in agreement, is one of the most difficult problems in research. We have attempted to approach this problem by drawing up a hierarchy of all biological and medical experiments (20). At the top are observations made on whole human beings, animals or plants, which are not affected significantly by their being measured. Then there are those experiments in vivo which are affected significantly by being measured. Next come experiments in isolated organs, tissues and homogenates. It is quite evident that the more treatment we give to a tissue with powerful physical or chemical reagents, the greater distance we are placing between our original source of information and our measurement of data ultimately originating from it. Whenever - as is common - we have to use systems in vitro, we should always try to define the relationship of our system in vitro to the organism in vivo. This is commonly done by comparing parameters like respiration, potassium ion concentration, electrical activity, arterio-venous differences, etc. In the same publication we have also included a hierarchy of intellectual instruments of data handling. The highest kind of measurement is one in which no assumptions are interposed between measurements and the data derived; then comes measurements with warranted assumptions; then measurements on the same systems bearing on the original experiments, measurements on similar systems. After measurements come extrapolations, then hypotheses, then diagrams. Each of these instruments has different epistemological dimensions, and we cannot have a sequence of reasoning either in our experimental design or our derivation of conclusions in which our different instruments are used together in haphazard sequence. Rules for what links are permissible and what are not are in the realm of philosophy, but, in general, hypotheses should be at the beginning or end of the logical sequence, and the particular experiment should be testing them. Furthermore, data compatible with, but not necessarily bearing on the logical sequence, have no value in discussion whatsoever.

In consonance with the above classification, we have adopted what we call a 'vertical approach' to experiments. If, for example, we want to study the biochemical relationship of potassium ions to brain, or the mechanism of the reaction of digitalis on the heart, one should study the effect of the ion or molecule in the following steps: its association with dialysed homogenised tissue without any small ions or molecules (4, p.113); gradual addition of the small ions or molecules to see how they affect the affinity of potassium ions or digitalis with the insoluble part of the tissue; study of their association with crude homogenates, then tissue slices, then isolated organs; examination of arteriovenous differences in the organs in situ; study of the injection of the potassium ion or the digitalis intravenously in vivo. The intention is to use the same concentration ranges under as similar conditions as possible in each of the preparations. Any difference of properties vertically between different preparations would indicate the mechanism of action of the ion

or drug. The research worker or the director of the group should not be satisfied until any apparent discrepancies between the actions of the ions or drugs on any of the preparations have been resolved in experiemnt, logic and explanation. We would maintain that, hitherto, research workers using different techniques have been far too tolerant of apparent discrepancies in findings. Often they have ignored them or accepted them without intellectual discomfort.

E. Many large drug companies have logical schemes to analyse the actions of drugs and develop them for marketing. In medical and biological research, for example, in studying the causation of disease, or the mechanism of action of drugs, such logical schemata do not seem to have been designed so far. There is no difficulty in principle in doing this, and we have attempted two such schemes, one for the mechanism of action of a drug and another for the mechanism of a disease process (21, 22). Our efforts have been very preliminary, and it is intended to elaborate and publish more precise and analytical schemes in the future. We would like to suggest that this approach could be embraced more widely to the advantage of research. The philosophy behind such schemes is that mechanisms, although complicated, are ultimately knowable, and should be accessible to logical analysis.

The other element of the philosophy behind these schemes is that all disease processes and drug actions act in a few sites localised anatomically and biochemically. The mechanisms are, on the whole, rather labile; evidence for this is the extraordinary care which has to be taken when transplanting grafts or growing tumours in culture, or preserving growth factors. Despite the enormous resources devoted to the analysis of the biochemistry of carcinogenesis or the chemical nature of growth promotion, remarkably little is known. We have suggested that the explanation for this is that very highly energetic techniques have been used to try to detect very labile processes (4, p.106). These may well have destroyed the pathways of reaction before the biochemical separation techniques could separate the factors responsible for them.

F. Drugs have specific effects in alleviating some diseases, for example, anticholinerases treat myasthenia gravis, and lithium ions work against manic depression. Much is known about the various actions of these drugs by pharmacologists, physiologists and biochemists; little is known about the mechanisms which are deranged when the diseases occur, although theories rise and fall with a frequency, which is demoralising. However, in many countries intensive research is going on into the location and mode of action of the drugs, as their specific actions must be the most important clues to the genesis of the diseases. Where the drug acts, the disease process starts. If this is amenable to analysis, the prevention of the disease will also be in the long run.

G. Finally, and perhaps most important, we would like to shift the emphasis of research from studying artefacts, like endoplasmic reticulum, histological sections, lipid extracts, inner mitochondrial membranes or homogenates, to the study of the cytoplasm of living cells, tissue by phase contrast microscopy, unextracted membranes, mitochondria in situ and isolated organs, respectively. The former techniques should only be used when their relationship to the latter ones has been determined quantitatively. The less destructive of life and the less productive

of artefacts a technique is, the more desirable its use must be in biological and medical research, whose aim is normally the analysis of events in living whole human beings, animals and plants.

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