THE LIVING CELL: a re-examination of its fine structure

The understanding of the structure of the living cell is of fundamental importance to research in all medical and biological sciences. Ideas about its internal architecture which had been developed by the use of the light microscope for about a century and a half were changed dramatically when the electron microscope was introduced to biology in the early 1940s.

Dr Harold Hillman and Mr Peter Sartory have examined the currently accepted views in the light of (a) the behaviour of living cells observed by light microscopy, (b) attempted building of three-dimensional models of electron micrographs and (c) the properties of tissues and the stains used to study them.

The authors have reluctantly concluded that the endoplasmic reticulum, the Golgi apparatus, the lysosomes, the nuclear pores, the cristae of the mitochondria and the 'trilaminar' appearance of the cell, nuclear and mitochondrial membranes—as opposed to the membranes themselves—could not exist in *living* cells; they must be artefacts arising during preparation for electron microscopy. This book examines in detail the evidence for the apparent existence of these structures, the reasons for the conclusions that they could not exist in life and the responses of cytologists to these conclusions. Explanations for their appearance in the electron microscope are suggested and the implications for life sciences of these views are examined.

THE LIVING CELL has been written so that it may be understandable and of interest to undergraduates, postgraduates and professional research workers in all biological and medical sciences. It can also be used as a teaching text. Special care has been taken to cite original and key references to modern concepts.

THE AUTHORS

Dr Harold Hillman is the Reader in Physiology at the University of Surrey, Guildford, and has degrees in physiology, biochemistry and medicine. He is Vice-President of the Queckett Microscopical Club and is author of many papers which have appeared in the *Journal of Microscopy, Microscopy,* the *Biochemical Journal,* the *Journal of Physiology, Nature* and others. He is the author of *Certainty and Uncertainty in Biochemical Techniques* (1972).

Mr Peter Sartory is a Fellow and a former Council Member of the Royal Microscopical Society. He is past President and an Honorary Member of the Queckett Microscopical Club and a Fellow of the Linnean Society. His publications have been on critical microscopy, transient phenomena using filters, interferometry and polarising light.

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Hillman & Sartory

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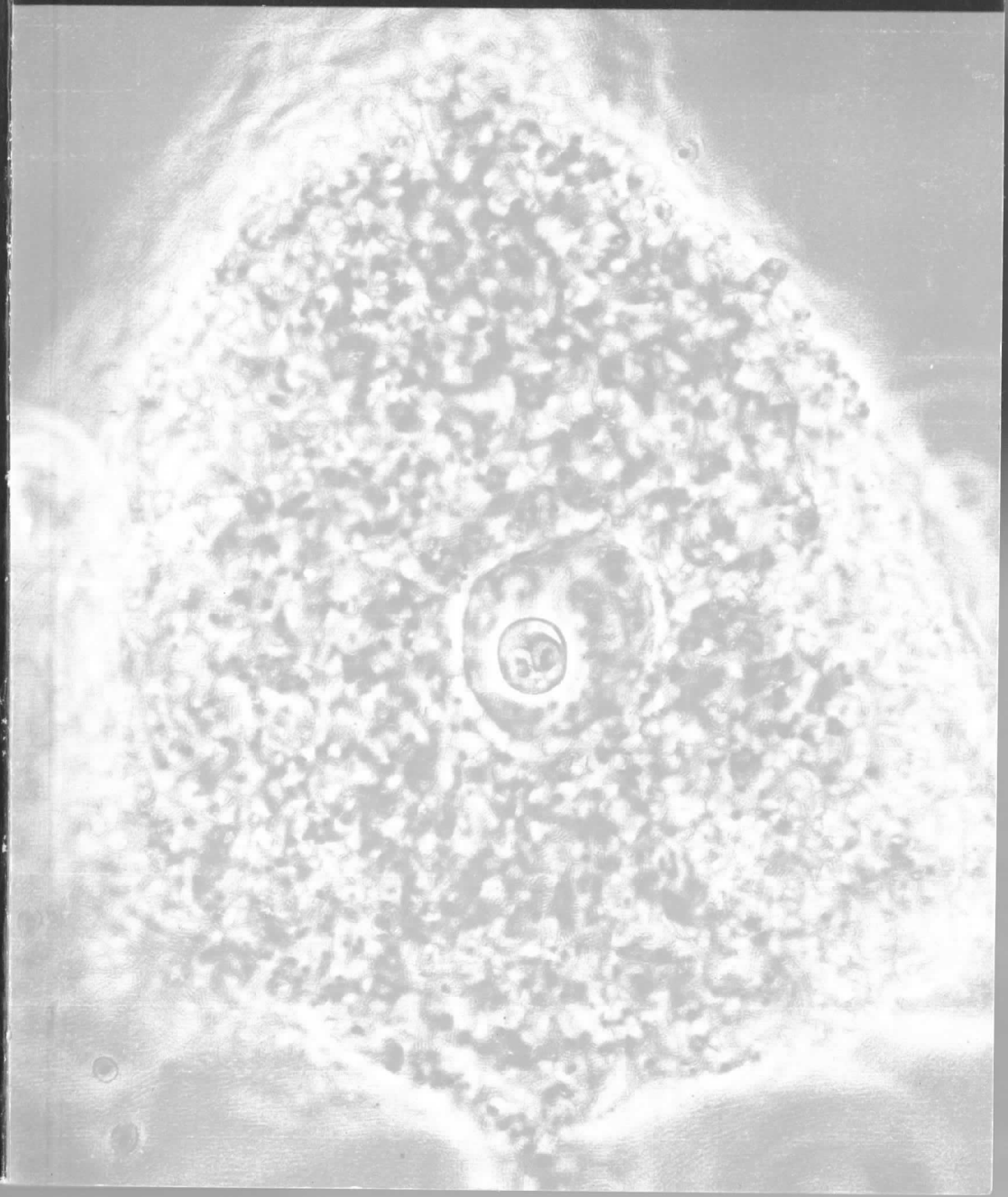
H. Hillman and P. Sartory



The Living Cell

Pluralitas non est pone sine necessitate William of Occam (1295 – 1348)

*It is a piece of idle sentimentality that truth, merely as truth, has any inherent power denied to error. . .*John Stuart Mill (1806 – 1873)

Faith is a fine invention
For gentlemen who see;
But microscopes are prudent
In an emergency! Emily Dickinson (1830 – 1886)

The Microbe is so very small
You cannot make him out at all,
But many sanguine people hope
To see him through a microscope.
His jointed tongue that lies beneath
A hundred curious rows of teeth;
His seven tufted tails with lots
Of lovely pink and purple spots,
On each of which a pattern stands,
Composed of forty separate bands;
His eyebrows of a tender green;
All these have never yet been seen—
But Scientists, who ought to know,
Assure us that they must be so. . .
Oh! let us never, never doubt
What nobody is sure about! Hilaire Belloc (1870 – 1953)

The illustration on the front cover is a drawing by Mr Henry Hunt of an unfixed rabbit neuron soma, isolated by hand dissection and viewed by phase contrast microscopy. The nuclear membrane has a diameter of about 5 μm.

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PREFACE

A modern scientist on hearing that one is seeking 'truth', may react initially by asking one to define it; we have defined what we mean by this term (please see page 99).

Another reaction is to deny that absolute truth exists in science, since often when new major discoveries are made, previous 'truths' are supervened by greater or more accurate descriptions of the same phenomena. Furthermore, elucidation of truth may be ultimately circumscribed by the changes induced by our measuring techniques, and by limitations of available resources, technical skills and human intellect.

At first glance, this philosophy may appear seductive. No scientist would claim that he has advanced his own discipline beyond the point at which it would merit further study. However, the danger of accepting the ephemeral nature of truth as a philosophical standpoint is that the research worker may feel no necessity to draw up criteria for deciding what at present constitutes the greatest approximation to truth; also, he may lack the intellectual drive to analyse all his own experiments by such chosen criteria.

In considering the general proposition that all our studies must be approximations to an unknowable truth, one should distinguish between two types of approximation. Firstly, current theories partially reflect the totality of knowledge to date; this seems a legitimate meaning for the term 'approximation'. Regrettably, the term is sometimes used for conclusions in which the laws of geometry, physics, chemistry, thermodynamics, or biology, have been disregarded – often, one suspects, unwittingly. This latter usage would be acceptable only if the application of these laws had been shown by experiment, mathematics or logic, to be too small to affect our conclusions significantly.

A third attitude is that many scientists in the real world are not pursuing abstract concepts like truth; they are engaged in the daily task of earning a living or achieving fulfilment in their work.

We would like to draw our readers' attention to an experience which has frequently befallen us. Up to the date of publication of this book, we have lectured on this subject or shown a film about its main findings at fourteen national or international meetings, and at seventeen universities in Britain and abroad, and to many student undergraduate societies. After nearly every occasion several members of the audiences have approached us privately to indicate their acceptance of our conclusions. We have asked these colleagues if they would be prepared to state in public their agreement with our views and their reasons for so doing. One lecturer has kindly agreed to do so. All the others have told us that they felt unable to support us in public, because they believed that such a stand might prejudice their prospects of obtaining funds for their research work or of advancement in their own careers. We sympathise with their predicament.

One of the commonest reactions to our demonstration of artefacts in cells has been to assert that everything one sees by light or electron microscopy is an artefact; therefore a logical consequence of our view would be that one would have to abandon the study of living tissues. We have listed a few examples of the enormous variety of experiments that can be done with minimal insult to the living structure or chemistry of tissue (please see page 84). Many of these kinds of experiments were carried out between the mid-nineteenth and mid-twentieth centuries. We also believe that this rather nihilist attitude is being used in an attempt to avoid the question of which of the artefacts reflect the properties of living cells – which we should be examining – and which arise only from reagents added during preparation – which are not native to the biological systems.

Members of audiences at a number of learned societies have stated either that no one now believes in the Robertson 'concept' of the 'unit' membrane, and, or, that it is not generally believed that the endoplasmic reticulum is attached to the cell membrane or to the nuclear membrane. We have shown that these assertions are simply not true in respect of the writers of many important modern textbooks (please see Appendix 3). The response of our critics to this demonstration has been that "no one seriously believes what is written in textbooks." Through the courtesy of the Editor of *Nature*, we have asked those who made this statement to justify it in print for the scientific public to consider, but so far no one has responded to our request (Hillman & Sartory, 1977a). Nevertheless, it is true that textbooks must be out of date to some extent, in view of the inevitable delay between production of new data, and its publication, review and incorporation into widely used textbooks. We have requested on several occasions to be informed of any references to any textbook, review, paper or lecture, which either denies the reality of the 'unit' membrane in the living cell, or the attachment of the reticulum to the cell and to the nuclear membranes. We ourselves have been unable to find any in the literature. However, we would like to express in no uncertain terms our extreme disquiet that senior scientists should doubt the veracity of so many textbooks, many of which they have written themselves, and also, that they should be recommending undergraduates to use textbooks containing important ideas in which they themselves do not believe.

In view of the unpopular nature of our conclusions, we would like to summarise briefly the philosophy behind our thinking. We believe that the following points would be entirely acceptable to most scientists.

1. We have employed Occam's razor unsparingly and without apology.
2. When evidence derived from living cells is in conflict with evidence from treated tissue, we generally prefer the former source (please see Appendix 2 and Hillman, 1976).
3. Geometry must be respected, however small the dimensions of the structures one is considering.
4. The constancy of an appearance is not evidence that it is not an artefact.
5. We embrace the attitude of Popper that a useful hypothesis must be open to disproof as well as to proof.
6. If for a phenomenon which is central to our teaching or research, there is not sufficient evidence which we are competent to assess, we should *not* accept its reality. A scientist should only adopt an agnostic position in subjects outside his own field of interest. In our view, all life scientists who teach about the fine structure of the cell are thereby obligated to take a positive stance about the present controversy, opposing or accepting our views.
7. We do not regard it as sufficient to pay lip service to all the artefacts of microscopy (Deutsch & Hillman, 1977; Hillman & Deutsch, 1978 and sources quoted there), while at the same time ignoring their effects on measurements, conclusions or theories. An experiment is only as good as its controls (Hillman, 1972).
8. A research worker has an intellectual obligation to examine not only his own experiments, but also all the previous experiments, upon which his final interpretations will depend, to see if the major and crucial assumptions stated or inherent in the latter are warranted. He may often find himself doing other people's control experiments.

* * * * *

We would also like to place on record the following further points:

(i) many cell biologists do not agree with our conclusions, but few have cast doubt on the evidence we have adduced;

(ii) it is our conclusion, *not our assumption,* that many of the structures visualised by electron microscopy must be artefacts;

(iii) so far, only one public debate of our views has taken place. Our offer to a public debate of current views about cell structure on equal terms before any scientific audience at any mutually convenient time (Hillman & Sartory, 1978 a, b) remains open indefinitely and for all countries;

(iv) no member of any audience or any reader of our publications has taken issue with our assertion that it would be impossible to construct a three-dimensional model of a living cell based on any electron micrograph of a whole cell, in which the model would show how intracellular movements could occur (Hillman & Sartory, 1977b). We wish to reiterate here this challenge;

(v) a film is available for loan from the authors summarising the main points of this book;

(vi) we undertake to respond to all serious correspondence from any quarter, however senior or junior, to modify our views, if necessary, and to acknowledge publicly that we have done so.

We wish to thank the Handicapped Children's Aid Committee, London, for considerable support. Mr. Russell Towns and the Audio-Visual Aids Department of Surrey University, helped us with the illustrations, Miss Nita Spektorov did the animation and produced the film. Mr. Henry J. Hunt drew the excellent picture on the cover.

Mr. Hung Kung Teh kindly executed some of the diagrams, and the following other members of the latter department did the photography: Mr. Colin Aggett, Mr. John Darby, Mr. Kevin Shaughnessy and Miss Frances Gibson-Smith. Librarians of the University of Surrey, University College London, the University of London, and the British Medical Association, have provided us with an excellent and indispensable service; we would like to mention particularly Mr. Glyn Davies and Miss Christine Smith, both of Surrey University.

Our colleague, Professor Karl Deutsch, has been kind enough to discuss electron microscopy in great depth and to comment on the manuscript. The many searching and intellectually hostile questions at meetings have helped us to formulate the problems. However, the responsibility for the views expressed is entirely our own.

Wherever reference is made in the text to a scientist or research worker in the male gender, this is intended to include female scientists and research workers.

This book is dedicated to all those who, in the pursuit of truth *as they see it*, have risked or will risk their careers, their liberty or even their lives.

CONTENTS

INTRODUCTION; UNDERSTANDING THE LIVING CELL

There has recently been an explosion of interest in biology, particularly in cytology, in universities, polytechnics, colleges and schools all over the world. The solution of the structure of desoxyribonucleic acid as well as the introduction of electron microscopy and subcellular fractionation since the 1940's has fired the imagination of the scientific world. Probably never before have so many people been engaged in the study of, and research in, cytology, biology, zoology, botany, biophysics and biochemistry.

While observing unfixed isolated neurons by phase contrast microscopy, we fell to discussing the structure of the living cell. We were struck by certain important anomalies between the structure and behaviour of the cell as seen by light microscopy in unfixed tissues, on the one hand, and the structure as deduced from electron microscopy in fixed tissue, on the other. The latter represents the common consensus among modern cytologists. Our further investigation of these anomalies and inconsistencies in the currently accepted views induced us to reappraise what is believed about the structure of the living cell.

Our basic axiom was that information derived from examination of living cells was likely to be more true than that from the study of stained sections or electron micrographs, whenever the two kinds of evidence were in conflict. We were very surprised to conclude that the commonly accepted model of the cell is impossible on geometrical and biological grounds, and in particular that the endoplasmic reticulum, the mitochondrial cristae, the Golgi body, the nuclear pores, as well as the unit membrane appearance – as opposed to the cell membrane itself – are artefacts; we are also very doubtful about lysosomes. We have discussed in what way these artefacts may have arisen during the preparation necessary for examination of tissue by electron microscopy.

The award of the Nobel Prize in 1974 to Professor Claude de Duve, Professor Albert Claude and Professor George Palade, renders more difficult an objective assessment of their conclusions, but should not, of course, deflect us or them from our common duty to analyse our own and their findings by the same criteria as we employ in relation to the research of less distinguished scientists.

We may consider it as axiomatic that research workers in life sciences aim to study the structures of the living cell and how it works. One must be continuously aware that any intervention which is necessary to examine the tissue may introduce artefacts (Toner and Carr, 1971, p. 117; Love, 1970; Hillman, 1972; Johnston and Roots, 1972). Everyone would agree that while we may use artefacts, we must make a clear distinction between the properties of living cells, and the artefacts which we may introduce deliberately or inadvertently when trying to increase our understanding of them. This becomes especially important when, firstly, the property being studied cannot be

observed directly in living cells, or, secondly, when two different findings from the same system one or both derived from indirect evidence or inductive reasoning are mutually incompatible. It would seem obvious that evidence collected from observations on living cells is more valid than that obtained by examination of dead, frozen, fixed, homogenised, extracted, inhibited or 'poisoned' tissues (Hillman, 1976). Studies on the structure of cells alive during examination have been carried out by light microscopy of plants, protozoa, tissue cultures, small transparent animals, tissue windows, and naturally large single cells like Mauthner cells, eggs, etc (please see page 28).

Chapter 1

THE STRUCTURE OF THE GENERALISED CELL

The generalised cell has been accepted as a useful teaching device for the last twenty or more years. Nearly every textbook and review of cytology, anatomy, physiology, biophysics, histology and genetics, has an early chapter showing it (see, for example, Brachet, 1961; Robertson, 1962; Bloom and Fawcett, 1966 p 22; Warwick and Williams, 1973 and references in Appendix 3). The concept is a useful one in that it describes the properties believed to be common to most living cells (fig 1.)

Although it is claimed that the structures are present in all cells, authors tend to choose specialised cells, like plasma cells of bone marrow (Fawcett, 1966; reproduced here as fig. 2), germinal epithelial cells (Fawcett and Ito, 1958) or smooth muscle cells (Robertson, 1960) in which particular structures can best be demonstrated. Unfortunately, the proponents of the generalised cell have never really clarified whether or not they believe that ribosomes, Golgi bodies, endoplasmic reticulum and lysosomes, are present in all cells but do not appear so clearly by standard techniques in many tissues, or whether they are present only in those cells in which they can be visualised clearly.

Until the early 1940's, it was agreed that all cells have an outer membrane, mitochondria, a nuclear membrane, a nucleolus, (fig. 3) and possibly a Golgi apparatus (Golgi, 1898: Cowdry, 1924; Kirkman and Severinghaus, 1938 a, b, c; Hirsch, 1939). The cell membrane, the nucleus, the nucleolus and the cytoplasm were known by the time of Schleiden and Schwann, (1847) (fig. 4) and Griffith and Henfrey (1861) whose drawings are reproduced here (figs. 5 – 8). Altmann (1890) added the mitochondria (fig. 9), although he probably was not the first person to see them (Hughes, 1959). (A variety of names has been used for these parts of the cells and we are using the simplest ones in this text). Estable and Sotelo (1951) described the nucleolonema in the nucleolus, and it has subsequently been seen in all nucleoli examined by light microscopy. We have detected a nucleolar membrane in several kinds of neurons (Hussain, Hillman and Sartory, 1974), but we do not know whether it exists in other kinds of cells.

The following further features have been added since the use of the electron microscope has increased the magnification possible by two to three orders (fig. 1,2).

The cell membrane, the nuclear membrane and the mitochondrial membranes now all appear as two lines with a space in between them; this appearance has been called the 'unit' membrane (Robertson, 1959, 1960, 1962, 1969, Lucy, 1975 and references in Appendix 3).

An *'endoplasmic reticulum'* permeating the cytoplasm three dimensionally has been described (Porter, Claude and Fullam, 1945; Porter, 1953; Palade

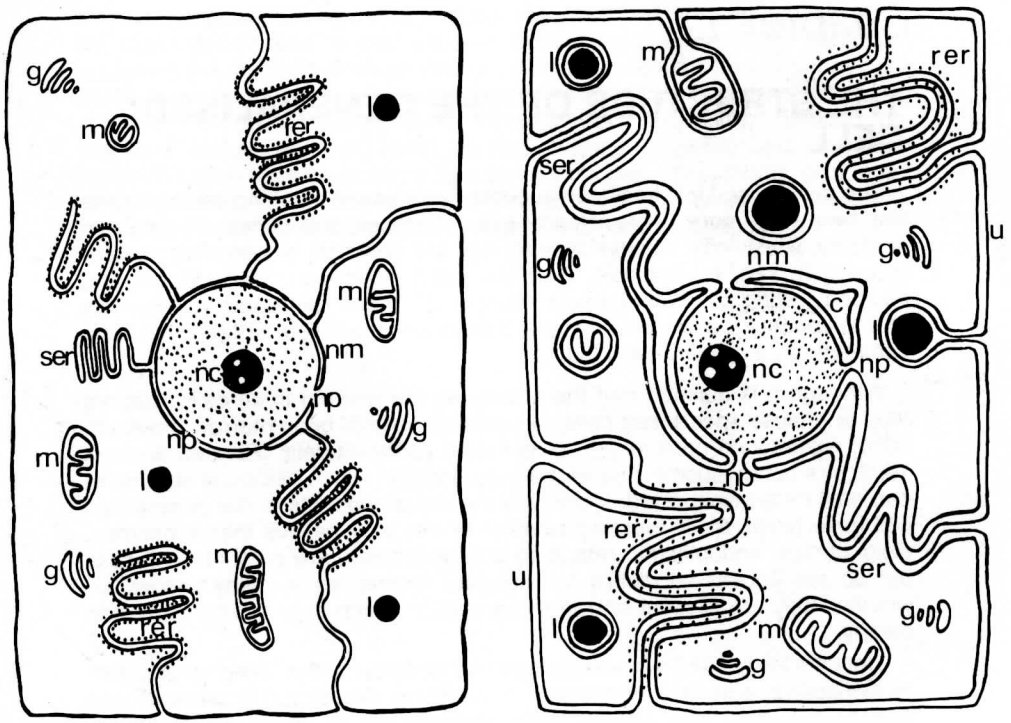
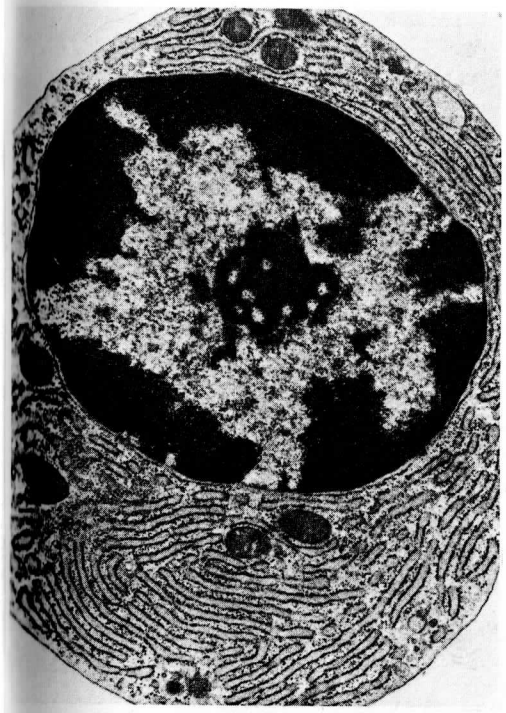


Fig 1. Diagrams of two of the most popular current representations of the structure of the generalised cell. In the left one, the membranes are depicted by single lines; in the right one, they all *appear double*, with c, *cisternae* between them. Other representations are similar to one of the above, but the *endoplasmic reticulum*, is often not attached to the cell membrane or, more rarely, it is not attached to the nuclear membrane. The following symbols are adjacent to the structures indicated: u, the '*unit*' membrane; rer, *the rough endoplasmic reticulum*, lined by *ribosomes*; ser, the *smooth endoplasmic reticulum*; g, the *Golgi bodies*; m, the mitochondria containing *cristae*; 1, the *lysosomes*; the nuclear membranes, nm are punctured by np, *nuclear pores*; the nucleus contains a nucleolus, nc sometimes with *vacuoles*. The structures in *italics* are those whose existence in the living cell are in question. Please compare this figure with Fig. 3.



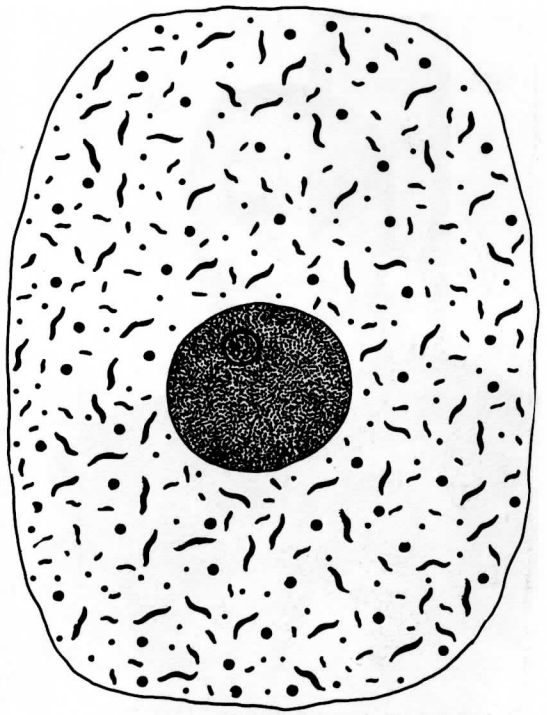
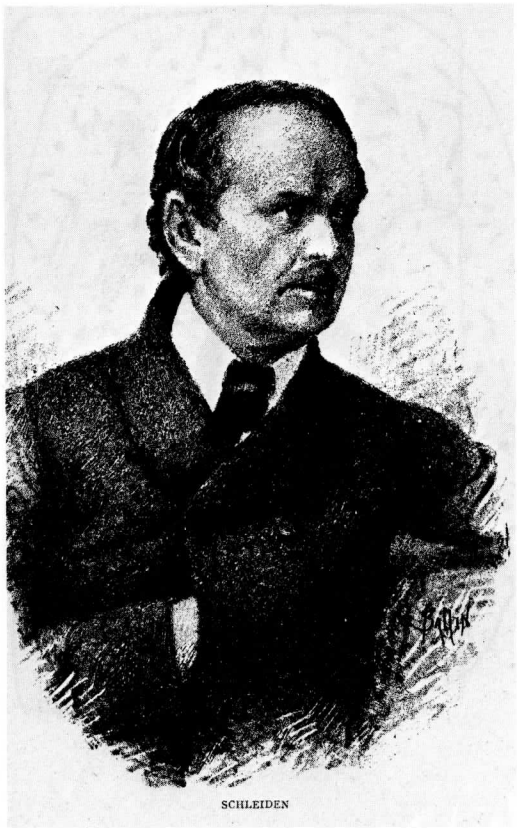


Fig. 2. (left) Electron micrograph of a plasma cell of bone marrow, from D.W. Fawcett, (1966) 'The Cell', Philadelphia, Saunders, p. 153, reproduced by kind permission of the Author and Publishers. Note the orientation of the endoplasmic reticulum in the plane of the section throughout the cell, and also the diameter of the mitochondria in relation to the 'weave' of the endoplasmic reticulum.

Fig. 3. (right) The structure of the generalised cell as believed in the early 1940's. Note the presence of granules and mitochondria in different orientations; the Golgi apparatus has not been drawn as its reality was not universally accepted.



SCHLEIDEN

SCHWANN

Fig. 4. (left) Matthias Jacob Schleiden, (1804 – 1881), had studied law and medicine, and held the chairs of botany at Jena, Dorpat and Frankfurt. He was co-author of Beitrage für Phytogenese, translated into English in 1847.

Fig. 4. (right) Theodor Schwann, (1810 – 1882), Prosector at Berlin, later Professor of Anatomy and Physiology at Louvain and then Liège. He was co-author with Schleiden of Beitrage für Phytogenese, in which the cell theory of tissue was proposed.

and Porter, 1954; Palay and Palade, 1955, see also fig. 2). It is also claimed that it is present in plant cells (Mercer, 1960 and references in Appendix 3). It also appears to have two layers with a space in between, and is often represented as being attached to the cell membrane and the nuclear membrane (Brachet, 1961; Robertson, 1962; Appendix 3).

'Ribosomes' line the endoplasmic reticulum (Palade, 1955, 1956; De Man and Noorduyn, 1969);

'Lysosomes' occur within the cytoplasm (De Duve and Wattiaux, 1966; Dingle and Fell, 1969; Dingle, 1973; Dingle and Dean, 1975, 1976).

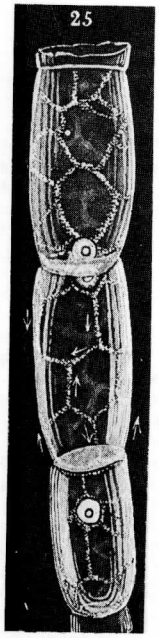


Fig. 5. Articulated hair of potato from Schleiden and Schwann (1847). The arrows indicating streaming are in the original drawing.

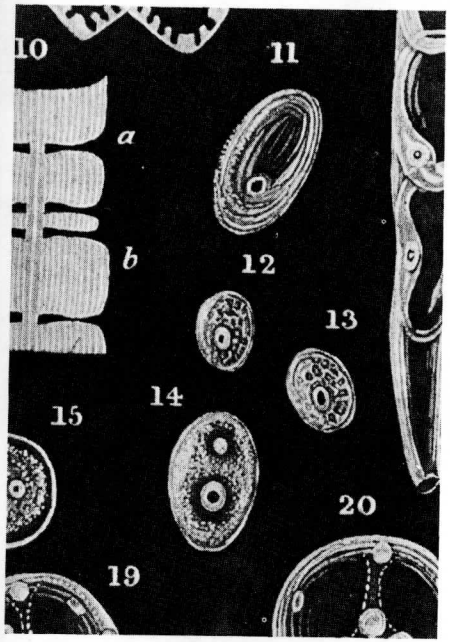


Fig. 6. Various cells drawn by Schleiden and Schwann (1847). Note that the nucleus and nucleolus were seen in most cells; 11 is a sporule from Rhizina laevigata, Fries; 12 – 14 are cytoblasts from the embryo sac of Pimelea drupacea; 20 is a sporule of Marchantia polymorpha.

Fig.7. Various normal cells from the first edition of the Micrographic Dictionary (Griffith and Henfrey, 1861, their Plate 40).

1. Mixtures of oil and water a, water in oil; b, c, oil in water.
2. Oceania cruciata (ACALEPHAE), epidermis of.
3. Oceania cruciata. a, b, stinging capsules with filament included; c d, with filament expelled.
4. Diphyes Kochii (ACALEPHAE); organs of adhesion upon tentacles.
5. Oceania cruciata, portion of margin of disk slightly magnified, a, ovary; b, muscular bundles; c, transverse vessel coming from the stomach; d, marginal vessel; e,f, tentacular filaments; g, auditory organs. Fig. 5. spermatozoa.
6. Infusorial embryos of ACALEPHAE.

7, 8, 9, 10. The same further developed.

11. Epidermis of Triton sristatus (water-newt).
12. Ciliated epithelium from frog's throat.
13. —apiculosa } Alders animalcules, considerably magnified (ALDERIA).
14. —ovata } This generic name being already in use, cannot be retained.
15. —pyriformis }
16. Haemocharis, epidermis of.
17. Haemocharis; transverse section of muscular fibres. } (ANNULATA)
18. Haemocharis; muscular fibe, showing the sarcolemma. }
19. Haemocharis; margin of cephalic disk, with branching muscular fibres c, and a, b, d, glands and ducts.
20. Aphrodita aculeata, hair of, treated with potash.
21. Blood corpuscles, human. a, d, surface view at different foci; c, side or edge view; b, colourless or lymph-corpuscle; e, coloured corpuscles altered, either spontaneously or by mixture with foreign matters, as urine, &c.
22. Blood-corpuscles of the goat (Capra hircus).
23. ,, ,, whale (Balaena).
24. ,, ,, ostrich (Struthio).
25. ,, ,, pigeon (Columba).
26. ,, ,, stickleback (Gasterosteus aculeatus).
27. ,, ,, loach (Cobitis fossilis) b, colourless corpuscle: c,d, the same, altered by water.
29. ,, ,, triton (Triton cristatus) ; b, colourless corpuscle; c, d, e, f, altered coloured corpuscles.
30. ,, ,, siren: b, colourless corpuscle.
31. ,, ,, crab (Carcinus).
32. ,, ,, spider (Tegenaria domestica).
33. ,, ,, cockroach (Blatta orientalis).
34. ,, ,, worm (Lumbricus terrestris). a, corpuscle partly drawn out, as occurs with the bodies of some Infusoria,
35. ,, ,, garden-snail (Helix aspersa).
36. ,, ,, human, coloured, undergoing division.
37. Blood, human, in coagulation; b, colourless corpuscle.
38. Cartilage of the ear of a mouse; the fat is partly removed from the cells.
39. Cartilage of human rib.
40. Cartilage of human epiglottis.
41. Areolar tissue, human, with fat-cells.
43. Formation of areolar tissue from cells.

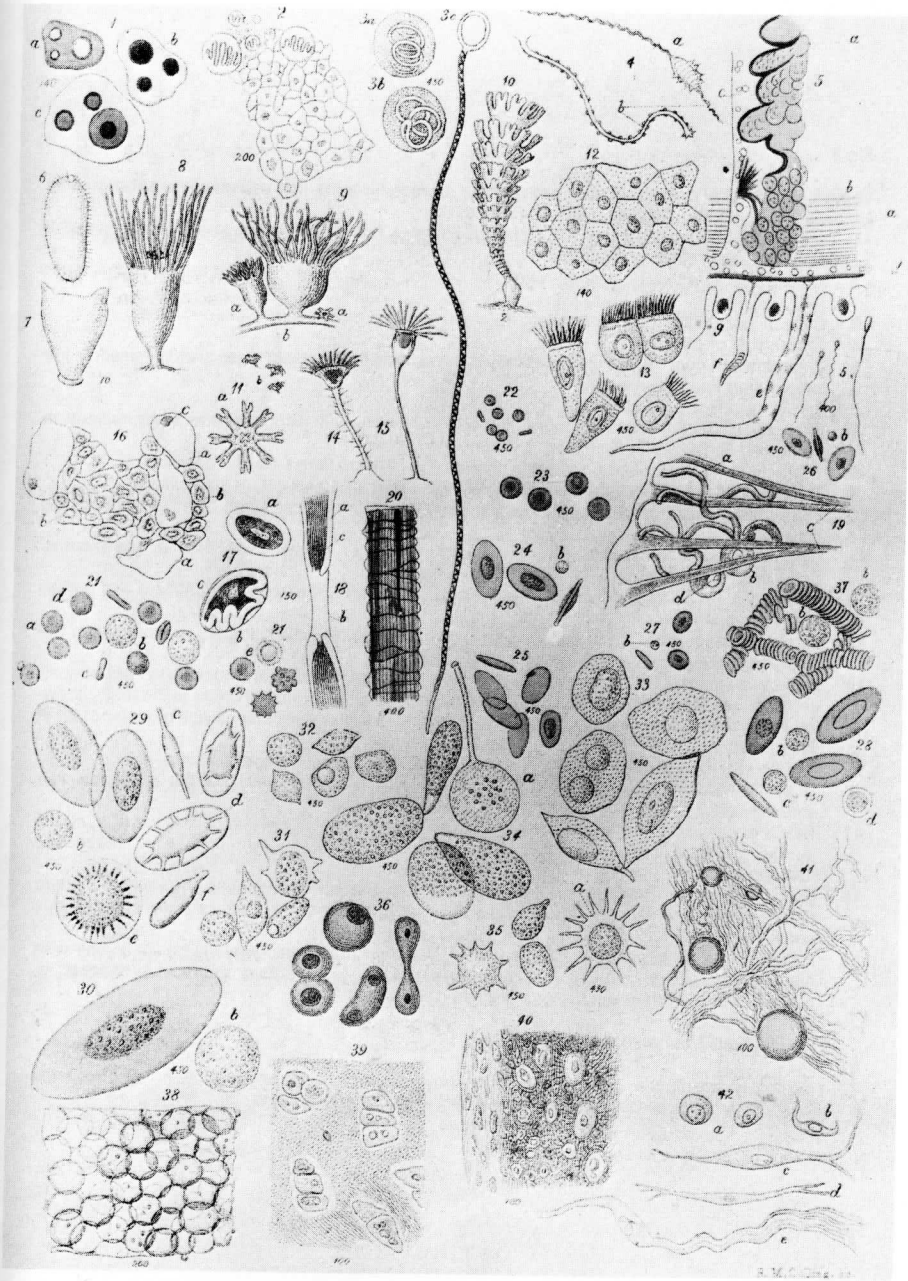


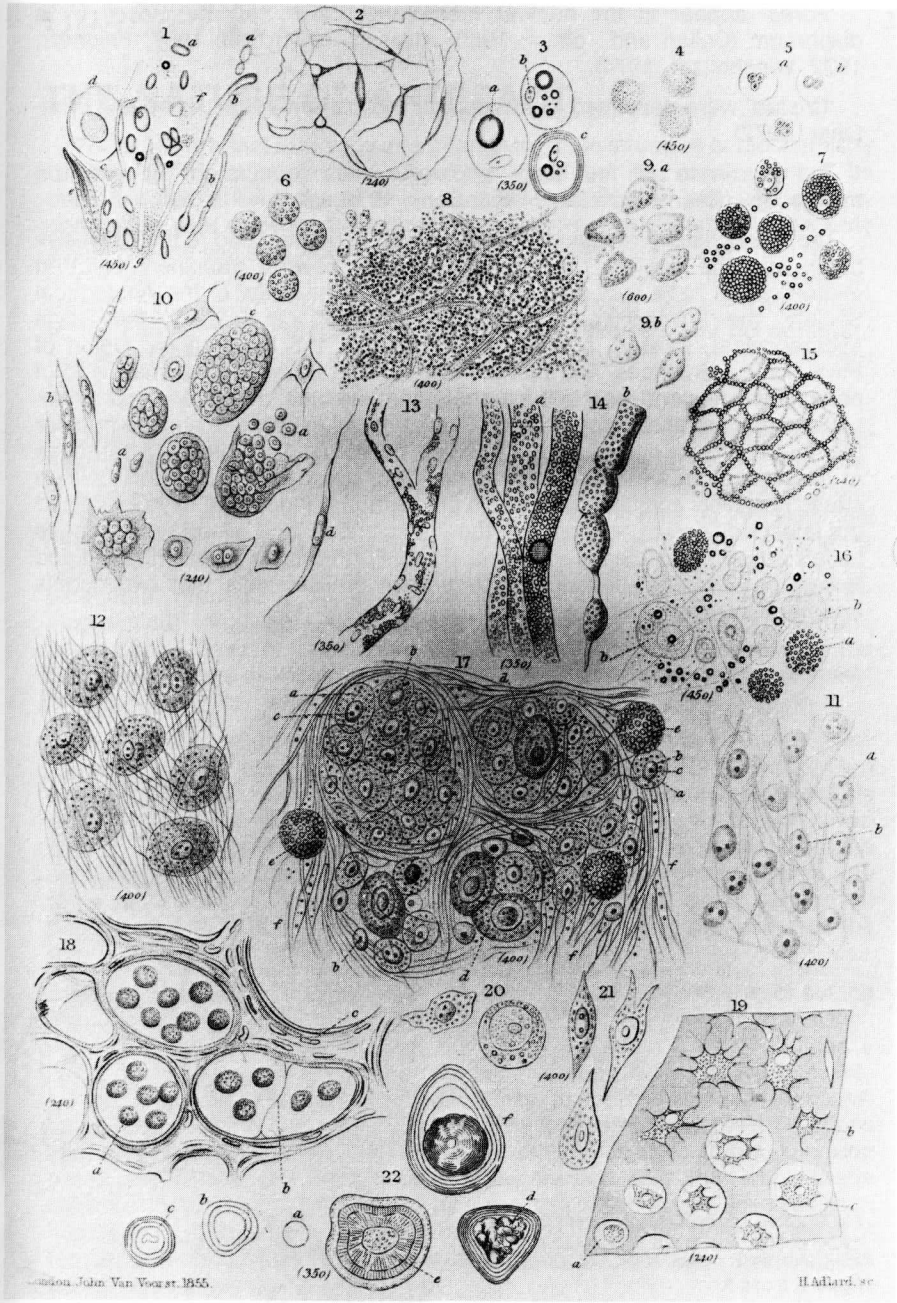
Fig 8

Various pathological cells associated with human disease from the first edition of the Micrographic Dictionary (Griffith and Henfrey, 1861, their Plate 30).

1. Aphtha. a, spores of fungus (OIDIUM); b, fibres; c and f, Bacterium termo; d, e, epithelial scales; g, early state of Bacterium.
2. Areolar tissue, with formative cells and homogeneous basis; from a fibroid tumour of the upper jaw.
3. Cells of fatty tissue in degeneration. a, fat; b, nucleus; c, cell with thickened walls.
4. Corpuscles of pus.
5. Corpuscles of pus, treated with acetic acid. a, nuclei with the object glass slightly raised; b, the same when this is depressed.
6. Pyroid corpuscles, of Lebert.
7. Granule-cells and loose fat-globules; some of the former with distinct cell-wall and nucleus, in the lowest these are absent; from a cutaneous cancer.
8. Tubercle in lung; showing pulmonary fibres, tubercle-corpuscles and fat-granules.
9. Tubercle-corpuscles more magnified. a, seen in water; b, treated with acetic acid.
10. Fibro-plastic cells from a sarcomatous tumour of the thigh. a, loose secondary cells; b, fusiform cells.
11. Cancerous tissue from a medulary cancer containing but a few and pale fibres. a, free nucleus; b, nucleus within a cell.
12. Cancerous tissue from a schirrous cancer; the fibres are numerous, but delicate and not arranged in bundles.
13. Capillary vessel in a state of fatty degeneration; showing the oblong nuclei, and the minute fat-globules in the substance of the wall of the vessel.
14. a, Fatty degeneration of the muscular bundles of the heart; the transverse striae are absent, and globules of fat are disseminated through the substance; b, from muscle of the thigh, showing collapse of sarcolemma and partial absorption of muscular substance with globules of fat in the remainder.
15. Intercellular fatty degeneration of encysted cutaneous tumour (cholesteatoma).
16. Tissue of medullary cancer of ovary. a, granule-cells; b, cancer cells; the fibres are very few and slender.
17. Tissue of cancer of the oesophagus. a, cancer-cells; b, their nuclei (secondary cells); c, nuclei (tertiary cells); d, cancer-cells with highly developed nuclei; e, granule-cells; f, fibres and fusiform cells.
18. Colloid or alveolar cancer of the peritoneum. a, nuclei or secondary cells, the walls of the two parent-cells are seen at b; c, nuclei of areolar tissue; the contents of the cells are of gelatinous consistence.
19. Portion of an enchondroma showing cells imbedded in a homogeneous basis. a, cell with nucleus (secondary cell) and nucleolus (tertiary cell); c, secondary cell with processes; b, secondary cell from which the primary has disappeared.

20. } Cancer-cells from medullary cancer.
21. }

22. Colloid corpuscles. a, simple; b, c, concentric or laminated corpuscles from hypertrophied heart; d, f, laminated corpuscles from a cyst in an atrophied kidney.



'Pores' appear in the nuclear membrane; these may be closed by a diaphragm (Callan and Tomlin, 1950; Afzelius, 1954; Gall, 1967; Feldherr, 1972: Wischnitzer, 1974).

'Cristae' were described in mitochondria (Sjöstrand, 1953; Tribe and Whittaker, 1972)

The existence of the *Golgi apparatus* was 'confirmed' by electron microscopy (Beams & Kessel, 1968; Cook, 1975; Whaley, 1975).

The 'endoplasmic reticulum' has been said to be attached to the cell membrane and the nuclear membrane. Recently, several prominent electron microscopists have indicated in private and at meetings of the Anatomical Society, the Physiological Society, the Biochemical Society, the Royal Microscopical Society, the Biophysical Society, the International Union of Physiological Sciences, and many universities in Britain and abroad, and in personal correspondence to the authors, that the 'unit' membrane and the attachment of the endoplasmic reticulum to the cell membrane and the nuclear membrane are not now generally believed, but they did not cite any references to this assertion, and we ourselves have not been able to find this opinion stated positively and unequivocally in the literature. Indeed we have examined the latest editions of textbooks in the life sciences, and *all* agree that these beliefs are still held (please see Appendix 3). Furthermore, unless Brachet and Robertson were each describing different 'generalised cells' their two models are mutually incompatible.

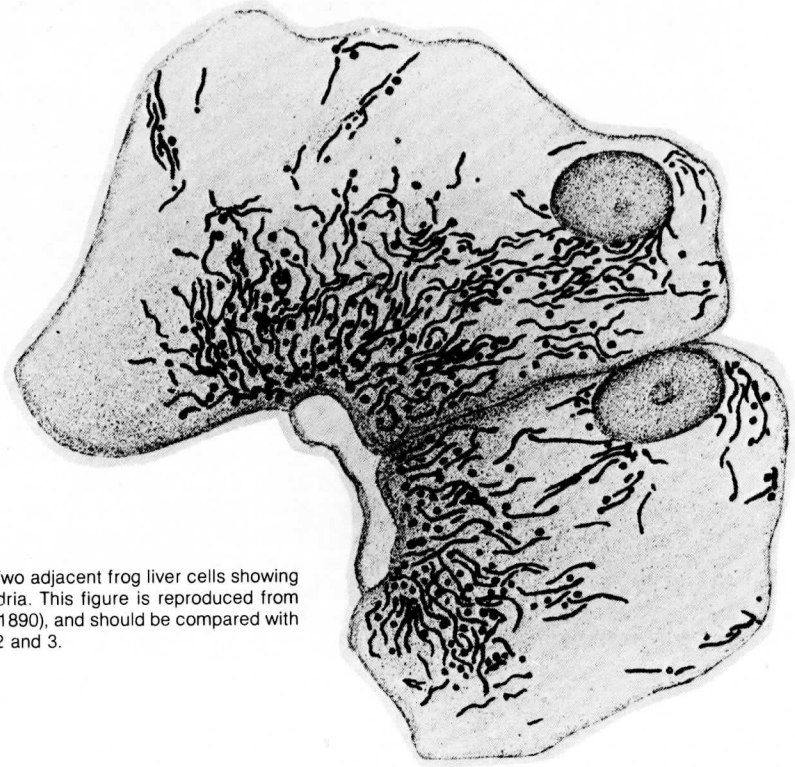


Fig. 9. Two adjacent frog liver cells showing mitochondria. This figure is reproduced from Altmann (1890), and should be compared with figure 1, 2 and 3.

Chapter 2.

THE NATURE OF ARTEFACT

We define artefact in biology as a change in tissue structure or biochemistry relative to its state in vivo, resulting in properties which cannot be demonstrated in living tissue, or – having been elucidated indirectly – are incompatible with evidence from direct observation or examination of the living tissue (please see also Appendix 1). We should also state quite explicitly that, in our view, tissue which is examined in the frozen, fixed, homogenised, extracted, inhibited or 'poisoned' state is not *normal* 'living' tissue – even if these states can be, have been, or are, subsequently reversed. Nor do we regard tissue subjected to x-rays, electron beams or low pressure as 'normal' or 'near living'.

Until recently all microscopic studies on the structure of cells *alive during examination* have been carried out by light microscopy of plants, protozoa, small animals and tissue cultures (but see Dupouy 1972; Parsons, 1974). Much other indirect information has been derived by experiments on living animals or metabolising tissue in vitro, often using radioactive techniques. In the latter experiments, the animal or tissue may be metabolically active during the experimental procedure, but often has to be killed, fixed or extracted for biochemical analysis. Alternatively, body fluids like blood, serum, cerebrospinal fluid or urine, may yield important indirect evidence of the biochemical state of the cells with which they had previously been in contact in vivo.

The difficulty implied in this discussion is what is meant by the term 'living tissue'. This has been dealt with elsewhere (Hillman, 1970). Obviously, an unanaesthetised unrestrained moving animal is living. If it is anaesthetised, its reflexes are gradually lost to a greater and greater extent – although reversibly. Its organs may be separated and perfused when the animal dies; the separated monkey brain can continue to show electroencephalographic patterns indistinguishable from normal; the isolated rabbit heart can go on beating for hours; the separated goat's udder continues to produce milk; the frog nerve muscle preparation can show tetanus.

However, when we start making tissue cultures, or isolating cells, or cutting tissue sections, it gradually becomes more difficult to define the term 'living'. A biologist can only measure its 'viability' by the number of major 'functions' a piece of tissue can carry out in vitro compared with its performance in vivo. Nevertheless, such tissue continues to respire, to accumulate potassium ions, to exclude some sodium ions, and to carry out many metabolic reactions characteristic of the living state. In many senses, tissue cultures, surviving slices, and isolated cells remain 'alive'. They are less 'alive' than whole tissue because they have been separated by a process which usually involves such manoeuvres as, severance of their normal blood supply, temporary change of temperature, application of pressure, subjection to shear and incubation in a foreign environment.

Fixed tissue, deeply frozen tissue, and freeze-substituted tissue metabolise minimally or not at all. When a tissue is homogenised, centrifuged, or extracted, its state resides somewhere between the living and dead tissue (Hillman, 1972, page 110). Furthermore, it must be insisted that fixed tissue is not living – it is dead. Therefore, the axiom can be restated that information derived from unfixed material must be truer than that derived from fixed tissue, stained tissue, frozen tissue or metal or salt deposits.

(There is a discussion on optical artefacts below.)

The essential elements of artefact are fairly clear and agreed by many authors. However, there is no common consensus on how much of an artefact is, or should be, acceptable to a research worker attempting to elucidate the structure of the living cell. Johnston and Roots, (1972, p. 4) say that the membrane structures seen in the electron microscope are the products of the interaction of biological molecules with a variety of fixative and staining reagents.

Toner and Carr (1971, p.117) note that fixation and staining of tissues are observations of systematic artefact, such as protein precipitation or the binding of dyes, but believe that they represent "a tolerably" close approach to their true structure. "The artefact of histology has come to be accepted because it is reproducible and consistent and because it has proved both meaningful and useful in the more general context of biology and medicine. In the same way, the artefact of electron microscopy has become accepted."

This section is given verbatim because it is crucial to a consideration of what we believe to be the excessive tolerance of artefact by the scientific community.

The criterion of reproducibility is hardly one that sheds light on whether or not a structure exists in vivo. All it tells one is that the same artefact can be produced consistently. A red blood cell or an oocyte appears under the light microscope to be several millimetres in diameter but no one would allege that these are dimensions in vivo. Nor can we accept without reservation that the observations are useful in the general context of biology and medicine. In respect of histology, we normally fix, stain and dehydrate tissue. The effects of each of these steps have been studied for over 100 years. Briefly, these include diminution of most enzyme activities, intracellular precipitation, shrinkage, distortion, tearing and dehydration of membranes. These effects are known or can be measured, so that we are bound to measure them, assess them by reference to the literature, or – at least – state why we think they would make very little change to the system we are characterising. For example, one cannot measure the size of subcellular organelles in dehydrated tissue without allowing for shrinkage; one cannot accept that precipitates exist in the cytoplasm of living cells when we know that the cytoplasm of unfixed cells appears devoid of precipitate, but fixation causes precipitation; fixation stops streaming in cells, so that one could not assert that such movement does not occur in vivo, because it cannot be seen occurring in histological sections.

Such an attitude would seem so obvious that it is hardly worth stating yet it is important to do so, in order to indicate that we are not satisfied with mere lip-service to those notions, while ignoring them in drawing conclusions from experiments. Johnston and Roots (1972) state that "provided one is aware of the hazards it seems to us that the use of information derived from all sources, *artefactual though some may be,* is justified" (our italics). We would urge that it is not good enough to be aware of the hazards – one must calculate them and take them into account. Furthermore, a fortiori, artefacts must not be considered as good evidence as direct observations. An accumulation of data from experiments containing many possible artefacts does not make an accurate contribution to knowledge any more than the assembly of a number of findings, each individually not statistically significant, can possibly add up to a significant conclusion.

Below are a few examples of the sort of experiments which fall into the above category:

(i) measurements without correction for shrinkage of membrane distances, synaptic clefts, mitochondrial dimensions, or fibre thickness, in histological or electron microscopic sections, which have been dehydrated during preparation;

(ii) assessment of the effects of acute hypoxia in histological sections which must be subjected to hypoxia when the animal is being killed for preparation of the tissue;

(iii) attempts to localise diffusible ions like Na^+ or K^+ in fixed tissue, while believing that the distribution of these ions is dependent on the active processes requiring an energy supply;

(iv) measurement of cerebral oedema in histological sections, which have been dehydrated.

If one did or could know such parameters as the degree of shrinkage of each organelle on dehydration, the cellular movements of ions during dying, the effects of homogenisation on the structure of the cells, etc., etc., it would be theoretically possible to make calculations indicating the relevant parameters in vivo (Hillman, Hussain and Sartory, 1976; Deutsch and Hillman, 1977; Hillman and Deutsch, 1978).

We are not implying that histological or electron microscopical observations are of no value, nor, indeed that that one should never study artefacts. These techniques can be used to describe the general shape of structures, the geographical relationship of visible structures to each other, and gross changes in those parts of the cells which react with the reagents, provided that one can show that the structures have not been distorted by being subjected to further external agents.

This is the sense in which Toner and Carr (1971, p. 117) are right in saying that the appearances "are taken to represent a tolerably close approach to their structures," and are useful in biology and medicine. Histology is an empirical science which developed pari passu with pathology. It serves as the control experiment for the pathologist, who knows, nevertheless, that in vivo

nuclei are not violet, nor cytoplasm pink. Baker (1942, p. 4) in discussing cytological techniques, makes the point that "the histologist calls a fixative 'good' when the tissues are evenly and slightly shrunken and the nuclei stand out sharply in the stained section, but the sharpness of the precipitated chromatin is no evidence of lifelike preservation, and the only evidence for what is good or bad is comparison with the living cell". The electron microscopist prefers 'beautiful' appearances, perhaps because he does not accept that irregularity and entropy also have their daises in the scientific pantheon.

There is no doubt that histology produces images that are reproducible, consistent and meaningful, in terms of the agreed 'normal' appearance of healthy tissue resulting from these specialised and complex preparation procedures. The pathology of acute or chronic disease can then be recognised as the distortion of this original pattern, resulting from the action of illness. We believe that there has been a failure to distinguish sufficiently clearly between the usefulness of histology as the point of reference for pathology, and its function as a source of knowledge about living tissue. Nevertheless, the use of smears, biopsies, and rapid freezing in diagnosis is also evidence of an awareness that the sooner, the nearer, and the less chemically, one embraces the living tissue, the more likely one is to find information about its state in vivo.

In classical histology, one is looking at a dead tissue devoid of some of its solutes, dehydrated and stained with elegant dyes. However, the insoluble portions of the tissue at least are still largely present. In electron microscopy, the 'specimen' – as it is called – is not the tissue: it is a trace of heavy metal or salt which has deposited on the originally mainly insoluble metal-loving parts of the tissue. Unstained tissue absorbs very few electrons and has hardly any contrast (Weakley, 1972, plate 2, reproduced here as figure 10). It is quite irrelevant how much of the tissue survives the preparation, as the electron microscopist is looking only at the heavy metal or its salt. Heavy metal salts of osmium, uranium, tungsten and lead are highly toxic, and the concentration in normal animal tissues is virtually zero. Therefore, by definition we are looking at an artefact under the electron microscope. Most histologists examine tissue plus artefact (stain) by transmitted light microscopy. One can look at unfixed tissue (with very much less artefact) by bright field, polarised, phase contrast, anopteral, or interference phase microscopy. Comparison of the *morphology* as visualised by histological or electron microscopic techniques of fixed tissue is the obvious way of assessing the validity of the information derived by the former methods. However, one cannot correlate accurately the morphology with the *biochemistry, biology* or *'function'* of fixed tissues since the intention of histology is to stop all 'functional' activity (see Appendix 1). Nor, indeed, could there be any meaning in the biochemistry, biology or 'function' of a heavy metal deposit or carbon replica.

Much of modern biology is concerned with the comparison of the properties of metabolising tissue slices or homogenates with heavy metal deposits. One is not comparing two attributes of the *same* material, rather the attributes of two quite different materials. When one watches pinocytosis in an amoeba, one is watching the 'structural' and 'functional' changes in the same object at the same time.

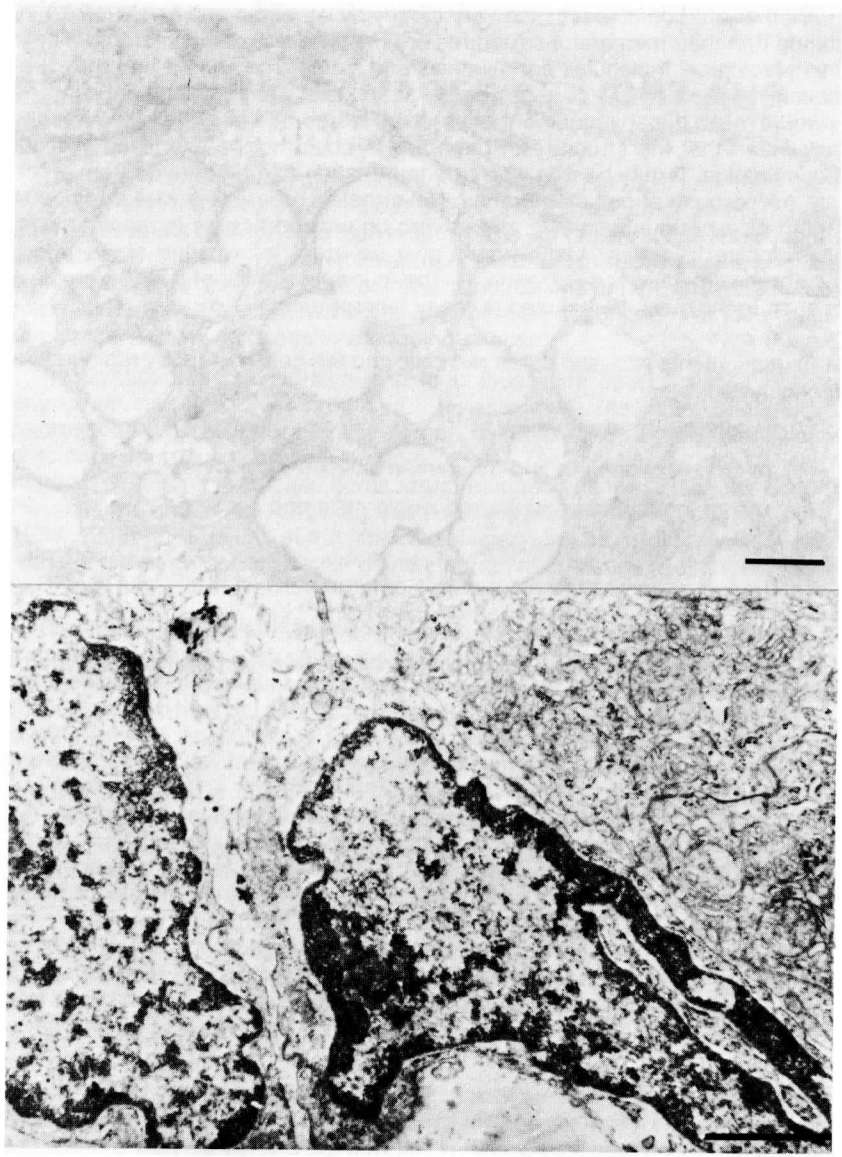


Fig. 10. Two electron micrographs from the same piece of ovarian steroid tissue, both fixed with glutaraldehyde and then osmium tetroxide; *above*, not subsequently stained, *below*, stained with uranyl acetate. Please note that no significant detail can be made out in the absence of stain. This figure is reproduced from Weakley, (1972, plate 2) by kind permission of the Authoress and Publishers, Churchill-Livingstone, Ltd.

Another attitude is expressed very clearly by Johnston and Roots (1972) in stating that their membrane structures are the products of the interaction between biological molecules and fixatives and stains. They admit that they are examining artefacts but consider further that, "to adopt these attitudes entirely would mean the abandonment of practical membrane studies." Presumably they mean that the structure of membranes could not be examined at high magnification. It may be that little true information can be derived from electron microscopy of cell membranes. Nevertheless, there is a vast armamentarium of experiments in vivo and in vitro on unfixed tissues in more or less physiological regimes, which would give us 'true' information about living membranes (please see Appendix 1). The following is a list of a few examples of such techniques, being used in many laboratories:

(i) movements into, and out of red cells and red cell 'ghosts' by radio-active techniques;

(ii) iontophoresis of nerve cells;

(iii) micro-injection into, and micro-manipulation of, oocytes;

(iv) use of incubated and cultured single cells and tissues;

(v) experiments on giant axons;

(vi) transport across the membranes of large cells in vitro;

(vii) model membrane systems made of biological extracts;

(viii) movements of water, sugars, amino-acids, etc., across frog skin, mammalian gut, etc.

Chapter 3

PRODUCTION OF IMAGES BY LIGHT AND ELECTRON MICROSCOPES

The light microscope was considered to be one of the most important tools in biology until the advent of the electron microscope but, unfortunately, today interest in its use is much less than hitherto. The light microscope should more correctly be called the photon microscope, since it is the action of the photons which produces the image. Electrons produce the image in an electron microscope, therefore the two terms are accurate descriptions of the properties of the two microscopes in producing images.

The light microscope works nearer to its theoretical limit of resolution than any other instrument yet produced. The maximum resolution of the light microscope is of the order of 0.25 microns, but this degree of resolution is only obtained with regular arrays (Carpenter, 1901). The resolution can be slightly better with random particles. Some structures such as the slits in the diatom Pleurosigma Angulatum, originally seen with the photon microscope (McClure, 1938; 1945) were denied at the time of discovery, but were later rediscovered with the greater resolving power of the electron microscope (Ockenden, 1945; Sartory, 1949).

The image is formed in the light microscope when the beam of photons is focussed in the same plane as the object to be observed. Photons are then stopped, which produces blackness; or they may be partially or selectively absorbed; or they may pass through the specimen without any modification, producing a white structureless field. Some photons are diffracted outwards by the object and away from the axis of the main or dioptric beam. These photons are gathered by the objective system of the instrument. Diffracted photons can be demonstrated easily if a regular array just within the resolving power of the instrument is examined first in focus, and then again when the ocular is removed while the array is kept in focus; the back focal plane of the objective can be examined and the various spectral orders of diffraction can then be seen with ease. The image thus formed is composed of photons in the central or dioptric beam which have passed through the instrument and have had their amplitude of vibration changed by the object on the stage, plus the first and second orders of the diffracted photons, all being united by the ocular; it gives a more accurate view of the object than can be achieved by any other method.

The light microscopist has at his disposal a large number of different methods of preparation and examination of his specimens, living, dead and fixed. Staining may be carried out not only in histological sections but also intra vitam in some organisms. Dye images thus obtained, like photographic transparencies, are grainless for all intents and purposes. The stream of photons may be directed from any angle through 360°, so that any natural incidence may be selected as if the object were being examined in the experimenter's hand. It is possible to obtain a very close idea of the real nature

of the specimen by gradation of tone or contrast, or by a change of the angle of incidence of the beam.

The photon microscope is not free from artefacts, mostly due to failure to respect, or lack of knowledge of, the physical requirements of the optical system. The diameter of the aperture of the substage condenser is often reduced too much in order to increase visibility, which produces the appearance of rings or capsules around single particles. Misalignment of the illuminating system can produce apparent doubling of single lines. In colour photo-micrography the use of ordinary oculars with apochromatic objectives, or compensating oculars with achromatic objectives, may produce colour where no colour exists in the specimens.

When using phase contrast microscopy, a common fault is to allow the condenser annulus and the objectives phase ring to come out of coincidence, thus producing a mixture of phase and annular illumination. The same condition is produced when viewing objects in cavity slides: departure from the centre of the slide produces a wedge effect, which throws the two parts of the system once again out of coincidence. In phase contrast microscopy the phase testing telescope should be used frequently to ensure that the phase conditions are being maintained.

In the electron microscope, on the other hand, the object is bombarded with electrons, which are particles having a high relative mass compared with the photon beam. A change of magnification in the electron instrument may be produced among other means by changing the accelerating voltage. The energy can be transmitted at many thousands of electron volts, which at once precludes the examination of living material; furthermore, a very high vacuum is necessary for the electrons to traverse the instrument at all. The electrons are particulate, so that diffraction can not take place in the instrument. The particles which have an enormous amount of energy are concentrated on the object by means of electromagnetic 'lenses'. The imaging 'lenses' are of small relative aperture and long focus compared with the light microscope in which the numerical aperture is relatively low. The electron instrument has a much higher resolution and magnification than the photon instrument, and the shortness of the wavelength used in some circumstances may compensate for its low angle.

In the electron microscope, the electrons which are not intercepted by the heavy metal atoms pass through to the fluorescent screen, whilst those which are intercepted give up their energy to the specimen, so that the image is a 'go-no-go' phenomenon. Other electrons which are deflected by the specimen add to the 'noise' or background. The capacity of electrons to pass through the heavy metal is small compared with the capacity of photons so that there is little graduation of tone in the electron microscopic image, which is composed of solid black particles or clear white spaces. This is the same principle as that of a shadowgraph, and, as the depth of focus of the electron beam is very large compared with its focal length, the electron microscope has no real plane of focus. Consequently, there is no possibility of discovering the spatial differences between the various elements of the image even though the specimen may be capable of a small degree of oscillation in the beam. Nevertheless, it is difficult to understand why subcellular structures hardly ever appear to overlap when viewed by electron microscopy (please see p.41).

The tissue is always dehydrated in preparation for electron microscopy, whether by transmission, scanning, or the use of freezing techniques (Moor, 1969; 1971: Weakley, 1972). Newer techniques subject the tissue to a very low partial pressure of water, and these remain to be evaluated (Parsons, 1974). Dehydration is done with alcohols, freezing (Love, 1966), vacuum treatment, or the heat induced by the electron beam. Much heat is dissipated in the specimen, and the temperature in metallic specimens under electron microscopy has been shown to rise by hundreds of degrees (Reimer, 1965; Reimer and Christenhusz, 1965: Watanabe, Someya and Nagahama, 1970; Grubb and Keller, 1972a, b); the low vacuum 10^{-5} to 10^{-4} torr not only increases evaporation but also prevents heat dissipation except by radiation; dissipation of heat by conduction can only take place in those regions where the embedded specimen is in contact with the grid. The grid is made of metal which conducts heat very well, and so it is intended that by this means the greater part of the heat will be dissipated rapidly. Unfortunately, this optimism cannot be justified for the following reasons.

Firstly, the metal grid is *in contact* with a plate which may or may not be cooled. It is not annealed or fused, as can be seen when it is removed from the electron microscope after examination. The real metallic contact of one metal object placed on another is extraordinarily poor. It could be compared to that of a 'dry soldering' in an electrical circuit; the heat generated in the metal deposit, the embedding film and the copper grid, could only escape at microscopic contacts between the metal grid and the holder, or by radiation, because of the high vacuum used. The poor contact between the grid and the holder also means that the effectiveness of the devices which are used to minimise the temperature rise must be very limited indeed.

Secondly, as soon as the electron beam strikes the grid, it will heat up the metal. Since the embedding medium has a greater heat capacity than the metal, the temperature will rise in the metal deposit on the tissue and in the metal grid *before* it does in the embedding medium. The metal deposit on the tissue is exceedingly small and is in intimate contact with the embedding medium so that its temperature would not be expected to remain significantly above that of the surrounding embedding medium. However, the relatively large volume of metal in the grid would reach a higher temperature during the electron bombardment, and therefore, heat could not pass from the embedding medium to the metal grid *during* the rise in temperature. When temperature equilibrium had been reached – if this were possible – the embedding medium and the metal grid would be at the same temperature, and therefore again heat would not pass from the embedding medium into the metal grid.

It may be argued that one does not achieve real equilibrium since heat is continuously being dissipated by the cooling near the grid. This would set up a gradient from the metal specimen through the embedding medium to the copper grid. However, its efficiency will always depend on the degree of contact of the metal grid with the specimen holder, which must always act as a bottleneck.

Thirdly, the electrons strike the specimen because they are aimed at it. The heat is generated whenever an electron hits the electron dense metal, which has been deposited on the tissue. The energy liberated at the point of impact will be largely independent of the temperature of the specimen: it will depend upon the energy of the beam – related to the magnification used – the time for which electrons bombard, and the hardness of the vacuum. All these parameters will determine the number of electrons absorbed by the metal 'stain'. It is also clear that the greatest energy will be liberated in those parts of the specimen which appear most electron dense, i.e. show the greatest contrast.

In recent years, electron microscopes with ever-increasing voltages, up to 3,000,000 V, have been developed (Dupouy, 1968; Favard and Carasso, 1972; Dupouy, 1972; Glauert and Mayo, 1972). The intention seems to have been that the faster electrons would pass through the metal stain so quickly that their radiation would damage the tissue less than electrons at a lower voltage would do. This seems to be an attempt to dissociate the fact that one sees a particle of metal on the electron microscope screen from the impact of the electron which makes it visible. The higher the voltage used, the greater the velocity of the electrons must be, and the greater will be the momentum that the electrons will possess; therefore, they will release more energy on impact with the heavy atoms of the stain. This will raise the temperature and the amount of radiation in the region of the metal atoms, which will damage and distort them, and make them oscillate. Furthermore, the higher the voltage used, the larger number of electrons are fired, and therefore the greater will be the quantity of heat dissipated. A biological science writer recently enthusing on the possibility of seeing living bacteria with this new high voltage electron microscope, noted that its only real disadvantage was the danger to the operatives standing nearby.

Fourthly, when one examines biological specimens with high magnification electron microscopy, the specimen is sometimes seen to disappear if the observation and photography is not carried out rapidly enough. Holes in the specimen can sometimes be seen on examining the grid afterwards under light microscopy.

The diminishing contrast is often attributed to 'contamination'; this may be due to decomposition of hydrocarbons, but is often said to arise from the oil used to prevent leaks of the instrument subjected to a hard vacuum. It is generally believed that this deposits on and obscures the specimen. Since the pump is continuously withdrawing gas through a port some distance *away* from the specimen, the pressure gradient would tend to prevent the deposit on the specimen. A more important consideration is that the region of the specimen being examined is the hottest part of the instrument since electrons are focussed on it. Therefore a deposit of oil is least likely to occur there, since heat cannot pass from a cold object to a hot object.

Fifthly, until now, the very high voltage microscopes have not given more information than the lower voltage electron instruments. The images are usually blurred. The employment of larger and more expensive instruments can only be justified if their use reveals hitherto unseen detail.

Although the local temperature rise is a function of the precise geometry and material of the specimen, the heat absorbed by organic materials has been assessed. Reimer (1965) gives the following example: at a magnification of 10,000 diameters with current densities in the specimen place of 10^{-2} amps cm^{-1} an object irradiated at 60 ke V absorbs approximately 6×10^{9} rads per second, which is equivalent to 6×10^{11} ergs.g^{-1}, or about 15,000 cal. $g^{-1}sec^{-1}$; this is a truly infernal dissipation of energy. The heat dissipated is a function of both the voltage and the magnification. A magnification by the electron microscope of 10,000 is considered small nowadays, and thus this example represents the minimal amount of energy absorbed by specimens.

It is often argued that freezing techniques are a separate source of information, which confirm the findings of transmission electron microscopy. The tissue is fixed not chemically but by rapid freezing to $-196°$ or $-150°$. After the tissue is frozen, platinum or carbon is deposited on the surface of the tissue, but it is difficult to be certain whether in a particular field the etching has gone through the extracellular or intracellular spaces. Furthermore, the appearance of the specimen is always interpreted by reference to transmission and scanning electron microscopy, so that the freezing techniques cannot be regarded as independent sources of evidence. Even if freezing techniques could be considered different sources of evidence, research workers who use them would then have to join the ranks of transmission electron microscopists and explain why the images of sections seen by these techniques *also* are two dimensional (see below.)

There is a widespread belief that freezing without the addition of cryoprotective agents can be carried out without causing dehydration. Sperm, red blood cells and seeds have been frozen very deeply in the presence of glycerol, and have been viable on rewarming (Smith, 1952; 1961; Mazur, Farrant, Leibo and Chu, 1969; Mazur, Miller and Leibo, 1974). Although it is often said that rapid freezing or previous immersion in glycerol prevents crystal formulation down to $-196°C$, we could find no direct light microscopic observation on solutions or tissues frozen to less than $-50°C$, in which crystals did not appear (Smith, 1952; 1961; Rapatz and Luyet, 1960; Persidsky and Luyet, 1960 a,b; Mazur, 1966; Luyet, 1966; Rapatz, Menz and Luyet, 1966; several papers in Wolstenholme and O'Connor, 1970). Unless glycerol has been used – which is not routinely done in preparation for electron microscopy – all tissues freeze when the temperature falls below $-50°C$ (Love, 1966). It would be interesting to see any photographs taken by light microscopy of any tissue at $-196°C$ in which there were no ice crystals. If ice crystals are present, the rest of the tissue must be dehydrated.

Two other serious problems with the interpretation of the image on electron microscopy are related to sampling. The first one is well-known and accepted by electron microscopists. A single section 100 – 1000 Angstrom units thick represents a very small sample of the whole tissue. A total field may only be 10's to 100's of microns in diameter. The result is that the sampling for assessment must be done by the electron microscopist, and is a relatively unique and unrepeatable event. This would not be a serious difficulty, if it were not compounded by a second one. In all electron microscopic fields there are con-

siderable areas which cannot be identified as known structures. These are not to be confused with those structures which have been given names by electron microscopists. These unidentifiable areas are sometimes spoken of as 'mush' colloquially, and have been compared to 'noise'. However, we would like to use the term 'non-information' (please see Appendix 1). The complete unclarity of absolute non-information is at one end of a spectrum of images, which go through a range of increasing clarity. In the middle of the range would be many images whose nature would be a matter of disagreement between different electron microscopists. At the other end of the range would be nuclei, mitochondria or nucleoli, about whose identity no two electron microscopists would be in dispute. This large problem due to 'non-information' in electron micrography is not shared with light microscopy, in which nearly all structures would be identified and agreed by competent histologists.

It would be generally agreed that the electron microscope has a depth of focus represented by the complete thickness of the specimen examined, yet there are two difficulties here. We shall adduce evidence – which can be seen by careful examination of any electron micrograph – that the images are largely two-dimensional; examination of pictures also shows that, even with thick sections, one never sees overlap of structures. We believe that the explanation for this apparent paradox is that the electron beam only 'develops' the surface of the specimen (please see page 40). It seems likely that electrons hitting heavy metals deeper within the specimen are scattered outside the field of observation. We put this forward as a tentative explanation, and would be interested in further reflections from electron microscopists or physicists on this problem.

In discussions about the solid geometry of tissues as understood by examination of electron micrographs, it has frequently been stated that electron microscopists choose the fields showing the features they wish to illustrate, so that one canot adduce the appearances seen in their pictures as evidence that they believe that these appearance may be regarded as general throughout the cell. For this reason, we have generally used illustrations showing whole cells. However, if we accept their assertion that their illustrations are selected, we must ask upon what basis. If the selection is made to show a *typical* view, then indeed we may quote that particular illustration. If the selection is made to show a particular appearance which is not typical, this is tantamount to saying that these authors are intellectually dishonest because they select 'typical' pictures subjectively, and their findings therefore must have little scientific validity. We do not wish to entertain the latter belief.

Chapter 4

CRITIQUE OF THE CURRENT VIEW

The cell membrane, the nucleus, the mitochondria, cytoplasmic inclusions and the nucleolus, can be seen by light microscopy in unfixed, unstained cells, and we regard the evidence that they exist in living cells to be beyond reasonable doubt. The rest of the currently accepted structure seems to be less certain.

A. THE CELL MEMBRANE

(a) **The appearance of two lines**

All the membranes appear on electron microscopy as two lines with a space in between, the latter sandwich being given the name 'unit' membrane by Robertson (1959). It is generally believed to be two protein layers sandwiching a lipid layer; the total thickness of a 'unit' membrane is given as 75Å to 95Å and as 210Å to 240Å for two opposed membranes (see, for example, Threadgold, 1976, page 64 – 75). These ranges were taken from electron micrographs of a number of authors and their variation is due to the fact that the thickness measured depends upon the fixative, the heavy metal used to stain, and on the embedding medium. The electron microscopists do not seem to have paid sufficient attention to the fact that the parameters depend upon the techniques of measurement – discrepancies which would not be tolerated in many other sciences. No allowance seems to have been made for shrinkage; this must be greater in the extracellular space, the cytoplasm and the nucleoplasm, which contain more water, than in the insoluble material of the membrane itself.

It should be emphasised that the idea of the 'unit' membrane, in which each unit *appears* as two lines (Robertson, 1969), would require that the membranes of two adjacent cells should appear as four lines with three spaces in between. This becomes important in consideration of the attachments of the endoplasmic reticulum. It also means that any electron or x-ray beams which were refracted or reflected at the surface of the two adjacent cells would meet at least *eight* interfaces from the inside of one cell to the inside of the other, and sixteen if they pased through two adjacent real cells or axons even without going through the nuclei; these would be:

1. incubating medium – protein
2. protein – lipid
3. lipid – protein
4. protein – cytoplasm
5. cytoplasm – protein
6. protein – lipid
7. lipid – protein
8. protein – extracellular fluid
9. extracellular fluid – protein
10. protein – lipid
11. lipid – protein

12. protein – cytoplasm
13. cytoplasm – protein
14. protein – lipid
15. lipid – protein
16. protein – incubating medium

In general, any membrane of n single but finite thicknesses, or n layers between two media of different refractive indices, will have 2n refracting surfaces, if each layer can be resolved by the electron or x-ray beam.

Low angle x-ray patterns of lipids and myelin sheaths indicate a repeating unit (Schmitt, Bear and Clark, 1935; Schmidt, 1936; Finean, 1959; Luzzati and Husson, 1962), and this has been regarded as evidence that the double appearance of myelin sheaths and all other membranes is not artefactual. However, one cannot know the number of sub-cellular organelles in the path of the x-rays in diffraction experiments. That is to say, the several interfaces of the cell membranes would account for the x-ray and electron diffraction patterns without the necessity of there being structures in the cytoplasm causing similar patterns. Many of these earlier experiments on myelin were carried out on dehydrated specimens, sometimes without correction being made for the effect of dehydration on membrane dimensions. Therefore, although one can conclude that there is a repeating pattern in usually partially dehydrated tissue, its microscopic location and precise dimensions cannot be deduced from such measurements. There is precious little critical evidence that it is not caused by large molecules and not necessarily membranes (Bragg, 1975; Wilson, 1966).

The double line appears in the following structures:

mitochondrial membranes
nuclear membranes
endoplasmic reticulum
cell membranes

The mitochondrial cristae and the lamellae of the Golgi apparatus have the same general appearance, i.e. two lines with a space about 25Å as seen on electron micrographs. It is of the utmost importance to our considerations that this distance between the particular two lines of a specific membrane always *appears* to be the same on the same electron micrograph and frequently on micrographs of many different tissues.

The concept of the 'unit' membrane has been broadened to include virtually all structures appearing as two lines on electron micrographs, and since the cell or 'plasma' membrane is generally believed to consist of protein and lipid molecules, the belief has gradually spread that all these 'membranes' are also composed of protein and lipid; furthermore, in many circles, it is regarded as necessary to demonstrate that a structure is of such a chemical nature before accepting it as a biological membrane. We would point out, (α) that we regard the evidence from the analysis of the chemistry of sub-cellular fractions to be too uncertain until the relevant control experiments have been done (Hillman, 1972, page 39); (β) in many cases, the belief that these 'membranes' are of such a nature has not even been demonstrated by the latter techniques, but

has been assumed by analogy of the structure with myelin sheaths or red cells on electron microscopy; (γ) we present evidence in this monograph that the endoplasmic reticulum is an artefact; (δ) we define 'membrane' in a broader sense than do electron microscopists (please see Appendix 1); (ε) in general, evidence from sub-cellular biochemistry is interpreted on the basis of the currently accepted view of cellular morphology, and cannot be used in support of it, and rarely bears upon it; (ζ) serious discrepancies between the electron microscopic appearances of structures in tissue sections and the fractions they are believed to represent are often obscured by saying that the homogenisation and sub-cellular preparation 'rounds off' the particles.

(b) **Evidence that the 'unit' membrane is an artefact**

The appearance of the 'unit' membrane must be artefactual for the following reasons:

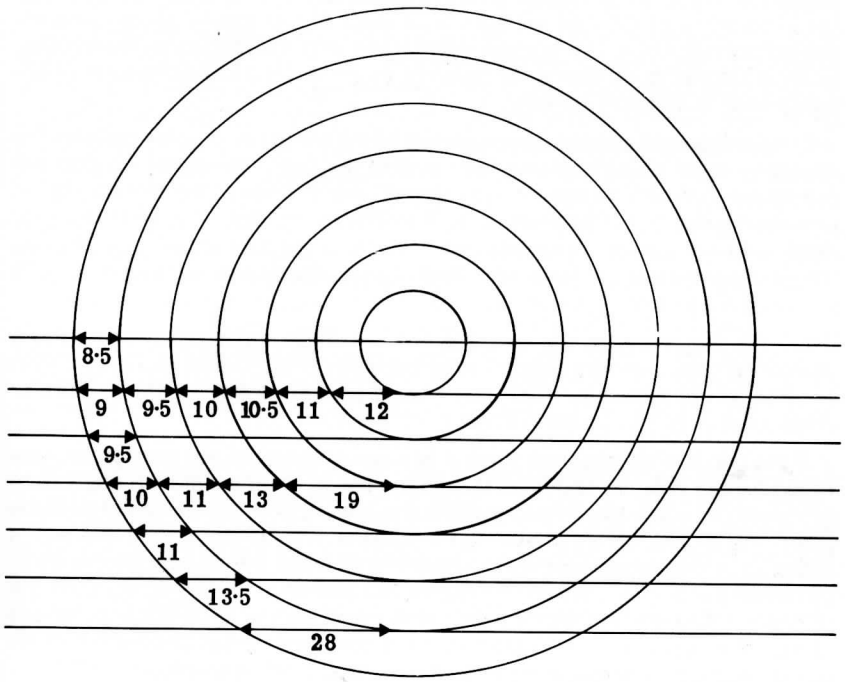


Fig. 11. Sections thought concentric spheres. It will be seen that as sections are made nearer the periphery, the distance between adjacent shells appears to increase. The shells only appear equidistant when the section is cut precisely through the centre of the spheres. This geometry applies to all the 'unit' membranes, the endoplasmic reticulum, the mitochondrial cristae, the Geren model of the myelin sheath, the synaptic cleft, the lamellae of chloroplasts and the lamellae of the Golgi apparatus.

(i) If one takes a series of concentric spheres whose radii increase by integral steps, and makes a section through the geometrical centre, the distance between each shell must appear to be equal (fig.11). However, sections made in all other planes not going through the centre must show apparently greater distances between the shells, as the sections are taken nearer the periphery. Even if in real structures all shells were equidistant, the distance apart between the two layers of which they were composed would appear on section to vary, depending upon the degree to which the section was central or tangential.

In respect of preparation for electron microscopy, the apparent equal spacing of all these membranes means perforce, firstly, that all the visible subcellular structures and their membranes in all the cells in a particular section would have to have been sited centrally and symmetrically at the time of section, and secondly, that the knife had cut through the centre of each of them in sequence. These conditions would have to be fulfilled simultaneously if the two lines indicative of the cell membrane, a layer of the myelin sheath, the endoplasmic reticulum, the nuclear membrane and mitochondrial membrane, were to appear to be the same distance apart. Such a concatenation of circumstance is absurdly unlikely.

The commonest explanation proferred by the electron microscopists for the apparent equal spacing of the two lines of the 'unit' membrane is that the membrane can only be seen clearly when it is in the line of the electron beam or within about 15° either side of it. If this were so, the mitochondria would rarely be seen clearly on longitudinal section, since they would only be seen where a section would have split them longitudinally precisely in their long axes and maximum dimensions.

For the same reason the layers of the myelin sheath should appear more unclear as they become peripheral to the axon in the Geren model (1954) in addition to the expectant that they should appear closer together. (Further consideration of this problem is given on page 41).

If the two line appearance were a genuine property of the membrane, one would expect that the two lines should appear to be any distance apart from complete overlap – when two adjacent shells were both normal to the electron beam – to a distance equivalent to the maximum arc between the two layers of the structure surrounded by the membrane, when the section happened to be tangential. Not only would one not expect that on electron micrographs the *two lines* representing the unit membrane would appear to be equal distances apart within any *one* section, neither would one expect that particular strands of the reticulum would appear to be equidistant from each other.

We would also like to stress that in extensive examinations of specimens of nervous tissue in the electron microscope, and of many thousands of electron micrographs in publications, we have been unable to find the following appearances:

(a) equally spaced layers of the myelin sheath should represent a section through the geometrical centre of the axon and sheath, therefore the axon should always be seen when the layers appear equally spaced;

(b) the lamellae of the Golgi body should also appear to be spreading out to different extents in different orientations;

(c) the synaptic cleft should appear to vary in width, and should only be seen when it is in the same plane as the electron beam, which should be a rare occurrence compared with the times the pre-and post synaptic thickening should be overlapping;

(d) we were also unable to find any significant incidence of expected variation of the spacing of lamellae in chloroplasts.

It is of interest that 'liposomes' or extracted lipids, which are deposited from suspensions, extracts or solutions (not sectioned, of course) do appear as equally spaced lamellae always in the same orientation (see, for example Luzzati and Husson, 1962; Bangham and Horne, 1964).

(ii) A membrane has a finite thickness and the stains must deposit on both sides of it. The stains react with both the membrane components, the proteins and lipids; they do not dissolve or replace them. The membrane in vivo is hydrated, so that its thickness may well be different from that of the dehydrated tissue prepared for the electron beam. Nevertheless, even after dehydration, it always appears to be of the order of 10's or 100's of Å units thick, which is well within the limit of the resolution of the electron microscope so that its two surfaces could always be seen on transverse section. The metal would deposit approximately symmetrically on both sides, as the membrane has two surfaces. A real line, having no thickness, is a geometrical abstraction, and would never be seen. A solid particle, like a glycogen granule, would appear to have a single layer round it, since it is not hollow, and the heavy metal salt would be deposited only round about its wall. With the possible exception of bubbles and 'lysosomes', the 'diaphragms' across the nuclear pores are the only other structures which appear as a single line (Novikoff, 1961).

It might be useful to suggest that much of the unidentifiable material seen under high power electron microscopy, which we have called 'non-information' (please see Appendix 1), might be due to the physical and chemical effects of the electron beam on the specimen. On the other hand, the contrast of membranes might be exaggerated, if the electron beam caused them to shrink, sublime, evaporate, or explode in a groove in the embedding medium (please see below).

(iii) The appearance of sub-cellular fractions on electron microscopy, particularly 'microsomes' is often used to support the findings by electron microscopy of the whole tissue. We would point out that these fractions – including those which are believed to originate from the cell membranes – also appear *linear*. They do not seem to be testudinal, gradually fading into the depth of the section, as one would expect if they really came from the cell membranes; they look like a series of bracelets laid out for sale, i.e. a number of rings in two dimensions only. The explanation has been offered that the membranes 'round off' during the preparation of the subcellular

fraction. Unfortunately, the circular or slightly collapsed circular appearance seems to be shared by all 'membrane' fractions. We are rather surprised that on the basis of the 'rounding off' explanation, biochemists are prepared to accept the identity of the membrane in sections with those after separation as subcellular fractions.

It is often stated that one only sees membranes which have been cut at right angles during preparation for electron microscopy, but calculations may be made for the frequency with which one can resolve clearly two layers separated by a distance of d in a section of thickness t (fig. 12).When the two lines overlap the space will be obliterated and the angle will be given by $\tan^{-1}\frac{d}{t}$. If the double layer (for example, the 'unit' membrane) can be viewed from any angle or if the double layers are randomly orientated, it becomes obvious that the lines can be resolved only in $4\tan^{-1}\frac{d}{t}/360$ fraction of observations. We can solve this equation for typical conditions. If the distances between the two layers are 30 Å, 70 Å or 200 Å in sections 100 Å thick, this will give incidences of observation of 0.19, 0.39, 0.70; if the sections are 600 Å thick, the incidence of separating the two lines will be 0.03, 0.07 and 0.20, of observations, respectively. These calculations imply the assumption that the resolution of the observing instrument – the electron microscope in this case – is significantly better than 30 Å; 10 Å is a figure usually quoted for microscopes used for observing membranes. These simple calculations indicate that any distance less than 70 Å will be seen on a minority of occasions, yet in many micrographs of sections 100 Å to 600 Å thick, electron

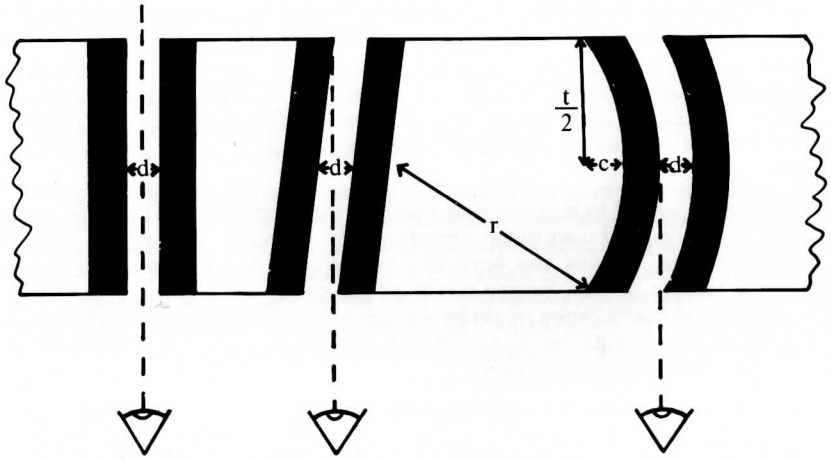


Fig. 12. This diagram shows two layers of a membrane separated by a distance d units in a section of thickness t units. It can be seen that the space between the two layers will be seen when the two layers are in the same axis as the observer (left), but will not be seen when the observer is at an angle to the two layers whose tan is given by $\frac{d}{t}$ (middle). The diagram on the right indicates that the space between two curved layers will not be seen when $c>d$. Since values of d and t are known, the radius of curvature which will not permit the space between the layer to be seen can be calculated. Please also see text.

microscopists are claiming to see the lipid layer of the Davson-Danielli model which appears to be smaller than this.

The latter calculations are made on the assumption that one is looking at a membrane which is straight as observed. However, cell, nuclear and mitochondrial membranes, will be curved (fig. 12). In the situation in which the curve of the membrane represented by its departure c from the arc exceeds d, the two lines of the membrane will not be able to be distinguished. We can calculate the minimum radius of curvature of an object which will permit us to observe its membranes as two lines.

If we ignore the thickness of the layers themselves, if $c = d$, and t is the thickness of the membrane we can calculate the minimum radii of curvature for separations of layers 30 Å, 70 Å and 200 Å apart in sections 100 Å and 600 Å thick. These radii are (to the nearest Å) 57, 53, and 106 for the thinner sections, and 1515, 678 and 325, for the thicker sections, respectively.

We may conclude that the curvature of the membrane would not inhibit significantly the resolution of two layers within the range of separations and tissue section thicknesses considered here. Most membranes would surround structures with diameters considerably in excess of 0.3 microns.

A cursory examination of any electron micrograph photographed at sufficient magnification to show the apparent spacing between the two lines of the 'unit' membranes indicates much greater incidences of the appearance of this space than the calculations above would permit. One possible explanation is that the 'unit' membrane does not penetrate through the whole thickness of the section; this may be true sometimes, but seems unlikely to explain the total discrepancy. A more likely possibility is that the electron beam is dissipated in the electron dense region near the suface, and so does not penetrate the section completely. Therefore, in the equation 4, $\tan^{-1}\frac{d}{t}/360$, the real value of t is much smaller than the total thickness of the section. This is compatible with the observation that one rarely sees overlap of any structures in 'thin' or in 'thick' electron micrographs.

(c) The nature of the 'unit' membrane artefact

When an embedded section is cut, heat is liberated at the cutting edge. One does not know the thermal coefficient of expansion, the elasticity, or the compressibility of the embedded membrane, but each of these coefficients would be extremely unlikely to be the same values as those of the embedding medium. Therefore, it would be virtually impossible for the embedded membrane after section to remain flush with the embedding medium, in microscopic dimensions.

After the section has been cut, the heavy metal or salt is deposited. The electron microscope is reduced to an extremely low pressure, and the specimen is subjected to the electron beam, which releases an enormous amount of energy.

The electron beam hits the preparation probably containing the following components, in unknown proportions:

(i) the tissue after it has been dehydrated, extracted with organic solvents, and subjected to 10^{-4} torr or less;

(ii) the embedding medium, for example, of Araldite or acrylic;
(iii) the heavy metal or salt deposit, for example of osmium, lead or tungsten, or a mixture of these;
(iv) other chemicals in the reagents such as salts of potassium, sodium, calcium, magnesium, etc.

Each of the components of these four major groups is quite different in respect of the following chemical and physical properties:

vapour pressure
volatility
stability
purity, including water content
thermal coefficient of linear expansion
heat conductivity
heat capacity,

All these parameters will vary to different extents with the temperature and the degree of vacuum present at any particular time during the preparation – and their rate of change. The thermal coefficients of linear expansion for some of the materials are known, and these are quoted as an example (Table 1). It can be seen that the coefficient of epoxy resins is approximately eight times as great as that of osmium.

Table 1. The thermal coefficients of linear expansion of materials exposed to the electron beam. Figures are not available for embedded biological tissue.

	Temperature range (°K)	Coefficient ($°C^{-1} \times 10^{-6}$)	References
Osmium	273 – 323	5.0 – 6.8	Lange (1952) Samsonov (1968) *Handbook of Chemistry & Physics* (1971)
Lead	273 – 593	26 – 33	Kaye & Laby (1966) Samsonov (1968) *Handbook of Chemistry & Physics*
Tungsten	293 – 2473	4.0 – 7.7	Lange (1952 Kaye & Laby (1966) Samsonov (1968)
Epoxy resins	343 – 623	48 – 67	Manufacturing Chemists' Assn. (1952) Touloukian (1967) Mark, Gaylord & Bikales (1970)

Subjection of this inhomogeneous specimen to the vacuum, to the large amount of energy in the electron beam, and to the radiation will cause grossly uneven changes in its properties. The following events could occur:

the tissue could evaporate or explode;
the embedding medium could rise or fall relative to the level of the metal deposit;
different components of the system would separate;
the specimen would be irradiated.

The first three of these changes would result in the appearance of two lines for every thickness of membrane. There is a considerable literature on the effects of electron beams on inorganic and organic materials and tissues (Deutsch, Fischer and Krause, 1964; Reimer, 1965; Favard and Carasso, 1972; Grubb and Keller, 1972 a,b).

It may be argued that one does not know the extent to which these changes occur. If this is true, it is a sad admission to have to make about a technique which has been used for thirty years in biological research. However, there can be no doubt that they must occur, as they arise from the well-known physical and chemical properties of the materials.

These points add weight to the proposition that the double line appearance of the membrane on electron microscopy is an artefact, but it should also be stressed that it is an inescapable consequence of geometrical optics that a transverse section of a layer whose thickness is within the limits of the resolution of the viewing system will always appear as two lines, if the magnification is high enough.

In recent years, there has been a tendency among biologists and electron microscopists in private conversation to regard the 'concept' of the 'unit' membrane as outmoded. The following remarks are pertinent in this respect:

(i) it cannot be a 'concept' – it is either present or it is not;
(ii) we have been quite unable to find a single *publication* unequivocally stating that the 'unit' membrane is an artefact;
(iii) those who agree that the 'concept is no longer widely believed' (in the words of the reviewer of one of the best known scientific publications) do not deny that on high power electron micrographs the membrane does appear as two lines, whether or not they have abandoned Robertson's terminology;
(iv) if they *now* believe that it does not appear as two lines, what is their explanation of the thousands of electron micrographs which have shown it as two lines hitherto.

B. THE ENDOPLASMIC RETICULUM

(a) Evidence for the existence of the reticulum

(please see Appendix 3)

The endoplasmic reticulum is regarded by many as one of the most important findings of electron miocroscopy. Diagrams of it are found in all life science textbooks used by schoolchildren as well as by university students, post graduates and career research workers. It is believed to be found in all plant and animal cells as a three dimensional net (Bernhard, Gautier and Oberling, 1951; Palade and Porter, 1954) or systems of flattened sacs or

vesicles (Sjöstrand and Hanzon, 1954; Sjöstrand, 1964). It appears so ubiquitously that it has been included in the generalised cell (Brachet, 1961, Robertson, 1962; Porter, 1966; Warwick and Williams, 1973 and references in Appendix 3). It has the appearance on electron micrographs – as in textbooks – of being two-layered (Porter, Claude and Fullam, 1945; Palade and Porter, 1954; Palade, 1955; 1956), or four-layered (Robertson, 1962). According to most descriptions it is attached to the cell membrance and the nuclear membrane and its 'lumen' is said to be continuous with the extracellular fluid at the outer end and the space between the two layers of the nuclear membrane at the inner end (fig. 1). As in the case of the 'unit' membrane we have been unable to find any modern textbook of cytology, biology, biophysics, physiology, biochemistry, zoology, botany, histology or anatomy, in which its existence in the living animal or plant cell has been doubted, although it has been questioned in private discussion with us. There has been no response to a request for information about any publications indicating that it might be an artefact (Hillman and Sartory, 1975).

Two kinds of reticulum are talked about, the 'rough' reticulum in which it appears as rather fuzzy lines, and the 'smooth' reticulum where the lines are clear and parallel. Sometimes, the reticulum appears to be lined by 'ribosomes' or small granules, which are believed to be composed largely of ribonucleic acid (please see below). On electron micrographs the reticulum appears to permeate part or whole of the volume of the cytoplasm. It is said to be particularly well represented in secreting cells. Most authors believe that the endoplasmic reticulum is connected to the Golgi body, in those cells where the latter structure can be seen.

The endoplasmic reticulum is obviously believed to exist in the living cell as in the electron micrographs, as is implied in the view that the ribosomes are involved in the synthesis of protein. It has generally been thought of as being solid, physically composed of strands or flattened sacs, although more recently sophisticated biologists have talked about a 'sol-gel' hypothesis, in which it is sometimes solid and sometimes fluid (Singer and Nicholson, 1972) (please see below). Not only has the existence of the endoplasmic reticulum been accepted universally among morphologists, but biochemists have separated subcellular fractions, which they identify as being from the endoplasmic reticulum and they have studied its biochemistry extensively (Hagenau, 1958; Baudhuin, Evrard and Berthet, 1967; Gran, 1968; Goldblatt, 1969; Cardell, 1977). Its 'function' has thus been correlated with its 'structure', which is regarded as confirmatory evidence for its existence in the living animal.

(b) **Evidence that the endoplasmic reticulum is an artefact**

Indications that it is an artefact of electron mocroscopy come from geometric and biological considerations.

Firstly, the endoplasmic reticulum seems to be only in the plane of the picture, whenever it can be seen clearly on a micrograph. If it were either a net or a series of flattened sacs, one would expect to see it in a number of orientations, beside that in the plane of the sections. A section would cut portions of the reticulum randomly orientated within a cell. We have represented some of the expected images in figure 13. Sections of the reticulum would cause the

same images whether it were flattened sacs or a real net, because of the depth of focus of the electron beam. It is extremely rare to see examples of such shapes as rectangles, rhomboids, Y-shapes, transverse sections of two layers, or regular patterns of the latter as dots in the electron microscope, on electron micrographs, in illustrations or in diagrams. It is quite impossible to conceive of a three dimensional object, which can always have the same appearance in two dimensions when section is made of it in any orientation.

Electron microscopists have proferred the following explanations in response to this difficulty:

(i) the above-mentioned shapes are rarer than one would expect because micrographs are usually selected to show the endoplasmic reticulum clearly; if one looked at other parts of cells on other mocrographs one would see it in other orientations. We have looked at electron micrographs of as many *whole* cells as we could find (for example, our fig. 2 from Fawcett, 1966; Porter, 1966; Cawley and Hayhoe, 1973), in which parts of the cell had not been selected to show the show the endoplasmic reticulum. We have examined relatively large areas of brain and liver tissues in the electron microscope including sections of many adjacent whole cells. We have observed the endoplasmic reticulum carefully in thousands of unpublished and published micrographs of cells in which the authors were drawing one's attention to structures other than the reticulum. We have requested electron microscopists in several countries publicly and privately to send us micrographs showing a significant incidence of these other orientations. So far, only two have done so; nevertheless, we still invite them to do so.

It is highly significant that – in complete contrast to the endoplasmic reticulum – sarcomeres and mitochondria (though not the cristae in them) do appear in electron micrographs in every orientation from the cylinder to the circle, depending on the plane of the section. These can be seen in any randomly selected electron micrograph, and the image of these structures represents an excellent control model for observations on the endoplasmic reticulum from this viewpoint. Of course, both sarcomeres and mitochondria can be seen by the light microscope, through their internal structure cannot be examined due to its lower magnification.

(ii) quite often, we have been shown blurred or mottled patterns which – if the contrast were better – might have shown up as these other orientations. This represented evidence of too poor quality, as it implied an interpretation which would be made if the blurring were not present, and was unacceptable (Appendix 2).

(iii) a well-known biologist gave as a reason for which the endoplasmic reticulum was usually seen in longitudinal section and hardly ever in transverse section, that it had 'poorer visibility' in the latter than in the former plane. If the reticulum were believed to be a single, extremely thin line, only visible at the limit of resolution of the microscope, it might be more difficult to see on transverse rather than longitudinal section. However, the reticulum can be resolved into two clear lines with a space in between it, so that the whole structure is clearly within the resolution of the electron microscope.

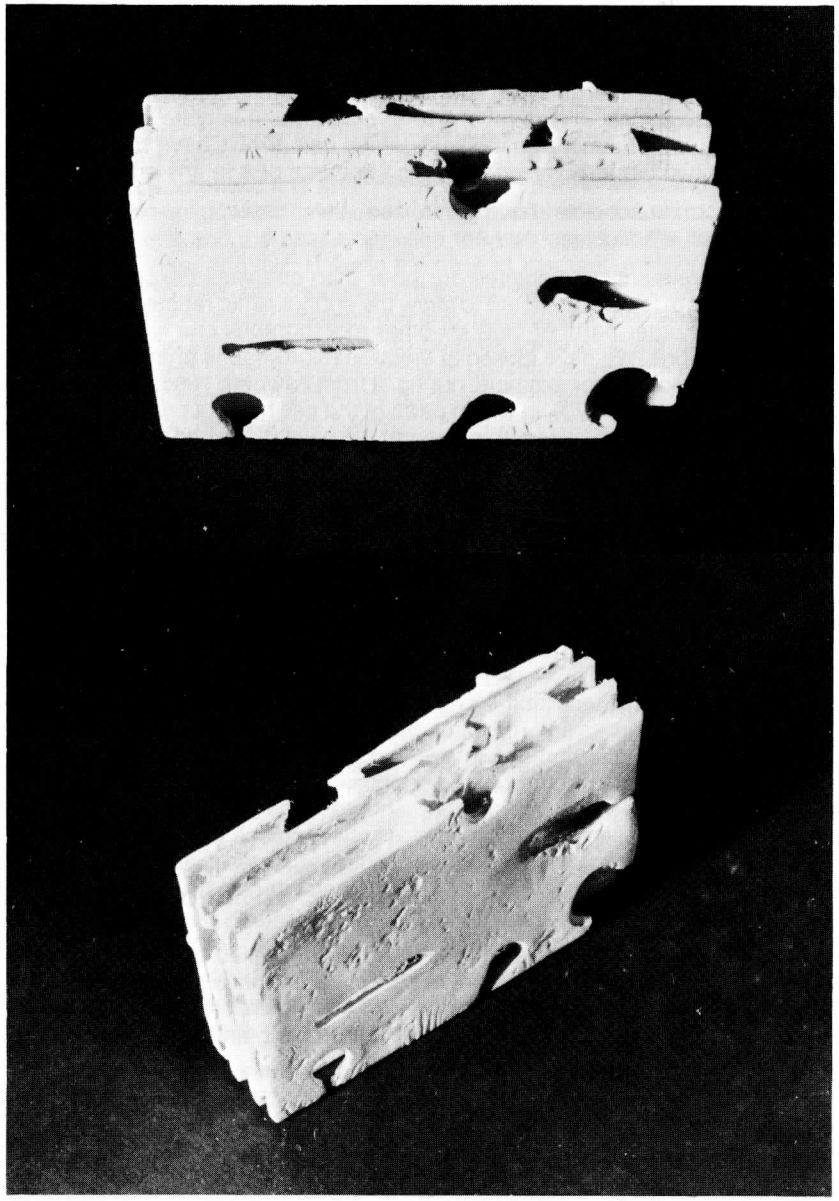
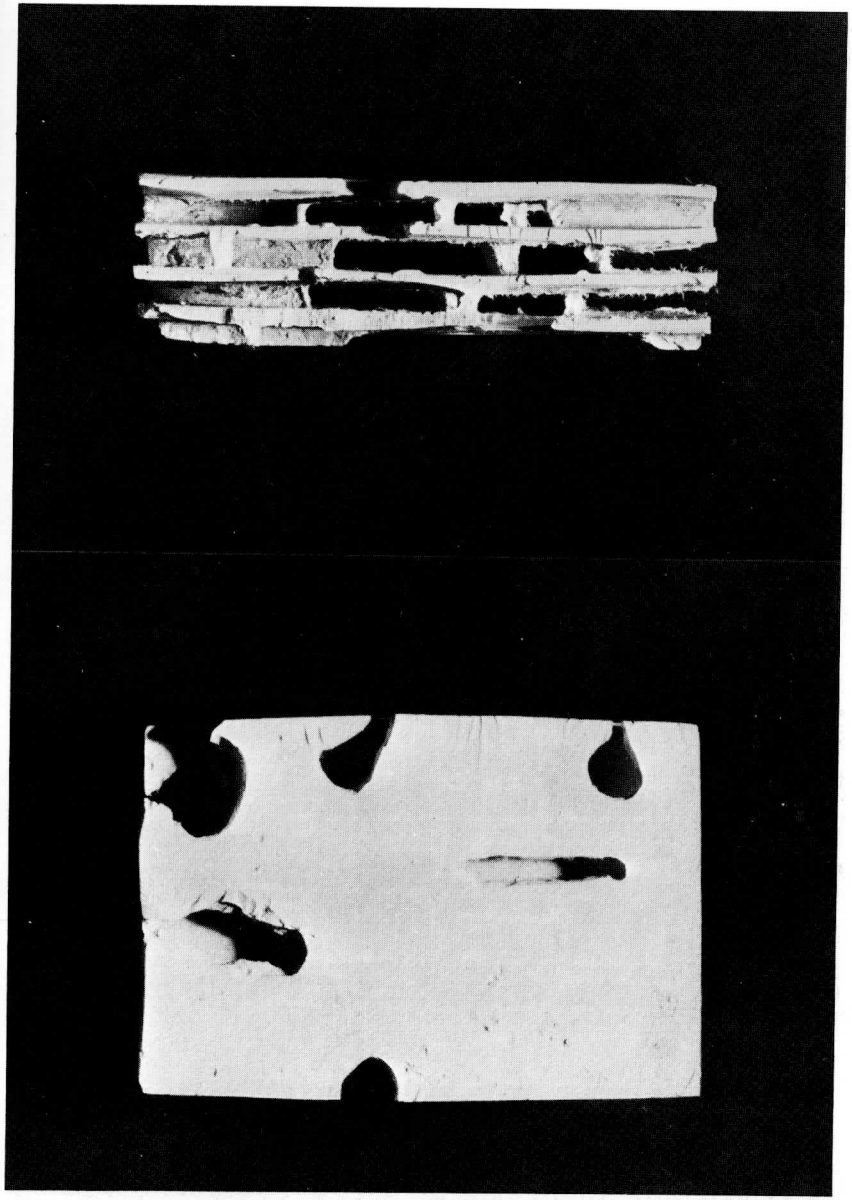


Fig. 13a. Diagrams of the appearance of an endoplasmic reticulum as flattened sacs or vesicles. If such structures were orientated at random three dimensionally in a cell, thin sections of the cell



would be expected to show a random selection of the shapes indicated in the other diagrams in this figure, as well as intermediate orientations of them.

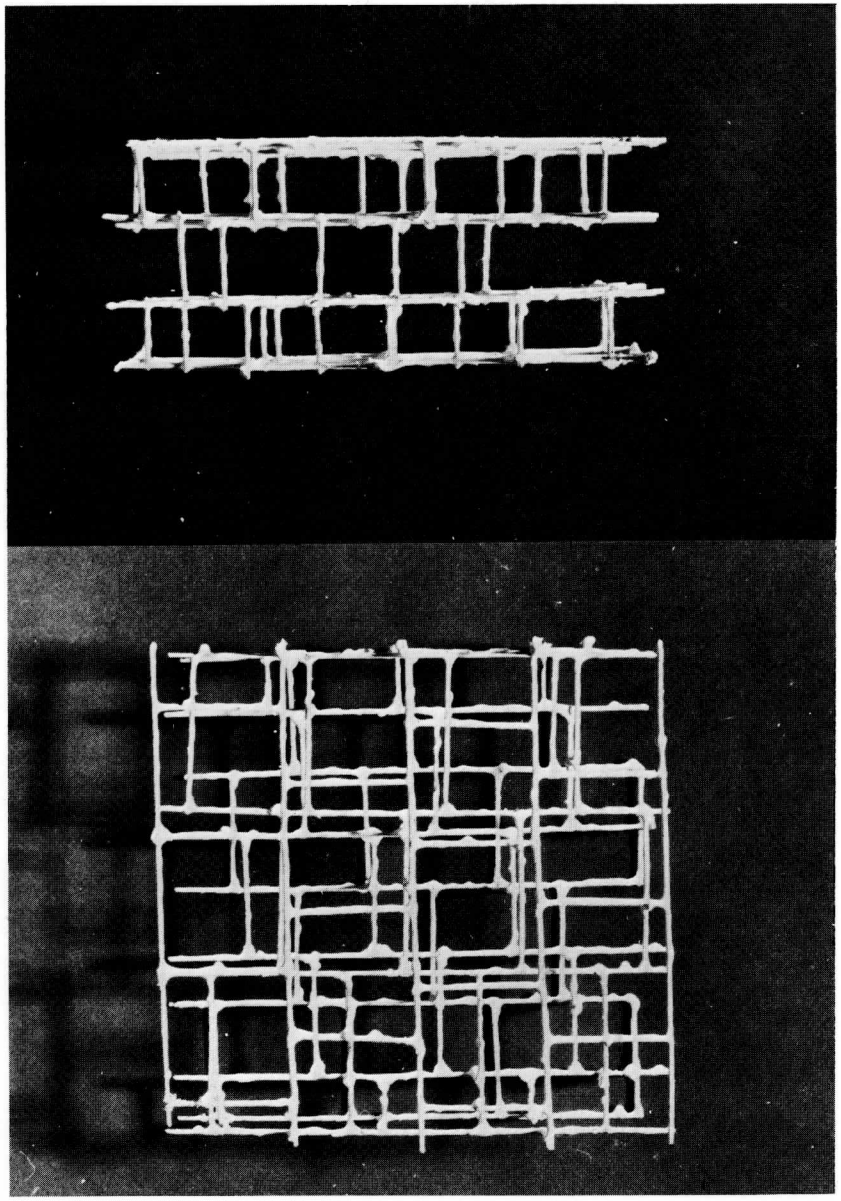
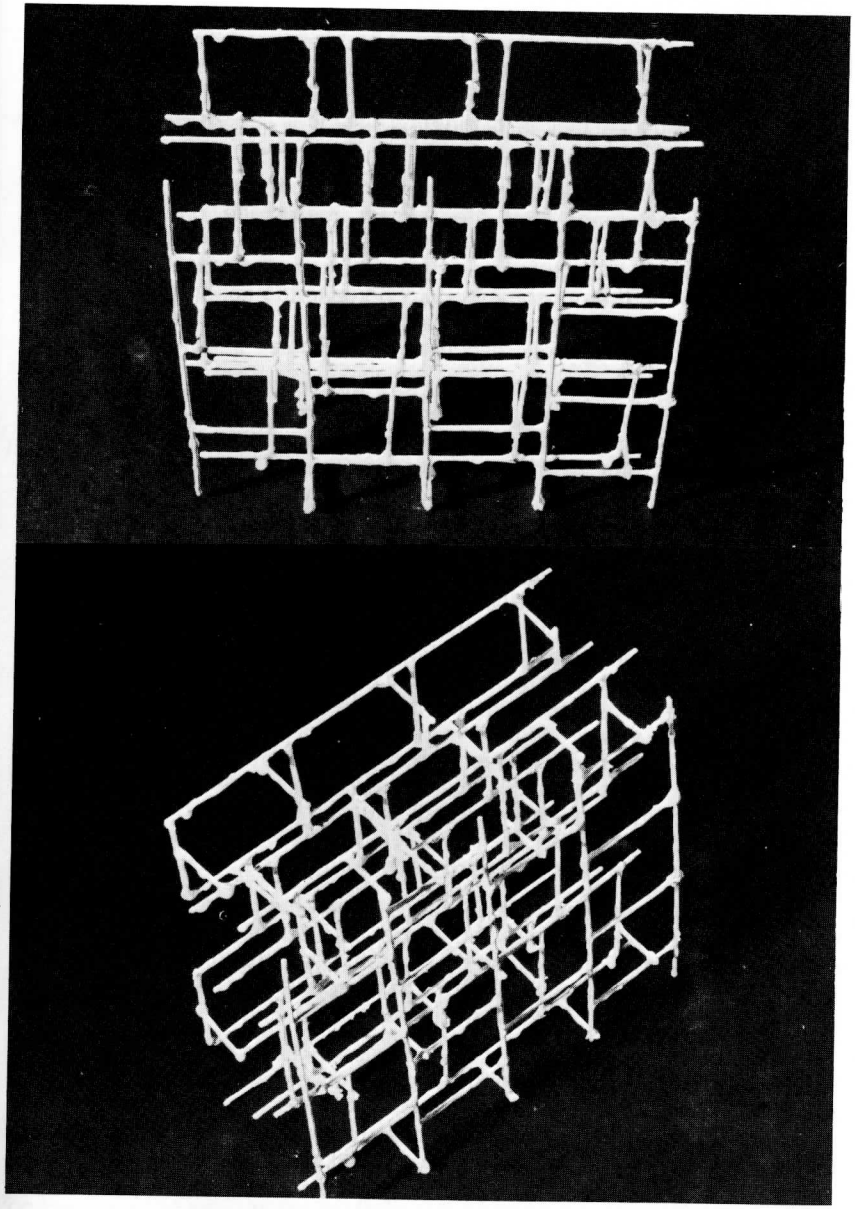


Fig. 13*b*. Diagrams of the appearance of an endoplasmic reticulum as a real reticulum.



A second reason why the reticulum must be an artefact was pointed out in connection with the two line appearance of the 'unit membrane'. Its two lines always appear the same distance apart as do the 'cisternae' of the Robertson model, and the two layers of each reticulum of the Brachet model. One would expect to see tangential pieces, of differing distances apart (please see figure 11).

Thirdly, the double layered endoplasmic reticulum appears to be thicker than the cell membrane and the nuclear membrane on the electron micrograph, yet the latter two membranes can be detected by light microscopy, so that the light microscope must have the resolution to detect them. Furthermore, the ribosomes would increase the apparent thickness of the reticulum. Why then is it so rarely claimed that the endoplasmic reticulum has been seen by the light microscope (but please see below)?

A fourth difficulty about the existence of the endoplasmic reticulum concerns its attachment to the two dense lines of the cell membrane and the nuclear membrane (Robertson, 1960; Stoeckenius, 1962), although Brachet (1961) has drawn the former as a single line. There are three possible ways this could happen. Either the outer line of the membrane could connect to the reticulum (fig. 14); this would require that the endoplasmic reticulum should always appear as four lines and it would require the existence of 'cisternae' which would permit the extracellular space to have access to the channels of the endoplasmic reticulum (Robertson, 1962). In the majority of electron micrographs the endoplasmic reticulum appears as two lines, and cisternae are extremely rare. A second possibility would allow the inner of the two layers of the cell and the nuclear membranes to be continuous with the reticulum, with no continuity of the reticulum with the extracellular space or the nucleus. As far as we are aware, electron micrographs of the region of attachment never show a continuous one-layered outer membrane at the junction of the cell membrane and the reticulum, with the single inner layer joining the reticulum. The third model, in which both layers of the reticulum make a hole in the outer membrane is often seen on diagrams, but we have been completely unable to locate it on electron micrographs.

Brachet (1961) may have drawn the outer cell membrane as a single line because he has not observed, or does not agree, that it appears double on electron micrographs, or because his diagram is supposed to represent a cell at relatively low magnification. The Brachet model is now much more popular in the textbooks than is the Robertson model.

How can nuclear rotation occur if the endoplasmic reticulum is moored both to the cell membrane and to the nuclear 'envelope', which is supposed to be crossed by pores (Paine and Feldherr, 1972; Franke and Scheer, 1974; Wischnitzer, 1974)? Under phase contrast microscopy of tissue cultures one can see individual thickenings in the nuclear membrane sticking into the nucleoplasm and cytoplasm rotate with it; these would have to be located on a separate membrane which would then be composed of three or four layers on electron microscopy. Such an appearance has also been difficult to find. Alternatively, the nuclear rotation could occur if the endoplasmic reticulum were not attached to the nuclear membrane.

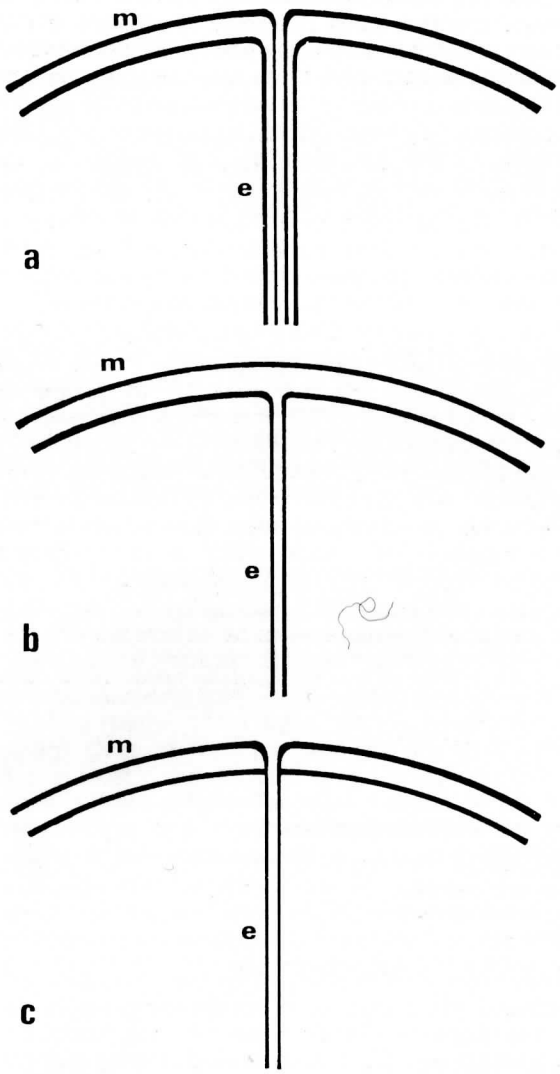


Fig. 14. Diagrams showing the possible modes of attachment of the endoplasmic reticulum (e) to the cell membrane (m); *a*, if both layers of the outer membrane connect to the reticulum, the latter would appear as 4 layers; *b*, if only the inner layer of the cell membrane is attached to the endoplasmic reticulum, its channel could not be continuous with the extracellular space; *c*, the channel in the endoplasmic reticulum represents a hole in the cell membrane.

Intracellular movements represent a fifth large and diverse portfolio of evidence that the endoplasmic reticulum is an artefact. Each of the following seven phenomena probably have different mechanisms but they are all movements of light microscopically visible structures. Some of them were first observed by different microscopists up to two centuries ago, and they have been reported both before and since the introduction of the use of the electron microscope for biology; they have received less attention since then. Although these movements cannot be seen by any histological or electron-microscopical technique which involves fixation, we are not aware that any histologist or electron microscopist doubts that they occur.

The exciting repertoire of movement is the normal criterion that a microscopist uses to diagnose life in plant cells, cancer tissue, protozoa or cultured cells. These intracellular movements have been observed by light microscopy at comparatively low magnification of 200 to 400 times. The endoplasmic reticulum and the 'cytoskeleton' are not usually seen with a magnification of less than 10 000 to 20 000. A three dimensional net connected at least with the nuclear membrane, and probably also with the cell membrane, is widely believed to permit particles of minimal diameters of one or two orders larger than the distance between the reticula to move vigorously (Table 2). The different kinds of intracellular movement are described by the following terms:

Table 2. Dimensions (Å) of the endoplasmic reticulum compared with the diameters of particles which are seen to be in motion in living cells. The diameter of the channel in the endoplasmic reticulum is taken from Callan and Tomlin (1950), Palade (1955) and Robertson (1960). The other dimensions are measured from figures in the literature, including Brachet and Mirsky (1961), Fawcett (1966), Toner & Carr (1971), Hurry (1972), Porter and Bonneville (1973), as well as figures in many issues of the *Journal of Microscopy*, the *Journal of Cell Biology*, the *Journal of Ultrastructure Research*, and *Experimental Cell Research*.

	Dimensions (Å)
Diameter of channel in endoplasmic reticulum	100 – 500
Thickness of reticulum	300 – 600
Distance between reticula	800 – 2000
Minimal mitochrondrial diameter	5000 – 20 000
Particles seen in Brownian motion	up to 5000
Nuclear diameter	50 000 – 200 000

(a) Streaming has been seen in plants since the 18th century (Corti, 1774). Brown demonstrated this phenomenon to Darwin before the voyage of the Beagle in 1831 (see Darwin, 1902). Schleiden (1847) illustrated his description of the cell theory with probably the first published drawing of streaming seen in a potato hair cell reproduced here in figure 5. Streaming of granules and chloroplasts has been observed in virtually all plant cells which have been examined (for review, please see Kamiya, 1962), and the endoplasmic reticulum is regarded as being present in all plant cells (Mercer, 1960 and references in Appendix 3). Nuclei, mitochondria and granules are seen moving continuously in protozoa and animal cells in culture (Lewis and Lewis, 1924; Canti, 1928; Costero and Pomerat, 1951; Jepps, 1956; Hansson and Sourander, 1964;

Jackson, 1966) but it is not always possible to differentiate between streaming and diffusion. Streaming in onion cell or amoebae is one of the first microscopical observations made by students of biology.

(b) Brownian movement occurs in all living cells and may continue after their death. Lewis and Lewis (1915) and Lewis (1923) observed particles in rapid oscillation in early tissue cultures. Indeed this property has been used for calculating the intracellular viscosity. Experimentally, it can be demonstrated by the introduction into cells of carbon particles, iron filings, organic particles and pinocytotic vesicles (Heilbrunn, 1956; Casley-Smith, 1963). In infected tissue cultures bacteria can sometimes be seen in staccato movement within cells. The rate of the movement depends upon the chemistry, charge and size of the particles, and the physico-chemical nature of the medium in which they move, especially its viscosity.

(c) Diffusion of small particles occurs, and it may be enhanced by convection currents caused by the heat of the illumination of the microscope. Bacteria were seen moving in leucocytes by Metchnikoff in 1883 (please see Metchnikoff, 1893, page 115). Particulate dyes injected into egg cells diffuse apparently freely throughout the cytoplasm (Chambers and Chambers, 1961). Mitochondria and granules of many kinds can be seen in continual motion in protozoa as well as in all animal tissue cultures (Lewis and Lewis, 1924; Canti, 1928; Costero and Pomerat, 1951; Jepps, 1956; Hansson and Sourander, 1964; Jackson, 1966; Chevremont, 1966; Chapman-Andresen, 1967).

'Axonal flow' is a term coined for movements of molecules and particles along the axon; it occurs in both directions. Enzymes, nucleotides, catecholamines and many other molecules and particles are seen to pass down the axon at a rate of 1.5 mm to 400 mm *per day*, which is considered to be rapid (Weiss and Hiscoe, 1948; Weiss, 1961; Lubinska, 1964; Sunderland, 1968; Bisby, 1976; Ochs, 1977). If the axoplasm is a liquid with low viscosity (Table 3) diffusion must occur. Streaming and Brownian movement are also evident in axons in tissue culture. Therefore if axonal flow is a different and 'specific' phenomenon one must demonstrate that the movement is significantly faster or slower than would result from these other well known mechanisms. Its rate would be determined by subtracting the rates of diffusion, streaming and Brownian movement, from the total rate of passage of the particles. Unfortunately, it is difficult to measure the rate of diffusion. The diameters of axons are so small, say between 2 microns and 20 microns, that it is very unlikely that flow would be Newtonian. The rate of diffusion would have to be measured at 37° to 38°. The axons are normally subject to the hydrostatic pressure from the surrounding tissues, which is increased during muscle contraction. During exercise the temperature rises in the surrounding muscle, and heat is generated by metabolism in the nerve. However, it is difficult to know how light-microscopically visible particles can avoid being trapped in the endoplasmic reticulum, filaments and fibrils which are commonly believed to course the axon (Fernandez-Moran, 1952; 1959; Schmitt, 1957; Shelanski and Fitt, 1972; Landon and Hall, 1976).

(d) Phagocytosis was first described by Haeckel (1862) and later by Metchnikoff in 1882 (see Metchnikoff, 1893). Embryonic clasmatocytes, fibro-

blasts, endothelial cells, leucocytes, epidermal cells, alveolar cells of lung, liver cells, kidney tubule cells, endodermal cells, pigment cells of retina, and smooth muscle cells, have been shown in tissue cultures and histological sections to ingest Indian ink, cellular debris, bacteria, iron filings, fat globules of diameters of 100 Å to 10 microns (for reviews, see Evans, 1915; Lewis and Lewis, 1924; Mudd, McCutcheon and Lucke, 1934; Cohn and Hirsch, 1960; Williams and Fudenberg, 1971). Many of the particles ingested during phagocytosis can be seen by relatively low power light microscopy.

(e) Pinocytosis is similar to phagocytosis, except that the substances ingested are often in solution. It is commonly seen in protozoa and many mammalian tissues in culture (Lewis, 1931; Holter, 1959; Chapman-Andresen, 1962; 1967). The cells sometimes extrude substances which they cannot ingest. Both phagocytosis and pinocytosis are often accompanied by the appearance of vacuoles; these have diameters between 4 microns and 56 microns; they are contractile and move continuously within cells (Kitching, 1938; Brandt, 1958; Casley-Smith, 1963; Chapman-Andresen, 1973).

(f) Nuclear rotation has been seen in tissue cultures. (Costero and Pomerat, 1951; Hansson and Sourander, 1964). Nuclear rotation occurs in either direction, and is most easily detected with time-lapse photography.

(g) The layering of subcellular organelles after centrifugation of single cells is rapidly reversed if the cells are not fixed (Lyon, 1907; Harris, 1935; Chapman-Andresen, 1967).

A hypothesis has been adumbrated to explain how these intracellular movements could be compatible with the existence of the endoplasmic reticulum in vivo. It is suggested that membranes may exist as a 'fluid mosaic' (Singer and Nicholson, 1972) – a proposition that has some similarities to the solgel hypothesis (see papers in Allen and Kamiya, 1964). This hypothesis has been extended to micro-tubules, and by implication to the endoplasmic reticulum, since both of these have been regarded as membranes (Allison, 1973). Let it be said at once that a hypothesis put forward to explain an apparent discrepancy between two incompatible findings cannot itself be used as *evidence* to make those findings compatible. Obviously, a hypothesis does not have anything like as much weight as findings, as it has different epistemological dimensions (please see Appendix 2).

If one believes in a more 'dynamic' view, presumably one must suppose either that the endoplasmic reticulum stretches considerably or that it dissolves in the line of the streaming chloroplasts, the granules in Brownian movement, the moving mitochondria, etc. Alternatively, the reticulum could be in a completely fluid state. How could one fluid apparently form sheets within another? If it were more dense than the cytoplasm, it would sink to the bottom: if it were less dense, it would float; if it were of the same density as the cytoplasm and insoluble in it, it would be in suspension. Furthermore, it is difficult to imagine how a fluid reticulum could be attached to a solid nuclear or external cell membrane. Although we are discussing the fluidity of the endoplasmic reticulum, the same reasoning would apply even more forcibly to the cell membrane. If any multicellular animal really consisted of cells with

fluid membranes, the whole animal would act as a fluid. The term fluid is sometimes used for materials which act mechanically like solids, but are physico-chemically fluids, e.g. glass is a solid without a crystalline structure. If this is what is really meant, one returns to the problem of how intracellular particules could move freely through such a solid mechanical structure.

If one were to believe that the reticulum is 'fluid' in vivo but solidifies during preparation, this is tantamount to admitting that its structure as seen in the electron micrograph is an artefact.

Tubulin is a protein extract believed to come from 'microtubules' of porcine brain (Weisenberg, Borisy and Taylor, 1968; Weisenberg, 1972). In the presence of adenosine triphosphate, magnesium ions and a strong chelating agent, it can be repolymerised. The resultant 'microtubules' can be seen on electron microscopy (Perry, 1976; Proc. Nat. Acad. Sci., 1977). Its solubility is very sensitive to the calcium ion concentration. This system has been suggested as a model for the solution and reappearance of the reticulum as a streaming granule arrives at the reticulum. This is a plausible hypothetical mechanism, but it has never been demonstrated directly to occur in living cells. Another experimental system which has been considered as a model is the group of amoebae whose cytoplasm becomes more viscous when they are subject to 457 kg per cm^2 pressure (6500 p.s.i.) (Marsland, 1964). This pressure is well in excess of any environment which animals even, those which inhabit the deep sea, are liable to have to live in.

A chemical or physical agent which disolves the reticulum could not be free in the cytoplasm, as it would prevent the reticulum being formed initially. Therefore, such an agent would have to be located in the moving particles and would have to be either secreted by them, or result from the interaction with them of a secretion with the cytoplasm.

This suggestion would require one of two situations: (i) that each particle had within it one agent to dissolve the reticulum and another to reform it; the particle would probably then have to have two compartments to keep the agents apart. While it might be possible that, say, a mitochondrion, could harbour two such agents, it would be grossly unlikely that a particle of carbon black or an iron filling would happen to contain them; (ii) the particle could contain one agent to dissolve the reticulum, and the cytoplasm could contain another to reform it. Again we are beset with the problem that carbon and iron particles probably do not contain chelating agents, or proteolytic or lipolytic enzymes. Every time a particle changed direction, as during Brownian movement, it would have to rotate through 180° so that its 'dissolving' secretion would be facing the direction of movement. It would also have to determine previously somehow in which direction it was going, and have enzymic mechanisms to synthesise the secretion. If, as most authors believe, the endoplasmic reticulum is a lipoprotein membrane, the cell membrane also would be at risk of being dissolved every time a particle came near it. Another difficulty resides in the reasonable expectation that at any instant of time a particle which dissolved material in front of it, and induced or permitted its precipitation behind it, would be expected to have a narrower gap between it and the reticulum fore than it would aft; one should expect to see a space

following every particle in the line of streaming, since streaming normally occurs in one direction near a particular cell wall. One would also expect that within one cell, all particles to one side would have their crowding fore and their space aft pointing in the same direction. None of these features are seen.

Unfortunately, the latter theoretical implications of the 'sol-gel' hypothesis, or of the co-existence with intracellular movements of neuro-filaments, neurofibrils, microtubules, or Golgi apparatus which permeate the whole cell, do not seem to have been discussed previously. One reason for this is that other authors do not seem to have considered the relative dimensions of the particles visible by light microscopy, and the 'weave' of the reticulum (Table 2). Some authors have assumed that the reticulum can stretch, but it will be immediately appreciated that it would be virtually impossible that the whole three-dimensional reticulum attached to the nuclear and cell membranes could stretch during, say, streaming, which is continuously in the same direction. It would have to form a series of concentric flattened membranes on cross section. When this point has been put to advocates of the endoplasmic reticulum, they have replied by saying that it is not now generally believed that the reticulum is attached to the cell membrane and the nuclear membrane (but please see Appendix 3).

If the reticulum were not attached to the latter two membranes, it could not act as the channel for 'exporting' the proteins believed to be synthesised, on the ribosomes. Furthermore, intracellular movement of relatively large particles would be possible, but the large particles would be trapped by the reticulum and their movements restricted by it. The inevitable consequences of this would be that all particles in a region would stream at approximately the same rate, irrespective of their size; also the random motions of Brownian movement could not occur. Particles introduced into the cytoplasm could not diffuse evenly in all directions as they are seen to do (Chambers and Chambers, 1961).

Another suggestion which has been made to justify the co-existence of these intracellular structures with intracellular movement is that the reticulum does not occupy a large part of the volume of the cytoplasm. This idea is not tenable, if the reticulum is connected to the cell membrane and the nuclear membrane, since it would represent a blockage of intracellular movement in part of the cell which would be rapidly followed by arrest of movement behind the piece of reticulum and throughout the cell. Furthermore, injections of dyes, or particulate material, into the living cell would be expected to show up regions of the cell to which they did not have access. We are not aware of reports of such findings.

A sixth reason which throws into doubt the existence of the endoplasmic reticulum concerns the viscosity of the cytoplasm (Table 3). This has been measured by a variety of techniques such as, introducing oil bubbles or iron filings, observing nuclei, centrifugation, and spin labelling. All authors (see, for example, those cited in Table 3), agree that the viscosity of cytoplasm is low, that it has a high negative temperature coefficient, and that it changes rapidly with damage to the tissue. It is obvious that low viscosity is incompatible with the existence of three-dimensional nets or sheets throughout the cytoplasm.

Table 3. The viscosity of the cytoplasm at 20 – 23°C of various cells compared with that of glycerol.

Tissue	Viscosity centipoises	References
Escherichia coli	12 – 15	Keith & Snipes (1974)
Amoeba	6	Pekarek (1933)
Echinus oocyte	10	Harris (1935)
Arbacia eggs	2 – 4	Heilbrunn (1956, p.22 – 24)
Lobster nerve	5.5	Rieser (1949a)
Frog muscle fibre	14 – 29	Rieser (1949b)
Red cell of man	30	Ponder (1934)
Human embryonic lung	120	Keith & Snipes (1974)
Glycerol	87	International Critical Tables (1930)

The single section studied in the electron microscope is a small part of the tissue, so that it should be possible to construct a three-dimensional view of the reticulum by following particular strands in serial section. We cannot find any pictures in the literature of such serial sections, and K.A. Deutsch (personal communication), who cut serial sections, found that successive sections did not fit together. It could be argued that it would be difficult to produce them, since the sharpened knife is altered each time a section is made, so that the faces of two successive sections might be sheared to different extents. However, the demonstration of serial sections of the endoplasmic reticulum would certainly add weight to belief in its existence.

If the protagonists of the endoplasmic reticulum now take the view that it is probably not joined to the cell membrane, then it would be agreed that it cannot regulate the passage of materials through the cytoplasm from the extracellular fluid or the nucleus. The only regulation possible across it would occur between the cytoplasm and the 'cisternae'. Therefore the endoplasmic reticulum cannot be regarded as a membrane, according to our definition, because it does not regulate, since the cytoplasm on both sides is similar (please see Appendix 1). It would be no more a membrane than a kite is in the wind. The only regulation it could have would be between the cytoplasm and the channel in the reticulum. Also, if it is accepted that a single finite thickness would appear as two layers on electron microscopy, there would be no cisternae or channels between the apparent two lines seen as a strand of the reticulum.

(c) The endoplasmic reticulum and light microscopy

It has been claimed that the endoplasmic reticulum has been seen by light microscopy in living testicular and pancreas cells (Sjöstrand, 1953; Fawcett

and Ito, 1958; Ito, 1962), and in cultures of adenocarcinomas and melanomas (Rose and Pomerat, 1960). These authors saw a number of folds, whose general form was similar to the appearance of the reticulum on electron microscopy, and they concluded that the images seen by the two methods of microscopy arose from identical structures. They then explained the reason for its visibility by light microscopy by suggesting that the curvature of the reticulum – which implicitly must consist of folds according to this view – permits them to see by light microscopy lines only 300 Å thick. Sjöstrand (1953) used Hirsch's method (1932) in which an anaesthetised mouse was placed on a microscope stage, and a piece of pancreas stretched to give the optimum optical conditions available. One can say beyond reasonable danger of contradiction that the illuminating conditions in such an experimental system would not be good enough to give the maximum resolution of the light microscope. For example, it is virtually impossible in vivo to stretch the pancreas so that one has a layer of only one cell thick. Transmitted light would be grossly diffracted by other cells in its path.

In the observations of Ito (1962), the appearance of the endoplasmic reticulum by light microscopy became clearer the longer the time that had elapsed since the spermatids were isolated, although such a delay did not seem to have been reported previously for preparations showing the image seen by electron microscopy. Furthermore, in all electron micrographs the mitochondrial diameters appear much larger than the 100 Å of the two layers of the reticulum, yet in Ito's light micrographs (1962) no clear mitochondria could be seen. It seems much more likely that the folds that appeared in the cytoplasm were due to protein being denatured and depositing during the dying of the cells.

The generally accepted maximum resolution of the transmitted light microscope is 2000 – 2500 Å (see for example, Carpenter, 1901; Needham, 1958; Lawson, 1972). The resolution possible with the phase contrast microscope is significantly less, because the numerical aperture in operation is of the ratio 7:12, compared with transmitted light microscopy. Thus those who claim to see the endoplasmic reticulum by light microscopy are endowing the light instrument with a resolution of at least six times that theoretically possible for it under the most favourable conditions. It becomes pertinent to repeat the questions: if the endoplasmic reticulum appears to be of the same thickness as the nuclear and cell membranes on electron microscopy, and the reticulum is also within the resolution of the light microscope, why it is not usually seen by light microscopy in mamalian liver and brain cells in which it can be seen so clearly by the electron microscope? Why is it not seen in thin cultured monolayers where the illuminating conditions are very good? The current view that 'ribosomes' line the endoplasmic reticulum in some cells would make the double layer apparently thicker, say to 600 – 800 Å, but this is still much below the resolution of the light microscope.

(d) **The nature of the artefact seen as the endoplasmic reticulum**

We have summarised a great deal of experimental evidence indicating that the endoplasmic reticulum must be an artefact. Yet there is no doubt that such an apparent structure can be seen on electron micrographs and so an explanation must be proferred for the image seen. Such an explanation is a hypothesis and may prove wrong, but even if it were, it would not affect the conclusion that the reticulum could not exist in vivo.

Preliminary consideration suggested that the appearance of the endoplasmic reticulum might have arisen from the precipitation of the cytoplasm by fixatives. During the last two decades of the 19th century there was a powerful controversy over over whether the cytoplasm contained fibrils or not (Hughes, 1959). Fibrillar networks were produced in filtered solutions of gelatin, collodion, peptone, egg albumin, silica gel, vanadium pentoxide, or india rubber, by the use of such fixatives as osmic acid, formalin, potassium bicarbonate, sulphocyanate, corrosive sublimate and heat (for reviews, see Bütschli, 1894; Hardy 1899; Walker, 1928; Frey-Wyssling, 1953). The spacing of the fibrils depended upon the concentration of the solutes, the pressure applied and their chemical nature; these authors showed that the fibrils looked very similar indeed to the appearance in cytoplasm on light microscopy produced by the same fixatives applied to epithelial cells, pancreas cells, bone marrow, etc. This was clear evidence to them that such a histological appearance could be artefactual. However, since fixation is the first step in electron microscopy, it would produce the appearance of a real three-dimensional reticulum. The endoplasmic reticulum appears to be only two-dimensional (see above) and so it must be concluded that it arises after sectioning of the tissue, and represents a surface and genuinely two-dimensional deposit.

Until the recent development of electron microscopic techniques which have attempted to examine cells in an aqueous atmosphere, all techniques used in electron microscopy have involved dehydration. This gives us the vital clue as to the nature of the appearance of the reticulum. It is a precipitate of the cytoplasm which had been in aqueous suspension in vivo. This precipitaion may occur (i) during fixation by cold or chemical fixatives, (ii) on dehydration of alcohols, (iii) on evacuation of the microscope and (iv) on its exposure to the electron beam.

Investigation of the literature on the effects of cold suggested the likely nature of the reticulum. Luyet and his collaborators have made micrographs of many frozen solutions of KCl, NaCl, albumin, gelatin, amino acids, and many others (Rapatz, Menz and Luyet, 1966; Mazur, 1966). One can summarise their many years of careful experiments with the following generalisations. Freezing to less than about $-10°$ of inorganic or organic solutions or mixtures produces an appearance of a regular pattern of lines, dendrites, spherulites or crazing paving, when examined by electron microscopy. A given pattern is characteristic of a solution of particular composition and concentration but also depends upon the rate of cooling and the geometry of the specimen. Very often a characteristic pattern of crystals is seen with equal spacing of the two lines. Provided a standardised procedure is carried out, very particular and apparently organised patterns may be revealed.

Since the living tissue is normally more than 60% water, the shrinkage due to dehydration must be considerable, even if the water is subsequently replaced by alcohols, organic reagents, and embedding media. These simple considerations do not seem to have been given any attention by microscopists. It

would seem to us that the appearance of the endoplasmic reticulum is due to the intense heat of the electron beam dehydrating and precipitating the metal-tissue complex, and etching the surface of the embedding medium.

Undoubtedly a proportion of cytoplasmic solutes is lost into the various fixatives, stains, dehydrating and embedding agents used (for reviews, see Ross, 1953; Baker, 1958; Hopwood, 1969; Hillman and Deutsch, 1978), but it would be reasonable to ask those who do not accept our view of the endoplasmic reticulum where most of the cytoplasmic solutes go when the tissue is dehydrated.

The low pressures (10^{-5} torr) used in freezing techniques, say to $-100°C$ (Moor, 1969; Robards, 1974) are often well below the vapour pressure of ice at that temperature (Meryman, 1966, page 615), so that even ice would evaporate very rapidly. The low temperature could not be maintained due to bombardment by the electron beam, therefore the temperature of the specimen would rise and so would the vapour pressure of the ice.

The only important difference between traditional electron microscopy and the newer freezing techniques is that initial fixation in the latter is induced by rapid freezing, instead of chemical fixatives. Freezing cannot be considered as independent and totally different series of methods for examining tissue, for example, in demonstrating the endoplasmic reticulum. The exponents of freezing electron microscopy would seem bound to attempt to resolve the objections to the existence of the reticulum, rather than regarding their findings as producing entirely independent confirmatory evidence for its reality in vivo.

The tissue shrinks on deep freezing to $-100°$ or $-196°$ not only because it dehydrates, but also because ice has a finite coefficient of thermal expansion between, say $-20°$ and $-100°$ or $-196°$. Even if, as many electron microscopists who carry out freeze-fixation believe, they can induce a rapid cooling to these extremely low temperatures which would not produce ice in the tissue, the 'hypercooled' cytoplasm would also shrink on cooling, as it presumably also has a coefficient of cooling; liquids generally have higher coefficients of thermal expansion than solids.

Nevertheless, we would like to reiterate that we know many references which allege that ice is not formed in tissue during rapid freezing to these temperatures, but we cannot find *any* publications in which this has been demonstrated.

C. RIBOSOMES

This name if given to granules seen on electron microscopy between the layers of the endoplasmic reticulum, and also to a particular subcellular fraction containing 40 – 50% RNA (De Man and Noorduyn, 1969). It is generally believed that RNA and its chemical 'function' reside in the former locality in vivo. The particles cannot be seen by light microscopy, and in our opinion there is insufficient evidence of subcellular localisation of biochemical activities (Hillman, 1972, page 98).

If the endoplasmic reticulum is an artefact due to deposit of the cytoplasm, it is very likely that the ribosomes are also a deposit. They may consist of RNA and protein in the separated fraction, but may well not be the same material as is seen on electron micrographs. If RNA were lining a reticulum one might see this on ultra-violet microscopy as dark channels permeating the cytoplasm. Where the endoplasmic reticulum is believed to be restricted to a small region of the cell, that part should absorb much more ultra-violet light, due to the higher concentration of RNA. We are not aware of such appearances having been seen (Caspersson, 1950), nor have we seen it ourselves in neurons (Hillman, Hussain and Sartory, 1973).

D. THE MITOCHONDRIA

Mitochondria appear as worm-like structures in continuous movements in the living cell, but are more difficult to see in fixed tissue, because the fixatives often precipitate the cytoplasm. Information about mitochondria came initially from observations on their general shape with Janus green or from their staining properties (Altmann, 1890; Bourne, 1942). Later on, they have been seen in tissue cultures. The big development of interest in their biochemistry came with the development of subcellular fractionation and electron microscopy (Bourne and Tewari, 1964; Borst, 1969; Birnie, 1972; Azzone, 1972; Wainio, 1976).

Two major morphological findings have been claimed by electron microscopy: the double-layered membrane and the cristae. Both of these structures share the same difficulties as have been considered above – namely, that they always appear on transverse section. In respect of the cristae, one should expect to see not only the characteristic lines crossing the structure in the plane of the picture, but also all the other views which one would see on viewing a disc (figure 16). Allusion has already been made to the beautiful crystal patterns which may result from cooling homogeneous solutions, tissues, bacteria and cells of many kinds (Meryman, 1958; Persidsky and Luyet, 1960 a, b; Luyet, Tanner and Rapatz, 1962; Rapatz, Menz and Luyet, 1966). Extracted phospholipids also appear on electron microscopy as fine parallel arrays (Bangham and Horne, 1962; Luzzati and Husson, 1962, Stoeckenius, 1962).

We would then regard the structure of the living mitochondrion as that of an elongate body with a single membrane and a homogeneous 'mitochondrioplasm', without any inclusions. During fixation, dehydration and straining, the 'mitochondrioplasm' would deposit in lines.

E. THE GOLGI APPARATUS

(a) The appearance of the Golgi apparatus

There is some uncertainty whether the Golgi apparatus was first seen by La Vallette St. George (1867), Platner (1885), or Hermann (1891), but it is generally agreed that it was first stained by Golgi (1898) in barn owl brain cells by the use of a modification of Cajal's method with osmic acid and silver nitrate. Its appearance has since been described in fixed tissue as 'a fibrous reticulum,

network or ring or cylinder, a very regular fenestrated plate, a more or less incomplete hollow sphere, vesicle or cup, a collection of small spheres, rodlets and platelets or discs, a series of anastemosing canals, a group of vacuoles, and a differentiated region of homogeneous cytoplasm crossed by irregular interfaces' (Kirkman and Severinghaus, 1938, a,b,c). In section, it has also been described as like 'rings, semicircles or banana shaped structures' (Moussa and Banhawy, 1960). It has been given over one hundred names (Hirsch, 1939; Gatenby, 1955; Dalton and Felix, 1956). A few examples of its appearance by light microscopy in different cells are given (Table 4). The Golgi apparatus usually appears either as a network throughout the whole or part of the cytoplasm, or as a large lumpy particle adjacent to and about the same diameter as the nucleus, in animal and plant cells (Symposium, 1956; Cameron, 1968; Hirsch, 1968; Northcote, 1971; Whaley, 1975).

Table 4. A few examples of the shapes and diameters of the Golgi apparatus from different cells seen by light microscopy. The diameters were measured from the illustrations in the papers. Please note that the diameters of the whole Golgi apparatus varied from 7 – 40 microns.

Kind of cell	Appearance	Approximate size (μ)	References
Spinal neuron of dog	Reticulum throughout cytoplasm	40 – 50	Golgi (1898)
Salamander neuron	Partial reticulum throughout cytoplasm	30 – 40	Holmgren (1902)
Pancreas cell of cat	A skein of tissue adjacent to the nucleus but larger than it	8 – 10	Von Bergen (1904)
Acinar cell of guinea pig	A skein of tissue adjacent to the nucleus	7 – 8	Cowdry (1924)
Guinea pig uterine gland cells	Large particles adjacent to the nucleus	10 – 15	Beams and King (1934)
Chick liver cell	Aggregate of particles	7 – 8	Richardson (1934)
Rat kidney cells	Large particles throughout cytoplasm	15 – 20	Hirsch (1939)
Cat adrenal cortical cell	Solid paranuclear mass	6 – 8	Bennett (1940)
Snail spermatocyte	Large particle adjacent to nucleus	8 – 12	Beams (1943)

Most light micrographs have been of stained tissue, although it has been claimed that the Golgi apparatus has been by light microscopy in unfixed cells (Ludford, 1935; Gatenby, 1955; Beams, Tahmisian, Devine and Anderson, 1957). In fixed tissue any cytoplasmic network or body of about the same size as the nucleus was regarded as the Golgi apparatus. However, in view of the relative difficulty of seeing the body in unfixed cells, and the vast preponderance of observations on fixed cells, several authors have suggested that the apparatus was an artefact (Parat, 1928; Baker, 1950; 1955; Shafiq, 1955).

There was a long and fierce controversy on the existence in neurons of the Golgi apparatus in vivo, in which the two main protagonists were Gatenby of Dublin and Baker of Oxford. In 1963, Baker in an honourable – but we believe mistaken – admission, conceded that the apparatus was real. "The author accepts after long hesitation", he wrote, "the view that the Golgi 'apparatus' in the neurons of vertebrates corresponds with the organelle of the same name in other cells . . ." (Baker, 1963). His grounds for changing his mind were (a) that the Golgi apparatus was peri-nuclear, while the Nissl substance was peripheral; (b) that S.L. Paley had shown him an electron micrograph of a Purkinje cell of a rat which satisfied him that the lamellar vacuolar fields were the Golgi apparatus; and (c) that Novikoff and Goldfischer (1961) had shown that thiamine diphosphate was present in the Golgi apparatus.

We do not wish to rehearse the whole historical and histological joust, which reached its high point at a Symposium in 1955 whose proceedings were published in an issue of the *Journal of the Royal Microscopical Society* in 1956. It was an interesting and illuminating disputation, of a kind which the modern tendency to conformity will probably never permit to be re-enacted. However, once Baker had acknowledged the existence of the Golgi apparatus, "a long period of controversy has apparently drawn to an end, and at the same time, a new era of investigation involving new techniques is well under way in studies involving the fine structure, origin, chemistry and function, of this interesting cellular organelle", Beams and Kessel wrote (1968).

With the use of the electron microscope, the Golgi apparatus was again seen in virtually every animal cell in which it was examined (Beams, Van Breenan, Newfang and Evans, 1952; Sjöstrand and Hanzon, 1954; Afzelius, 1955; Burgos and Fawcett, 1955; Gatenby, 1955; Lacey and Rogers, 1956; Dalton and Felix, 1956; Cook, 1975 and many others). Its general shape was similar in the electron micrographs of all cells described. It appeared as a series of crescent-shaped or horse-shoe lamellae, with vacuoles, vesicles or lipid droplets in the region. It was usually thought of as being connected to the endoplasmic reticulum. The maximum diameters of the Golgi apparatus measured from several hundred electron micrographs in the literature were between 0.5 and 2 microns. The lamellae always appeared equal distances apart, and their walls were also equal distances apart, except in localised regions where small vacuoles could be see.

(b) Evidence that the Golgi apparatus is an artefact

The Golgi apparatus must be an artefact for the following reasons, which were not at issue during the controversy quoted above:

(i) if the Golgi apparatus is a net throughout the cytoplasm as originally described by Golgi, it would present the same impediment to the intracellular movements as are cilted in connection with the endoplasmic reticulum. Obviously, the smaller the extent of its ramification throughout the cytoplasm, the less these difficulties become relevant;
(ii) the Golgi apparatus under the electron microscope always appears like the side view of a hemisected onion, i.e. in only one orientation. One would expect to see it in several other orientations, such as a circle, concentric circles, and at tangents. We have been unable to detect any significant incidence of these other appearances in published electron micrographs;
(iii) on electron micrographs one would expect the spacing of lamellae to vary, if they were three dimensional (please see fig.11);
(iv) the demonstration of the Golgi apparatus by electron microscopy is generally regarded as very strong confirmation of the reality of the Golgi apparatus as seen by light microscopy. The following disturbing discrepancies between the two types of image appear:

(a) the *minimum* diameter seen with the light instrument was 7 – 8 microns (Table 4) while the *maximum* diameter on the electron micrographs was 2 microns. Thus the volume of the apparatus enclosed by the latter technique appeared to be about 2.5% of that enclosed by the former one. Even if one supposed that there was greater shrinkage during preparation for electron microscopy, it is highly doubtful if *any* object could shrink to one fortieth of its original volume. This is by no means the maximum discrepancy. Furthermore, it cannot be argued that one is examining only the mean diameters as this would apply to both light and electron micrographs, and sections would be cut through each of them randomly;

(b) in light microscopy the shapes of the bodies are seen either: as nets in part of, or throughout, the cytoplasm; or as large single roughly spherical particles; or as a chain of particles around the nucleus. In electron micrographs, they are seen as 'half-moon' lamellae. Clearly the histologist sees three different shaped objects, and the electron microscopist sees a fourth one;

(v) if it contained a substantial concentration of lipids, these would be extracted by the organic solvents used during dehydration and embedding;
(vi) If it is attached to the endoplasmic reticulum, and especially if it arises from it, it is likely to have the same refractive index. Therefore, the light microscopists like Fawcett and Ito (1958) and Rose and Pomerat (1960) who claim to see the reticulum by phase contrast microscopy in unfixed tissue should also see the Golgi body under the same circumstances.

A consideration of these discrepancies depends to some extent on whether one believes that the Golgi apparatus does occur in all cells, whether one accepts that it can be seen in unfixed cells, and whether or not one is of the opnion that the different appearances do indeed represent the same structure in the living cell.

In summary, we would point out that the light microscopically visible Golgi apparatus is much more polymorphous than the electron microscopically visible one, and is so different in volume that it could not be the identical structure.

(c) **The nature of the Golgi artefact**

There is a long history of the production of fibrils and droplets by compression between microscope slides of different mixtures of oils, gelatins, etc., by Carpenter, Hardy, Bütschli and, more recently, Walker and Allen (1927) and Walker (1928). Baker (1942, page 137) showed an early electron micrograph of a silver granule apparently homogeneous under the light microscope; it looked remarkably like the 'paranuclear' type of Golgi body. Walker and Allen (1927) and Walker (1928) made microscopic models resembling the Golgi apparatus with various concentrations of gelatin, albumin and lecithin. As with the production of patterns during cooling of tissue, one can observe that a very large variety of shapes may be produced by the manipulation of immiscible materials. It is extremely difficult – if not impossible – to precipitate out a mixture of materials of difficult solubility in such a way that the precipitate would appear to be uniform under high magnification.

The tissue has a finite volume, and fixative, stains, dehydrating agents, and heavy metal salts, will all diffuse from the extracellular fluid towards the nucleus. This will itself create considerable inhomogeneities within the cell of those cytoplasmic substances which cannot diffuse out. The cytoplasmic solutes will gravitate to a position next to the nucleus. Yet, if one looks back again at *living* protozoa or tissue cultures, it is remarkable how homogeneous the cytoplasm appears, except for a few crystals and mitochondria. It seems that until the use of the electron microscope any mass seen under the light microscope in the cytoplasm of unfixed or fixed cells, which was not the nucleus or mitochondria, was given the name Golgi apparatus. (Nowadays such masses are called lysosomes). Nevertheless, the reticular appearance to which Golgi gave the name was definitely not the same as the 'paranuclear' body of later Golgi observers, but both of these were probably identified as being the same structure, as there was no other name they could be given. We would suggest that the same may be true of the structure identified in the electron microscope, though its appearance is undoubtedly much more uniform than the light microscopic mass – even if it must be different.

The simplest hypothesis is that the Golgi body is a precipitate of metal or metal salts with the cytoplasm. This hypothesis could be tested by carrying out careful studies of the appearance under the electron microscope of mixtures of emulsions of these salts with albumin, gelatin, lecithin, and other naturally occurring protein, lipid, and carbohydrate mixtures. If one is to maintain that the Golgi apparatus exists in the living cell, it seems necessary to explore and exhaust all the appearances to which these simpler systems are heir. Then one could 'subtract' these from the appearance of cells under the electron microscope.

Although uneven metal precipitates may be responsible for the appearance of the Golgi apparatus as seen in fixed cells, we have no explanation for the reported appearance of the Golgi body in unfixed or living cells (Ludford, 1935; Gatenby, 1955; Beams et al., 1956; Hirsch, 1968). However, once again, we would like to emphasize that, in our view, the incompatibility of the findings of different observers cited in the previous section makes the Golgi apparatus as a single entity very unlikely. That unlikelihood would not be affected by

disproof or proof of the truth of our hypotheses about what causes the artefact to appear.

F. LYSOSOMES

This term was originally applied to a subcellular fraction, prepared in a particular way, which had very little enzyme activity until treated with sonication, fat solvents, detergents, extra homogenisation or freezing and thawing, all of which resulted in it exhibiting many acid hydrolase enzyme activities (de Duve, Pressman, Gianetto, Wattiaux and Appelmans, 1955; de Duve, 1963). The 'purity' of preparations is assessed by electron microscopy of the fractions (Stahn, Maier and Hannig, 1970; Baggliolini, Hirsch and de Duve, 1970; Daems, Wisse and Brederoo, 1972), and by measurements of their enzyme activities (Tappel, 1969; Barrett, 1972). A reading of some of the enormous volume of literature on these particles (see, for example, de Reuck and Cameron, 1963; Dingle and Fell, 1969, vols. 1 – 3; Wattiaux, 1969; Dingle, 1972; Dingle & Dean, 1973; 1976) permits one to make the following generalisations. Nearly every single particle which is not attributable on light or electron microscopic examination to another named subcellular organelle has been given the name lysosome. This includes 'natural' particles ingested by phagocytosis or pinocytosis, or unnatural ones which can be so ingested. All the enzymes which are regarded as being classically lysosomal also occur in other fractions under particular conditions. They are often called 'contaminants' when they are found in fractions in which the research worker does not believe them to be in vivo. The isolated 'undamaged' lysosomes have little enzyme activity, but the addition of powerful agents like sonication, detergents or fat solvents – agents often used deliberately by enzymologists to alter enzyme activity – 'damages' the lysosome and allows its enzymes to be 'liberated'.

This poses two questions. What does 'structure-linked' mean? How much of the increased enzyme activity is due to the addition of these powerful agents themselves? The apparent 'liberation' of enzyme may be merely that greater activity is measured in the presence of particular reagents.

An extraordinarily complicated vocabulary of metaphorical and metaphysical terms has been adumbrated with the 'lysosome concept'. It describes the geography of an 'intracellular digestive tract', which is the apparent pathway of phagocytosis and pinocytosis. The process of 'intracellular digestion' is described in the following neo-Grecian language (de Duve, 1963):

'endocytosis'
formation of a 'phagosome' (a kind of lysosome)
formation of a 'storage' granule (also a lysosome)
an 'autophagic' vacuole (another lysosome)
a 'residual body' (a fourth kind of lysosome)
'exocytosis' 'excretion' or 'defaecation'.

If one translates this into simpler descriptive terms, much of the impressiveness of the terminology disappears.

A bacterium is engulfed by a macrophage ('endocytosis'). When it enters it is relatively large ('phagosome'). It is broken down (in a 'digestive vacuole') into larger pieces (in an 'autophagic vacuole'), smaller pieces ('residual bodies'), or insoluble pieces ('storage granules'). The vacuole eventually discharges at the cell membrane ('exocytosis, excretion or defaecation').

To view the process with naive eyes – particles which enter cells are broken down; those that do not ultimately dissolve in the cytoplasm leave the cell.

It is worth reflecting that the later stages of phagocytosis and pinocytosis after the particles have been taken into vacuoles could occur by simple well known mechanisms. A vacuole containing a particle must change its osmotic pressure as the large insoluble particle is broken down to smaller pieces and subsequently to smaller molecules. The fact that vacuoles can be seen changing in volume indicates that their osmotic pressure is probably changing. Nevertheless, there will be a natural tendency for vacuoles to leave the cells, because, firstly, they are suspended droplets of different chemical composition than the cytoplasm; secondly, by random diffusion they would be likely to come into contact with the surface sooner or later; thirdly, when several adjacent vacuoles accumulate, they coalesce to form larger ones; fourthly, the chemical reactions involved in digestion would probably be exothermic, which would increase their kinetic energy; fifthly, the chemical reactions could well form products which would alter the surface tension of the vacuoles; sixthly, after all the 'digestible' material of the particles has been solubilised and absorbed, the insoluble residue will exert no osmotic pressure, so that the water might well diffuse into the cytoplasm leaving an insoluble particle of a different density, which would drop out; seventhly, many protozoa are motile, and if the residue is of a different density to their cytoplasm, it will tend to move at a different velocity; eighthly, many protozoa rotate when they move, which would tend to induce centrifugal forces, which would move particles towards the periphery.

On electron microscopy, the lysosomes – when they are seen – look about the same diameter as mitochondria, but they are rarely seen on light microscropy. Mitochondria are always seen. What is the explanation for this difference?

We would not accept that the evidence that the enzyme activities are located within the particles seen by the electron microscopist is sufficiently rigorous to be persuasive (Hillman 1972). Unfortunately, there is a large volume of complex and often uncontrolled experiments on the subject of lysosomes.

G. THE NUCLEAR MEMBRANE

(a) The appearance of the nuclear pores

Nuclei have been seen for over 150 years. More recently nuclear pores have been reported in the nuclear membranes of many animal and plant cells by electron microscopy, though not – as far as we are aware – by light microscopy. A few examples of some of the diameters of the pores which have been reported in various nuclear membranes, are given in Table 5. A pore ap-

pears on transverse section of the nuclear membrane as a discontinuity, and on tangential view as a circle or octagon. Originally, they were conceived as simple hiatuses in the two line appearance of the nuclear membrane but in recent years more and more detail has been described. Nowadays, they are part of the nuclear pore complexes. These consist of a 'collar' 600 Å long and 240 Å thick, with a pore now of about 500 Å . Some immature cells have pores covered by a thin diaphragm or 'fenestration'. These features have been indicated in many pictures and diagrams (please see Gall, 1967; Wischnitzer, 1974; Threadgold, 1976, pages 98 – 105 and the references given in Table 5). The pores are said to occupy 3% – 32% of the nuclear surface (for review, please see Feldherr, 1972).

Table 5. Nuclear pore diameters.

Type of cell	Pore diameter Å	Reference
Rat cerebellar cells	800 – 1000	Toner & Carr (1971, page 147)
Rat neurons	280 – 360	Palay & Palade (1955)
Rat ganglion cells	700	Hartmann (1953)
Mouse pancreatic acinar cells	200 – 400	Watson (1954)
Amphibian oocytes	500	Callan & Tomlin (1950)
Sea urchin oocytes	1000	Afzelius (1955)
Several types of cells	400 – 1000	Feldherr (1965, 1972)

(b) **Evidence that nuclear pores are artefacts**

Nuclear pores with or without 'diaphragms' must be artefacts for the following reasons:

(i) on transverse section of the nuclear membrane they appear as hiatuses in a line, and tangential to the nucleus they appear as a circle or octagon. They are rarely, if ever, seen as any of the intermediate shapes, which would be expected. They should preserve their maximum diameters, but become more slit-like towards the periphery of the nucleus (fig. 15);
(ii) if one draws a tangential section of the nuclear membrane and then a transverse section of a pore (figs. 16 and 17) one arrives at the following conclusions: the pore will not be seen on transverse section unless its diameter is greater than the section thickness *and* the section cuts both faces of it; the pore on transverse section will always appear to have the diameter of the hole in the smaller face of the pore cut by the section; the pore diameters will vary from zero to a maximum on transverse section of the nuclear membrane, and the pores will not always appear to be of the

same diameter in the same cell or tissue. In the earlier literature it was often claimed that the diameters of pores were measured and found to be less than the thickness of sections which were 500 – 1000 Å thick; often a single figure was given for the diameter of the pores, because it appeared to be constant;

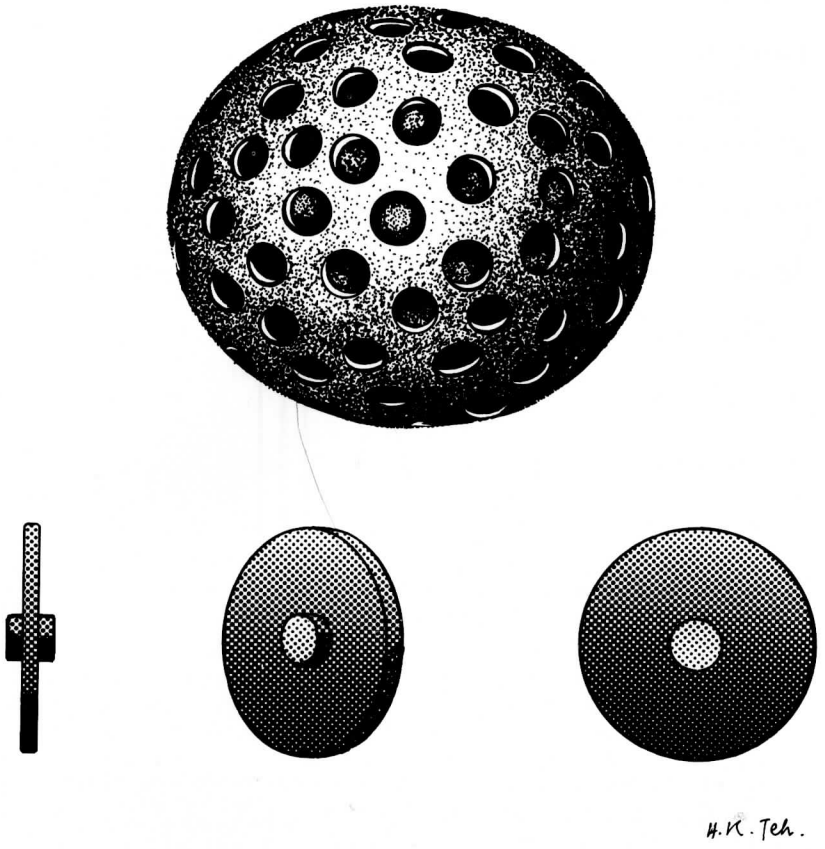


Fig. 15. Upper; a diagram of a surface view of the nucleus covered by 'pores'. It should be noted that pores away from the centre should appear as slits. Lower; a disc showing three orientations similar to the expected orientations of pores in the nuclear membrane. The question is raised why are the first and third orientations the only ones to be seen on electron micrographs?

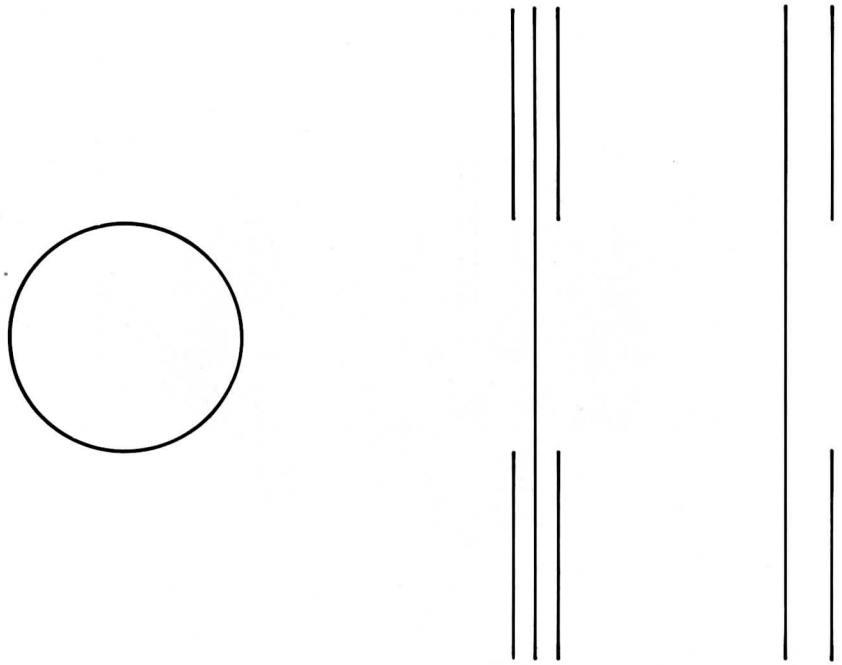


Fig. 16. Left diagram; plane view of a nuclear pore; middle diagram; appearance of a 'diaphragm' on transverse section which arises from between the two apparent layers of the nuclear membrane; right diagram, the 'diaphragm' arises from one of the layers of the nuclear membrane.

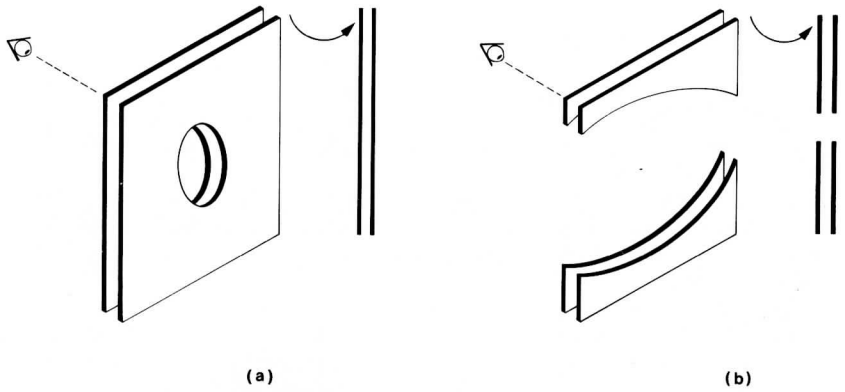


Fig. 17. A diagram of a pore in a nuclear membrane (a) in a tissue section which is thicker than the pore diameter; (b) in a tissue section in which the pore diameter is larger than the tissue thickness *and* the pore is in such a position that it cuts the anterior and posterior faces of the section. Note that in (a) the pore will not appear on transverse section of the membrane, and in (b) it will always appear to have a diameter smaller than the real one, when viewed on transverse section.

(iii) the distance between the apparent two layers of the pore complex tube, as of the two layers of the nuclear membrane, should not be constant as most sections would not be cut equatorially or tangentially through individual pores;

(iv) if pores occupied 3 – 32% of the nuclear membrane (Feldherr, 1972) an enormous amount of energy would be necessary to regulate the passage of water, ions and small molecules between the nucleus and the cytoplasm;

(v) one may ask those who accept the view that the endoplasmic reticulum is visible by light microscopy, why one cannot see the nuclear pores, which are claimed to have diameters up to three times the thickness of the reticulum, by light microscopy (cf. tables 2 and 4).

(vi) on electron micrographs (loc. cit.) one sees arrays of circles which the authors claim to be nuclear pores. However, examination of many cells reveals circles of the same diameter *in the cytoplasm.* Of course, these would not be considered to be nuclear pores. Indeed the criterion of a circle of relatively uniform diameter on the nucleus appears to be the only way of identifying pores;

(vii) if the nuclear pore complexes rivet the apparent two lines of the nuclear membrane together, and if the nuclear membrane is attached by the endoplasmic reticulum to the outer cell membrane, how could nuclear rotation occur?

(viii) if there were perforations of even only 3% of the nuclear membrane, it seems difficult to understand how the nucleus could maintain an approximately spherical shape after homogenisation. Maintenance of a spherical shape would require either a pressure gradient across the wall – which could not be maintained if it were punctured – or an internal structure maintaining its shape as a sphere. Furthermore, many authors have carried out electron microscopy of nuclear fractions, of which the nuclei, after having been subjected to high pressures, have apparently retained their spherical shapes after having been centrifuged;

(ix) with the possible exception of the frog oocyte, there is a potential difference across the nuclear membrane in all living cells examined (Lowenstein and Kanno, 1962; 1963 a, b; Naora et al., 1962). This potential difference has been attributed to the gradient of the Na^+ ions, and the other ions listed in Table 6 could also affect it. Pores occupying such a large percentage of the area of the nuclear membrane would cause short-circuiting of the potential difference across the membrane;

(x) pores vary in diameter from 280 Å to 1000 Å (Table 5). They apparently permit molecules or particles of 45 – 75 Å diameter to pass through them (Paine and Feldherr, 1972). How could pores of 280 Å in diameter prevent ions less than 18 Å in diameter passing through (Table 6)? It would be virtually impossible for regulatory processes on the edge of the pores to affect the passage of these ions, even if the hypothesis that the pores are charged were accepted. The nuclear membranes of single living EL 2 cells and frog oocytes have been demonstrated most elegantly to act as a diffusion barrier by direct measurement (Fry, 1973; Kohn, Siebert and Kohn, 1971; Dick and Fry, 1973). The nucleus contains more Na^+ than the cytoplasm (Allfrey, Meudt, Hopkins and Mirsky, 1961).

Table 6. *Ionic or molecular diameters. The ionic diameters are taken from Harris, (1960), and the molecules from Sobotka, (1944).*

	Diameters of hydrated species (Å)
K^+, Na^+, Cl^-, H^+, Ca^{++}, Mg^{++}	<9
Simple proteins	7 – 18
Globular proteins	40
Chlorophyll	59
Molecules of m. wt. <10,000	35

It has been suggested that large molecules could cross the nuclear membrane in pinocytotic vesicles, but, of course, these would carry within them a sample of cytoplasm containing the small ions as well. Such an explanation would also require evidence that pinocytosis had been seen occurring across the nuclear membrane;

(xi) the contrast and detail seen by electron microscopy of the nuclear membrane is extremely poor, and there are many areas of non-information (Appendix 1). We wish to put on record quite unequivocally our view that the diagrams seen in papers and textbooks of the 'nuclear pore complexes', 'the nuclear pore apparatus' and the 'nuclear envelope' represent considerable extrapolations beyond what may be clearly seen under the electron microscope, or in electron micrographs. Some working electron microscopists have admitted to us privately that the diagrams represent a mixture of observation, construct and supposition. We believe that the proportion of observation in these models is too little;

(xii) the small granules which appear clearly in the centre of the 'nuclear pore apparatus' on plan view of the nuclear membrane (Franke & Scheer, 1974) do not seem to occur so frequently on *transverse* sections of nuclear pores in the literature.

(c) **The nature of the artefact appearing as a nuclear pore**

In electron micrographs there is so much unidentifiable material that a variety of descriptive terms, such as 'bodies', 'somes', 'invaginations' and 'vesicles' have been coined in the hope that in the future their 'function' may be elucidated. Such is the hope with the nuclear pores.

The preparation necessary to isolate particles changes them chemically too much, and we have to fall back on terms describing the geometry of the heavy metal deposit. With these reservations in mind, it might still be useful to speculate upon the real nature of the artefact seen as nuclear pores, because this will suggest experiments capable of testing the theories of their nature.

The pores could be:

(i) cracks in the nuclear membrane occurring during fixation, dehydration, freezing, or subjection to the electron beam, due to uneven or sudden changes caused by alteration of state during these procedures;

(ii) cracks due to the fact that the membrane is chemically inhomogenous and the different materials of which it is composed have different water contents, thermal coefficients of expansion, and heat conductivities; these are related to (i);

(iii) shearing of the membrane due to differential shrinkage of the cytoplasm and nucleoplasm, as the latter are physicochemically different in the same respects as are mentioned in the previous paragraph;

(iv) the linear coefficient of expansion of acrylic, Araldite and other epoxy resins is much higher than that of heavy metals, so that strains in the embedding medium might crack the membrane;

(v) during section of embedded tissue any structure including the nuclear membrane might be cut slightly. Cutting artefacts are well known to histologists and the much higher magnification with electron instruments would reveal many more. Most of the organic solvents used in the preparation for electron microscopy extract lipids (Hopwood, 1969) so that one would expect the nuclear membrane – if it is mainly composed of lipids as is currently believed – to become more fragile;

(vi) it is possible that sometimes the pores are due to uneveness of the heavy metal deposit, but this is unlikely as the nuclear membrane otherwise appears clear and continuous, and also these discontinuities are not seen in electron microscopy of pure salt or amino acid preparations;

(vii) it could be a portion of the nuclear membrane flattened from the transverse section through which the knife has cut. This seems unlikely to occur in such a small localised region;

(vii) circles on or near the nuclear membrane are attributed to nuclear pores, but in the cytoplasm one can see many circles, of the same size, and both smaller and bigger. Electron microscopists do not call these pores. However, this orientation and the similarity of circles in so many parts of some electron micrographs suggests the possibility that the pores represent shrunken particles, which had organic material in their centres until the heat of the electron beam exploded them, and left a crater;

(ix) the appearance of pores could be due to failure of the heavy metals to gain access to the whole of the nuclear membrane. This could be due to:

(a) dust, bacteria or chemical impurities, depositing on the tissue during the preparation for electron microscopy. Feldherr (1965) saw, in amoebae, gold particles in the regions of nuclear pores, which he regarded as contributory evidence to the idea that these particles pass through the pores. An alternative explanation would be that the gold prevents the deposit of the heavy metal stain at that region;

(b) substances from the tissue precipitating near the nuclear membrane as a result of dehydration during the preparation;

(c) particles of material from the knife during section.

The symmetry of the pores through both lines representing the nuclear membrane could be explained as being due to the real nuclear membrane being only single layered in vivo, so that a single wall with a heavy metal deposit on both sides would produce a symmetrical hole.

H. THE NUCLEOPLASM

In uniform eukaryotic cells viewed by light microscopy, the nucleoplasm appears fairly homogeneous, although a certain amount of particulate material is seen moving between the nucleolus, the nucleoplasm and the cytoplasm (Costero and Pomerat, 1951; Hansson and Sourander, 1964; Sartory, Fasham and Hillman, 1971). The nucleolus of neurons absorbs ultraviolet light uniformly (Caspersson, 1950; Hillman, Hussain, and Sartory, 1973). In the living cell, the nucleus in tissue cultures examined by time-lapse photography is apparently rotating, sometimes in one direction, sometimes in the opposite direction. Its position in the living culture is not fixed relative to the cell membrane, although it normally remains fairly central.

The nucleolus can also be seen moving rather slowly. Nevertheless, one should bear in mind that the energy imparted to the cells by the degree of illumination necessary for high power microscopy of single cells, or monolayers, might cause the intracellular movements observed in living cells. Obviously, since one cannot see the structures without illumination, this question cannot be answered directly.

I. THE NUCLEOLUS

The nucleolus was probably first described by Fontana in 1781; he saw 'une tache' within the nucleus or epithelial cells from the slime of an eel (Hughes, 1959, page 33). By the middle of the 19th century it was easily seen and had been drawn in many cells examined by the microscope (figures 6 – 8).

In 1951, Estable and Sotelo observed thread-like structures in the nucleolus, and they gave them the name 'nucleolonema'; the rest of the nucleolus was given the name 'pars amorpha' (fig. 18). In unfixed mammalian neurons viewed by phase contrast microscopy, the nucleolonema is seen to have a changeable shape, including rosettes and lozenges, which dissolve and reprecipitate within the nucleolus (Sartory, Fasham and Hillman, 1971).

More recently, a nucleolar membrane has been detected by the use of transmitted light, vertical illumination, phase contrast, anopteral phase contrast, interference, and dark ground illumination microscopy; it has been seen in all the medullary neurons, ventral horn cells, dorsal ganglion, and sympathetic ganglion cells of rat, rabbit, guinea-pig and frog (Hussain, Hillman and Sartory, 1974). It is not known whether it is present in cells other than neurons.

There has been a certain amount of resistance to the belief in the existence of a nucleolar membrane, even among anatomists, who have looked at preparations and admit to seeing an 'interface' between the nucleolonema and the nucleoplasm. The gounds for their doubts have been:

(a) it has not been demonstrated by electron microscopy;

(b) it has not been shown to be composed of proteins and lipids;

(c) it has not been seen before.

At present, the definition and contrast of the nucleolus examined by the electron microscope is extremely poor. The nucleolonema appears as one or several amorphous masses (Toner and Carr, 1971, page 145; Busch and Smetana, 1970, pages 18 – 21; Threadgold, 1976, pages 105 – 107), in contrast to the fine threads seen with the light microscope (Montgomery, 1898; Hertl, 1957; Hussain, Hillman and Sartory, 1974; fig. 18). Since examination of unfixed cells by the latter technique involves many fewer steps in preparation, it is more likely to yield truer conclusions (appendix 1).

If one does not accept findings from light microscopy, unless they have also been demonstrated by electron microscopy, one must reject *all* information derived by light microscopy for the century between the 1840's, when the achromatic microscope was introduced into use, until the electron microscope was first used in biology in the 1940's. One must also reject many properties which can only be seen occurring in living cells. These include such phenomena as transport, streaming, Brownian movement, phagocytosis, pinocytosis, diffusion, mitosis and immuno-fluorescence. While sometimes the existence of these phenomena may be deduced from examining electron micrographs of different cells before and after these processes, they can only be observed *while they are occurring* by use of the optical microscope. Therefore, the inescapable implication of a refusal to admit any phenomena which have not been 'confirmed' by the electron microscope is that one should not believe in the existence in the living animal of any of the above phenomena. Such an attitude would diminish understanding of biology very much, and would undoubtedly make one disbelieve much classical experimental cytology, cytopathology and immunology.

Some doubts have been expressed because no one has yet sought to elucidate whether the nucleolar interface is composed of proteins and lipids; this implies that all membranes are composed of these two substances. Much of the evidence of the protein-lipid view comes either from interpretation of low angle diffraction studies which are compatible with such a model but in our opinion do not prove that it is the state in the living animal; or it has been derived from the use of subcellular fractionation which necessarily implies many unwarrantable and wrong assumptions (Hillman, 1972, pages 33 – 34). Nor do we accept it as a necessary hypothesis that membranes have to be of the above chemistry (for definition of a 'membrane' see Appendix 1). Usually electron micrographs alleging to show the cell membrane have been prepared with the use of fixatives, stains and dehydrating agents, which may extract much of the protein or lipid present (McGee-Russell and De Bruijn, 1968; Hopwood, 1969).

We believe that the reason for which the nucleolar membrane had not been seen before in neurons was that when cells were separated in 250 mM sucrose the contrast of the nucleolus was poorer than when they were separated in saline(Hussain, Hillman and Sartory, 1974). This suggested that sucrose changes the refractive index of the 'pars amorpha'. In saline, the 'pars amorpha' appeared more translucent, and the nucleolar membrane could be seen as distinct from the nucleolonema and the 'pars amorpha'. A similar appearance can be seen in the nucleoli of Hertl (1957).

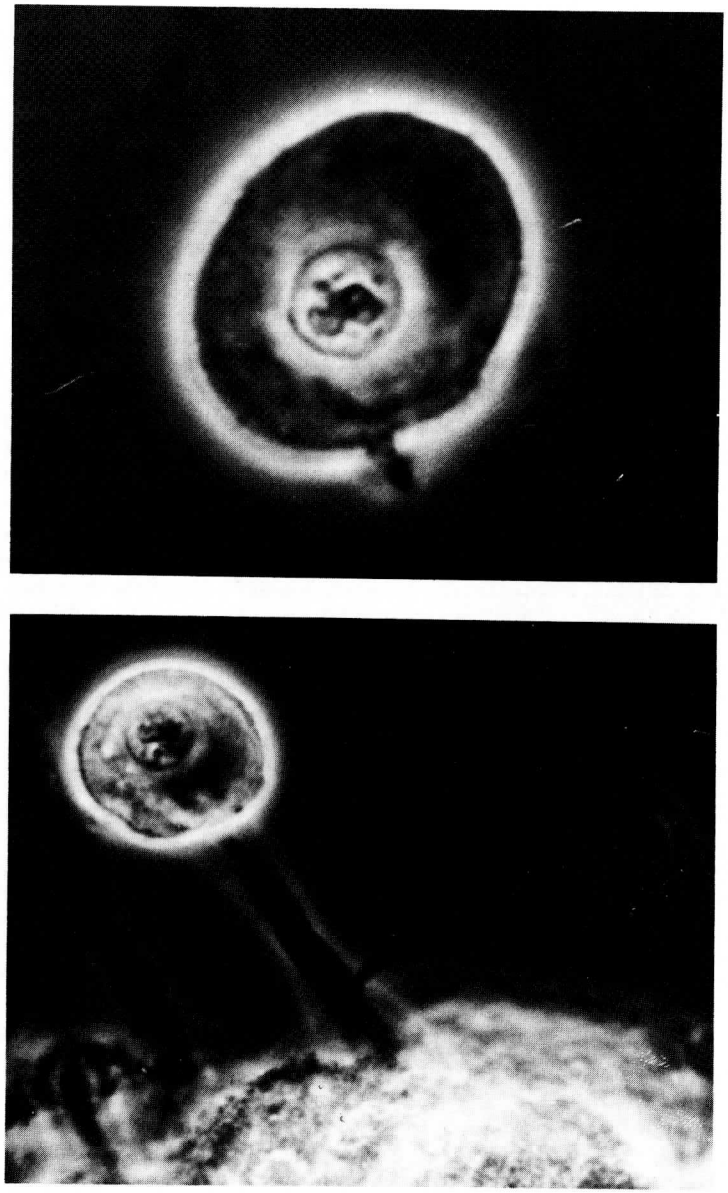


Fig. 18. Nuclei isolated in saline by hand dissection from rabbit medullary neurons; they are not fixed. The nuclei are 15 – 20 microns in diameter. Note the nucleolar membranes and the nucleolonema.

Professor A. Shahar, who has observed many tissue cultures, has pointed out that the definition of nucleoli improves and a ring appears around them, when a culture is dying; this appearance is similar to that of a nucleolar membrane (personal communication). If such a membrane did not exist round the nucleoli of 'healthy' cells, it would be unlikely to arise so clearly, regularly and spherically, while the cell was dying. As the membrane can be clearly seen by light microscopy at a magnification of 200 times, it is fairly thick.

It should also be noted that, in living cells, the nucleolus, like the nucleus and the whole cell, does not have a constant shape; it is changing continuously. This property of the nucleolus has not been widely reported in the literature.

At present, it is not known whether a nucleolar membrane exists in cells other than neurons, although ring-shaped nucleoli have been seen in some normal red cell precursors, spermatocytes, oocytes and egg cells (Austin and Braden, 1953; Brinkley, 1969) and in Hela cells from animals treated by starvation with antibiotics (Journey and Goldstein, 1961; Wessing, 1965; Potmesil and Smetana, 1968). Nevetheless, it would be desirable to observe the frequency of appearance of a nucleolar membrane in other cells subjected to minimal handling.

Nucleolus-associated chromatin can be seen in many cells (Hyden and Hamberger, 1945; Hertl, 1957). It is possible that during fixing and staining the apparent chromatin is deposited on the nucleolar membrane. Certainly, it is sometimes difficult to distinguish on which side of a thin surface a relatively thick deposit is located.

It is not easy to differentiate between a membrane and an interface in a biological system. The two criteria normally used for the former – the appearance of a single or double line on light or electron microscopy, and the existence of a potential difference between the phases – apply equally to an interface. The most critical criterion is the ability to dissect away the membrane and lift it away, as has been done with giant axons, oocytes and isolated mammalian neurons (Cummins and Hyden, 1962). Obviously, this could not be done with an oil-water interface, for example. However, in practice, the tissue which one can dissect thus is almost certainly thicker than the few hundred Angstroms that the electron microscopists see as the 'unit' membrane, not only because it can be picked up, but also because the resolution of the light microscope would not permit us to see a layer as thin as the one they identify as the cell membrane.

Interference measurements of fully hydrated tissue would probably show a membrane as being much thicker than an interface.

Chapter 5

THE REAL STRUCTURE OF THE LIVING CELL; IMPLICATIONS OF PRESENT CONSIDERATIONS

Having employed Occam's Razor unapologetically, one can now look again at the living cell. Our conclusion is that its structure is very similar to that generally accepted in the early 1940's (figure 3). There is a surrounding membrane apparently one layer thick. It probably contains proteins and lipids, which may be orientated in layers (Danielli and Davson, 1936; Finean, 1959) or may be more random (Bennett, 1969; Toner and Carr, 1971, page 6; Van Bruggen, 1971). Despite much controversy, it is difficult to interpret unequivocally experiments confirming either model. The extracellular space is very small. The shapes of the nuclei and nucleoli are changing continuously, but often slightly, in living cells.

The cytoplasm is a fairly translucent suspension of low refractive index, containing mitochondria, granules, droplets and sometimes vacuoles, all in constant motion. There are relatively few cytoplasmic inclusions in cells which do not secrete, or act as phagocytes, or live on particulate material. There is no endoplasmic reticulum or Golgi apparatus. The mitochondria are elongated tubes; they have a thin single-layered uniform membrane, and probably contain a homogeneous liquid – the 'mitochondrioplasm'; there are no cristae. The granules and droplets in the cytoplasm may consist of, or contain, crystals, enzymes, ingested particles, insoluble macromolecules, or bacteria. In plant cells, protozoa, cells in tissue culture, and cells of the reticuloendothelial system; the particles may occur in vacuoles.

The nuclear membrane is one layer thick and imperforate. The nucleoplasm appears fairly homogeneous and surrounds the nucleolus. The nucleolus in neurons is surrounded by a membrane but it is not known whether or not such a membrane is present in other normal cells. The nucleolonema is also in continuous slow motion and also parts of it are slowly redissolving and reprecipitating. The apparently clear space between the strands of the nucleolonema is called the 'pars amorpha'.

The present suggested structure differs from that seen by Altmann (1890) by the presence of the nucleolonema, and possibly the nucleolar membrane (figure 18).

The appearance of artefacts

There should be extensive studies of the appearances and artefacts found in photon and electron microscopy by as many completely independent techniques employing different physical principles as possible.

How each step may produce artefacts

Any new techniques should be analysed to see in what way each step might produce artefacts. The aim of such analysis should be to assess quantitatively, as well as qualitatively, the effect that each manipulation of the animal, each

step of the experimental procedure, and each chemical reagent, would have on the final appearance of the tissue. The empirical histology originating in the nineteenth century still serves an important pathological purpose, but does not necessarily inform us about the state of the cells within the living animal (Baker, 1958; Hillman and Deutsch, 1978).

Hierarchy of evidence

If our suggested hierarchy of evidence (Appendix 2) is not acceptable, every research worker should design a personal hierarchy in order to analyse his or her own experiments, as well as published papers. This would create a logical framework for the objective assessment of experimental evidence. In designing new experimental schedules, experiments yielding evidence nearer the top of the hierarchy should be adopted in preference to those lower down (Hillman, 1976).

The vertical approach to biology

In experiments in biology, biochemistry and especially in pharmacology, we would recommend the following attitude, which we call the 'vertical approach to the examination of mechanisms'. One may construct a pyramid consisting of the following blocks from the base going upwards:

fixed tissue
dialysed tissue (Hillman, Stollery and Joanny, 1974; Hillman, 1975)
crude tissue homogenate
tissue slices
tissue cultures
isolated functional organs
the same organs in vivo
the whole animal

For example, if one is studying the mechanism of the sodium ion pump, we should first see how the sodium ion associates with dialysed tissue from which all the small solutes have been removed. Then one replaces identified small molecules, one by one, in the experimental mixtures, and sees if and how each of them affects the association of the sodium ion with tissue. Next, the mixture is warmed to body temperature, substrates and co-factors are added, and the effect of each of them on the reaction in question is observed. The next step is to test the crude tissue homogenate.

Any mechanism found in the dialysis residue or the crude homogenate must be a property of the stable, insoluble chemical materials of which the tissue is composed. One then proceeds to examine it in tissue slices. Additional properties of tissue slices not present in the homogenate may be concluded to be related to the integrity of at least part of the tissue. A tissue culture which is growing has properties obviously nearer the state in vivo than those possessed by surviving tissue slices. Its activities can be directly observed by optical microscopy during experiments. The optimum isolated organ has an arteriovenous difference similar to that found in vivo, and can synthesise similar products; for example, an isolated udder produces milk, an isolated heart contracts and does work. Of course, isolated organs do not have the normal neural or hormonal influences, so the difference between findings from them

and findings in vivo would indicate how these functions are affected by the latter influences. Though it would be unfair to expect any postgraduate student to climb the whole of this pyramid himself, directors of institutes or research groups could examine structures, mechanisms and drug actions, systematically in this way. Obviously, it would be their task to ensure that any apparent anomalies found between the different levels of the hierarchy should be analysed exhaustively and resolved. Also, while a biochemical or drug mechanism could not be simpler in a system higher in the pyramid that would be found lower down, its effect would have to be 'subtracted' from the findings in the more complex systems to define the nervous and hormonal influences in vivo. The paradox is that one is usually examining the 'function' of living animals or tissues at one end of the hierarchy and attempting to correlate this with the structure which is at the other. By analogy with dimensional analysis of physics and chemistry, the different preparations may be considered to be dimensionally different. This paradox makes the relation of 'structure' to 'function' particularly difficult.

Unacceptability of incompatible evidence

The current compacency of accepting that mutually incompatible findings in cell biology and electron microscopy, for example, derived by using different techniques, can co-exist or be ignored, should be abandoned. Vague disparaging or imprecise remarks about the techniques used by other workers, or refusal to consider their findings, should not be acceptable as part of normal scientific intercourse. Nor should one accept incompatibilities between findings at different levels of the experimental hierarchy.

Light microscopy and living cells

A number of important findings have been made by electron microscopy about the structure of cells. Two such are the examination of muscle which led to the sliding filament hypothesis of muscle contraction (Huxley, 1957; 1972; Moore, Huxley and de Rosier, 1970) and also the elegant pictures of the surface of diatoms (Hendey, 1959; Moss and Gibbs, 1974). In the former study, geometry was respected, in that the shape of the filaments was demonstrated in three dimensions; in the latter study, the diatoms are composed of extremely stable silica, and the electron microscopists were examining its surface. Nevertheless, until the electron microscope will have been shown to be productive of information about *living* cells, we would strongly advocate a revival of interest in light microscopy.

The corollary of this is that there should be more teaching of light microscopy in schools and colleges. In our view, there is no better way of studying biological function in relation to structure than by sitting patiently and curiously for several hours at a time observing the undisturbed behaviour of living organisims.

Correlation of structure and function

Structure and function should be examined in the same preparation whenever possible. If not possible, one should keep to a minimum the number of interventions with the tissue between the application of the two different techniques.

Suggested new techniques

In a study of living systems, one can concentrate on the use of non-destructive, non-invasive techniques; one can also use minimally disturbing ones, such as gastric pouches, brain windows; in-dwelling cannulae and ear chambers; the latter group sometimes requires operation on animals, which are permitted to recover completely before further examination. We would like to suggest two new series of techniques, which we have not yet tried ourselves, but which we can propose as two examples of simple approaches. One is the development of chromatographic techniques in the living animals, which would not require powerful and unphysiological extracting agents. Minute columns could be placed in the mesentery, the subdural space or in the blood vessels, and would indicate the chromatographic and therefore chemical activities of the substances in vivo, and in a physiological chemical environment.

The second system consists of a re-examination of histological techniques and biochemical findings using only reagent systems, which would not be expected to change tissue irreversibly. Strong reagents denature proteins (Joly, 1965), and therefore their use should be avoided if possible. Such 'physiological' reagents as isotonic NaCl, Krebs-Ringer solution, serum, cerebrospinal fluid, etc. should be preferred. Ideally, they should be the kind of reagents that the research biologist would be prepared to put into his own conjunctiva in the belief that they would neither damage nor discomfort him. If this seems slightly hazardous, one could test reagents on mammalian tissue cultures. These are balanced on the knife edge between culture and decay, and any slightly unbiological addition pushes them towards decay. Perhaps the point here does not require labouring; we wish to examine the biology of living tissues in media which do not change them biochemically. This is hardly more radical than *incubating* tissues in solutions in which they can metabolise and grow, and studying their physiological properties.

It might be argued that the new techniques suggested above could be useful to aid the understanding of the biochemistry of the tissue, but not necessarily its fine structure. Of course, the conformation of the molecules at a biochemical and physical level determines the microscopic and macroscopic appearance of the tissue. Muscle contraction, wool processing, cryopreservation, and fruit ripening, are all examples of techniques in which changes in the chemistry are reflected in different macroscopic appearances. We believe that many biologists have assumed that one can induce gross biochemical change with powerful reagents, but leave the overall appearance or fine structure in its status quo ante. It may seem banal to have to insist that there must be a close and precise relationship in cells between the molecular orientation and their histological architecture, but it seems to us to be a satisfactory interpretaion of the relationship of structure to function.

Does subcellular localisation matter?

It may well be argued that the desire to localise biochemical activities in subcellular organelles is a laudable aim in itself in that it may help one to correlate the shape of an organelle with its biochemical activity. Yet although the physiological actions of the lens, the stomach and most other organs, are apparently clearly related to their shape, there seems to be no reason why an enzyme activity should be related to the particular shape of a cell or one of the organelles. Supposing that one were not persuaded that one could find out by current techniques whether or not oxidative phosphorylation does indeed occur in mitochondria in vivo; supposing that in the whole living cell it occurs in the cytoplasm. This would not alter our view of cellular biochemistry, since all the reagents believed to be involved in oxidative phosphorylation could be present in the cytoplasm. Since the mitochondria are separated from other cellular constituents for study by homogenisation and centrifugation – both involving considerable pressure – which would affect their permeability, one can say little about their permeability in vivo. The simplest proposition would be that mitochondria in vivo do enclose large molecules, which may not be able to cross their membranes. However, since bacteria do not appear to have nuclei or mitochondria, but can carry out most metabolic activities, it is clear that compartmentation as detected by light or electron microscopy – as opposed to such a phenomenon at a molecular level – is not necessary, since all their multiplicity of quite different biochemical activities must be located within the same single compartments in these microbes. In metazoa, everyone would agree that there are several thousand enzyme activities, yet no one claims more than about eight electron microscopically visible compartments, compared with our five – the nucleolus, the nucleus, the cytoplasm, the mitochondria and the extracellular space; each of these can be seen in unfixed tissues histologically and histochemically. The provision of a few further compartments, like the cisternae in the reticulum, or the lysosomes, does not contribute much to solving the housing problem of the overcrowded enzymes, substrates or reactions. Thus compartmentation can not be used as an argument *necessitating* the existence of double layered membranes, endoplasmic reticulum, lysosomes, peroxisomes, nuclear pore apparatuses, etc.

Homogenisation and compartmentation

It might be useful to add a note about the philosophy of homogenisation as applied to a biochemical cytology. If one were to view the matter dispassionately, it would seem to be a rather illogical manoeuvre to attempt to discover the localisation of activities within what are presumed to be separate compartments, by deliberately destroying the walls between them and compressing the contents of all the compartments, and then trying to find out afterwards what was the previous distribution of substances within them. Distances within cells are so small and diffusion must occur, so that it is not necessary to adduce further mechanisms, limiting membranes, or intracellular transport systems, to explain how the products of a chemical reaction in one part of a bacterial cell can move to another. A fortiori, if such small organisms at the at the bottom of the evolutionary tree can perform such magic, why cannot larger or multicellular ones do it also? We stand in risk of trading verbal certainty for intellectual insecurity. The former makes our textbooks look tidier and our teaching easier, but does not stimulate us to do the more searching or accurate experiments engendered by intellectual insecurity.

The changes in distribution of the organelles take place; firstly, at the time of homogenisation; secondly, after the additon of the homogenate to gradients in a centrifuge tube; thirdly, during centrifugation; and fourthly, by diffusion

throughout the preparation. Unfortunately, no amount of circumlocution, coining of new terms, or refusal by research workers to admit these simple and unchallengeable technical facts, can prevent diffusion and thus defy the second law of thermodynamics. The ways by which one could prevent relocation of enzymes would be, either to examine the organelles in vivo, or to separate them before they could mix, by such techniques as the following:

(a) split-beam spectrophotometry of preparations of metabolising tissue;
(b) the isolation of parts of cells by hand dissection;
(c) the observation of living hand dissected cells by optical microscopy;
(d) the transplantation of nuclei;
(e) destructive radiation focussed on particular organelles;
(f) intracytoplasmic and intranuclear injections;
(g) direct withdrawal of cytoplasm, nucleoplasm or axoplasm with micropipettes;
(h) the comparison of the properties of cells lacking a particular organelle like mitochondria, with cells from animals of similar species possessing them;
(i) the use of radioactive precursors with autoradiography;
(j) intracytoplasmic and intranuclear recording of ion activities;
(k) the use of the naturally 'large' cells, like egg cells, medullary neurons, Mauthner cells, squid axons, myxicola, arbacia eggs, etc.

The few examples illustrate the wealth of techniques of the kind already in use, which one may expect would cause minimal redistribution of intracellular organelles or their contents.

The teaching of semantics

The teaching of semantics is not normally part of scientific courses. So many concepts, like rôle, structure, function, mechanism, transport, membrane, lack agreed universal meaning and therefore one should define them when using them.

The situation is more serious when an unclear definition obscures a real inconsistency within a concept (Appendix 1 and Hillman, 1972, pages 115 – 120). It is useful to attempt to define such vague quasiphilosophical terms whenever one comes across them. These expressions are not quite meaningless, but they only have a meaning in relation to the precise measurements which they are being used to correlate. In general, we would urge a much greater attention to the *measurements* made by an experimenter than the vague generic terms used by the interpreters to make accounts of them coherent.

Advisability of the teaching of logic

Logic used to be an important introductory subject to many branches of the natural and physical sciences, but it has been largely dropped from syllabuses, at least in Great Britain. We are continuously disturbed at the inability of many professional workers to distinguish between findings, assumptions, speculations, etc. The importance of logic cannot be exaggerated because the fundamental value of an experiment in advancing knowledge is dependent on the degree to which every major assumption implied in its use is

valid, especially the *weakest* assumption. The findings of any experiment which contain even one important unwarrantable, illogical, or disproved assumption, should be ignored if it has a crucial rôle in the conclusion.

New kind of discussion

In order to improve the quality of experiments, a new kind of discussion group should be initiated, with the following rules. All participants should speak in a way that is comprehensible to any other physical, chemical or biological scientist. The speaker should be prepared to answer and discuss fully any question, however simple or naive it may appear to be. The discussion should be completely democratic. Subjects should be discussed to the limit of knowledge or opinion. Problems arising from lack of knowledge or differences of opinion should be investigated by members and reported on to a subsequent meeting. Such subjects which receive little treatment in biological books should be discussed, for example, the theory and assumptions of measurements, experimental evidence for well-established concepts, and the experiments of the individual research worker.

The current view

We would request cytologists to look down their electron microscopes or examine clear micrographs of any cells in which a substantial proportion of the field consists of double-layered membranes, endoplasmic reticulum and nuclear pores. Eschewing any temptation to be guided other than by their own senses and reasoning, they should ask themselves anew the simple question – could these images really be representations of genuinely three dimensional objects?

POSTCRIPT

It is reasonable to ask why cytology has been so unproductive of new and significant findings in view of the very large number of research workers involved in it all over the world. Not that total production has been small, rather that overall productivity has been poor. This is not an academic question in view of the vast resources with which society furnishes research workers, and the likely diminution of these resources in future.

We believe that the reason for the poor productivity has been that biologists have lacked the chemical and analytical approach to their experiments. They have plunged into complicated and difficult experiments, in which modern equipment has obscured the complexity, often because they cannot see their preparations for long periods of the experiment. They have been satisfied with photographs, traces, print-outs, and other surrogate and alienated information, which psychologically becomes confused with the living tissue itself. In the case of electron microscopy, the research worker may instruct the expert technician to embed, cut, stain and photograph a preparation, and then hand him the photographs. He has failed to make the direct contact with the source of his information, unlike the natural historian who watches his specimen while it is performing. It is our contention that the automatic nature of the machinery, added to the compexity of the experiments carried out, has led research workers to ignore the effects of their preparative techniques, as well as the assumptions necessarily but often unknowingly implied by their use. His inability to see the tissue directly has permitted the research worker to ignore the gross transformations from its state in vivo due to the physical manipulations and chemical reagents to which it is being subjected. It is interesting to reflect on how so many of the important discoveries of cytology have been the result of *direct observations*, where the words are used in their literal sense.

There is insufficient interest in the effects of preparation on the supposed results of experiment, and often attempts to kindle it are resisted strongly. One reviewer of the *Biochemical Journal* wrote that to suggest that a physical technique could have biochemical effects was revolutionary. Nevertheless, an experimental finding can never express a greater truth than the experimental technique used to arrive at it, the relevant test used to control it, or the validity of the assumptions implied in its use.

The approach suggested here is not nihilist. It is designed to improve the general standard by encouraging people to do simpler and more fundamental experiments. One must be very patient. We would suggest that either the 'vertical approach' to experiment, or the execution of empirical applied experiments, would be most advantageous. The efficacy of the latter is tested by their usefulness in application. The empirical complex experiments, in which category the majority carried out today must be placed, are the most dangerous. We enter then a plea for simplicity of assumption, technique, and experiment.

In consonance with the above attitude, we would stress the importance of intellectual honesty. It seems to us that the very greatest priority should be given to training research workers in exercising their critical faculties, irrespective of the popularity of their views or the 'diplomatic' damage which may result to them. Intellectual diplomacy should be a heresy in scientific circles and publications. No subject can be advanced by anyone who is not prepared to make the intellectual leap beyond the bounds of currently accepted belief. This is not to imply that disbelief is a virtue in itself, but the questioning undoubtedly is. Questioning should be pursued until either a rationally satisfying answer has been found, or a way has been thought out how to define the question so that it may be examined experimentally. We would like to reiterate that no professional scientist should allow to co-exist in his discipline or his spirit incompatible views in an area which is central to his interest. This may involve abandonment of well-accepted current beliefs, or it may necessitate frank and loud statements to encourage other workers to attempt to resolve discrepancies. Under any circumstance, we all have a duty to the public which believes in our honesty, and usually pays for our work.

Those of us who are engaged in research work which has any bearing at all on treatment of patients – even in the very long run – must frequently remind ourselves of the winding queues outside our consulting rooms waiting very patiently for the results of our endeavours.

* * * * *

If one accepts that the endoplasmic reticulum, the Golgi apparatus, the cristae of the mitochondria and the nuclear pores, do not exist in living cells, one is driven to the conclusion that all reseach workers studying these artefacts in health or disease – in healthy human beings, patients, animals, or plants – would be more profitably engaged on research in other areas, to which their present massive resources should be diverted.

We forecast that our views will be widely accepted by the time two more generations of research workers have matured.

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APPENDIX 1

A. Definitions and Comments

1. *An artefact is a statistically significant structural or quantitative change in a tissue occurring between its state in the living animal and its state during examination.*
An artefact is undesirable by its nature, but can be used, (i) if its relationship to the original state can be defined, and (ii) if it does not change its relationship to the original material or the measuring system during the experiment.
2. *A dead tissue is one which has lost irreversibly its ability to exhibit enzyme activity and respire.*
By definition, information derived from it is of less value than that derived from living tissue.
3. *Dehydration is the removal of water from a tissue by chemical agents, heat, cold, or low pressure.*
It always results in shrinkage of tissue, unless or until the water is replaced by another substance, which the tissue absorbs to the same extent. If often results in denaturation of the protein.
4. *Fixation is the attempt to arrest physical and biochemical change within a tissue after its removal from an animal for histology.*
It is intended that the structure of the tissue will not be altered grossly from its state in vivo, or after it has been excised; in histochemistry, it is hoped to maintain the biochemical activities of enzyme in a state close to that in vivo.
5. *The functions of a tissue are the series of biochemical and physiological properties which it has in vivo and which survive during its state in vitro.*
It should not imply a teleological meaning, or purpose for which that issue exists.
6. *An image is what can be seen or photographed of the original tissue or that changed by preparation before viewing it.*
The more the tissue has been prepared, the less its image represents its appearance or state in vivo.
7. *An interface is the orientation of molecules between two phases, or it is a physical structure between two phases at least one of which it encloses.*
It becomes apparent when the two phases have different optical properties or there is a membrane between them. All membranes are interfaces.
8. *A living tissue can perform work against its environment, and it respires.*
Living separated tissue does not necessarily react completely similarly to its state in vivo.
9. *A biological membrane is a thin continuous mechanically solid structure between two phases, which are usually chemically different.*
10. *Non-information in microscopy is an image which cannot be identified with certainty as being the whole or part of a known structure.*
It must be considered as noise of the system, and no suppositions can be made as to what structure it might contain, if it were to be seen more clearly. It has no value as evidence.
11. *The resolution of a microscope is the minimal angle subtended on the retina by two points which can be seen as separate.*
On a picture it means the minimum distance between two points which can be distinguished.
12. *A specimen is the sample being examined.*
In histology it is normally a piece of tissue which has been impregnated with a stain or stains. In electron microscopy, it is the heavy metal deposit struck by electrons from the beam.
13. *A stain in light microscopy is a dye which is used to colour part of a tissue. In electron microscopy, it is a heavy metal deposit on the tissue.*
It is an artefact by nature.

14. *The structure of the tissue is the closest approximation to its architecture in the living animal which may be achieved.*
15. *A truthful conclusion in biology is the closest description of the state in the living animal, which we may approach, which respects the natural laws, and is consistent with the maximum volume of experimental findings from living animals.*

B. Loose or misleading use of terms

16. *Contamination* is an enzymic activity or a chemical substance found in association with a subcellular fraction, which one does not wish to be found there, so that the research worker often continues to subject it to further procedures until it is no longer found in that fraction. The use of the term implies that there are other independent ways of determining the location of the particular enzyme activity or chemical substance.
17. *Damage* to a cell means nothing unless it is defined by measurement of a particular property relative to the state of the tissue in vivo. It is of doubtful usefulness in relation to mitochondria, nuclei or 'lysosomes', when their preparation was initiated by homogenising the tissue and subjecting it to high pressure.
18. Any structure or reaction of a cell is *important* if the cell cannot remain in dynamic equilibrium in its absence or impairment. However, one must beware of the danger of using it to mean important to our understanding; this is obviously teleological.
19. *Role* means effect.
20. *Significance* in a statistical sense has a precise meaning, but in descriptive cytology or subcellular biochemistry there is a danger of it having a teleological implication.
21. *Storage* of a material in a particle means that it is present in that particle.
22. A *putative* transmitter is a substance which is believed to be a transmitter before enough evidence has been accumulated to prove that it is.

APPENDIX 2

Problems of interpretation of experiments

The electron microcopist, like the light microscopist, derives a great deal more information from looking into his instrument than can be seen by perusing photographs in books. However, one must avoid the temptation to interpret 'non-information' (see Appendex 1). Observations made from the fuzzy parts of the field cannot be cited as evidence for, or against, observations of the clearer parts of the image. The nuclear pore apparatus is a good example of a complex structure which has been deduced from a rather furry line and some sub-cellular biochemical observations. Clearly, however, the *hypothesis* that more optimal conditions might reveal other structures cannot be used as an argument for, or against, the *findings* which have already been made (Hillman, 1976). There are many biological intepreters who have been unable to distinguish the value as evidence between hypotheses and data. The "sol-gel" hypothesis of cytoplasm is an example where an explanation of a difficulty of interpretation has been regarded as experimental evidence bearing upon it; this is a circular argument.

We do not intend to review the semantics or logic of science or biology, but would request our readers in the interpretation of biological experiments to ponder on the following quite different instruments of interpretation: these are listed in decreasing order of validity. (Measurements are implied in the term observations).
(i) observations of an *organism in its natural environment*;
(ii) observations on an untreated organism in an *unnatural* environment;
(iii) observations made on *treated* unrestrained animals;

(iv) observations made on *restrained* or *treated* animals;
(v) observations of '*functional*' separated organs;
(vi) observations on *metabolising* organisms with partial 'functional' deficit;
(vii) observations on tissue from animals *treated* before the isolation of the tissue;
(viii) secondary *calculations* involving tested assumptions from observations (i) – (vii);
(ix) secondary *calculations* involving testable assumptions from (i) – (vii);
(x) *deductions* or *extrapolations* from (i) – (ix);
(xi) data derived from other experiments on the *same system* in the same tissue;
(xii) data from other biologically *similar* systems;
(xiii) evidence from *biologically analogous* systems;
(xiv) evidence in the same systems totally *compatible* with present findings;
(xv) *testable* hypotheses, assumptions or explanations upon which depend interpretations of present experiments;
(xvi) explanations *not incompatible* with results of present experiments;
(xvii) *untestable hypotheses*, assumptions or explanations, related to the present experiments;
(xviii) *speculations*.

While there may be some differences of opinion about to which category part of an experiment should be assigned, we believe that nearly all scientific statements should be classifiable into one or other of these categories. We would regard (xvi, xvii and xviii) as unacceptable as evidence. All below (ix) would be unacceptable if the testable hypotheses, assumptions or explanations form an important element in the experimental evidence or theory.

This hierarchy is a pragmatic one arrived at as a result of many discussions by the authors. It may not be universally agreed, but we hope that it will be a useful attempt to classify findings in respect of their validity as evidence. We would be pleased to receive comments and criticisms to improve it. Please also see Hillman (1976).

Appendix 3 appears on pages 102 and 103.

APPENDIX 3

List of references of recent editions of textbooks showing: (a) the 'unit' membrane, (b) the attachment of the endoplasmic reticulum to the nuclear and cell membranes

A. ANIMAL CELLS

Author and year	Title	Publisher and edition	(a) Unit membrane page	(b) Attachment to nuclear and cell membranes, page
Kurtz, S.M. (ed.) (1964)	Electron Microscopic Anatomy	Academic Press	8	16
Fawcett, D. (1966)	The Cell, its Organelles	Saunders	341	135
Rogers, H.J. & Perkins, H.R. (1968)	Cell Walls & Membrane	Spon	335	365
Davson, H. (1970)	Textbook of General Physiology	Churchill (4)	480	23
Loewy, A.G., & Siekevitz, P. (1970)	Cell Structure & Function	Holt, Rinehart & Winston	41	49
Toner, P.G. & Carr, K.E. (1971)	Cell Structure	Churchill-Livingstone (2)	5	28
Best, C.H. & Taylor, N. (1973)	Physiological Basis of Medicale Practice	Wilkins (9)	1 – 3	1 – 2
Howland, J.L. (1973)	Cell Physiology	Macmillan	258	26
Warwick, R. & Williams, P. (1973)	Gray's Anatomy	Longman (35)	4	4
Arey, L.B. (1974)	Human Histology	Saunders (4)	20	21
Bevelander, G. & Ramaley, J.A. (1974)	Essentials of Histology	Mosby (7)	4	3
Ham, A.W. (1974)	Histology	Lippincott (7)	102	No connection with outer membrane shown
Mountcastle, V.B., (1974)	Medical Physiology	Mosby (13)	6	Endoplasmic reticulum not discussed
Porter, K.R. & Bonneville, M.A. (1974)	Fine Structure of Tissues and Cells	Lea & Febiger (4)	13	not mentioned
Tedeschi, H. (1974)	Cell Physiology	Academic Press	462	57
De Robertis, E.D.P., Nowinski, W.W. & Saez, F.A. (1975)	Cell Biology	Saunders (6)	150 – 151	178
Rhodin, J.A.G. (1975)	Atlas of Histology	Oxford University Press	Throughout book	Not shown
Bloom, W. & Fawcett, D. (1976)	Textbook of Histology	Saunders (10)	32	33
Guyton, A.W. (1976)	Textbook of Medical Physiology	Saunders (5)	15	16
Leeson, T.A., & Leeson, C.R. (1976)	Histology	Saunders (3)	22	32
McElroy, W. & Swanson, C.P. (1976)	Modern Cell Biology	Prentice Hall (2)	42	38 Not connected to cell membrane
Novikoff, A.B. & Holzman, E. (1976)	Cells & Organelles	Holt, Rinehart (2)	43	xii, 88 – 93
Roberts, M.B.V., (1976)	Biology – A Functional Approach	Nelson (2)	22	57
Windle, W. (1976)	Textbook of Histology	McGraw Hill (5)	19 – 20	28
Ambrose, E.J., & Easty, D.M. (1977)	Cell Biology	Nelson (2)	173 – 175	178
Gardner, W.D. & Osborne, W.A. (1978)	Anatomy of Human Body	Saunders (3)	5	9
Hopkins, C.R. (1978)	Structure and Function of Cells	Saunders	59 (term not used)	143
Jacob, S.W. Francoine, C.A., & Lossow, W.J. (1978)	Structure and Function in Man	Saunders (4)	28 (term not used)	28
White, A. Handler, P., Smith, E., Hill, R.L. & Lehman, I.R. (1978)	Principles of Biochemistry	McGraw Hill (6)	301	303
Giese, A.C. (1979)	Cell Physiology	Saunders (5)	185	111
Robertson, J.D. (1979) in Lima de Faria, A. (ed.)	Handbook of Molecular Biology	North Holland	1407 – 1425	Not shown
B. PLANT CELLS				
Hill, J.B., Popp, H.W. & Grove, A.R. (1967)	Botany	McGraw Hill (4)	24	25
Weier, T.E., Stocking, C.R., & Barbour, M.G. (1975)	Botany, Introduction to Plant Biology	Wiley (4)	43	45
Meier, B.S., Anderson, D.B., Bohning, R.H., & Frantianne, D.G. (1973)	Introduction to Plant Physiology	Van Nostrand (2)	22	30
Denferr, B., Bell, P.R. & Coombe, D. (1976)	Strasburger's Textbook of Botany	Longman (30)	22	17
Hufford, T.L. (1978)	Botany, Basic Concepts in Plant Biology	Harper & Row	38	43

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