Br. J. exp. Path. (1974) **55**, 221.

POST-MORTEM CHANGES AT ELECTRON MICROSCOPE LEVEL IN THE TYPE II CELLS OF THE LUNG

R. E. PATTLE, C. SCHOCK AND J. M. CREASEY

From the Chemical Defence Establishment, Porton Down, Salisbury, Wilts SP4 0JQ, England

Received for publication January 8, 1974

Summary.—In the type II cells of the lungs of mice, left in the cadaver after death, no post-mortem changes at electron microscope level have been found after 30 minutes. At 1 hour cytoplasmic vesicles have appeared and the microvilli have altered. Vesiculation and nuclear contrast then increase, and after 8 hours at 25° most of the cell is occupied by large vesicles and the cell boundary is disrupted. Meanwhile nuclei, mitochondria and lamellated osmiophilic bodies (LOPBs) remain intact. The timing of the changes is irregular but in these cells they are less rapid than the literature (on other cells) would suggest. Useful information about mitochondria and LOPBs can be obtained even after some hours' delay in fixation. The changes seem to be more rapid if the tissues are immersed in saline than if they are left in the cadaver.

INFORMATION about post-mortem changes in the type II cells of the lung was required for various reasons. Recent work (Gil and Reiss, 1973) has confirmed the long-standing supposition that these cells secrete lung surfactant in the form of lamellated osmiophilic bodies (LOPBs); these are of diameter 0·5–2·0 μm and their internal structure can only be examined with the electron microscope. The surfactant is deficient in the respiratory distress syndrome of the newborn human (Avery and Mead, 1959), which has not so far been reproduced in laboratory animals. An excess of surfactant appears sometimes to be produced as a reaction to inhaled silica (Heppleston and Young, 1972). The type II cells are therefore of particular interest in human material, in which there is often delay in fixation. It has been found that the LOPBs of man and of monkeys differ in structure from those of the non-simian species so far studied, and it was desired to know what information about further species differences could be obtained from non-laboratory animals whose autopsy had been delayed.

The literature on post-mortem changes at electron microscope level is sparse and inconclusive. Many workers (see Discussion) stress the necessity for extremely rapid fixation, while Ito (1962*a*) found little change in liver cells at 2 hours post mortem. The results of organ grafting procedures, experiments on nerve muscle preparations etc, suggest that some cells may remain viable for hours after removal, even though their ultrastructure may have changed during that period.

MATERIALS AND METHODS

Mice were killed with intraperitoneal pentobarbitone sodium (Nembutal). Without the chest being opened, the cadavers were transferred to an oven at 37° or to a cold room at 4°, or kept at room temperature (about 25°). Cat cadavers which had been the subject of other

experiments were also used. At intervals a cadaver was withdrawn and a small piece was cut from the angle between the diaphragmatic and pleural surfaces of the lung. Each specimen was obtained from a separate animal. It was fixed in 2% glutaraldehyde, in 1% osmium tetroxide and then in tricomplex fixative (10 vol M/40 $Pb(NO_3)_2$ with 10·5 vol M/60 K_3 $Fe(CN)_6$). It was embedded in Araldite using acetone as dehydrant and thinner (Schock *et al.*, 1973). It is supposed that the lead and ferricyanide ions form a "tricomplex" with the phosphate and choline groups of the LOPBs; the excess ferricyanide prevents blotchy precipitation. The lead probably contributes some heavy metal staining and makes section staining unnecessary. Alcohol and epoxy propane seriously erode the LOPBs, and acetone is therefore used in embedding. Sections 50–100 nm thick were cut for electron microscopy. From the same blocks sections 1 μm thick were cut for optical microscopy. These were stained with p-phenylene diamine by the method of Estable-Puig, Bauer and Blumberg (1965) and counterstained with methylene blue. In some cases adjacent sections, for light and electron microscopy, were taken without removing the block from the ultramicrotome. This enabled bodies seen on one to be identified on the other.

Some thin (1 mm) strips of lung were also kept for 30 min at 4° in various solutions. They were then fixed and treated as usual. We also examined other specimens in which some post-mortem change had been inevitable.

RESULTS

The results of our experiments are detailed in Table I and summarized in Table II. Figure 1 represents part of a normal type II cell stained by our methods.

TABLE I.—*Post-mortem Changes in Type II Cells of Lung in Mouse Cadavers Stored in Various Temperatures*

Storage temperatures	4°	4°	4°	4°	4°	4°	25°	25°	25°	25°	25°	25°	37°	37°	37°	37°	37°
Time interval	30 min	1 h	2 h	4 h	8 h	24 h	15 min	30 min	1 h	2 h	4 h	8 h	30 min	1 h	2 h	4 h	8 h
Microvilli																	
Thinned	−	−	+	±	−	−	−	−	+	−	−	−	−	−	−	−	−
Absent	−	−	−	−	−	+	−	−	−	+	+	+	−	+	+	+	+
Mitochondria																	
Swollen	−	−	+	+	+	++	+	+	+	−	+	−	−	+	−	−	+
LOPBs																	
Visible damage	−	−	−	+	+	+	−	−	−	−	+	+	−	−	−	+	+
Myelinated	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	+	−
Vacuoles with wisps	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
Vacuoles from EPR																	
Detectable	−	+	+	+	+	+	−	±	+	+	+	+	−	+	+	+	+
Filling cell	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	+	+
Nuclei																	
Memb. separate	−	−	−	+	+	+	−	−	−	−	+	+	−	+	+	−	+
Contrast up	−	+	+	+	+	+	−	−	−	+	±	+	−	+	+	+	+
Cells																	
Disrupted with bare organelles	−	−	−	−	−	±	−	−	−	−	−	+	−	−	−	+	+
Optical microscope																	
Visible vacuoles	−*	−*	−*	−*	−*	−*	†	†	†	†	†	+‡	−‡	−‡	−‡	+‡	+‡
Nuclei pyknotic	−*	−*	−*	−*	−*	−*	†	†	†	†	†	+‡	−‡	−‡	−‡	±‡	+‡
PM change rating	0	A	AB	B	B	B	0	0	A	AB	B	C	0	A	B	BC	C
Figure										2	3						

Changes are indicated by + signs, † = not examined, * Paraffin sections, H. & E. ‡ 1 μm Araldite sections.

TABLE II.—*Summary of Post-mortem Changes in Lungs*

Time	Stage of change at storage temperature 4°	25°	37°
	Mouse lung		
30 min	O	O	O
1 h	A	A	A
2 h	AB	AB	B
4 h	B	B	BC
8 h	B	C	C
24 h	B	†	†
	Cat lung		
2 h	†	C	†
16 h	AB	†	†

† Not examined.

We have found that in general post-mortem changes at electron microscope level are irregular, and that one cannot predict from time and temperature exactly what changes will occur. In large animals body heat will be retained longer than in small animals. The description of our results can therefore be taken only as a rough guide to what might happen. We have graded the changes O (normal), A, B, and C, with intermediate stages AB and BC.

Control pieces of tissue taken from mice in each batch immediately after death and processed along with the specimens whose fixation was delayed, were of uniform appearance (Fig.1). This was taken as evidence that the changes observed after delayed fixation were not due to faulty fixative or pre-existing disease.

Post-mortem changes when lung was left in the cadaver

The first sign of post-mortem change was a slight swelling of the vesicles of the endoplasmic reticulum. This could be detected after 1 hour at all temperatures. At the same time the microvilli sometimes became less regular in thickness (50–150 nm, as against 90–110 nm in fresh material). Mitochondria sometimes appeared swollen, but at 25° and 37° this was inconstant. At 4°, after 2 hours or more, the mitochondria were always swollen. At this stage the LOPBs were unaffected, the nuclear membranes showed little change and there was no obvious increase in intranuclear contrast. This we call stage A (Fig. 2, 4).

Later on the same processes continued. Microvilli could no longer be seen, except after storage at 4°. Vacuoles, apparently derived from swollen endoplasmic reticulum, were a prominent feature and sometimes took the form of long, wide channels. There were no further changes in the mitochondria. In one case a mitochondrion with an internal osmiophilic whorl (of a type common in animals which have been stressed) (Pattle *et al.*, 1972, 1974) could be recognized (Fig. 3). Homogeneous areas were sometimes found in the LOPBs, but 4 nm spacing could still be seen in the lamellae. Sometimes the LOPBs had swollen, leaving a core of osmiophilic material or a number of wisps, sometimes showing 4 nm spacing, inside a membrane bounded space. The gap between the nuclear membranes was prominent and the outer membrane bulged between the mitochondria, the LOPBs and the vacuoles; these last took up much of the space in the extranuclear part of the cell. The type II cell was sometimes detached from its basement membrane.

The contrast within the nucleus was more marked, giving it a " black and white " appearance in the electron micrograph. At this stage vacuoles in the cytoplasm could sometimes be seen in 1 μm sections with the light microscope; these were larger than the vacuoles (representing eroded LOPBs) which give the type II cell its alternative name of " vacuolated alveolar cell ". In paraffin sections (stained with haematoxylin and eosin) of specimens kept in the cadaver for 24 hours at 4°, no sign of post-mortem change could be found with the light microscope (D. Gall). In the EM the cytoplasm outside the vacuoles and organelles had a mottled appearance. This stage of change we call B (see Fig. 2, 3, 4).

In the next stage, the vacuoles had grown so that they were the most prominent feature of the type II cells. The boundary of the cell was often untraceable; the nuclei, LOPBs and mitochondria usually survived and often appeared bare and detached from any cytoplasm. The nuclei retained their " black and white " appearance or else were homogeneous and dark; under the light microscope the latter type appeared pyknotic. The nature of the pyknotic nuclei was not always obvious under the EM as they resembled red blood corpuscles, but in adjacent 1 μm sections they could be identified with the light microscope. In some cases the LOPBs showed a spacing greater than 4 nm and a tendency to myelinization. The membranes of the mitochondrial cristae were still visible. Vacuoles were prominent in the light microscope. This stage we call stage C.

In general, refrigeration delayed, but did not prevent, post-mortem change. At 4° the microvilli persisted much longer but swelling of the mitochondria was more prominent. The difference between mouse and cat was perhaps caused by the cat cadaver retaining its original body heat longer.

Practical examples

Having found that the LOPBs in a specimen of human lung obtained at lobectomy were concentric while those of mice, etc., usually have a cross-barred or arcuate pattern of lamellae, we wished to know whether this was due to pathological changes or to a species difference. A piece of human lung fixed 15 hours after death from trauma became available. This confirmed the species difference (Creasey, Pattle and Schock, 1974). A specimen of lung from a Bennett's wallaby (*Protemnodon rufogrisea*) became available; this had been fixed about 12 hours after death. It confirmed that in this species the LOPBs were mainly of the cross-barred or arcuate (non-simian) type (Fig. 4). Similar information was obtained from other specimens in which autopsy had not been immediate.

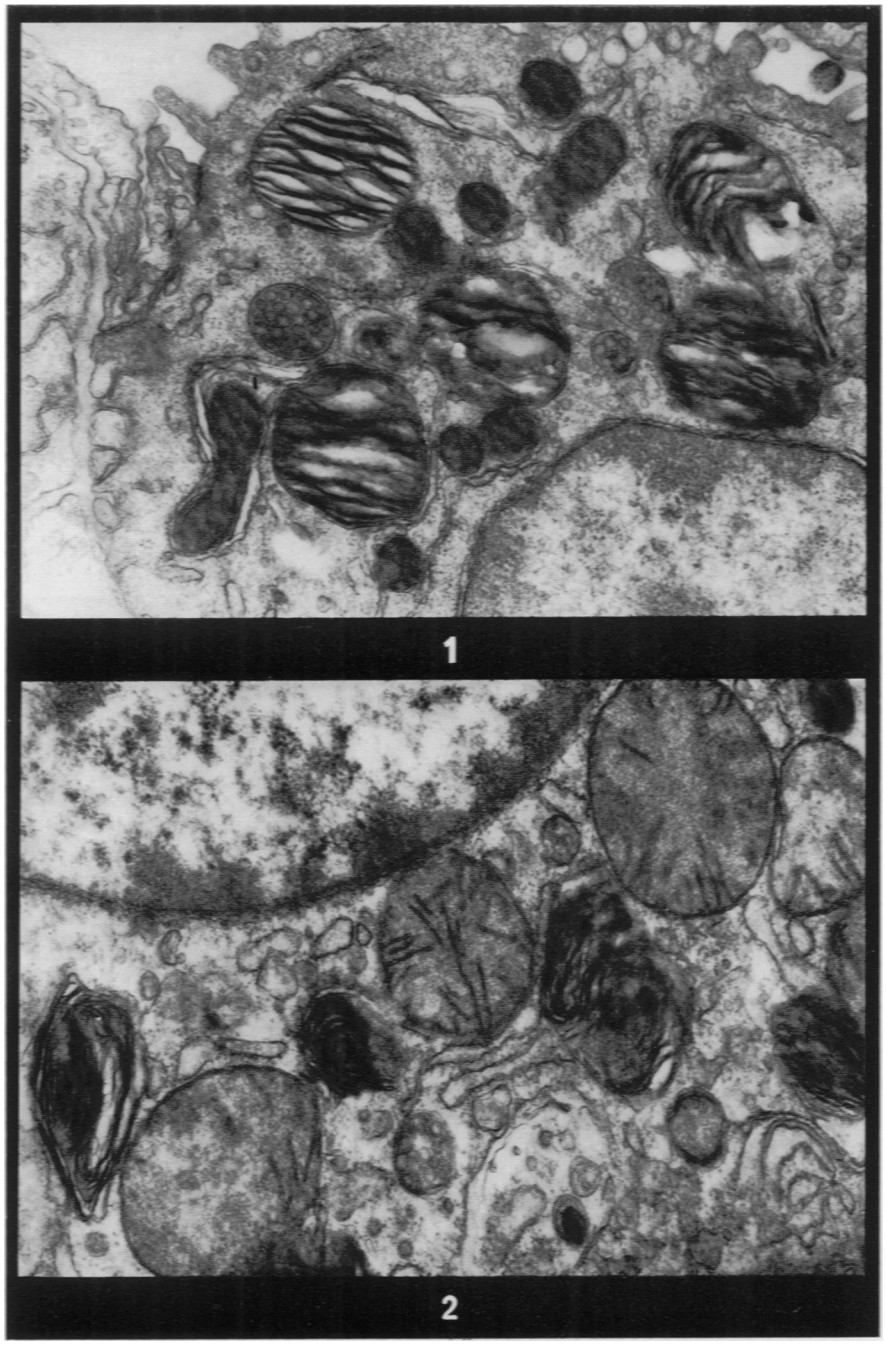
EXPLANATION OF PLATES

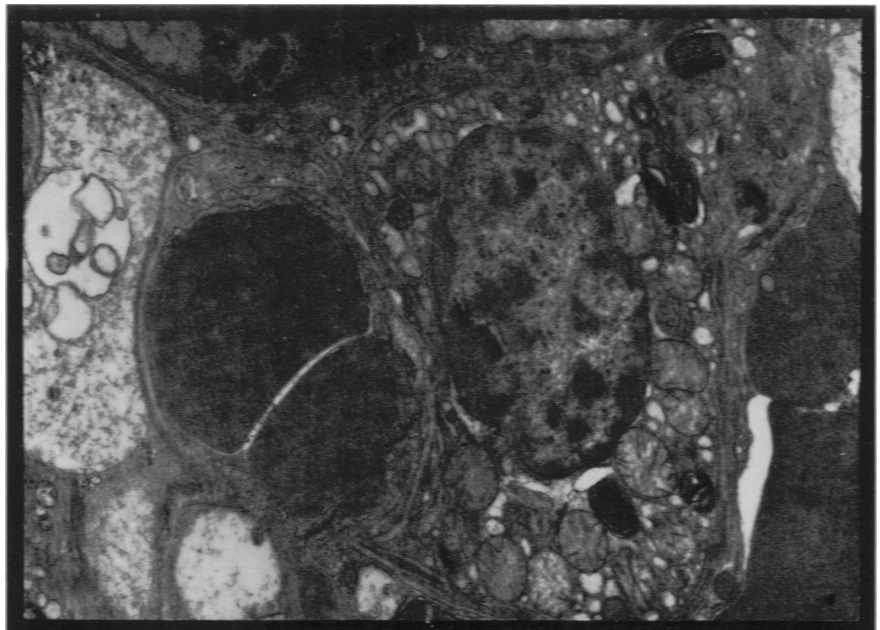
FIG. 1.—Mouse lung. Part of normal type II cell with LOPBs and mitochondria. Multivesicular bodies present. Stage O changes. × 16,200.

FIG. 2.—Mouse lung. Stored in cadaver 2 hours at 25°. Vacuoles beginning to grow in cytoplasm. Nuclear contrast enhanced. Some changes in LOPBs. Some mitochondria have fewer cristae than normal. Stage AB changes. × 16,200.

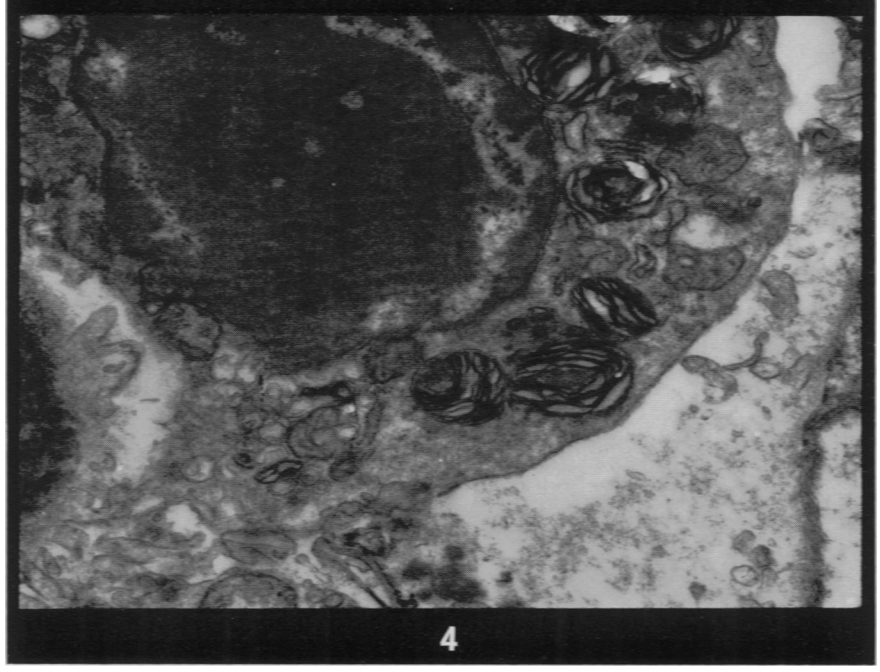
FIG. 3.—Mouse lung. Stored in cadaver 4 hours at 25°. Many vacuoles in cytoplasm. LOPBs show homogeneous areas. Mitochondria intact. Space between nuclear membranes is enlarged. Stage B changes. × 6,600.

FIG. 4.—Wallaby lung. About 12 hours delay in fixation. Nuclear contrast is enhanced, mitochondria are distorted and cell boundary is blurred but pattern of cross barred and arcuate LOPBs is clearly visible. Stage AB changes. × 13,800.


1
2



3



4

DISCUSSION

Our results refer only to the type II cells of the lung under the conditions of our experiments and in no way contradict the findings of other workers on other cells.

It has been stated by Policard, Collett and Pregermain (1957) that with lung it is necessary to start with tissues fixed immediately after removal " as the electron microscope reveals the smallest artefacts ". Sjöstrand and Hanzon (1954), working on the mouse pancreas, killed the animals in a cold room and aimed to complete dissection within 10–30 seconds. They reported marked structural changes in pieces fixed 10 minutes post mortem. These changes included swelling of the mitochondria, which we find an inconstant phenomenon. Rhodin (1954) found in the kidney normal mitochondria after 5 minutes at room temperature, but swelling of the mitochondria and changes in the brush border of the cells at 60 minutes. Maunsbach, Madden and Latta (1962) found closure of the proximal tubules of the kidney unless fixative was dripped onto the organ *in vivo*. Aggregation of nuclear chromatin and separation of the nuclear membranes have been reported (Karlsson and Schulz, 1966) from the rat nervous system after 15 minutes at room temperature; widened elongated cisternae appeared in the cytoplasm at 60 minutes.

By contrast, Ito (1962*a*), studying pieces of rat liver kept at room temperature, found hardly any change at 2 hours; at 24 hours most of the fine structure of the mitochondria was present though markedly distorted. Vesiculation of the agranular cytoplasmic reticulum took place within a few hours of death. In a bat kidney preserved at 14° for 6 days the mitochondria were little altered but the cell boundaries were indistinct. The microvilli of a bat gastric parietal cell (Ito, 1962*b*) had disappeared after 3 days at 5° but the mitochondria were still recognizable.

We found much the same changes, occurring in the same order, as other workers; the time scale in our experiments was rather faster than that found by Ito (1962*a*, *b*) but slower than that of any of the other workers. Like Ito (1962*a*), we find that membrane bound organelles (nucleus, mitochondria, LOPBs) may outlast the cell structure and that microvilli are particularly sensitive to a change.

The results on cat lung (see Table II) could not have been predicted from those on mouse lung and serve to emphasize the irregularity of post-mortem change.

Application to human and other material after delayed autopsy

The present findings suggest that autopsy specimens of lung will show some changes at electron microscope level within an hour of death. After refrigeration overnight serious changes are to be expected. This does not, however, mean that no information can be derived from such specimens and potentially useful material should not be discarded on the ground that immediate autopsy was not possible.

The nuclei, mitochondria and mitochondria with internal osmiophilic whorls (Pattle *et al.*, 1972, 1974) are likely to be equally durable in all species. The LOPBs of non-simians usually have cross-barred or arcuate lamellae and a single bounding membrane. These are therefore likely to be durable. The LOPBs of man and monkeys are of concentric conformation; it is at present doubtful whether they are membrane bound. It is therefore possible that they may be less durable than the LOPBs of non-simians, but (as mentioned above) useful information has been obtained about them after delayed fixation.

SUBSIDIARY EXPERIMENTS

Specimens kept in liquids

In all the experiments described above the lungs were left in the cadaver. In other experiments strips of lung about 1 mm across were kept at 4° for 30 minutes or 1 hour in distilled water, saline or cacodylate buffer at pH 7·2 (0·05 mol/l $(CH_3)_2$ AsO_2Na with 0·0042 mol/l HCl). They were then fixed in glutaraldehyde and treated as before.

The changes in all these experiments were more serious than those in lungs left in the cadaver. For instance, after 1 hour's refrigeration in 0·85% saline there was heavy vacuolation; this contrasts with Baker's finding (1965) that under these conditions there is no change in mouse pancreas. After 30 minutes in distilled water the mitochondria had swollen and there were large vacuoles. By contrast, the LOPBs were compressed so that the lamellations were no longer visible. After 30 minutes in 1·8% saline the LOPBs had swollen so that the lamellations were torn; 4 nm spacing was, however, still visible. After 30 minutes in 0·9% saline vacuolation was also prominent and there were gaps between the nuclear membranes.

It is evident that the processes are complex and that much more work would be necessary to elucidate them. Water movements are obviously involved. Baker (1958) denies that there is any virtue in having a fixative isotonic with body fluids, but does not discuss the effect of introducing colloids into fixatives. In the present experiments the serum proteins in the cadaver probably assisted preservation by reducing the thermodynamic activity of water and thus hindering its transport across membranes impermeable to colloids.

We are grateful to Dr Margaret W. Stanier for the specimen of wallaby lung.

REFERENCES

AVERY, M. E. & MEAD, J. (1959) Surface Properties in Relation to Atelectasis and Hyaline Membrane Disease. *A.M. Ass. J. Dis. Child.*, **97**, 517.

BAKER, J. R. (1958) *Principles of Biological Microtechnique. A Study of Fixation and Dyeing.* London: Methuen. p. 83.

BAKER, J. R. (1965) The Fine Structure Produced in Cells in Fixatives. *J. R. Microsc. Soc.* **84**, 115.

CREASEY, J. M., PATTLE, R. E. & SCHOCK, C. (1974) Ultrastructure of Inclusion Bodies in Type II Cells of Lung, Human and Sub-simian. *J. Physiol.*, **237**, 35P.

ESTABLE-PUIG, J. F., BAUER, W. C. & BLUMBERG, J. M. (1965) Paraphenylenediamine Staining of Osmium-fixed Plastic-embedded Tissue for Light and Phase Microscopy. *J. Neuropath. exp. Neurol.*, **24**, 531.

GIL, J. & REISS, O. K. (1973) Isolation and Characterisation of Lamellar Bodies and Tubular Myelin from Rat Lung Homogenates. *J. cell Biol.*, **58**, 152.

HEPPLESTON, A. G. & YOUNG, A. E. (1972) Alveolar Lipoproteinoses: an Ultrastructural Comparison of the Experimental and Human Forms. *J. Path.*, **107**, 107.

ITO, S. (1962*a*) Light and Electron Microscopic Study of Membranous Cytoplasmic Organelles. In *The Interpretation of Ultrastructure.* Ed. R. J. C. Harris. New York and London: Academic Press. p. 129.

ITO, S. (1962*b*) *Post-mortem Changes of the Plasma Membrane.* 5th Internat. Cong. Electron Microsc., L-5. New York: Academic Press.

KARLSSON, U. & SCHULZ, R. L. (1966) Fixation of the Central Nervous System for Electron Microscopy by Aldehyde Perfusion. III. Structural Changes after Exsanguination and Delayed Perfusion. *J. Ultrastruct. Res.*, **14**, 47.

MAUNSBACH, A. B., MADDEN, S. C. & LATTA, H. (1962) Variations in Fine Structure of Renal Tubular Epithelium under Different Conditions of Fixation. *J. Ultrastruct. Res.*, **6**, 511.

PATTLE, R. E., SCHOCK, C., DIRNHUBER, P. & CREASEY, J. M. (1972) Lamellar Transformation of Lung Mitochondria under Conditions of Stress. *Nature, Lond.*, **240**, 468.

PATTLE, R. E., SCHOCK, C., DIRNHUBER, P. & CREASEY, J. M. (1974) Lung Surfactant and Organelles after Exposure to Dibenzoxazepine (CR). *Br. J. exp. Path.*, **55**, 213.

POLICARD, A., COLLET, A. & PREGARMAIN, S. (1957) Structures alveólaires normales du poumon examinées au microscope électronique. *Path. Biol.*, **33**, 385.

RHODIN, J. (1954) *Correlation of Ultrastructural Organization and Function in Normal and Experimentally Changed Proximal Convoluted Tubule Cells of the Mouse Kidney.* Stockholm: Aktiebolaget Godvil.

SCHOCK, C., PATTLE, R. E. & CREASEY, J. M. (1973) Methods for the Electron Microscopy of the Lamellated Osmiophilic Bodies of the Lung. *J. Microsc.* **97**, 321.

SJÖSTRAND, F. S. & HANZON, V. (1954) Membrane Structures of Cytoplasm and Mitochondria in Exocrine Cells of Mouse Pancreas as Revealed by High Resolution Electron Microscopy. *Expl cell Res.*, **7**, 393.