Vol. 55 No. 2 (April, 1974) was issued 17.5.74

THE BRITISH JOURNAL OF
EXPERIMENTAL PATHOLOGY

VOL. 55 JUNE, 1974 NO. 3

LUNG SURFACTANT AND ORGANELLES AFTER AN EXPOSURE TO DIBENZOXAZEPINE (CR)

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Received for publication December 21, 1973

Summary.—Rats were exposed to a heavy dosage of the sensory irritant dibenz (b.f.)—1,4 oxazepine (CR). No change in the lung surfactant could be detected by the methods used. Electron micrography showed that the ordinary lamellated osmiophilic bodies (LOPBs) and their precursors were unaffected. Bodies containing both mitochondrial cristae and dense osmiophilic whorls (" mitochondrial lamellated bodies ", or MLBs) were found in the type II cells of some animals up to 15 days after the exposure. These whorls originate from the bounding membranes and cristae; serial sections show that they usually abut on the boundary of the organelle. A large proportion of the mitochondria in any cell may be affected by this process. Unequivocal evidence that the MLBs finally evolve into LOPBs without cristae was not obtained in this series; the ultimate fate of the MLBs and the cells containing them is uncertain. The MLBs may perhaps act as an emergency source of surfactant.

THE LUNG surfactant is a lipoprotein, rich in fully saturated lecithin, which lowers the tension of the alveolar surface so as, under normal circumstances, to prevent collapse and transudation (Pattle, 1965). It is of particular interest because it is deficient in the respiratory distress syndrome of the human newborn (Avery and Mead, 1959; Pattle *et al.*, 1962). The bulk of the evidence (Meyrick and Reid, 1973; Gil and Reiss, 1973) suggests that the surfactant originates in the type II cells of the alveoli from the lamellated osmiophilic bodies (LOPBs). These are usually derived *via* transitional (Sorokin) bodies from the multivesicular bodies (MVBs), (Sorokin, 1967) rather than from mitochondria.

In animals which have undergone various stresses, however, bodies appear which contain both mitochondrial cristae and strongly osmiophilic whorls with a 4 nm spacing resembling that of LOPBs (Pattle *et al.*, 1972). These we here call mitochondrial lamellated bodies (MLBs). The present paper describes the status of the surfactant and the occurrence and morphology of MLBs after a heavy dosage of an aerosol of dibenz (b.f.)-1,4 oxazepine (CR); this is a powerful sensory irritant of low toxicity (Higginbottom and Suschitzky, 1962; Ballantyne, Beswick and Price-Thomas, 1973).

MATERIALS AND METHODS

Exposure.—Ten specific pathogen-free rats were exposed to a dosage of 115,000 mg.min/m^3 (6·9 kg.s.m^{-3}) of CR, put up as an aerosol by pyrotechnic means, over a period of 1 hour. They were killed with intraperitoneal pentobarbitone sodium (Nembutal) at intervals after the experiment, as shown in Table I.

Tests for surfactant.—No satisfactory method for the routine quantitative determination of lung surfactant has so far been devised. Two qualitative methods for detecting the presence of surfactant are commonly used: one depends on the low surface tension of bubbles squeezed from a lung fragment and the other on the low surface tension obtained in a trough of lung extracts when the surface area is reduced. The former appears to bear more relation to the formation of a surface film at the lung lining than does the latter. The bubble method was used in the present study. It is simple to use and will be described in detail.

The apparatus necessary consists of a flat microscope slide, a hollow slide or small jar, and a microscope with an eyepiece graticule. The flat slide must be thin enough for bubbles resting against its under surface to be viewed with the ×40 objective. A drop of water is placed on the slide; the drop must not spread out over the slide as this will cause bubbles in it to drift about; it is therefore desirable that the upper slide should be slightly greasy.

If the lung to be examined contains air, a piece about 2 mm across is cut off. If the lung is atelectatic, a part must be aerated before the piece is cut off, by blowing air into the lung tissue through a fine hypodermic needle or by inflation through a bronchus. The slide is inverted so that the drop hangs from it and the piece of lung is squeezed into it with narrow-bladed forceps. This releases bubbles, with the associated lung lining film, into the drop.

The slide with its hanging drop is then placed over the hollow slide and sealed to it with a drop of water. The bubbles are then viewed through the ×40 objective and a sketch is drawn showing 10 bubbles whose diameters, as measured with the eyepiece graticule (to within one division or about 3 μm), are between 30 and 60 μm. Alternatively, if a large number of tests are being made, a photograph may be taken. If the lung specimen is normal, the bubbles contract a little and then remain stable. They are measured or photographed 20 minutes later or when they have definitely stopped contracting. Meanwhile, a search is made for smaller bubbles (3 μm or less in diameter) undergoing Brownian motion; these constitute signs that normal surfactant is present. From the initial and final diameters the stability ratio (s.r.) of each bubble is read off from a table. The mean s.r. found for normal lung will depend a little on the speed of the initial sketching, as contraction starts as soon as the bubbles are formed. The mean s.r. will therefore be rather less if photography is used instead of sketching. Normal lung usually shows a mean s.r. of about 0·7; values less than 0·6 suggest deficiency of surfactant, while uniformly high values (0·9 and above) are found in oedematous lung. There is no reason to suppose that high values of s.r. indicate an excess of surfactant; they arise either through slow sketching or from geometrical factors associated with oedema. Examples of the use of this method are given by Pattle and Burgess (1961), Pattle *et al.* (1962) and Benzer *et al.* (1969).

When bubbles derived from the lung are made to contract by dissolving their air content, they sometimes execute a peculiar regular rhythmic change of shape known as " clicking ", which is highly characteristic of normal lung lining; the authors know of no other substance which shows this. There are two methods which may be used to test for it. In one, bubbles are squeezed into a hanging drop as above. The hollow of the lower slide is filled with water which has been de-aerated by boiling and cooled. The upper slide is then placed over the lower so that the whole of the hollow is filled with water and the bubbles remain below the upper slide. With practice this can be done without losing the small bubbles or including a large one; it is easier if the lower slide is slightly greasy. The bubbles are then inspected with the ×10 objective. If the lung is normal, bubbles 100 μm or more in diameter will be seen slowly to increase in horizontal diameter and suddenly to contract; this process is repeated every few seconds. Actually the bubbles are slowly flattening, and then suddenly shedding part of their surface film and resuming spherical shape.

Another method of inducing " clicking " is to place in the hollow slide a drop of volatile saturated fluorocarbon, such as perfluorohexane (Flutec PP1). The fluorocarbon vapour is highly insoluble so that air diffuses out of the bubbles much faster than fluorocarbon diffuses in, and the bubbles dissolve; as they do, so they exhibit " clicking ".

The bubble methods can be used with very small tissues such as atelectatic patches re-inflated. In the present work 3 batches of 10 bubbles from each animal were tested but,

unless variation from one part of the lung to another is suspected, normal s.r., Brownian motion and clicking in one batch of 10 are sufficient for a diagnosis of a normal lining film. The authors have never found a naturally aerated lung of normal appearance which showed any sign of abnormality of the lining film; in such a case the test may be superfluous.

Electron microscopy.—Specimens were taken from the lung and fixed in glutaraldehyde and osmium tetroxide solutions; they were then further treated with a mixture of 10 vol M/40 lead nitrate and 10·5 vol M/60 potassium ferricyanide. This fixes and stains lecithin. They were embedded in Araldite with acetone as dehydrating and thinning fluid. These methods are described by Schock, Pattle and Creasey (1973). Sections about 75 nm thick were cut for electron microscopy and adjacent sections 1000 nm thick were taken for optical microscopy. The latter were stained with p-phenylenediamine (Estable-Puig, Bauer and Blumberg, 1965) and counterstained with methylene blue.

Serial sections about 75 nm thick were cut from the specimen taken at 15d, which showed well developed MLBs. The areas least obscured by the grid bars were chosen from low magnification micrographs, and these areas were re-examined at high magnification. Various factors prevented more than 14 consecutive sections being taken; in the best series 10 of these were unobscured by the grid.

RESULTS

General observations

The results are summarized in Table I. There were no fatalities from CR among the animals treated, but one of those sacrificed had respiratory distress.

TABLE I.—*Characteristics of Lungs of Rats after Exposure to Dosage of* 115,000 *mg.min/m³ of CR over* 1 *Hour, showing Incidence of Mitochondrial Lamellated Bodies*

Serial number 42 B	1	2(1)	2(2)	3	4	5	6	7	8	9	10
Time from end of exposure to death	45 min	5 hours		28 hours	48 hours	75 hours	8 days	13 days	15 days	43 days	93 days
Respiratory distress present at time of death	−	+		−	−	−	−	−	−	−	−
Lung atelectasis present at death	−	+		+	+ +	+ +	+ + +	−	−	−	−
Normal bubble stability	+	+		+	+	+	+	+	+	*	*
Normal bubble clicking	+	+		+	+	+	+	+	+	*	*
Normal bubble Brownian motion	+	+		+	+	+	+	+	+	*	*
Atelectasis of E.M. specimen	−	+	−	−	+	+	+	−	−	−	−
Normal LOPBs present	+	+	+	+	+	+	+	+	+	+	+
Number of cells with MLBs/number of cells examined	4/5	2/2	7/7	0/4	4/5	6/6	0/5	0/5	4/5	0/1	0/5
Grossly vacuolated cells present	−	+	+	+	+	+	+	−	−	−	−
Number of sections included in Col. 2, Table II.	0	2	1	0	1	3	0	0	3	0	0

* Not tested.

This is not discordant with a dosage of 0·57 LCt50 having been given (Ballantyne *et al.*, 1974, in preparation). At 5 and 28 hours there were small atelectatic spots on the lungs; at 48 and 75 hours there were larger atelectatic patches. At 8 days the right upper lobe was collapsed and froth oozed from a bronchus on cutting. At 13 days the lungs appeared slightly emphysematous and at 15, 43 and 93 days they appeared normal. A full account of the pathology caused by CR will be given elsewhere (Ballantyne *et al.*, 1974). In every case tests on bubbles, whether obtained from an atelectatic portion of lung or from one of normal appearance, gave no sign that the surfactant was abnormal.

Electron microscopic observations

These were mainly directed to the type II cells. In all the specimens examined by electron microscopy there were numerous LOPBs of normal appearance in

these cells; this was so whether the specimen was macroscopically of normal appearance or was atelectatic. MLBs were seen in tissue from the animals killed 45 minutes, 48 and 75 hours, and 15 days after the end of the exposure. By contrast, they could not be found at 28 hours, 8, 13, 43 and 93 days. When the bodies were present at all, it was easy to find them in almost every type II cell, whether from aerated or atelectatic lung. In the 45 minute specimen the osmiophilic whorls were small and possibly at an early stage of their evolution; in the 5, 48, and 75 hours and the 15 day specimens they were thicker and resembled LOPBs growing inside mitochondria. A typical view of part of a type II cell containing MLBs is shown in Fig. 1. In some of the specimens vacuolated cells, probably dead, were prominent.

Deposits of osmiophilic matter were noted among the Golgi apparatus in one case (Fig. 1), and in the endoplasmic reticulum in another (Fig. 9), but the irritant appeared to give no obvious general stimulus to the laying down of osmiophilic material elsewhere than in the mitochondria. The barrier between the capillary blood and the air or exudates filling the alveolar space appeared normal where it was sectioned. In the thicker parts of the barrier caveolae (pinocytotic vesicles) were present in normal numbers.

Morphology of mitochondrial lamellated bodies (MLBs)

In general, the MLBs resembled mitochondria in size and shape. The osmiophilic whorls within them might be of any kind, from a simple ring or loop among the cristae to a dark concentric mass filling most of the section; 4 nm spacing, similar to that of the lamellated osmiophilic bodies, could often be seen.

Serial sections showed large and irregular variations in the shape and density of a whorl from one section to the next. In this, the whorls within mitochondria differed from the ordinary LOPBs; the shape and pattern of the latter varied in a much more regular manner. No definite bounding membrane could be found round the whorls, though the whole MLB was surrounded by a double membrane like that of a mitochondrion; ordinary LOPBs have a single bounding membrane.

Figures 2–7 show 6 successive serial sections through an MLB and Fig. 8 shows a section through another. The proportions in which the MLBs were filled with osmiophilic matter or cristae varied from section to section; some sections showed mainly whorls, others only cristae. The whorls appeared always to be associated with the bounding membranes of the MLBs. A thorough search was made for any whorl which serial sections showed to be completely detached in the interior of the

EXPLANATION OF PLATES

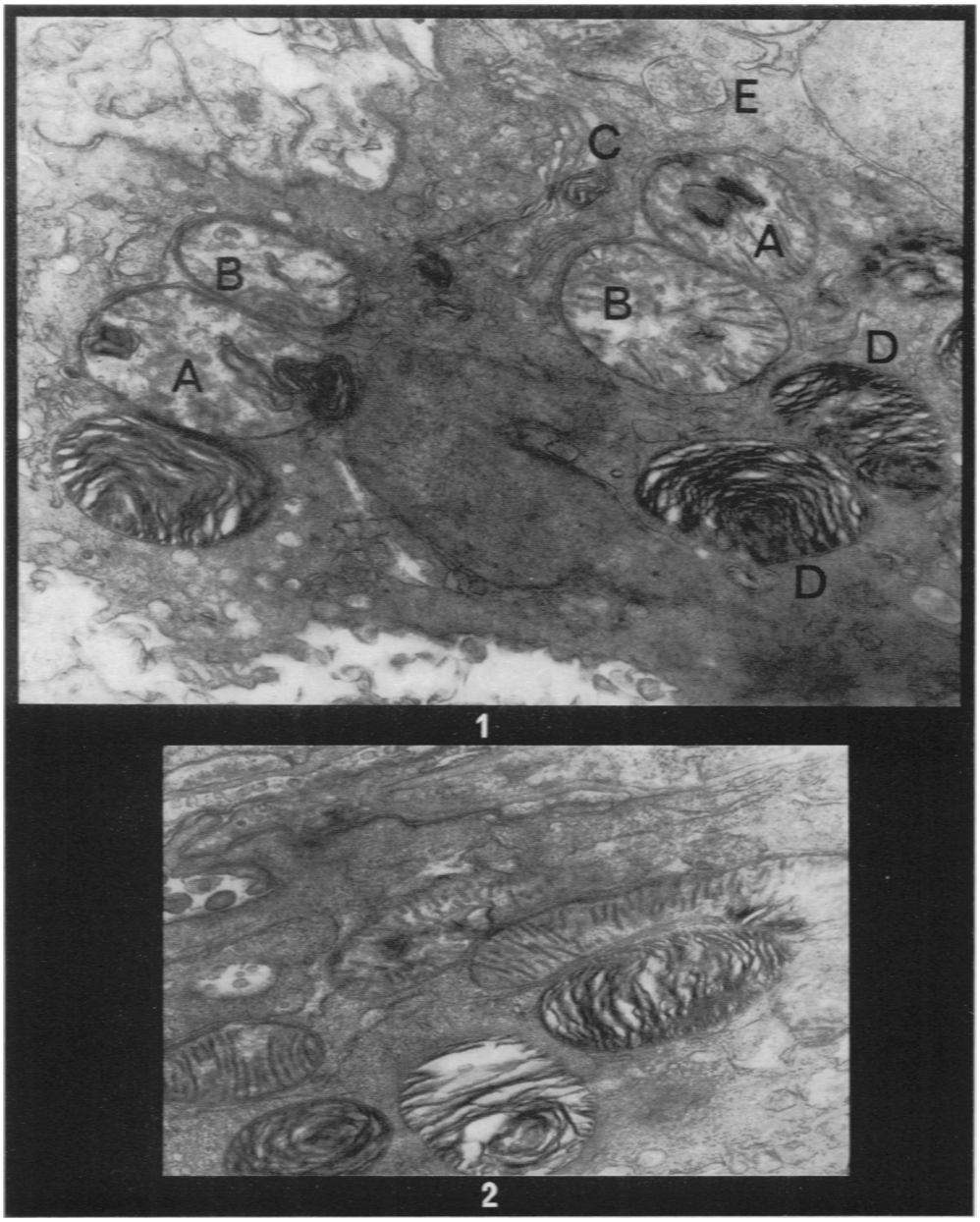
FIG. 1.—General view of part of a type II cell showing mitochondrial lamellated bodies AA, mitochondria BB with small whorls, osmiophilic matter C deposited in Golgi apparatus, normal LOPBs DD, and multivesicular body E. No normal mitochondrion is visible. 15 days after exposure. × 18,400.

FIGS. 2–7.—Six successive sections (about 75 nm thick) through a mitochondrial lamellated body. 15 days. Fig. 2 × 18,400. Fig. 3–7 × 11,500. Note: Fig. 2 and 4 are inverted in relation to sections 3, 5, 6 and 7.

FIG. 8.—Mitochondrial lamellated body with whorl abutting against its boundary. A normal arcuate LOPB is present. 15 days. × 21,000.

FIG. 9.—Mitochondria with light and dark backgrounds. Bodies A and C show whorls in another section, body B does not. Just outside A is a vesicle of endoplasmic reticulum with osmiophilic stripes. 15 days. × 25,500.

FIG. 10.—Part of a type II cell with irregular swollen LOPBs, some with double bounding membranes; these may be an end-product of MLBs. 5 hours. × 9,600.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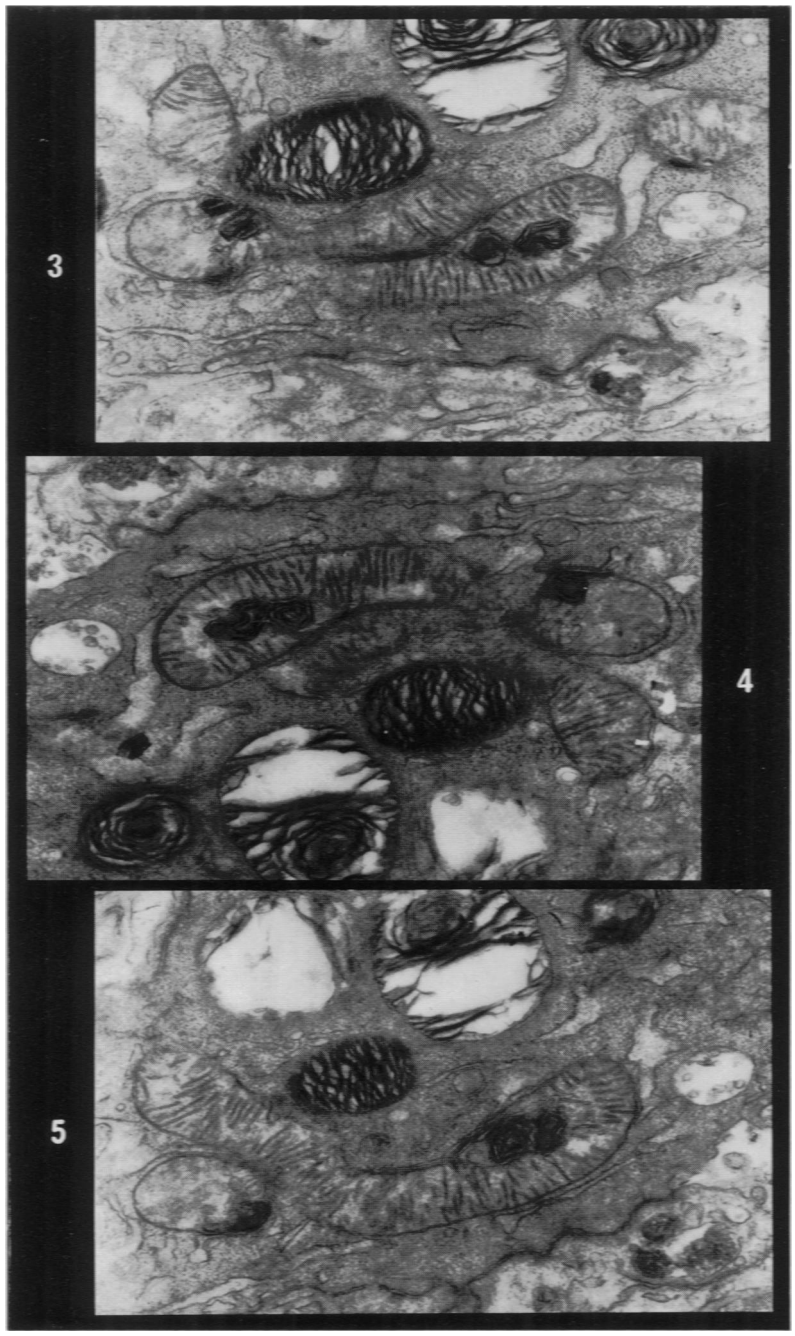
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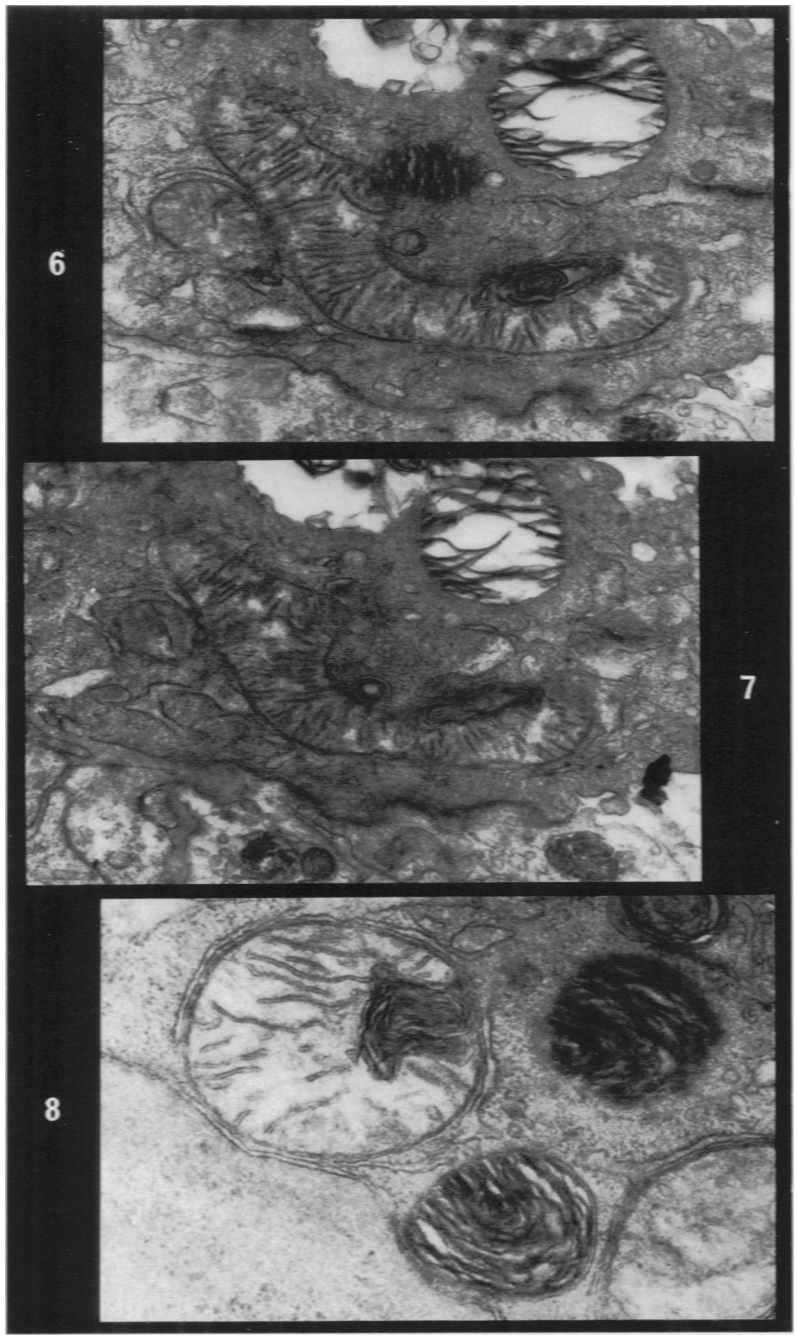
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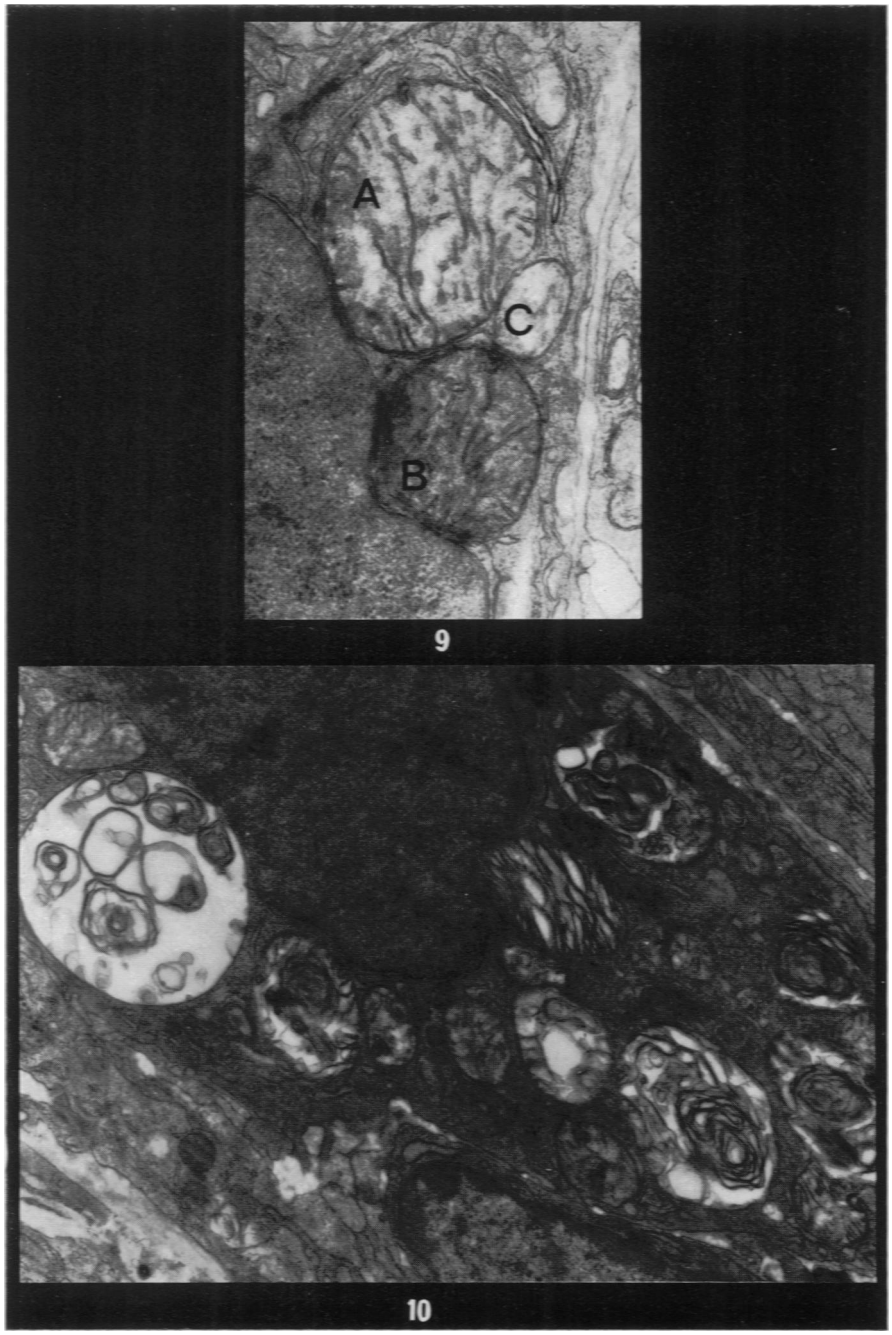

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10

MLB; none was found. In many cases a whorl which appeared in one section as isolated in the middle of an MLB (Fig. 2–5) was seen in other sections to be based on the bounding membrane (as in Fig. 6, 7, 8).

No unequivocal case was found in serially sectioned cells of an LOPB with a double bounding membrane of mitochondrial type but no cristae. In one case an MLB showed cristae in some sections but only osmiophilic whorls in others. The existence of bodies of mitochondrial origin, completely filled with osmiophilic lamellae, therefore remains doubtful.

In one cell in the 5 hour specimen, LOPBs of irregular pattern showed double bounding membranes and appeared to be grossly swollen. It may be that in this cell most of the mitochondria had been transformed into MLBs and that the cell was dying (Fig. 10). This cell was not serially sectioned.

Electron density of backgrounds of mitochondria and MLBs

The background of mitochondria containing irregularities or whorls is generally less dense than that of ordinary mitochondria. An example is shown in Fig. 9. Mitochondrion A appeared on every unobscured member of a set of 14 serial sections; it showed no irregularities. Its background is much denser than that of mitochondrion B. The latter, in Fig. 9, shows a fold in its boundary and in other sections it contained a small whorl. Mitochondrion C also has a pale background and showed an osmiophilic whorl in another section.

Relative numbers of different types of body

Table II shows the frequency of LOPBs, mitochondria and other bodies in sections through type II cells in normal and treated rats. The sections were chosen

TABLE II.—*Average Numbers per Section of Organelles of Various Types in Rat Type II Cells, from Normal Animals and from Animals Exposed to CR and Showing MLBs*

	Normals	Exposed, showing MLBs
Number of animals examined	5	4
Number of cells examined	10	10
Number of sections examined	10	10
Numbers of organelles		
Mitochondria		
Normal	8·4	2·8
Irregular	0·1	1·6
LOPBs		
Cross-barred and arcuate	3·5	3·2
Concentric	0·6	1·0
Irregular	0·3	1·5
Unclassified	2·3	1·3
Total	6·7	6·0
Compound bodies		
Multivesicular (Sorokin)	0·7	0·4
Mitochondrial lamellated	0·3	3·1
Other	0·3	0·1
Multivesicular bodies	1·2	0·5

so as to pass through the nucleus and the region where LOPBs were massed; they included 2 median sections of sets of serials. The classification is that of Pattle, Schock and Creasey (1974, in preparation). It must be emphasized that these are the numbers per section chosen as above, not numbers per cell. To ascertain the numbers per cell would require either serial sectioning of a complete cell or examination of a number of truly random sections through type II cells. The former process is not at present feasible. The latter would require a means of recognizing type II cells from sections which contained no LOPBs; allowance would then be made for the fact that the probability of a section cutting a body increases with the size of that body.

DISCUSSION

Surfactant tests

The bubble tests show that in every case sufficient surfactant was present to form a lining film resembling that of normal lungs; they give no indication as to whether the amount of surfactant was greater or less than that normally present. The results are in line with the finding that in most conditions in which lung has become atelectatic it forms a normal lining film on re-inflation (cf. Pattle and Burgess, 1961).

Origin of MLBs

The view has sometimes been propounded that the MLBs are artefacts caused by the superposition of a mitochondrion on an LOPB, so that the latter bulges into the former, and that a section through the two shows part of the LOPB inside the mitochondrion. The following arguments, however, show that this is not the case and that the osmiophilic whorls arise from the mitochondrion itself.

(i) In sections through a type II cell, either a normal one or one in which MLBs appear, it is unusual for an LOPB to be seen bulging into a mitochondrion. MLBs, when they do occur in a section, occur frequently.

(ii) As has been shown in the present work, the profiles of the osmiophilic whorls show irregular variations from one serial section to another. The shapes of the osmiophilic whorls as seen in section are much less regular than those of LOPBs.

(iii) An LOPB bulging into a mitochondrion should show, at least in some sections, osmiophilic matter surrounded by the outer bounding membrane of the LOPB and also by the double bounding membrane of the mitochondrion. There is no sign of this.

(iv) In section, the osmiophilic matter often appears to be based on the outer boundary of the mitochondrion and extends inwards but not outwards from the boundary (Fig. 8).

(v) In non-simian lung, the LOPBs are usually of cross-barred pattern (Creasey, Pattle and Schock, 1974). The osmiophilic portions of MLBs usually have a concentric or irregular pattern.

(vi) Similar bodies are found in tissues other than lung in which LOPBs similar to those of lung do not occur. They have been recorded, for instance, by Spiro, Shy and Gonatas (1966); in human muscle in myotubular myopathy; by Pannese (1966); by Candiollo and Filogamo (1966) in neuroblasts in the developing chick; and by Sacktor and Shimada (1972) in flight muscle from ageing blowflies.

Pannese (1966) considered that in the chick neuroblast osmiophilic whorls, formed at the nuclear membrane, became detached and unfolded to form new mitochondria. The hypothesis that MLBs represent LOPBs unfolding to form mitochondria would not accord with the difference between the internal arrangement of LOPBs and that of the osmiophilic portion of MLBs (see (v) above). It is also contradicted by the fact that where MLBs are present the number of normal mitochondria is reduced, not increased (Table II).

It thus appears that the osmiophilic whorls are formed by the mitochondria themselves and that this formation (called by some authors " degeneration ") is a potentiality of mitochondria as a whole, and not only of those of the lung. It may be that the relative electron opacity of mitochondrion A in Fig. 9 is due to the presence in its matrix of phospholipid which in the bodies B and C has been incorporated into the whorls.

MLBs are found either in large numbers in one animal or hardly at all (Table I). They have been found in animals treated with the irritant o-chlorobenzylidene malononitrile (CS) or with the glucocorticoid substitute 9-fluoroprednisolone (Pattle *et al.*, 1972). They have also been found in untreated animals and in animals prematurely born (Pattle *et al.*, unpublished data). In the present series they appeared in a lung of normal appearance (15 days) but not in the most atelectatic lung (8 days), and their appearance was not strictly correlated with the interval between exposure and death. These facts accord with the hypothesis that they are produced under the influence of a process, possibly involving the adrenal cortex, which affects the whole animal, and are not produced by local exposure of the lung to irritant. Meanwhile normal LOPBs continue to be produced.

Function and final fate of MLBs

The evidence that ordinary LOPBs consist of surfactant is strong (Gil and Reiss, 1973); no such evidence exists in the case of the MLBs. One may postulate either that two kinds of phospholipid are being laid down in the same cell or that surfactant is being laid down both in LOPBs and MLBs. In the latter case it might be that the cell is being stimulated to produce an emergency supply of surfactant. Further work is needed to ascertain the sites of synthesis of surfactant in the type II cells and the chemical and surfactant properties of the mitochondrial whorls.

It is known that the contents of the LOPBs are extruded into the alveolar space; whether this happens to MLBs is uncertain. Mitochondria completely filled with lamellae, if present at all, are certainly outnumbered by those which still contain cristae. It may be that when a sufficient number of mitochondria have been affected the cell dies and the osmiophilic matter is released by holocrine secretion. The cell shown in Fig. 10, with its grossly swollen inclusions, may be about to undergo this process.

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