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A cytochemical study of the granular pneumonocytes in hamster lung

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INTRODUCTION

In a wide range of mammalian species the granular pneumonocytes (type II cells) of the alveolar epithelium lining contain large lamellar inclusion bodies (Schulz, 1959; Policard, Collet & Pregermain, 1959; Campiche, 1960). A considerable body of evidence has accumulated which suggests that the contents of the inclusion bodies are secreted into the alveolar lumen and that they are probably a source of pulmonary surfactant (Sorokin, 1967; Kuhn, 1968; Kikkawa, Motomaya & Gluck, 1968). The morphological evidence of this secretory process, although circumstantial, is impressive and is in agreement with the results of a number of experimental and biochemical studies (Bensch, Schaefer & Avery, 1964; Buckingham, Heineman, Sommers & McNary, 1966; Goldenberg, Buckingham & Sommers, 1969).

Early attempts to demonstrate enzyme activity in granular pneumonocytes (Bensch *et al.* 1964; Buckingham, McNary & Sommers, 1964) proved disappointing, but recently several workers have had considerable success in localizing acid phosphatase activity using specially modified techniques (Kuhn, 1968; Corrin, Clark & Spencer, 1969; Etherton & Botham, 1970). Acid phosphatase reaction is present in the inclusion vacuoles, multivesicular bodies, and channels of the Golgi apparatus. According to Goldfischer, Kikkawa & Hoffman (1968) and Corrin & Clark (1968), the inclusions also contain aryl sulphatase, another acid hydrolase, and it has been postulated that the inclusion bodies are lysosomal in nature. Kuhn (1968) demonstrated alkaline phosphatase activity in the apical cell membrane of granular pneumonocytes in rat and human lung. However, the single other ultrastructural study only mentioned localization of alkaline phosphatase in the inclusion bodies (Buckingham *et al.* 1964).

The present report describes the cytochemical features of the granular pneumonocytes in hamster lung.

MATERIALS AND METHODS

Young adult hamsters weighing 150–200 g were killed by cervical dislocation and small blocks of lung tissue were excised and fixed in ice-cold 2·5 % glutaraldehyde in 0·1 M cacodylate buffer containing 5 % sucrose, pH 7·3. After fixation for 90–120 minutes the blocks were washed for 30 minutes in cacodylate buffer containing 5 % sucrose. Tissue sections (about 0·5 mm thick) were then prepared freehand or alternatively 50 μm sections were cut in a cryostat. The tissue sections were incubated to demonstrate the following enzymes:

Acid phosphatase, using Smith & Farquhar's (1966) modification of the Gomori technique. Sodium β-glycerophosphate and naphthol AS-MX phosphate were used as substrates.

Aryl sulphatase, using the *p*-nitrocatechol-barium chloride medium of Hugon & Borgers (1967) at pH 4·2, 5·5 and 7·3.

E-600-resistant esterase. Sections were incubated in diethyl-*p*-nitrophenylphosphate (E-600) 10^{-4} M in 0·2 M acetate buffer at pH 6·0 for 1 hour at 37 °C. They were then transferred to the medium of Wachstein, Meisel & Falcon (1961) for 15–90 minutes. The incubation temperatures were 37 and 4 °C.

Alkaline phosphatase, using the glycerophosphate-lead method of Hugon & Borgers (1966) and the glycerophosphate-calcium method of Sabatini, Bensch & Barrnett (1963). Test sections of kidney were incubated under similar conditions.

Adenosine triphosphatase, using the medium of Wachstein & Meisel (1957) at pH 7·3. Control sections were incubated in a medium in which ATP was replaced by an equimolar concentration of sodium β-glycerophosphate.

Control sections for all enzymes were incubated in substrate-free media.

Following incubation the tissues were washed briefly in cacodylate buffer, post-fixed for 45 minutes in cacodylate buffered 1 % osmium tetroxide at pH 7·3, dehydrated in graded alcohols and processed through propylene oxide to Araldite. Small blocks of lung tissue fixed directly in osmium tetroxide were processed in a similar manner. Ultrathin sections for electron microscopy were generally examined without counterstaining to avoid confusion with enzyme reaction product.

RESULTS

Acid phosphatase

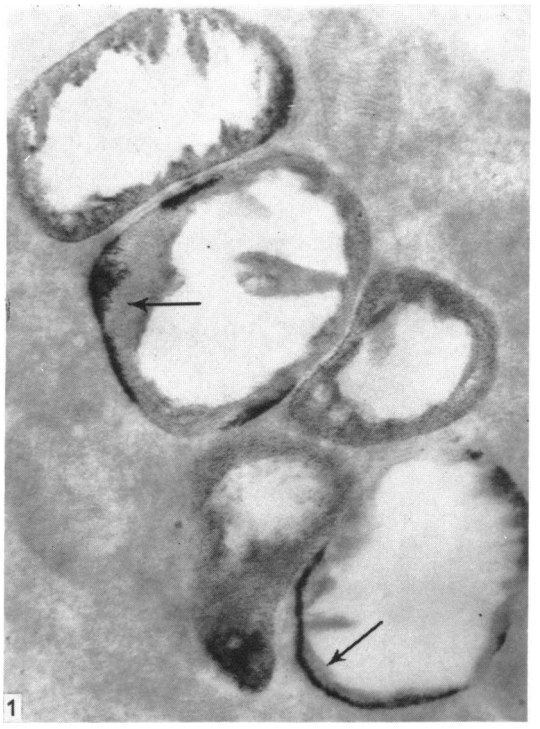
Acid phosphatase preparations using naphthol AS-MX phosphate as substrate were, in general, more satisfactory than those using sodium β-glycerophosphate. Non-specific staining and enzyme leakage were much more pronounced in the sodium β-glycerophosphate preparations. Lead phosphate deposits were seen in the lamellar inclusion bodies of the granular pneumonocytes, the deposition being most intense in the smaller inclusions (Fig. 1). The reaction product was mainly localized in the limiting membrane of the inclusions; occasionally small quantities of reaction product also appeared in the lamellated contents. In addition, less marked enzyme activity was observed in multivesicular bodies and Golgi lamellae (Fig. 2). Non-enzymic deposition of lead occurred principally in the nuclear membranes and the apical plasma membranes of cells incubated for longer than 100 minutes.

Fig. 1. Acid phosphatase reaction in the lining membrane of inclusion bodies (arrows) in granular pneumonocyte. Naphthol AS-MX phosphate substrate. No counterstain. × 50000.

Fig. 2. Acid phosphatase reaction in an inclusion body (*Ib*) and lamellae of Golgi apparatus (*Ga*). Naphthol AS-MX phosphate substrate. No counterstain. × 37500.

Fig. 3. Aryl sulphatase A activity localized in the walls of inclusion bodies. No counterstain. × 34000.

Fig. 4. Aryl sulphatase B activity in walls of inclusion bodies. No counterstain. × 28500.



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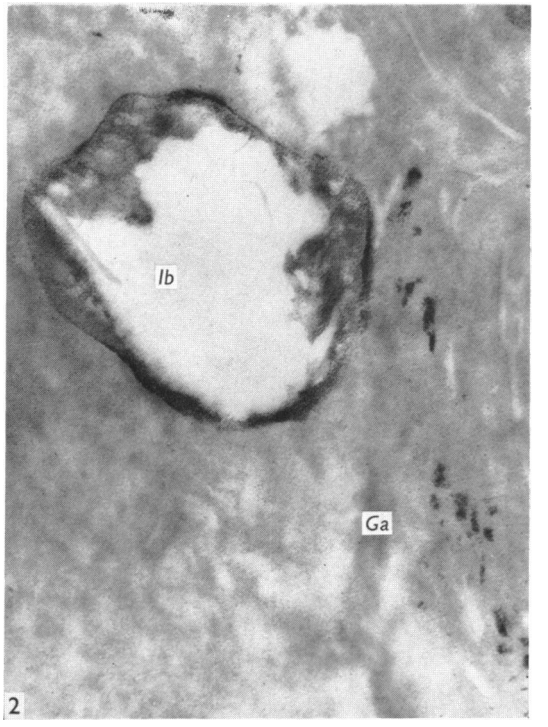

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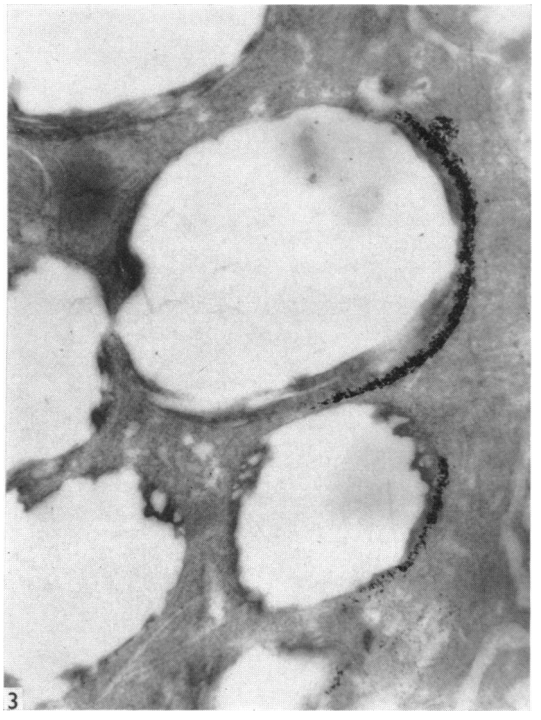

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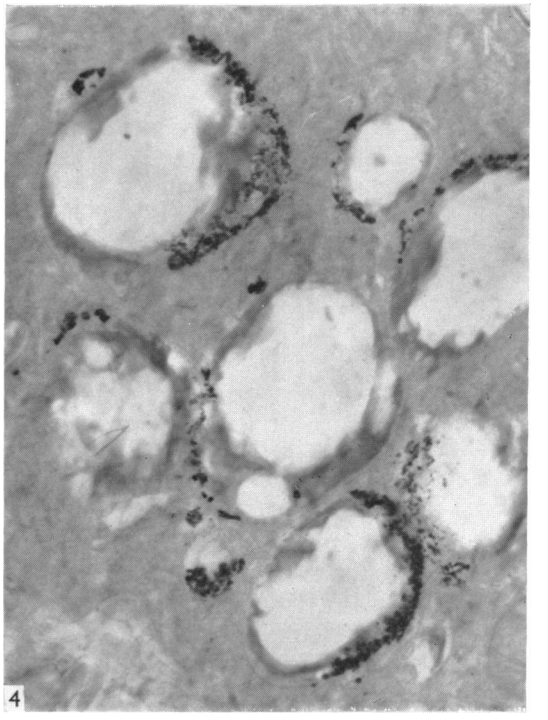

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Aryl sulphatase

Aryl sulphatase A, active at pH 4·2, was present in small amounts in the occasional granular pneumonocyte. The barium sulphate reaction product was located in the limiting membrane of the inclusion vacuoles and in the matrix of the multivesicular bodies (Fig. 3). Aryl sulphatase B, visualized at pH 5·5, had a similar distribution, but the reaction was much more intense and a greater number of cells showed enzyme activity (Fig. 4). There was no evidence of aryl sulphatase C, the ribosomal enzyme active at pH 7·3 (Roy, 1960).

E-600-resistant esterase

Enzymic hydrolysis of thioacetic acid in the presence of free lead ions results in an insoluble electron-opaque deposit of lead sulphide. Initially the esterase preparations proved to be unsatisfactory because of irregular heavy lead sulphide precipitation throughout the tissues. It was found that incubation of the tissues at 4 °C, instead of 37 °C as in the original procedure of Wachstein *et al.* (1961), greatly reduced this non-specific precipitation without altering the sensitivity of the method. Most granular pneumonocytes showed concentrations of reaction product in or near the limiting membrane of the inclusion vacuoles (Fig. 5). This deposit was frequently coarsely granular in character and usually extended into the lamellated contents of the inclusions (Fig. 6). Golgi zones and mitochondria were unstained (Fig. 5). Osmiophilic myelin figures present in the alveolar spaces, presumably representing surface-active material discharged from granular pneumonocytes, showed a strong reaction (Fig. 7).

Alkaline phosphatase

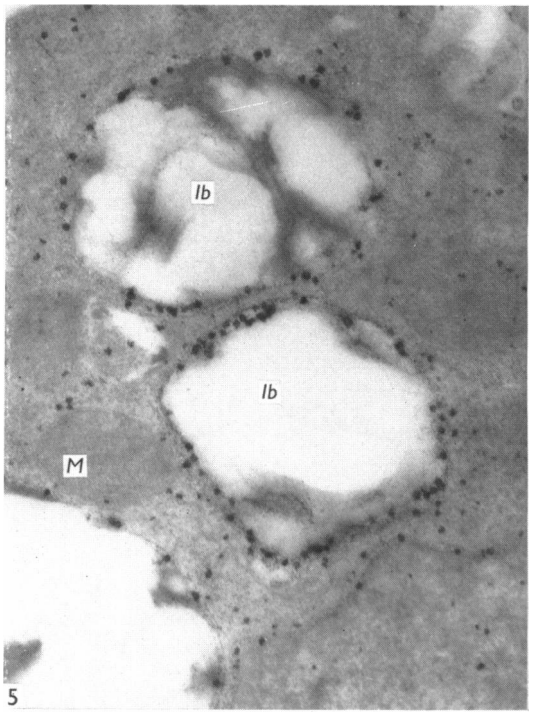
There was no cytochemical evidence for the occurrence of alkaline phosphatase in granular pneumonocytes (Fig. 8); both the lead-glycerophosphate method of Hugon & Borgers (1966) and the calcium-glycerophosphate method of Sabatini *et al.* (1963) failed to produce any consistent pattern of staining, even after prolonged incubation (90 minutes). A variable quantity of lead was deposited irregularly over cell nuclei in tissues incubated in the medium of Hugon & Borgers, but this was equally prominent in control sections incubated in a medium which lacked sodium β-glycerophosphate. Test sections of renal cortex incubated in complete media showed strong enzyme activity associated with the brush border and basal membrane indentations of the convoluted tubule cells.

Fig. 5. E-600-resistant esterase activity in the lining membrane of inclusion bodies (*Ib*). The mitochondrion (*M*) is unstained. No counterstain. × 37500.

Fig. 6. E-600-resistant esterase reaction in lining membrane of inclusion bodies and also in the lamellated contents. No counterstain. × 37500.

Fig. 7. Inclusion vacuole of granular pneumonocyte communicating with alveolar lumen. E-600-resistant esterase activity is present in the inclusion contents and on membranous material (*Mm*) lying free in the alveolus. No counterstain. × 34000.

Fig. 8. Granular pneumonocyte in an alkaline phosphatase preparation (lead-glycerophosphate technique). Incubation 120 minutes. No reaction product is seen. No counterstain. × 15000.



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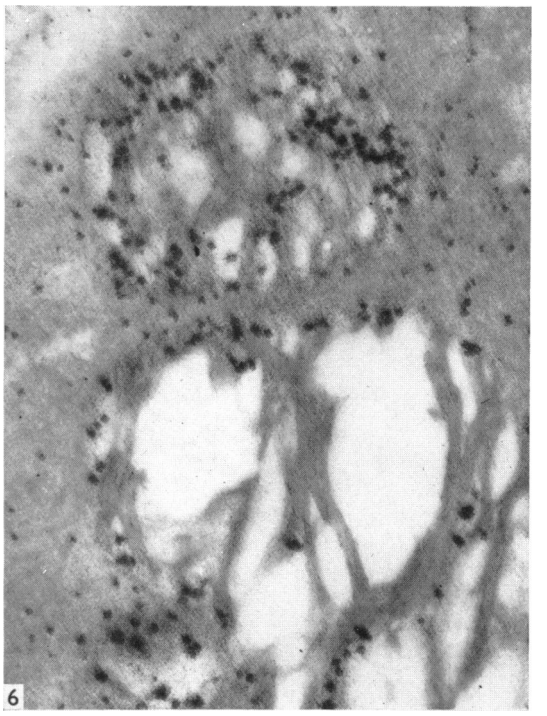
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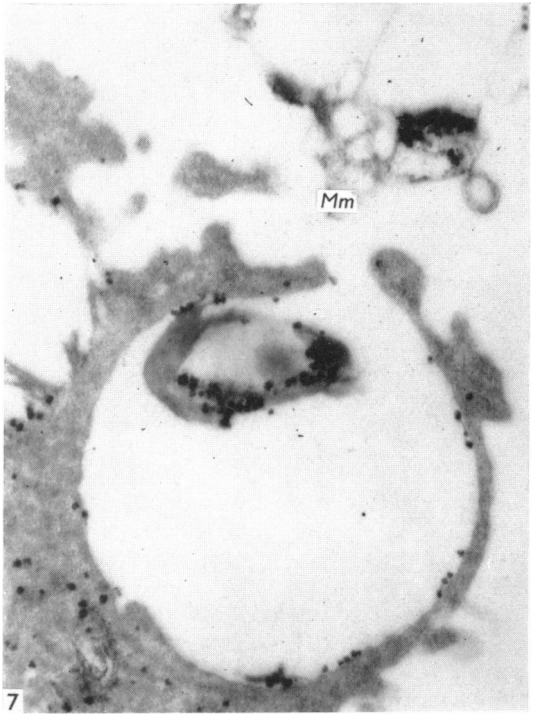

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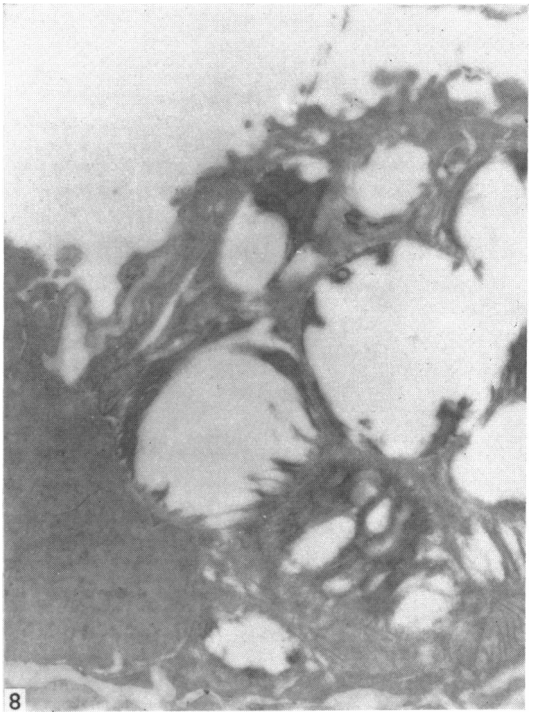
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Adenosine triphosphatase (ATPase)

The lead phosphate reaction product was frequently observed in the apical plasma membranes and at the outer surface of the microvilli of granular pneumonocytes (Fig. 9). There was no localization within the filamentous internal structure of the microvilli. The exact relationship of reaction product to the plasma membrane was impossible to determine because of the low contrast of membranous structures in incubated tissues. ATPase activity did not extend to the lateral or basal plasma membranes. The intensity of the enzymic reaction in granular pneumonocytes showed considerable variation, even in cells situated within the walls of the same alveolus; activity ranged from a heavy deposit to no detectable quantity of reaction product (Fig. 10). Most cell nuclei showed some non-specific deposition of lead phosphate. Mitochondria and endoplasmic reticulum were non-reactive even after prolonged incubation. In a few instances inclusion bodies and other cytoplasmic vacuoles showed enzyme activity (Fig. 11), although examination of serial sections usually confirmed that such structures communicated with the apical plasma membrane. No reaction was obtained when sodium β-glycerophosphate was substituted for ATP in the incubation medium (Fig. 12).

DISCUSSION

The present investigation has demonstrated the presence of acid phosphatase in the lamellar inclusion bodies, multivesicular bodies and Golgi apparatus of granular pneumonocytes in hamster lung. A similar pattern of acid phosphatase activity has recently been described in rat lung (Corrin *et al.* 1969) and rabbit lung (Goldfischer *et al.* 1968). Enzyme activity was only demonstrated after incubation for more than 60 min. As Kuhn (1968) has pointed out, the need for prolonged incubation probably explains the failure of early electron microscopical studies to localize acid phosphatase (Bensch *et al.* 1964; Buckingham *et al.* 1964).

The ultrastructural studies in rabbit lung by Goldfischer *et al.* (1968) and in rat lung by Corrin & Clark (1968) have demonstrated the presence of aryl sulphatase activity in multivesicular bodies and inclusion vesicles of the granular pneumonocytes. The present investigation of hamster lung has revealed a similar distribution for both aryl sulphatases A and B, the latter enzyme being the more active. Aryl sulphatase C, the ribosomal enzyme active at pH 7·3, was not detected. It should be noted that enzyme activity was localized predominantly in the periphery of the

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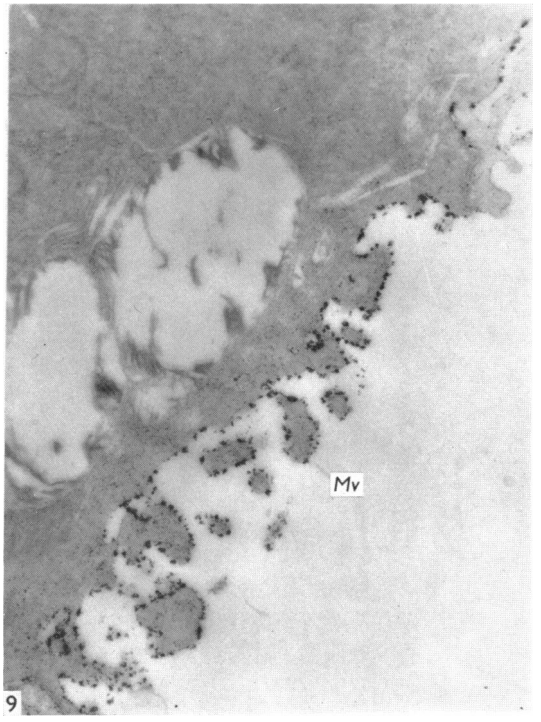
Fig. 9. ATPase preparation. Reaction product localized in apical plasma membrane and microvilli (*Mv*) of granular pneumonocyte. No counterstain. ×40000.

Fig. 10. ATPase preparation. Granular pneumonocyte from wall of same alveolus as the cell shown in Fig. 9. No reaction present. Mitochondria (*M*) are not reactive. Uranyl acetate. ×15000.

Fig. 11. ATPase reaction in inclusion body (*Ib*) of granular pneumonocyte. No counterstain. ×28000.

Fig. 12. Control section incubated in a medium in which Na-β-glycerophosphate was substituted for ATP. No reaction product formed. Uranyl acetate. ×28000.



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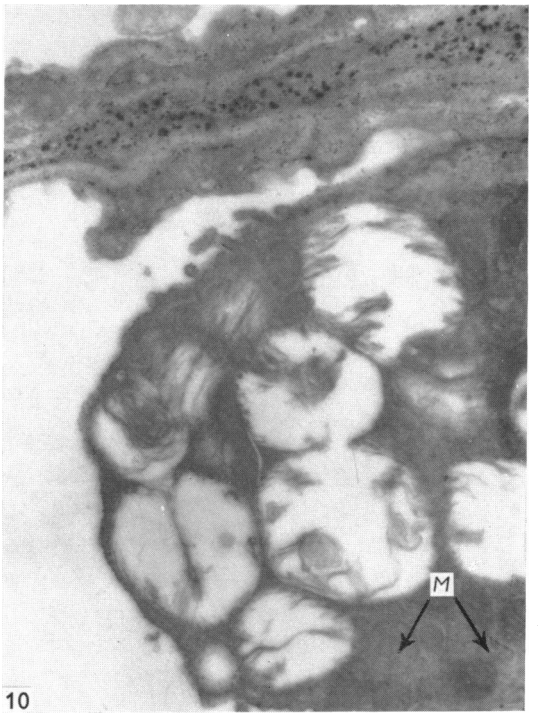

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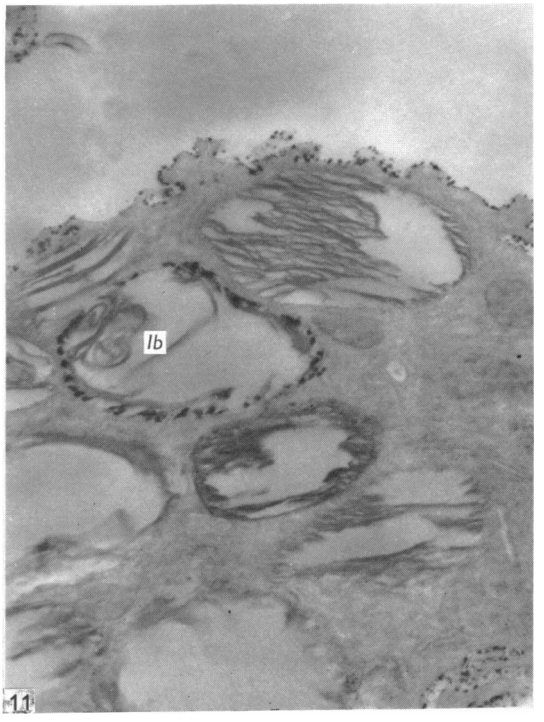

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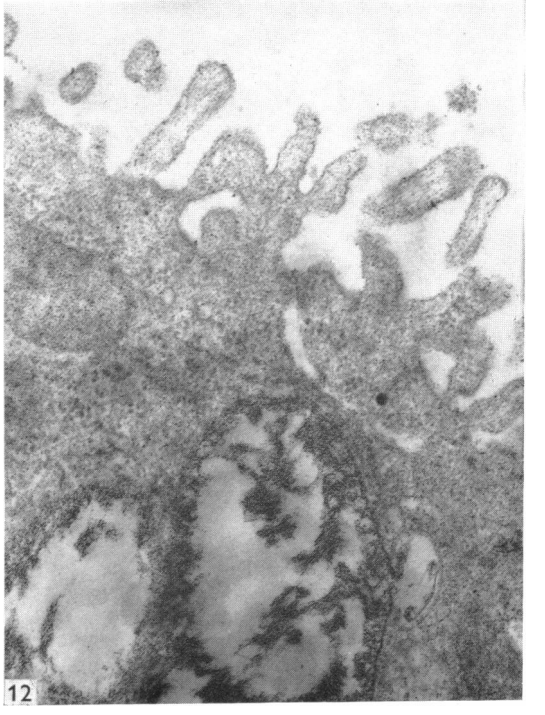

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inclusion vesicles and that many vesicles, particularly the largest of them, failed to show any aryl sulphatase activity. It is possible that this appearance may be due, at least in part, to the rapid extraction of inclusion body contents which occurs during incubation of tissues in acid media.

E-600-resistant esterase activity has been studied in duodenal epithelium (Hugon & Borgers, 1967) and liver cells (Arstila & Trump, 1968) by electron microscopy, but no previous ultrastructural investigation of this enzyme in lung appears to have been undertaken. Recently, O'Hare, Reiss & Vatter (1971) studied the enzymes responsible for the hydrolysis of *p*-nitrophenylthiol acetate and *p*-nitrophenylthiol butyrate in developing and adult rat lung. They found that thiol esterase activity increased greatly just prior to the development of surface tension activity in lung homogenates. The demonstration of E-600-resistant esterase in the lamellated inclusion bodies and in association with membranous material lying free in the alveolar spaces lends support to the hypothesis of O'Hare *et al.* (1971) that esterases play a key role in the biosynthesis or degradation of pulmonary surfactant.

Reported studies of alkaline phosphatase activity in granular pneumonocytes have divergent results. Buckingham, McNary & Sommers (1964) described alkaline phosphatase activity in the lining membrane of inclusion bodies but no experimental details were given. Kuhn (1968), using the method of Hugon & Borgers (1966), demonstrated alkaline phosphatase in the apical plasma membrane of granular pneumonocytes; the few inclusions that contained reaction product were always situated in the apical cytoplasm, very close to the plasma membrane. In the present study, which also used the lead-glycerophosphate method of Hugon & Borgers (1966) together with the calcium-glycerophosphate method of Sabatini *et al.* (1963), there was no evidence of alkaline phosphatase activity even after prolonged incubation. The strong reaction obtained when test sections of kidney were processed by the same techniques tends to exclude the possibility that the negative results were due to defective technique.

The reaction product produced by the hydrolysis of ATP was localized within the apical plasma membrane and microvilli of granular pneumonocytes. The absence of reactivity when sodium β-glycerophosphate was substituted for ATP in the incubation medium suggests that the hydrolysis of ATP is not due to the action of non-specific alkaline phosphatase, although this possibility cannot be excluded. In this context, Novikoff, Hausman & Podber (1958) maintained that the enzymic activity demonstrated by the Wachstein-Meisel technique using ATP as substrate is most likely due to a specific ATPase rather than a non-specific phosphatase. Marchesi & Barrnett (1963) were of the same opinion. ATPase has previously been demonstrated on the microvilli of intestinal epithelium (Ashworth, Luibel & Stewart, 1963), the endothelium of capillaries (Marchesi & Barrnett, 1962), the basal infoldings of cell membranes in kidney tubules (Spater, Novikoff & Masek, 1958), and in the microvilli of bile canaliculi (Essner, Novikoff & Masek, 1958). Furthermore, there is a considerable body of biochemical evidence which suggests that ATPase plays a fundamental role in the transport of ions across the cell membranes of a number of tissues (Dunham & Glynn, 1961; Auditore, 1962). It appears likely, therefore, that ATPase is involved in active transport mechanisms in granular pneumonocytes, and in particular with the extrusion of surface-active agents from these cells.

A non-uniform distribution of ATPase activity throughout the population of granular pneumonocytes was observed in the present study. Differences have been reported in the staining responses of the surface membranes of bile canaliculi and sinusoids in liver (Essner, Novikoff & Masek, 1958) and in cultured HeLa cells (Epstein & Holt, 1963). The latter workers postulated that such heterogeneity could reflect cyclic changes in cell function. In granular pneumonocytes the intensity of the ATPase reaction could not be related to any particular morphological appearance of the microvilli or inclusion vesicles.

Acid hydrolases are commonly associated with the lysosomes of phagocytic cells (de Duve, 1963). The presence of acid phosphatase, aryl sulphatase and E-600-resistant esterase in the inclusion bodies of granular pneumonocytes is therefore unusual in view of the fact that these structures are generally regarded as being secretory vesicles. Nevertheless, the presence of such enzymes in secretory organelles is not restricted to the present case, since acid phosphatase has been demonstrated in secretory vesicles of paneth cells (Goldfischer, Essner & Novikoff, 1964) and anterior pituitary cells (Smith & Farquhar, 1966). The inclusion bodies of granular pneumonocytes would appear to provide a further example of lysosomal structures assuming a secretory role.

SUMMARY

The localization of acid phosphatase, aryl sulphatase, E-600-resistant esterase, alkaline phosphatase and ATPase in granular pneumonocytes of hamster lung was studied using electron microscope techniques.

Acid phosphatase, aryl sulphatase and E-600-resistant esterase activities were present in varying amounts in the lamellar inclusion bodies, while acid phosphatase and aryl sulphatase were also present in multivesicular bodies. ATPase was prominent in the apical plasma membrane and microvilli. Alkaline phosphatase activity was not detected.

The results support the hypothesis that the inclusion bodies of granular pneumonocytes are involved in the synthesis of pulmonary surfactant.

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