NORMAL RABBIT ALVEOLAR MACROPHAGES
II. Their Primary and Secondary Lysosomes as Revealed by Electron Microscopy and Cytochemistry*

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In addition to their well-recognized function in the phagocytosis and digestion of foreign materials which enter their environment, alveolar macrophages may also participate in the turnover of surfactant, as indicated by recent evidence from kinetic investigations (1–3). Moreover, it has recently been clearly documented that macrophages of the lung ingest tubular myelin and incorporate it into cytoplasmic vacuoles (4). But whether these are ultimately destined to become secondary lysosomes has not yet been established beyond question, since, to date, the intracellular distribution of digestive enzymes in these phagocytes has not been carefully analyzed. Thus, the possibility that there are cytochemical differences between the inclusions of macrophages cannot yet be ruled out. This project was undertaken, therefore, to investigate the subcellular localization of two lysosomal enzymes, acid phosphatase and arylsulfatase, in normal rabbit alveolar macrophages. The major objective of this study was twofold: (*a*) to ascertain whether the vacuoles containing surfactant are indeed secondary lysosomes or whether subpopulations of inclusions exist, and (*b*) to define the pathway of lysosomal enzyme transport from sites of production to sites of utilization in digestive vacuoles.

Materials and Methods

Materials. Alveolar macrophages were obtained from the lungs of five New Zealand albino rabbits.

Reagents for cytochemical procedures (grade I β-glycerophosphate and *p*-nitro-catechol sulfate) were obtained from Sigma Chemical Company, St. Louis, Mo., and pentobarbital (Beuthanasia Special) was procured from H. C. Burns Pharmaceuticals, Oakland, Calif.

Methods, Collection of Tissues. Glutaraldehyde fixative was used to wash the cells from the lung according to the procedure described in the preceding paper (4).

Fixation. For cytochemical procedures, cells were fixed for 10–30 min in 1.5% glutaraldehyde in 0.1 M sodium cacodylate-HCl buffer (pH 7.4) with 1% sucrose at room temperature.

Enzyme Procedures. After three washes in sodium cacodylate-HCl buffer, specimens were incubated in the following enzyme media, each containing 5% sucrose: (*a*) modified Gomori's medium (5) for acid phosphatase (ACPase),[1] pH 5.0, using β-glycerophosphate as substrate; (*b*)

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[1] *Abbreviations used in this paper:* ACPase, acid phosphatase; GERL, Golgi-endoplasmic reticulum-lysosomes; RER, rough endoplasmic reticulum.

Goldfischer's medium (6), pH 5.5, for arylsulfatase, with a subsequent treatment with 2% ammonium sulfide (7). Some enzyme incubations were conducted at 37°C for 30–90 min. Variations at 30°C for 2–3 h resulted in the same enzyme localizations but better tissue preservation and cleaner specimens.

Control Preparations. Control material for both ACPase and arylsulfatase consisted of incubations from which the substrates had been omitted. Additional control specimens for ACPase contained 0.01 M NaF as an inhibitor in the complete incubation medium.

Subsequent Processing. Specimens were then postfixed for 1 h at 4°C in 1% osmium tetroxide in acetate-Veronal buffer, pH 7.4, and 5% sucrose, and stained in block for 30 or 60 min at room temperature in 0.5% uranyl acetate in acetate-Veronal buffer plus 4% sucrose. Subsequent processing and examination was carried out as described in the companion paper (4).

Results

Localization of Lysosomal Enzymes Within Alveolar Macrophages

ACID PHOSPHATASE. Dense deposits of lead phosphate, the cytochemical marker for the enzyme, are localized within (*a*) many cisternae of the rough endoplasmic reticulum (RER) (Figs. 1 and 2); (*b*) many cisternae of the Golgi complex in most cells (Figs. 1 and 3); (*c*) smooth tubules and cisternae (designated Golgi-endoplasmic reticulum-lysosomes [GERL] by Novikoff [8]) in the Golgi region (Fig. 4); (*d*) small vesicles, sometimes coated, about 60–100 nm in diameter (Fig. 4); and (*e*) virtually all of the digestive vacuoles (Figs. 1, 2, and 4), in some of which tubular myelin (Fig. 5) and myelin figures (Fig. 9), apparently recently ingested, are clearly discernible. In other vacuoles, however, morphological detail was obscured by the sheer density of the precipitated reaction product. The intensity of the overall reaction varied from cell to cell.

ARYLSULFATASE. Dense precipitates of lead sulfide resulting from the tests for the enzyme are limited to (*a*) a rare short segment of the RER (inset Fig. 6); (*b*) an occasional outer cisterna of the Golgi complex; (*c*) smooth profiles of GERL in the Golgi region (Fig. 7); (*d*) small vesicles 60–100 nm in diameter (Fig. 8); and (*e*) almost all of the many secondary lysosomes (Fig. 6) in which on rare occasions tubular myelin was visible (inset Fig. 9). Reactive bristle-coated vesicles (approximately 60 nm in diameter) were occasionally seen in continuity with GERL elements in the Golgi region (Fig. 7). Reactive vesicles of similar size, but devoid of the bristle-coat were also seen in continuity with digestive vacuoles (Fig. 8).

Our results demonstrating the localization of enzymes in the endoplasmic reticulum are consistent with the biochemical findings of other investigators that alveolar macrophages are actively producing lysosomal enzymes (9, 10). The transport of these enzymes to digestive vacuoles appears to be carried out by 60-nm vesicles which appear in the Golgi region. Such vesicles, recognizable by their lysosomal enzyme content, are in continuity with elements of GERL and with digestive vacuoles.[2] Since these vesicles are membrane-limited bodies which contain lysosomal enzymes that have not yet met substrate, the vesicles qualify as primary lysosomes (11). No deposits of reaction-product for either enzyme were found over the extracellular tubular myelin or myelin figures.

[2] Although the Golgi cisternae were reactive for acid phosphatase, no evidence was obtained for the derivation of enzyme-filled vesicles from the Golgi cisternae proper, and in morphological preparations, vesicles were seldom seen in continuity with the Golgi cisternae.

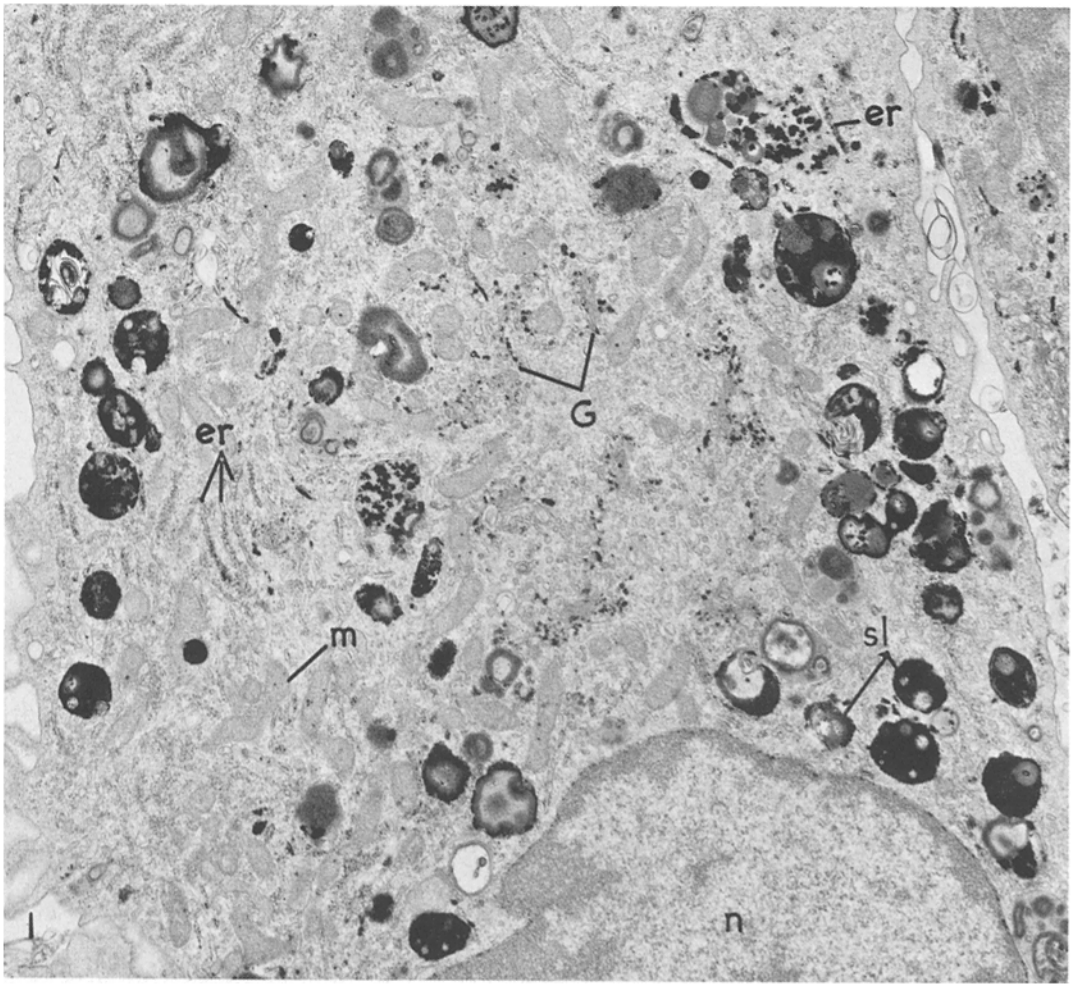


FIG. 1. Portion of a macrophage tested for the localization of acid phosphatase. Reaction produce for the enzyme is particularly prominent in the secondary lysosomes (sl). It is also demonstrable in some cisternae of the rough endoplasmic reticulum (er) and the Golgi complex (G). Mitochondria (m) are moderately numerous. Specimen fixed 15 min in 1.5% buffered glutaraldehyde, incubated in a modified Gomori's medium for 2 h at 30°C, postfixed 1 h at 4°C in Palade's OsO_4, stained in block 30 min in buffered uranyl acetate, and on grid briefly with lead citrate. × 11,200.

The presence of reaction-product for the lysosomal enzymes, acid phosphatase and arylsulfatase, in vacuoles containing tubular myelin indicates that these vacuoles are secondary lysosomes. Thus, our results indicate that phagocytized tubular myelin travels the same intracellular route as other ingested materials destined for digestion in alveolar macrophages.

Control Preparations. No reaction product for either ACPase or arylsulfatase was found in those specimens that were incubated in media lacking substrate. In tests for ACPase in which sodium fluoride was added to the complete incubation medium, the reaction was totally inhibited.

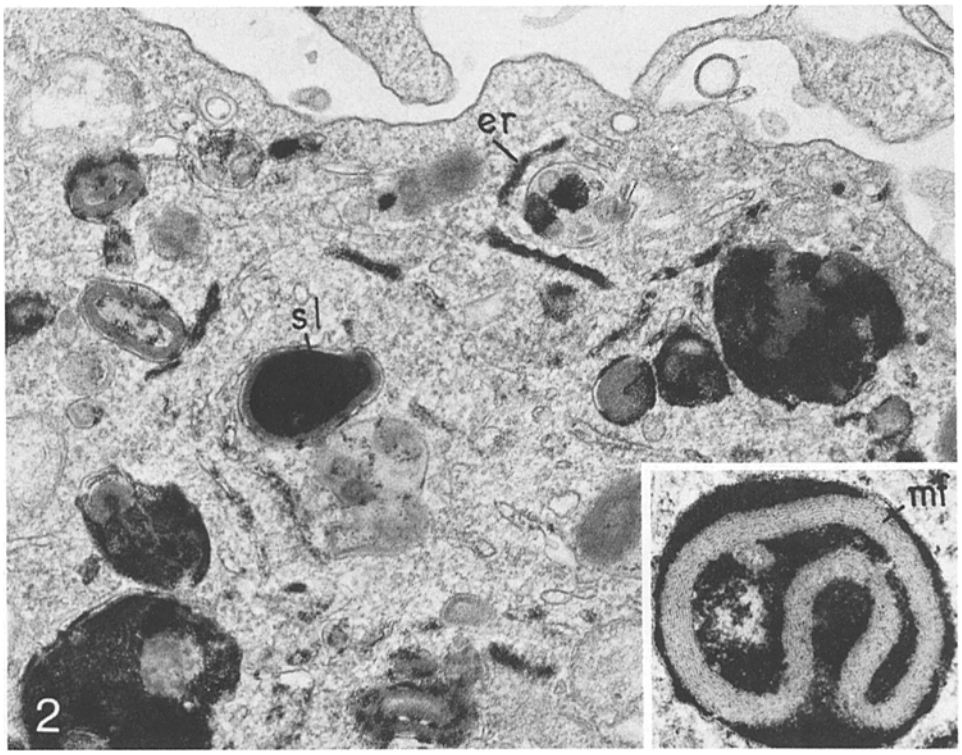


FIG. 2. Portion of a macrophage tested for acid phosphatase. This higher magnification view illustrates to better advantage the density of the reaction product in the RER (er). The internal structure of the secondary lysosomes (sl) is obscured by the lead precipitate. Inset. Higher magnification of a typical inclusion from a similar specimen. The densely reactive matrix provides sharp contrast to the curving myelin figure (mf). Its evenly spaced membranes display only a minimal amount of finely scattered reaction product. Specimen preparation as in Fig. 1. × 31,000. Inset, × 60,000.

Discussion

Tubular Myelin in the Secondary Lysosomes of Macrophages. That the cytoplasmic inclusions which contain tubular myelin are true secondary lysosomes in macrophages has been proved by our observations in this study, for these organelles manifest digestive enzyme (acid phosphatase and arylsulfatase) activity. Moreover, Franson and Waite (12) isolated similar lysosomes in a fraction from rabbit alveolar macrophages and found that they contain phospholipases A1 and A2, which hydrolyze phospholipids such as surfactant at an acid pH (also see 3). In earlier investigations, lysophospholipase (13) and other lipases (14, 15) were additionally demonstrated biochemically in alveolar macrophage homogenates. Evidently, then, these phagocytes possess the enzymatic equipment to digest surfactant after it is engulfed and incorporated into secondary lysosomes. Yet the relative importance of macrophages in disposing of surfactant as opposed to alternatively proposed mechanisms (2) remains to be resolved.

The Transport of Digestive Enzymes to Phagosomes. The primary lysosomes

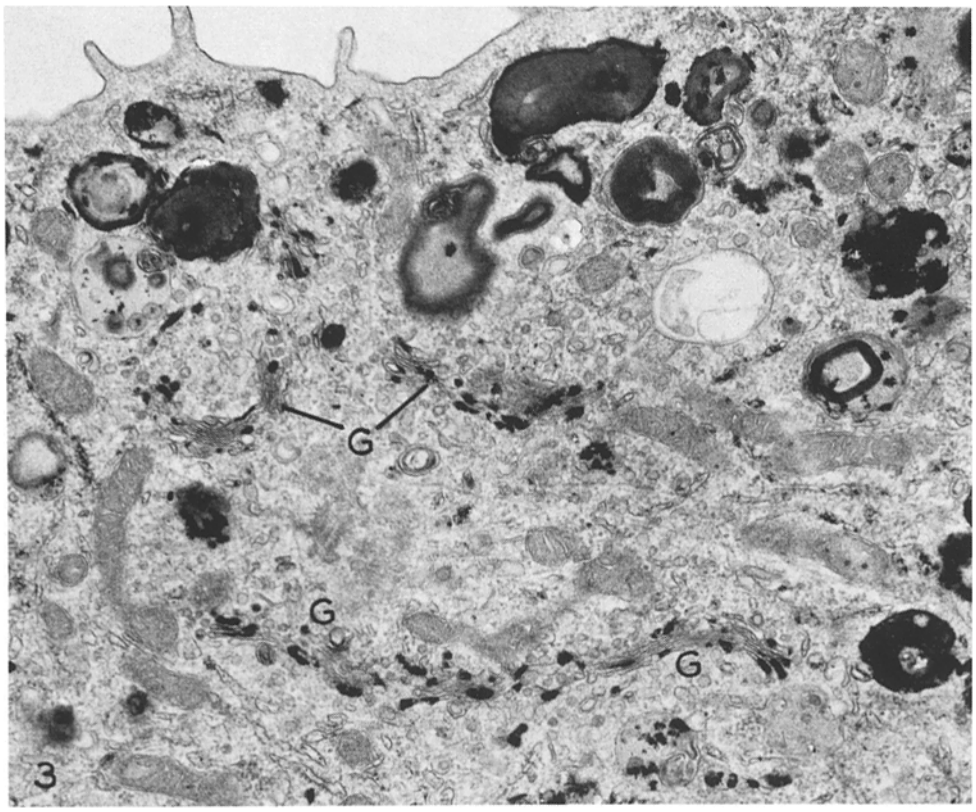


FIG. 3. Portion of a macrophage tested for acid phosphatase. This view of the Golgi region demonstrates that all of the cisternae within each Golgi stack (G) contain some enzyme reaction product but its distribution is spotty and it appears to be more concentrated at the ends of the saccules. Specimen prepared as in Fig. 1. × 20,000.

of alveolar macrophages are small vesicles (60 nm in diameter) originating in GERL (16). Recently, Essner and Haimes (17) reported a similar source of primary lysosomes in alveolar macrophages of the beige-mutant mouse. The vesicles, identified by their enzyme content, sometimes bear bristle coats when in continuity with GERL. Other small coated vesicles, devoid of enzyme reactivity, presumably result from pinocytic activity (18).

In addition to the primary-lysosomal route, there is another avenue of digestive enzyme transport to phagosomes in alveolar macrophages, that is, through fusion with already existent secondary lysosomes. In the experiments of Stossel et al. (19) involving macrophages with ingested albumin-coated paraffin-oil droplets, electron microscopy of the lysosomal fraction disclosed that some of the large, dense, heterogeneous inclusions had fused with phagocytic vacuoles. We observed a related phenomenon in our experiments when we occasionally found two secondary lysosomes in continuity, apparently after fusion of their membranes.

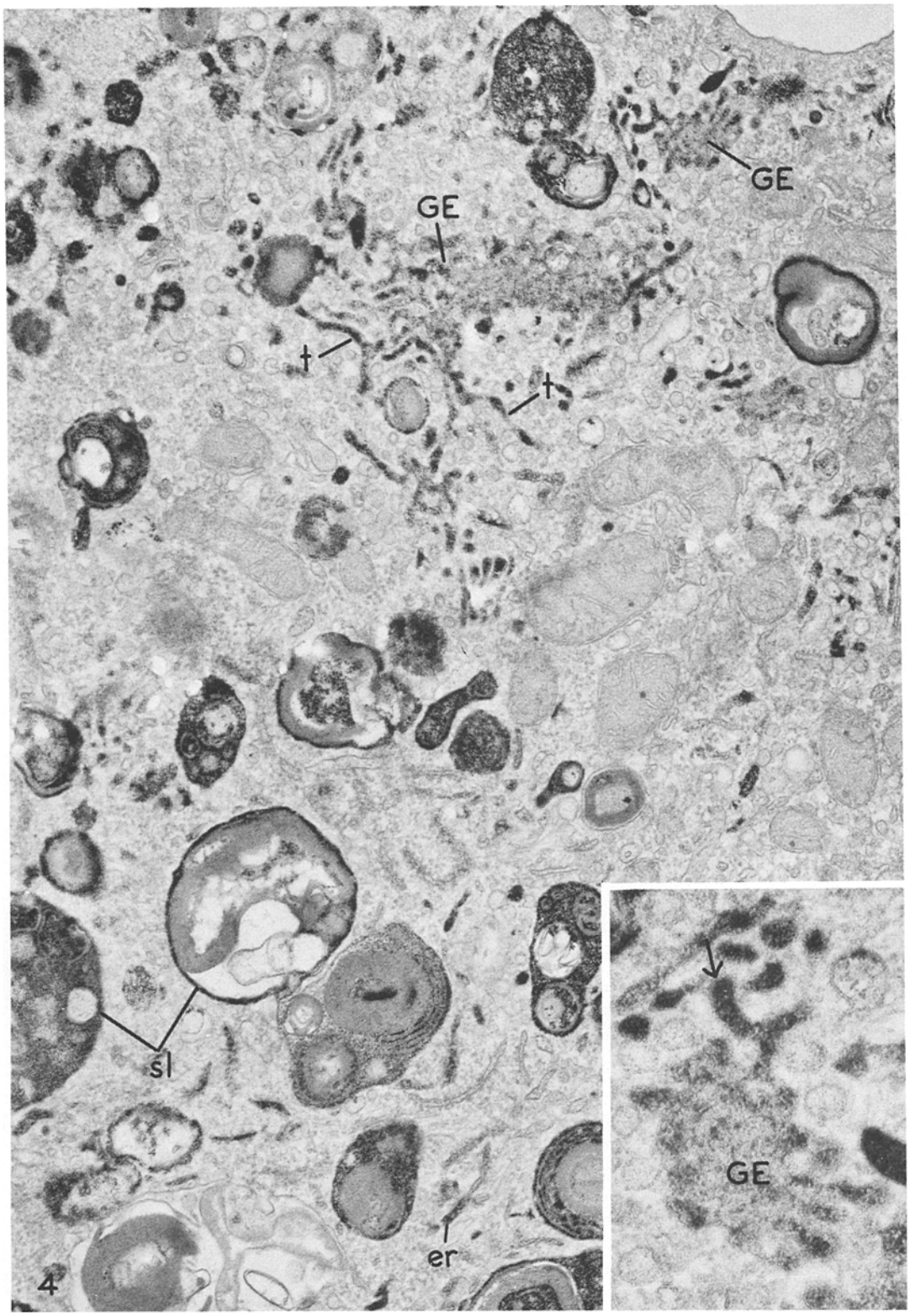
GERL. The constellation of cisternae and tubules known as GERL is particularly well-delineated in alveolar macrophages. Novikoff (16) first described

GERL in neurons, and it has since been discovered in many diverse tissues (7, 20–23). Several aspects of form and enzymatic properties are considered to establish this as an entity related to the Golgi complex yet distinct from it (8). GERL lies near the inner face of the Golgi complex. Its structure comprises unfenestrated cisternal portions from which anastomosing tubules extend irregularly into neighboring areas of cytoplasm where they may be in continuity with the rough endoplasmic reticulum (ER). For this reason, Novikoff believes the tubular part of GERL to be a specialization of the smooth ER. ACPase and other acid hydrolases are concentrated in GERL and packaged into primary lysosomes. Thus, GERL resembles both rough ER and lysosomes in enzyme content. Because of its intimate spatial relationship to these cytoplasmic constituents, Novikoff considers it to serve as an intermediate point in the pathway of enzyme transport from sites of synthesis to those of utilization. In the present study of alveolar macrophages, we have demonstrated a structure much resembling GERL (according to his description) in location, morphology, and enzyme content, supporting his hypothesis that GERL is indeed a unique entity.

Differences in the Localization of the Two Lysosomal Enzymes. The distribution of the two lysosomal enzymes was found to differ slightly, i.e., the more abundant ACPase was demonstrable in Golgi cisternae as well as in GERL, whereas arylsulfatase appeared in GERL alone. Several factors should be borne in mind in evaluating these results: First, the two enzymes may truly vary in localization by following divergent intracellular pathways after synthesis on the RER. For example, perhaps ACPase requires some processing step in the Golgi complex not required by arylsulfatase. And second, the variation in distribution may have a technical basis. It is well-known that the fixation procedure destroys some enzymatic activity.[3] Therefore levels of arylsulfatase in the Golgi cisternae may be so low that adequate amounts for demonstration fail to survive fixation, whereas ACPase remains detectable. Even more difficult to determine is the specificity of the phosphatase reaction: some phosphatases with acid pH optima are nonlysosomal (25, 26). These include acid phosphatases which have been localized at pH 5.0 in the Golgi cisternae of certain secretory cells (27). This dilemma cannot be resolved using current cytochemical techniques. Hence on the basis of past experience, localization of ACPase in the Golgi cisternae is not easy to interpret. However, no positive evidence has emerged for the production of primary lysosomes from the Golgi cisternae, although the data clearly indicate that they arise from GERL.

The Lysosomal Enzyme Content of Lamellar Bodies. Paradoxically, in type II alveolar cells, surfactant is produced in organelles with digestive enzyme activity. The enzymatic constituents of lamellar bodies have been investigated by means of cytochemical techniques suitable for light (28) and electron microscopy (29). Although these experiments have documented the presence of ACPase (28, 29) and arylsulfatase (29), surfactant is apparently not digested but rather synthesized in lamellar bodies. Several theories may suffice to explain this

[3] In studying the effects of fixation on ACPase survival, Seeman and Palade (24) found 80% retention of enzyme after 10-min fixation in 1 or 2% glutaraldehyde, but levels decreased to 20% after 90 min.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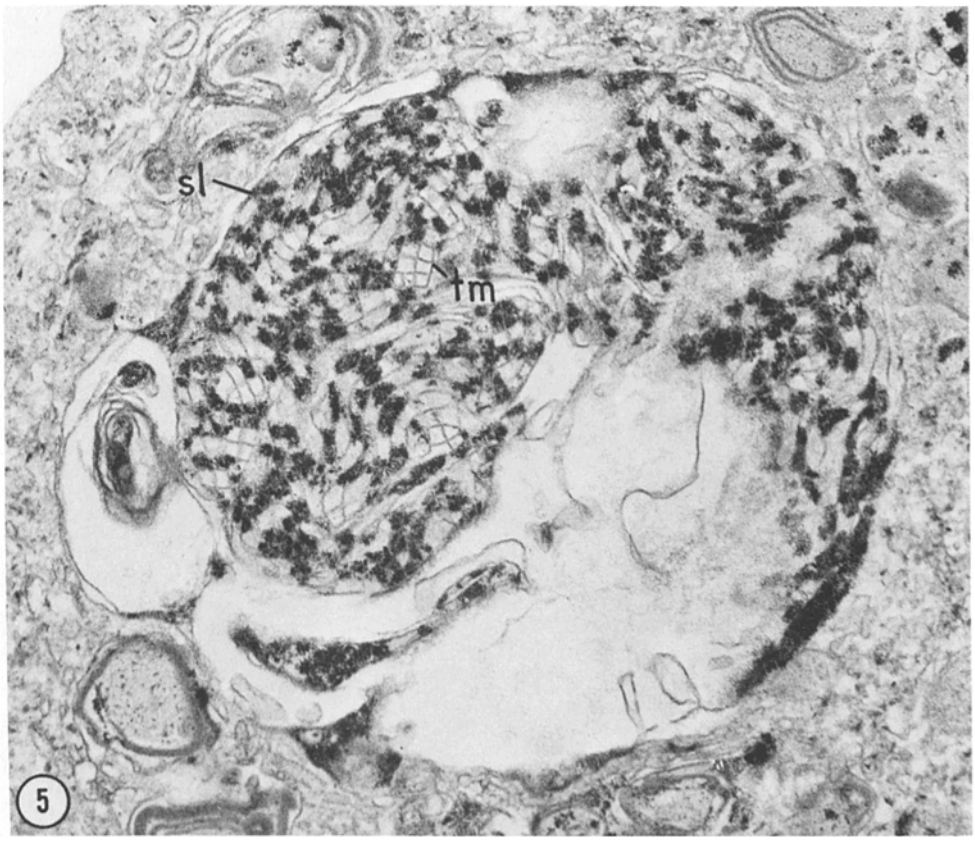

FIG. 5. Portion of a macrophage tested for acid phosphatase. A large secondary lysosome (sl) densely reactive for acid phosphatase contains a disorganized array of tubular myelin (tm) in which some squares of the latticework are still intact. The constant spacings between some of the tubules are still evident. Specimen prepared as in Fig. 1. × 40,500.

enigma. Possibly surfactant is not susceptible to digestion until some extracellular alteration has taken place in the substance. Actually, tubular myelin phagocytized by macrophages may represent used or denatured surfactant. Or in lamellar bodies there may be other, internal regulatory mechanisms such as inhibitors or temporal changes in pH. It is also conceivably possible that the hydrolases function by cleaving certain materials with products that are subsequently needed for synthetic pathways. For example, phospholipases them-

FIG. 4. Portion of a macrophage tested for acid phosphatase, illustrating dense deposits of reaction product in the RER (er) and secondary lysosomes (sl). Two reactive GERL cisternae (GE) are viewed *en face*. (It was difficult to identify the larger reactive cisterna since it is sectioned somewhat obliquely. However, it is interpreted as GERL (GE) because it contains reaction product throughout. The Golgi cisternae (See Fig. 3) differed in appearance because they have a discontinuous distribution of reaction product.) Smooth tubules (t) extend from the cisterna and ramify into the neighboring cytoplasm, but no continuities with the RER (er) or secondary lysosomes are observed. When a portion of this cell is viewed at higher magnification, (Inset) a coated vesicle is seen in continuity with one of the GERL tubules (arrow). Specimen preparation as in Fig. 1. × 27,000. Inset, × 80,000.

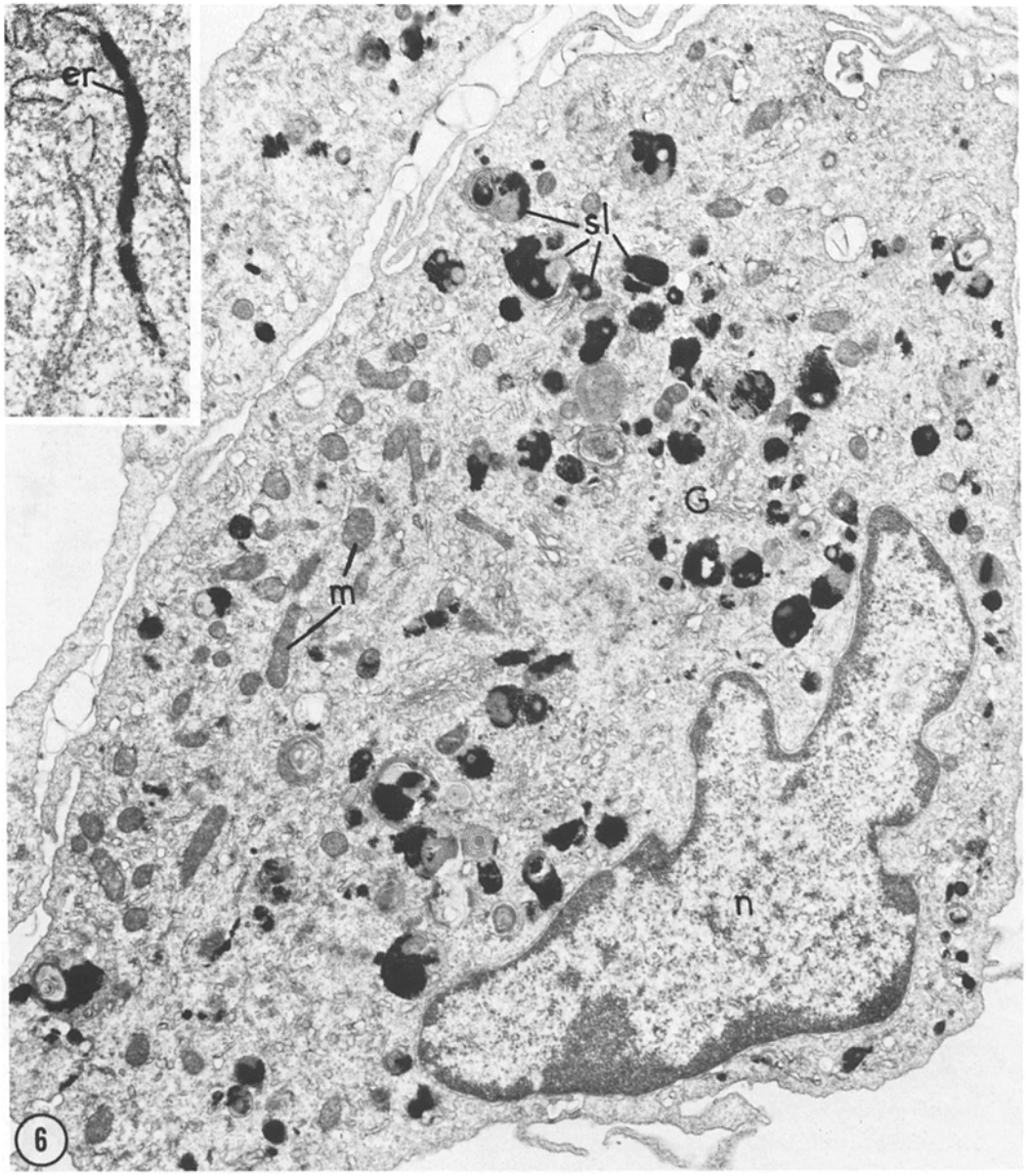

FIG. 6. Alveolar macrophage prepared for the demonstration of arylsulfatase activity. Many, but not all, of the secondary lysosomes (sl) display dense deposits of reaction product for the enzyme. Rarely, a short segment of RER (er) is reactive (Inset). Golgi complex is seen at (G) and mitochondria are seen at (m). Specimen briefly fixed in glutaraldehyde, incubated 2 h at 30°C in Goldfischer's medium for arylsulfatase. The reaction product was converted to lead sulfide by ammonium sulfide treatment. Subsequent processing was the same as for the specimen in Fig. 1. × 10,500. Inset, × 48,000.

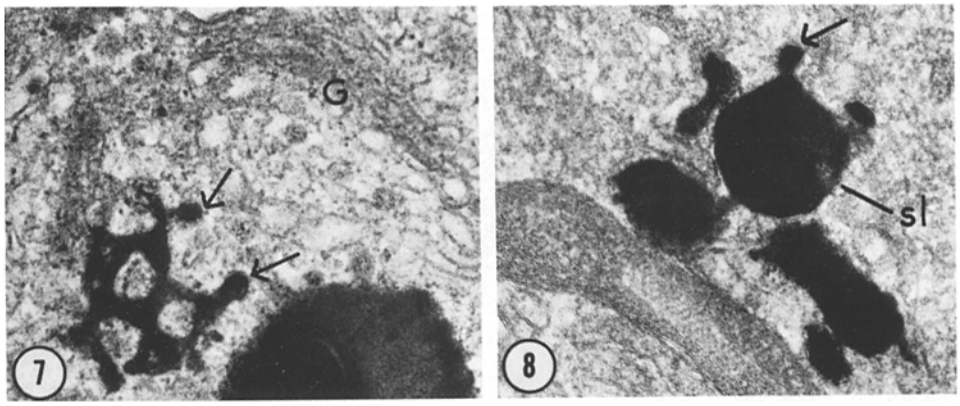

FIG. 7. Golgi region from a macrophage tested for arylsulfatase. Dense deposits of reaction product are present in a smooth tubular system (t) adjacent to the unreactive Golgi complex (G) which is sectioned obliquely. Note the bristle coats on two vesicles (arrows) which are in continuity with the GERL tubules (t). Specimen was prepared as in Fig. 6. × 57,500.

FIG. 8. Small part of a macrophage tested for arylsulfatase, illustrating a reactive vesicle (arrow) (in size and enzyme content like those apparently budding from GERL in Fig. 7) in continuity with a secondary lysosome (sl). Specimen prepared as in Fig. 6. × 55,000.

selves may participate in phospholipid cycles leading to synthesis as well as hydrolysis (30). In any event, solutions to these problems must await more extensive biochemical analyses of isolated lamellar bodies.

A Shift in Primary Lysosome Packaging within Mononuclear Phagocytes. Our earlier work has established that the cells that precede alveolar macrophages in development, the blood monocytes, contain primary lysosomes differing from those of the macrophages (31). In the monocyte, they consist of preformed storage granules fashioned during maturation in the bone marrow. Relatively few cell types (notably leukocytes) store hydrolytic enzymes before their use in digestion (32). In the course of phagocytosis, leukocyte granules become depleted by fusion with phagosomes, and neutrophils and eosinophils then terminate their brief life-span. Monocytes are unusual in this connection: as they degranulate, they enlarge and differentiate into macrophages. As this happens, they shift to a variant mode of packaging lysosomal enzymes. Like most other cells in which primary lysosomes have been explored, enzyme transit then occurs via small vesicles originating in the Golgi region. And the source of such vesicles in GERL has now been determined in increasing numbers of cell types (as we have found here in alveolar macrophages).

Summary

In this investigation, vacuoles containing tubular myelin proved to be digestive compartments with cytochemical reactivity for acid phosphatase and arylsulfatase. These cytochemical markers identify the secondary lysosomes, known to contain enzymes capable of hydrolyzing phospholipids like surfactant. Therefore, it appears that alveolar macrophages possess the enzymatic machinery for the degradation of the tubular myelin found in their digestive vacuoles. Although it thus appears evident that alveolar macrophages participate in the

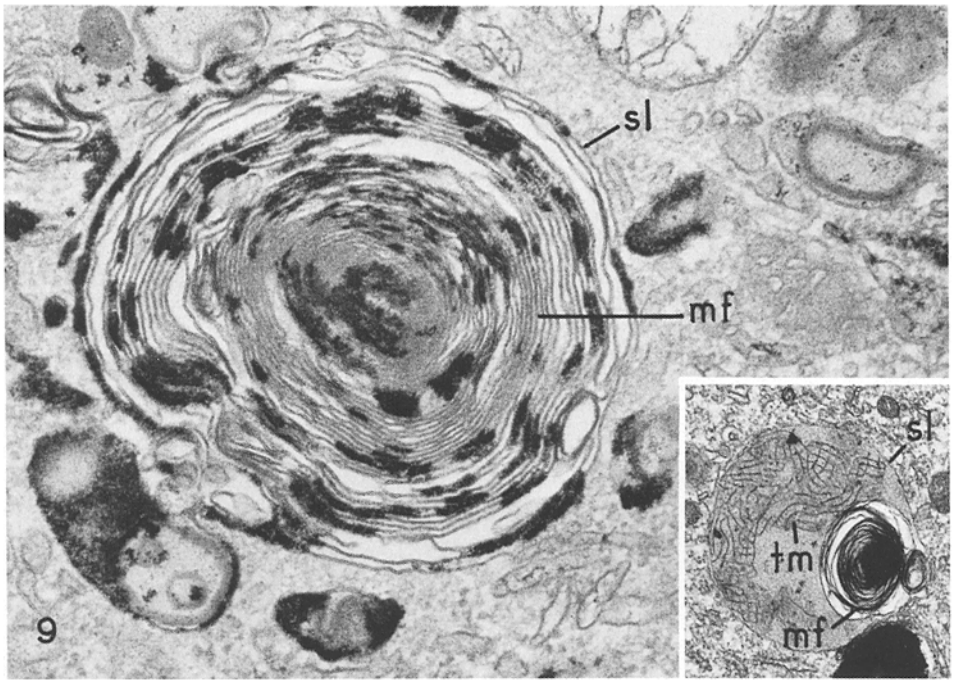

FIG. 9. Portion of a macrophage tested for acid phosphatase. The secondary lysosome (sl) contains a large myelin figure (mf), possibly only recently ingested. In structure, it resembles the extracellular myelin figures which are sometimes in continuity with the tubular myelin. The membranes of the myelin figure form an irregular pattern against the matrix of low density. Abundant reaction product for acid phosphatase is widely distributed throughout the digestive vacuole. Inset. Portion of a macrophage tested for arylsulfatase. Residues such as tubular myelin (tm) and myelin figures (mf) are also seen within vacuoles containing arylsulfatase activity, suggesting the digestion of these bodies within macrophages. Fig. 9, specimen prepared as in Fig. 1. × 50,000. Inset, specimen prepared as in Fig. 6. × 65,000.

turnover of surfactant, the quantitative significance of this route of disposal is undetermined.

This investigation has also established that acid hydrolases, so prominently displayed in the secondary lysosomes, are also localized in the rough endoplasmic reticulum and in Golgi-endoplasmic reticulum-lysosomes (GERL). Moreover, small vesicles which are produced from GERL serve as primary lysosomes in transporting digestive enzymes to the vacuoles.

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