Isolation and Properties of Phagocytic Vesicles

II. ALVEOLAR MACROPHAGES

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ABSTRACT Phagocytic vesicles were obtained by density gradient centrifugation of homogenized rabbit alveolar macrophages that had ingested emulsified paraffin oil contained Oil Red O. The phagocyte vesicles floated and thereby were separated from the soluble fraction and from other cell components which sedimented. The purity of the isolated vesicles was documented by electron microscopy, chemical and enzyme analysis. The vesicles contained 87% of the cell-associated Oil Red O, and were essentially free of DNA, RNA, succinic dehydrogenase, and glucose-6-phosphatase. Acid phosphatase, β-glucuronidase, and catalase were transferred from the sedimenting fraction to the phagocytic vesicle fraction during phagocytosis, whereas enzyme activities of the soluble fraction remained unchanged. Half of the catalase of resting macrophages was in the pellet fraction and, compared with acid phosphatase, greater amounts of digitonin were required to release full activity. Such differential latency has been described for enzymes of peroxisomes vs. those of lysosomes. Compared with polymorphonuclear leukocyte vesicles studied previously, phagocytic vesicles of macrophages had more electron-dense material and lower Oil Red O: protein, phospholipid: protein, and enzyme: protein ratios. It is thus probable that secondary lysosomes become part of the macrophage vesicle. When paraffin oil particles, the stimulus for phagocytic vesicle formation, were washed away from the macrophages, acquisition of hydrolases by preformed vesicles ceased, i.e. transfer of these enzymes into phagocytic vesicles occurred only during or shortly after the formation of new vesicles. As noted previously by others, the content of acid hydrolases of stimulated alveolar macrophages was doubled in comparison to normal cells. The difference between stimulated and normal macrophages was even more marked when isolated phagocytic vesicles were analyzed. Vesicles from stimulated macrophages had 3–5 times more enzyme activity (per milligram of vesicle protein or per amount of paraffin oil ingested) than did vesicles from normal cells.

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INTRODUCTION

Alveolar macrophages contain acid hydrolases which are compartmentalized within small vesicles and electron-dense granules (1). Some of the latter structures are probably secondary lysosomes, residua of earlier endocytic events. During phagocytosis there is an intracellular rearrangement of these enzymes in which they are transferred to phagocytic vesicles, a process similar to the degranulation phenomenon of polymorphonuclear leukocytes (2, 3). Alveolar macrophages collected from animals previously challenged with certain bacterial products (stimulated macrophages) have higher concentrations of acid hydrolases (4) and greater bactericidal activity than do cells from untreated animals (5).

We have recently reported quantitative biochemical studies of degranulation in polymorphonuclear leukocytes using a new technique for collecting phagocytic vesicles in an isolated state (6). We describe here some characteristics of phagocytic vesicles obtained in a high state of purity from rabbit alveolar macrophages by a similar procedure and a comparison of the enzyme content of phagocytic vesicles from stimulated macrophages and from normal cells.

METHODS

Alveolar macrophages were obtained from male New Zealand rabbits by pulmonary lavage with 0.15 M NaCl (7). In order to increase the yield of cells, most of the rabbits were injected intravenously with 1 ml of Freund's adjuvant (Difco Laboratories, Detroit, Mich.) approximately 4 wk before cells were harvested (stimulated macrophages). The cells were washed once with ice-cold Krebs-Ringer phosphate medium, pH 7.4, containing one-third the usual concentration of $CaCl_2$. This medium is referred to below as Krebs-Ringer phosphate medium. The cells were filtered through a silk screen and suspended in the same medium.

Droplets of heavy paraffin oil, the substrate for phagocytosis, were prepared by sonic emulsification of the hydrocarbon (Fisher Scientific Company, Pittsburgh, Pa.) in Krebs-Ringer phosphate medium with bovine albumin (Fraction V from bovine serum, Armour Pharmaceutical Co., Chicago, Ill., lot D-26304), 20 mg/ml, as previously described (6). When paraffin oil particles without protein were desired, starch (soluble starch for iodimetry, Merck Chemical Division, Merck & Co., Inc., Rahway, N. J.) was substituted for albumin as the emulsifier. All data, unless otherwise specified, were obtained using albumin-coated droplets. To measure the rate and extent of phagocytosis, the paraffin oil was colored with Oil Red O (Allied Chemical Corp., New York) as described previously (1).

Measurement of the oxidation of glucose-1-^{14}C (47.8 mCi/mmole) and glucose-6-^{14}C (44.9 mCi/mmole to $^{14}CO_2$ by alveolar macrophages was performed as previously described (6). Radioactive glucose substrates were purchased from New England Nuclear Corp., Boston, Mass.

Alveolar macrophages were incubated in Krebs-Ringer phosphate medium containing 4.5 mM glucose, with 0.2 vol of paraffin oil emulsion (phagocytosis) or, for control cells, 0.2 vol of a solution of bovine albumin or soluble starch, 2 mg/ml, depending on the composition of the emulsion. Usually the concentration of cells was about 15% (v/v) or approximately 10 mg cell protein per ml. Incubations were terminated by addition of ice-cold phosphate buffered saline, pH 7.4, and cells were washed as described for polymorphonuclear leukocytes (6). The rate of phagocytosis was determined by extraction of Oil Red O from washed cells, and Oil Red O is expressed in terms of micromoles of Oil Red O or milligrams of paraffin oil, as previously described (6).

Procedures for homogenization and fractionation of washed alveolar macrophages were slightly modified from those used for polymorphonuclear leukocytes (6). Washed cells were suspended in 0.34 M sucrose containing 1 mM Tris, pH 7.5, and 500 U heparin/ml (usually 6 times the pellet volume), and paraffin oil emulsion was added to the control macrophages. Cells were homogenized with a tight fitting Dounce homogenizer maintained at ice bath temperature until all macrophages were disrupted, as determined by phase contrast microscopic examination. Alveolar macrophages required more vigorous homogenization (up to 50 strokes) for total cell breakage than did polymorphonuclear leukocytes, and it was difficult to avoid generating small bubbles during this step. The marked change in viscosity which signaled complete disruption of granulocytes was much less evident with macrophages.

4 ml of homogenate was layered over 3 ml of 0.45 M sucrose containing 1 mM Tris, pH 7.5, in a 12 ml cellulose nitrate ultracentrifuge tube, and was covered with 4 ml of 0.25 M sucrose with 1 mM Tris, pH 7.5. The concentrations of sucrose in the homogenizing medium and in lower wash layer were slightly higher than those used in fractionating polymorphonuclear leukocytes, because the macrophage homogenates had a tendency to stream upward into the upper wash layer due to the bubbles produced during homogenization. By increasing the sucrose concentrations, or interposing a small amount of 0.30 M sucrose at the 0.36–0.25 M sucrose interface, this difficulty was largely eliminated. After centrifugation at 100,000 *g* for 1.5 hr, the phagocytic vesicles, control floating fraction, pellets, and supernatants fluid fractions were collected and stored essentially as described for polymorphonuclear leukocytes (6). These fractions differed somewhat in gross appearance from the polymorphonuclear leukocyte gradient fractions. The macrophage phagocytic vesicles were not packed as tightly as the granulocyte floating fractions, and hence were often aspirated from the top of the gradient with a Pasteur pipette rather than lifted off with a spatula. Small fragments of floating material adhered to the pellet by a "string" and tended to collect at the interface of the supernatant fluid fraction and the upper wash layer. These remnants were collected and pooled with the phagocytic vesicle fractions. Prolongation of the centrifugation to 2 hr decreased but did not totally eliminate this tendency.

Protein, DNA, RNA, lipid phosphorus and Oil Red O, acid phosphatase, alkaline phosphatase, β-glucuronidase, peroxidase, catalase, and succinic dehydrogenase were determined as described previously (6). Urate oxidase was measured by the method of Schneider and Hogeboom (8) and glucose-6-phosphatase by the method of Swanson (9). Samples were obtained and processed for electron microscopy as previously described (6).

RESULTS

Giemsa stained smears of cell suspensions obtained from rabbits previously injected with Freund's adjuvant revealed that 90% of the cells were large mononuclear cells with small round nuclei, apparently alveolar macrophages. The remainder of the cells were heterophils. Similarly, in smears of cells collected from uninjected rabbits, over 90% were macrophages. Rabbit alveolar macrophages reportedly lack alkaline phosphatase (7), and homogenates prepared from the cells used in this study also were lacking in this enzyme. Since rabbit heterophils have alkaline phosphatase (10), the fact that we did not detect this enzyme in our preparations provides biochemical evidence that heterophil contamination did not substantially affect our results.

Uptake of paraffin oil emulsion particles. Alveolar macrophages ingested albumin-coated or starch-coated oil droplets at similar rates. Phagocytosis of paraffin oil emulsion was inhibited by 1 mM *N*-ethylmaleimide and by 1 mM KCN. Fresh rabbit serum markedly enhanced the rate of uptake of these particles. Serum was not used in the studies reported here in order to facilitate comparison of the macrophage phagocytic vesicles with those from polymorphonuclear leukocytes (6). Serum had no effect on the ingestion of paraffin oil emulsion by polymorphonuclear leukocytes. Phagocytosis of paraffin oil particles elicited stimulation of glucose oxidation by alveolar macrophages. The rate of

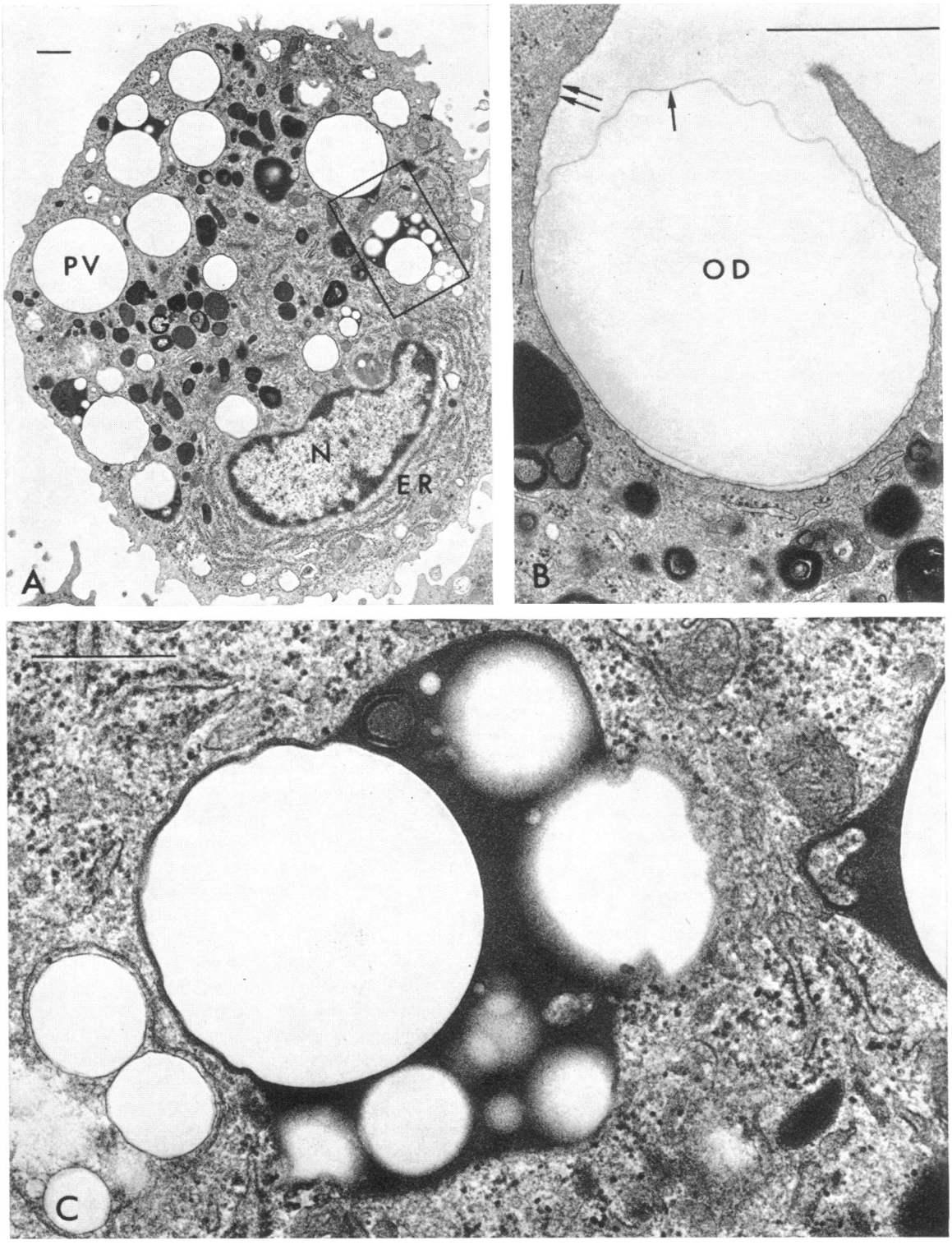

FIGURE 1 Electron micrographs of thin sections of rabbit alveolar macrophages which had ingested droplets of oil emulsion for 45 min. The oil has been extracted during the preparation for electron microscopy. *A*. This is a low magnification view of a macrophage containing

oxidation of glucose-1-^{14}C was increased from 2- to 9-fold, and of glucose-6-^{14}C from 8- to 17-fold.

Morphology of macrophages ingesting oil droplets and of isolated phagocytic vesicles. Stimulated alveolar macrophages contained numerous electron-dense bodies of varying size and shape. Many of these bodies which contained myelin figures and other debris are probably secondary lysosomes. After ingestion of paraffin oil droplets, the macrophages contained membrane-bounded cytoplasmic vesicles enclosing one or more of these droplets and a variable amount of dense material identical in appearance to that found in the cell's secondary lysosomes (Fig. 1). The paraffin oil was extracted during preparation of the cells for electron microscopy so that the space formerly occupied by the oil droplet was electron lucent. The electron-dense perimeters of the lucent areas presumably correspond to the albumin coating of the emulsion droplets. As shown in Fig. 1, both the dense lysosomal contents and the debris associated with the secondary lysosomes was incorporated into the newly formed droplet-containing phagocytic vesicles.

The floating fraction from the macrophage homogenates consisted of membrane-bounded phagocytic vesicles (Fig. 2). All of the isolated phagocytic vesicles contained oil droplets (as defined above) and most contained, in addition, dense material and secondary lysosomal debris. Their appearance was identical to that of the phagocytic vesicles in intact cells. The trilaminar membrane surrounding these isolated phagocytic vesicles was frequently intact, but it was often broken in one or more places. Rupture of these membranes may have occurred during processing for electron microscopy or possibly during homogenization or fractionation of the cells. The only significant morphologic contamination of the isolated phagocytic vesicles in the floating fraction was a few mitochondria and a very rare smooth membrane, presumably from the Golgi complex.

Distribution of chemical components among subcellular fractions. Homogenates prepared from macrophages that had engaged in phagocytosis for 45 min invariably contained less total protein, DNA, RNA, and phospholipid than did homogenates of resting cells (up to 21%). The percentage losses of all these cellular components were of equal magnitude (Table I).

TABLE I

Chemical Composition and Enzyme Specific Activities of Rabbit Alveolar Macrophage Homogenates

	Control	Phagocytosis	No. preparations
Protein	8.86 ±0.73	6.88 ±0.79	6
Phospholipid	6.56 ±0.49	6.48 ±0.38	4
DNA	28.47 ±1.24	26.87 ±0.94	4
RNA	64.30 ±0.20	59.30 ±6.80	2
Oil Red O	—	10.70 ±1.80	6
Acid phosphatase I	0.267 ±0.11	0.264 ±0.29	2
Acid phosphatase II	0.205 ±0.16	0.211 ±0.17	6
β-Glucuronidase	2.67 ±0.29	2.82 ±0.48	4
Catalase	0.199 ±0.023	0.197 ±0.027	6
Succinic dehydrogenase	2.06 ±0.16	2.13 ±0.23	4
Glucose-6-phosphatase	0.163 ±0.245	0.153 ±0.292	2

Homogenates were prepared from macrophages incubated for 45 min with or without paraffin oil emulsion. Protein content is expressed as milligrams per milliliter of homogenate; phospholipid in micrograms lipid phosphorus per milligram protein; DNA and RNA in micrograms per milligram protein; Oil Red O in micromoles per milligram protein; acid phosphatase I in micromoles orthophosphate released from β-glycerophosphate/minute per milligram protein; acid phosphatase II in micromoles *p*-nitrophenol released from *p*-nitrophenyl phosphate/minute per milligram protein; β-glucuronidase in nanomoles of product produced/minute per milligram protein; catalase in units per milligram of protein; succinic dehydrogenase in micromoles of succinate oxidized/minute per milligram of protein; glucose-6-phosphatase in micromoles of orthophosphate released/15 min per milligram of protein.

* Means ±SEM are given where more than two preparations were analyzed; otherwise means and ranges are given.

The phagocytic vesicle fraction contained 83–94% of the cell-associated Oil Red O (Table II). The remainder was in the pellet fraction. The floating vesicle fraction was free of contamination by nucleic acids (Table II) and mitochondrial or microsomal enzymes (Table III). The recovery of a large percentage of nucleic acids in the supernatant fluid reaction was presumably a result of destruction of nuclei during homogenization. Phagocytosis did not change the distribution of DNA or RNA. As shown in Table II, the supernatant fluid fractions from macrophages contained small amounts of phospholipid (fractions from polymorphonuclear leukocytes contained none), perhaps due to the presence of microsomal fragments. Some glucose-6-

numerous phagocytic vesicles (PV) enclosing one or more oil droplets and some dense material. Other prominent features of the cell are numerous dense granules (G), endoplasmic reticulum (ER), and the nucleus (N). Bar represents 1 μm, × 5,600. *B.* This micrograph shows a macrophage in the process of ingesting an oil droplet. The center of the droplet appears clear because the oil has been extracted, but the albumin coating its surface has been preserved as a single dark line (single arrow). The plasma membrane of the macrophage is marked with double arrows. The cytoplasm contains several dense granules, some of which have myelin figures and other debris, and therefore, are thought to be secondary lysosomes. Bar represents 1 μm, × 28,000. *C.* This is a higher magnification of the area enclosed in the box in *A*, showing phagocytic vesicles containing oil droplets, dense material, and some debris characteristic of secondary lysosomes. Bar represents 0.5 μm, × 46,000.

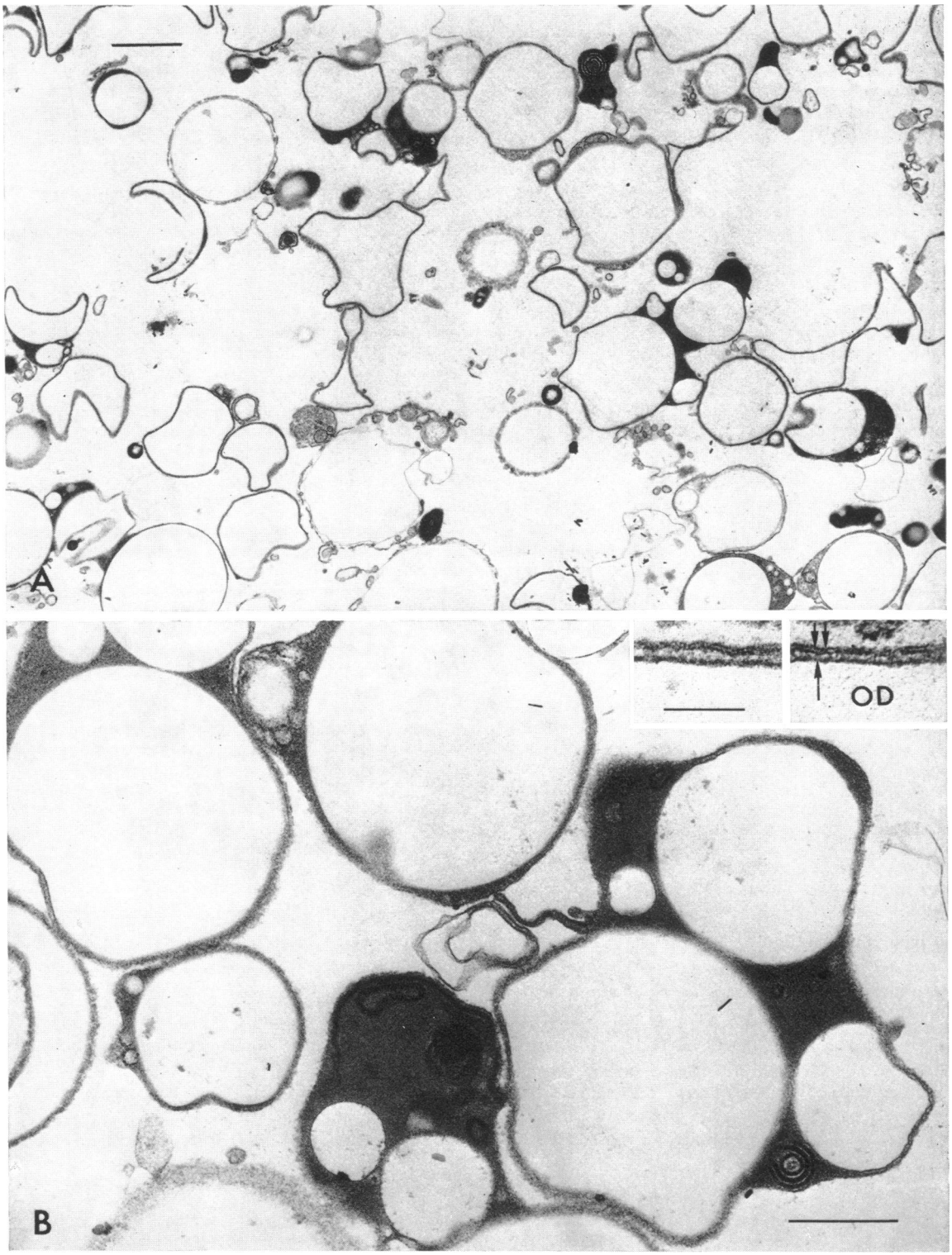

FIGURE 2 Electron micrographs of representative thin sections of the floating fraction containing isolated phagocytic vesicles. *A*. This micrograph shows that the fraction consists of highly purified phagocytic vesicles containing oil droplets, dense material, and some debris. Many of

TABLE II

Distribution of Chemical Components in Subcellular Fractions of Rabbit Alveolar Macrophages

	Supernatant fluid		Pellet		Phagocytic vesicles		Total recovered		No. of preparations
	Control	Phagocytosis	Control	Phagocytosis	Control	Phagocytosis	Control	Phagocytosis	
	% of total recovered						%		
Protein	58.1 ±1.5*	58.8 ±2.0	41.9 ±1.5	35.6 ±2.1	—	5.6 ±0.5	87.9 ±4.8	85.3 ±3.3	6
Oil Red O	0	0	—	12.8 ±2.0	—	87.2 ±2.0	—	91.3 ±4.2	6
DNA	80.9 ±2.9	81.3 ±1.1	19.1 ±2.9	18.7 ±1.1	0	0	72.2 ±5.9	80.1 ±9.4	4
RNA	46.5 ±1.1	60.3 ±12.3	53.5 ±1.1	39.7 ±12.3	0	0	98.8 ±0.8	91.7 ±2.5	2
Phospholipid	6.1 ±0.8	5.5 ±0.6	93.5 ±0.7	85.6 ±1.4	0.4 ±0.1	8.9 ±1.0	88.1 ±10.0	85.5 ±7.5	4

The fractions were prepared from alveolar macrophages incubated for 45 min with or without paraffin oil emulsion.

* Percentage recovered $= \frac{\text{Amount in vesicle + fluid + pellet fractions}}{\text{Amount in whole homogenate}} \times 100$.

‡ Means ±SE are given where more than two preparations were analyzed; otherwise the means and ranges are given.

phosphatase was also found in these fractions (Table III). The floating fractions from resting cells contained essentially no phospholipid.

The mean Oil Red O to protein ratio of macrophage phagocytic vesicles, 166 ±13.7 μmoles/mg (mean ±SE, n = 6), was 56% of that in vesicles of polymorphonuclear leukocytes (296 ±15.3 μmoles/mg, n = 6). The findings are consistent with the morphologic observation that the phagocytic vesicles of the macrophages contained more electron-dense material, probably in large part protein, than did the vesicles of polymorphonuclear leukocytes. The Oil Red O to protein ratio of macrophage phagocytic vesicles containing particles emulsified with starch was 24% higher than that of vesicles containing particles emulsified with albumin. This finding suggests that albumin constituted 20% of the protein in the phagocytic vesicles prepared with albumin-coated emulsions. The protein to phospholipid weight ratio of phagocytic vesicles of alveolar macrophages (without correction for albumin) was 4.15.

With the appearance of protein and phospholipid in the phagocytic vesicle fraction, there was a corresponding decrease of these components in the pellet fraction (Table II). The content of protein and phospholipid in the supernatant fluid fraction was not altered by phagocytosis (Table II).

Distribution of enzymes in stimulated macrophages. The specific activities of all enzymes measured in homogenates of alveolar macrophages were unaltered by phagocytosis (Table I). Thus, the decrease in total activity of all enzymes was commensurate with the loss of total protein (and other cell components) during phagocytosis. No alkaline phosphatase, peroxidase, or urate oxidase activity was detected in macrophage homogenates.

The phagocytic vesicle fractions were not significantly contaminated with succinic dehydrogenase (a mitochondrial enzyme) or with glucose-6-phosphatase (a marker enzyme for endoplasmic reticulum) (Table III). Most of the activity of each of these enzymes was in the pellet fraction, and this was not altered by phagocytosis. The control floating fraction contained essentially no enzymes. The granule-associated enzymes, acid phosphatase and β-glucuronidase were found in phagocytic vesicles and were apparently transferred from the pellet fractions (Table III). The activities of these enzymes in the supernatant fluid fractions were not affected by phagocytosis (Table I). The fraction of acid phosphatase activity recovered in the phagocytic vesicle fraction was the same whether *p*-nitrophenyl phosphate or β-glycerophosphate was used as substrate (Table III). Almost half of the catalase activity of resting alveolar macrophages was in the pellet fraction, and a portion of that activity was shifted to the vesicle fraction during phagocytosis (Table III).

When acid phosphatase activity of freshly prepared phagocytic vesicles was assayed in the presence of 0.25 M sucrose, enzyme activity was increased 45–70% by the addition of 0.1% Triton X-100. The activity of the pellet fraction was increased up to fourfold. These degrees of enzyme latency are less than those previously described for isolated phagocytic vesicles of polymorphonuclear leukocytes (6), for polymorphonuclear leukocyte granules (11), or for macrophage particles (1). The relatively low latency observed in our

the vesicles are intact, but the surrounding membrane of some is broken. Bar represents 1 μm, × 11,000. *B.* This micrograph shows several isolated phagocytic vesicles. The surrounding membrane is not visible here, but can be seen clearly at higher magnification in the inserts. The single arrow marks the albumin coat surrounding the oil droplet (OD) and the double arrows mark the trilaminar membrane of the vesicle. Bar represents 0.5 μm, × 38,000; insert bar represents 0.1 μm, × 130,000.

TABLE III

Distribution of Enzymes in Subcellular Fractions of Rabbit Alveolar Macrophages

	Supernatant fluid		Pellet		Phagocytic vesicles		Total recovered		No. of preparations
	Control	Phagocytosis	Control	Phagocytosis	Control	Phagocytosis	Control	Phagocytosis	
	% of total recovered						%*		
Acid phosphatase I	14.5 ±0.5‡	13.3 ±0.5	85.5 ±0.8	67.6 ±0.1	0	19.1 ±0.5	94.5 ±2.2	95.6 ±3.8	2
Acid phosphatase II	14.2 ±1.5	11.7 ±1.5	84.4 ±1.9	69.3 ±2.9	1.4 ±0.4	19.0 ±2.5	96.2 ±5.7	99.6 ±9.6	6
β-Glucuronidase	21.2 ±1.6	21.5 ±3.4	78.7 ±1.6	64.0 ±2.9	0.1 ±0.1	14.5 ±2.2	78.0 ±4.8	87.2 ±9.5	4
Catalase	51.7 ±4.4	54.7 ±5.3	47.9 ±3.9	37.3 ±5.2	0.4 ±0.3	8.0 ±0.7	79.7 ±4.9	84.7 ±7.5	6
Succinic dehydrogenase	16.9 ±3.5	12.8 ±4.6	81.5 ±3.2	83.7 ±3.9	1.6 ±0.9	3.5 ±1.9	111.5 ±3.7	113.0 ±3.5	4
Glucose-6-phosphatase	12.5 ±6.9	14.4 ±8.5	87.5 ±6.9	83.1 ±8.7	0	2.5 ±0.3	91.2 ±2.0	94.8 ±2.0	2

The fractions were prepared from alveolar macrophages incubated for 45 min with or without paraffin oil emulsion.

*The data are presented as in Table II.

‡ Means ±SE are given where more than two preparations were analyzed; otherwise means and ranges are given.

experiments may be due to homogenization used to disrupt the alveolar macrophages. Nevertheless, the distribution of protein and enzymes between particulate and soluble fractions of resting macrophages reported here (Table III) is similar to that found in a study in which greater enzyme latency was observed (1). The latency of catalase in the pellet fraction was investigated using digitonin (Fig. 3). Very low concentrations of this agent released all of the acid phosphatase activity, whereas considerably larger amounts were required to solubilize catalase entirely. The differential latency of these two enzymes is identical to that described for enzymes of liver lysosomes and peroxisomes (12).

Enzymes in unstimulated macrophages. Two preparations of unstimulated alveolar macrophages obtained from the lungs of rabbits that had not been treated with Freund's adjuvant were studied. The specific activities of acid phosphatase (0.108 and 0.114) and of β-glucuronidase (1.45 tnd 1.51) in homogenates of the unstimulated macrophages were approximately one-half of those in homogenates of stimulated macrophages (see Table II for units and activities of stimulated cells). The specific activities of these enzymes in phagocytic vesicles isolated from unstimulated macrophages were only 20–30% of the corresponding activities in vesicles from stimulated macrophages (Table IV). This relationship was observed whether activities were expressed per ingested material (Oil Red O) or per total vesicle protein.

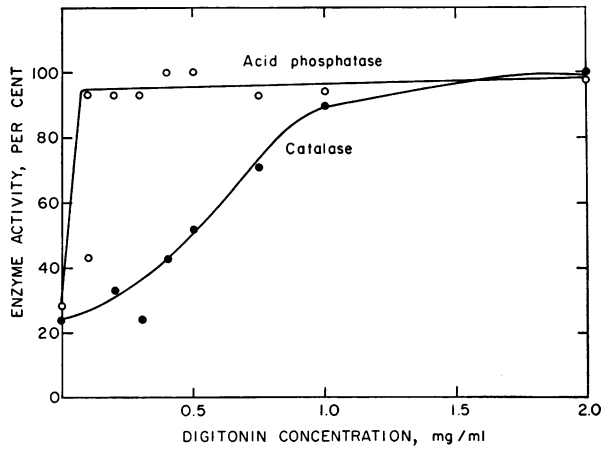

FIGURE 3 Effect of digitonin concentration on the latency of acid phosphatase and of catalase in an alveolar macrophage pellet fraction.

Since stimulated macrophages have a higher concentration of granule enzymes than do resting cells, artifactual adsorption of enzymes to the vesicles during homogenization and fractionation could contribute to the higher specific activity of enzymes in the vesicles from stimulated cells. In order to investigate this possibility, granules were prepared from stimulated alveolar macrophages (1) and divided into two portions, one of which was disrupted by repeated freezing and thawing. Washed alveolar macrophages (stimulated) which had ingested paraffin oil droplets were suspended in sucrose and divided into halves; the intact and broken granules were added to one half, while appropriate diluent was added to the other. Homogenization and fractionation were then carried out in the standard manner. The homogenate enriched with particulate and solubilized granule enzymes was found to have twice the acid phosphatase and β-glucuronidase activity of the homogenate which did not have added granule material. Yet the specific activities of enzymes in the phagocytic vesicles isolated from these homogenates were equal. Thus, it seems unlikely that adsorption of granules or their enzymes to vesicles contributed significantly to the enzyme content of these fractions.

Kinetics of enzyme transfer to phagocytic vesicles. In polymorphonuclear leukocytes, particle uptake and transfer of enzymes to the phagocytic vesicles were closely integrated temporally (6). As shown in Fig. 4, a similar relationship obtained in alveolar macrophages.

TABLE IV

Stimulated and Unstimulated Alveolar Macrophages: Specific Activities of Hydrolytic Enzymes in Isolated Phagocytic Vesicles

Enzyme activity	Macrophages	
	Stimulated*	Unstimulated‡
Acid phosphatase		
Micromoles *p*-nitrophenyl phosphate hydrolyzed:		
per milligram protein	0.99 (0.83–1.29)	0.18 (0.17–0.18)
per micromole Oil Red O	0.15 (0.10–0.29)	0.045 (0.028–0.053)
β-Glucuronidase		
Nanomoles *p*-nitrophenyl β-D-glucuronide hydrolyzed:		
per milligram protein	8.92 (5.16–11.32)	1.85 (1.50–2.25)
per micromole Oil Red O	1.20 (0.89–1.39)	0.30 (0.24–0.35)

* The mean and range of six preparations are given.

‡ The mean and range of two preparations are given.

After incubation with paraffin oil emulsion for 10 min, the cells were washed free of particles with ice-cold buffered incubation medium and divided into two portions. The sample incubated again without particles did not incorporate more enzyme into phagocytic vesicles. The macrophages given oil droplets were clearly capable of vigorous phagocytosis during the second incubation period, and transferred acid phosphatase into phagocytic vesicles.

DISCUSSION

Engulfment of a variety of kinds of particles by alveolar macrophages causes increased oxidation of glucose labeled in the first and sixth carbon positions, presumably reflecting augmentation of Krebs' cycle and hexose monophosphate shunt activity (13, 14). Phagocytosis of paraffin oil droplets similarly enhanced the oxidation of glucose-1-^{14}C and glucose-6-^{14}C by alveolar macrophages. Relative to polymorphonuclear leukocytes studied previously (6), alveolar macrophages (per milligram protein) ingested paraffin oil emulsified with albumin at slower and more variable rates.

Homogenates of macrophages which had phagocytized emulsion particles contained less protein, phospholipid, DNA, RNA, and enzymes than did homogenates of paired resting control cells. Although the incubating medium was not analyzed in this study, other investigators have documented the accumulation of macrophage enzymes in the extracellular medium during phagocytosis (2). It is likely that lysis of a certain fraction of cells accounts for these findings, but differences in cell recovery during washing or efficiency of homogenization between control and ingesting cells have not been ruled out.

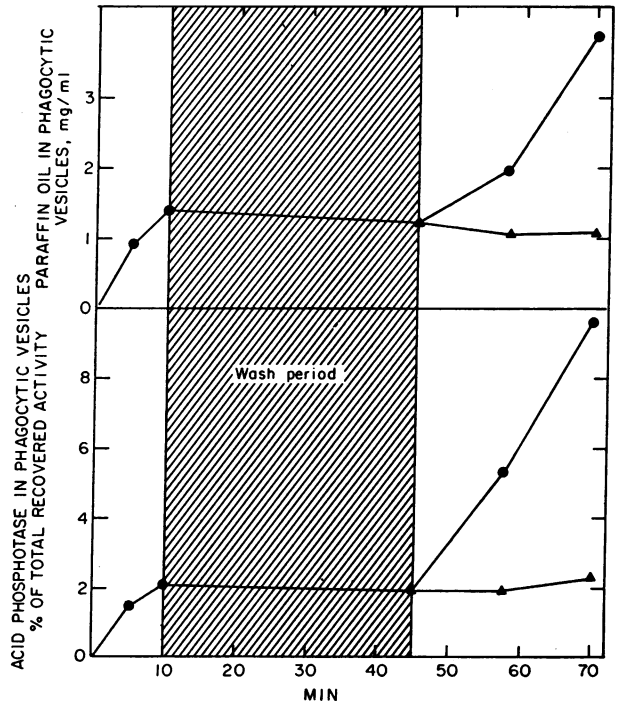

FIGURE 4 The effect of abrupt termination of phagocytosis on acid phosphatase content of phagocytic vesicles. Phagocytosis by rabbit alveolar macrophages was allowed to proceed for 10 min in Krebs-Ringer phosphate medium at 37°C, at which time the paraffin oil particles were removed from the cells by washing with ice-cold Krebs-Ringer phosphate medium (centrifugation at 100 *g* for 10 min; total duration of wash period 35 min). The cell suspension was divided into two equal portions. One portion was reincubated at 37°C with paraffin oil emulsion (●) and the other without paraffin oil emulsion (▲).

The pellet fraction from alveolar macrophages contained some phagocytic vesicles, and the supernatant fluid fraction may have had vesicle membranes as well as others. The composition of these fractions was, in addition, more variable than that of the corresponding fractions from polymorphonuclear leukocytes. The phagocytic vesicles from the macrophages, however, were apparently as pure as those isolated from polymorphonuclear leukocytes, as evidenced by electron microscopy and by the absence of nucleic acids and mitochondrial and microsomal enzymes. Nachman, Ferris, and Hirsch (15) have analyzed membranes of phagocytic vesicles isolated from alveolar macrophages by the technique developed by Wetzel and Korn for *Acanthamoeba castellanii* (neff) (16). That method, in which vesicles are collected by density gradient centrifugation from homogenates of cells that have ingested polystyrene spheres, may well be adequate for obtaining vesicle membranes. Homogenization of alveolar macrophages containing rigid particles has been shown, however, to result in release of particulate enzymes into a soluble fraction, presumably a result of damage to phagocytic vesicles (2). Our method may be more appropriate for the study of enzymes in phagocytic vesicles.

Acid phosphatase and β-glucuronidase were transferred from the pellet to the phagocytic vesicle fraction of the macrophages during phagocytosis just as was shown with polymorphonuclear leukocytes (6), although in the macrophages it is more difficult to ascertain from what structures in the pellet the vesicle enzymes originate. On the basis of electron micrographs included here and in other publications (3, 17, 18) it would appear that granules and other dense structures, possibly secondary lysosomes, which may contain sizeable amounts of nonenzyme proteins, fuse with and become part of phagocytic vesicles. The composition of the macrophage vesicle fraction is consistent with this view. Relative to polymorphonuclear leukocyte vesicles, the phagocytic vesicles of alveolar macrophages contained greater amounts of electron-dense material. They had higher ratios of protein to Oil Red O and protein to phospholipid, and a lower ratio of enzyme to Oil Red O. The quantity of protein in phagocytic vesicles of alveolar macrophages may increase their average density such that some oil-laden vesicles sediment rather than float during centrifugation.

Other electron microscopists have suggested that the large dense cytoplasmic particles of macrophages, which may be secondary lysosomes, fuse with but do not penetrate the phagocytic vesicle membranes (3, 18, 19). Perhaps the apparently different properties of the phagocytic vesicles obtained in this study are related to the fact that they contain droplets of inert oil rather than bacteria (which were used in the studies mentioned above).

Whereas in polymorphonuclear leukocyte, catalase is found only in the supernatant fluid fraction (6, 11), almost half of the catalase of resting macrophages was found in the pellet fraction. A portion of this activity was transferred to the phagocytic vesicle fraction during phagocytosis. In mammalian liver and kidney, the catalase that is particle-associated sediments, together with hydrogen peroxide–producing oxidases, at a slightly lower equilibrium density than do lysosomes (20). In rat liver, the particles containing oxidases and catalase (named peroxisomes) are identified morphologically by the presence of a dense crystalline core. This crystalline core is believed to represent urate oxidase. It is not seen in human liver or rat kidney peroxisomes which lack this enzyme (20). We detected no urate oxidase activity in homogenates of alveolar macrophages, and no particles with characteristic crystalline cores were seen in electron micrographs of these cells. D-amino acid oxidase activity previously demonstrated in homogenates of alveolar macrophages (14) was found in the pellet, supernatant fluid, and phagocytic vesicle fractions. Whether this or other oxidases are associated with catalase in peroxisome-like structures in alveolar macrophages remains to be determined. The similarity between liver peroxisomes and the alveolar macrophage pellet in terms of the effects of digitonin on acid phosphatase and catalase activities is certainly consistent with the presence of peroxisomes in the macrophages. Acid phosphatase and catalase differed not only in distribution in the resting macrophage and in treatment needed for activation or release but also differed significantly in the per cent of total, but not of pellet, activity transferred to the phagocytic vesicle fraction (Table III).

The function of peroxisomes is unknown. It is tempting to speculate that in alveolar macrophages they play a role in delivery of the enzymatic components of a bactericidal system (oxidases and catalase) to the phagocytic vesicles. In polymorphonuclear leukocytes granule-associated peroxidase enters phagocytic vesicles (6). Peroxidase, in combination with hydrogen peroxide, is a potent antimicrobial agent (21). Alveolar macrophages lack peroxidase (1, 14). Catalase can, however, act in a peroxidative manner (22), and catalase and hydrogen peroxide together have a bactericidal effect in vitro (23). Since alveolar macrophages do generate hydrogen peroxide during phagocytosis (24), catalase in the phagocytic vesicle of the macrophage may serve the function assigned to peroxidase in the phagocytic vesicle of the polymorphonuclear leukocyte.

Alveolar macrophages contain greater amounts of hydrolytic enzymes than do peritoneal macrophages (25), and if the alveolar macrophages have been elicited with Bacillus Calmette-Guérin, this difference is more pronounced (4). Increased synthesis of hydrolytic enzymes by peritoneal macrophages in tissue culture after stimulation by phagocytosis or pinocytosis has been convincingly demonstrated (*26, 27*). Greater efficiency of bacterial killing by stimulated macrophages has also been observed (5). We found higher levels of hydrolytic enzymes in stimulated alveolar macrophages than in unstimulated cells. Because of variability in rates of phagocytosis and the small number of unstimulated preparations investigated, we could not ascertain whether stimulated macrophages also had a greater rate of particle uptake. Whether the lesser accumulation of enzymes into phagocytic vesicles of unstimulated macrophages is due to a numerical deficiency of granules or secondary lysosomes (i.e. less enzyme available per cell) or to defective mechanisms for fusion and discharge of enzymes into the vesicle, or both, cannot be deduced from the experimental findings. It is probable that truly unstimulated alveolar macrophages were not obtained in this study. The pulmonary alveolar macrophages presumably constantly ingest microorganisms (28), and levels of hydrolytic enzymes in these cells have been shown to vary with a seasonal periodicity that matched fluctuations in challenge by pulmonary infection (4).

When uptake of paraffin oil and thereby the formation of new phagocytic vesicles was halted by washing oil particles from the cells, fusion of hydrolase-containing structures with phagocytic vesicles apparently ceased. Similar results were obtained in our previous study of polymorphonuclear leukocyte degranulation (6), although a brief interval was observed after cessation of phagocytosis during which hydrolase accumulation continued. The results suggest that either the initiating stimulus or metabolic requirement for phagocytic vesicle formation is necessary to induce granule fusion with the vesicle or alternatively that the hydrolase content of newly formed phagocytic vesicles is strictly regulated. In any event, the simple presence of phagocytic vesicles in the cytoplasm is not sufficient to cause active hydrolase transfer.

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