THE PARTICULATE HYDROLASES OF MACROPHAGES

I. Comparative Enzymology, Isolation, and Properties*

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The two most important groups of phagocytic cells are polymorphonuclear leucocytes and the variety of mononuclear elements which comprise the reticuloendothelial system. These latter cells, which include the Kupffer cells of the liver, glia of the central nervous system, tissue histiocytes, and "dust cells" of the alveoli, are functionally related by their phagocytic properties. Their role in host defense reactions, inflammatory processes, immune mechanisms, and erythrocyte destruction has been amply documented (1–3). In general, their function is considered to be the intracellular breakdown and disposal of particulate elements. In this regard, they have been reported to contain a wide variety of hydrolytic enzymes.

Previous studies from this laboratory have indicated that the polymorphonuclear (PMN) leucocyte obtained from rabbit peritoneal exudates contains hydrolases associated with their specific cytoplasmic granules (4). This data as well as the observations of de Duve (5) on analogous particles in rat liver prompted a more detailed study of the macrophage enzymes. This report will deal with the content of selected hydrolases in three types of macrophages as well as with their intracellular localization and properties.

Materials and Methods

Cell Types.—

Rabbit peritoneal macrophage: Peritoneal macrophages were obtained from 3 to 4 kg rabbits injected 4 days previously with 50 ml of sterile mineral oil (nujol, extra heavy, Plough Inc., Hackensack, New Jersey). Animals were sacrificed and 100 ml of pyrogen-free saline was injected forcefully into the peritoneal cavity. The abdomen was then opened, and the exudate fluid was removed with a broad tipped 25 ml pipette, and dispensed to sterile centrifuge tubes. The exudate was then centrifuged at 800 RPM (International centrifuge, type 1) for 5 minutes at room temperature, and the upper oil layer and intermediate supernatant fluid discarded. The pellet of cells was then washed and suspended to the appropriate concen-

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tration. More than 90 per cent of the cells, in the preparations employed for this study, were large mononuclear cells, about 20 to 30 per cent of which contained small oil droplets. The yield from a single rabbit ranged between 1 to 2 $\times$ 10^8 macrophages.

Normal Alveolar Macrophages: Macrophages from rabbit lungs were obtained by the general method of Myrvik *et al.* (6). Rabbits weighing 3 to 4 kg were killed by the injection of 50 ml of air in the marginal ear vein. The upper end of the trachea was clamped and the lungs, heart, and trachea removed *in toto*. The heart, lymph nodes, and connective tissue were then carefully dissected, blood expelled from the pulmonary circulation, and the external surface of the lungs rinsed with saline. These steps were of some importance in obtaining erythrocyte-free preparations. Twenty-five ml of pyrogen-free saline was then injected into each main bronchus, the lungs wrapped in moist gauze and gently kneaded to expel the washings. This process was repeated once more in the case of normal rabbits and the washings pooled. An average of 2 $\times$ 10^7 cells were obtained from normal rabbits of which more than 95 per cent were large, uniformly sized macrophages.

BCG-Induced Alveolar Macrophages: These cells were obtained by a modification of the procedure described by Myrvik *et al.* (7). BCG (Aranson) was obtained from Dr. R. Schaedler, The Rockefeller Institute, as a water-washed lyophilized powder. The dead bacilli were suspended in saline containing 0.01 per cent tween 80 and injected intravenously in the marginal ear vein. Two injections of 20 mg each, in a volume of 1.0 ml, were given on successive days. This produced a marked granulomatous response in the lungs and an enormous increase in the number of macrophages. Lungs from BCG-injected rabbits were harvested at 3.5 to 4.5 weeks according to the above procedure. Three wash-outs, of 50 ml each, were performed as a routine and pooled. The total number of cells obtained from such animals varied from 1 to 3 $\times$ 10^9. Differential counts on these preparations usually showed 85 per cent large mononuclear cells, 8 to 15 per cent small mononuclear cells, and 1 to 15 per cent polymorphonuclear leucocytes. Occasional lots contained as many as 40 per cent lymphocyte-PMN mixtures and were not employed for fractionation studies. There was considerable variation in the size of the macrophages, and binucleate and multinucleated giant cells were present.

Preparation, Quantitation, and Staining of Cells.—Each type of macrophage was collected by centrifugation at 800 RPM for 5 minutes and washed twice with large volumes of either saline or balanced salt solution. Total cell counts were performed in a hemocytometer according to usual procedures. Differential counts were made either on Giemsa-stained smears or as wet mounts in the hemocytometer chamber. Vital staining with neutral red and Janus green was accomplished by the method of Sabin (8). Examination of cell fractions was performed with the phase microscope.

Electron Microscopy.—

Electron microscopy of intact BCG-induced alveolar macrophages was kindly performed by Dr. David J. L. Luck, of The Rockefeller Institute. Cells obtained directly from the lung were fixed for 1 hour in buffered 10 per cent osmic acid, dehydrated, and imbedded in epon. Thin sections were examined in the RCA electron microscope.

Enzymatic and Chemical Assays.—

Alkaline phosphatase was assayed by a previously described method (4), in veronal buffer at pH 9.9. Incubation was carried out at 38°C in water bath for 60 minutes and the liberated inorganic phosphate determined.

Acid phosphatase was determined in either veronal or acetate buffer at pH 5.0 (4). A 0.1 M solution of sodium β-glycerophosphate (Eastman Kodak Company, Rochester) served as the substrate, and the reaction was carried out at 38°C for 60 minutes. Aliquots of the protein-free filtrate were then analyzed for inorganic phosphorus.

Cathepsin: Catheptic activity was assayed employing a 2 per cent solution of denatured

hemoglobin (Nutritional Biochemical Corp., Cleveland) in 0.05 M acetate buffer at a final pH of 3.5. Reactions were carried out at 38°C for 60 minutes. Aliquots of the TCA filtrate were assayed for chromogen by the method of Lowry (9). The method was calibrated with a preparation of crystalline egg white lysozyme and calculated as protein equivalents. One unit of enzyme activity was proportional to an optical density increment of 0.1 unit at 660 mμ/hour and corresponded to 20 μg of lysozyme equivalents.

Acid ribonuclease was measured by a modification of the method of Schneider and Hogeboom (10). Purified yeast RNA (freed from nucleotides by a 48 hour dialysis against 0.1 M acetate buffer) served as substrate and was suspended in 0.1 M acetate buffer at a final pH of 5.0. Reactions were carried out at 38°C for 10 minutes and stopped by the addition of 2.5 ml perchloric acid containing 0.25 per cent uranyl acetate. The filtrate was diluted with water and the optical density read at 260 mμ in a Beckmann model DU spectrophotometer. Units are as described previously (4).

Acid deoxyribonuclease was determined by a previously described method (4).

Beta glucuronidase was assayed by the procedures of Follette *et al.* and Fishman as described previously (4). Phenolphthalein-β-glucuronide was obtained from Sigma Chemical Company, St. Louis as a 0.01 M solution. Reactions were carried out in 0.1 M acetate buffer, pH 4.5 at 38°C.

Esterase: Assays were performed by a modification of the method of Nachlas and Seligman (11) and Hardin *et al.* (12). Naphthol acetate served as substrate and was obtained from Dajac Laboratories, Philadelphia. Reactions were carried out in 0.1 M tris buffer, pH 7.4 at 22°C for 30 minutes. Results are expressed as the μmoles naphthol liberated per hour at 22°C. Taurocholate (8×10^{-2} M) inhibited the activity of this enzyme by 85 per cent.

Lipase was measured by a similar method but employing naphthol laurate as substrate (Dajac Laboratories). Sodium taurocholate was present in the reaction mixture at a concentration of 8×10^{-2} M. Reactions were carried out at 38°C for 30 minutes and the results expressed as μmoles of naphthol liberated per hour. The pH optimum of this enzyme was found to be 6.0, although tests were as a routine performed at 7.4. Taurocholate was found to increase the activity of the enzyme by 10 to 15 per cent in whole homogenates and by 200 to 300 per cent in the isolated dense granule fractions.

Lysozyme: Determinations were performed as described previously (4), employing suspensions of *Micrococcus leisodykticus* as substrate. Tests were standardized with crystalline egg white lysozyme and expressed as micrograms of lysozyme activity.

Cytochrome oxidase was assayed by the procedure of Nielsen and Lehninger (13). Cytochrome c (Sigma Chemical Company, type III, horse heart) was reduced with sodium borohydride as described by Martin *et al.* (14). Enzymatic activities were calculated from the initial zero order rates during the first 3 minutes. All preparations were frozen and thawed twice before assay.

Aryl sulfatase was assayed by method II as described by Fromageot (15).

Peroxidase was assayed by the method of Maehly (16) using uric acid as substrate.

Unless otherwise stated cells and cell fractions were frozen and thawed six times in an alcohol dry ice bath prior to assay. Appropriate controls were included in each determination.

Chemical analyses: Total nitrogen was determined colorimetrically with Nessler's reagent after digestion with 5 N sulfuric acid, copper selenite, and hydrogen peroxide (17). The Fiske and Subbarow method (18) was used to measure inorganic phosphorus. Protein was determined by the method of Lowry (9) against a standard of crystalline egg white lysozyme.

Preparation of Continuous Sucrose Density Gradients.—Linear sucrose gradients were prepared using the machine described by Britten and Roberts (19). Concentrations of sucrose ranging from 15 to 70 per cent w/w were employed to load the apparatus. Most gradients were prepared in a volume of 30 ml at 4°C either the night before or the morning of the experiment.

Smaller 4.0 ml gradients were occasionally employed. Dye dilution techniques were used to assure linearity and showed stability of the system for at least 18 hours.

Disruption of Macrophages.—Macrophages which had been washed twice with saline were counted and 300 to 400 × 10^6 cells sedimented at 800 RPM for 5 minutes. The supernatant fluid was decanted and the sides of the tubes carefully wiped with cotton swabs. Forty ml of ice cold 0.25 M sucrose was added to the cell pellet and the macrophages gently resuspended by means of pipetting. The cells were then centrifuged at 1100 RPM for 7 minutes. This constituted the initial sucrose wash. The resulting pellet was then taken up in cold isotonic sucrose to a cell concentration of 50 to 60 × 10^6/ml and transferred to a chilled homogenizer tube. Homogenization was carried out with a teflon pestle for three, 2-minute periods, with equal intervals in an ice bath. This procedure was found to give adequate but not complete disruption of the cells as checked by phase microscopy. Within these time periods 75 to 90 per cent of the cells were disrupted, liberating their cytoplasmic granules. More prolonged homogenization, although more efficient in terms of cell breakage, resulted in the solubilization of increasing amounts of the hydrolases. It should be stressed that these are relative periods of homogenization, depending upon the technique of the individual operator, so that cell breakage should be checked continually with phase optics.

More recent experiments have been conducted with 0.25 M sucrose containing 0.002 M versene (disodium ethylenediaminetetraacetate, pH 7.5), in both the initial sucrose wash and homogenization steps. The addition of versene has facilitated cell disruption without influencing the subsequent distribution of enzymes.

Differential Centrifugation of Macrophage Homogenates.—Following cell breakage the contents of the homogenization flask were transferred to conical centrifuge tubes and diluted 1:2 with cold isotonic sucrose. The diluted homogenate was then mixed and centrifuged at 500 *g* for 12 minutes at 0°C. The milky supernatant fluid was carefully removed by means of a fine tipped Pasteur pipette and transferred to lusteroid centrifuge tubes. The initial low speed pellet was then taken to volume with sucrose and constituted the "nuclear" fraction. The supernate was then centrifuged in a high speed, angle head centrifuge (Lourdes, rotor 9RA) at 12,000 to 15,000 *g* for 12 minutes. This resulted in a lipid pellicle, a slightly opalescent supernatant fluid, and a tan pellet. The lipid layer and supernatant fluid were removed, adjusted to volume with sucrose, and constituted the "supernatant" fraction. The pellet resuspended easily in sucrose and represented the "15 *g*" pellet. Aliquots of the total homogenate, nuclear, 15 *g*, and supernatant fractions were then assayed for enzymatic activity.

In other experiments pellets were obtained by progressively higher centrifugal forces ranging from 2,000 to 25,000 *g* for 12 minutes.

RESULTS

Characteristics of Macrophages.—The three types of macrophages employed in this study were actively phagocytic cells which differed somewhat in morphological properties. Each was a large mononuclear cell which demonstrated little in the way of cytoplasmic structure when stained by either the Wrights or Giemsa method. However, major differences were noted when viewed under darkfield or phase illumination. There was a large number of morphologically heterogeneous cytoplasmic organelles in the alveolar cells, and a relative paucity of such structures in the peritoneal macrophage. When stained supravitally with neutral red, the alveolar cells exhibited rosette-like granules in a large perinuclear area which corresponded to the granules seen with phase optics. This accumulation of neutral red–positive structures corresponds to the centrosphere described by earlier investigators (20, 21). Figs. 1 and 2

show thin sections of a BCG-induced alveolar macrophage and illustrate the variety of organelles forming the neutral red rosette. In addition to the neutral red positive bodies, cells from both alveolus and peritoneum demonstrated Janus green–positive structures, presumably mitochondria. The number of these organelles differed markedly in individual alveolar macrophages. Acid-fast bacilli were never seen in the BCG-induced alveolar macrophage, although the organisms were easily recognized in the center of interstitial granulomata from sectioned lungs. The granules of the centrosphere region were periodic acid-Schiff–positive.

TABLE I

Hydrolase Activity of Peritoneal and Alveolar Macrophages

Enzyme	Peritoneal		Normal alveolar		BCG alveolar	
	Act./10^6 cells	Act./mg N	Act./10^6 cells	Act./mg N	Act./10^6 cells	Act./mg N
β-Glucuronidase	3.8	172	6.5	189	7.9	230
Acid phosphatase	2.6	118	20.7	600	37.0	1073
Cathepsin	64	2905	224	6496	235	6815
Acid ribonuclease	1.0	45.4	4.0	116	3.8	110
Lysozyme	0.4	18.2	3.2	93	9.2	266
Esterase	—	—	0.81	21.3	0.80	23.3
Lipase	0.12	5.5	0.31	8.2	0.83	24.1

Activity Units (Act.)

β-glucuronidase	μg phenolphthalein/hr. 38°C
Acid phosphatase	μg phosphorus/hr. 38°C
Cathepsin	units/hr. 38°C
Acid ribonuclease	OD 260 mμ/hr. 38°C
Lysozyme	μg egg white lysozyme equivalents. 22°C
Esterase	μmoles naphthol/hr. 22°C
Lipase	μmoles naphthol/hr. 38°C

Comparative Enzymology.—Initial studies were conducted on the distribution and content of seven hydrolytic enzymes in the three types of macrophages from the rabbit. These results are presented in Table I and compare the enzymes on a per/cell and nitrogen basis. The values represent the means of 5 to 10 separate lots of each type of cell. It is evident that on a specific activity basis the content of hydrolases was considerably higher in the cells obtained from the lung. Other investigators have obtained similar results with certain of the hydrolases (22). This was most strikingly illustrated with acid phosphatase, cathepsin, acid ribonuclease, lysozyme, and lipase and was less impressive for beta glucuronidase. The activity on a cell basis was similarly increased, in part the result of the greater size of the alveolar cell.

A more interesting comparison existed between the hydrolase activity of the

normal and BCG-induced alveolar macrophage. Cells stimulated by the injection of dead BCG exhibited a 2- to 3-fold increase in the activities of acid phosphatase, lysozyme, and lipase. The activities of cathepsin and beta glucuronidase showed slight increases, and no significant alteration of acid ribonuclease or esterase was noted. It should be stressed that the origin of these macrophages is uncertain, so that increased enzymatic activity could result either from a new population of cells or through the stimulation of preexisting alveolar macrophages. It is of interest that certain of the "lysosomal" hydrolases varied independently of others.

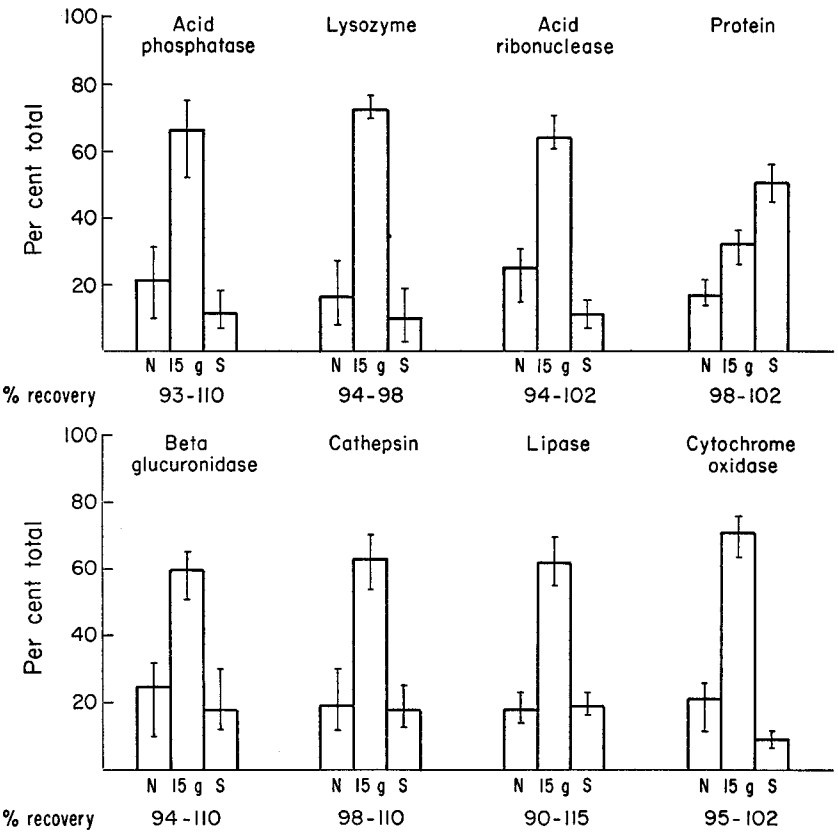
A study of the pH optima of acid phosphatase, cathepsin, lipase, and beta glucuronidase showed no differences between the normal and BCG-induced alveolar macrophages. Assays for alkaline phosphatase and peroxidase were uniformly negative in alveolar cells when assayed at three times the normal cell concentration.

The Intracellular Distribution of Macrophage Hydrolases.—In general, the cytoplasm of the macrophage contained a wider variety of cytoplasmic organelles than was present in the adult polymorphonuclear leucocyte. Whereas the leucocyte cytoplasm contained little else but the specific granules, the macrophage had many more mitochondria and a more highly developed Golgi and endoplasmic reticulum. In addition, many particles of varying size and density have been reported in electron microscopic studies (23, 24).

With this data in mind quantitative studies were conducted on macrophage fractions separated by differential centrifugation. Initial experiments were made with oil-induced peritoneal macrophages. These cells, some of which contained oil droplets, were homogenized and their organelles separated by differential centrifugation. These studies indicated that at least 50 per cent of the total content of acid phosphatase, cathepsin, beta glucuronidase, and acid ribonuclease was bound to particles which sedimented at 12,000 *g* for 15 minutes. The majority of the remaining enzyme was found in the supernatant fraction. More detailed studies on this cell type were not conducted because of the relatively small yield of cells and the possibility of artefacts related to the presence of mineral oil droplets.

Attention was next turned to the BCG-induced alveolar macrophage. This cell was available in huge numbers and contained large quantities of the enzymes under study. Text-fig. 1 shows the distribution of the hydrolases, protein, and cytochrome oxidase in the three fractions obtained from cell homogenates. The values presented are the means of five experiments along with maximum and minimum values for each fraction. The per cent recovery refers to the sum of enzymatic activity present in the three fractions as compared to the initial homogenate. Approximately 60 to 80 per cent of the total cell content of acid phosphatase, lysozyme, acid ribonuclease, beta glucuronidase, cathepsin, and lipase was found in the "15 *g*" pellet. Supernatant enzyme

varied from experiment to experiment. It was of some interest, however, that in any given fractionation, lysozyme always exhibited the greatest localization to the 15 *g* pellet and less of this enzyme was found in the supernatant. Although lipase was found primarily in the 15 *g* fraction, an esterase which split



TEXT-FIG. 1. The distribution and recovery of six hydrolytic enzymes, protein and cytochrome oxidase in the nuclear (N), 15g and supernatant (S) fractions obtained from BCG-induced alveolar macrophage homogenates by differential centrifugation.

naphthol acetate was distributed equally in the soluble and particulate fractions. A number of experiments have been performed with aryl sulfatase and acid deoxyribonuclease, and both enzymes are primarily localized in the high speed pellet.

Considerable enzyme was present in the "nuclear" fraction. This was most likely due to the presence of unbroken cells and a small number of trapped larger granules, a speculation verified by direct phase microscopy. Experiments

in which more prolonged homogenization was carried out showed that the nuclear and 15 *g* fractions contained progressively less enzyme with a corresponding increase in the activity of the "supernatant" fraction. This was presumably the result of progressive trauma to the hydrolase-containing particles and liberation of soluble enzyme.

Since macrophages contained numerous mitochondria, cytochrome oxidase was employed as a marker for these organelles. It was apparent that this enzyme was present in the 15 *g* pellet along with the acid hydrolases. Examination of this fraction with both Janus green and neutral red revealed that a mixed population of particles was present, the majority of which took up neutral red. Attempts at separating the mitochondria from the hydrolase particles by differential centrifugation were not successful. Fractions collected between 2000 and 12,000 *g* (15 minutes) showed no significant increase in specific activity above what was obtained with the above schedule. In general, the 15 *g* fraction demonstrated a twofold increase in activity (activity/mg N) over that of the initial homogenate for all the enzymes localized in this fraction. Centrifugal forces of 20,000 to 50,000 *g* for 15 minutes sedimented only 1 to 2 per cent of the enzyme in the supernatant fraction, and this had low specific activity.

Activation and Release of Particulate Hydrolases.—Previous data with the specific granules from polymorphonuclear leucocytes (4) indicated that marked increases in enzymatic activity occurred following the treatment of the particle with surface active agents, mechanical trauma, or freezing and thawing. Unless such treatments were employed, the total activity of certain fractions was vastly underestimated and quantitative recoveries impaired. The particulate hydrolases of macrophages demonstrated similar properties. Initial studies indicated that marked activation occurred in the total homogenate, nuclear, and 15 *g* fractions, whereas no change in activity resulted after treatment of the supernatant fraction. This was the case with both surface active agents and six cycles of freezing and thawing. The results with freezing and thawing were more reproducible for all the enzymes studied and this procedure was the method of choice.

The 15 *g* pellet was washed once with sucrose (0.25 M) and resuspended in the same medium. The suspended particles were then dispensed to two tubes, one of which was frozen and thawed six times and the other maintained in a ice bath. Different quantities of each preparation were then assayed for enzyme, employing a 15 minute and 60 minute incubation period. The frozen and thawed preparation was taken as 100 per cent activity since further treatment caused no additional increase in activity.

Table II illustrates the result of one such experiment. Untreated granules incubated for 15 minutes exhibited only 20 per cent or less of total activity as compared with a frozen and thawed control. This value rose to 50 to 60 per cent when a 1 hour assay was conducted. Progressive activation no doubt

occurred in the acidic buffer systems at 38°C. These data are analogous to the results of Beaufay and de Duve (25) with rat liver lysosomes and illustrate the enzymatic latency of the macrophage particles.

Treatment of the granules by means of a Waring blendor or an all glass homogenizer disrupted the particles and liberated 86 to 95 per cent of acid phosphatase, cathepsin, beta glucuronidase, lysozyme, acid ribonuclease, and acid deoxyribonuclease in a soluble, non-particulate form which did not sediment at 30,000 *g* for 30 minutes.

TABLE II

The Effect of Freezing and Thawing on the Enzymatic Activity of the 15g Fraction

Enzyme	Length of enzymatic assay	Total activity*	
		Untreated particles	Frozen and thawed
	min.	*per cent*	*per cent*
Lysozyme	3	24	100
Acid phosphatase	15	18	100
" "	60	57	100
β-Glucuronidase	15	22	100
"	60	44	100
Acid ribonuclease	15	16	100
" "	60	52	100
Cathepsin	15	27	100
"	60	63	100

* Enzyme activity after 6 cycles of freezing and thawing.

The Separation of Macrophage Cytoplasmic Organelles by Isopycnic Sucrose Gradient Centrifugation.—It was clear from the previous results with differential centrifugation that the 15 *g* fraction contained a mixture of both mitochondria and hydrolase-containing particles. Since it was not possible to separate these elements by differential centrifugation, aqueous sucrose gradients were next employed.

Homogenates of BCG-induced alveolar macrophages were prepared as described previously employing 300 to 400 × 10^6 cells. The homogenate was then centrifuged at 500 *g* for 12 minutes to sediment the nuclei and unbroken cells. The postnuclear supernatant fluid was removed by means of a Pasteur pipette and served as the starting material which was layered over the gradient. This fraction therefore contained both soluble and particle-bound enzyme.

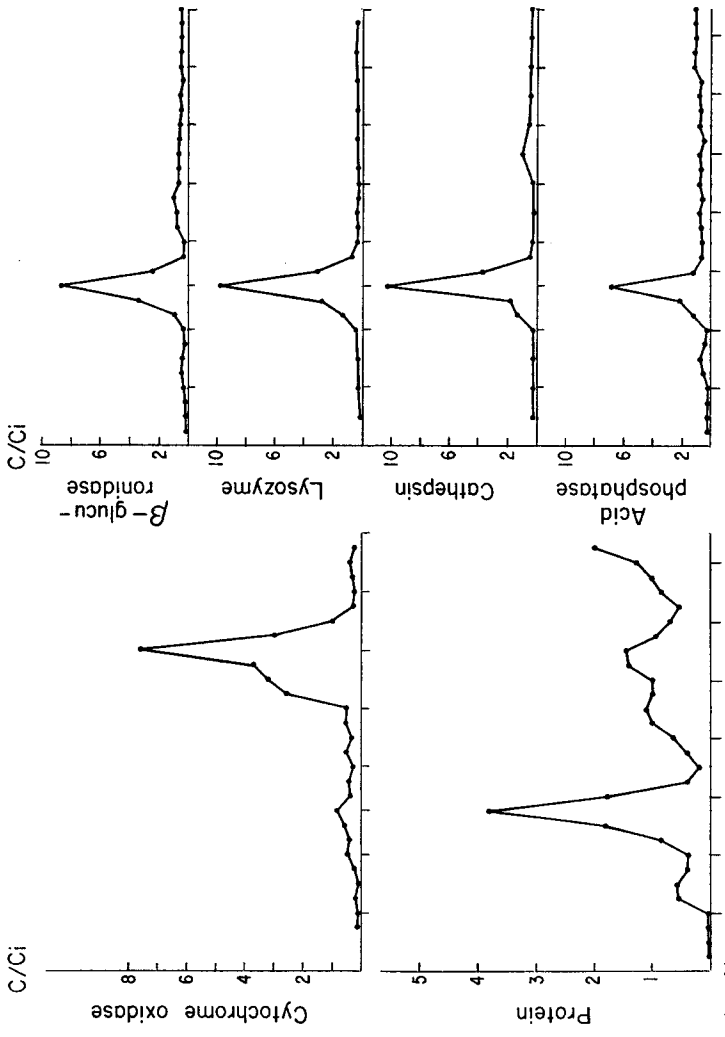
Linear sucrose gradients were prepared using 30 and 70 per cent sucrose (w/w) in the

chambers of the apparatus. This range was employed in the majority of the experiments although the results with other concentrations will be described. The total volume of the gradients was 30 ml. Three ml of the postnuclear fraction was then carefully layered over the gradient and centrifuged in the SW 25 rotor of the Spinco model L ultracentrifuge at 25,000 RPM (53,500 *g*) for 5 to 7 hours at 0°C. After centrifugation the bottom of the tube was perforated with a needle and 33 1-ml fractions collected in calibrated tubes. Each of these fractions was frozen and thawed six times and then assayed for protein and enzyme activity. The results are expressed as the ratio *C/Ci* in which *C* represents the sum of activities of all fractions (exclusive of load volume) divided by the number of fractions and *Ci* the activity of an individual fraction. Experiments in which the load volume was thoroughly mixed with a gradient and then assayed gave similar values for *C*.

A total of seven gradients have been assayed for the distribution of hydrolases, protein, and cytochrome oxidase. Although some variation in particle density and distribution was noted, the procedure gave fairly reproducible results. Text-fig. 2 represents a gradient in which four of the hydrolases were studied simultaneously. Following 6 to 7 hours of centrifugation (19.5 to 22.6 $\times$ 10^6 *g* per minute) two clearly visible bands were present in the centrifuge tube. The lowest band was tightly packed, somewhat granular in appearance, and light tan in color. Examination of this fraction under the phase microscope showed the presence of phase-dense granules of varying sizes. Essentially all the particles in this fraction could be stained with neutral red. The other major band was less dense, more diffuse in appearance and was composed of particles which did not take up neutral red. The two macroscopic bands corresponded to the distribution of protein; the first with a sharp peak at a density of 1.26 to 1.27 and a more diffuse region at 1.19 to 1.20. The less dense peak was bimodal and contained the majority of the cytochrome oxidase activity of the cell and presumably most of the mitochondria. In contrast, the dense protein peak corresponded to the position of acid phosphatase, cathepsin, lysozyme, and beta glucuronidase. Each of these enzymes had maximal activity in the same fraction with C/Ci ratios ranging from 7 to 10. It was noted that a low baseline of acid phosphatase activity was distributed in a uniform way throughout most of the fractions, resulting in a somewhat lower relative activity.

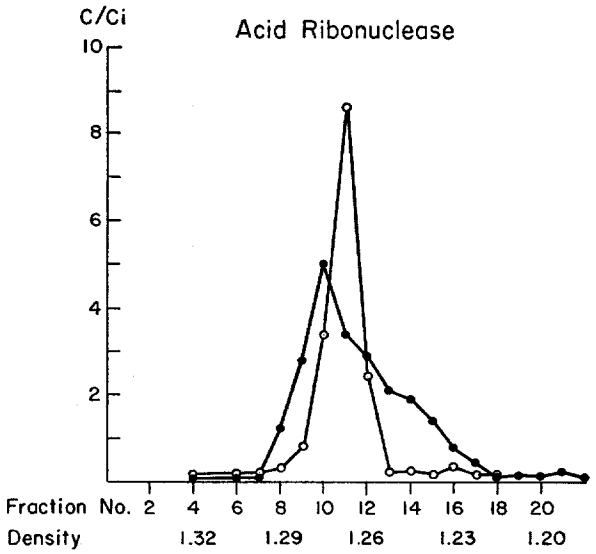
Although not presented in the chart, there was considerable enzymatic activity present in the load volume. This represented soluble enzyme which did not migrate in the centrifugal field, possibly the result of the disruption of granules during the prolonged centrifugation. The dense granule fraction accounted for approximately 50 per cent of the total activity of the cell and contained 9 to 13 per cent of the total nitrogen. The specific activity of each of the enzymes in the dense granule fraction was therefore 3.5- to 4.5-fold higher than the original homogenate.

The equilibrium density of the hydrolase-containing particles was examined by altering the time of centrifugation as well as the concentration of sucrose.



TEXT-FIG. 2. Density equilibration of BCG-induced alveolar macrophage postnuclear fraction in a linear, aqueous sucrose gradient (6.5 hours at 53,500 *g*).

Centrifugation for as long as 10 hours did not alter their position in the gradient, whereas periods of 3 to 4 hours resulted in tailing with activity present in the lighter fractions. Varying the sucrose concentration between 15 and 70 per cent while maintaining the length of centrifugation at 6.5 hours resulted in bands with corresponding densities of 1.26 to 1.27. Nevertheless, some variation was noted with different preparations and this is illustrated in Text-fig. 3. This shows two experiments employing acid ribonuclease as the indicator enzyme and indicates the limits of variation found in the study. Four other hydrolases



TEXT-FIG. 3. A portion of two density equilibrium experiments illustrating the variation in distribution of acid ribonuclease.

followed the same pattern as ribonuclease. It is not known whether this variation in particle density is the result of different granule populations or artefacts inherent in the technique.

It was occasionally noted that a smaller protein peak was present at sucrose densities of 1.31 to 1.32. This component was thought to represent another group of particles, possibly from contaminating polymorphonuclear leucocytes. A single density gradient in which mixed populations of cells were employed (60 per cent macrophages and 40 per cent PMN) showed an increase in the protein content of the fraction. This population of particles contained the alkaline phosphatase and peroxidase activity of the preparation and may have been composed of the granules of PMN leucocytes, since no significant activity of either enzyme was found in the BCG-induced alveolar macrophage.

Discontinuous Sucrose Gradients.—Once it had been ascertained that the hydrolase-containing particles of the macrophage were considerably denser than mitochondria, it was possible to arrange discontinuous gradients for the separation of these particles in bulk. This was found to be considerably simpler to perform, although it did not result in the quantitative isolation of the granules.

Discontinuous sucrose gradients were prepared with solutions ranging from 30 to 70 per cent w/w sucrose. One ml of the postnuclear fraction was layered over a tube containing: 2.0 ml 30 per cent, 3.0 ml 40 per cent, 3.0 ml 50 per cent, and 2.0 ml 70 per cent sucrose. The tube was then centrifuged at 25,000 *g* for 150 minutes in the SCR rotor of the Lourdes LRA centrifuge. After centrifugation, fractions were collected at each interphase by means of a curved pipette or through a pin hole in the bottom of the tube.

TABLE III

Specific Activities of Hydrolases in Postnuclear and Dense Granule Fraction

Enzyme	Activity*/mg N		Dense granule / Postnuclear
	Postnuclear	Dense granule	
Lysozyme	139	600	4.3
Acid phosphatase	0.910	3.98	4.4
Acid ribonuclease	52	202	3.9
Cathepsin	6620	27,142	4.1
β-Glucuronidase	242	1020	4.2
Lipase	25.2	96.2	3.8

* Activity units described previously.

Under these conditions a portion of the dense granules sedimented through the 50 per cent sucrose layer and collected at the 50 to 70 per cent interphase. This represented about 25 to 35 per cent of the total enzyme in the postnuclear supernatant fluid. Considerable purification was achieved as evidenced by the specific activities of the hydrolases which were approximately 4-fold higher than the starting material (Table III). The increase in specific activity was comparable with the results obtained with isopycnic centrifugation in continuous gradients. This fraction was probably composed of the larger granules with higher sedimentation coefficients, whereas smaller structures remained in the upper fractions. Less than 5 per cent of the cytochrome oxidase was found in the dense granule fraction.

Preliminary electron microscopic examination of a granule pellet, which sedimented through 55 per cent sucrose, has revealed the presence of a heterogeneous population of granules similar in structure to those seen in the intact cell (Figs. 1 and 2). Further studies on the morphology of these fractions is in progress.

Fractionation of Vitally Stained Alveolar Macrophages.—It was noted in the previous experiments that the isolated hydrolase-containing granules were avidly stained when neutral red was added to the fraction. This suggested that these granules corresponded to the neutral red–positive granules seen in intact supravitally stained cells. This point was of some importance in following the fate of these granules under various physiological conditions and was examined by the fractionation of supravitally stained macrophages.

350×10^6 BCG-induced alveolar macrophages were washed twice with saline and resuspended in 9.0 ml of the same medium. One ml of a 1 mg/ml solution of neutral red (neutral red, vital, National Aniline Division, Allied Chemical and Dye Corp., New York) in 8.5 per cent sucrose was added to the cells and allowed to remain at 22°C for 5 minutes. This resulted in the uptake of 98 per cent of the dye from the extracellular medium. Microscopic examination showed brightly stained granules in the perinuclear region. The cells were then washed twice with large volumes of saline and once with isotonic sucrose. Homogenization was carried out and the cellular organelles isolated both by differential centrifugation and sucrose gradients. The quantity of neutral red was assayed spectrophotometrically in the acid state at 530 mμ against standard solutions. The dye contained in cell fractions was liberated after extraction with 0.1 N HCl at 40°C for 1 hour.

Fractionation of the stained macrophages by differential centrifugation resulted in a 15 *g* pellet which was deeply stained and contained 74 per cent of the total cell content of neutral red. The supernatant fraction contained some dye but this was less than 20 per cent of the total. The dense particles isolated in sucrose gradients were deeply stained and their position was essentially the same as that of the hydrolases. It was of some interest that the addition of weak mineral acids (0.01 N HCl) to suspensions of stained granules resulted in the prompt release of the dye and a marked decrease in the opacity of the suspension. This was analogous to the effect of acids on isolated PMN granules.

DISCUSSION

It seems clear from the data presented in this study, as well as from that of other investigators (26), that macrophages constitute an array of cells which differ, at least quantitatively, in their content of hydrolytic enzymes. The nature of this variation is unclear but at least two possibilities seem attractive. First, these cells may have different origins and therefore have genetically distinct levels of enzymes. Little or no evidence is available to support this supposition. Second, these phagocytes may arise from common monocytic stem cells and differentiate under the influence of their environment. A variety of *in vitro* and *in vivo* studies suggest that this may occur. In many instances the conversion of the blood monocyte to the mature macrophage or clasmatocyte (27, 28) has been associated with the accumulation of cytoplasmic inclusions, usually arranged in the perinuclear area and often in a rosette-like

formation. These organelles, although varying in both size and shape, take up the vital stain neutral red. In one instance they were shown to be the site of acid phosphatase activity (29). In this regard they resemble the inclusions described in the alveolar macrophage, Kupffer cell, and other tissue histiocytes (24, 30).

Fractionation of the alveolar macrophage homogenates by both differential and gradient centrifugation showed that the hydrolases of the cell were primarily associated with cytoplasmic granules which demonstrated the properties of latency. The majority of these granules were considerably denser than mitochondria and could be isolated as a sharp band in the gradients. A comparison with the studies of Beaufay *et al.* (31, 32) on rat liver lysosomes, a portion of which originate from Kupffer cells, indicates that the alveolar macrophage granules are denser and more homogeneous in aqueous sucrose gradients. This, however, may reflect the cell of origin, the influence of BCG induction, and other technical factors. In addition, these particles are associated with a lipase and lysozyme, two enzymes not described in liver preparations but also found in the PMN granule (33). Another difference in the preparations is the relatively large number of hydrolase-containing particles in reference to other cytoplasmic organelles. These phagocytes therefore represent a particularly rich source of lysosomes, whose separation is facilitated by the availability of a relatively homogeneous cell population.

The granules of the macrophage also differ in many respects from those of the PMN leucocyte. These include their morphological heterogeneity, quantitative and qualitative content of enzymes (4), and the lack of any demonstrable bactericidal material in the macrophage granules. Another factor may relate to the origin of these organelles. Although poorly understood in both cell types, it is likely that the PMN granule arises during the maturation of the granulocyte in a manner similar to the formation of zymogen granules in the pancreas. This process occurs in the bone marrow and is usually complete at the time of liberation of the cell into the circulation. In contrast, the macrophage differentiates in the tissues in response to inflammatory stimuli and under environmental conditions in which phagocytosis and pinocytosis may be taking place. Many of the macrophage granules may therefore represent "phagosomes" or "residual bodies" (34) remaining from previous endocytic events and containing foreign materials. In addition, there may be "primary lysosomes" present which are produced by the cell without the stimulus of endocytosis, possibly in the region of the Golgi and arranged about it as a portion of the neutral red rosette. It should be pointed out that it is not certain whether all of the particles visualized by electron microscopy and vital staining contain the hydrolases, nor whether a full complement of enzymes is present in each active particle. These questions will remain unanswered until more definitive morphological and histochemical evidence is available.

SUMMARY

The contents of selected hydrolytic enzymes of oil-induced peritoneal, normal alveolar, and BCG-induced alveolar macrophages have been studied. On a per cell or nitrogen basis the normal alveolar cells contained considerably more acid phosphatase, cathepsin, acid ribonuclease, lysozyme, and lipase than peritoneal cells. The BCG-induced alveolar macrophage exhibited increased levels of acid phosphatase, lysozyme, and lipase as compared to alveolar macrophages from unstimulated rabbits. The morphological differences between these cells was discussed and electron micrographs of the BCG-induced macrophage presented.

Fractionation of the BCG-induced macrophage by differential centrifugation showed that 60 to 80 per cent of the total cell content of acid phosphatase, cathepsin, beta glucuronidase, acid ribonuclease, acid deoxyribonuclease, aryl sulfatase, lysozyme, and lipase were localized in a postnuclear fraction which sedimented at 15,000 *g*. This fraction also contained the majority of the mitochondria as evidenced by its content of cytochrome oxidase. Non-specific esterase was not localized to this fraction.

A separation of the hydrolase-containing particles and mitochondria was achieved by isopycnic sucrose gradient centrifugation. Under the conditions employed, the mitochondria distributed at densities of 1.19 to 1.20, whereas the hydrolase particles sedimented to a density of 1.26 to 1.27. Each of the hydrolases including acid phosphatase, beta glucuronidase, cathepsin, lysozyme, and acid ribonuclease exhibited maximum activities in the same gradient fraction.

The isolated granules exhibited enzymatic latency, and activation could be achieved by cycles of freezing and thawing or surface active agents. The majority of each of the hydrolytic enzymes could be liberated in a non-particulate form by mechanical trauma.

Macrophages which had been stained supravitally with neutral red were fractionated by differential and gradient centrifugation. More than 70 per cent of the dye could be recovered in the particulate hydrolase fraction. The isolated, stained granules resembled those seen in the intact cell.

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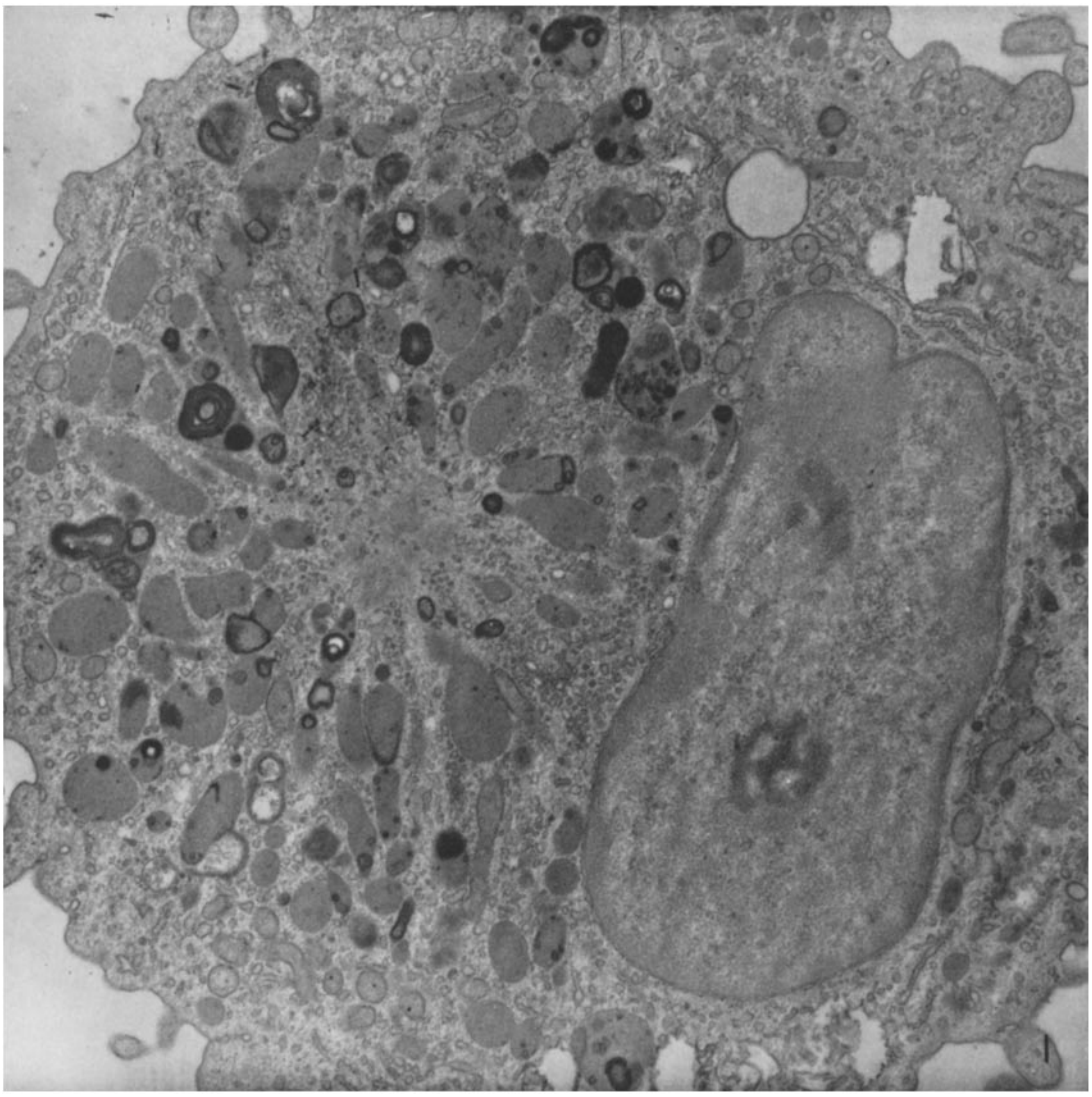
EXPLANATION OF PLATES

PLATE 85

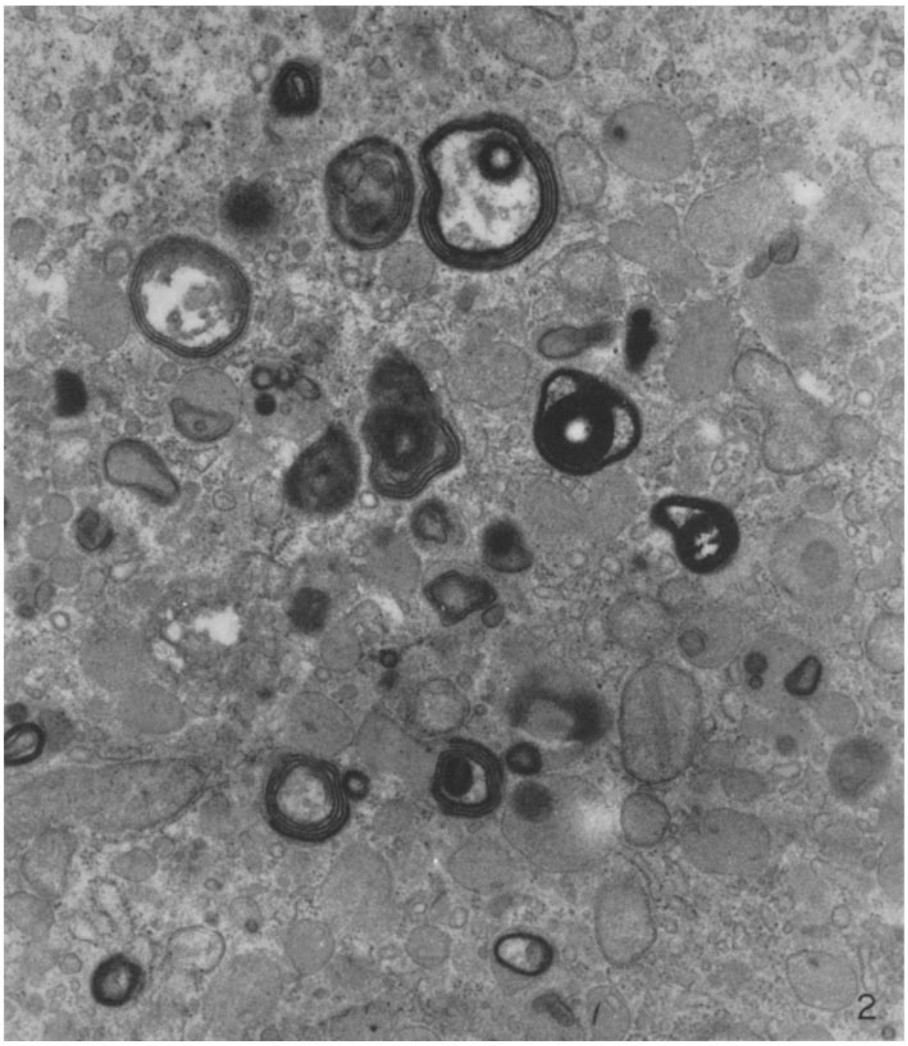
FIG. 1. An electron micrograph of a typical BCG-induced alveolar macrophage. Granules of various size, density, and internal structure are arranged in a rosette-like fashion in the perinuclear region. These particles vary from a uniform electron density to multivesiculated structures and myelin figures. Numerous small, smooth surfaced vesicles can be seen extending from the nucleus to the center of the rosette. × 12,000.



(Cohn and Wiener: Macrophage hydrolases. I)

PLATE 86

FIG. 2. An enlarged area of another BCG-induced alveolar macrophage illustrating the heterogeneity of granule structure. × 24,000.



(Cohn and Wiener: Macrophage hydrolases. I)