

## THE DIFFERENTIATION OF MONONUCLEAR PHAGOCYTES

### MORPHOLOGY, CYTOCHEMISTRY, AND BIOCHEMISTRY\*

By ZANVIL A. COHN, M.D., AND BELINDA BENSON

(From *The Rockefeller Institute*)

PLATES 5 TO 14

(Received for publication, August 27, 1964)

The mononuclear phagocytes comprise a spectrum of cell types ranging from the blood monocyte to the tissue macrophage, epithelioid, and giant cell (1). Their distribution in the body is widespread with particular localization in organs such as spleen, liver, and lymph nodes. In these and other tissue sites they form the reticuloendothelial system of Aschoff (2) and are intimately associated with defense to infectious agents (3), inflammation and tissue repair (4), immunological reactions (5), the destruction of effete erythrocytes (6), and the metabolism of lipid and bone (7, 8). Many of these physiologic parameters are related to their ability to phagocytose particulate elements and dispose of them intracellularly. In large measure, their origin, turnover, and interrelationships remain obscure.

Previous findings from this and other laboratories indicated that three types of mononuclear phagocytes obtained from the rabbit differed markedly in their content of hydrolytic enzymes (9). These results suggested either that distinct populations of macrophages existed or that the cells could alter their morphology and content of enzymes. The latter possibility was supported by earlier cytological data (10-12). These investigations demonstrated that the culture of mixed blood cells resulted in morphological alterations of the monocytes with the progressive appearance of large macrophages, epithelioid, and giant cells. In addition, Weiss and Fawcett (13) and more recently others (14, 15) have described the appearance of acid phosphatase under various experimental conditions. These studies as well as in the *in vivo* investigations of Ebert and Florey (16) indicated that the blood monocyte could alter its morphology and perhaps its content of enzymes in both *in vivo* and *in vitro* environments. To study this situation in more detail a system was required in which homogeneous populations of mononuclear phagocytes could be manipulated so as to obtain both morphological and biochemical data.

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\* This investigation was supported in part by research grant E-1831 from the National Institute of Allergy and Infectious Disease, Public Health Service.

This report will deal with the differentiation of monocyte-like cells in both an *in vitro* and *in vivo* milieu.

#### *Materials and Methods*

*Animals.*—Mice of the NCS strain (pathogen-free), maintained at The Rockefeller Institute were employed for all studies. Animals of both sexes weighing 25 gm were used as routine.

*Harvesting, Quantitation, and Processing of Mouse Mononuclear Phagocytes.*—Mice were killed rapidly with ether and the abdominal skin reflected. One ml of medium 199 (Microbiological Associates, Bethesda) was then injected intraperitoneally. The peritoneum was then perforated with a Pasteur pipette and the fluid withdrawn. This did not result in the quantitative recovery of all cells but yielded a more concentrated suspension containing  $6$  to  $10 \times 10^6$ /ml. Heparin was occasionally incorporated into the wash out fluid when large numbers of animals were to be harvested. Total leucocyte counts were performed with a hemocytometer and Turks solution. The cells were then sedimented at 500 RPM (International centrifuge, type 1) for 5 minutes, the supernatant fluid decanted and the pellet gently re-suspended in complete medium to a concentration of  $2 \times 10^6$ /ml. The tissue culture medium employed for all experiments to be presented in this paper consisted of medium 199, 20 per cent new born calf serum, and penicillin at a concentration of 200 units/ml. Components of the complete medium were obtained from Microbiological Associates.

Differential cell counts performed either as wet preparations in the hemocytometer or on Giemsa-stained smears revealed 50 to 60 per cent small monocyte-like cells with reniform nuclei and the remainder were typical lymphocytes with an occasional mast cell.

#### *Tissue Culture Techniques.*—

*Leighton tubes:* For routine morphological observations cells were cultured in Leighton tubes containing  $10 \times 35$  mm flying coverslips. One ml of cells suspended in complete medium ( $2 \times 10^6$ /ml) were dispensed to each Leighton tube. The tubes were gassed with a 5 per cent  $\text{CO}_2$ -air mixture and closed with white rubber stoppers. Tubes were then allowed to stand at  $37^\circ\text{C}$  for 30 minutes. This allowed the attachment of the monocyte-like mononuclear phagocytes to the coverslip. At this time the medium was agitated vigorously and withdrawn. The cell layer was next washed once with 2.0 ml of complete medium and agitated. The wash fluid was removed and the cells overlaid with 1.0 ml complete medium, gassed, and incubated. The agitation and wash procedure removed more than 95 per cent of the lymphocytes and in most preparations it was difficult to find such a cell on the coverslip. If lymphocytes were not removed by these steps, the monocytes engulfed many of them whereas the remainder died within 24 hours. The medium was changed at intervals of 3 days although most observations were made during the first 72 hours.

*T flasks:* When large numbers of cells were required for biochemical determinations these were cultured in pyrex T flasks with a mean surface area of  $30 \text{ cm}^2$ . Ten ml of cell suspension ( $2 \times 10^6$ /ml) was employed for each flask. In contrast to Leighton tube cultures the mononuclear phagocytes were allowed to adhere to glass for 60 minutes, agitated, washed with 10 ml of medium, and subsequently incubated with 10 ml of complete medium after gassing. These flasks had adequate optical properties so that gross morphology of the cell sheet could be followed. Medium was changed at intervals of 72 hours.

After varying periods of incubation ranging from 3 to 144 hours the cells were harvested in the following manner. The medium was completely removed by suction and the flask gently rinsed with five 15-ml aliquots of warm saline. This was found to remove all medium protein leaving the cells firmly attached to the glass surface. The last saline wash was removed carefully and 3 ml of saline was added to each flask. This volume was found to cover

the cell sheet and was necessary for further manipulations. The flasks were then placed on a flat surface and their contents frozen and thawed 4 times. This resulted in the detachment of all cells from the glass. The suspension was then removed and stored at  $-20^{\circ}\text{C}$  for biochemical determinations. This method of removing cells quantitatively was necessitated by the inadequacy of high concentrations of both trypsin and versene. Biochemical observations began after 4 hours of incubation since prior to that time some cells were not firmly attached and were removed by the saline washes.

*Microscopy and Cytochemistry.*—After varying periods of *in vitro* incubation coverslips were removed from the Leighton tubes and processed. The majority of observations were made by oil immersion phase contrast microscopy employing a Zeiss ultraphot model II.

Living cells were observed by inverting coverslips over shallow troughs of medium and sealing with a vaseline-paraffin mixture. When incubated in a warm chamber cells were well maintained for at least 24 hours with active membrane movement and pinocytosis.

*Fixation with OsO<sub>4</sub>.*—For the photography of typical cells after various periods of cultivation the following method gave the best internal structure under phase contrast. Leighton tubes were removed from the incubator, the medium withdrawn by means of a Pasteur pipette and 0.5 to 1.0 ml of cold buffered (pH 7.4) 1 per cent OsO<sub>4</sub> introduced over the coverslip. Immediate fixation occurred and this was allowed to proceed for 5 to 8 minutes at  $4^{\circ}\text{C}$ . The osmium tetroxide solution was then removed and the tube rinsed twice with 5.0 ml of distilled water. The coverslip was then withdrawn and while still wet inverted over a drop of water and the edges sealed. Such preparations could then be observed for periods up to 4 to 5 hours without cell disintegration.

*Acid phosphatase.*—A standardized procedure was employed for all cells illustrated in this article. The medium was removed and 1.0 ml of 1.25 per cent glutaraldehyde (pH 7.4) added. Fixation was allowed to proceed for 30 minutes at  $4^{\circ}\text{C}$ . The coverslips were extracted from the tubes and processed for acid phosphatase staining according to a previously described procedure (17). Incubation in the  $\beta$ -glycerophosphate substrate at  $37^{\circ}$  took place for 30 minutes. After the reaction was completed the coverslips were air-dried. They were then observed after mounting in distilled water. Cells which underwent this procedure were usually smaller in diameter than osmium tetroxide-fixed preparations. All photographs were made with phase contrast illumination to bring out internal structure.

*Staining.*—On occasion coverslips were fixed in methanol and stained by the Giemsa method. Supravital staining was performed with neutral red (neutral red, vital, Allied Chemical & Dye Corp., New York). A stock solution of dye (1 mg/ml) was made up in 8.5 per cent sucrose. This was diluted 1:10 with complete medium and a drop placed on a microscope slide. A coverslip containing viable cells was then inverted, sealed with paraffin-vaseline and promptly examined.

*Photography.*—All photographs were taken with the Zeiss ultraphot apparatus using 4 x 5 Panatomic x film at a magnification of approximately 1000. The cells were subsequently enlarged 2.5 times upon printing. All photographs were taken with phase contrast illumination.

*Biochemical Determination.*—Prior to enzymatic and chemical analysis the cell suspensions harvested from the T flask were frozen and thawed 6 times in an alcohol-dry ice bath to fully activate the hydrolases.

Protein and nitrogen were determined as described previously (9). All analyses were performed in duplicate.

*Acid phosphatase assays* (9) were performed using 0.5 to 1.0 ml aliquots of the activated suspension and incubated for 4 hours at  $37^{\circ}\text{C}$ . Activity is calculated as either  $\mu\text{g}$  phosphorus liberated from  $\beta$ -glycerophosphate/mg N/hour or as total activity per flask. The latter value represents activity/hour/ml of suspension  $\times 3$  (the total harvest volume).

*Cathepsin* determinations and activity units have been reported previously (9). Usually 0.5 ml of cell suspension was incubated for 2 hours at 37 C.

$\beta$ -Glucuronidase was assayed with phenolphthalein- $\beta$ -glucuronide (Sigma Chemical Company, St. Louis) as substrate at pH 5.0 (9). Routine assays employed 0.5 to 1.0 ml of cell suspension and were incubated at 37°C for 4 hours.

*Acid ribonuclease* determinations were described before (9) and were assayed after 3 hours' incubation at 37°C.

All enzymatic assays were linear for the time periods employed and the usual blanks were performed.

*Lipopolysaccharide (LPS) Stimulation in Vivo.*—A purified lipopolysaccharide endotoxin from *Salmonella abortus equi* was obtained originally from Dr. Otto Westphal. Twenty  $\mu$ g of LPS suspended in saline was injected intraperitoneally in a volume of 0.2 ml. At intervals ranging from 1 to 30 days groups of animals were sacrificed and peritoneal cells harvested.

Peritoneal cells from stimulated mice were employed for cultural studies as outlined in a previous section. Screening of cells for acid phosphatase activity and morphology was performed as follows. Cells were harvested as routine and drops placed on glass slides which were incubated in moist chambers for 30 minutes at 37°C. After cell adherence had occurred excess fluid was removed and the slide fixed in glutaraldehyde or with absolute methanol.

Peritoneal cells to be used for culture were obtained four days after challenge with LPS. Lymphocytes were removed by the usual washing technique and the cultures assayed. Analyses on T 4-hour cultures represented the initial level of enzyme in the stimulated cells.

## RESULTS

*General Considerations and Observations.*—For a detailed analysis of the differentiation of mononuclear phagocytes a model system was required which had: (a) Homogeneous populations of young monocyte-like cells, (b) the absence of degenerating cells, e.g. granulocytes, platelets, as a source of phagocytatable particles, (c) large numbers of easily available cells for both morphological and biochemical determinations, (d) a phased temporal "maturation" *in vitro*.

Preliminary observations were made with preparations of BCG-induced alveolar macrophages. These cells, although available in large numbers, were mature macrophages with large amounts of hydrolytic enzymes. In culture these cells did not exhibit increases in hydrolases but in many instances formed syncytial giant cells. It was subsequently found that the mononuclear phagocytes in the normal mouse peritoneal cavity met the above criteria.

Initial observations indicated that a medium composed of No. 199, newborn calf serum, and penicillin yielded excellent cell survival and maturation. The calf serum was as effective as mouse serum and was employed as routine, since it was readily available. During the course of *in vitro* cultivation, for periods up to 6 days, there was no evidence of cell division as ascertained by quantitative cell counts. In addition, following the use of colchicine at 0.01 to 0.05  $\mu$ g/ml, no metaphase figures were observed in appropriately stained cover slips.

*The Temporal Sequence of Morphological Differentiation.*—The introduction

of unstimulated mouse peritoneal cells into a tissue culture environment resulted in a reproducible series of alterations. The initial population of cells (after washing) was composed entirely of monocyte-like phagocytes which adhered readily to glass. At 3 to 4 hours of incubation the cells were well attached and were beginning to flatten out. At this time the majority exhibited a single nucleus which was indented and centrally located. A small percentage were binucleated and in general appeared to be somewhat large in diameter. Figs. 1 *a* to 1 *c* illustrate the appearance of three typical cells at 3 to 4 hours of incubation. After osmium tetroxide fixation the mitochondria appeared as phase-dense rods scattered through the cytoplasm. A number of spherical dense bodies were also apparent which in many instances were found near the nucleus. In addition, Figs. 1 *a* and 1 *b* illustrate less distinct lamellae present in the peripheral cytoplasm which corresponded to the endoplasmic reticulum as seen in the electron microscope. These cells were thus typical of the starting population and resembled mouse blood monocytes in size and phase contrast appearance.

After 24 hours of *in vitro* incubation the cells had increased in diameter, as shown in Figs. 2 *a* to 2 *c*. As illustrated in Figs. 2 *a* and 2 *b* the cells had become flatter and had developed larger numbers of phase-dense, osmiophilic bodies in the perinuclear area. In addition, highly refractile lipid droplets were beginning to form at this time. Fig. 2 *c* is a binucleate cell which is much larger in size but shows similar changes. The mitochondria appear to be larger and give the impression of being more numerous. Typically, mitochondria were found in the peripheral cytoplasm.

Fig. 2 *d* illustrates a cell at approximately 48 hours of cultivation. At this time long projections of the cytoplasm were present and a well developed cluster of dense granules had appeared, which in this case were oriented to one side of the nucleus.

In Figs. 3 *a* to 3 *c* there are three cells which are typical of 48 to 72 hour cultures. Long cytoplasmic processes containing mitochondria were present. Cell diameter had increased and more refractile lipid droplets were found. The lipid droplets usually accumulated on the side of the nucleus opposite to that which contained the dense granules, although they also may be seen on the periphery of the dense granule area. An obvious increase in the number of phase-dense granules had occurred and in some cases the granules were smaller in size than at earlier time periods.

After 6 days of cultivation the cells had continued to grow in size and the majority of the cytoplasm was filled with spherical phase-dense granules (Figs. 4 *a* and 4 *b*). The granules appeared smaller in size than on previous occasions and were not confined to a definite centrosphere. Many more mitochondria were present but most were obscured by the large number of granules. In comparing the initial cell population to the cells at 6 days there was roughly

a 4- to 5-fold increase in surface area. A polarization in cell outline was often seen at this time (see Figs. 8 *a* and 8 *b*) with a leading and trailing edge suggesting active locomotion. More lipid droplets were usually seen although these are not present in the illustrations.

If cultures were maintained for longer intervals (11 days) some of the cells lost their dense granules and were seen to be filled with mitochondria. A portion of one such cell is illustrated in Fig. 5. This change became particularly apparent if the medium was changed at intervals of more than 72 hours. Associated with the morphological alteration many of these epithelioid-like cells began to fuse and form large syncytia. Giant cell formation was very uncommon prior to this time.

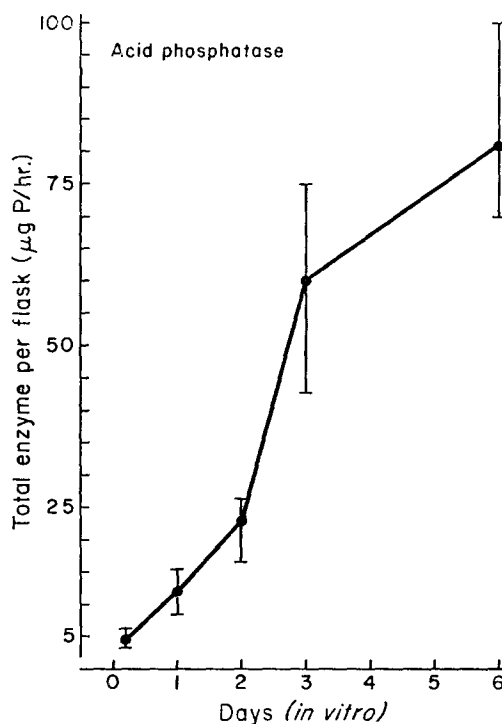
*The Temporal Sequence and Localization of Cytochemically Demonstrable Acid Phosphatase.*—Associated with the changes in cell diameter and the accumulation of phase-dense, osmiophilic granules there were also alterations in the content of cytochemically demonstrable acid phosphatase. These changes are illustrated in Figs. 6 to 8. The cells presented in these 3 figures have all been manipulated in an identical manner except for the time of *in vitro* cultivation.

Figs. 6 *a* to 6 *c* represent three cells stained for acid phosphatase after 4, 24, and 60 hours of incubation respectively. Fig. 6 *a* shows a small amount of reaction product in the perinuclear area, some of which appears localized to tiny granules. After 24 hours of incubation and thereafter the reaction product was found in the newly formed granules which appeared in the perinuclear region. Cells in Figs. 6 *b* and 6 *c* show the progressive increase in acid phosphatase-positive granules. Mitochondria were uniformly negative although they appear somewhat dark because of the phase optics. In addition there were other structures, seen both in glutaraldehyde fixed preparations and in living specimens, which were not so apparent in osmium fixed materials. These were pinocytic vesicles which appeared as clear vesicles both in the peripheral cytoplasm and in amongst the acid phosphatase-positive granules in the centrosphere. These clear vesicles were uniformly negative for acid phosphatase. Figs. 7 *a* and 7 *b* illustrate two other cells which show good localization of acid phosphatase to the dense granules of the centrosphere. In contrast Fig. 7 *c* represents a control cell fixed with glutaraldehyde but incubated in the absence of substrate. In this instance granules were not very evident and appeared phase-lucent. In all instances lipid bodies were negative for the enzyme.

After 6 days of cultivation (Figs. 8 *a* and 8 *b*) the cytoplasm of the cell was filled with smaller, acid phosphatase-containing granules which corresponded to the dense structures seen in Figs. 4 *a* and 4 *b*. Pinocytic vesicles were again apparent at this time. The positive granules were less prominently arranged in the centrosphere and were distributed throughout the cytoplasm. Although not illustrated, cells of the type shown in Fig. 5 demonstrated a reduced num-

ber of acid phosphatase-positive granules, when compared to earlier time periods.

From these observations it appeared that with prolonged *in vitro* cultivation there was a progressive increase in the number of acid phosphatase-positive granules. Whether or not individual granules exhibited more intense staining was difficult to evaluate although in some instances this seemed to be the case.



TEXT-FIG. 1. The production of acid phosphatase by normal mouse mononuclear phagocytes cultivated *in vitro*.

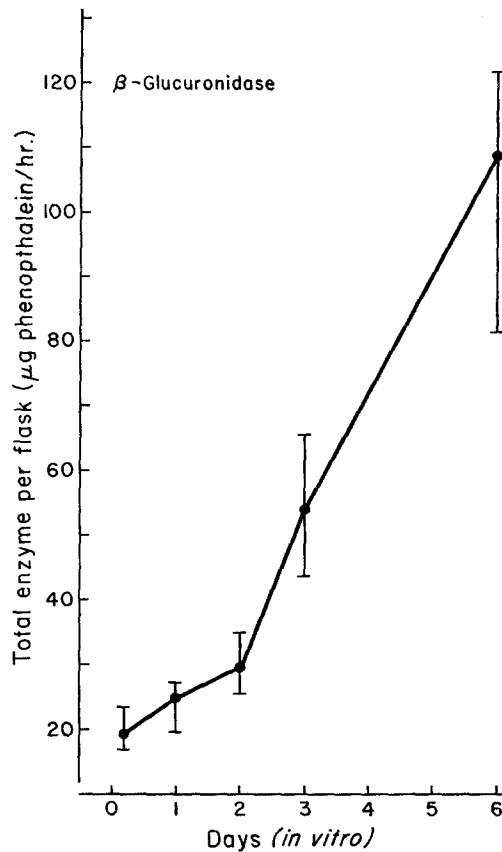
This is illustrated in cells of Figs. 7 *a* and 7 *b* in which the reaction was so intense that a halo appeared about the cluster of granules,—this had the yellowish-brown appearance of lead sulfide under phase optics.

The granules which exhibited acid phosphatase by the Gomori procedure were similar in number and distribution to neutral red-positive granules in supravital preparations.

*The Temporal Sequence of Hydrolytic Enzyme Production as Determined Biochemically.*—In view of the progressive increase in the number of acid phosphatase-positive granules it was of importance to study the fluxes of this enzyme with more quantitative biochemical techniques. Since acid phosphatase

is often used as an indicator of lysosomes, two other typical lysosomal hydrolases were also examined. All of the subsequent experiments were derived from mass cultures maintained in T flasks.

Text-fig. 1 shows the increase in the total acid phosphatase content of cells



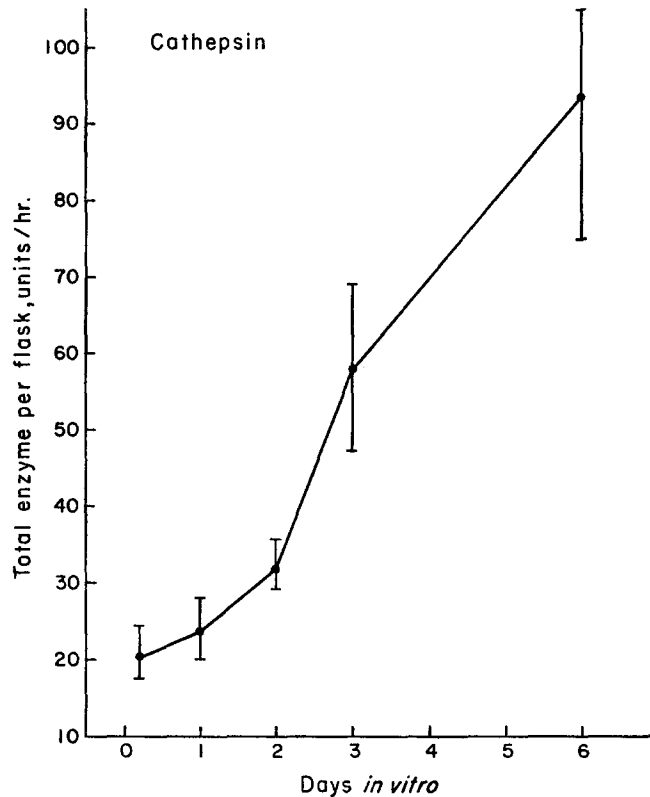
TEXT-FIG. 2. The production of  $\beta$ -glucuronidase by normal mouse mononuclear phagocytes cultivated *in vitro*.

after periods of incubation ranging from 4 hours to 6 days. The solid circles represent the mean values of 10 separate experiments and the vertical bars the maximum and minimum values. Within the first 2 days there was a 4-fold increase in enzyme. At 72 hours of cultivation there was a 13-fold increase and at 6 days total enzyme was approximately 20-fold above the starting value.

Text-figs. 2 and 3 illustrate the results with  $\beta$ -glucuronidase and cathepsin respectively. Over the 6 day period a  $5\frac{1}{2}$ -fold increase in the total activity of



$\beta$ -glucuronidase and a  $4\frac{1}{2}$ -fold increase in cathepsin took place. The rates of production of both enzymes was somewhat different than that of acid phosphatase. In each instance little increase in total enzyme occurred during the first 48 hours, thereafter the level of these hydrolases rose sharply with major increments during the latter 72 hours of incubation.



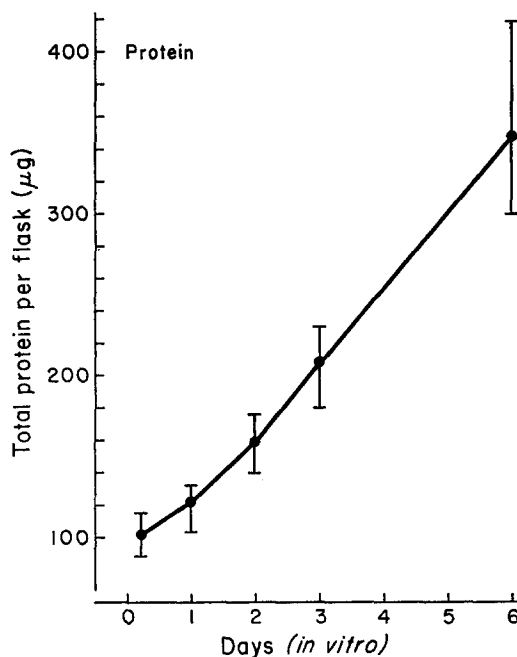
TEXT-FIG. 3. The production of cathepsin by normal mouse mononuclear phagocytes cultivated *in vitro*.

In keeping with the progressive enlargement of the cultured cells there was an associated increase in cell protein. This is illustrated in Text-fig. 4 and occurred without a demonstrable difference in cell numbers. After the first 24 hours there was a relatively linear increase in recoverable protein. At 72 hours, a 2-fold change had occurred and this rose to a value 3 times above the base-line after 6 days.

In addition to the differences in the total enzyme content of cultures there were also variations in their specific activities. Text-fig. 5 indicates the rapid

increase in the activity of acid phosphatase on a nitrogen basis. Within a 72 hour period specific activity of the enzyme rose 20- to 30-fold. After this time period the values leveled off even though more total enzyme was being produced.

Less striking increments occurred in the specific activities of  $\beta$ -glucuronidase and cathepsin (Table I). These enzymes increased 2- to 3-fold during the incu-



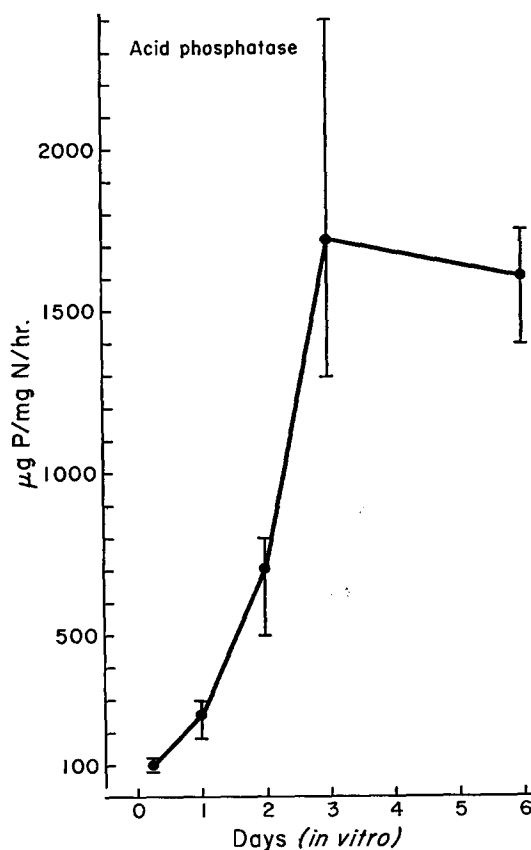
TEXT-FIG. 4. The increase in protein content of normal mouse mononuclear phagocytes cultivated *in vitro*.

bation period and the temporal changes were again different from that of acid phosphatase. Examination of acid-ribonuclease, however, showed a reduction in specific activity although a small increase in total enzyme was noted.

*The in Vivo Differentiation of Mouse Peritoneal Phagocytes.*—The previous morphological and biochemical events occurred in an artificial environment in the presence of heterologous serum and one in which the cells were attached to a glass surface. It was therefore of importance to compare this with an *in vivo* situation. This was accomplished by following a controlled inflammatory process within the peritoneal cavity.

Following the administration of 20  $\mu$ g of a bacterial lipopolysaccharide intraperitoneally a variety of cellular events occur in the cavity (18). Within 24

hours circulating PMN and possibly other blood cells emigrate into the peritoneum. Total cell counts rise to 2- to 3-fold above normal levels and are maintained for a week or more. For the first 72 hours there are a mixture of PMN and mononuclear elements while thereafter changes occur in the morphology



TEXT-FIG. 5. The increase in specific activity of acid phosphatase upon *in vitro* cultivation

of the mononuclear phagocytes. At 48 hours, large macrophages appear, which in settle preparations, immediately flatten out on glass surfaces and demonstrate amoeboid motion. These are larger cells containing increased amounts of cytochemically demonstrable acid phosphatase (19, 20). By the 4th to 6th day, they constitute the majority of the mononuclear phagocytes. Of interest is the fact that they are still present at 2, 3, and 4 weeks after a single intraperitoneal injection of LPS. Administration of LPS by the intravenous route results in less striking effects and 2 to 3 times more material is required to alter the characteristics of a portion of the peritoneal cells.

Figs. 9 and 10 illustrate the appearance of the large macrophages obtained from mice 4 days following the administration of LPS and shortly after attachment to coverslips. These cells illustrate the presence of numerous phase-dense

TABLE I  
*Specific Activities of Selected Mouse Macrophage Hydrolases under in Vitro Conditions*

Enzyme	<i>In vitro</i> cultivation		
	4 hrs.	72 hrs.	144 hrs.
Cathepsin*	668 (415-874)	1190 (1008-1325)	1401 (1247-1612)
$\beta$ -Glucuronidase†	736 (607-820)	1420 (1248-1550)	1965 (1716-2295)
Acid ribonuclease‡	114 (78-130)	65 (58-79)	74 (66-97)

Mean values of 7 to 12 experiments with minima and maxima.

\* Cathepsin, units/mg N/hour.

†  $\beta$ -Glucuronidase,  $\mu$ g phenolphthalein/mg N/hour.

‡ Ribonuclease, OD units 260 m $\mu$ /mg N/hour.

TABLE II  
*Hydrolase Activities\* of Normal and Lipopolysaccharide-Stimulated† Mouse Mononuclear Phagocytes in Vitro*

	4 hrs. <i>in vitro</i>				72 hrs. <i>in vitro</i>			
	Control		LPS		Control		LPS	
	Specific activity	Total enzyme	Specific activity	Total enzyme	Specific activity	Total enzyme	Specific activity	Total enzyme
Acid phosphatase.....	98	4	670	46	1380	55	1145	42
$\beta$ -Glucuronidase.....	650	15	1163	49	1297	52	2240	75
Cathepsin.....	480	21	840	44	1225	43	1520	65

\* Specific activity and total enzyme in units according to previous Figures. Mean value of 5 experiments.

† Cells harvested 4 days following the intraperitoneal injection of 20  $\mu$ g LPS.

granules and lipid droplets similar to cells which have differentiated *in vitro*. In addition, Figs. 9 a to 9 c demonstrate the well developed granule rosette, also seen in rabbit alveolar macrophages but not as apparent in *in vitro* cultivated cells. Although not illustrated, these granules stain positively for acid phosphatase. In contrast to *in vitro* maintained cells, the stimulated macro-

phages often contain fragments of phagocytosed cells (Figs. 10 *a* and 10 *b*). In general, cells from LPS-stimulated mice show more variation in size and granule content.

Quantitative biochemical studies were performed on (*a*) macrophages obtained 4 days following intraperitoneal LPS, and (*b*) after their subsequent cultivation *in vitro* for 72 hours (Table II). It is apparent that these cells initially contained (4 hours *in vitro*) larger quantities of the three hydrolases than cells from normal mice. Following *in vitro* cultivation these enzymes continued to increase although the rate was somewhat slower than that found in cells obtained from unstimulated animals. It is not certain under these conditions to what extent the initial monocyte-like cell population was fortified by intravascular monocytes or by local elements. However, irrespective of origin, the morphological and biochemical differentiation taking place *in vivo* was similar to the *in vitro* stimulated events.

#### DISCUSSION

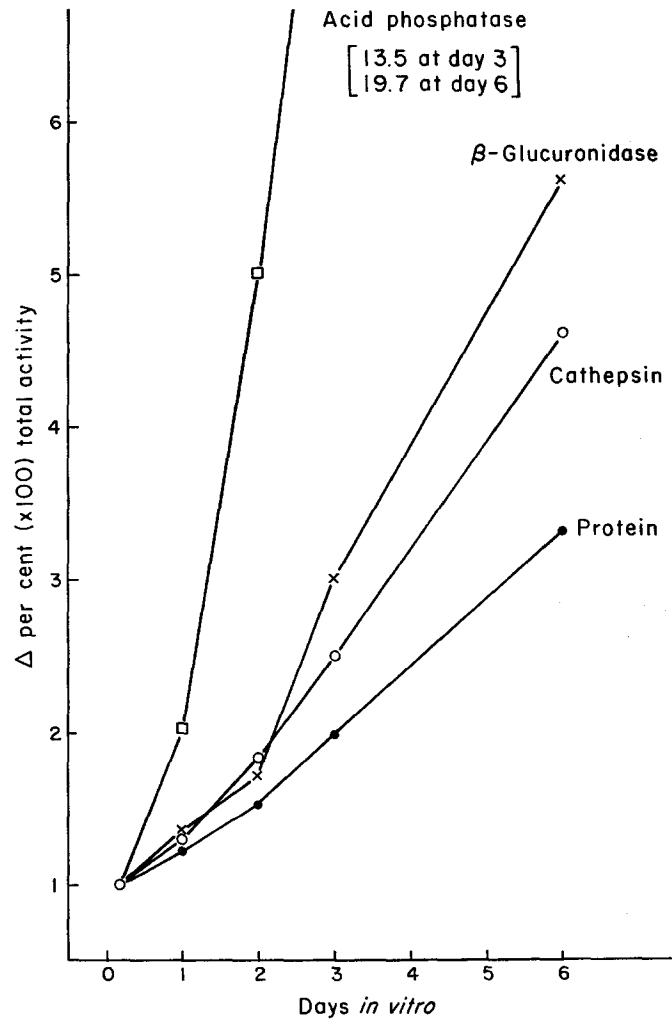
The stimulus for the differentiation of mononuclear phagocytes is as yet unknown. Certainly this occurs continually in the body and becomes apparent during episodes of inflammation when large populations differentiate at a given time. In addition, it proceeds *in vitro* upon exposure to an artificial environment and progresses in a fashion which is similar to that described *in vivo*. The engulfment of solid particles (phagocytosis) by the cell was not a necessary stimulus in the present experiments although it may play some role under the usual inflammatory conditions.

During differentiation, both *in vivo* and *in vitro*, there was a progressive increase in cell size and protein content. Associated with cell growth there was the development of phase-dense granules which accumulated in the perinuclear region. The majority of these organelles reacted strongly for acid phosphatase and thereby satisfied an important criterion for being recognized as a form of lysosome (21, 22). With the accumulation of cytochemically demonstrable acid phosphatase there were also large increases in biochemically assayable enzyme. In addition, considerably more  $\beta$ -glucuronidase and cathepsin were present although these enzymes have not been localized to the same granules by cytochemical techniques. However, these hydrolases are associated with acid phosphatase-positive granules in both rat liver (23) and alveolar macrophages (9) as ascertained by isopycnic sucrose gradient centrifugation.

The rate of hydrolase production during differentiation was not the same for all enzymes. Text-fig. 6 clarifies this difference and illustrates the more rapid rate of production of acid phosphatase as compared to cathepsin and  $\beta$ -glucuronidase. This suggests that the granules formed during the early phase of differentiation contain different ratios of the three hydrolases as compared to the granules produced at later periods. Another interpretation would be

that cathepsin and  $\beta$ -glucuronidase are synthesized at a later date and then enter all preformed and newly formed granules. Further evidence on the mode of formation and turnover of both granule membrane and granule contents is therefore required.

Another morphological change during differentiation was the apparent increase in the number of mitochondria. As yet this alteration has not been corroborated by biochemical data. It does, however, suggest that a change in



TEXT-FIG. 6. The relative rates of production of three hydrolytic enzymes by mouse mononuclear phagocytes *in vitro*.

the pattern of energy metabolism may be occurring during the process. In this regard, more mature macrophages, with higher concentrations of lysosomal hydrolases, depend to a greater extent on oxidative phosphorylation for their phagocytic activity (24, 17).

What role does macrophage development play in the economy of the host? No clear cut answer is available although circumstantial evidence appears in a number of fields. One of these is in the process of "cellular immunity" in which certain macrophages can be shown to be more resistant to infectious agents (25, 26). Since it has been demonstrated that macrophages liberate some or all of their hydrolases in response to particle engulfment (17), a greater number of lysosomes may have some advantage for the host. Another possibility arises in the more efficient disposal of inflammatory products and damaged tissues. The studies of Weber (27) suggest that during the thyroxine-induced regression of the tadpole tail, macrophages which are engulfing muscle fragments develop increased levels of acid phosphatase. Further speculation is unwarranted at this time and demands more careful functional studies at different stages in the process. These data do suggest, however, that levels of macrophage digestive enzymes may be governed by environmental stimuli, and serve as an adaptive response to the inflammatory state.

Many questions remain unanswered regarding the mechanisms and stimuli which evoke these biochemical and morphological phenomena. These will no doubt be of considerable interest in terms of our understanding of the mononuclear phagocytes, pinocytosis and the formation and turnover of lysosomes. Studies on these topics as well as the ultrastructure of differentiation are currently in progress and will be reported separately.

#### SUMMARY

The *in vitro* differentiation of homogeneous populations of monocyte-like cells from the unstimulated mouse peritoneal cavity is described. Under the conditions employed, a progressive increase in cell size occurs without significant cell division. This process is characterized morphologically by the accumulation of phase-dense and neutral red-positive granules, mitochondria, and lipid droplets. The phase-dense granules react strongly for acid phosphatase. Biochemical determinations indicate marked increases in the total content and specific activity of acid phosphatase, cathepsin, and  $\beta$ -glucuronidase. The production of acid phosphatase is more rapid and extensive than that of the other two hydrolases. From these data it appears that the conversion of a monocyte-like cell to a mature macrophage is accompanied by the formation of increased numbers of lysosome-like cytoplasmic organelles.

Mouse peritoneal phagocytes stimulated *in vivo* with a bacterial lipopolysaccharide undergo a similar series of morphological and biochemical events.

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## EXPLANATION OF PLATES

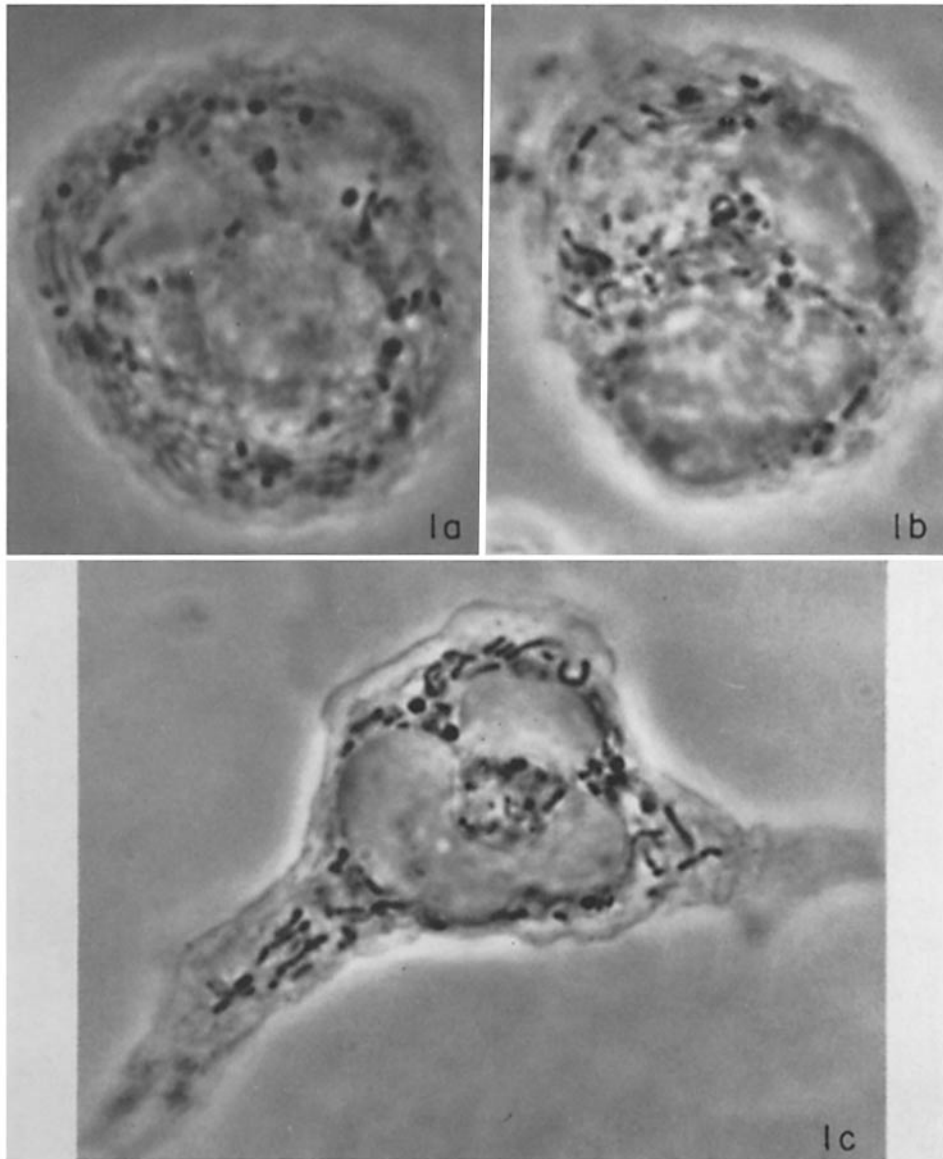
FIGS. 1 to 5. Normal mouse peritoneal phagocytes during the course of *in vitro* cultivation. All cells fixed in 1 per cent buffered osmium tetroxide and viewed with phase contrast.  $\times 2500$ .

FIGS. 6 to 8. Acid phosphatase-stained normal mouse peritoneal phagocytes during the course of *in vitro* incubation. All cells fixed in 1.25 per cent buffered glutaraldehyde and incubated in substrate for 30 minutes. Phase microscopy.  $\times 2500$ .

FIGS. 9 and 10. Cells obtained from the peritoneal cavity of mice injected 4 days previously with 20  $\mu\text{g}$  lipopolysaccharide. Four hours *in vitro*. Osmium tetroxide fixation, phase contrast.  $\times 2500$ .

## PLATE 5

FIGS. 1 *a* to *c*. Characteristic cells at 3 to 4 hours of cultivation illustrating the range of size and appearance. The cytoplasm contains a few phase-dense granules and rod-like mitochondria. Cell in Fig. 1 *c* has flattened out more rapidly but exhibits similar cytoplasmic organization.

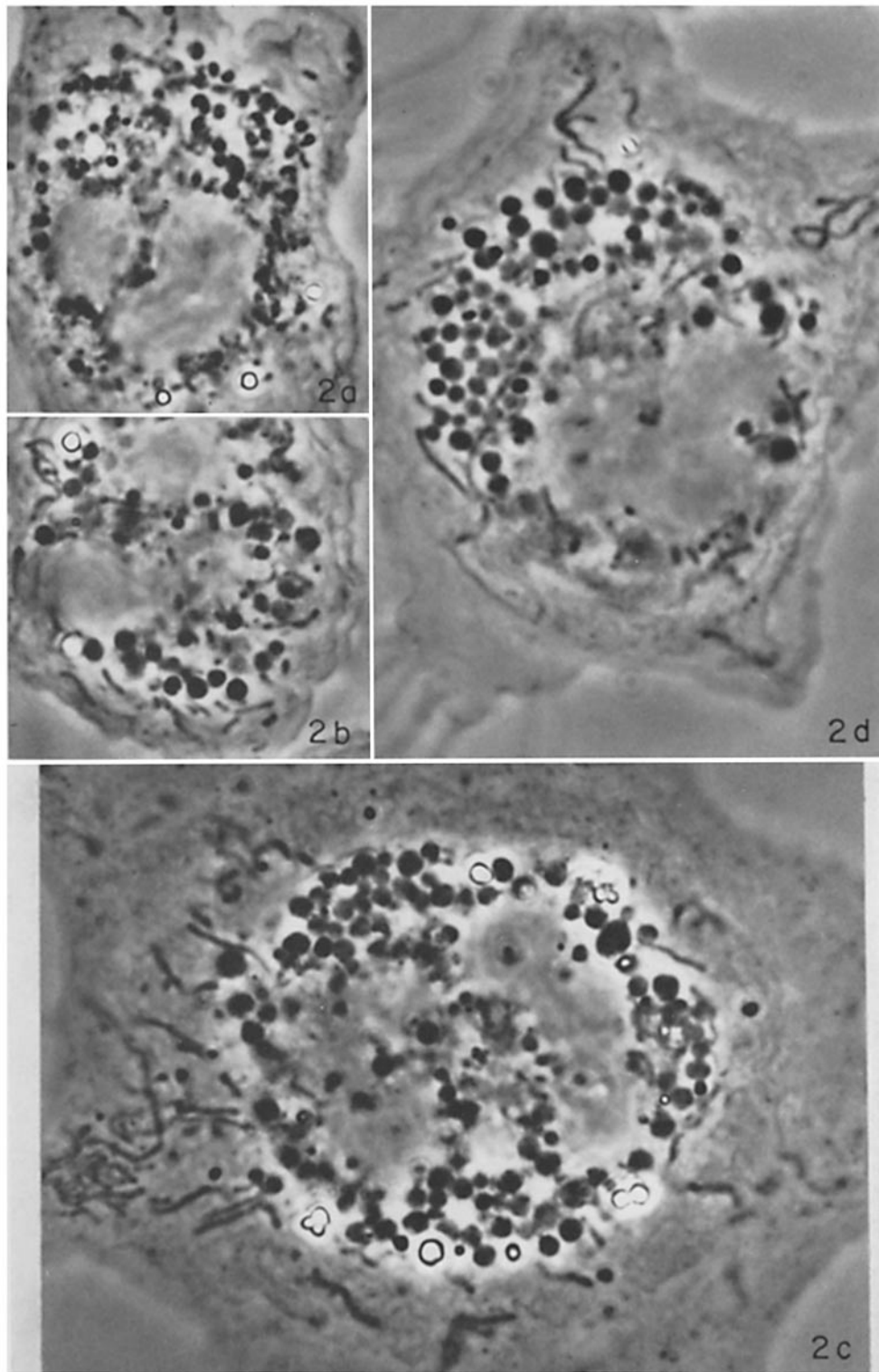


(Cohn and Benson: Macrophage differentiation)

PLATE 6

FIGS. 2 *a* to 2 *d*. Cells were osmium tetroxide-fixed after 24 hours of *in vitro* cultivation. They show the increased number of osmiophilic, phase-dense granules in the cytoplasm. Cell in Fig. 2 *c* is binucleate, large in diameter, and contains granules in a halo about the nuclei. A few highly refractile lipid droplets are present as well as more peripherally located mitochondria.

Cell in Fig. 2 *d* is typical of the 48 hour period. The majority of the granules are sharply demarcated in the centrosphere area, whereas mitochondria extend into the cytoplasmic projections.

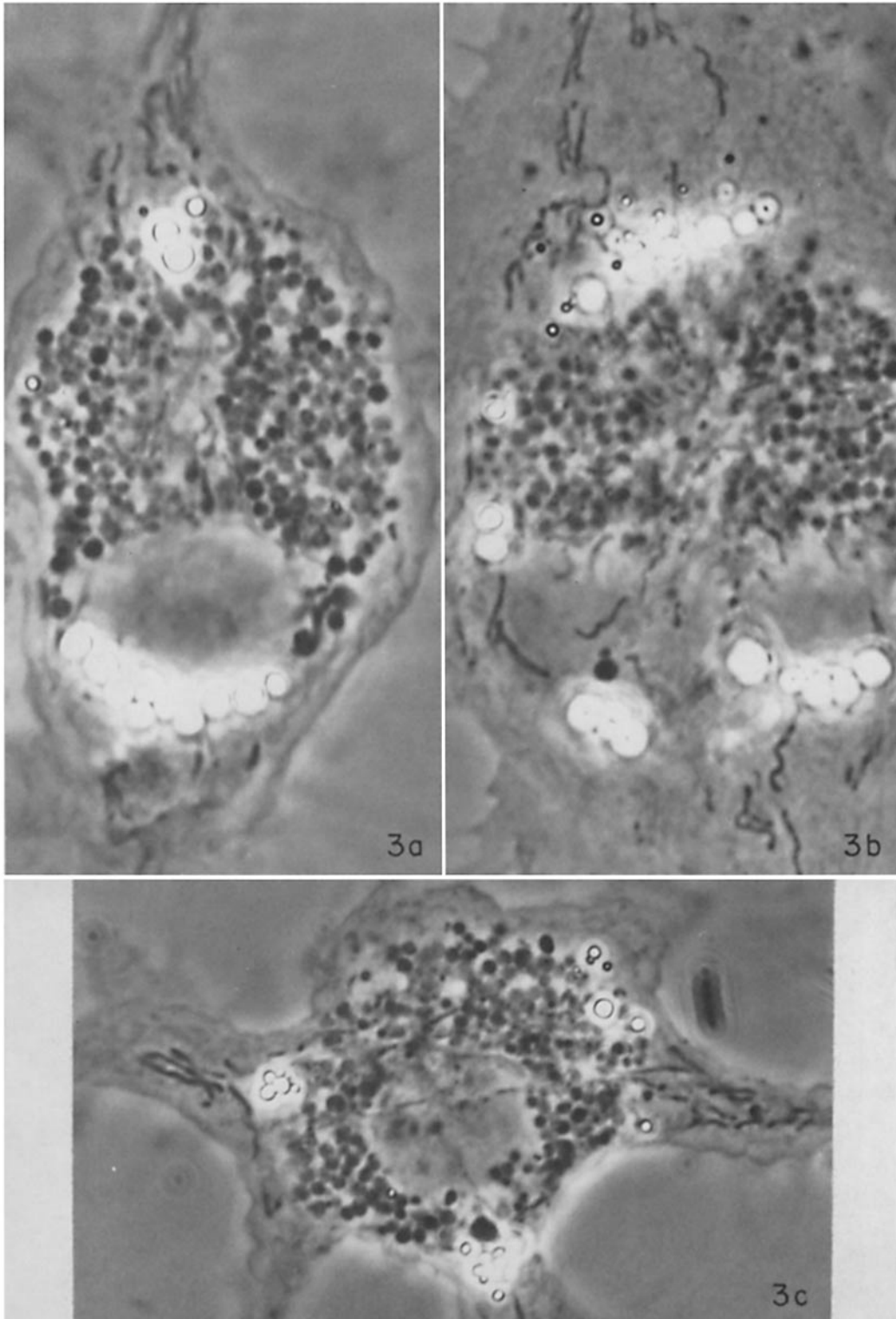


(Cohn and Benson: Macrophage differentiation)

PLATE 7

FIGS. 3 *a* to 3 *c*. The appearance of *in vitro* cultivated cells at 72 hours.

Cell diameter has increased with the accumulation of more dense granules in the perinuclear region. Lipid droplets are more numerous and are characteristically found on one side of the nucleus or at the periphery of the granule region. Mitochondria are apparent in the long pseudopods.

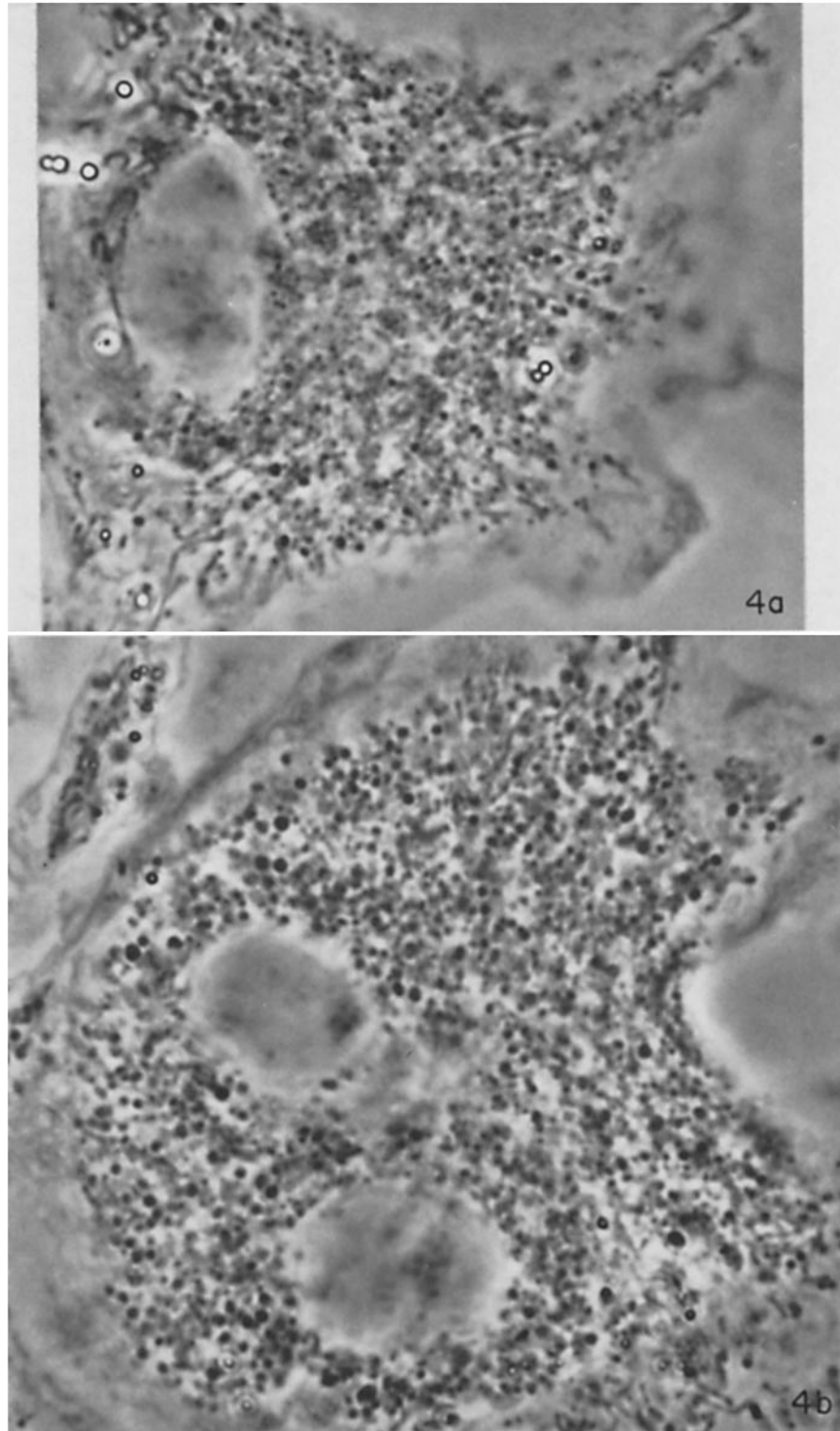


(Cohn and Benson: Macrophage differentiation)

PLATE 8

FIGS. 4 *a* and 4 *b*. Cells after 144 hours of *in vitro* cultivation. A progressive increase in cell size and granules has occurred. Almost the entire cytoplasm contains granules which are somewhat smaller than at 48 and 72 hours. Mitochondria are interspersed among the granules but are difficult to visualize in these photographs.

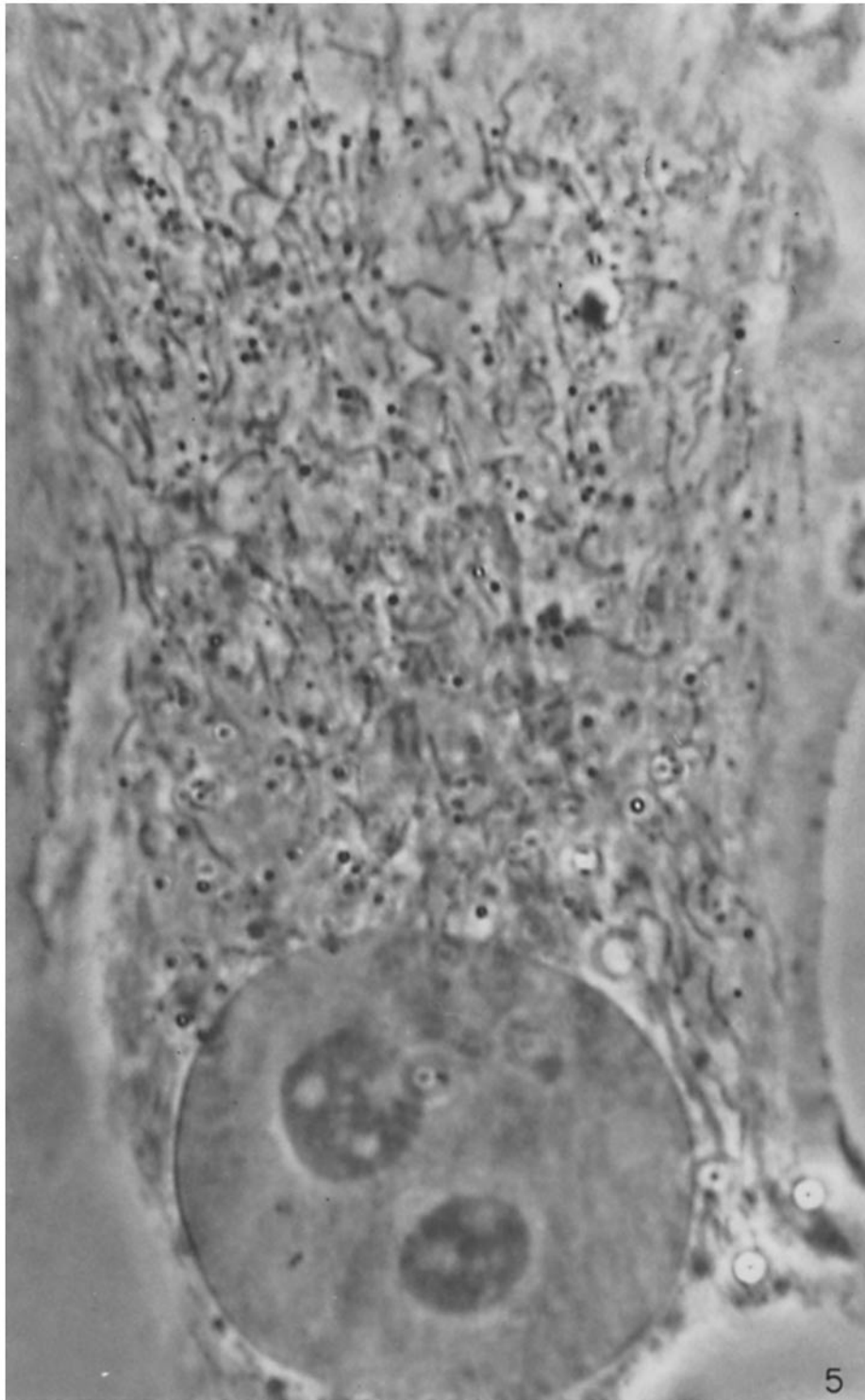




(Cohn and Benson: Macrophage differentiation)

PLATE 9

FIG. 5. A portion of one cell after 11 days of *in vitro* cultivation. The granules are much smaller and considerably reduced in number. The striking morphological picture is that of numerous mitochondria which fill the cytoplasm.



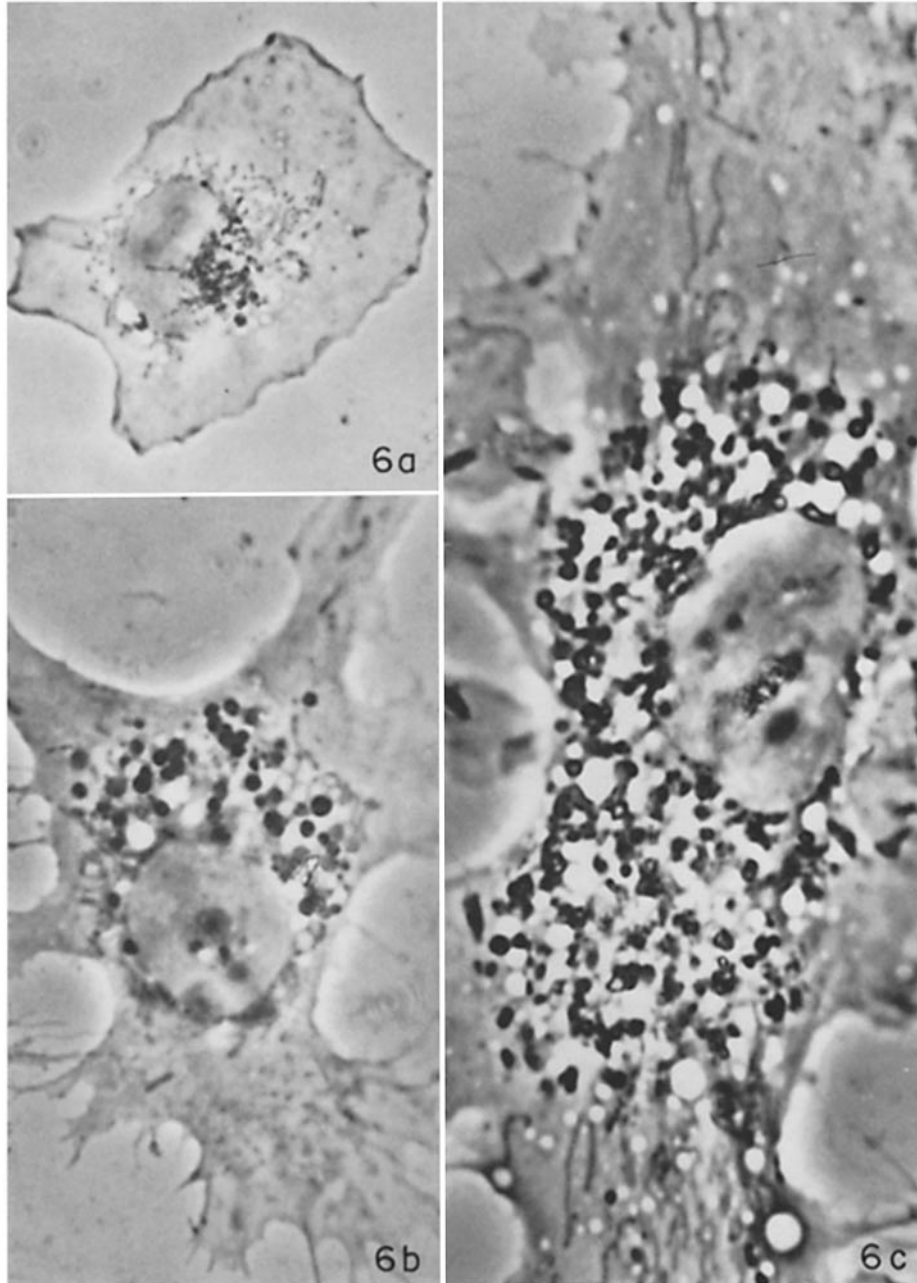
(Cohn and Benson: Macrophage differentiation)

PLATE 10

FIG. 6 *a*. Four hours *in vitro*. A small amount of reaction product in perinuclear area.

FIG. 6 *b*. Twenty-four hours *in vitro*. Black reaction product in granules of the centrosphere area. Clear pinocytotic vesicles are also present in the perinuclear region and are negative. Mitochondria are uniformly negative.

FIG. 6 *c*. Sixty hours *in vitro*. Increased number of acid phosphatase-positive granules. Pinocytotic vesicles can be seen in the pseudopods. These fuse and become larger as they reach centrosphere area.

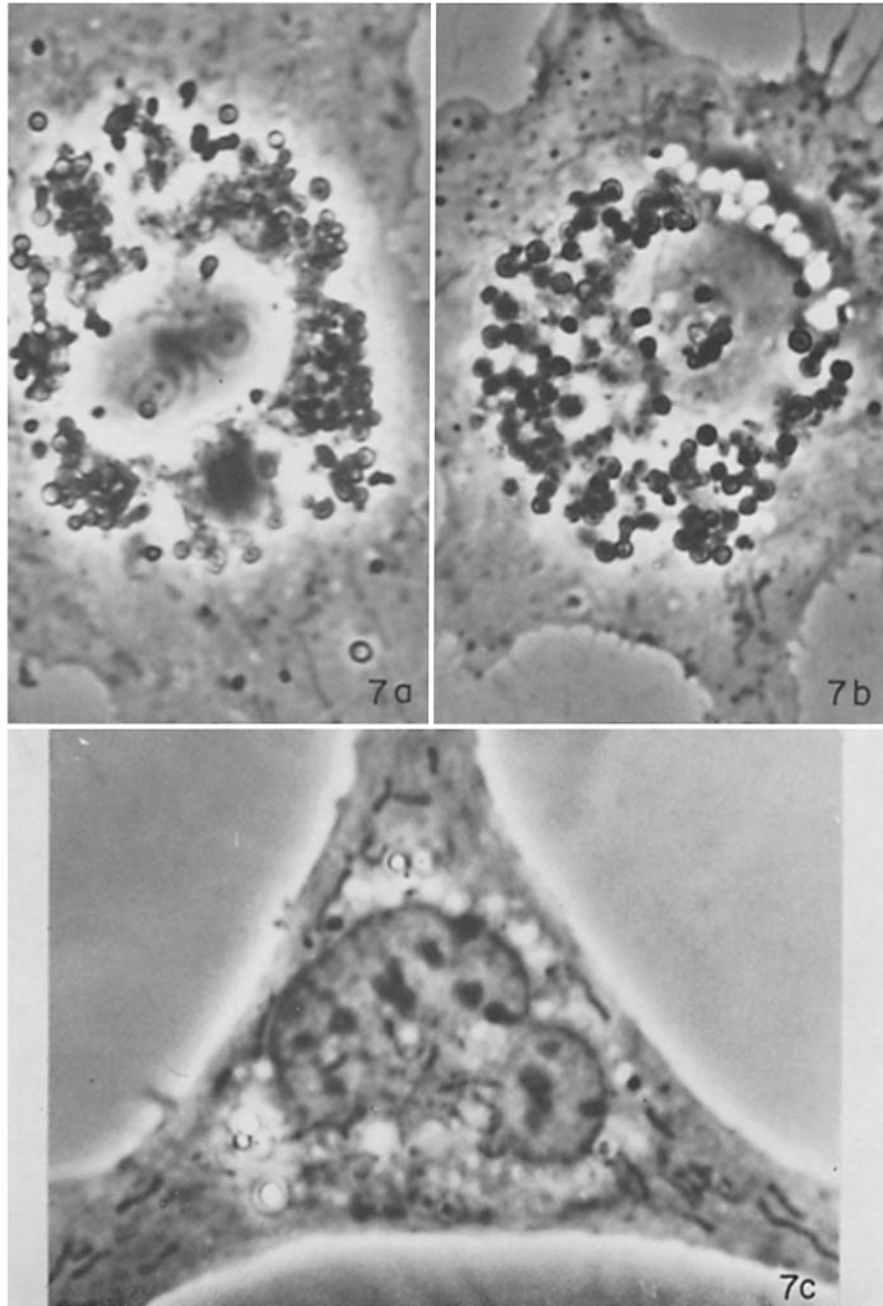


(Cohn and Benson: Macrophage differentiation)

PLATE 11

FIGS. 7 *a* and 7 *b*. Portions of two cells at 72 hours of incubation. Strongly positive granules are present in perinuclear area. Cell in Fig. 7 *b* shows a cluster of negative lipid droplets in the right upper quadrant of the perinuclear zone.

FIG. 7 *c*. Control cell incubated in absence of substrate showing a few phase-dense granules.

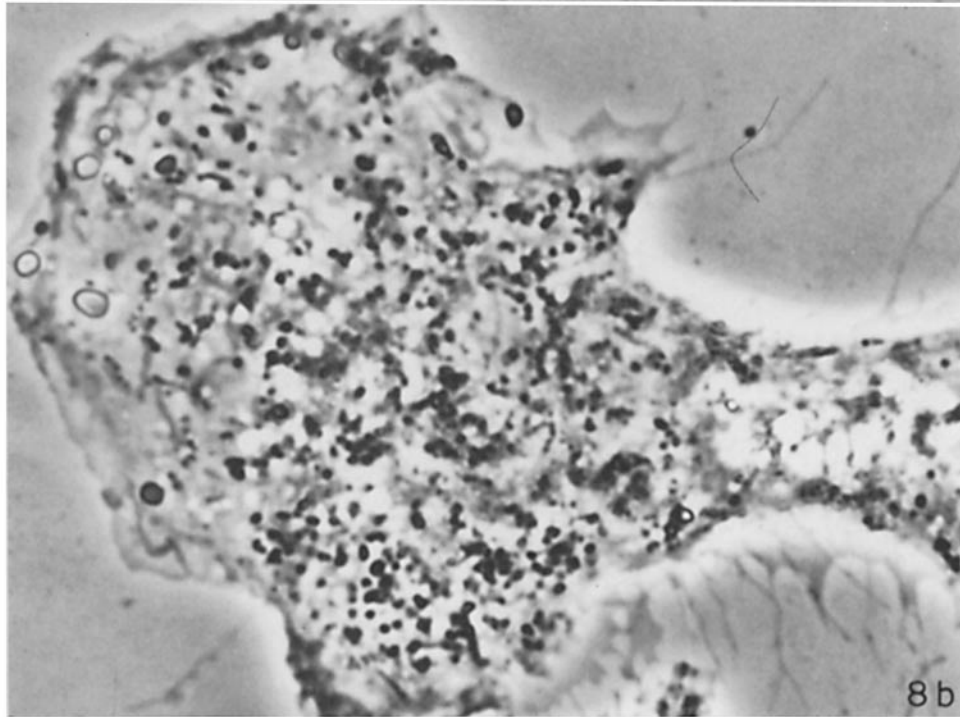
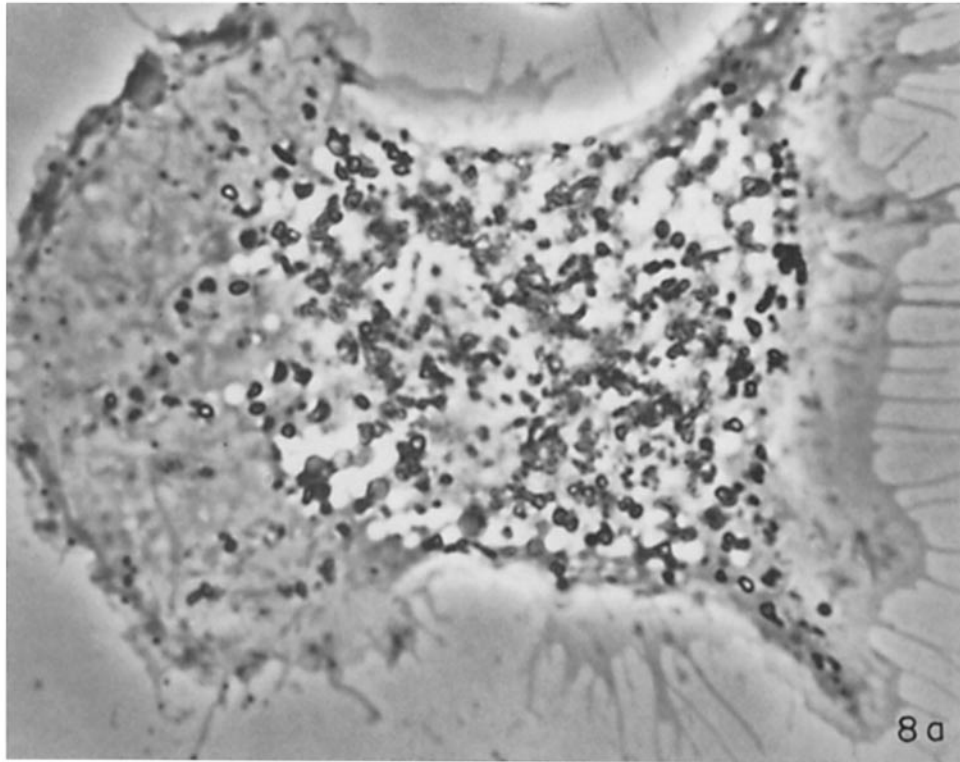


(Cohn and Benson: Macrophage differentiation)

PLATE 12

FIGS. 8 *a* and 8 *b*. Cells at 144 hours of cultivation. Many small acid phosphatase positive granules are scattered throughout the cytoplasm.

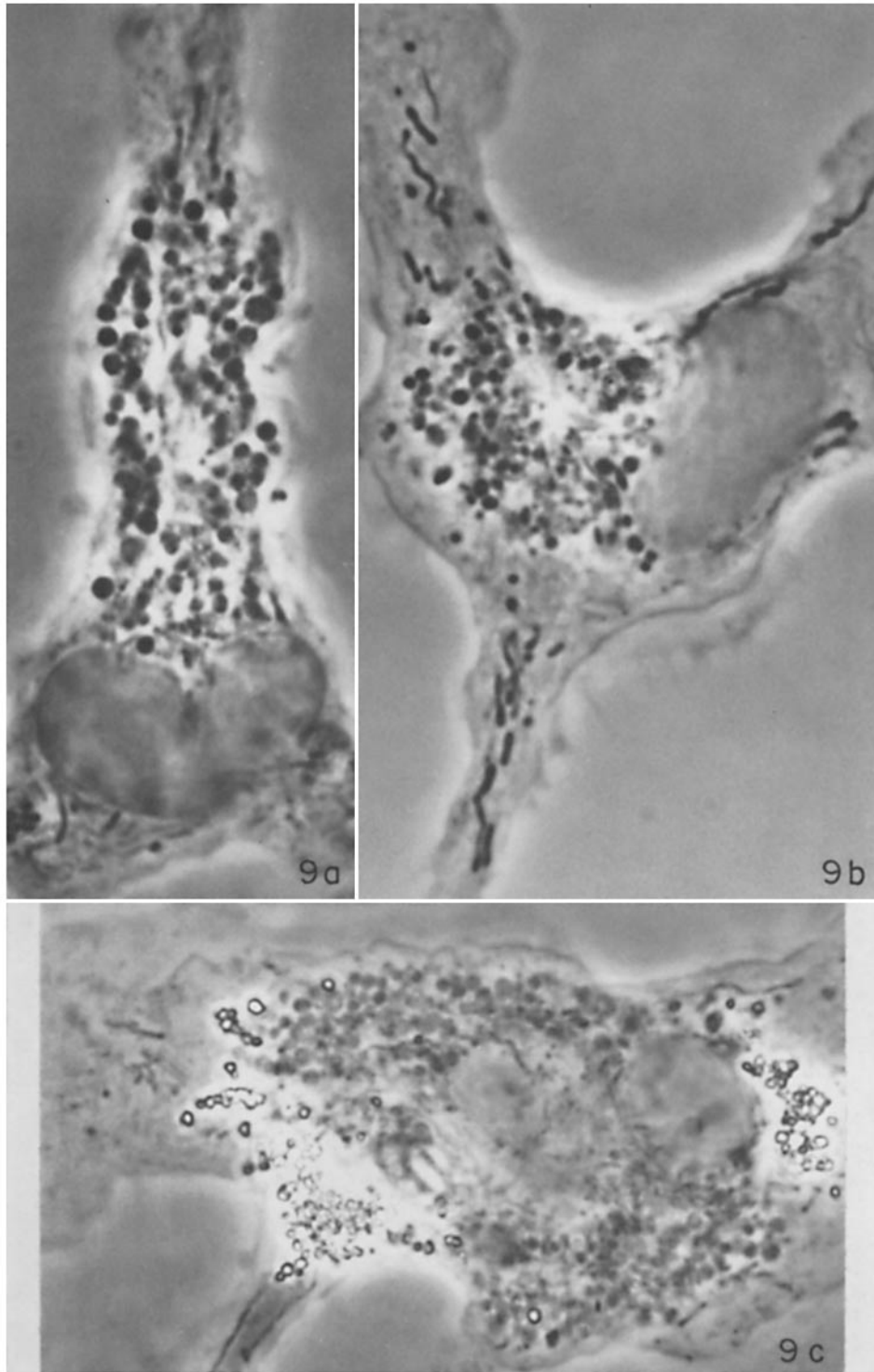




(Cohn and Benson: Macrophage differentiation)

PLATE 13

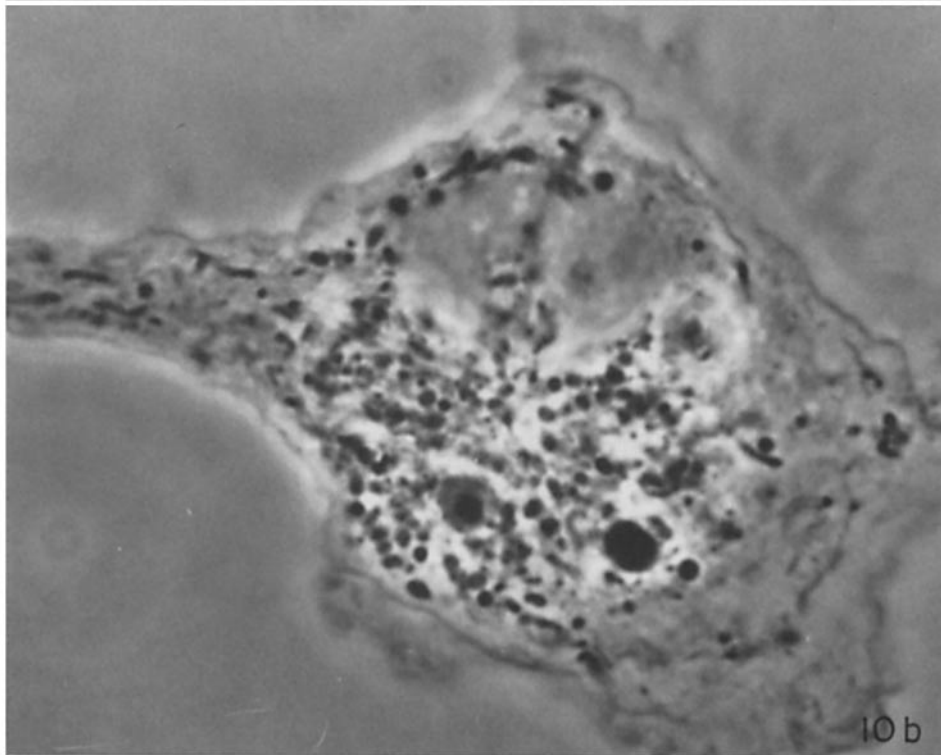
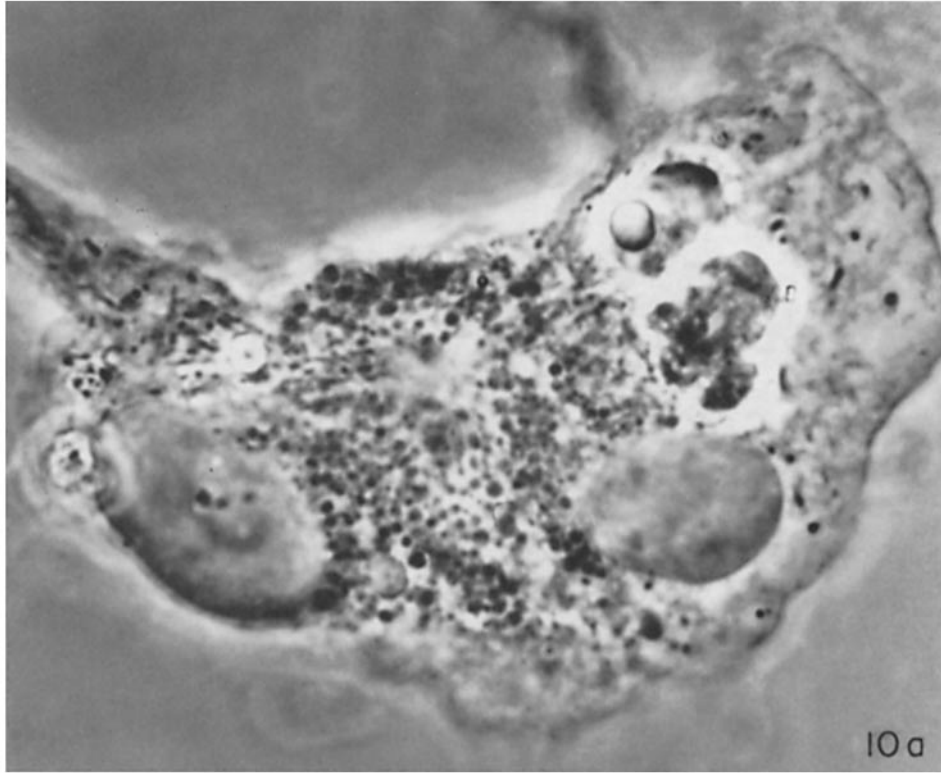
FIGS. 9 *a* to 9 *c*. Illustrating range in cell size and number of dense granules. Cell in Fig. 9 *b* exhibits well defined rosettes of granules with central clear zone. Cell in Fig. 9 *c* shows smaller more numerous granules with accumulations of lipid droplets.



(Cohn and Benson: Macrophage differentiation)

PLATE 14

FIGS. 10 *a* and 10 *b*. Two large cells, which in addition to granules, contain large irregular fragments of phagocytized cellular debris. Although not illustrated both the granules and the phagosomes stain positively for acid phosphatase.



(Cohn and Benson: Macrophage differentiation)