THE IN VITRO DIFFERENTIATION OF MONONUCLEAR PHAGOCYTES

III. The Reversibility of Granule and Hydrolytic Enzyme Formation and the Turnover of Granule Constituents*

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Plates 32 to 35

(Received for publication, April 24, 1965)

The *in vitro* cultivation of mouse mononuclear phagocytes leads to the formation of cytoplasmic organelles with the properties of lysosomes (1). The production of these granules as well as three hydrolytic enzymes was blocked by inhibitors of protein synthesis (2). Further analysis revealed that the phase-dense granules arose from pinocytic vacuoles which accumulated in the centrosphere region (3). The rate of formation and yield of granules and hydrolytic enzymes was dependent upon the concentration of serum in the medium. In addition, there was a direct relationship between serum concentration and the rate of pinocytosis. These data suggested that the regulation of granule formation and hydrolytic enzyme production was controlled in some fashion by the pinocytic process (3).

This article will be concerned with the reversibility of macrophage differentiation by means of alterations in the external medium. In addition, the uptake and turnover of selected granule constituents has been examined.

Materials and Methods

The technology of harvesting and cultivating populations of mouse peritoneal phagocytes has been reported (1). Enzymatic and chemical analyses, cell fixatives, and photographic procedures were identical to those in prior publications (1). Newborn calf serum was employed for all experiments.

Qualitative and Quantitative Studies Employing Colloidal Gold.—Colloidal gold was obtained from Abbott Laboratories, North Chicago, Illinois as a non-radioactive, stabilized suspension. Prior to use in a cell system it was exhaustively dialyzed against phosphate-buffered saline (ph 7.5) at 4°C. After dialysis the suspension was stable for at least 7 days at 4°C.

The qualitative uptake of colloidal gold was studied by direct morphological studies. Cells cultivated on coverslips in Leighton tubes were routinely employed. After 24 hours of *in vitro* growth colloidal gold was added to the medium at concentrations of 5 to 10 μg/ml. The cells were then exposed for 2 to 24 hours and either fixed or washed and placed in fresh medium.

* This investigation was supported (in part) by Public Health Service Grant No. E-1831 from the National Institute of Allergy and Infectious Diseases.

The fixative of choice was 1.25 per cent buffered glutaraldehyde. Under these conditions the granules of the centrosphere region remained phase lucent and any increased density was related to the incorporation of gold. Granules containing gold appeared as red spheres under the light microscope or as grey or black granules with phase-contrast illumination.

The quantitative incorporation and subsequent fate of colloidal gold was studied with mass cultures maintained in T-flasks. Cells were grown for 24 hours in the presence of 50 per cent newborn calf serum. New medium containing 10 μg/ml of colloidal gold was then added and the cells exposed for 2 hours. At the time of harvest, the medium was removed and the cells washed three times with warm saline. The flasks were then drained over filter paper, 2 to 3 ml of 0.5 N NaOH added to the cell sheet and incubated for 2 hours at 37°C. This dissolved the cells and resulted in a clear solution containing colloidal gold. The spectra of each sample was then determined between 500 to 600 mμ and compared to standards of gold in alkali. The concentration of gold followed Beer's Law between 0.1 to 5.0 μg/ml and demonstrated an absorption maximum at 520 mμ. If gold resided in cells longer than 12 hours its spectral properties changed and the absorption maximum became broader with a peak between 520 to 550 mμ. The optical densities of the suspensions were compared at 525 mμ for quantitation.

The Uptake and Fate of Lysozyme.—Crystalline (3X) egg white lysozyme was obtained from Calbiochem, Los Angeles. Cells were grown in T-flasks for 12 hours in the 50 per cent NBCS medium. They were then exposed to 150 μg/ml of lysozyme in 50 per cent NBCS for 12 hours. At the end of this time the cell sheet was washed three times with 8.0 ml of 10 per cent NBCS—No. 199 and placed in appropriate fresh medium. Flasks were also harvested after the 12 hour pulse to obtain initial lysozyme levels. Controls in which lysozyme was added immediately before harvesting were consistently negative for enzyme activity.

At the time of harvesting, the cells were washed three times in warm saline, drained, and 2 to 3 ml of saline added. The cells were then detached from the glass by cycles of freezing and thawing and assayed for the enzyme employing freshly prepared suspensions of *Micrococcus lysodeikticus* as substrate (4). The results are expressed in terms of the crystalline egg white lysozyme standard.

Fluorescein Labeling of NBCS Proteins and Anti-NBCS Rabbit Serum.—Newborn calf serum was obtained from Microbiological Associates, Bethesda, Maryland. Three fractions were labeled with fluorescein. The first represented all the proteins of serum and was obtained by dialyzing serum exhaustively against phosphate-buffered saline at ph 7.5. The second was a globulin fraction isolated at 18 per cent saturation with sodium sulfate, and the third was an albumin fraction consisting of the precipitate obtained between 60 to 69 per cent ammonium sulfate. Each preparation was adjusted to a concentration of 20 mg/ml and labeled with fluorescein isothiocyanate (50 μg/mg protein) according to the procedure of Riggs *et al.* (5). After removing free fluorescein by dialysis and sephadex filtration the preparations were adjusted to a protein concentration of 20 mg/ml. The preparations were stored without preservative in the frozen state at −20°C.

Anti-NBCS antibody was obtained by injecting rabbits with whole serum. Adult rabbits weighing 4 to 5 kg were injected intravenously *via* the marginal ear vein with 1.0 ml of serum, three times per week for a total of 9 injections. The animals were bled by cardiac puncture 2 weeks after the last injection. A gamma globulin fraction obtained at 18 per cent saturation with sodium sulfate was isolated from 40 ml of serum. A portion of this was conjugated with fluorescein isothiocyanate through the kindness of Dr. John Zabriskie of The Rockefeller Institute.

Microscopic Observations on the Uptake and Localization of Fluorescein-Conjugated Proteins.—All observations were performed on cells which were growing on coverslips in Leighton tubes. The majority of observations were made on cells growing in a medium of 50 per cent NBCS—No. 199. All fluoresceinated proteins were used at a final concentration of 0.17 to 0.20

mg/ml in the growth medium. Fluoresceinated proteins were added at *in vitro* periods ranging from T2 hours to T48 hours. The exposure of cells to the labeled protein was from 3 to 24 hours.

The uptake of fluoresceinated derivatives of calf serum was examined by direct microscopy employing the Zeiss ultraphot II and a high pressure mercury arc lamp. Cells were fixed with 1.25 per cent buffered glutaraldehyde for 15 minutes at 4°C and washed repeatedly with saline. The coverslips were then mounted in distilled water, sealed, and examined for fluorescence with the dark field condenser and filters BG-12 and OG-4. Photographs were taken with 4 x 5 Polaroid, 3000 speed, type 57 film using exposure times of 2 minutes. For reproduction in this article, negatives were prepared from the polaroid prints. All photographs were taken at a magnification of 1000 ×.

The procedure for staining mouse cells with fluorescein-tagged anti-NBCS rabbit serum was somewhat different. Cells grown in 50 per cent NBCS for 24 hours were washed four times with 5.0 ml of warm medium 199. They were then fixed for 30 minutes with 1.25 per cent glutaraldehyde and rinsed with saline. The cells were then incubated in the presence of tagged antibody for 30 minutes at 37°C, rinsed four times with saline, and mounted in distilled water.

RESULTS

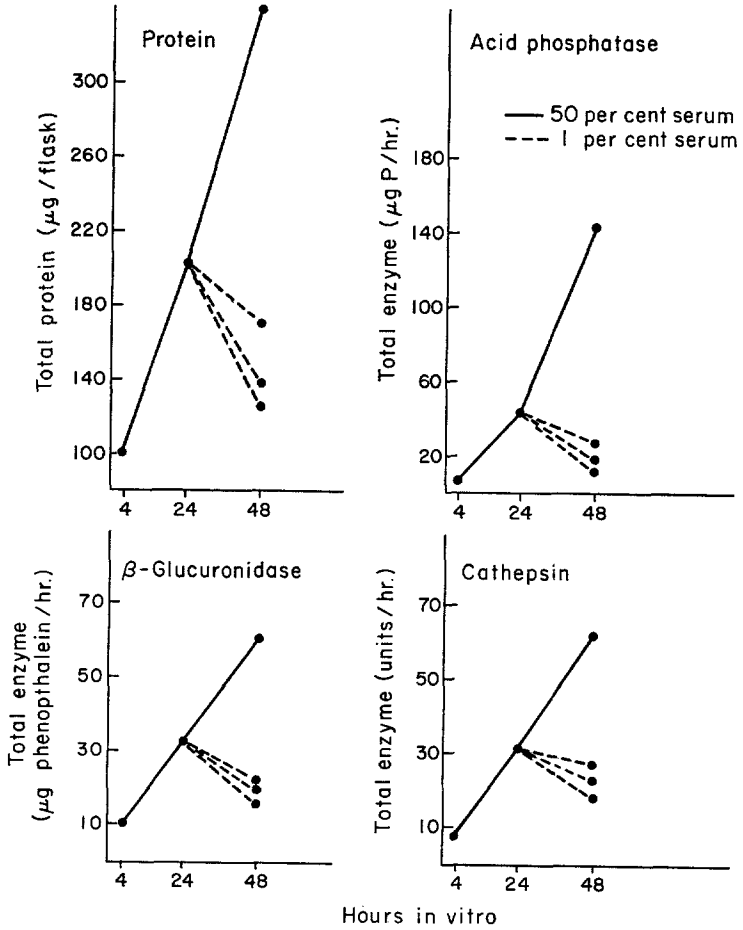
The Reversibility of Granule Formation and Hydrolase Production.—The previous paper in this series illustrated the correspondence between granule formation, enzyme production, and the concentration of serum in the medium. The influence of serum was on the rate of pinocytosis and it was apparent that the cells pinocytized actively when placed in a high serum medium. It was also evident that the rate of pinocytosis could be markedly diminished at any point during *in vitro* cultivation by reducing the serum concentration. This response allowed a more detailed analysis of the fate of granule contents.

Cells employed for these studies were cultivated in both Leighton tubes and T-flasks. In the first group of experiments the cells were cultured in the presence of 50 per cent newborn calf serum (NBCS)—No. 199 for 24 hours. The medium was then changed to one containing 1 per cent NBCS and maintained for another 24 hours. For quantitative studies groups of flasks were harvested at 4, 24, and 48 hours.

The results of a group of such experiments are presented in Text-fig. 1. Cells maintained in 50 per cent serum medium (solid line) for the full 48 hours demonstrated marked increases in the total amounts of cellular protein, acid phosphatase, β-glucuronidase, and cathepsin. In contrast, changing the medium at 24 hours to the low serum level (dotted line) resulted in a loss in these substances. Pinocytic vesicle formation was reduced to less than 10 per cent of that observed in 50 per cent serum.

Morphological changes accompanied the biochemical results just mentioned. Within 24 hours after being placed in low serum medium there was a striking decrease in the size of the phase-dense granules of the centrosphere region (Figs. 1 *a* and 1 *b*). Concomitantly the level of cytochemically demonstrable acid phosphatase was reduced and reaction product was now associated with smaller

granules (Figs. 2 *a* and 2 *b*). The size of these reaction sites can be compared to the granules in osmium-fixed cells (Fig. 2 *d*). It could not be ascertained from these studies whether or not the *number* of granules had declined. However, examination of individual cells for periods up to 12 hours, did not reveal

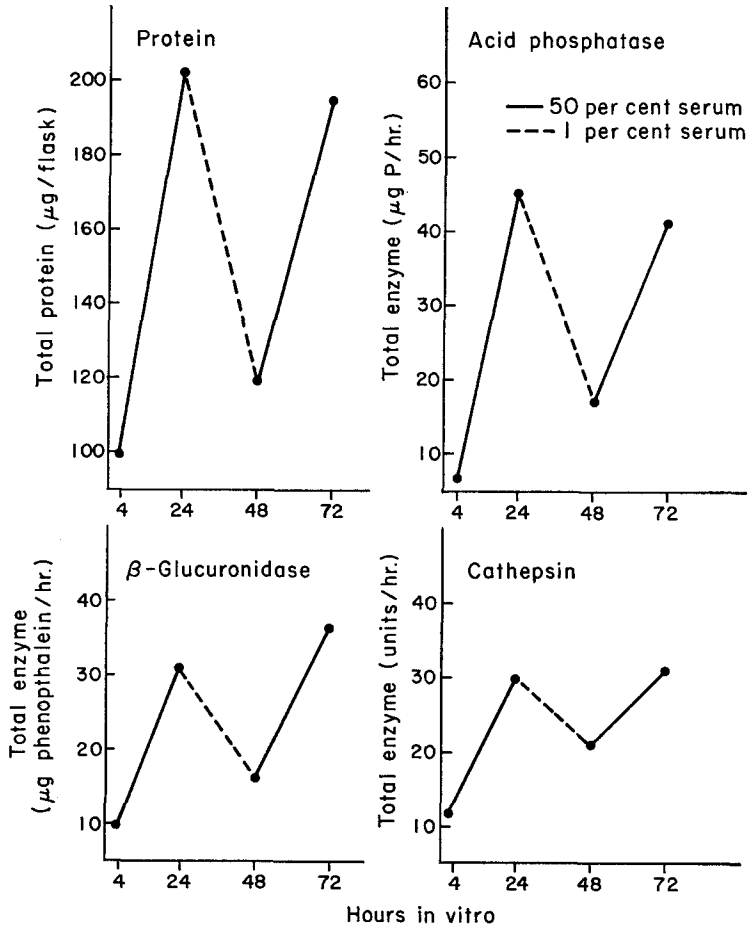


TEXT-FIG. 1. The behavior of cellular protein and hydrolases upon reducing the serum concentration of the medium from 50 to 1 per cent.

the extrusion of granules into the medium. In addition, assays on the low serum medium did not reveal the presence of demonstrable acid phosphatase, cathepsin, or β-glucuronidase.

The loss in granule size and the cellular content of protein and enzymes could be reversed by again placing the cells in high serum medium. Biochemical data are presented in Text-fig. 2.

Cells were grown in T-flasks for 24 hours in 50 per cent serum media. They were then washed twice with 10.0 ml of warm No. 199 and incubated for 24 hours in 1 per cent serum medium. After 48 hours *in vitro* the medium was again changed to 50 per cent serum. Flasks were harvested at each time point. The data on long term cultivation in 1 or 50 per cent serum can be found in a previous publication (3).



TEXT-FIG. 2. The reversible formation of macrophage hydrolases.

Upon reconstituting the serum concentration to 50 per cent the cells again accumulated protein, acid phosphatase, β-glucuronidase, and cathepsin to the levels present at 24 hours. Similarly, granule size increased, pinocytic vesicles became prominent, and cytochemically demonstrable acid phosphatase was again evident (Fig. 2 *c*).

The Uptake and Fate of Fluorescein-Labeled Protein.—The importance of

pinocytosis in granule formation suggested that exogenous cell constituents might be taken up and concentrated in granules. This possibility was initially investigated by employing fluorescein-tagged components of the normal growth medium and examining the cells by fluorescence microscopy. A study on the uptake of fluorescein-conjugated proteins by cells has been published by Holtzer and Holtzer (6).

The exposure of cells to either labeled albumin, globulin, or total protein fractions of NBCS for 12 hours in the 50 per cent serum medium resulted in fluorescent granules of the type seen in Figs. 3 *a* and 3 *b*. Only large granules were fluorescent and other cell structures such as peripherally located pinocytic vesicles were without label. The uptake of the three preparations, as judged by the brightness of the granules was essentially the same. As far as could be judged by observations of individual cells by both phase contrast and fluorescence microscopy, the vast majority of the granules were labeled.

The exposure of cells to a shorter pulse of fluorescein-tagged protein also resulted in the labeling of granules (Figs. 3 *c* and 3 *d*). Within 3 hours, the majority of granules were fluorescent, although the intensity of fluorescence was somewhat less than with longer pulses. This implied that newly formed pinocytic vacuoles in the centrosphere, as well as preformed dense granules contained the label. The general appearance of these cells was the same as those in contact with tagged protein for the entire 24 hours of *in vitro* cultivation, a situation in which all granules should have contained fluorescein.

The longevity of fluorescein-tagged proteins within the granules was examined.

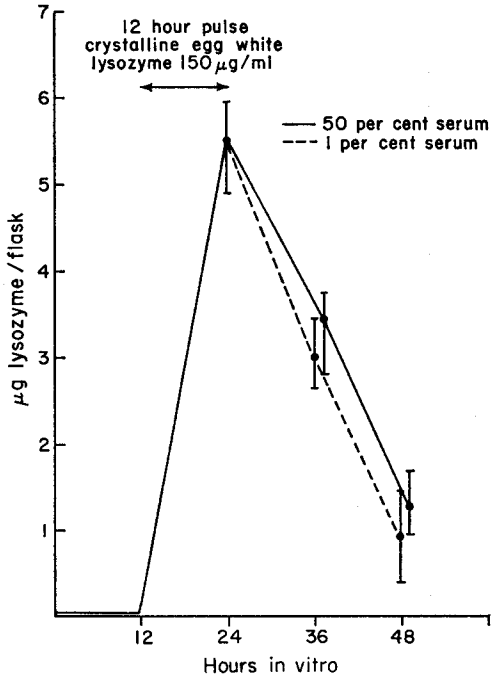
Cells were pulsed for 6 hours with the fluorescein-tagged proteins of newborn calf serum. They were then washed three times with 3.0 ml of 50 per cent NBCS 199 and incubated in the presence of 50 per cent NBCS medium. At intervals of 6 hours, coverslips were fixed and examined by fluorescence microscopy.

Within 12 hours after placing labeled cells in "cold" medium the majority of granules had begun to lose their fluorescence. This process was complete by the 18th hour. This indicated that granule contents were being turned over at a relatively rapid rate and were lost from the cell after intracellular digestion or through the extrusion of granules.

Cells stained with anti-NBCS rabbit serum exhibited a somewhat different appearance. In addition to the staining of granules, tiny fluorescent vesicles were observed in the periphery of the cell, which corresponded to pinocytic vesicles. In most cases there was also a rim of fluorescent material which outlined the cell surface. It should be noted that the addition of fresh anti-NBCS rabbit serum to washed cells grown in NBCS resulted in a cytotoxic effect with rapid cell disintegration.

The Uptake and Fate of Crystalline Egg White Lysozyme.—The prior results

suggested that exogenous cell constituents were concentrated within granules and underwent a discernible turnover. The experiments with fluorescein-tagged proteins were of a qualitative nature and a more quantitative assay was required. Previous studies on the enzymatic constitution of mouse cells differentiating *in vitro* revealed the absence of any lysozyme activity. However, the addition of lysozyme to the medium resulted in detectable cell associated concentrations of this polysaccharidase.



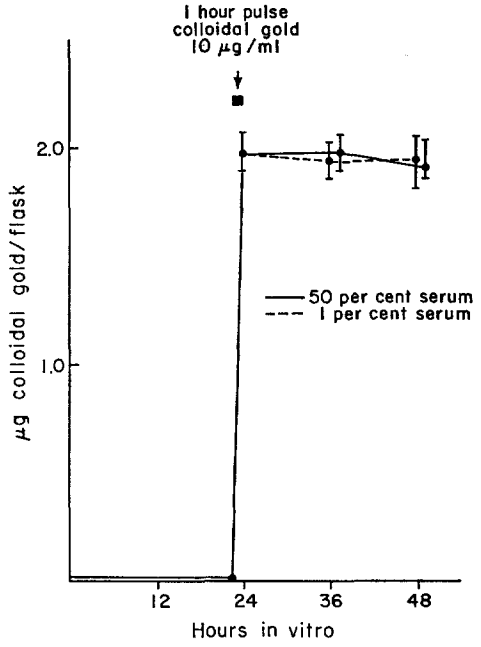
TEXT-FIG. 3. The uptake and fate of crystalline egg white lysozyme.

Cells growing in 50 per cent NBCS—No. 199 medium for 12 hours were exposed to 150 μg/ml of crystalline egg white lysozyme. After a 12 hour pulse, the cells were washed with three 10 ml aliquots of medium 199, and incubated in fresh 50 and 1 per cent serum medium for another 12 and 24 hours. Flasks were also harvested at the end of the 12 hour pulse to ascertain intracellular activity.

The results of experiments on the uptake and subsequent fate of lysozyme are presented in Text-fig. 3. Within 12 hours the cells had accumulated considerable amounts of lysozyme. After this pulse, cells were then placed in either 50 per cent serum or 1 per cent serum and incubated for another 24 hours. During this wash out period the majority of lysozyme was lost from the cell. There was no significant difference in lysozyme disappearance from cells which were

actively making hydrolases (50 per cent serum medium) or those which were losing endogenous hydrolases (1 per cent medium). No detectable levels of lysozyme could be found in the extracellular medium at the 48 hour period. It is assumed that lysozyme is segregated in granules in a manner similar to fluorescein-tagged proteins.

The Uptake and Fate of Colloidal Gold.—The loss of endogenous hydrolases, fluorescent protein, and lysozyme suggested turnover of granule constituents. To distinguish between extrusion of granules or intragranule digestion with



TEXT-FIG. 4. The uptake and fate of colloidal gold.

subsequent release of small molecular weight products, a "non-digestible" substance was required. The previous studies of Gosselin (7) and Harford *et al.* (8) suggested that colloidal gold might be an ideal candidate.

The addition of 5 to 10 μg/ml of gold to macrophages for short periods resulted in the labeling of the granules (Figs. 4 *a* to 4 *c*). Initially, gold was observed outlining the periphery of the granule and in smaller dense bodies (Fig. 4 *a*). With prolonged incubation the gold was more homogeneously distributed resulting in a reddish granule with the light microscope or a black organelle with phase-contrast illumination. The subsequent fate of gold after a short pulse was then examined microscopically and with quantitative techniques.

Cells were grown for 24 hours in 50 per cent NBCS medium. They were then exposed to 10 μg/ml colloidal gold for 2 hours, and washed extensively with warm No. 199 to remove extra-

cellular gold. Flasks were sampled immediately after the 2 hour pulse and at 12 and 24 hours in "cold medium." A similar experiment was performed with cells growing in Leighton tubes.

The appearance of cells at 24 hours after the pulse of gold is shown in Figs. 4 *b* and 4 *c*. In the presence of 50 per cent serum (Fig. 4 *b*) gold was present in large granules scattered throughout the centrosphere. In addition, clear, phase-lucent granules, indicative of new granule formation, were also observed. There were, therefore, a mixed population of labeled and unlabeled granules. The appearance of cells in 1 per cent serum was quite different (Fig. 4 *c*). In this case the gold was distributed in tiny, very dense granules and no new phase-lucent granules were observed. This correlated with the absence of pinocytic vacuoles in the presence of 1 per cent serum.

The results of the quantitative studies are shown in Text-fig. 4 and are in striking contrast to the analogous experiment performed with egg white lysozyme. During the wash out period of 24 hours, the concentration of cell-associated gold remained constant in both 1 and 50 per cent serum medium. This indicated that there was quantitative conservation of gold, under conditions in which granule size was reduced and cell protein and hydrolases were being lost from the cell. Prior experiments had revealed that gold did not influence the loss of hydrolases in 1 per cent serum medium. If one assumes that colloidal gold had no unusual stabilizing effect on the granule then these experiments exclude the possibility that a major portion of the granules were extruded from the cell.

DISCUSSION

The experiments presented in this article indicate that the formation of macrophage granules is a reversible process. In the presence of high levels of calf serum, pinocytosis is stimulated and this results in the formation of dense granules and hydrolytic enzymes. If pinocytosis is now depressed, the granules shrink in size and there is a loss of cellular protein as well as the three enzymes. Apparently the cell's sojourn in low serum medium is not particularly damaging since reintroduction into high concentrations of serum again results in granule formation and enzyme production. It is likely that a similar process occurs *in vivo* so that fluctuations in macrophage activity and composition may be dependent upon the nature of its immediate milieu.

The turnover of granule contents occurred at a relatively rapid rate when compared with the estimates reported for liver lysosomes (9). Under these *in vitro* conditions it appeared that the mechanisms for endogenous enzyme loss resulted from the "digestion" of granule contents with release of degradation products. This was based upon the shrinkage in granule size, the absence of detectable enzymes in the external medium, and the conservation of a "non-digestible" granule marker. The resulting small granule with reduced enzyme content would then be analogous to the residual body. Namely, an organelle which previously contained lysosomal hydrolases and substrates and now is

composed of poorly digestible material. It is not certain whether hydrolases are released from the cell in an active form or degraded prior to their exit. The presence of inhibitors in the extracellular medium might mask the presence of active enzyme.

A comparison between the loss of endogenous and exogenous granule constituents is given in Table I. Under these conditions, the loss of newly formed acid phosphatase, β-glucuronidase, and total protein as well as incorporated lysozyme, ranged between 70 to 80 per cent within a 24 hour period after the cessation of pinocytosis. It was of interest that the level of cathepsin was more stable and its activity was retained to a greater extent. These data suggest that

TABLE I

*The Loss of Endogenous and Exogenous Cell Components as Influenced by a Reduction in Serum Concentration from 50 to 1 Per Cent**

Component	Total activity			Loss‡
	T4	T24	T48	
				per cent
Acid phosphatase	5	45	16	72
Cathepsin	12	30	20	55
β-Glucuronidase	10	33	16	73
Protein	100	200	120	80
Lysozyme	—	5.5	1.3	76
Colloidal gold	—	1.98	1.96	2

* Cells cultured in 50 per cent NBCS for 24 hours, washed with No. 199, and maintained in 1 per cent serum No. 199.

‡ $\frac{T24 - T48}{T24 - T4} \times 100$ = per cent loss.

the stability of the hydrolases within the granule was not greatly different from that of cellular "protein" or exogenous lysozyme. If this is true then the synthesis and degradation of hydrolases is probably occurring constantly so that the assayable enzyme level reflects the differential rates of these processes.

The qualitative studies employing fluorescein-conjugated proteins gave no indication of a preferential uptake of albumin or globulin. More exacting studies with purified fractions are in order. One wonders, however, the extent to which antibody globulin is taken up in the intact animal. In view of the uptake and turnover of granule proteins, the concentration of intracellular antibody would depend upon the concentration in the external environment as well as its rate of degradation within the granule. Antibody which is segregated within granules would be expected to interact with soluble or particulate antigens through the process of membrane fusion.

The uptake, storage, and concentration of exogenous macromolecules by the pinocytic process raises the question of the origin of certain lysosome-associated enzymes. The rapid uptake of lysozyme by the mouse cell *in vitro* may reflect on the previously described disparity in the lysozyme content of peritoneal and alveolar macrophages (10). One might expect that the cell obtained from the lung would be exposed to high concentrations of lysozyme present in the respiratory secretions and incorporate this enzyme into similar dense granules.

SUMMARY

Mouse mononuclear phagocytes cultivated in 50 per cent newborn calf serum medium pinocytize actively and form large numbers of phase-dense granules as well as three hydrolytic enzymes. When such cells are then placed in 1 per cent newborn calf serum they illustrate (*a*) a low level of pinocytic activity, (*b*) a shrinkage in granule size, and (*c*) a loss in cell protein, acid phosphatase, β-glucuronidase, and cathepsin. Examination of the extracellular medium revealed no detectable hydrolase activity. The reintroduction of cells into high levels of serum again resulted in granule and enzyme formation.

Cells rapidly incorporated fluorescein-conjugated calf serum proteins into the phase-dense granules. The fluorescence of labeled granules was lost during an 18 hour period in non-fluorescein–containing medium.

Crystalline egg white lysozyme was concentrated in the macrophages. Approximately 80 per cent of the cell-associated enzyme was lost during a 24 hour washout period in either 1 or 50 per cent serum medium. No enzymatic activity could be recovered in the medium.

Colloidal gold was taken up and concentrated in macrophage granules. Quantitative assays revealed this particle to be conserved during a 24 hour washout period.

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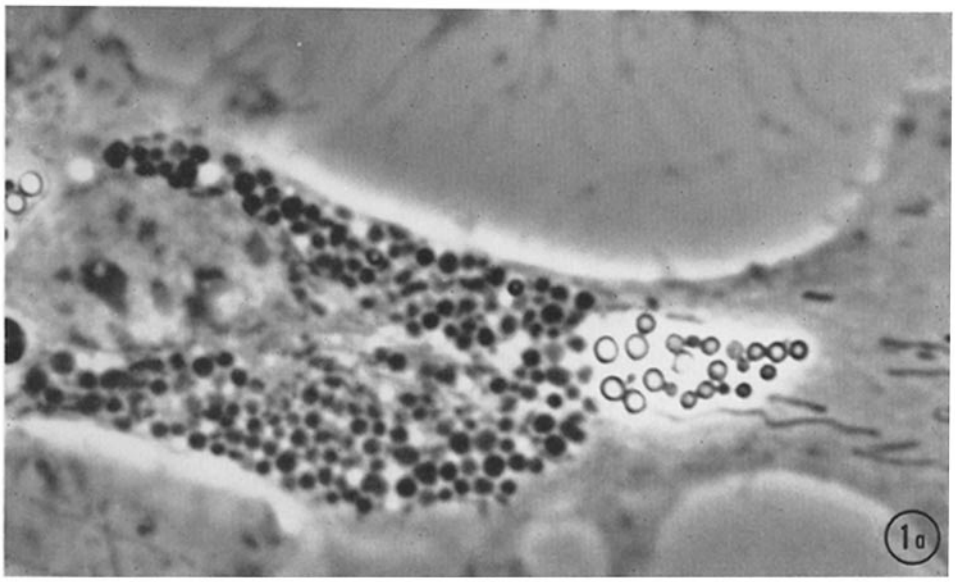
EXPLANATION OF PLATES

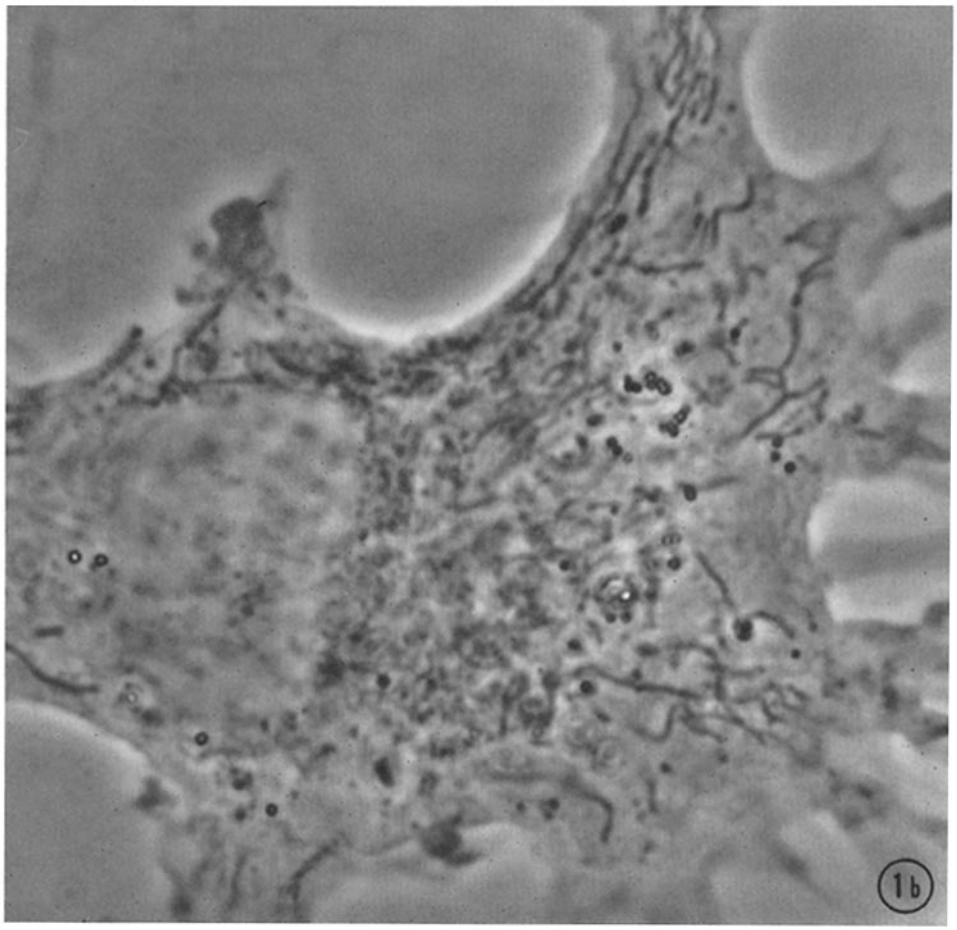
PLATE 32

FIGS. 1 *a* and 1 *b*. Cells fixed in 1.25 per cent glutaraldehyde and postfixed in 1 per cent OsO_4. Phase contrast, × 2500.

FIG. 1 *a*. Cell cultivated in 50 per cent serum medium for 48 hours. A well defined rosette of large granules is present in the perinuclear region. A number of clear pinocytic vacuoles are also apparent.

FIG. 1 *b*. Cell cultivated in 50 per cent serum medium for 24 hours, washed and incubated another 24 hours in 1 per cent serum medium. The granules are much smaller in size and are less osmiophilic. Numerous mitochondria are visible and occasional highly refractile lipid droplets are scattered in the cytoplasm.





(Cohn and Benson: Macrophage differentiation)

PLATE 33

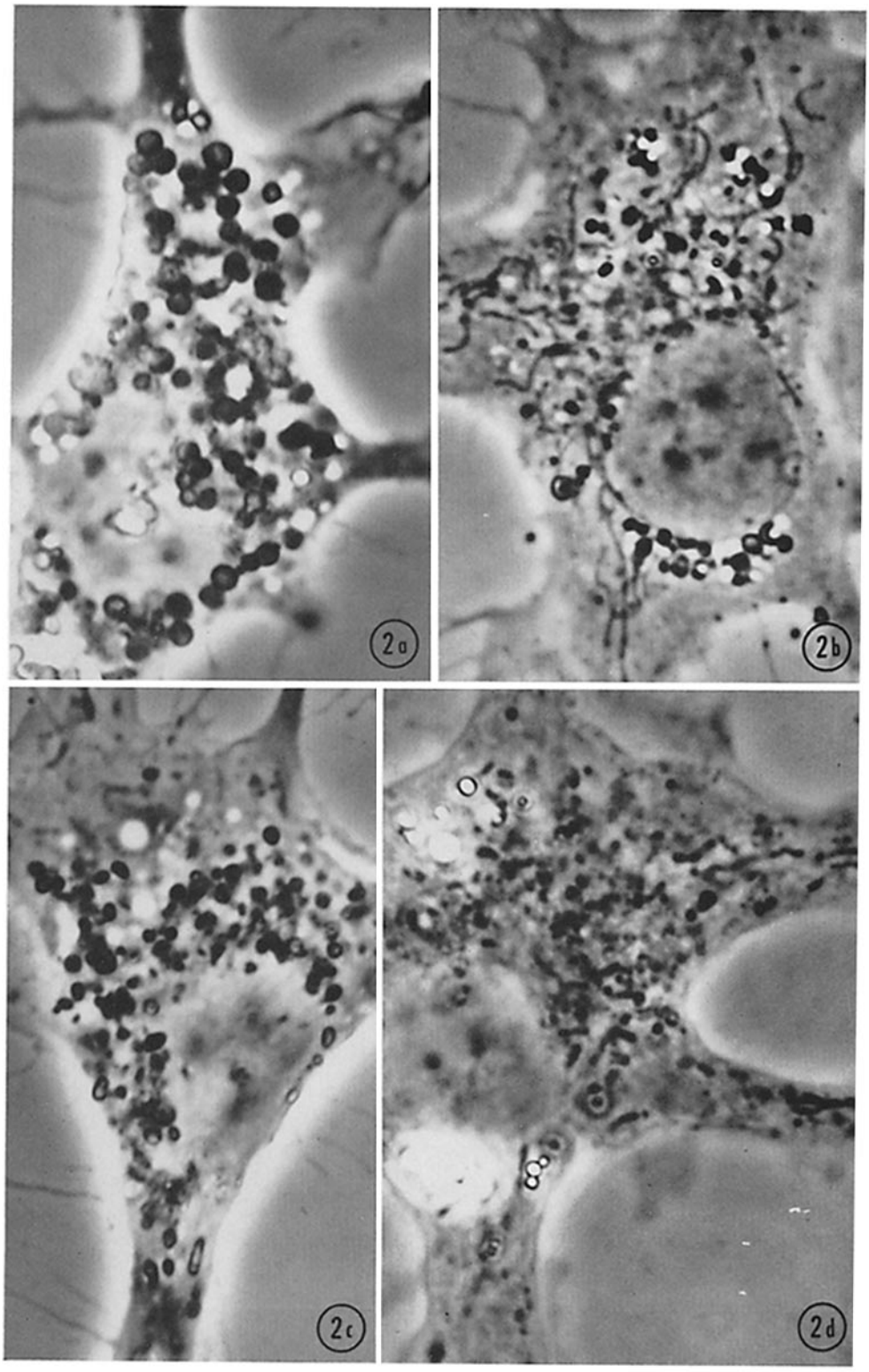
FIGS. 2 *a* to 2 *c*. Cells fixed in 1.25 per cent glutaraldehyde and stained for acid phosphatase. Phase contrast, × 2500.

FIG. 2 *a*. Cell cultivated in 50 per cent serum medium for 24 hours. Lead sulfide reaction product outlines the large dense bodies. Clear pinocytic vacuoles are also present.

FIG. 2 *b*. Cell cultivated in 50 per cent serum medium for 24 hours, then washed and placed in 1 per cent serum medium for 24 hours. Small amounts of reaction product associated with shrunken granules. This represents the maximum activity apparent at this time.

FIG. 2 *c*. Same as Fig. 2 *b* except reintroduced into 50 per cent serum medium for 8 hours and then fixed. Reactive sites are larger and more numerous. After 24 hours the cells resemble those seen in Fig. 2 *a*.

FIG. 2 *d*. Same as Fig. 2 *b* but fixed with 1 per cent buffered OsO_4. Decrease in granule size is apparent. Phase contrast, × 2500.



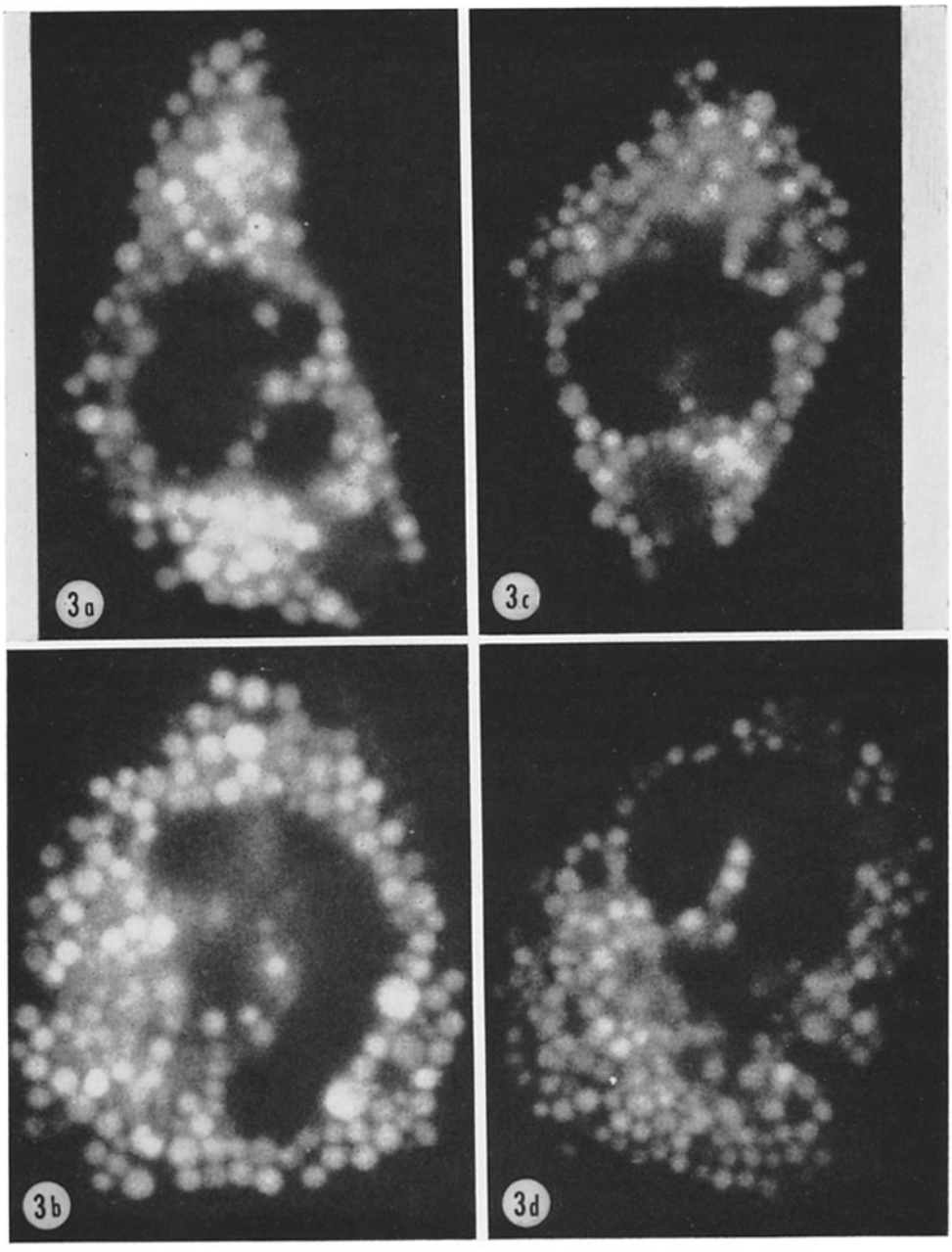
(Cohn and Benson: Macrophage differentiation)

PLATE 34

FIGS. 3 *a* to 3 *d*. Concentration of fluorescein-conjugated proteins by macrophages. Darkfield fluorescence microscopy, × 2500.

FIGS. 3 *a* and 3 *b*. Cells exposed to 0.17 mg/ml fluorescein-conjugated newborn calf serum protein for 12 hours. The perinuclear rosette of granules is intensely labeled. The remainder of the cell is negative.

FIGS. 3 *c* and 3 *d*. Cells exposed for 3 hours to conjugated protein. Localization of label is similar but fluorescence is less intense.



(Cohn and Benson: Macrophage differentiation)

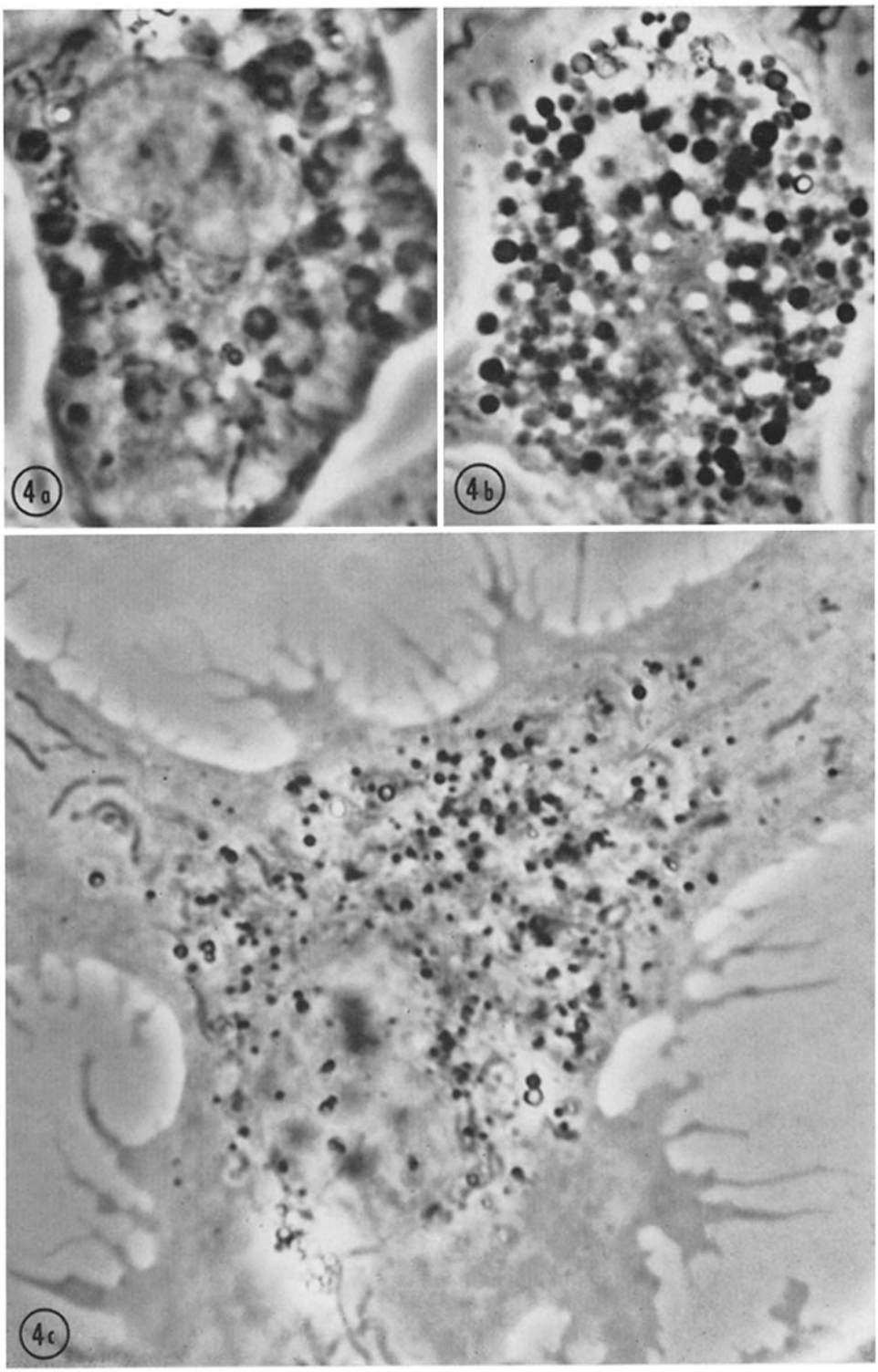
PLATE 35

FIGS. 4 *a* to 4 *c*. Uptake of colloidal gold by macrophages. Cells fixed in 1.25 per cent buffered glutaraldehyde. Phase contrast, × 2500.

FIG. 4 *a*. Cell grown for 24 hours in 50 per cent serum medium and exposed to 5 μg/ml colloidal gold for 1 hour. A rim of gold is seen outlining many of the phase-lucent granules. Occasional, tiny, dense bodies are also seen.

FIG. 4 *b*. Same as Fig. 4 *a* but washed and incubated in 50 per cent serum medium for 24 hours in the absence of colloidal gold. Gold is now more homogeneously distributed in old granules whereas new granules are phase lucent.

FIG. 4 *c*. Same as Fig. 4 *a* but washed and incubated in 1 per cent serum medium for 24 hours. The gold is now distributed in tiny, very dense granules.



(Cohn and Benson: Macrophage differentiation)