# THE IN VITRO DIFFERENTIATION OF MONONUCLEAR PHAGOCYTES

## II. The Influence of Serum on Granule Formation, Hydrolase Production, and Pinocytosis*

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Plate 63 to 67

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Previous communications from this laboratory delineated the events associated with the *in vitro* cultivation of unstimulated mouse mononuclear phagocytes (1, 2). These processes included a progressive increase in lysosome-like cytoplasmic granules. In addition, it was shown that two inhibitors of protein synthesis blocked the formation of both granules and hydrolytic enzymes. These data as well as the autoradiographic evidence of newly formed protein in the granule region suggested that this was an active process associated with the formation of new hydrolytic enzymes.

This report is an extension of these findings in which the origin, rate of formation, and controlling factors connected with granule and hydrolase production have been examined in more detail.

### *Materials and Methods*

*General Methods.*—The methods for harvesting and cultivating mouse peritoneal phagocytes in Leighton tubes and T-flasks have been outlined previously (1). All chemical and enzymatic methods, cytochemical techniques, and fixatives have been described (1). Photography was performed with phase contrast illumination employing the Zeiss ultraphot II microscope.

Newborn calf serum and bovine serum as well as medium 199 were obtained from Microbiological Associates, Bethesda, Maryland. Horse serum was obtained from an animal maintained at The Rockefeller Institute and bled by jugular puncture. Normal rabbit serum was processed from cardiac blood and heat inactivated at 56°C/30 minutes.

*The Estimation of Pinocytosis.*—An estimate of the rate of pinocytosis was obtained by phase contrast microscopy. Cells growing on coverslips in Leighton tubes were employed for all observations. The routine procedure was as follows. The medium was removed and the cells fixed for 10 minutes at 4°C with 1.25 per cent buffered glutaraldehyde. The coverslips were then washed twice with distilled water, mounted in water, sealed with paraffin, and observed the same day. This fixative preserved pinocytic vesicles which had the same size and distribu-

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tion as those seen in living specimens. The number of pinocytic vesicles visible under oil immersion phase contrast (× 1000) were counted in 300 cells in each preparation.

Further details are given in Results.

## RESULTS

*The Influence of Newborn Calf Serum Concentration on Granule Formation.*— All the previous studies on the *in vitro* differentiation of the mouse mononuclear phagocyte had been performed with a medium composed of 20 per cent newborn calf serum (NBCS) and No. 199. This medium had been arrived at by studies employing Wright stained preparations of cells in which 20 per cent serum represented an adequate environment for attachment, spreading, and long-term survival. Upon more careful examination it was found, that the serum concentration had a dramatic effect upon both the size and number of cytoplasmic granules and their rate of formation. A typical experiment will serve to illustrate this point.

Leighton tube cultures were prepared and the initial attachment of cells was allowed to proceed for 30 minutes in a medium containing 20 per cent newborn calf serum. At the end of this time the coverslips were washed with three, 5.0 ml aliquots of warm No. 199. Groups of tubes were then incubated with 1.0 ml of medium containing 1, 10, 20, and 50 per cent of newborn calf serum. At 24-hour intervals coverslips were removed and processed for morphology and acid phosphatase cytochemistry.

Cells incubated in 1 per cent NBCS took considerably longer to spread out on the glass surface. This process required 48 hours and at this time the cells were well attached, exhibited thin, short mitochondria, and lacked a perinuclear rosette of granules. At 72 hours, osmium tetroxide–fixed cells showed a small number of tiny osmiophilic, phase-dense granules scattered in the cytoplasm, very rare lipid droplets and were usually spindle shaped. One such cell is illustrated in Fig. 1 *a*. Other than the lack of granules, the cells were viable and cell numbers remained constant for at least 72 hours of cultivation.

In contrast, cells incubated in 50 per cent serum exhibited much more rapid spreading than at a concentration of 20 per cent. At 24 hours of incubation the cells were filled with granules which were larger and more numerous than those seen with 20 per cent serum (Figs. 2 *a*, and 2 *b*). At 48 and 72 hours they showed a progressive increase in size and in the number of granules. The accumulation of refractile lipid droplets was greater in the higher serum concentration. A typical cell at the 72 hour period is seen in Fig. 1 *b*. At this time the granules were usually smaller than at the 24 and 48 hour period and in this respect resembled the process in 20 per cent serum.

The general pattern of acid phosphatase staining followed the accumulation of phase-dense granules. Cells maintained in 1 per cent serum medium exhibited occasional reactive sites which in number and intensity were only slightly greater than in control cells at 4 hours of cultivation. This was quite different

than cells maintained in 50 per cent serum which were strongly reactive at 18 hours. The lead sulfide reaction product was strictly localized to the cytoplasmic, phase-dense granules as described previously (1) and was greater than in cells maintained in 20 per cent serum medium.

From these results it appeared that both the rate of development and size of acid phosphatase–positive cytoplasmic granules was dependent upon the concentration of serum in the medium.

*The Influence of Serum Concentration on Protein Content and Hydrolytic Enzyme Production.*—The previous morphological findings strongly suggested that the concentration of serum played a decisive role in the *in vitro* formation of the phase-dense granule. To examine this situation in more detail quantitative studies were carried out on mass cultures maintained in T-flasks.

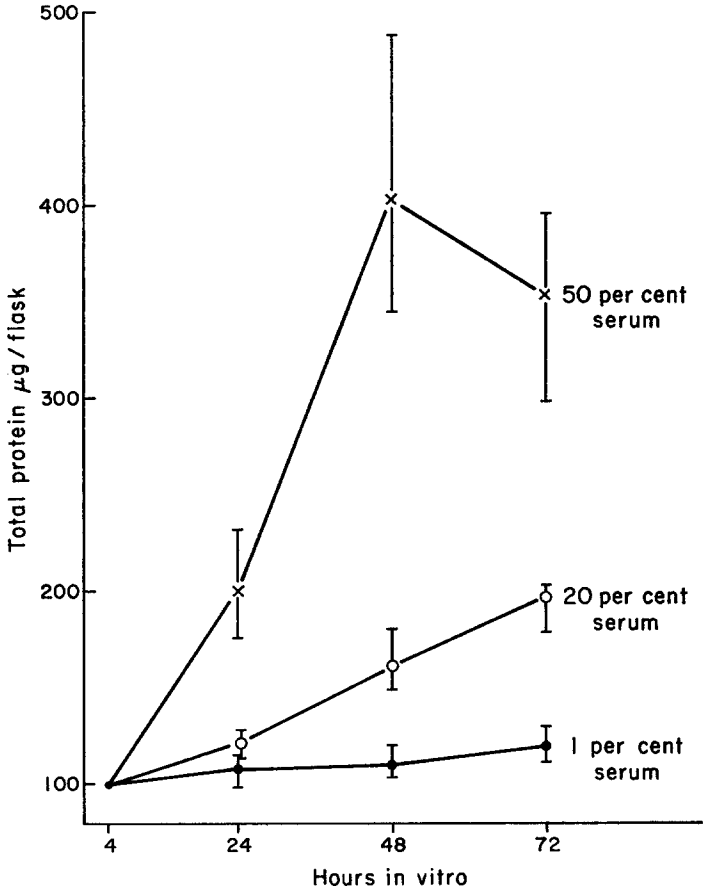
Unstimulated mouse peritoneal cells were routinely processed and allowed to adhere to the surface of T-flasks for 1 hour at 37°C in a medium composed of 20 per cent newborn calf serum, medium 199, and penicillin. At the end of this time and medium was removed and the cell sheet flushed three times with 8 ml of warm medium 199. This removed the lymphocyte population as well as the serum constituents. Groups of flasks were then maintained in the presence of 1, 20, and 50 per cent serum with No. 199 and penicillin for periods of 4 to 72 hours. Cells were harvested in the usual manner, activated by freezing and, assayed.

The content of cellular protein reflected the concentration of serum in the medium and is shown in Text-fig. 1. In the presence of 1 per cent serum only a slight change in protein content was noted and this occurred at the 72 hour period. A concentration of 20 per cent serum yielded a linear increase so that at 72 hours the cells contained twice as much protein as compared with the initial value. With 50 per cent serum a doubling of protein content was reached at 24 hours and by 48 hours this value was 4-fold higher than the initial level. Between 48 and 72 hours there was usually a slight fall in protein content. Cell numbers remained constant throughout this interval.

Text-fig. 2 illustrates the changes in specific activity of acid phosphatase in different concentrations of serum. These data generally parallelled the increment in cellular protein and the cytochemical studies. With 50 per cent serum the activity of acid phosphatase rose much more rapidly and reached peak levels at 48 hours. Activity at this period was higher than that reached in 20 per cent serum medium at any time of *in vitro* incubation. In 1 per cent serum medium only a slight increase occurred and this took place during the last 24 hours of incubation. It appeared, therefore, that serum increased both the rate of production and the amount of this enzyme. The specific activity of acid phosphatase in mouse cells cultured in 50 per cent serum was higher than in any other phagocyte so far studied in this laboratory.

Text-fig. 3 illustrates the effect of serum concentration on the total activities of the three hydrolases. Under these conditions 50 per cent serum resulted in a 35- to 40-fold increase in total acid phosphatase and parallelled the changes in

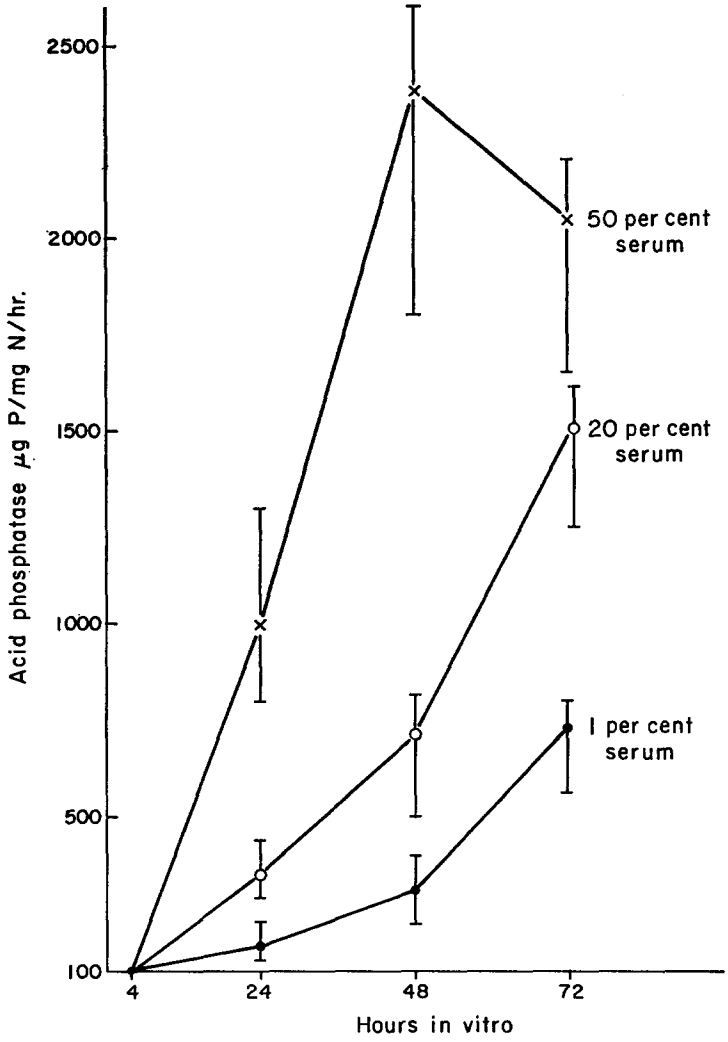
specific activity. The results with β-glucuronidase and cathepsin are illustrated on the right side of the Figure and show a similar serum effect. The increase in these two enzymes is, however, more linear with 50 per cent serum. Serum




TEXT-FIG. 1. The effect of newborn calf serum (NBCS) concentration on the protein content of mouse mononuclear phagocytes.

concentration, therefore, also affected the rate of production and total amount of β-glucuronidase and cathepsin, but quantitative differences are apparent when compared to acid phosphatase production.

*Direct Observations on Granule Formation with Phase Contrast Microscopy.*— The rapid production of phase-dense granules in the presence of 50 per cent newborn calf serum medium facilitated direct phase contrast observations on the formation of these organelles.

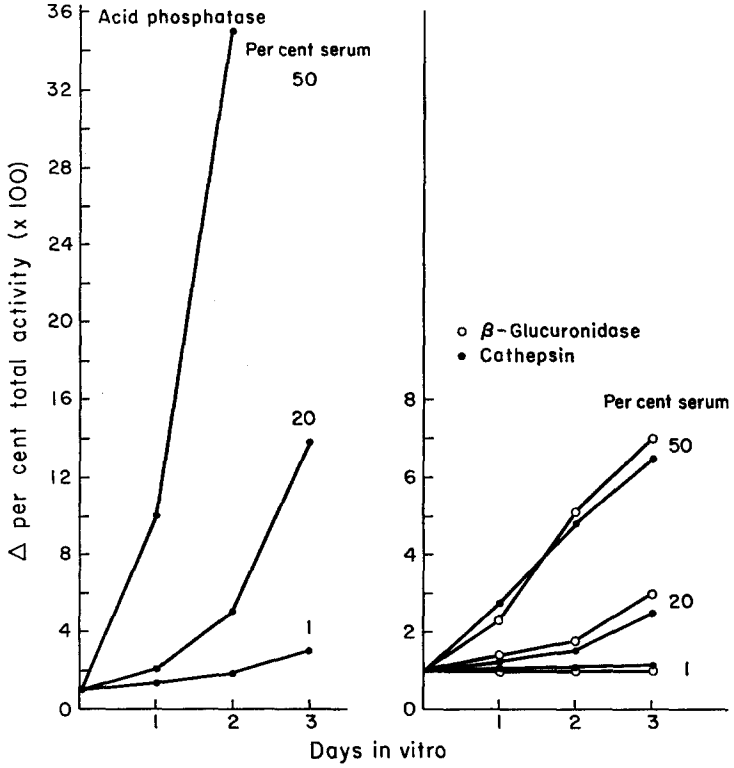



TEXT-FIG. 2. The effect of NBCS concentration on the specific activity of acid phosphatase.

Two systems were employed for the long-term study of granule formation. The first was to allow cells to adhere to coverslips in Leighton tubes. The coverslips were then inverted over shallow troughs (2 to 3 mm) of paraffin which had been built up on a microscope slide and filled with warm medium (50 per cent serum). The coverslip was then sealed in place with paraffin-beeswax mixture and placed on a warm stage. This type of preparation could be viewed directly with the oil immersion objective and was suitable for time periods of up to 5 to 6 hours.

The second system employed the Sykes chamber purchased from Bellco Glass, Inc., Vineland, New Jersey. This is a unit in which two coverslips are separated by a silicon rubber gasket and accommodates 0.8 ml of fluid. Cells were allowed to adhere on one side of the chamber,

washed, and the medium replaced by 50 per cent serum medium which had previously been gassed with 5 per cent $CO_2$-air. The cells were then viewed with an inverted phase contrast microscope in a warm chamber which was continually flushed with 5 per cent $CO_2$-air. This unit as well as the time lapse photography equipment was kindly supplied by Dr. Cecil Taylor of The Rockefeller Institute. Under these conditions cells were observed and continually photographed for 24-hour periods without a change in medium. The authors are indebted to Dr. James G. Hirsch for his assistance in taking time lapse cinematographs.




TEXT-FIG. 3. The influence of NBCS concentration on the total increment of acid phosphatase, cathepsin, and β-glucuronidase.

A composite view of granule formation as obtained from direct observation and the analysis of time lapse cinematography will be presented. Shortly after adherence to glass in the 50 per cent serum medium the cells began to pinocytize very actively. Small phase-lucent vesicles appeared at the ruffled border and moved centripetally to the perinuclear zone. As these vesicles streamed towards the centrosphere they fused with each other and increased in size. Within a few hours large numbers of clear pinocytic vacuoles had arranged themselves in a perinuclear rosette and with time their number gradually increased (Figs.

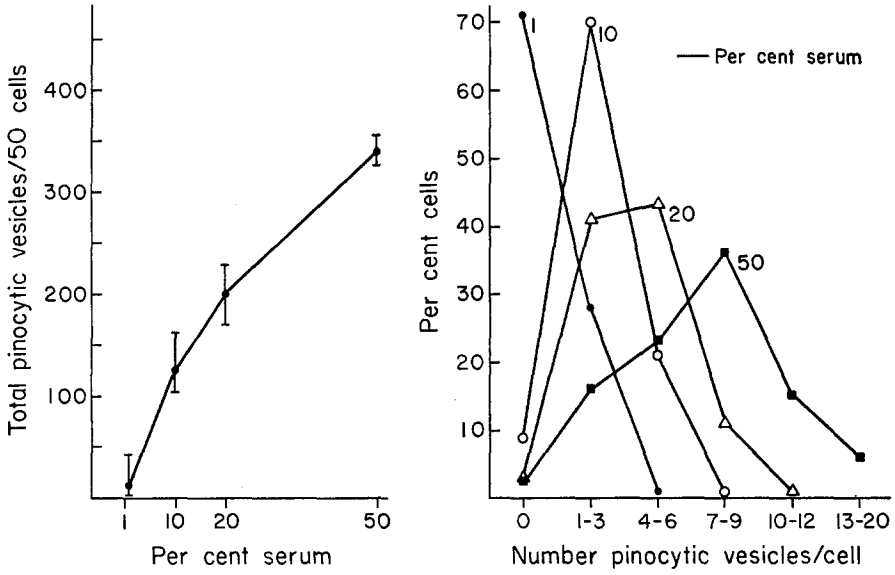
3 *a* to 3 *c*). During this period changes occurred in the phase density of the pinocytic vacuoles (Figs. 3 *a* to 3 *c*). This was characterized by a gradual increase in phase density, the end result being a very dark granule of the type illustrated in Figs. 2 *c* and 4 *a*. Examination of time lapse photographs indicated that the conversion of a phase-lucent vacuole to the phase-dense granule could occur within 90 minutes. The change in density only took place in the centrosphere region. The dense granules varied in size and were often smaller than the initial clear vacuoles. Dense bodies which remained in the perinuclear region have been seen to enter, rather than fuse with, newly formed and larger, clear vacuoles. In this location they exhibited rapid intravacuolar motion which gradually ceased as the phase density of the vacuole increased. Throughout this time there was a continuing stream of pinocytic vesicles from all portions of the cell into the perinuclear region. These most often fused with the larger preformed clear vacuoles although they also were seen to fuse with granules which had already become phase-dense. By 18 hours the cell had accumulated large numbers of dense granules and this process continued with longer periods of incubation but at a slower rate. The overall impression was of constant fusion of new and old pinocytic vesicles in the centrosphere with the gradual accumulation of the dense granules which reacted positively for acid phosphatase (1). It had previously been demonstrated that the phase-lucent pinocytic vacuoles were uniformly negative for acid phosphatase (1). The origin of the macrophage granule is, therefore, in large measure the result of pinocytic activity. These results are similar to the formation of the neutral red positive granules described by Lewis in macrophages (3, 4) and to the observations of Rose on certain variant HeLa cells (5).

*The Estimation of Pinocytic Activity and the Influence of Serum.*—In view of the close relationship between pinocytic vesicles and the mature granule it was of importance to be able to quantitate the rate of pinocytosis. This was difficult in living cells because of the constant membrane activity and the continual formation of vesicles. However, it was found that fixation with glutaraldehyde preserved the vesicles and allowed relatively accurate microscopic observations (Figs. 4 *a* and 4 *b*). It should be mentioned that the rate of vesicle formation in living preparations was not a continuous process but waxed and waned in a given cell. In addition, these counts enumerate only those vesicles large enough to be seen at a magnification of 1000. It is clear from electron microscopic observations that there are many micropinocytic vesicles too small to be resolved at this magnification. Only those vesicles were counted which were clearly outside the centrosphere region and were presumably in transit. With these limitations in mind quantitative counts were then performed on cells maintained in different media.

Two types of preparations were employed which gave essentially the same quantitative results. In the first, cells were cultivated in 50 per cent serum medium for a period of 20 hours

on coverslips. At the end of this time groups of tubes were washed three times with 3.0 ml of warm medium 199. This removed the majority of serum constituents. One ml of medium containing 1, 10, 20, and 50 per cent serum respectively was added, the tubes gassed with 5 per cent $CO_2$-air and allowed to incubate for 4 hours at 37°C. The cells were then fixed and examined. Under these conditions cell diameter and general morphology was the identical in all tubes.

The second technique was to culture cells in medium containing the same four serum concentrations. At the end of 24 hours the cells were then fixed and examined. Under these conditions cell size was considerably greater in the media with high concentrations of serum. A total of 300 cells were counted for each serum concentration.




TEXT-FIG. 4. The effect of NBCS concentration on the rate of pinocytosis and the cellular distribution of pinocytic vesicles.

The composite data are presented in Text-fig. 4. The influence of serum on the total number of pinocytic vesicles is presented on the left side. It is apparent that as the concentration of serum in the medium is elevated the number of pinocytic vesicles, and presumably the rate of pinocytosis, is increased in a curvilinear fashion. At a concentration of 1 per cent serum, only rare vesicles were seen in living preparations whereas many were apparent at the 50 per cent level. Similarly, the number of phase-lucent vacuoles, which accumulated in the centrosphere and were not counted by this method, were increased at the higher concentrations of serum.

The distribution of pinocytic vesicles in cells, at each serum concentration, is presented on the right side of Text-fig. 4. At 1 per cent serum, slightly more than 70 per cent of the cells contained no visible pinocytic vesicles and the remainder had only 1 to 3 per cell. As the concentration of serum was increased,

the distribution curves were shifted to the right. In addition, there was a greater variability in the number of vesicles per cell at the higher levels of serum as evidenced by the broader peak with 50 per cent serum. Examination of cultures at 48 and 72 hours in the presence of 50 per cent serum revealed that a high level of pinocytic activity was maintained on the basis of vesicle counts. At each time interval the number of pinocytic vesicles per 50 cells was 300 or greater.

These data indicate that there is a direct association between the concentration of newborn calf serum in the medium and the rate of pinocytosis as evaluated by microscopic analysis.

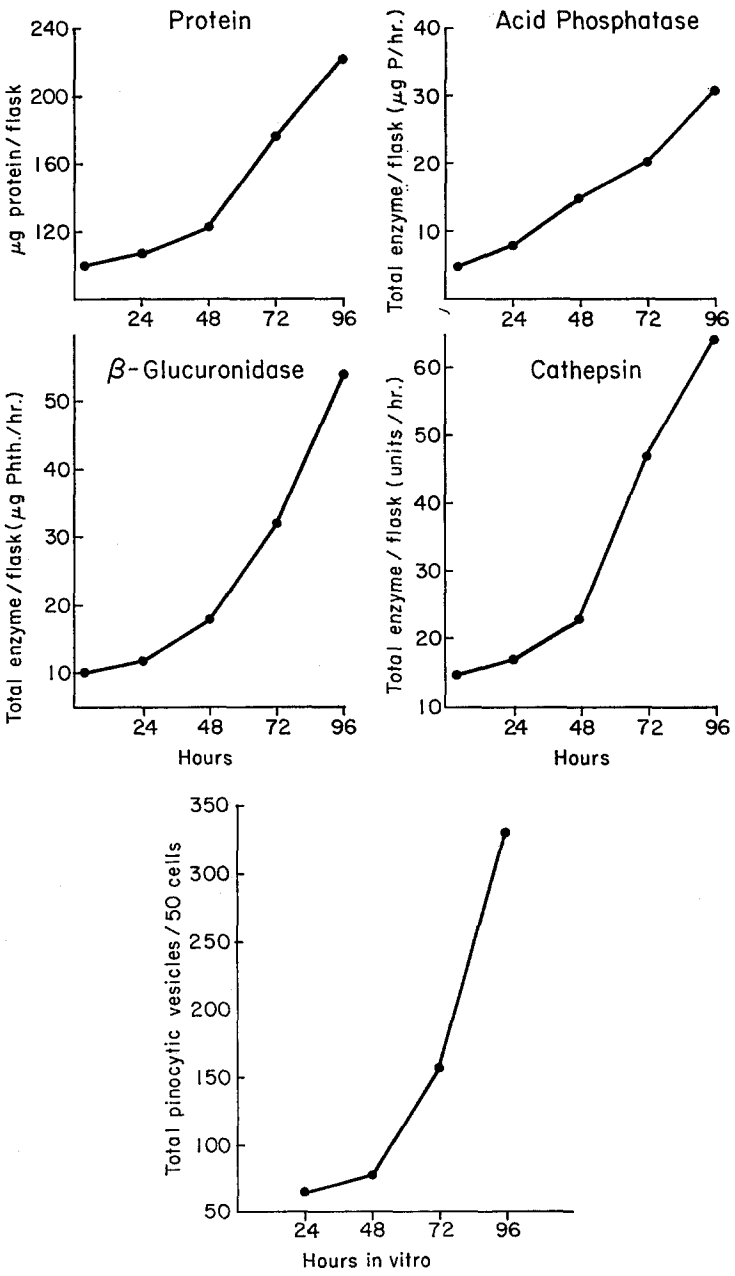
TABLE I

*The Influence of Different Sera on Mononuclear Phagocyte Hydrolases after 72 Hours' Incubation in Vitro*

| Serum | | Protein | Acid phosphatase | | β-Glucuronidase | | Cathepsin | |
|---|---|---|---|---|---|---|---|---|
| Species | Conc. | μg/Flask | Total μg P/hr. | Activity μg P/mg N/hr. | Total μg PHTH/hr | Activity μg PHTH/mg N/hr. | Total units/hr. | Activity units/mg N/hr. |
| | *per cent* | | | | | | | |
| Horse | 50 | 183 | 20 | 670 | 33 | 1235 | 50 | 1820 |
| Bovine | 50 | 215 | 40 | 1200 | 42 | 1251 | 58 | 1707 |
| Rabbit | 50 | 118 | 12 | 520 | 20 | 1040 | 28 | 1410 |
| Newborn calf | 50 | 300 | 112 | 2444 | 57 | 1200 | 52 | 1084 |
| 4 hr. control | | 97 | 6 | 87 | 12 | 720 | 13 | 630 |

*The Influence of Different Sera on Hydrolase Production and Granule Formation.*—All previous studies had utilized newborn calf serum in the medium. It was next of interest to determine the influence of other sera on the qualitative and quantitative pattern of hydrolase production, morphology, and pinocytic process. Each of these sera were employed at a final concentration of 50 per cent and studied at time points ranging from 24 to 96 hours. Data concerning the hydrolase content of cells at the 72 hour period are presented in Table I.

It is apparent that newborn calf serum stimulated the production of hydrolytic enzymes to a greater extent than the other sera tested. Cells cultivated in bovine serum (adult) accumulated less protein and produced less enzymes although the general pattern of enzyme activity was similar to a lower concentration of newborn serum, *i.e.* 20 per cent. Examination of the morphology of cells in 50 per cent bovine serum showed that the rate of granule formation and granule size closely resembled that seen in 20 per cent newborn calf serum. In addition, the evaluation of pinocytosis at 24 and 48 hours yielded consistent values of 150 to 160 pinocytic vesicles per 50 cells. It appeared, therefore, that




TEXT-FIG. 5. The influence of horse serum on hydrolase production and pinocytosis by mouse macrophages. Phth, Phenophthalein

bovine serum influenced the maturation of these cells in a qualitatively similar fashion but was less potent in stimulating pinocytosis, granule formation, and hydrolase production than newborn calf serum.

Rabbit serum yielded the poorest response. Since fresh serum proved to be cytotoxic the experiments were performed with preparations heated at 56°C/30 minutes. This resulted in the formation of spindle shaped cells with a few small granules. Total enzyme production was at a low level and was consistent with the morphological observations. It should be noted that the properties of newborn calf serum were not affected by heating at 56°C/30 minutes.

Horse serum represented an interesting situation which was examined in more detail. At a concentration of 50 per cent the mouse cells produced considerable amounts of both cathepsin and β-glucuronidase with relatively high specific activities. In contrast, the total amount and specific activity of acid phosphatase was unusually low at this time period when compared to calf serum.

A more complete study of the formation of hydrolases in the presence of 50 per cent horse serum is shown in Text-fig. 5. It is evident that only small amounts of enzyme are found during the first 48 hours of cultivation. Following this period the amount of cathepsin and β-glucuronidase rose rapidly with a major increment between 72 and 96 hours. In contrast to newborn calf serum, the amount of acid phosphatase increased at a much slower rate.

An examination of the pinocytic process was performed at daily intervals and is shown on the right side of Text-fig. 5. During the first 48 hours the rate was relatively low and then exhibited a sharp rise. The shape of this curve appeared similar to those of protein content, cathepsin, and β-glucuronidase. It should be noted that the size of peripherally located pinocytic vesicles as well as those in the centrosphere region were considerably smaller than seen in calf serum.

The presence of horse serum resulted in the formation of cells with distinct morphological properties. Examples of the cells can be found in Figs. 5 *a* to 5 *c*. Throughout the course of incubation the cells maintained a more fibroblastic appearance and accumulated only small amounts of lipid droplets. The granules were always small in size and became quite numerous by 48 hours of incubation. A well defined perinuclear localization was not prominent and the granules were often scattered throughout the cytoplasm. Staining for acid phosphatase showed a much less intense reaction than that seen at a comparable period with calf serum.

## DISCUSSION

It seems clear that pinocytosis plays a major role in the formation of macrophage lysosomes under these *in vitro* conditions. In the presence of serum the

cell began to pinocytize and transferred large amounts of surface membrane and probably extracellular constituents into the centrosphere region. The rate of this transfer as well as the subsequent size of the granules was dependent upon the level of serum. In the presence of high concentrations of newborn calf serum the pinocytic rate was stimulated and in turn large numbers of vacuoles accumulated in the centrosphere zone. Shortly after the localization of pinocytic vacuoles to the perinuclear region these structures became progressively phase-dense resulting in the osmiophilic, acid phosphatase–positive organelles described in this and previous communications (1, 2). This transition in density could arise from a concentration of intravacuolar contents derived from the medium as well as the transfer of endogenous materials, *i.e.* hydrolases, from some point in the cytoplasm. It is likely that both processes occur thereby mixing substrates and enzymes in the same membrane-enclosed granule. This schema assumes that the three hydrolases under study are not incorporated from the medium. Such an uptake is quite unlikely since it would require more than a millionfold concentration of calf serum enzymes to approach intracellular activities. Another assumption would be that the three hydrolytic enzymes under study are produced, transferred, and segregated in a similar fashion.

In addition to the formation of cytoplasmic granules, the level of calf serum also governed the production of hydrolytic enzymes. Although no accurate comparison is possible, there seemed to be a direct relationship between serum concentration, pinocytosis, and hydrolase accumulation. From this association we may speculate that the flux of pinocytic vesicles and/or the nature of their contents stimulated the synthesis of hydrolytic enzymes. This implies an adaptive cellular response in which the rate of uptake or level of exogenous molecules entering the cell, controls the production of hydrolases. These enzymes by their very nature, would be expected to attack a variety of serum constituents thereby making them available either for cell metabolism or excretion into the medium. In this regard the macrophage represents a much more versatile phagocyte than the adult polymorphonuclear leucocyte which is limited in synthetic capabilities (6). Portions of this general schema are implicit in a number of previous hypotheses put forth by de Duve (7), Novikoff (8), Strauss (9), and Miller and Palade (10).

At the present time it is uncertain whether the components of the medium induce the formation of specific hydrolases or whether this represents a controlled species response to a variety of exogenous stimuli. The complexity of the serum medium makes it difficult to answer this question and studies with specific substrates and other enzymes are in order. It is of interest, nevertheless, that the same pattern of enzymes was found in cells which matured in an *in vivo* inflammatory response and in the presence of a number of different sera *in vitro* (1). In this regard, one might expect that macrophages in the inflamed

peritoneum would be exposed to a variety of macromolecules arising from the breakdown of other cell types. The one exception to this general pattern of enzymes was obtained with horse serum and resulted in a much lower level of acid phosphatase than would be expected on the basis of cell protein and the levels of cathepsin and β-glucuronidase.

If, as we believe, pinocytosis is a prime regulator of granule formation and hydrolase production, it will be of considerable interest to ascertain the responsible components in serum. Preliminary observations indicate that this may not be a simple problem since both dialyzable and non-dialyzable factors are required for *in vitro* macrophage differentiation. Are analogous determinants operative *in vivo* and are they responsible for the monocyte to macrophage transition in areas of inflammation? If so, then pinocytosis stimulating factors arising either from humoral or cellular components might play an important role in inflammation.

Many questions related to the formation and turnover of macrophage lysosomes remain to be answered. Some of these include (*a*) the mechanism of hydrolase transfer from the synthesizing apparatus to the pinocytic vacuole, (*b*) the turnover of granule components, (*c*) the eventual fate of the granule, and (*d*) the reversibility of the process. Certain of these problems will be discussed in the subsequent papers of this series.

## SUMMARY

The concentration of newborn calf serum in the medium has marked effects on the morphological and biochemical properties of mouse mononuclear phagocytes. At a low serum concentration, the cells developed small numbers of tiny cytoplasmic granules and little or no increase in acid phosphatase, cathepsin, and β-glucuronidase. As the serum concentration was raised, granules were formed at a more rapid rate and were larger in size. The rate of production and total amount of three hydrolytic enzymes was increased at higher levels of serum.

Observations on living cells indicated that the phase-dense granules which accumulated in the perinuclear region were derived from pinocytic vesicles. These clear vesicles fused and migrated to the centrosphere where they underwent a gradual increase in phase density and reacted positively for acid phosphatase.

A microscopic technique was described for the evaluation of the pinocytic process. When this method was employed, the rate of pinocytosis increased curvilinearly with elevations in the calf serum concentration of the medium.

The comparative influence of bovine, horse, and rabbit serum on mouse cells was evaluated.

It is suggested that pinocytosis is a major regulator of granule formation and hydrolytic enzyme production by the mouse macrophage.

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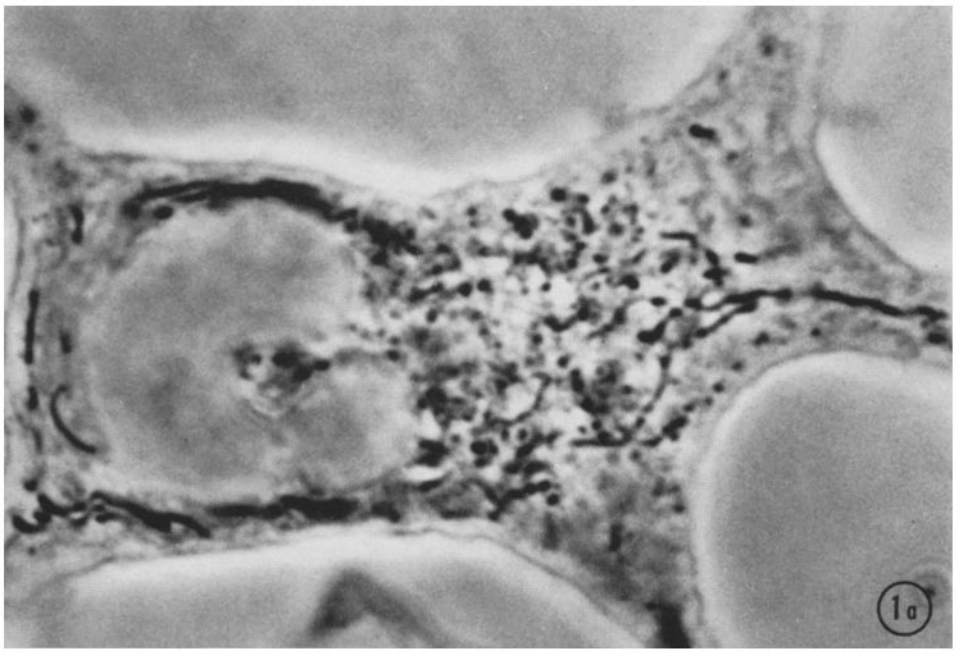
## EXPLANATION OF PLATES

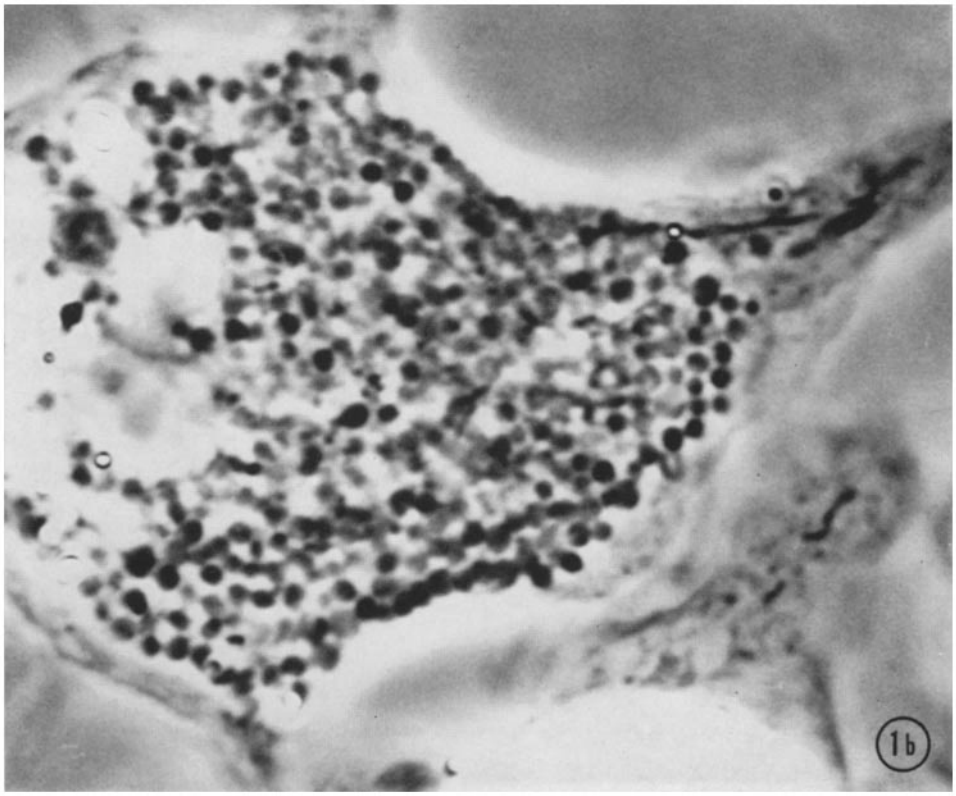
### PLATE 63

FIGS. 1 *a* and *b*. Mouse cells after 72 hours' incubation *in vitro*. Fixed with 1 per cent $OsO_4$. Phase contrast, × 2500.

FIG. 1. *a*. Maintained in 1 per cent NBCS, No. 199. Occasional small granules distributed in cytoplasm without a circumscribed centrosphere region. Few lipid droplets are seen. Mitochondria are scattered through the cell and are more apparent because of the absence of granules.

FIG. 1 *b*. Maintained in 50 per cent NBCS, No. 199. The majority of the cytoplasm is filled with large, osmiophilic, phase-dense granules. Many highly refractile lipid droplets are present.



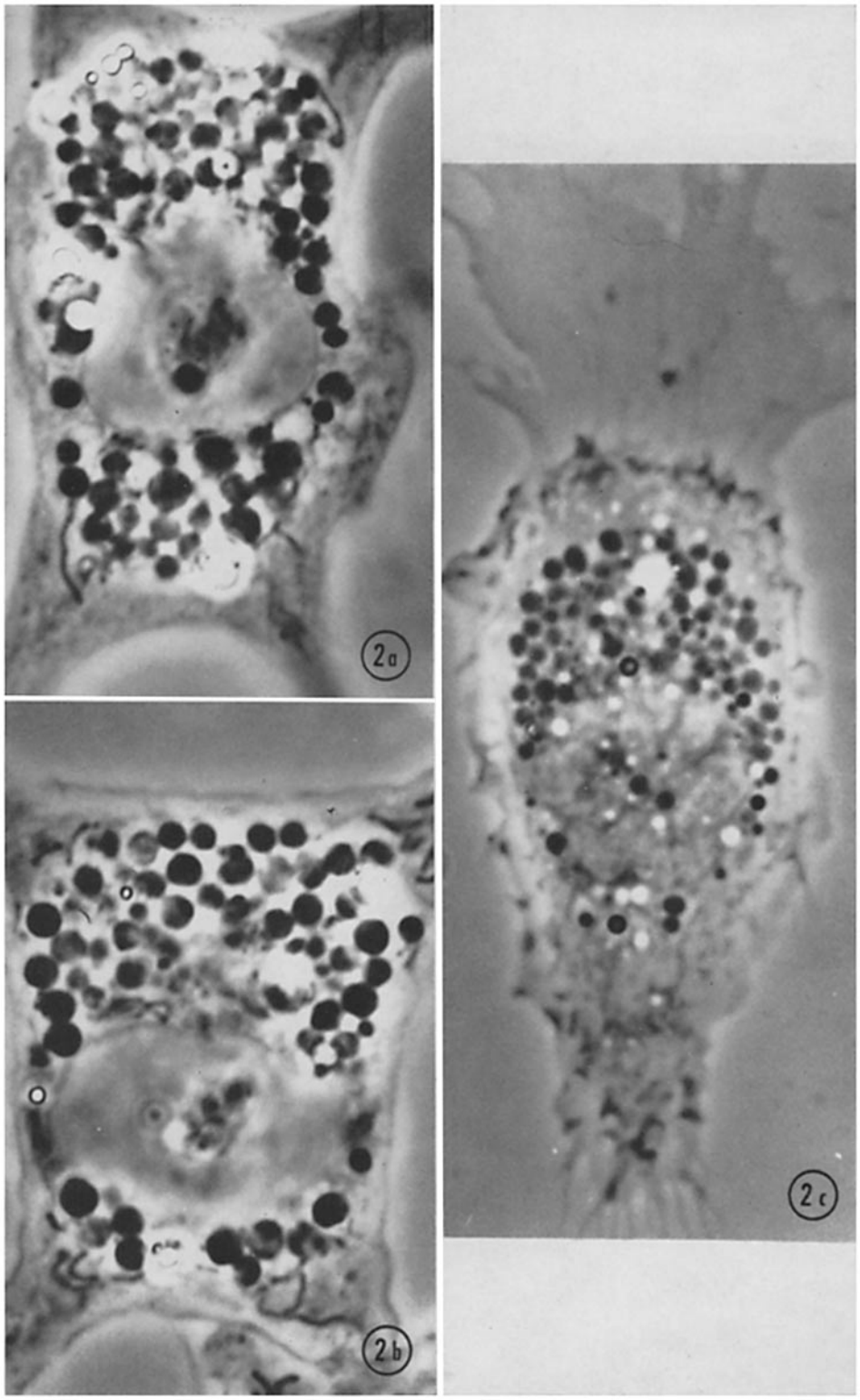

(Cohn and Benson: Macrophage differentiation)

## PLATE 64

FIGS. 2 *a* to 2 *c*. Mouse cells maintained in 50 per cent newborn calf serum. Phase contrast, × 2500.

FIGS. 2 *a* and 2 *b*. Fixed in 1 per cent $OsO_4$ after 24 hour's incubation. Very large osmiophilic granules filling the cytoplasm. These are generally larger than at the 72–hour period. Individual granules vary in density suggesting an inhomogeneous ultrastructure.

FIG. 2 *c*. A living cell photographed in a warm chamber preparation after 18 hours' incubation. The cell is polarized with the majority of phase-dense granules on the upper side of the nucleus. Clear pinocytic vesicles are scattered amongst the granules and are arising from the area of the ruffled membrane. Pinocytosis appears more active in the perigranular region above the nucleus.




(Cohn and Benson: Macrophage differentiation)

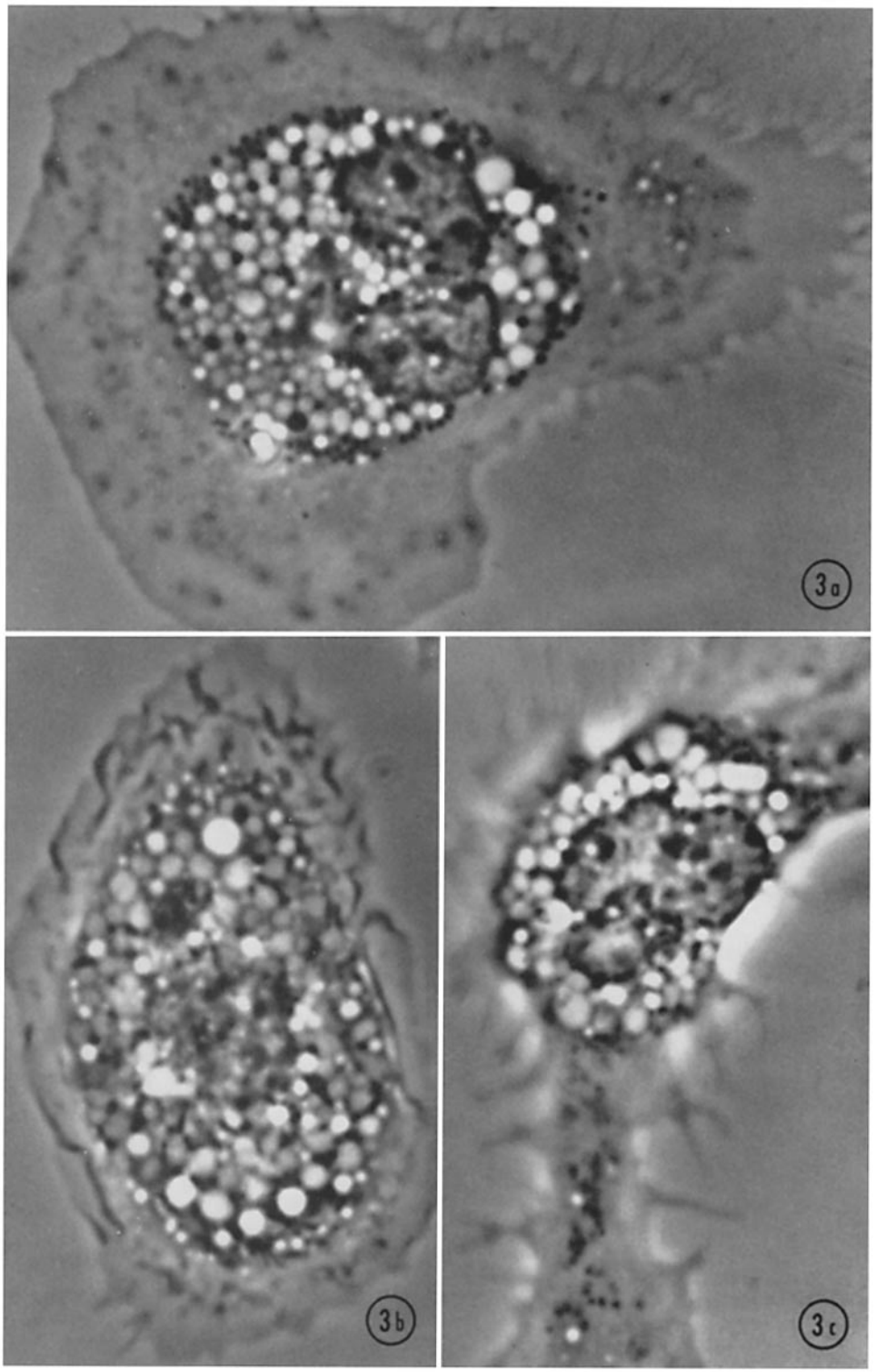
PLATE 65

FIGS. 3 *a* to 3 *c*. Intermediate stages in the formation of phase-dense granules. Living cells in warm stage preparations photographed after 5 to 6 hours of *in vitro* cultivation in 50 per cent NBCS medium. Phase contrast, × 2500.

FIG. 3 *a*. Perinuclear accumulation of large vacuoles has occurred. Some of the vacuoles are phase-lucent having the density of the smaller pinocytic vesicles in the peripheral cytoplasm. Other vacuoles illustrate various stages of increasing density. A few are already very dense and resemble those seen at later stages. Surrounding the vacuole area there is a halo of smaller dense bodies which are spherical or rod-like in shape. The majority of these are small mitochondria.

FIG. 3 *b*. Another cell illustrating transitional forms of granules. The limiting membrane is very ruffled and pinocytic vesicles were seen arising at the very edge of the vacuole area.

FIG. 3 *c*. A cell which has already extended a pseudopod. Clear pinocytic vesicles in the pseudopod were seen moving towards the vacuole region. Small mitochondria have already entered the pseudopod and can be contrasted with those seen in Figs. 4 *a* and *b*.

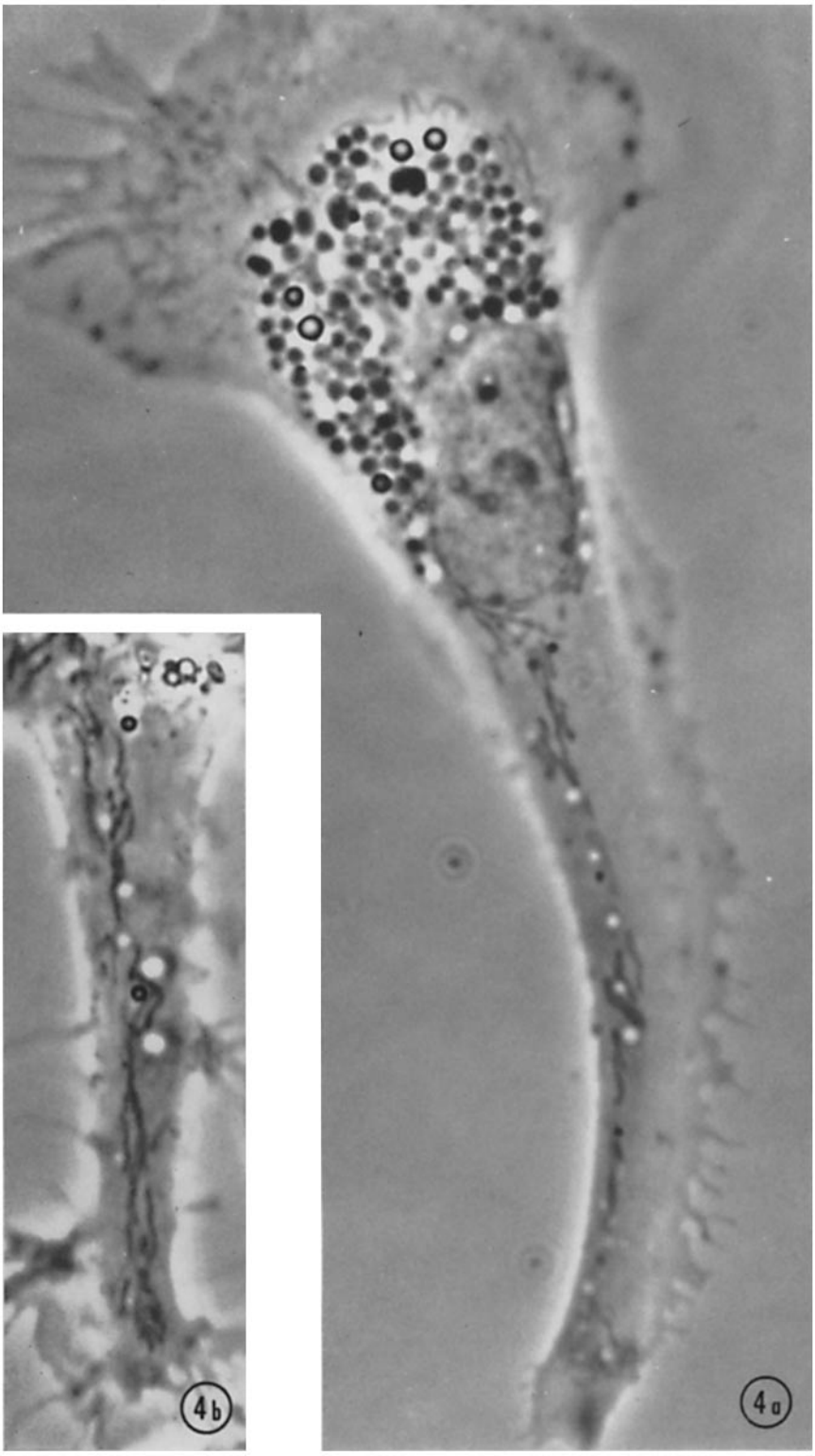



(Cohn and Benson: Macrophage differentiation)

PLATE 66

FIG. 4 *a*. Appearance of living cell after 18 hours of cultivation in 50 per cent NBCS medium. The majority of organelles in the centrosphere are now phase-dense. Pinocytic vesicles were seen arising in the tip of the pseudopod and moved centripetally toward the granule region. During this process they fused to form larger vacuoles, which then either fused with preexisting structures or became progressively phase-dense. The mitochondria present in the pseudopod are much larger than at 6 hours of cultivation. × 2500.

FIG. 4 *b*. The appearance of a pseudopod fixed with buffered 1.25 per cent glutaraldehyde. The pinocytic vesicles in this cell are larger than in Fig. 4 *a* and clearly visible. Phase contrast, × 2500.




(Cohn and Benson: Macrophage differentiation)

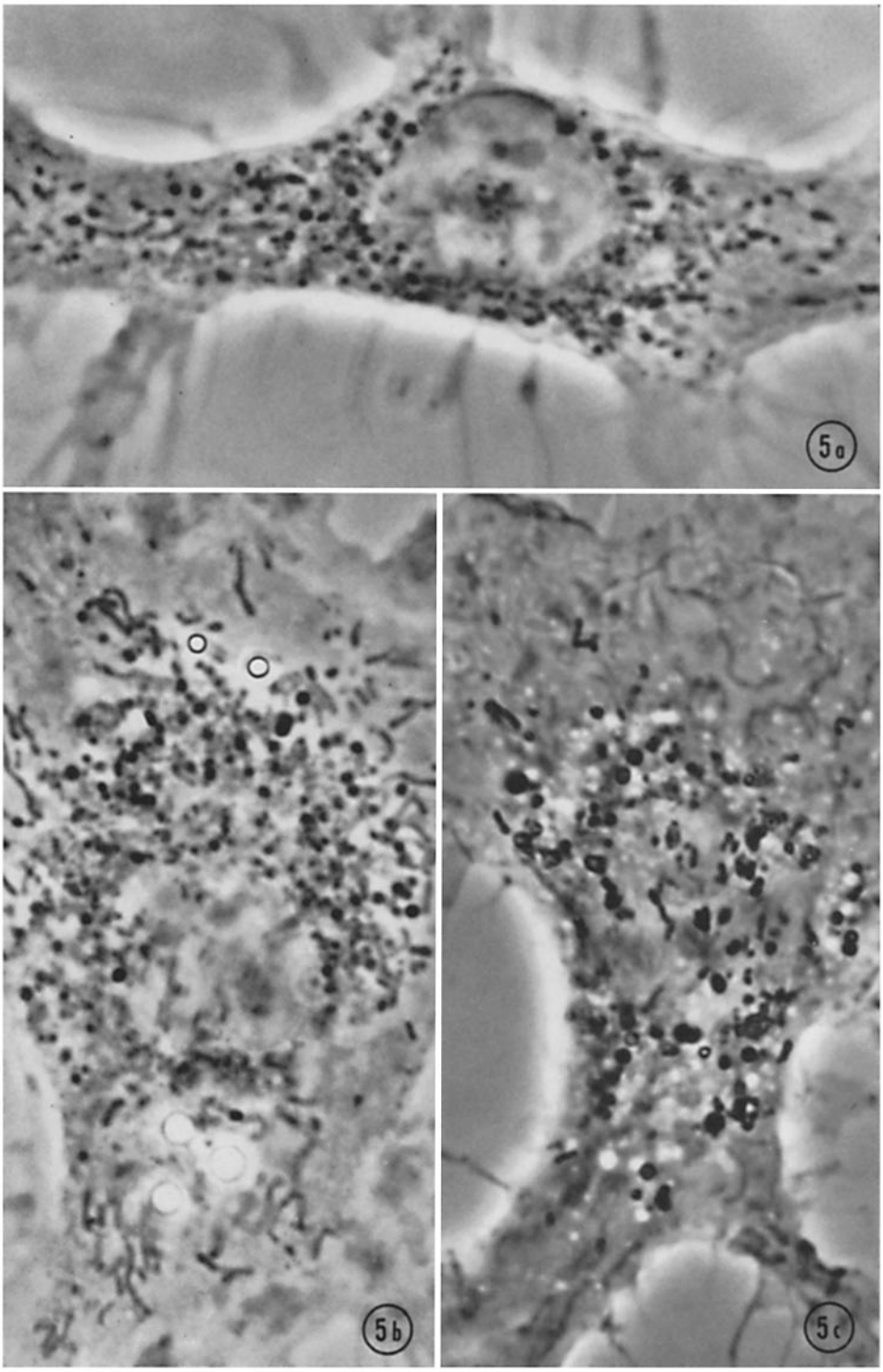
## PLATE 67

FIGS. 5 *a* to 5 *c*. The appearance of mouse phagocytes cultivated in 50 per cent horse serum. Phase contrast, × 2500.

FIG. 5 *a*. Osmium tetroxide-fixed cell at 52 hours. The granules are smaller than seen in NBCS medium. There is a less clearly demarcated centrosphere and little lipid accumulation.

FIG. 5 *b*. Osmium tetroxide–fixed cell at 84 hours. Cell size and the number of small granules increased progressively. Lipid accumulation was minimal even at this late stage.

FIG. 5 *c*. Acid phosphatase stained cell at 72 hours. The reactive granules are smaller and fewer in number than comparable cells cultivated in newborn calf serum.




(Cohn and Benson: Macrophage differentiation)