THE IN VITRO DIFFERENTIATION OF MONONUCLEAR PHAGOCYTES

I. The Influence of Inhibitors and the Results of Autoradiography*

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

(From The Rockefeller Institute)

Plates 22 to 26

(Received for publication, October 20, 1964)

A previous publication documented the morphological and biochemical events which occurred when normal mouse peritoneal phagocytes were exposed to an *in vitro* culture environment (1). These changes were characterized by a progressive enlargement of the cell with the accumulation of phase-dense granules in the centrosphere region. The granules reacted strongly for acid phosphatase, and this enzyme as well as two other acid hydrolases were shown to increase in both total amount and in specific activity. On the basis of these data it was suggested that the differentiation of monocytes to macrophages was accompanied by the production of lysosome-like cytoplasmic organelles.

This article will be concerned with the influence of selected inhibitors of protein and nucleic acid synthesis on the morphological and biochemical aspects of *in vitro* development. In addition, autoradiographic studies concerning the early localization of incorporated leucine and choline will be presented.

Materials and Methods

A full description of the methods for cultivating, harvesting, and assaying normal mouse peritoneal phagocytes has been reported (1). The complete medium employed for all studies consisted of 20 per cent newborn calf serum, medium 199 and 200 units/ml of penicillin. Assays of the complete medium for the three hydrolases have been consistently negative.

Autoradiography.—DL-Leucine-4,5-H^3 and choline-methyl-H^3 were purchased from New England Nuclear Corp., Boston, as sterile solutions. Both the tritiated leucine (5.45 c/mmole and choline (98 mc/mmole) were diluted directly into complete medium.

Cells growing on coverslips in Leighton tubes were employed for all experiments. Although labeling was performed on cells after 24, 48, and 72 hours of cultivation, the majority of work was conducted at the 48 hour period. At this time the cells had developed a well defined centrosphere, were spread out, and had not accumulated excessive lipid. When cells were to be labeled, the old medium was removed and prewarmed fresh complete medium containing tracer in a volume of 1.0 ml was added to the tube. After periods ranging from 2 to 120 minutes

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

cells were either fixed or the isotope removed, the coverslips washed thoroughly with complete medium and then incubated in medium containing 200 μg/ml of either leucine or choline as a cold chaser.

The preparations were fixed in the following manner to preserve internal structure and incorporated isotope. After removal of the isotope-containing media, 1.0 ml of 1 per cent buffered (pH 7.4) OsO_4 was added and the cells allowed to fix for 10 minutes at 4°C. The tube containing the coverslip was then washed twice with distilled water and briefly drained. This was followed by fixation with 1.25 per cent glutaraldehyde (pH 7.4) for 10 minutes. The tubes were then washed five times with 5.0 ml of water, the coverslips removed and air-dried. This method of dual fixation yielded the best morphology for the localization of label.

The coverslips were then processed according to the general plan of Caro and van Tubergen (2). Both Ilford Nuclear Research emulsions K-5 and L-4 have been employed. Emulsion L-4 with its smaller grain size gave somewhat better resolution and was used as routine. Coverslips were dipped in L-4 (1 gm/ml) at room temperature and air-dried. They were then mounted on glass slides with paraffin and exposed in light proof slide boxes containing a packet of drierite. In the majority of instances when 10 μc/ml of label was employed an exposure of 18 hours was sufficient. The coverslips were developed for 2 minutes in Kodak D-19, fixed for 5 minutes, water washed, and mounted in distilled water. The coverslips could be repeatedly hydrated without loss of morphology and were photographed under oil immersion phase contrast illumination.

Studies with Inhibitors.—Cells were harvested from normal mice and processed for both Leighton tube and T-flask cultures (1). Initial adherence to glass took place in the complete medium without inhibitor. After washing, to remove lymphocytes, complete medium containing inhibitor was added, the cultures gassed with 5 per cent CO_2-air and incubated at 37°C. In each experiment control preparations without inhibitor were always included. The general plan of the experiments was to test various concentrations of inhibitor in Leighton tube cultures. At intervals of 24 to 72 hours, coverslips were removed and fixed with 1 per cent OsO_4 for phase contrast morphology and with 1.25 per cent glutaraldehyde for acid phosphatase staining. After an effective concentration of inhibitor had been determined, which altered morphology but allowed survival of cells for 72 hours, T-flasks were prepared for quantitative biochemical determinations. All data on biochemical determinations relate to control flasks prepared on the same day and processed in an identical manner.

Puromycin and DL-*p*-fluorophenylalanine were obtained from Nutritional Biochemicals Corp., Cleveland, and colchicine, U.S.P. from Amend Drug and Chemical Co. Inc., New York. Actinomycin D was kindly supplied by Dr. E. Reich, The Rockefeller Institute.

RESULTS

The present experiments were designed to examine the influence of selected inhibitors of protein and nucleic acid synthesis on the morphology and biochemistry of developing mononuclear phagocytes.

DL-*parafluorophenylalanine.*—The inhibitor was dissolved in medium 199 and tested at final concentrations ranging from 25 to 500 μg/ml. No attempt was made to prepare media deficient in phenylalanine and this amino acid was present in both medium 199 and as a constituent of serum. Preliminary trials revealed that concentrations of 200 and 250 μg/ml inhibited the increase in size of the cells. At these concentrations cell numbers remained constant and at 72 hours were essentially the same as controls. A concentration of 500 μg/ml produced cell death and the majority of cells had detached from the glass surface at 24 to 48 hours. In contrast, a dose of 50 to 100 μg/ml was without

demonstrable effect on cell size, morphology, or cytochemically demonstrable acid phosphatase when compared to controls.

Figs. 1 *a* and 1 *b* illustrate cells maintained in the presence of 250 μg/ml of DL-parafluorophenylalanine for 72 hours. Fig. 1 *a* is a cell fixed with 1 per cent OsO_4 and is of a characteristic appearance. The cell is well spread out and contains short mitochondria in the two cytoplasmic projections. The most striking feature is the absence of osmiophilic, phase-dense granules in the perinuclear zone. Control cells which have been described in a previous publication (1) contain numerous granules in the centrosphere region at this time and cell diameter is much larger.

Fig. 1 *b* is a glutaraldehyde-fixed cell incubated for acid phosphatase. An occasional black granule is found in the perinuclear zone and this is the type of staining seen in the peritoneal cells at the onset of *in vitro* incubation. There are, however, many acid phosphatase negative-clear vesicles in the centrosphere as well as smaller ones in the peripheral cytoplasm. These represent pinocytic vesicles. From this morphological evidence it appeared that PFϕA inhibited the formation of acid phosphatase–positive granules as well as a general increase in cell size.

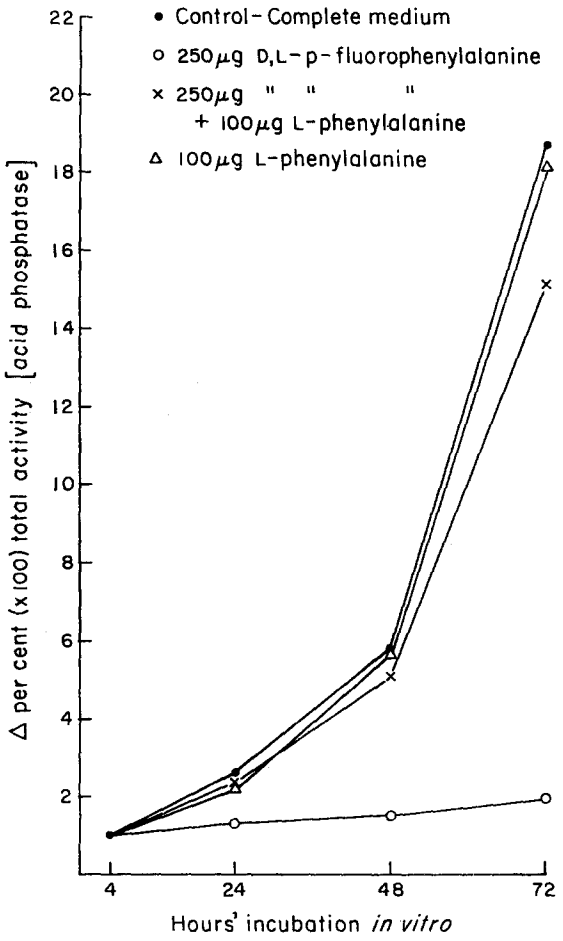
The same concentration of inhibitor (250 μg/ml) was then employed in T-flask cultures. Under these conditions the effect on the quantitative levels of hydrolytic enzymes and protein content could be ascertained. Text-fig. 1 illustrates these results for acid phosphatase during periods of cultivation ranging from 4 to 72 hours. The solid circles delineate the increase in total acid phosphatase and show the typical 20-fold increase in enzyme. In the presence of PFϕA only a slight increase was noted indicating the marked inhibition in active enzyme production. This effect of the analogue could be completely reversed if the natural amino acid L-phenylalanine were present in the medium. It was found that 100 μg/ml of L-phenylalanine was sufficient for this purpose. The addition of phenylalanine alone to the medium had no stimulatory effect on enzyme production.

Text-fig. 2 illustrates the inhibitory influence of the analogue on other parameters of macrophage development. When compared to the complete medium control, there was marked inhibition in the total quantities of protein, β-glucuronidase and cathepsin as well. The inhibitory effect on the three hydrolases was essentially the same.

Table I shows the effect on specific activities of the three enzymes calculated on a nitrogen basis. The 4 hour control represents the starting level of enzyme at the onset of cultivation. Almost no increase occurred in the specific activities of cathepsin and β-glucuronidase. In contrast, a 4- to 5-fold increase was noted in acid phosphatase even though total enzyme production was markedly inhibited. It appeared, therefore, that some new enzyme was being formed but this was only a small proportion of that occurring in normal cells.

Puromycin.—Puromycin represents another form of inhibitor of protein

synthesis active at the RNA level. Experiments were performed at concentrations ranging from 0.01 to 5.0 μg/ml. In Leighton tubes, concentrations of 0.2 to 0.4 μg/ml inhibited cell development whereas concentrations of 1.0 μg/ml

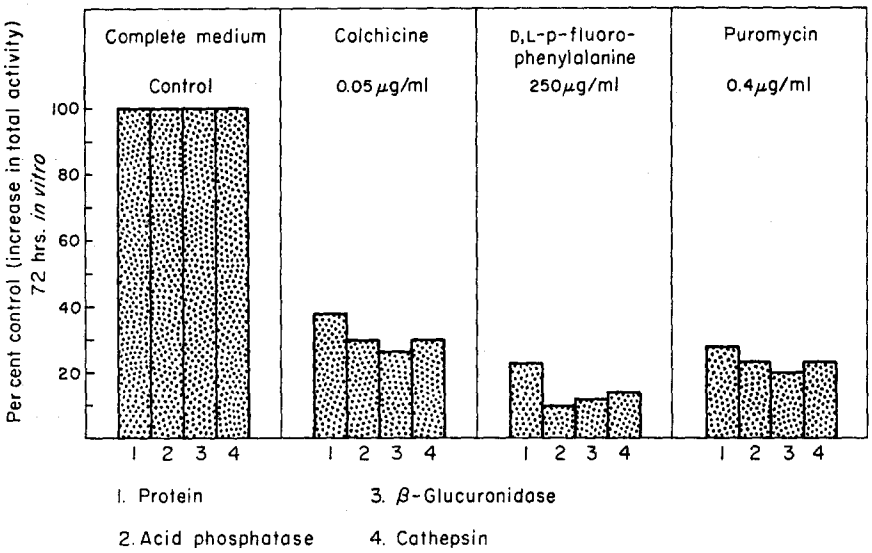


TEXT-FIG. 1. The inhibition of acid phosphatase production by DL-*p*-fluorophenylalanine and its reversal with L-phenylalanine.

produced cell death. Figs. 2 *a* to 2 *c* show cells maintained for 72 hours in the presence of 0.4 μg/ml. Figs. 2 *a* and 2 *b* are osmium tetroxide–fixed cells and show the paucity of phase-dense granules and the absence of a well defined centrosphere. A number of empty looking granules are seen and mitochondria are apparent. In general, these cells are much smaller in size and contain fewer lipid droplets than controls. Fig. 2 *c* is a glutaraldehyde-fixed cell stained for

acid phosphatase. The number and size of reacting sites was considerably less than similarly treated controls (1).

Biochemical data were obtained from T-flask cultures maintained in the



TEXT-FIG. 2. The influence of three inhibitors on the formation of enzymes and protein by mouse phagocytes cultivated for 72 hours.

TABLE I

*The Influence of Selected Inhibitors on the Specific Activities of Mononuclear Phagocyte Enzymes after 72 Hours' Cultivation**

Medium	Conc.	Acid phosphatase	β-Glucuronidase	Cathepsin
	μg/ml	*μg P/mg N/hr.*	*μg phenolphthalein/mg N/hr.*	*units/mg N/hr.*
Complete medium	—	1680	1520	1296
Puromycin	0.4	564	824	756
DL-*p*-fluorophenylalanine	250	489	862	760
Colchicine	0.05	1084	890	614
4 hr. control	—	92	760	650

* Inhibitors added to complete medium at T zero. Mean of 6 determinations.

presence of 0.4 μg/ml of inhibitor for 72 hours. The results are presented in both Text-fig. 2 and Table I. In the presence of puromycin the total increase in protein, acid phosphatase, β-glucuronidase, and cathepsin was reduced to 20 to 30 per cent of the control flasks. As in the case of *p*-flourophenylalanine small

increases in specific activity were noted, suggesting the presence of some protein synthesis.

Colchicine.—Colchicine was employed in the course of early experiments to evaluate the mitotic potential of mouse mononuclear phagocytes (1). Although metaphase figures were not seen it was noted that the alkaloid inhibited the growth in cell size and produced bizarre alterations in the outline of the cytoplasmic projections. Figs. 3 *a* and 3 *b* show two examples of cells maintained in 0.05 μg/ml of colchicine for 72 hours. Following osmium tetroxide fixation many small filamentous projections were noted on the cell surface as well as bulbous knobs on the larger pseudopods. In addition, there appeared to be fewer granules, and these were smaller in size. Mitochondrial appearance was not unusual but fewer lipid droplets had accumulated. Cytochemical tests for acid phosphatase indicated a somewhat smaller number of reactive sites although staining intensity of the granules approached that of controls.

Quantitative assays of T-flask cultures are found in Text-fig. 2 and Table I. The increase in total protein and the three hydrolases was significantly lower than controls and corresponded to the inhibition in cell growth. The specific activity of acid phosphatase rose over 10-fold, a level much above that obtained with puromycin and PFϕA.

Actinomycin D.—The use of Actinomycin D was complicated by its toxicity and the variable response of different cells in the culture. At levels of 0.005 μg/ml approximately 50 per cent of the cells were dead at 24 hours. The cytoplasm was practically absent and the nucleus small and pyknotic. The majority of the remaining cells contained fewer granules and were smaller in size. However, the dose dependance varied from day to day and was not reproducible. Because of the lack of a uniform cellular response further studies were not conducted. Levels higher than 0.005 μg/ml uniformly killed all cells in the cultures.

Autoradiography.—The previous studies suggested that active protein synthesis was necessary for the sequential morphological and biochemical changes occurring *in vitro*. It was, therefore, of interest to localize newly formed protein within the cell. This was accomplished by short pulses of tritiated leucine according to the general plan of Caro and Palade (3). In addition, choline was employed as a precursor of phospholipid and presumably of newly formed membrane (4).

A number of isotope concentrations and pulse ranges were employed. In general, maintaining isotope at 10 μc/ml, a high level, pulses of 2 minutes gave sufficient grains to be seen after overnight exposure with Ilford emulsion L-4. The illustrations in this article were photographed after pulses of 2 to 3 minutes and 60 minutes respectively. With the short pulses there was more non-specific absorption of the isotope to the cell surface. This could not be removed by washing and was also present in controls when the cells were fixed

immediately after the addition of tracer. After coating with emulsion, much of the phase contrast definition of the cytoplasm was lost.

Figs. 4 *a* and 4 *b* illustrate the localization of grains after a 2 to 3 minute exposure to leucine-H^3. Except for what appear to be random counts over the peripheral cytoplasm, the majority of grains are found in the centrosphere area which contains the phase-dense granules. The grains appear both light and dark in color because of intermediate focussing. Highly refractile lipid droplets on the other side of the nucleus remain free of label as does the nucleus.

Fig. 4 *b* is a cell exposed for 60 minutes to leucine-H^3. The major difference is the larger number of grains in the centrosphere region. Again lipid droplets and nucleus are free of isotope. These data suggest that newly formed protein accumulates in the granule area of the perinuclear zone.

Figs. 5 *a* and 5 *b* represent a similar experiment but this time performed with choline-H^3. Fig. 5 *a* represents a cell after a 3 minute pulse whereas Fig. 5 *b* shows localization of grains after 60 minutes. In both cases the majority of grains are found in the perinuclear granule region. With time the number of grains increases proportionately. No particular localization was noted over mitochondria, lipid droplets, or nucleus. These data suggest that early incorporation into phospholipid or its accumulation also takes place in the centrosphere region. The possibility of transmethylation has not, however, been ruled out.

Other experiments were conducted on cells exposed to leucine-H^3 for 5 minutes, washed, and then placed in cold medium for 2 hours. Under these conditions, grain counts remained constant for 2 hours and were well localized to the granule area. This suggested the storage and stability of new protein for at least this period.

DISCUSSION

The present experiments indicate that two inhibitors of protein synthesis interfere with the sequential differentiation of mouse mononuclear phagocytes *in vitro*. This was shown by the inhibition of both newly formed hydrolytic enzymes and granules in the centrosphere zone. The two agents presumably act at different sites of protein synthesis. DL-Parafluorophenylalanine is an analogue of the natural amino acid and is incorporated into protein, thereby stopping subsequent synthesis and/or the biological activity of the protein (5). In contrast, puromycin inhibits charged sRNA with splitting of the ribosome-bound peptidyl-sRNA (6). In these experiments it is likely that partial inhibition was achieved, since the cell survived for 72 hours, but new enzyme formation was more profoundly affected. The influence of colchicine is less definite although some evidence suggests an action at the RNA level (7).

The mechanism by which these agents inhibit the formation of enzyme-containing granules depends to a large extent on the natural mode of granule

production. Although many possibilities exist we will discuss one hypothesis currently under study. One can consider that granule or lysosome formation under these conditions is a two-stage process. One stage is the influx of phase-lucent vesicles into the centrosphere region by means of pinocytosis. This is consistent with observations on living macrophages in which pinocytic vesicles, formed at the cell surface, continually fuse and migrate centripetally into the perinuclear region (8, 9). These vesicles are the same size as the phase-dense granules which contain acid phosphatase. The second stage would represent the subsequent transfer of hydrolytic enzymes into the enlarged pinocytic vesicle. Since the Golgi apparatus has been implicated as an intermediate in the transport of newly synthesized protein (3), this could be accomplished by the constant flow of enzyme containing Golgi vesicles into the larger structures of pinocytic origin. With this mechanism in mind, puromycin and PFϕA might influence the process at a ribosomal level as well as inhibiting pinocytosis and the flux of large membranes into the centrosphere.

The autoradiographic evidence presented suggests that newly formed protein and possibly phospholipid containing membranes accumulate in the perinuclear granule zone. Whether this represents the initial site of formation or merely a storage area will require further study. In addition, the exact localization of label is uncertain and necessitates similar experiments at the electron microscope level. Examination of the ultrastructure of these cells, which is currently under investigation, reveals that the centrosphere region contains in addition to large granules of varying electron density, scattered Golgi lamellae and a profusion of small vesicles.

SUMMARY

The influence of selected inhibitors of protein synthesis on the *in vitro* differentiation of mouse mononuclear phagocytes has been investigated. DL-*p*-Fluorophenylalanine at concentrations of 250 μg/ml inhibits the formation of three lysosomal hydrolases, cytochemically demonstrable acid phosphatase and osmiophilic, phase-dense granules. These effects occur in the absence of cell death and are reversed by L-phenylalanine. Puromycin at concentrations of 0.2 to 0.4 μg/ml has a similar effect on both the morphology and biochemistry of cell maturation.

Colchicine at a concentration of 0.05 μg/ml inhibits the growth in cell diameter and has less of an effect on enzyme production.

Cells exposed to leucine-H^3 for 2 to 3 minutes exhibit the localization of grains in the perinuclear-dense granule region. After an exposure of 60 minutes a similar localization is evident but with a correspondingly greater number of grains. A similar localization of grains occurs when choline-methyl-H^3 is employed as a tracer. The data suggest the storage of newly formed protein and possibly phospholipid in the centrosphere region.

BIBLIOGRAPHY

1. Cohn, Z. A., and Benson, B., The differentiation of mononuclear phagocytes. Morphology, cytochemistry, and biochemistry, *J. Exp. Med.*, 1965, **121,** 153.
2. Caro, L. G., and van Tubergen, R. P., High resolution autoradiography. I. Methods, *J. Cell Biol.*, 1962, **15,** 173.
3. Caro, L. G., and Palade, G. E., Protein synthesis, storage, and discharge in the pancreatic exocrine cell. An autoradiographic study, *J. Cell Biol.*, 1964, **20,** 473.
4. Luck, D. J. L., Formation of mitochondria in neurospora crassa. A quantitative radioautographic study, *J. Cell Biol.*, 1963, **16,** 483.
5. Shive, W., and Skinner, C. G., Amino acid analogues, *in* Metabolic Inhibitors, (R. M. Hochster and J. H. Quastel, editors), New York, Academic Press, **1, 2,** 1963.
6. Nathans, D., Puromycin inhibition of protein synthesis: incorporation of puromycin into peptide chains, *Proc. Nat. Acad. Sc.*, 1964, **51,** 585.
7. Davidson, J. N., Leslie, I., and Waymouth, C., The nucleoprotein content of fibroblasts growing *in vitro*. IV. Changes in the RNA phosphorus and DNA phosphorus content, *Biochem. J.*, 1949, **44,** 5.
8. Lewis, W. H., Pinocytosis, *Johns Hopkins Hosp. Bull.*, 1931, **49,** 17.
9. Lewis, W. H., Pinocytosis by malignant cells, *Am. J. Cancer*, 1937, **29,** 666.

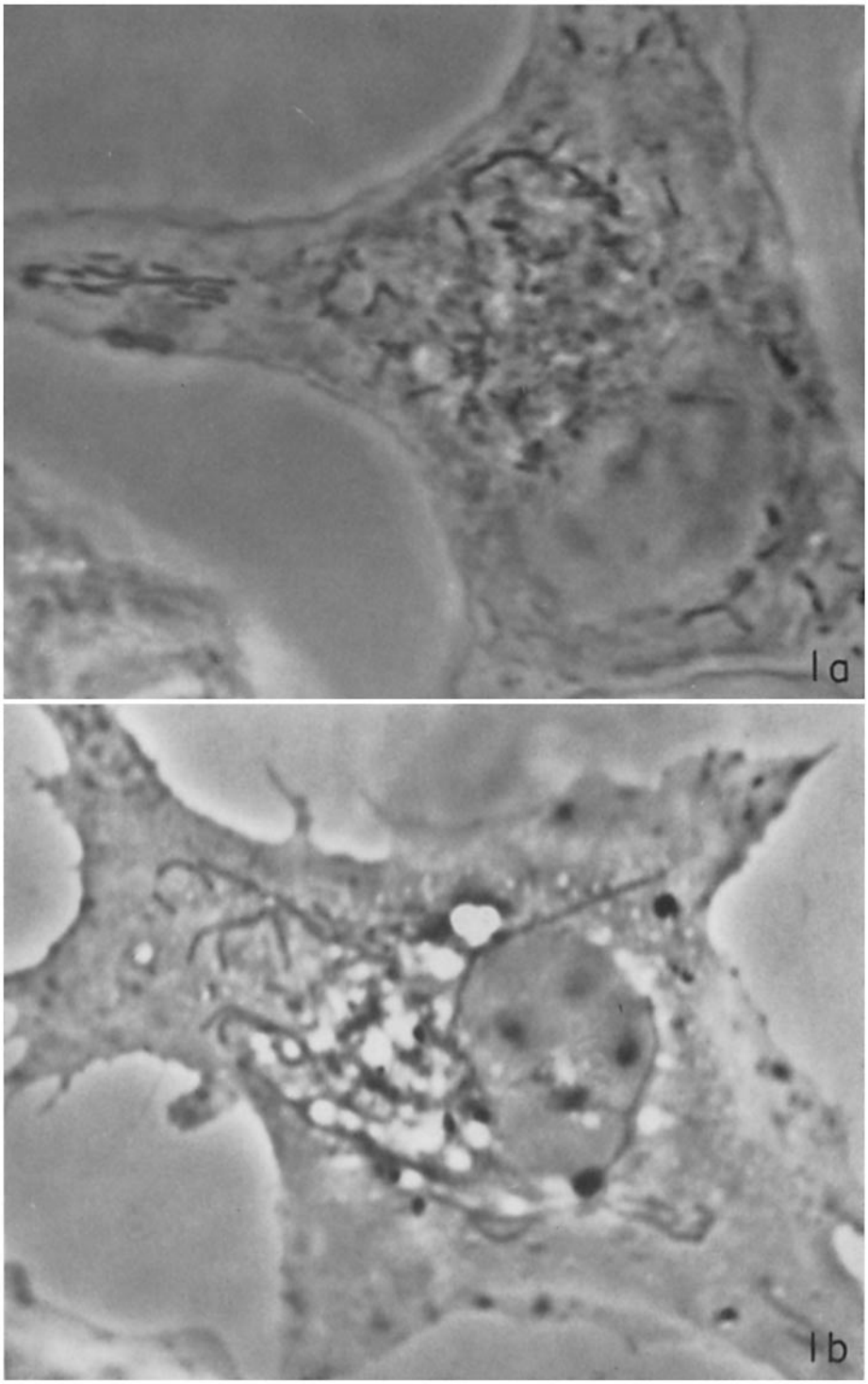
EXPLANATION OF PLATES

PLATE 22

FIGS. 1 *a* and 1 *b*. Cells cultivated in presence of 250 μg/ml DL-*p*-fluorophenylalanine for 72 hours.

FIG. 1 *a*. Mitochondria are present but there is an absence of large, phase-dense granules in the centrosphere region. Fixed with 1 per cent OsO_4, phase contrast. × 2500.

FIG. 1 *b*. Acid phosphatase–stained cell. Small amounts of black reaction product scattered between large, clear vesicles. The clear vesicles are pinocytic in origin. Phase contrast. × 2500.



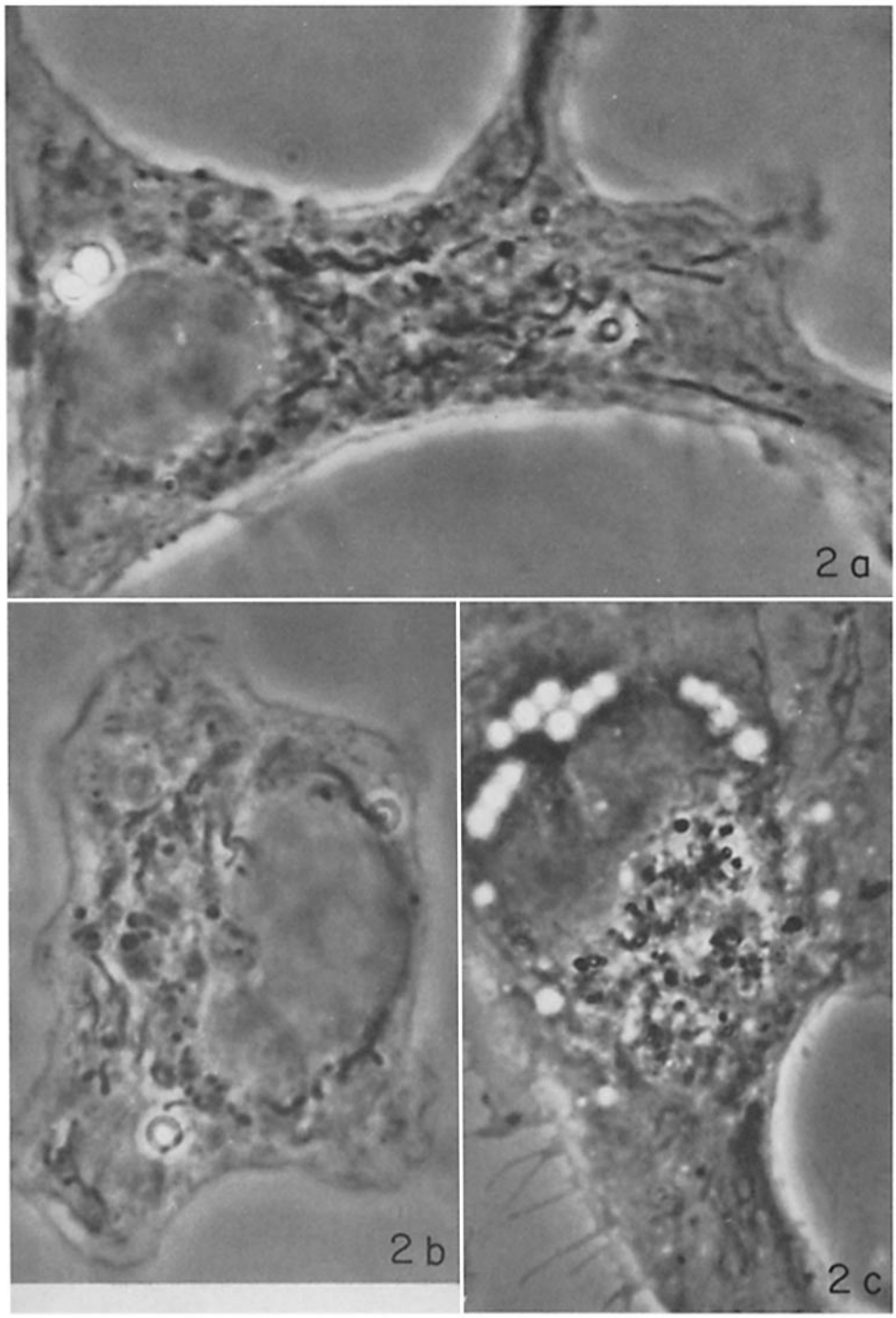
(Cohn and Benson: Macrophage differentiation)

PLATE 23

FIGS. 2 *a* to 2 *c*. Cells cultivated in presence of 0.4 μg/ml of puromycin for 72 hours.

FIGS. 2 *a* and 2 *b*. 1 per cent OsO_4-fixed cells. Cell diameter is considerably smaller than controls. A few dense or empty appearing granules are scattered throughout the cytoplasm. Phase contrast. × 2500.

FIG. 2 *c*. Acid phosphatase–stained cell. Small numbers of reactive granules in centrosphere area. This cell represents the upper limit of staining observed in these preparations. × 2500.

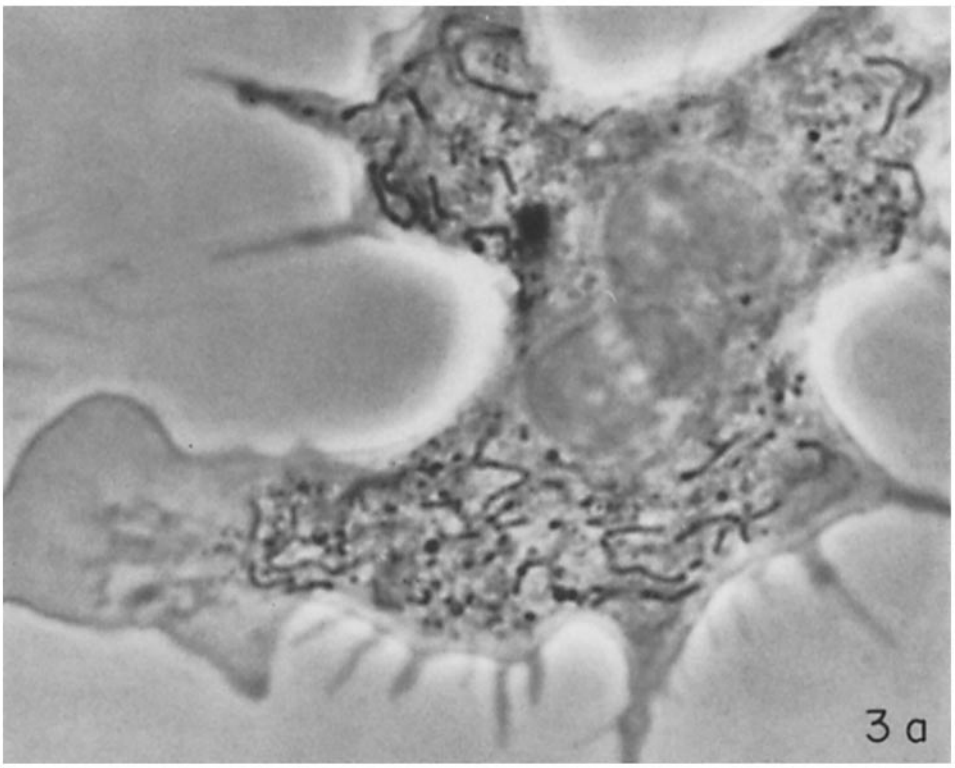


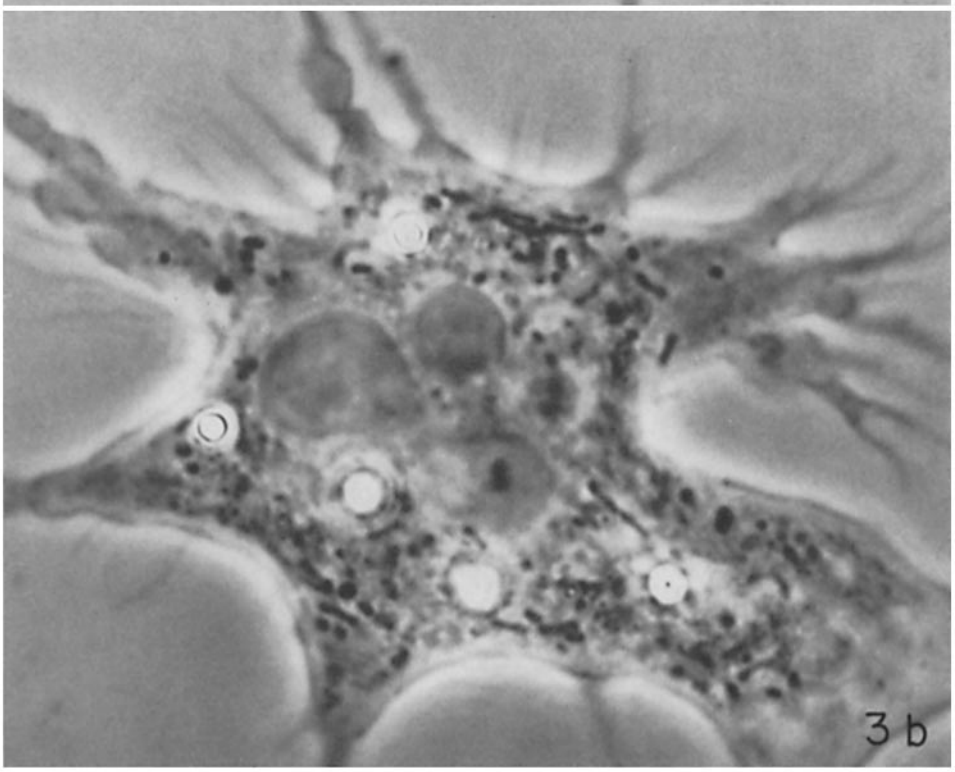
(Cohn and Benson: Macrophage differentiation)

PLATE 24

FIGS. 3 *a* and 3 *b*. Cells cultivated in presence of 0.05 μg/ml of colchicine for 72 hours. Many dense rod-like mitochondria apparent. Lipid accumulation less than normal at this time period. No well defined centrosphere but a scattering of small dense granules in the cytoplasm. Surface of the cell shows many small projections with bulbous formations of the larger pseudopods. × 2500.

FIG. 3 *b*. Fixed in 1 per cent OsO_4, phase contrast.

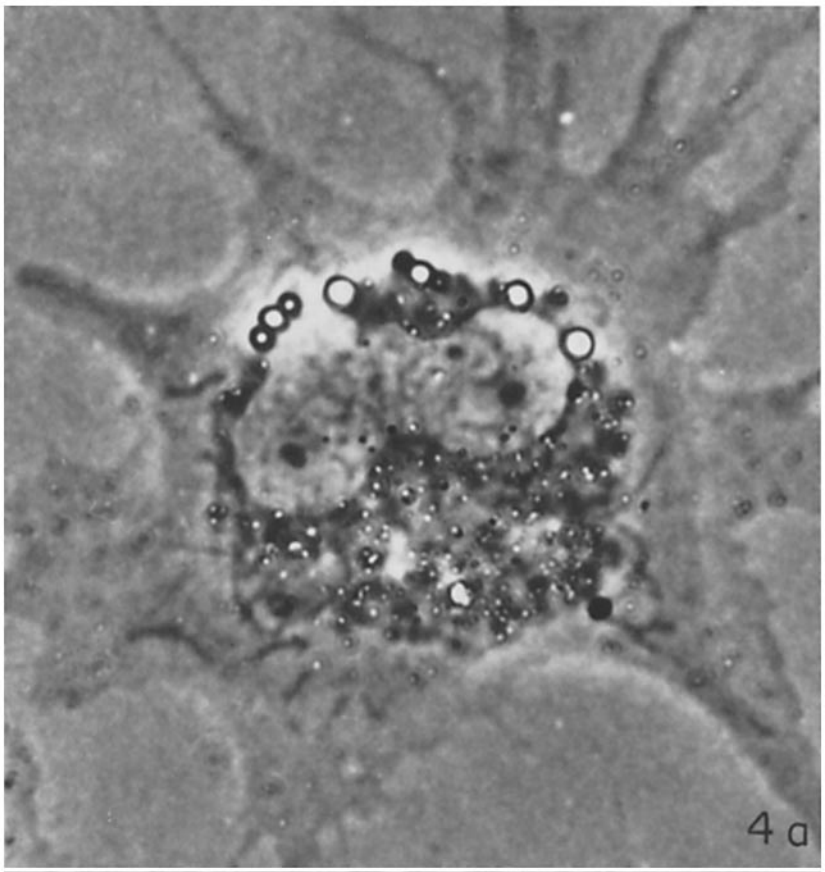


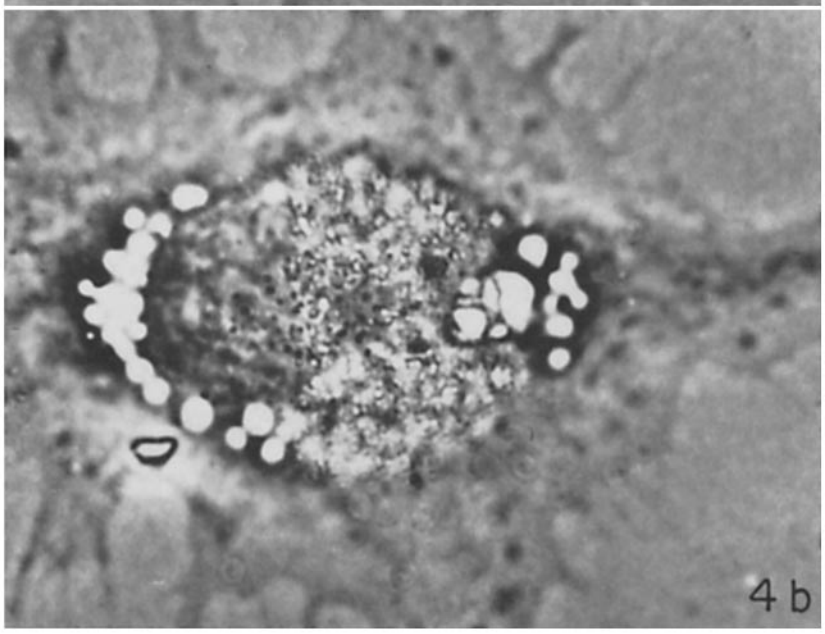


(Cohn and Benson: Macrophage differentiation)

PLATE 25

FIGS. 4 *a* and 4 *b*. Autoradiographs of cells cultivated for 48 hours *in vitro*. Cell in Fig. 4 *a* exposed to 10 μc/ml of tritiated leucine for 3 minutes whereas cell in Fig. 4 *b* exposed for 60 minutes. Grains appear as light or dark uniform spherules primarily located in the centrosphere region. Dense granules are poorly defined. Highly refractile lipid droplets are present and appear non-radioactive. × 2500.

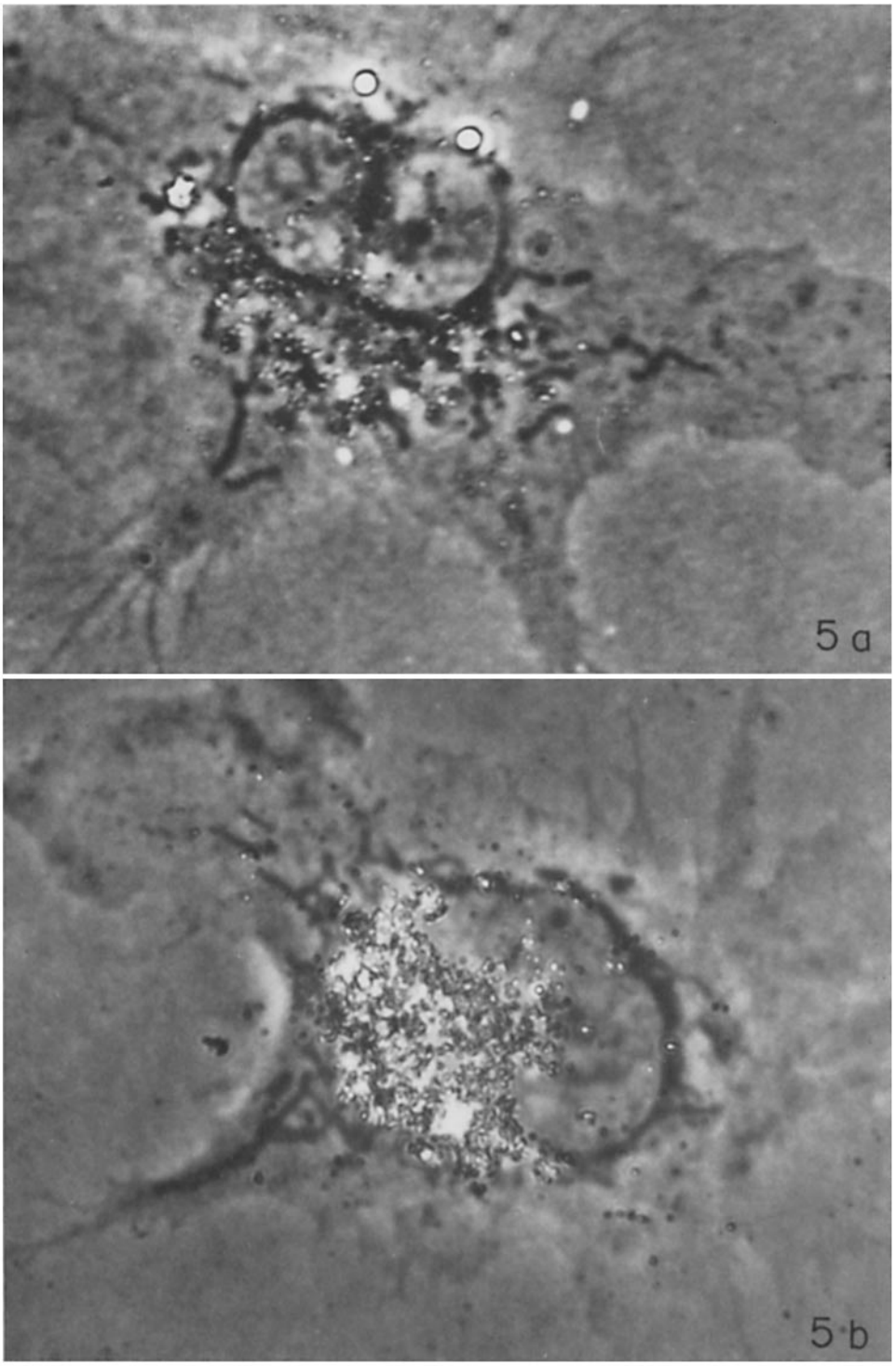




(Cohn and Benson: Macrophage differentiation)

PLATE 26

FIGS. 5 *a* and 5 *b*. Autoradiographs of cells cultivated for 48 hours *in vitro*. Cell in Fig. 5 *a* exposed to tritiated choline for 3 minutes whereas cell in Fig. 5 *b* has received a 60 minute pulse. Both cells exhibit localization of grains to the perinuclear granule region. Random grains over peripheral cytoplasm without apparent localization to mitochondria. Lipid droplets are not labeled with choline. × 2500.



(Cohn and Benson: Macrophage differentiation)