THE REGULATION OF PINOCYTOSIS IN MOUSE MACROPHAGES

IV. The Immunological Induction of Pinocytic Vesicles, Secondary Lysosomes, and Hydrolytic Enzymes*

By ZANVIL A. COHN, M.D., and EILEEN PARKS

(From the Rockefeller University, New York)

Plates 105–108

(Received for publication 14 February 1967)

During the course of studies on the induction of pinocytosis, it was noted that anionic species were more active stimulators than either neutral or cationic agents (1). This work was initiated by the presence in newborn calf serum (NBCS) of potent inducers, the nature of which were then unknown. Under the conditions of the assay procedure, certain components of NBCS, i.e. albumin and fetuin, proved to be good inducers of vesicle formation whereas bovine gamma globulin, obtained as fraction II, yielded a modest response and then only at high concentrations. These results were interpreted to indicate that the γ-globulins present in fraction II probably played little role in the vesicle formation induced by NBCS.

Subsequent experiments, however, indicated that NBCS as well as sera from adult cattle contained a specific γ-globulin which was an extremely potent initiator of pinocytosis. This article will describe properties of this material as well as the influence of the isolated γ-globulin on the pinocytic activity, morphology, and lysosomal enzyme production of cultivated mouse macrophages.

Materials and Methods

Cultivation of Mouse Macrophages.—

Leighton tube cultures: The peritoneal contents of unstimulated female NCS mice were obtained after a lavage with medium No. 199. The details of the method are given in previous publications (2). After incubation in a medium containing various concentrations of serum and medium 199, the cells were fixed with 1.25% glutaraldehyde and examined with phase-contrast optics.

Mass cultures for biochemical analysis: The method for cultivation in T flasks has been described previously (3). After incubation at 37°C the medium was removed, the cell sheet washed four times with 10 ml warm saline, drained, and 3 ml of saline added to the flasks. The contents of the flask were then frozen and thawed six times, harvested, and maintained at −20°C for assay. Analyses for protein, acid phosphatase, β-glucuronidase, and cathepsin were then performed by methods already described (3).

* This investigation was supported in part by Research Grants AI 01831-08 and AI 07012-01, United States Public Health Service, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md.

Evaluation of Pinocytic Activity—The peripheral pinocytic vesicles of glutaraldehyde-fixed cover slip preparations were enumerated with oil immersion phase-contrast microscopy at a magnification of ×1000. The details of this method have been presented in a previous article (2). Cells were exposed to various serum-containing media for 120 min prior to fixation.

Hemagglutination Titrations.—Mouse blood was harvested from the axillary fold into heparinized Pasteur pipettes. The pooled blood was then centrifuged at 2000 rpm (type 2 centrifuge, International Equipment Co., Needham Heights, Mass.), the supernatant plasma decanted, and the packed erythrocytes washed four times with 10 volumes of isotonic sodium chloride. They were then resuspended in phosphate-buffered isotonic saline (pH 7.5) to a concentration of 5%. 2-fold dilutions of serum or other plasma fractions were prepared in plastic depression trays using saline as the diluent. Washed erythrocytes were then added to a final concentration of 0.5% and incubated for 1 hr at 37°C and overnight at 4°C. The last dilution macroscopic hemagglutination was taken as the end point.

Studies on Bovine Serum.—Samples of fetal, precolostral, and postcolostral newborn calf serum were obtained from Microbiological Associates, Bethesda, Md.; Grand Island Biological Co., Grand Island, N. Y., and Colorado Serum Co., Denver, Colo. Samples of fresh, adult bovine serum were obtained from Hereford cattle maintained at The Rockefeller University and bled by jugular puncture. We are indebted to Dr. George Padgett for procuring the adult bovine blood.

Separation of γ-Globulin by Na_2SO_4 and $(NH_4)_2SO_4$ Fractionation.—Fresh bovine serum was fractionated with Na_2SO_4 by the general procedure described by Thurston et al. (4). The precipitation with 18% Na_2SO_4 was repeated four times and the material dissolved in saline. It was then dialyzed exhaustively against buffered saline (pH 7.5) at 4°C, concentrated to a known volume by ultrafiltration, and sterilized by means of a millipore filter, pore size 0.45 μ. The $(NH_4)_2SO_4$ procedure of Kendall (5) was also used and gave a similar result. Subsequent discussion of bovine γ-globulin will refer to the salt-fractionated product.

Zone Electrophoresis of Bovine Serum.—This was conducted by a modification of the techniques of Kunkel and Slater (6). 1 ml of serum was placed on a Pevikon block and electrophoresed in 0.1 μ barbital buffer, pH 8.6, for 18 hr at 250 v, 50 ma, and at a temperature of 4°C. 1-cm strips were cut and serum fractions were filtered through a coarse glass filter with 3.0 ml of saline. Protein and hemagglutination assays were performed on the eluates.

Sucrose Gradient Centrifugation of Bovine Serum and Bovine γ-Globulin.—Continuous sucrose gradients were constructed with a Britten machine (7), between 10 and 40% (w/w) sucrose. 0.5 ml of concentrated bovine serum or γ-globulin was layered on the gradient and centrifuged at 35,000 rpm in the SW 39 rotor for 18 hr at 4°C. 0.5-ml fractions were collected through a basal pin hole into calibrated tubes. The fractions were dialyzed against saline and assayed for mouse RBC–hemagglutinating activity and for pinocytic-inducing properties when applied to Leighton tube cultures of mouse macrophages.

Sephadex Gel Filtration.—We are indebted to Mrs. Barbara Ehrenreich of The Rockefeller University for the preparation of a calibrated Sephadex G-200 column. 1 ml of a Na_2SO_4-fractionated preparation of bovine γ-globulin containing 10 mg of protein was employed. This was loaded on a 48 × 1.5 cm column and eluted with 0.05 M phosphate saline (pH 7.7). Fractions were assayed for hemagglutinating and pinocytosis-inducing activities.

Treatment of Sera with 2-Mercaptoethanol.—2-Mercaptoethanol was added to a final concentration of 0.1 M. The mixture was incubated at 25°C for 2 hr and then dialyzed overnight at 4°C. Samples of sera which were incubated in the absence of mercaptoethanol and dialyzed served as controls. Treated and untreated sera were then assayed for both mouse-hemagglutinating activity and pinocytosis-stimulating properties.

Preparation of Rabbit Antimouse Erythrocyte Antiserum.—Rabbits weighing 3 kg were injected with 1.0 ml of 50% mouse erythrocytes which had been washed five times with large

volumes of saline. Injections were given via the lateral ear vein on 2 successive days and the animals bled 8 days after the first injection.

Absorbed Serum Preparations.—Aged postcolostral newborn, and fetal calf serum were employed. Mouse or bovine erythrocytes were washed five times with phosphate-buffered saline (pH 7.5) and packed at a speed of 2000 rpm for 20 min. 1 volume of serum was added to 0.75 volume of packed erythrocytes and incubated at 25°C for 60 min with occasional agitation. The red cells were sedimented by centrifugation and the procedure repeated once more. The absorbtion with bovine erythrocytes served as a dilution control.

Newborn calf serum was also absorbed with packed mouse spleen cells. Mouse spleens were removed by sterile procedure, minced with a razor blade, and separated by the method of Blinkoff (8). The cell suspension was washed twice with medium 199 and packed by centrifugation. Two absorptions with equal volumes of packed spleen cells were employed.

Cytotoxicity Assays.—Fresh, frozen serum was employed. Mouse peritoneal cells were cultivated on cover slips in 50% NBCS medium from 1 to 72 hr. After appropriate periods of

TABLE I

Mouse Erythrocyte-Agglutinating Activity of Bovine Serum and Serum Fractions

Material	Reciprocal of HA titer*
Fetal calf serum	0
Precolostral NBCS	2–4
Postcolostral NBCS	8–16
Adult bovine serum	32–128
Bovine γ-globulin‡	16–64
Bovine fraction II 1–50 mg/ml	0
Fetuin 1–50 mg/ml	0

* Aged sera, range of four samples.

‡ Sodium sulfate–fractionated and taken to original serum volume.

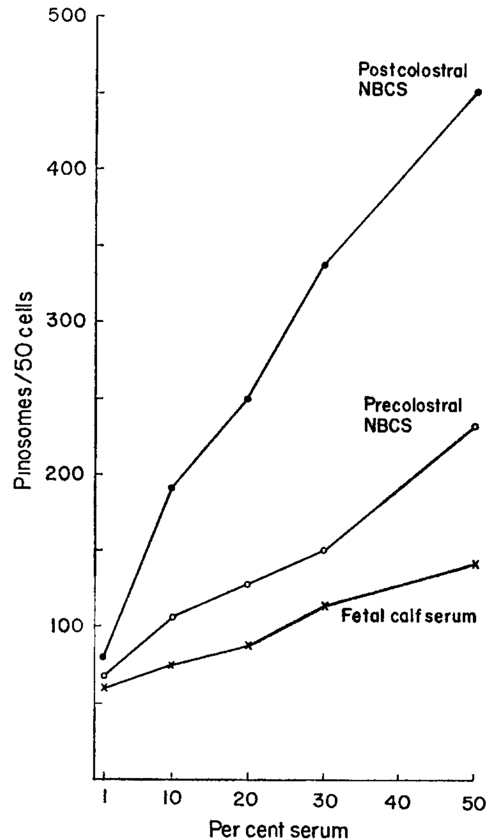
incubation, the medium was removed and the cell sheet washed twice with 3.0 ml of warm medium 199. Dilutions of fresh serum in medium 199 were then added and the cells incubated at 37°C for 5 min to 2 hr. The macrophages were then fixed in glutaraldehyde and examined with phase-contrast optics. 200 cells were enumerated and the percentage of lysed and intact macrophages calculated.

RESULTS

General Considerations.—During the course of investigations on the adherence and phagocytosis of erythrocytes by mouse peritoneal macrophages, Dr. Michel Rabinovitch of this laboratory discovered that commercial preparation of newborn calf serum contained a hemagglutinin which agglutinated mouse erythrocytes. This finding prompted us to study this reaction more carefully since it seemed possible that an agglutinin for erythrocytes might also interact with and have an influence on the surface properties of mouse macrophages.

The Hemagglutination and Hemolysis of Mouse Erythrocytes.—Table I illustrates the range of hemagglutinating titers of bovine serum obtained at different

ages as well as selected serum fractions. It was clear that hemagglutinating activity was present and that the titer increased with the age of the animal. No hemagglutinating activity was found in fetal serum and only low titers in precolostral newborn calf serum. Shortly after the neonatal period, these titers rose sharply and at 1 yr were at maximum levels.

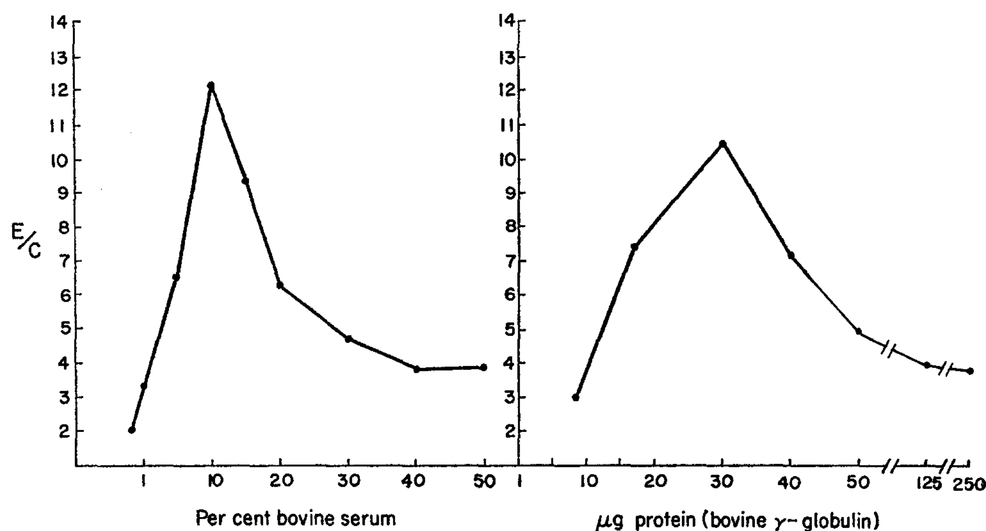


TEXT-FIG. 1. The pinocytic response of macrophages to fetal and newborn calf serum.

The major portion of the hemagglutinin could be found in a salt-prepared γ-globulin fraction. In marked contrast, commerical preparations of adult bovine γ-globulin (fraction II) prepared by ethanol fractionation were devoid of hemagglutinating activity. In addition, the α-globulin fetuin did not have any detectable hemagglutinating activity.

When fresh bovine sera were employed, hemolysis of mouse erythrocytes occurred. With adult sera the hemolytic titer ranged from 1/8–1/20. Lower titers were obtained when fresh newborn calf sera were employed and none was seen

with fresh fetal calf serum. These data suggested an antibody- and complement-dependent hemolytic reaction. The hemolytic activity of fresh adult bovine serum was only partially destroyed at 56°C/30 min. When higher temperatures were used (60–65°C/30 min) hemolytic activity was lost but there was a significant reduction in hemagglutinating titer (±4-fold) as well. Subsequent studies revealed that hemolytic activity could be preferentially destroyed by storage of sterile serum at room temperature for 1½–2 wk and for longer times at 4°C. Such aged sera retained full hemagglutinating activity and were used for subsequent studies on cultured macrophages. These data suggested the



TEXT-FIG. 2. The pinocytic response to adult bovine serum and bovine γ-globulin.

presence of a "natural" mouse erythrocyte–agglutinating γ-globulin, developing in the neonatal period, which was heat-sensitive and absent from bovine fraction II.

The Influence of Bovine Sera on the Pinocytic Activity of Mouse Macrophages.— Prior studies had described the stimulating effect of newborn calf serum on the pinocytic activity of mouse macrophages (9). In view of the different levels of mouse erythrocyte agglutinins present in bovine sera, this parameter of endocytic function was reinvestigated. Text-fig. 1 illustrates the influence of various concentrations of fetal as well as pre- and postcolostral NBCS on pinosome formation. The same sera employed in Table I were utilized. Postcolostral NBCS, which corresponds to the usual commercial preparation employed in the past, evoked vigorous vesicle formation. A curvilinear response obtained at concentrations from 1 to 50%. Precolostral NBCS which had a mouse erythrocyte titer of 1/4 produced a much less vigorous pinocytic response. It was of

interest that modest numbers of vesicles appeared at a concentration of 50% serum but then fell off rapidly as the serum level was lowered.

Fetal calf serum produced only small numbers of pinocytic vesicles and the dose-response curve was quite flat.

The left portion of Text-fig. 2 shows the results of a titration performed with aged, adult bovine serum. The data are presented somewhat differently as the ratio of pinosomes per 50 cells in the experimental tube (E) to a control (C) which contained 1% NBCS. It is quite apparent that dose-response relationships are markedly different than with the previously employed sera.

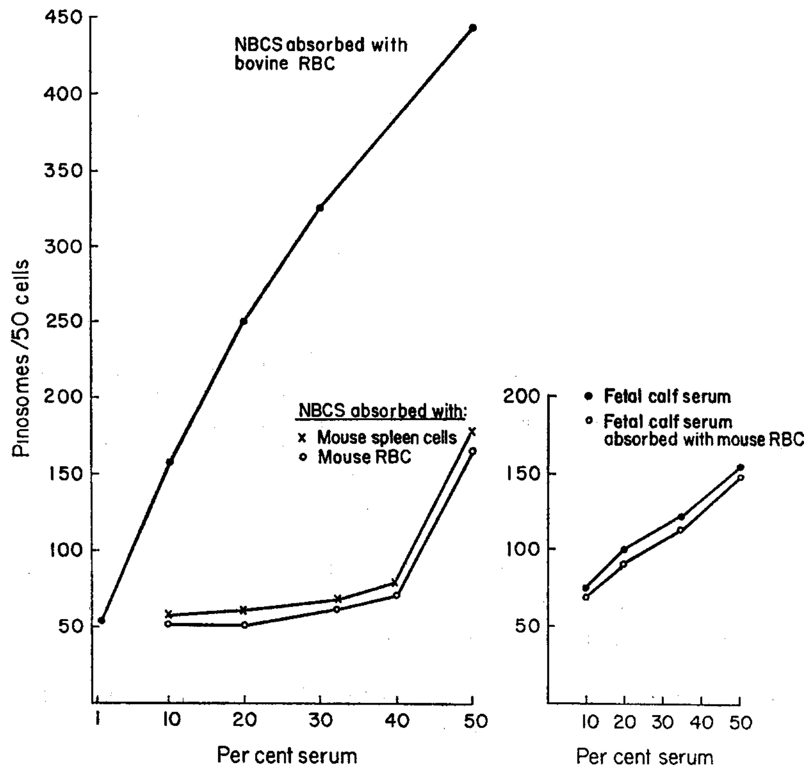
With the adult sera a striking prozone effect was observed with many fewer vesicles at higher serum concentration. As the level was reduced to approximately 20%, pinosome formation occurred and reached a peak at 10% serum. Thereafter, a linear reduction in pinocytic vesicle occurred. In addition, the maximum level of vesicles was greater than with sera from younger animals. Examination of macrophages exposed to 30–50% adult bovine serum revealed extreme ruffling of the limiting membrane with relatively little formation of vesicles.

The right portion of Text-fig. 2 illustrates the results obtained with a γ-globulin preparation prepared from adult bovine serum and adjusted to the same mouse red cell agglutination titer (64–128) as the serum sample used in the left portion of the figure. The shape of the pinocytosis dose-response curve is very similar to that obtained with complete adult bovine serum, with a pronounced prozone effect. It was of interest that small amounts of this γ-globulin preparation were capable of inducing high levels of vesicle formation. A peak response was reached at 30 μg of protein per milliliter and a demonstrable effect was still present at 10 μg/ml. This is particularly striking in view of the probable heterogeneity of the preparation and in terms of previous studies in which much larger quantities of other proteins were necessary. On a weight basis the salt-fractionated product was at least 1000 times more active than bovine fraction II.

The Absorbtion of the Hemagglutinating and Pinocytosis-Stimulating Properties of Bovine Serum.—In view of the close correlation between the mouse erythrocyte agglutinating and pinocytosis-inducing properties of various bovine sera, additional studies were conducted to examine the specificity of the reaction. For this purpose NBCS and fetal calf serum were absorbed with mouse erythrocytes and spleen cells. The control sera for these studies were preparations absorbed with washed autologous bovine erythrocytes. Samples were then tested for both hemagglutinating and pinocytosis-stimulating activities.

Absorbtion of postcolostral NBCS with either mouse erythrocytes or spleen cells removed all detectable mouse erythrocyte-agglutinating activity. Absorbtion with bovine erythrocytes did not alter the titer of mouse RBC agglutinins. The absence of agglutinins in fetal calf serum precluded similar studies with this specimen.

The effect of absorbtion on pinocytic vesicle formation is shown in the titrations of Text-fig. 3. On the left are the results with newborn calf serum. Unabsorbed serum promoted vigorous vesicle formation with the usual dose-response relationship. In contrast, sera absorbed with mouse erythrocytes or spleen cells exhibited much less activity and a markedly different dose-response curve. A modest number of vesicles were formed at 50% serum levels, and a prompt loss



TEXT-FIG. 3. The effect of absorbtion on the pinosome-inducing properties of newborn and fetal calf serum.

of activity occurred at levels of 40% and under. Under these conditions, therefore, there was a concomitant loss of both red cell–agglutinating and pinocytosis-stimulating activity of the sera.

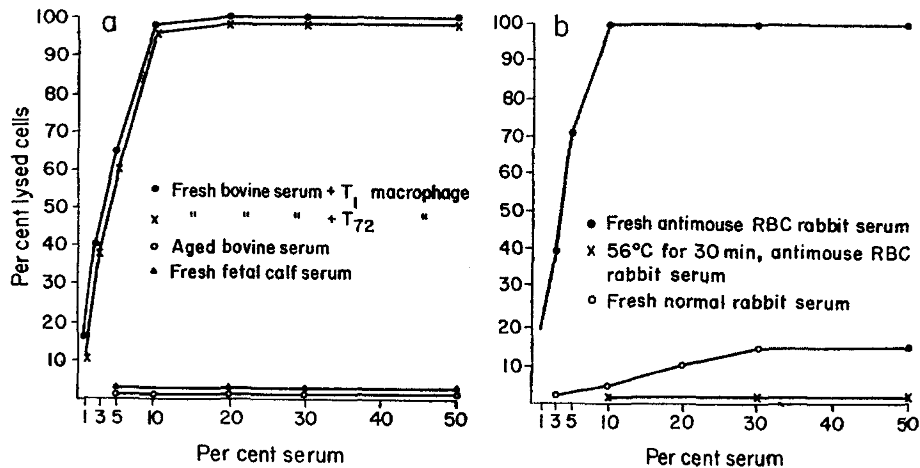
Studies with fetal calf serum are shown on the right side of Text-fig. 3. It is apparent that absorbtion had no effect on the inducing properties of fetal serum. The absence of detectable hemagglutinating activity and the ineffectiveness of absorbtion indicated that the pinocytosis-stimulating factors of fetal serum were different from those of NBCS.

These data suggested the presence of an antibody in bovine serum directed

against antigens common to the surface of mouse erythrocytes and macrophages.

Cytotoxicity of Fresh Sera for Mouse Macrophages.—The prior studies indicated that fresh, adult bovine sera were capable of hemolyzing mouse erythrocytes. This suggested that, if an antibody in bovine serum were interacting with the macrophage surface, a cytolytic effect might result in the presence of fresh serum or complement.

The results of such experiments are shown in Text-fig. 4. When fresh bovine serum was added to mouse macrophages (Text-fig. 4 *a*) there was a rapid cytolytic effect. This involved essentially all cells in the culture and reached completion 30 min after the addition of fresh serum. In contrast, fresh fetal serum



TEXT-FIG. 4. The cytotoxicity of fresh sera for mouse macrophages.

had no cytotoxic effect. Cells cultivated for 1 and 72 hr (T_1 and T_{72}) in vitro exhibited the same susceptibility to dilutions of serum. No cytotoxic effect was noted with aged sera which had lost its properties to lyse mouse erythrocytes.

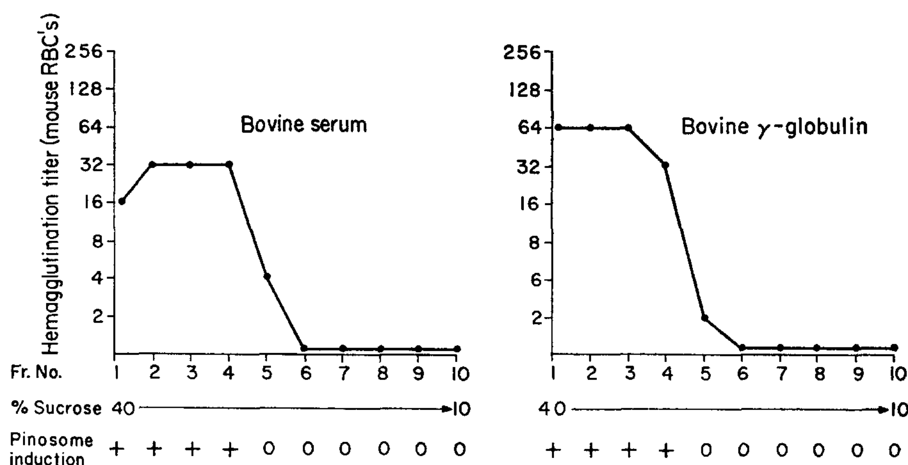
The influence of normal and immune rabbit sera are presented in Text-fig. 4 *b*. Certain normal rabbit sera with low mouse hemagglutin titers had a slight cytolytic effect on mouse macrophages. Sera from rabbits immunized with mouse erythrocytes and which had a mouse RBC agglutination titer of 1/256 were able to lyse cultured mouse macrophages. This property was destroyed by heating the sera to 56°C for 30 min.

Studies on the Globulin from Adult Bovine Sera.—

Zone electrophoresis: Samples of fresh sera and salt-fractionated γ-globulin were separated by electrophoresis on Pevikon. In each case, the mouse-hemagglutinating activity corresponded to a protein peak which was found between 0 to 6 cm from the origin and corresponded to a "slow" globulin. The fractions were toxic for macrophages and could not be tested for pinosome induction.

Treatment with 2-mercaptoethanol: Exposure of postcolostral NBCS, adult bovine serum, and the γ-globulin fraction from adult serum to 0.1 M 2-mercaptoethanol resulted in the complete loss of mouse-hemagglutinating activity. Experiments with dialyzed samples, at 50% serum concentrations, revealed that the pinocytosis-inducing properties were also destroyed. Mercaptoethanol-treated NBCS gave values of 50–93 pinosomes/50 cells, a value similar to the absorbed serum preparations and to 1% untreated NBCS.

Ultracentrifugation of adult bovine sera and γ-globulin: Preparations of serum and salt-fractionated γ-globulin were centrifuged in continuous sucrose gradients. The results of these studies are shown in Text-fig. 5. In both cases, the



TEXT-FIG. 5. The behavior of the hemagglutinating and pinosome-inducing activities of bovine serum and γ-globulin in continuous sucrose gradients.

mouse erythrocyte–agglutinating activity and pinocytosis-inducing properties were found in the lower portion of the tube and corresponded to the position of a macroglobulin.

Gel filtration: Fractions were collected from the G-200 column and assayed. Both hemagglutinating and pinocytosis-inducing activities were found in a sharp peak which corresponded to the void volume and were therefore excluded by the gel. No activity was found in later fractions. This again suggested that the hemagglutinating and pinosome-inducing activity of bovine serum were the property of a macroglobulin.

The Morphology of Macrophages Cultivated in Various Bovine Sera.—The cultivation of mouse mononuclear phagocytes in NBCS resulted in many structural changes including the formation of phase-dense, acid phosphatase–positive granules in the centrosphere region (9). The rate of formation, size, and number of granules increased with elevated NBCS concentrations, a finding correlated with increased pinosome formation. A typical cell grown in 50%

NBCS is illustrated in Fig. 1 *a*. When such sera were absorbed with mouse erythrocytes, a procedure which reduced pinosome formation, many fewer dense granules were present in the cell (Figs. 1 *b*, 1 *c*). Under these conditions, therefore, the reduction of pinocytic activity was reflected in the formation of smaller numbers of secondary lysosomes.

Fetal calf serum, which stimulated vesicle formation to a relatively small extent, was next employed. Cells cultivated in concentrations of 30–50% exhibited small numbers of phase-lucent pinosomes and dense granules (Fig. 2 *a*). The ability of fetal serum to support the formation of dense granules was strikingly increased by the addition of small quantities of γ-globulin isolated from adult bovine serum. These granules were positive for acid phosphatase as determined by the Gomori technique. The appearance of two cells cultured in 50% fetal serum plus γ-globulin is shown in Figs. 2 *b* and 2 *c*. These cells have the appearance of macrophages cultivated in 50% NBCS. An increase in secondary lysosomes occurred, therefore, upon the addition of this inducer of pinocytosis.

A similar relationship between pinocytosis and dense granule formation was studied with rabbit sera. Samples of normal rabbit sera, which contained little mouse erythrocyte agglutinin, supported the survival of mouse macrophages and lead to the formation of only small numbers of dense granules and hydrolytic enzymes (9). An example is shown in Fig. 3 *a*. In contrast, sera obtained from rabbits immunized with mouse erythrocytes produced active pinocytosis and resulted in dense granule formation (Fig. 3 *b*).

In the presence of fresh, adult bovine serum and antimouse erythrocyte rabbit serum, mouse macrophages were rapidly destroyed. Time course experiments revealed a typical series of cytolytic events.

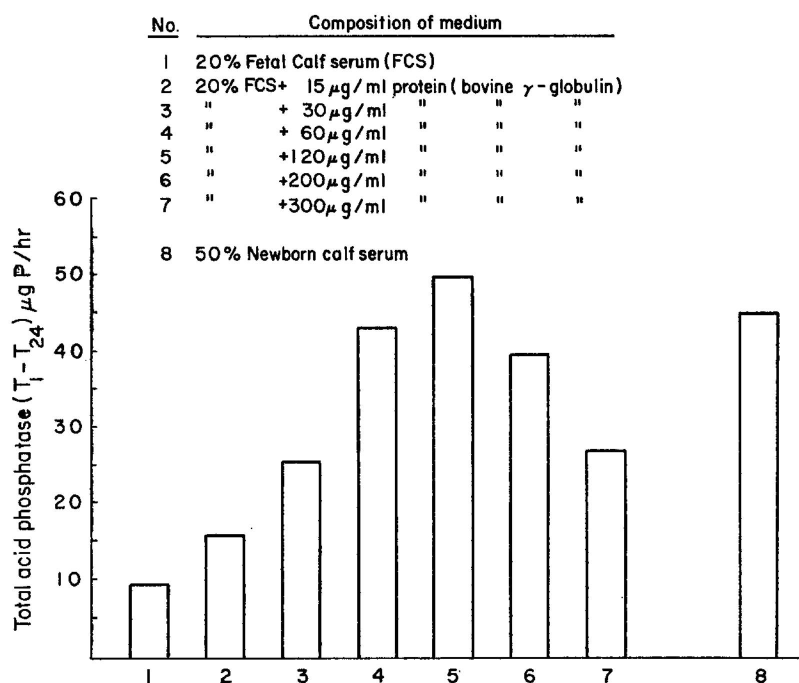
Cells were cultivated in 50% NBCS for 24 hr. The medium was removed and the cell sheet washed twice with 2.0 ml volumes of warm medium 199. A medium consisting of 20% fresh adult bovine serum in medium 199 (1.0 ml) was added, the tubes gassed with 5% CO_2-air, and incubated at 37°C. Individual cover slips were fixed with glutaraldehyde at 0, 5, 10, 15, 30, 45, and 60 min and examined under the phase microscope.

Morphological alterations were evident as early as 5 min. These consisted in a loss of phase density of the peripheral cytoplasm and granules and a shortening of mitochondria. By 10 min, dendritic projections were present on the cell surface and obvious swelling of the cell body had begun. At 15 minutes, approximately 50% of the cells were swollen, a process which also involved phase-lucent pinocytic vacuoles. Essentially all of the cells in the culture were involved by 30 min with obvious fragmentation of the limiting membrane, a reduction in nuclear diameter, and the pinching off of micronuclei. Certain of these changes are illustrated in Figs. 4 *a*–4 *c*.

The Influence of Bovine γ-Globulin in Stimulating the Production of Acid Hydrolases of Cells Cultured in Fetal Calf Serum.—The morphological studies had indicated that the addition of bovine γ-globulin to fetal calf serum resulted

in the formation of larger numbers of phase-dense granules. The influence of γ-globulin on the formation of acid hydrolases was next studied by quantitative biochemical techniques in mass cultures.

Mass cultures of mouse peritoneal phagocytes were prepared in T flasks. After a 1-hr pre-incubation period in 20% fetal calf serum, the unattached lymphocytes were removed by washing the monolayer. The medium was then replaced with 20% fetal calf serum–medium 199. Additions of salt-fractionated γ-globulin were made to this medium, the flasks gassed with 5% CO_2-Air and incubated for 24 hr at 37°C. The T flasks were harvested immediately



TEXT-FIG. 6. The stimulation of macrophage acid phosphatase by bovine γ-globulin.

after the 1-hr attachment and were assayed for the initial level of enzymes. The data is presented as the difference in activity between cells at 1 and 24 hr (T_1 and T_{24}). Control cultures contained either 20% fetal calf serum alone or 50% NBCS to give a maximum enzyme response. The preparation of γ-globulin was the same lot as used in Text-Fig. 2. Assay of this preparation for acid phosphatase yielded a value of 0.07 γP/mg of protein per hour.

The results of one such experiment are presented in Text-fig. 6. Cells cultured in 20% fetal calf serum produced only small quantities of acid phosphatase during the 24 hr of incubation. In the presence of added γ-globulin, there was a progressive increase in enzyme content with a maximal value reached at 120 μg of added protein per milliliter. The addition of larger amounts of γ-globulin resulted in a progressive decrease in the amount of acid phosphatase. The maximal values obtained with fetal calf serum plus γ-globulin

were slightly greater than with the 50% NBCS medium. When these data were calculated on a specific activity basis (μg P/mg of cell protein per hour) a similar response was noted. Preparations cultivated in 20% fetal calf serum with 120 μg/ml of γ-globulin had specific activities which were 3 to 4-fold higher than the 20% fetal calf serum control. The differences between total and specific activity were related to the fact that cells incubated with γ-globulin accumulated more protein.

Examination of the content of β-glucuronidase and cathepsin revealed a maximal stimulatory effect at 120 μg/ml of γ-globulin and resulted in total values which were 2.3- and 2.5-fold greater than the controls.

DISCUSSION

These studies suggest that the naturally occurring macroglobulin of bovine sera is directed against common surface antigens of both mouse erythrocytes and macrophages. In the absence of heat-labile factors, which are assumed to be complement, this antibody-like activity is expressed as hemagglutination, whereas in their presence the lysis of erythrocytes takes place. The action of the γM-like globulin on the macrophage also appears directed toward surface components. This interaction is observed as a stimulation of the limiting membrane and, depending upon the intensity of the stimulus, may result in vigorous vesicle formation and the flow of pinosomes to the perinuclear region. In the presence of heat-labile factors, a prompt disruption of the macrophage membrane occurred, which was similar to the influence of anticellular antibodies reported by other investigators (10, 11, 12). These phenomena suggest the binding of an interspecies macroglobulin to antigenic determinants on the mouse macrophage-limiting membrane.

The macrophage-directed macroglobulin in bovine serum appears to be a major determinant of pinocytosis under these in vitro conditions. This is based upon the relatively small numbers of pinosomes seen in cells exposed to antibody-deficient sera, i.e. fetal or specifically absorbed sera, and the reconstitution of pinosome induction by the addition of the globulin. Certainly many other serum components may play a role in the absence of the specific globulin, as illustrated by the prior studies with albumin, fetuin, and other macromolecules. However, this "immunological inducer," although quite different in charge from the macroanions, may function effectively by virtue of its greater affinity and binding to membrane receptors. In the presence of antibody-containing media, macrophages continually react with the macroglobulin and in the process appear to interiorize large amounts of limiting membrane. Presumably, as new membrane is formed, it undergoes a similar series of events, a process which may continue for a number of days. In view of the similar lysis curves obtained with freshly explanted and cultivated macrophages, the newly synthesized membrane may have similar antigenic determinants.

It is not known at this time whether other types of specific immunoglobulins have a related influence on pinocytosis. This seems likely in view of experiments

in which hyperimmune, rabbit antibovine serum albumin (γ-G) evoked a brief but intensive period of vesicle formation when applied to bovine serum albumin–coated macrophages. Another obscure point is the relationship of these in vitro findings to in vivo events. It is conceivable that macrophages coated either with antigens or antibodies may react with their respective counterparts. This could lead either to enhanced endocytic activity or, in the presence of a complement fixing system, to the destruction of the cell. In any event, further studies are required to evaluate the role of humoral factors in enhancing the cellular activities involved in immunological reactions and the uptake of soluble macromolecules.

These studies also reemphasize the relationship between pinocytosis, the subsequent formation of dense granules, and the accumulation of acid hydrolases. This was illustrated by the good correlation between the ability of sera to initiate vesicle formation in short-term assays and their effectiveness in producing dense granules in long-term cultures. Furthermore, the addition of small amounts of macroglobulin to cells maintained in antibody-deficient serum resulted in as much as a 5-fold increase in the cellular content of acid phosphatase. A definite prozone effect was again noted, and the shape of the acid phosphatase dose-response titration was similar to that observed by the enumeration of pinocytic vesicles (see Text-fig. 2). In addition, the relative amounts of the three hydrolases evoked by globulin were similar to those seen with complete 50% NBCS. Other inducers of pinocytosis also stimulate hydrolytic enzyme production by mouse macrophages and will be reported in subsequent communications.

SUMMARY

Bovine sera contain factors which are capable of agglutinating mouse erythrocytes and stimulating the pinocytic activity of cultivated mouse macrophages. The hemagglutinating and vesicle-inducing activities of sera increase with the age of the animal and are absent in fetal calf serum.

The majority of this material is recovered in globulin fractions prepared with Na_2SO_4–$(NH_4)_2SO_4$ and is absent in bovine fraction II. It behaves as a macroglobulin in studies employing zone electrophoresis, Sephadex G-200 filtration, sucrose density gradient centrifugation, and in its sensitivity to 2-mercaptoethanol and heat.

Absorption of bovine sera with either mouse erythrocytes or spleen cells removes the hemagglutinating and pinosome-inducing properties of the sera.

The addition of small quantities of bovine macroglobulin to mouse macrophages results in a stimulation of pinocytic activity, phase-dense granule formation and the cellular content of three acid hydrolases.

In the presence of heat-labile factors, the macroglobulin initiates the hemolysis of mouse erythrocytes and the cytolysis of mouse macrophages.

This material is thought to represent an interspecies γM-type antibody di-

rected against common antigenic determinants on the mouse erythrocyte and macrophage surface.

BIBLIOGRAPHY

1. Cohn, Z. A., and E. Parks, 1967. The regulation of pinocytosis in mouse macrophages. II. Factors inducing vesicle formation. *J. Exptl. Med.* **125:**213.
2. Cohn, Z. A. 1966. The regulation of pinocytosis in mouse macrophages. I. Metabolic requirements as defined by the use of inhibitors. *J. Exptl. Med.* **124:**557.
3. Cohn, Z. A., and B. Benson. 1965. The differentiation of mononuclear phagocytes. Morphology, cytochemistry, and biochemistry. *J. Exptl. Med.* **121:**153.
4. Thurston, J. R., M. S. Rheins, and E. V. Buehler. 1957. A rapid method for recovering serologically active globulins by sodium sulfate precipitation. *J. Lab. Clin. Med.* **49:**647.
5. Kendall, F. E. 1937. Studies on serum proteins; identification of single serum globulin by immunological means. *J. Lab. Clin. Med.* **16:**921.
6. Kunkel, H. G., and R. J. Slater. 1952. Zone electrophoresis in a starch supporting medium. *Proc. Soc. Exptl. Biol. Med.* **80:**42.
7. Britten, R. J., and R. B. Roberts. 1960. High-resolution density gradient sedimentation analysis. *Science* **131:**32.
8. Blinkoff, R. C. 1966. γM and γG antibodies in mice: Dissociation of the normal immunoglobulin sequence. *J. Immunol.* **97:**736.
9. Cohn, Z. A., and B. Benson. 1965. The in vitro differentiation of mononuclear phagocytes. II. The influence of serum on granule formation, hydrolase production and pinocytosis. *J. Exptl. Med.* **121:**835.
10. Goldberg, B., and H. Green. 1959. The cytotoxic action of immune gamma globulin and complement on Krebs ascites tumor cells. I. Ultrastructural studies. *J. Exptl. Med.* **112:**699.
11. Easton, J. M., B. Goldberg, and H. Green. 1962. Immune cytolysis: Electron microscopic localization of cellular antigens with ferritin-antibody conjugates. *J. Exptl. Med.* **115:**275.
12. Easton, J. M., B. Goldberg, and H. Green. 1962. Demonstration of surface antigens and pinocytosis in mammalian cells with ferritin-antibody conjugates. *J. Cell Biol.* **12:**437.

EXPLANATION OF PLATES

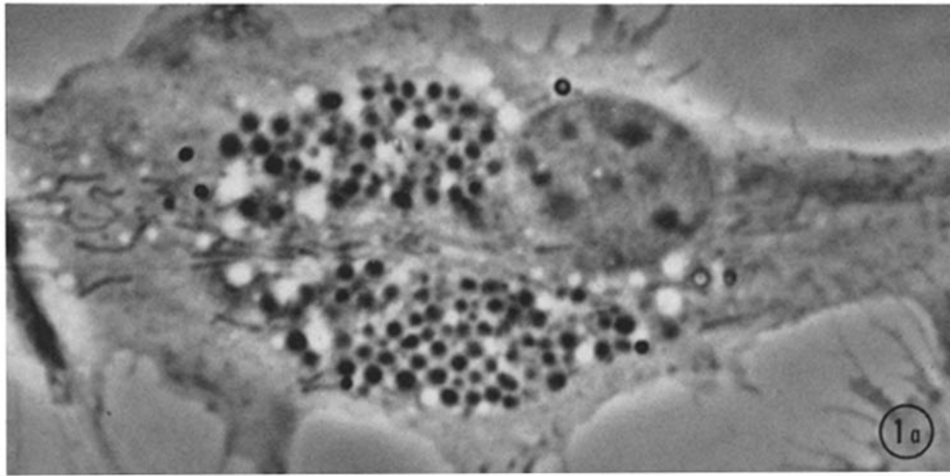
Plate 105

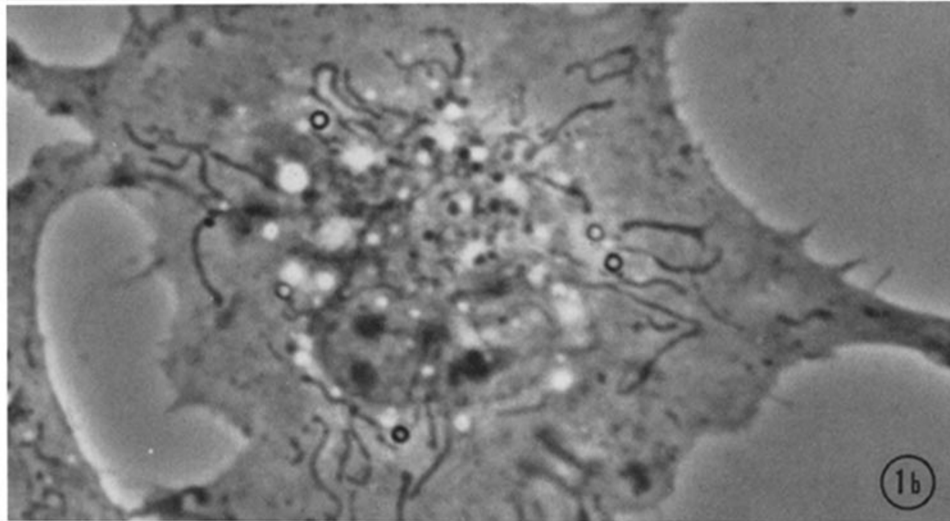
Figs. 1 *a*–1 *c*. Macrophages cultivated for 24 hr and fixed with glutaraldehyde. Phase-contrast. × 2700.

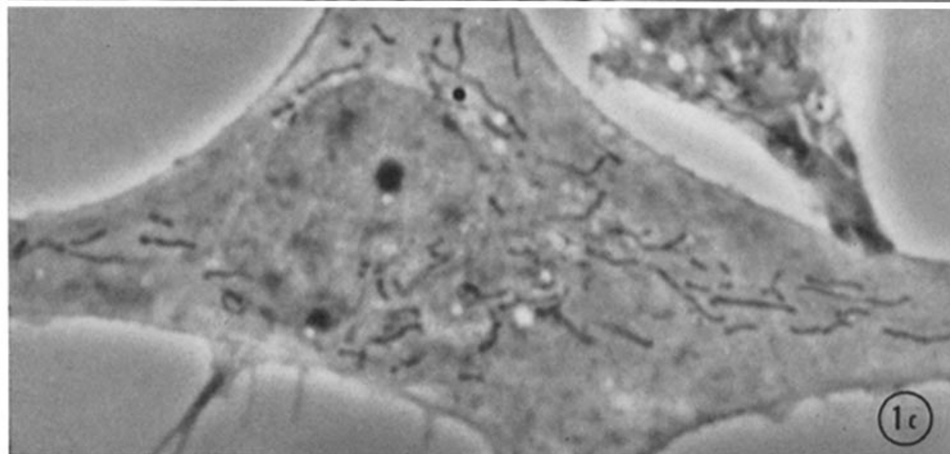
Fig. 1 *a*. A typical cell cultivated in 50% NBCS which exhibits a prominent centrosphere containing phase-dense granules. A number of phase-lucent pinosomes are seen on the left margin, amongst the mitochondria.

Fig. 1 *b*. A cell cultivated in 50% NBCS which was absorbed with mouse erythrocytes. The cell is well spread and contains numerous mitochondria. Only a few dense granules are present in the perinuclear region, and no peripheral pinosomes are present.

Fig. 1 *c*. A cell cultivated in 40% NBCS which was absorbed with mouse erythrocytes. Similar to cell in Fig. 1 *b* but with almost no apparent dense bodies.







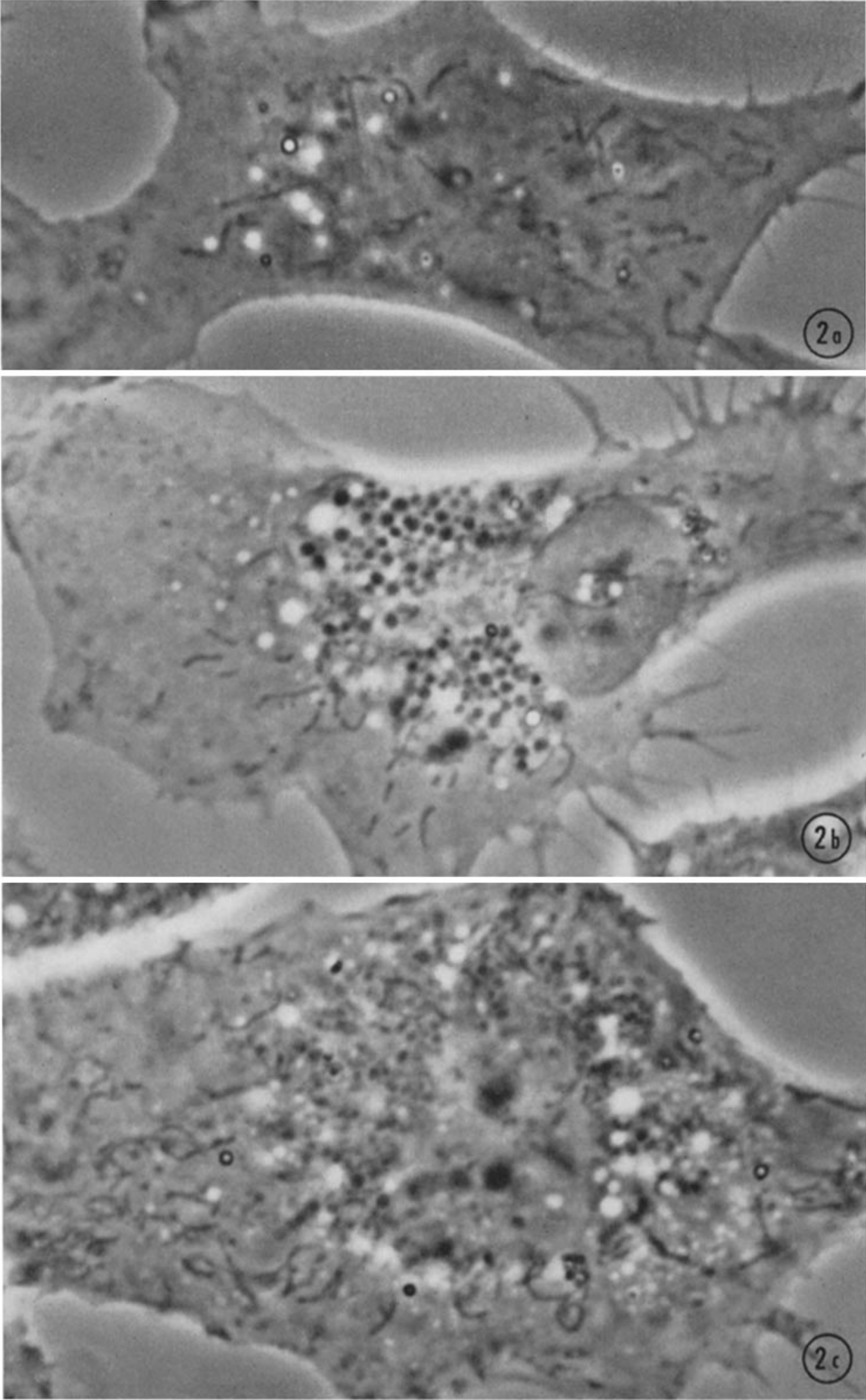
(Cohn and Parks: Pinocytosis)

PLATE 106

FIGS. 2 *a*–2 *c*. Macrophages cultivated for 24 hr and fixed with glutaraldehyde. Phase-contrast × 2700.

FIG. 2 *a*. A cell cultivated in 20% fetal calf serum. The cell is flat and well spread without ruffling of peripheral membranes and contains few dense bodies and pinosomes.

FIGS. 2 *b* and 2 *c*. Cells cultivated in 20% fetal calf serum in the presence of 80 μg/ml (protein) of bovine γ-globulin. Active pinocytosis and dense granule formation have occurred.



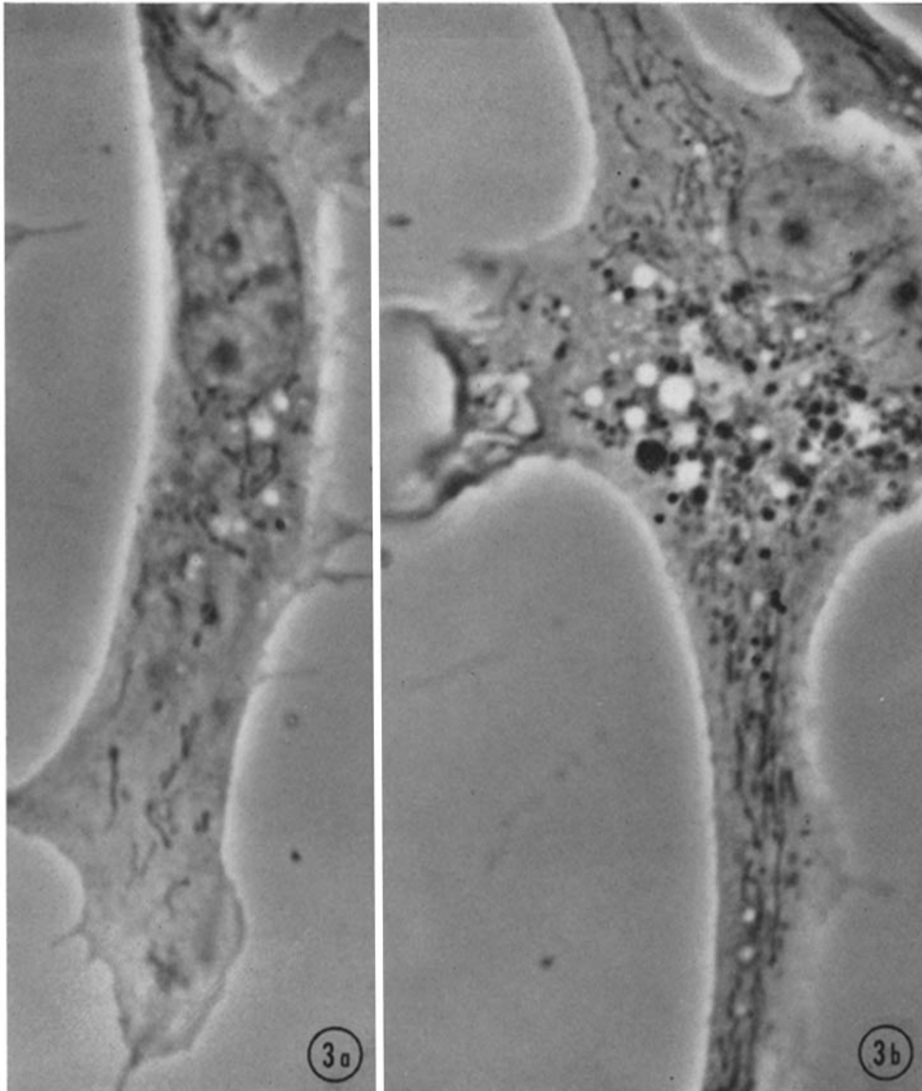
(Cohn and Parks: Pinocytosis)

PLATE 107

FIGS. 3 *a* and 3 *b*. Cells cultivated for 24 hr in heated rabbit sera and fixed with glutaraldehyde. Phase-contrast × 2700.

FIG. 3 *a*. Characteristic appearance of cell cultivated in 10% normal rabbit serum. Only a few phase-lucent vacuoles and phase-dense granules are seen in the juxtanuclear region.

FIG. 3 *b*. Cell cultivated in 10% antimouse erythrocyte rabbit serum. Extensive pinocytosis and secondary lysosome formation has taken place.



(Cohn and Parks: Pinocytosis)

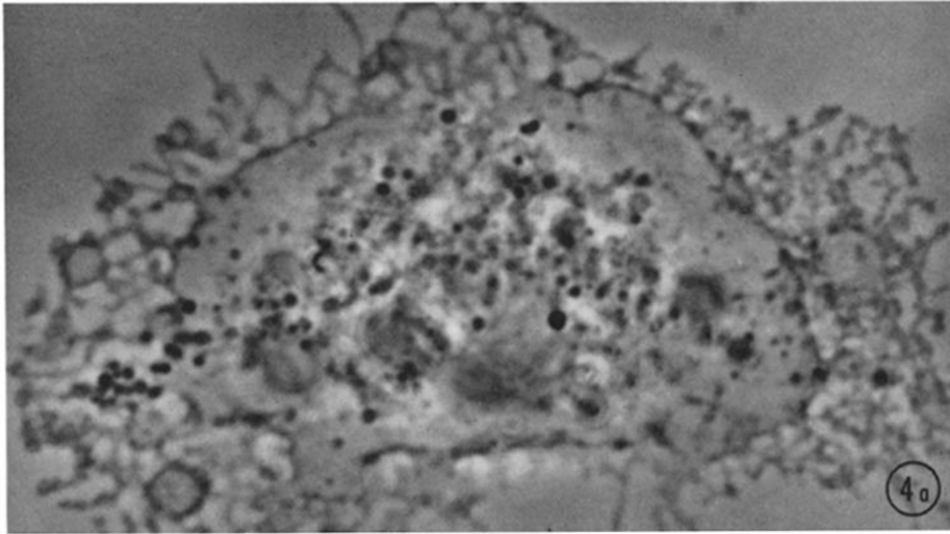
Plate 108

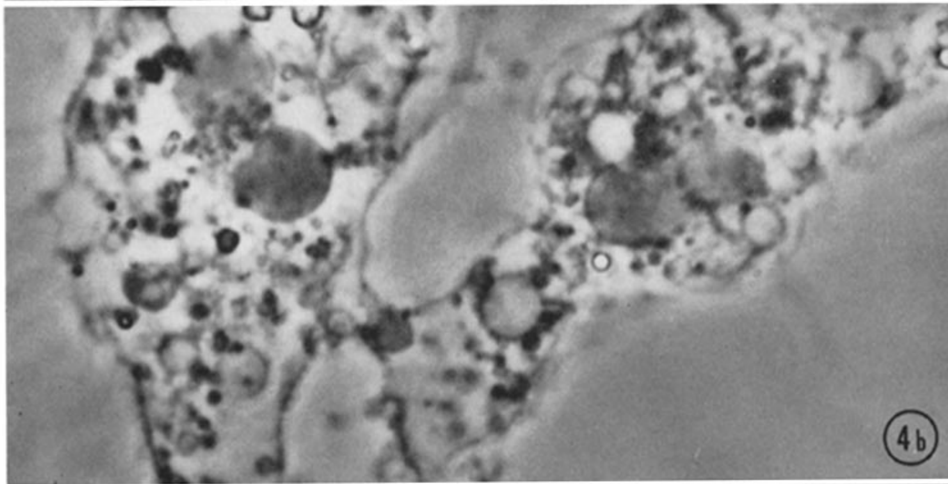
Figs. 4 *a*–4 *c*. The cytotoxic effect of 20% fresh, adult bovine serum on mouse macrophages. Cells cultivated for 24 hr in 50% NBCS, washed and incubated in fresh serum for 60 min prior to fixation. Phase-contrast × 2700.

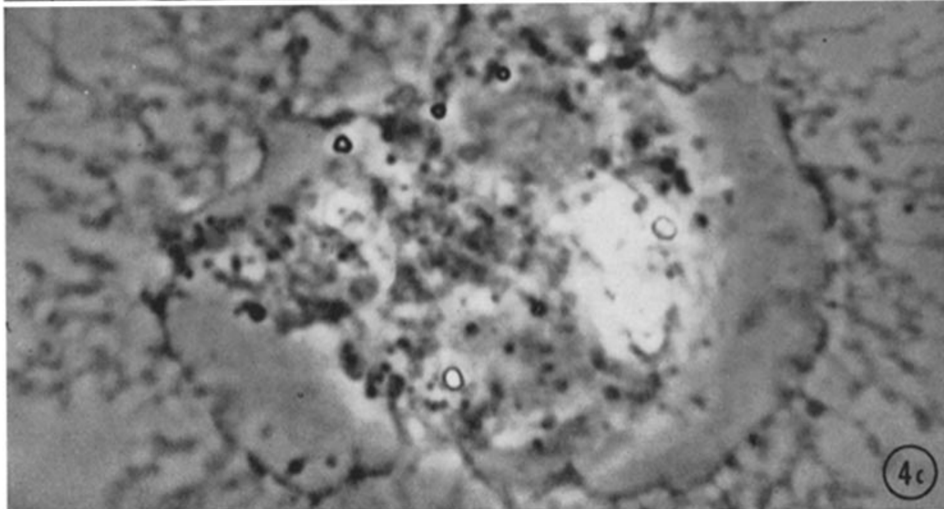
Fig. 4 *a*. Typical dendritic surface extensions, disruption of limiting membrane, and disorganization of internal structures.

Fig. 4 *b*. These cells illustrate the constriction and separation of nuclei as well as swelling of internal vesicular structures. The cells are attached by a cytoplasmic bridge; fusion of this type occurs in a small percentage of cells.

Fig. 4 *c*. Extensive swelling and loss of cytoplasmic organelles.







(Cohn and Parks: Pinocytosis)