THE REGULATION OF PINOCYTOSIS IN MOUSE MACROPHAGES

II. Factors Inducing Vesicle Formation*

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Plates 19–21

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A prior study outlined a microscopic technique with which one could quantitate the formation of pinocytic vesicles in cultured mouse macrophages (1). Under these conditions it was evident that vesicle formation responded promptly to factors contained in newborn calf serum. In addition, through the use of metabolic inhibitors, it was possible to examine certain of the determinants involved in pinosome formation.

Largely through the efforts of Holter and his colleagues an increasing body of knowledge has accumulated on the mechanism of pinocytosis in the amoeba (2, 3). A portion of these investigations has been concerned with the induction of channel formation and the resulting uptake of both small and large molecules from the external environment (4). In general, only charged molecules function as effective stimulators of pinocytosis and these are all thought to bind to the surface coat of the amoeba (5).

With the previous studies of the Carlsberg group as a guideline and with the knowledge that newborn calf serum contained pinocytosis-stimulating factors, experiments were undertaken to examine those factors which induced the formation of pinocytic vesicles in mammalian cells. This report will deal with various molecular species which appear to be nonspecific inducers of pinocytic vesicles in cultured mouse macrophages.

Materials and Methods

The basic techniques for the preparation of macrophage cultures, phase-contrast microscopy and the enumeration of pinocytic vesicles, have been reported in a previous article (1).

The Study of Inducers of Pinocytosis.—Macrophage cultures were incubated in a 50% newborn calf serum (NBCS)–No. 199 medium for either 24 or 48 hr at 37°C. At the time of an experiment the medium was removed from Leighton tubes by means of a fine tipped pipette. Residual serum components were then washed from the culture vessel by two lavages with warm, medium 199. After the second washing, 1.0 ml of 1% NBCS–No. 199 was added to the cultures. This was the basic medium to which all inducers were then added. It was the usual practice to wash and add 1% serum medium to all the tubes of a given experiment. This pro-

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cedure usually took 30 min for 12 cultures and under these conditions vesicle counts were at low levels, see Figs. 1 *a* and 1 *b* (1). Experimental compounds were then added to the tubes, gassed with 5% CO_2-air and incubated at 37°C for 150 min. The cells were then fixed with glutaraldehyde, mounted, and pinocytic vesicles enumerated. Control tubes were run for each experiment and were maintained in 1% serum–No. 199 for the course of 150 min. Excellent cell morphology was preserved in 1% serum for this time period and control vesicle counts were 10–20% of those found with 50% NBCS medium. The protein content of NBCS was approximately 6 g/100 ml so that the 1% serum medium contained ± 0.6 mg/ml. This small concentration of protein was required to maintain cell integrity during the 150 min of incubation.

The experimental results are reported as either the number of pinosomes/50 cells or else as the ratio E/C in which E = pinosomes/50 cells in the experimental culture, and C = pinosomes/50 cells in the 1% NBCS control culture. Although C varied with different batches of serum, cells and age of the culture, E/C was relatively constant. The value of C in a series of 100 experiments ranged from 34 to 90 pinosomes/50 cells. Cells cultivated for 48 hr usually had higher basal pinocytic vesicle counts than after 24 hr. All points of subsequent figures were derived from 3 or more separate experiments and from counts on more than 200 individual cells.

Preparation and Source of Inducing Molecules.—All materials to be tested for inducing properties were dissolved in No. 199 (Microbiological Associates, Inc., Bethesda, Md.) at concentrations 10-fold or greater than the highest used in the cell system. The pH was adjusted to 7.3 with NaOH or HCl. All dilutions of stock solutions were also prepared in No. 199 and added to culture tubes in volumes of 0.05–0.1 ml. In most instances various concentrations of a given inducer were titrated using the same lot of cultures.

Bovine plasma fractions, prepared by the Cohn ethanol procedure were obtained from various commerical sources. Crystalline bovine plasma albumin (3 ×) from Armour Pharmaceutical Co., Kankakee, Ill., and crystalline β-lactoglobulin (3 ×) Nutritional Biochemicals Corporation, Cleveland, Ohio. Histone was a purified preparation (Worthington Biochemical Co., Freehold, N. J.) and protamine sulfate, USP was obtained from Mann Research Laboratories, N. Y.

A preparation of poly-L-glutamic acid of bacterial origin was a gift from Dr. René Dubos, The Rockefeller University, and was originally obtained from Merck Sharp and Dohme, Rahway, N. J. Synthetic poly-L-glutamic acid, mol wt ~ 38,000; poly-D-glutamic acid, mol wt ~ 40,000 and poly-DL-lysine, mol wt ~ 21,000 were obtained from Yeda, Rehovoth, Israel.

Purified heparin was purchased from Connaught Laboratories, Toronto, Canada, and chondroitin sulfate C from Calbiochem, Los Angeles, Calif. Hyaluronic acid purified from vitreous humor was from the Worthington Biochemical Co. *Staphylococcus epidermidis* teichoic acid and the lipopolysaccharide from *Salmonella abortus-equi* were gifts from Dr. S. I. Morse and Dr. O. Westphal respectively. Calf thymus deoxyribonucleic acid was a high molecular weight preparation from Worthington Biochemical Co., and ribonucleic acid was an ethanol purified preparation from yeast.

A purified preparation of *N*-acetylneuraminic acid was purchased from Calbiochem.

Oleic and linoleic acids were chromatographically pure, vacuum-sealed preparations from Mann Research Laboratories.

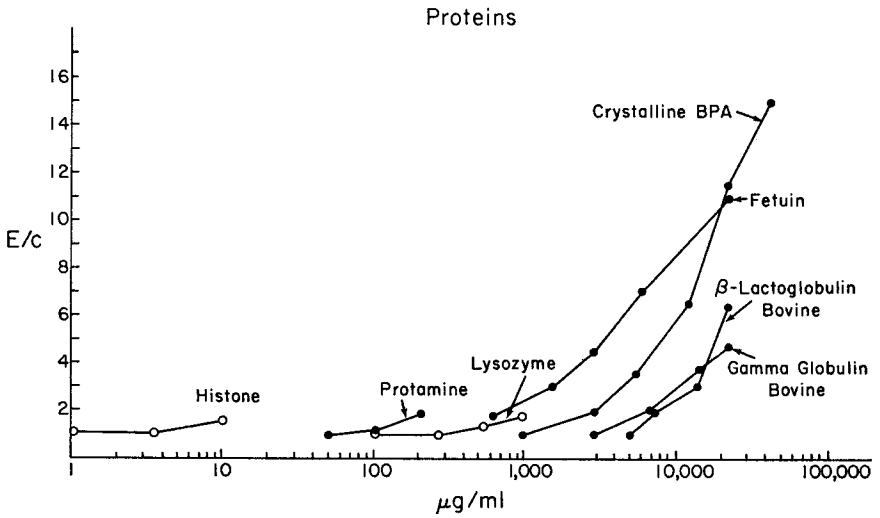
Preparations of ficoll (mol wt 400,000), dextran (mol wt 2×10^6), dextran-SO_4 (mol wt 5×10^5 and 2×10^6) were obtained from Pharmacia, Upsala, Sweden.

Other modification involving inducing agents will be presented in Results.

RESULTS

Considerations.—Under the present conditions macrophages were exposed to various concentrations of inducing agents for a period of 150 min at 37°C.

After fixation, vesicle counts were performed and compared to control cells which had been incubated in 1% NBCS–No. 199. Each agent must therefore be able to stimulate and maintain pinosome formation for this time period without compromising the morphology of the macrophages. If substances produced only a short burst of vesicle formation this may have been missed with the assay. However, an intensive period of pinocytic activity was usually reflected in the accumulation of new phase-lucent vacuoles in the centrosphere region. This will be illustrated in a subsequent section. A second consideration was the cytotoxic effect of certain molecules, which was concentration dependent, and which limited the range of their study.



TEXT-FIG. 1. The influence of proteins on pinocytic vesicle formation in mouse macrophages.

In most instances, the effect of agents which enhanced pinocytic vesicle formation were examined on living cells in a warm chamber (6). In all cases, an intracytoplasmic flow of pinosomes was observed with their ultimate localization to the centrosphere region.

Proteins.—Initial studies were conducted with proteins, some of which were constituents of newborn calf serum. Text-fig. 1 illustrates the influence of various concentrations of proteins on the intensity of pinocytosis in mouse macrophages. It was apparent that the stimulatory effect of the compounds tested was dose-dependent and that the intensity of the response (E/C) varied markedly with different classes of agents. Bovine gamma globulin (fraction II) gave only a modest response at the high levels of 25 mg/ml. β-Lactoglobulin produced an intermediate effect in large doses, whereas both fetuin and

crystalline BPA yielded major increases in the vesicle counts of treated macrophages. The exposure of cells to both fetuin and BPA resulted in vesicle counts which were higher than those obtained with 50% NBCS. The response to both fetuin and BPA was relatively linear over most of the dose-response range although the slope of the BPA response was much steeper. The incubation of macrophages with each of the four proteins did not result in visible cell damage at the reported concentrations. At stimulatory doses, the cells exhibited ruffling of the limiting membranes and the size of the new pinosomes in the peripheral cytoplasm and centrosphere region were quite large, many being 4 μ in diameter.

Three basic proteins with isoelectric points in the alkaline range were also examined, i.e., histone, protamine, and lysozyme. Each of these agents had marked cytotoxic effects at concentrations 2-fold greater than the highest dose reported in Text-fig. 1. This was characterized by lysis of areas of the limiting membrane as well as swelling, dissolution, and loss of phase density of the granules in the juxtanuclear region. None of the three agents had any significant stimulatory effect at subtoxic doses.

Three separate experiments were performed with crystalline bovine insulin but are not presented. The observations were complicated by the small size of the pinosomes which made accurate counts impossible. However, a definite stimulation occurred between 0.05–5 units (2–200 μg/ml) with E/C values as high as 4.2.

It appeared, therefore, that proteins having isoelectric points in the acid range were better inducers of vesicle formation. Further studies were conducted with two of the more potent agents; i.e., BPA and fetuin.

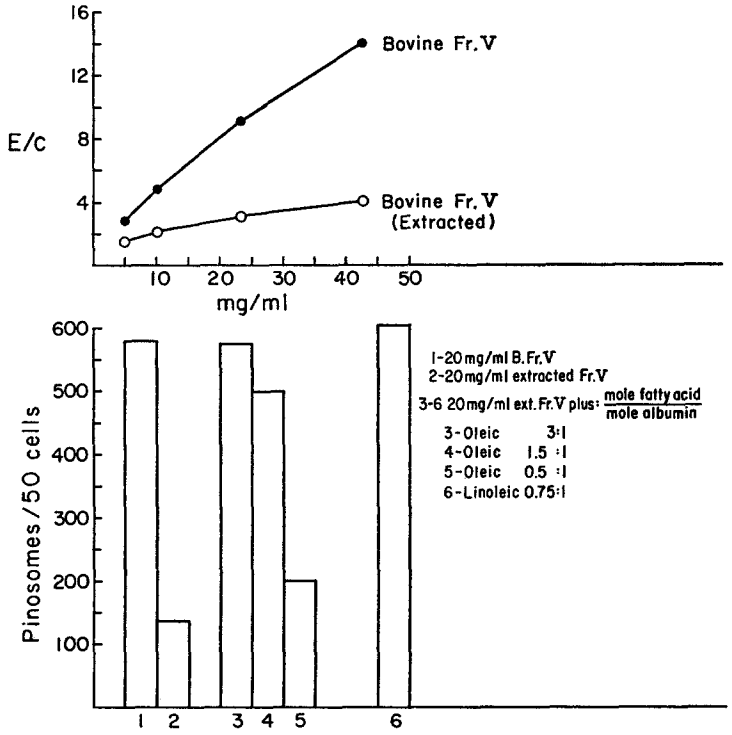
A. *Studies with Bovine Albumin.*—In view of the possible involvement of anionic groups in the induction of vesicle formation, and the well known binding of fatty acids by albumin, further experiments were performed with de-fatted preparations. For these studies bovine fraction V was employed which gave the same pinocytosis stimulation as crystalline BPA.

Bovine fraction V (Pentex) was treated according to the method of Goodman (7) with 5% glacial acetic acid–isooctane to remove the majority of the bound fatty acids. The product was then exhaustively dialyzed and adjusted to a concentration of 200 mg/ml. An initial preparation of de-fatted albumin was kindly supplied by Dr. Martin Rizack of The Rockefeller University. Untreated bovine fraction V was also dialyzed, adjusted to 200 mg/ml, and both preparations were tested for their ability to stimulate pinocytosis.

The results of such experiments are shown in the upper portion of Text-fig. 2. Untreated albumin gave the usual linear dose-response relationship whereas the extracted samples exhibited only slight activity. It appeared, therefore, that extraction with glacial acetic acid–isooctane removed the pinocytosis-stimulating activity of albumin. It was possible, however, that the extraction process resulted in alterations of the albumin molecule other than the removal

of fatty acids. Experiments were then performed to replace the fatty acids of the extracted albumin and examine the stimulatory activity of the reconstituted molecule.

Purified preparations of oleic and linoleic acids were dissolved in acetone and small aliquots dispensed to test tubes. The acetone was then evaporated under a stream of warm nitrogen and a film of fatty acid was left in the container. 1 ml volumes of glacial acetic acid–isooctane



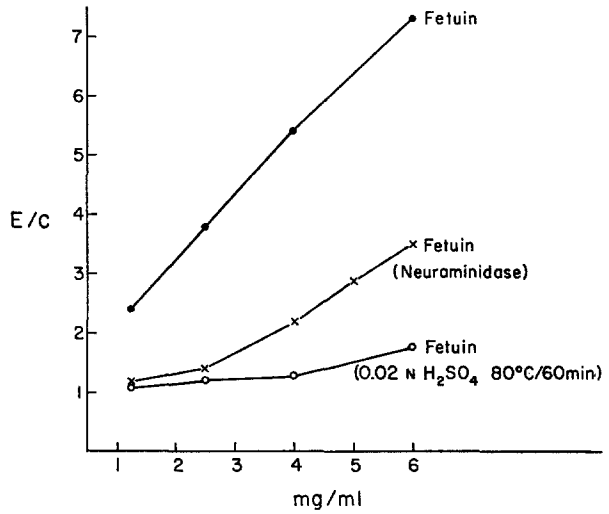
TEXT-FIG. 2. The pinosome response to de-fatted albumin and its reversal with fatty acids.

extracted bovine fraction V were added to the tubes and allowed to equilibrate overnight. The concentrations of fatty acid and albumin were adjusted so that a range of 0.5–6.0 moles of fatty acid per mole of extracted albumin were examined. The inducing capacity of each of these mixtures was then compared with native fraction V at a constant albumin concentration of 20 mg/ml. The data are expressed as the number of pinocytic vesicles/50 cells.

The results of these experiments are illustrated in the lower portion of Text-fig. 2. Columns 1 and 2 show the marked difference in pinocytic vesicle counts of cells exposed to native and extracted bovine fraction V. When extracted albumin was exposed to a ratio of 1.5–3.0 moles of oleic acid/mole albumin the resulting molecule induced pinocytic vesicle formation to the

same extent as the native unextracted substance. A much less striking effect was noted at molar ratios of 0.5. Although not shown, a ratio of 6 moles fatty acid/mole albumin resulted in a mixture which was cytotoxic to macrophages. This was related to free, unbound fatty acid in the suspension and could be reversed by the addition of more albumin. Linoleic acid appeared to bind less avidly to extracted albumin and mixtures containing 1.8 moles/mole albumin were cytotoxic. As the ratio was reduced to about 1.0, a marked stimulation of pinosome formation took place.

From these results it appeared that a major factor in the pinocytosis-inducing properties of albumin was related to its content of bound fatty acids.



TEXT-FIG. 3. The effects of mild acid hydrolysis and neuraminidase digestion on the pinosome inducing properties of fetuin.

It was also of interest that small quantities of oleic acid (10 μg/ml) could be added to the 1% serum medium. This was presumably bound to proteins and increased vesicle counts to E/C values of 8.0.

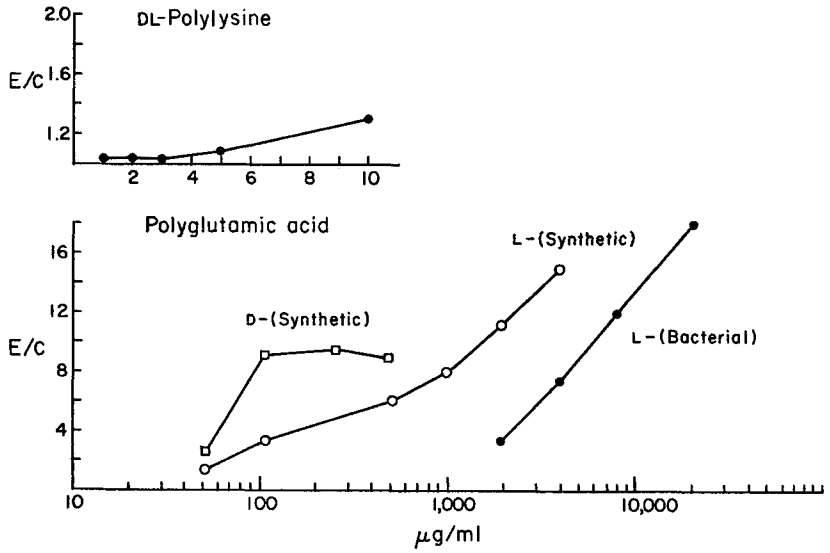
B. *Studies with Fetuin.*—Fetuin is an acid glycoprotein present in fetal and newborn calf serum which contains approximately 10% sialic acid (8). In view of its anionic nature, which is primarily related to *N*-acetylneuraminic acid residues, experiments were performed to remove sialic acid and study this alteration on the pinocytosis-inducing properties of the molecule.

Two methods were available to remove sialic acid. The first was described by Spiro (8). 25 mg of fetuin was dissolved in saline, made 0.025 N with respect to H_2SO_4 and heated at 80°C for 60 min in a water bath. The solution was neutralized with $Ba(OH)_2$, filtered, and the supernatant dialyzed. This resulted in the removal of 87% of the sialic acid content of the

preparation. This procedure was reported by Spiro to raise the isoelectric point of fetuin from 3.3 to 5.2 without other significant structural alterations of the molecule.

The second procedure was to digest fetuin enzymatically with neuraminidase. 25 mg of fetuin were dissolved in No. 199 and *Vibrio cholera* neuraminidase (Calbiochem) added to a final concentration of 200 units/ml. Digestion took place at 38°C for 2 hr. The preparation was then dialyzed at 4°C against frequent changes of phosphate-buffered saline at pH 7.5 for 24 hr. This treatment removed 72% of the sialic acid of the starting preparation. A sample of BPA was neuraminidase digested and processed in an identical fashion.

Both the acid-hydrolyzed and enzyme-treated fetuin, as well as an untreated dialyzed sample were adjusted to the same protein concentration. Dilutions of each were then tested for pinocytosis-inducing activity at concentrations between 1.25–6.0 mg/ml.



TEXT-FIG. 4. The induction of vesicle formation by homopolymers of polyamino acids.

The results of these experiments are shown in Text-fig. 3. The untreated preparation yielded a linear dose-response relationship and enhanced pinocytic activity. In contrast, the acid hydrolyzed and neuraminidase-digested specimens produced only a minor stimulating effect at higher concentrations. These data suggest that sialic acid plays an important role in the pinocytosis-inducing properties of fetuin. A neuraminidase-digested sample of crystalline bovine plasma albumin retained full inducing activity.

Polyamino Acids.—The relative efficiency of smaller polypeptides was examined in the next series of experiments. Two classes of homopolymers were studied and the results presented in Text-fig. 4. The lower portion illustrates the marked stimulatory activity of polyglutamic acid. The L form of this compound obtained from both bacterial and synthetic sources yielded values

of E/C which were as much as 18-fold higher than the 1% NBCS control. A sample of polyglutamic prepared with the unnatural D isomer also gave a high level of pinocytic vesicle formation. The increased activity of the L-synthetic polymer over that exhibited by the bacterial product is unexplained. The purity of the bacterial-derived compound as well as its exact molecular weight is unknown.

In contrast to the brisk vesicle formation with polyglutamic acid, little or no effect was obtained with poly-DL-lysine. This cationic polymer proved to

TABLE I

The Effect of Amino Acids on Pinosome Formation

Amino acid	Concentration	E/C*
	M	
L-glutamic	0.02	6.5
L-aspartic	0.02	7.2
L-glutamine	0.02	1.1
L-asparagine	0.02	1.2
L-leucine	0.02	1.1
L-phenylalanine	0.02	1.0
Glycine	0.02	1.0
Taurine	0.03	1.1
L-histidine	0.02	2.1
L-arginine	0.002	1.0
L-lysine	0.002	1.0
D-glutamic	0.02	0.2
D-aspartic	0.02	0.3

* E/C of 1% NBCS–No. 199 control = 1.0.

be strongly cytotoxic at concentrations above 25 μg/ml and resulted in the swelling and subsequent disintegration of cells. At concentrations from 0.1 to 10 μg/ml, vesicle counts of exposed cells were barely above control values.

Amino Acids.—The influence of a number of amino acids was examined and the results shown in Table I. Each of these agents was tested over a 100-fold range of concentrations. The data in Table I are at concentrations giving maximal stimulation and without cytotoxic effects. It was of interest that the only two amino acids that stimulated pinosome formation were the L isomers of the dicarboxylic acids glutamic and aspartic. The amides of both were without significant effect at the same concentration. Neutral amino acids were not stimulatory. Again, the more strongly cationic agents, i.e. lysine and arginine,

were cytotoxic at higher levels and were without stimulatory effects at sublethal doses.

One unusual outcome of these experiments was the inhibitory effects of the D isomers of glutamic and aspartic acids. When these agents were added to the 1% NBCS medium, the exposed cells were almost devoid of pinosomes and exhibited flattening of the limiting membranes with the absence of undulations. This picture was quite similar to the addition of inhibitors of protein synthesis; i.e., *p*-fluorophenylalanine and puromycin (1).

Because of the small number of vesicles present in 1% NBCS medium, additional experiments on the inhibitory effect of D amino acids were performed in 50% NBCS medium (1).

TABLE II

The Inhibition of Pinocytic Vesicle Formation by D-*glutamic acid*

D glutamic acid	Per cent of* 50% NBCS control
M	
0.04	9
0.03	12
0.02	31
0.01	55
0.005	84
0.0025	97
0.0010	100

* 50% NBCS control averaged 425 pinosomes/50 cells.

Cells were cultivated for either 24 or 48 hr, the medium aspirated and replaced with fresh 50% NBCS–No. 199. Various concentrations of either D or L amino acids were then added, the tubes gassed with 5% CO_2-air and the cells incubated for 150 min at 37°C.

Table II illustrates the ability of D-glutamic acid to inhibit the formation of pinocytic vesicles in the presence of 50% NBCS. Between the concentrations of 0.0025–0.03 M there was an almost linear reduction in vesicle formation. Although not illustrated, the D isomers of both leucine and phenylalanine, at concentrations of 0.02 M also inhibited vesicle formation to a similar extent. The inhibition by D isomers was therefore not a property of the dicarboxylic amino acids. It was of interest that poly-D-glutamic acid had a stimulatory effect whereas the monomeric isomer inhibited pinocytosis. The lack of inhibition by the polymer may reflect the inability of macrophages to degrade this compound.

Table III shows the results of experiments in which L-glutamate reversed the inhibitory effect of the D isomer. This response was most prominent at D/L ratios of 3/1–6/1 whereas at 15/1 only a partial reversal took place. These

data are no doubt influenced by the concentrations of L-glutamic acid in both medium 199 and 50% NBCS.

Polysaccharides.—The next series of macromolecules to be tested were the polysaccharides. In view of the increasing evidence for the stimulatory effects of anions, our attention was focused on the acidic polysaccharides. The results of dose-response titrations are shown in Text-fig. 5.

Ficoll, a polysucrose with a molecular weight of approximately 400,000 enhanced vesicle formation but only at very high concentrations. Maximal counts occurred at 50 mg/ml and within a 5-fold reduction in dose had fallen to control values. Since this preparation had not been dialyzed it is possible that minor contaminants could have contributed to the response. A lipopolysaccharide endotoxin (LPS) was examined at concentrations from 1 to 10 μg/ml and was without enhancing properties under these conditions.

TABLE III

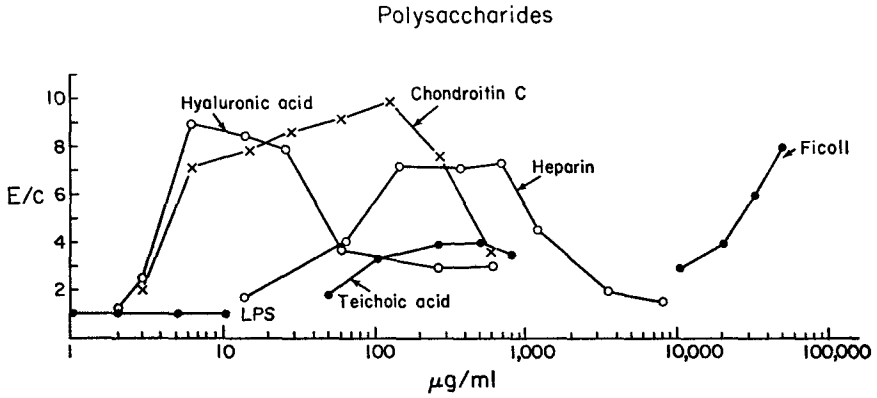
The Inhibition of Pinosome Formation by D-glutamic Acid and its Reversal with L-glutamic Acid

D glutamic	L glutamic	Per cent of 50% NBCS control
M	M	
—	—	100
0.03	—	12
—	0.03	94
0.03	0.01	87
0.03	0.008	84
0.03	0.005	86
0.03	0.002	31

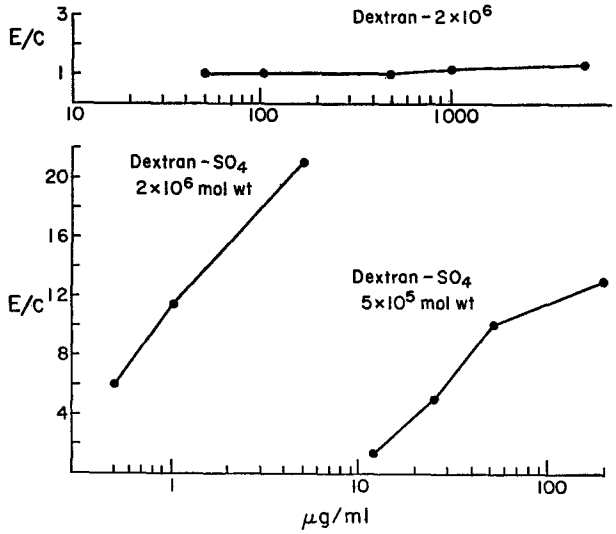
The most striking increase in vesicle formation occurred with acidic polysaccharides including heparin, chondroitin sulfate C, and hyaluronic acid. Each of these agents stimulated the production of pinosomes in microgram quantities and hyaluronic acid was effective at levels as low as 5 μg/ml. The shape of the dose-response curves was of interest in that a definite prozone occurred at higher, noncytotoxic levels. This was not unique to the mucopolysaccharides and has been observed with other classes of macromolecules. A specimen of *S. epidermidis* teichoic acid (glycerophosphate-glucose polymer) of low molecular weight (±8,000) gave less intense vesicle formation.

The availability of different forms of dextran made possible an interesting comparison of molecular weight and charge on pinosome formation. These data are presented in Text-fig. 6. The upper portion of the graph illustrates the absence of any significant stimulatory effect of high molecular weight dextran (2×10^6) at concentrations as high as 5000 μg/ml.

The use of dextran sulfate (mol wt 2×10^6), which contains 17% S, resulted in a tremendous stimulation of vesicle formation. Values for E/C were as high as 20 times the control reading and pronounced activity occurred at less than



TEXT-FIG. 5. The pinocytic response of macrophages to various polysaccharides.



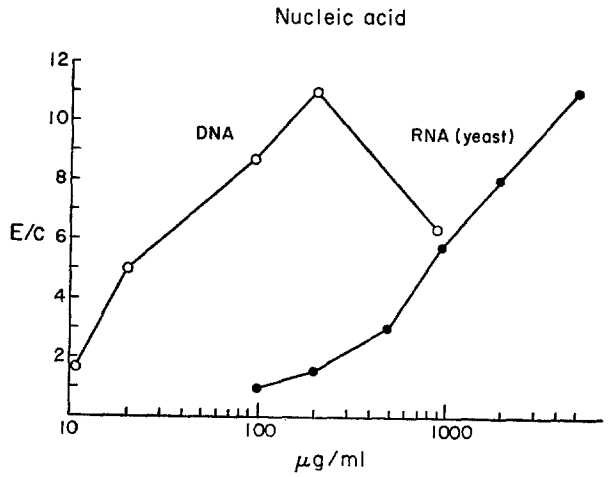
TEXT-FIG. 6. The comparative effects of high molecular weight dextran and dextran sulfate on vesicle formation.

1.0 μg/ml. The sulfated product was therefore at least 10,000 × more active than dextran of the same molecular weight.

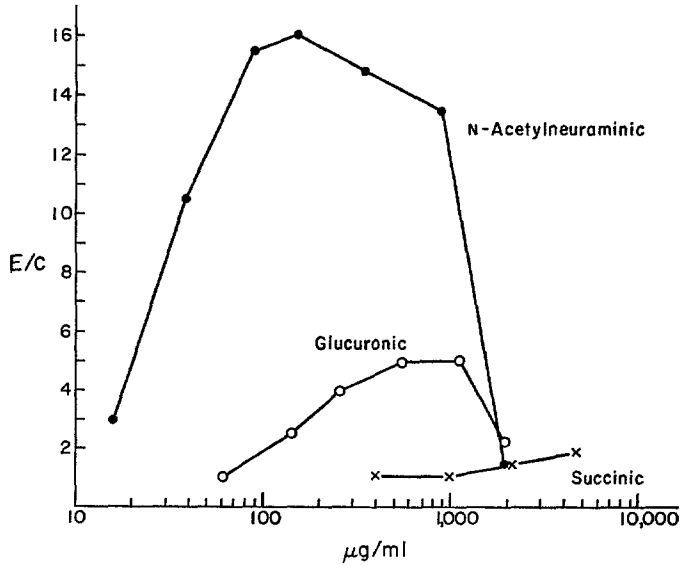
When another preparation of Dextran sulfate was employed, which differed only in being ¼ of the molecular weight (500,000 mol wt), stimulation was achieved, but the compound was less active on a weight basis. However, when data were calculated on a molar basis it was found that each molecular species of dextran sulfate gave a 2-fold or greater stimulation at about 1×10^{-8} M.

Nucleic Acids.—The nucleic acids represented another group of highly

charged macromolecules which depend upon phosphate groups for their anionic nature. Experiments in which various concentrations of both DNA and RNA were tested are presented in Text-fig. 7. DNA was more active on a weight basis, and demonstrated a prozone effect. The lowest concentration which gave a detectable stimulus was about 10 μg/ml. The preparation of RNA



TEXT-FIG. 7. The response to nucleic acids.



TEXT-FIG. 8. The response of mouse macrophages to selected low molecular weight anions.

stimulated pinosome formation at higher concentrations and exhibited a similar slope. A 2-fold increase in vesicle counts occurred at RNA levels of 300 μg/ml and it therefore appeared that DNA was 30 times as active employing this criterion. The exact molecular weights of these preparations is not known.

Small Molecules.—The prior studies on fetuin and other macromolecules suggested that the anionic moieties of these agents were responsible for the

TABLE IV

The Influence of Selected Small Molecules on Pinosome Formation

Compound	E/C*	Morphology
NaCl		
3–25 mg/ml	—	Cytotoxic
<3 mg/ml	1.0–1.5	Intact cells
Sucrose		
25–100 mg/ml	—	Cytotoxic
12.0 "	4.2	Intact cells with large pinosomes
6.0 "	3.5	" " " "
3.0 "	2.5	" " " " "
1.5 "	1.2	" " " " "
Saponin		
5–25 μg/ml		Liquified cytoplasm with empty granules
2.0 "	2.7	Intact cells with tiny pinosomes
1.0 "	5.8	" " " " "
0.5 "	3.2	" " " " "
0.1 "	1.5	" " " " "
Desoxycholate		
3–25 μg/ml	—	Disrupted cells
1.0 " —	2.4	Intact cells with blunted pseudopods
0.5 "	4.2	" " " " "
0.1 "	3.8	" " " " "
0.01 "	1.3	" " " " "

* E/C of 1% NBCS control = 1.0.

induction of pinocytic vesicle formation. The effects of a few low molecular weight anions were next investigated and are shown in Text-fig. 8. It was of particular interest, in view of the prior studies with fetuin, that *N*-acetylneuraminic acid produced a striking stimulation of vesicle formation at low concentrations. A clear cut prozone effect occurred with little or no activity at 2.0 mg/ml. As the concentration was reduced, values for E/C rose to 16, and then fell to low levels at a dose of 15 μg/ml. This degree of vesicle formation was considerably higher than that obtained with glucuronic acid. In contrast, succinic acid did not enhance pinosome production at these levels.

Table IV presents data obtained with a few selected molecules. Sodium

chloride was without demonstrable effect whereas sucrose produced a modest increase in vesicle formation at concentrations of 3–12 mg/ml.

Two surfactants were examined (Table IV) and both saponin and desoxycholate induced the formation of small numbers of vesicles at subtoxic levels.

The Morphology of Stimulated Macrophages.—Shortly after placing macrophages in the control medium, consisting of 1% NBCS–No. 199, they had the appearance illustrated in Figs. 1 *a* and 1 *b*. The well defined centrosphere region was filled with phase-dense granules as well as a few lucent vacuoles. These granules surrounded a central clear zone which is the location of the Golgi apparatus. A few pinocytic vesicles were seen in the peripheral cytoplasm.

Fig. 2 *a* shows a portion of a highly stimulated cell which had been exposed to poly-L-glutamic acid 2½ hr prior to fixation. Many small vesicles were seen in the cytoplasm and these were arising from multiple peripheral sites. The centrosphere region of the cell was now distended with many new lucent vacuoles. When such cells were observed in living preparation, it was apparent that this was the result of the influx of new pinosomes, many of which had fused with the preexisting dense granules. Fig. 2 *b* shows a peripheral pseudopod from a similarly treated cell. Many pinosomes were seen arising from the ruffled, somewhat bulbous tip of the pseudopod. Their linear, centripetal flow, in close association to mitochondria was apparent.

Figs. 3 *a*–3 *c* illustrate a cell exposed for 2½ hr to 20 μg/ml of hyaluronic acid. The peripheral cytoplasm in this case was quite flat and numerous, small pinosomes were arising from a single focus. The centrosphere region was filled with smaller vacuoles and its outer limits were indistinct.

Figs. 3 *b* and 3 *c* illustrate two stages of the response to fetuin. Fig. 3 *b* was taken 30 min after exposure to 5 mg/ml, when only small numbers of lucent vacuoles had accumulated in the juxtanuclear zone. This cell also illustrated the wide fluctuation in size of the peripheral pinosomes. The pseudopod on the left contains larger vesicles whereas that on the right has tiny structures. After exposure for 120 min (Fig. 3 *c*), the centrosphere region was now filled with large vacuoles.

DISCUSSION

The evidence presented in this article indicates that the mammalian macrophage responds to a wide variety of anionic molecules by increasing the formation of pinocytic vesicles, interiorizing large amounts of limiting membrane, and segregating this material in the juxtanuclear region. Little or no response occurred with neutral or cationic molecules of both low and high molecular weights. This response occurred in a complex medium containing small amounts of serum, at physiological hydrogen ion concentrations and over a period of 150 min. The overall response was qualitatively different from that observed

in the amoeba, although charged molecules were still required. Certainly, the surface membrane of the amoeba which is adapted to a free living existence, must be somewhat different from that of mammalian cells. The large and complex mucous coat, composed of mucopolysaccharides, is one such example which no doubt influences the response to inducing agents (9).

Many of the inducing agents which were not cytotoxic exhibited a rather interesting dose-response relationship. This was characterized by a prozone region at higher concentrations in which vesicle formation was at a low level. Phase-contrast observations of such cells revealed the presence of healthy macrophages often with extremely ruffled limiting membranes, but without evidence of significant vesicle formation. As the inducer level was lowered, a maximal pinosome response was achieved which again diminished at limiting inducer concentrations. The latter response was often quite linear but differed in slope for the various inducers. These data suggest that the process of pinosome interiorization, although correlated with the "activation" of the limiting membrane, occurs only at limited levels of the stimulus. Another salient feature of the inducer response was the number of cytoplasmic sites involved in vesicle formation. With 50% serum, the majority of vesicles were formed at the tips of the pseudopods and usually only one pseudopod was active at any given time. In contrast, cells stimulated with anionic inducers usually illustrated multiple cytoplasmic loci.

The response of the macrophage membrane to an inducing agent is a complex interaction which is poorly understood at this time. Presumably, some binding of the anionic stimulatory agent takes place, the exact receptor group being unknown. In view of the fact that most macroanions are effective at $\pm 10^{-8}$ M this is probably a nonspecific charge effect which in some way activates the energy dependent interiorization process. The well known difficulty of charged anions to enter cells is in sharp contrast to the relative ease with which basic or neutral agents enter and are concentrated within cells either by diffusion or active transport. Pinocytosis may, therefore, play a significant role in the uptake of negatively charged molecules. In contrast, many small cationic molecules may enter the cytoplasm by a mechanism unrelated to vesicle formation. Certain basic, vital dyes, i.e. neutral red, appear to diffuse immediately into the cytoplasm of viable macrophages and their uptake is a nonenergy dependent process (Z. A. Cohn. Unpublished observations). The stimulation of albumin uptake by histones and basic polyamino acids as reported by Ryser and Hancock (10), would be in direct contrast to the present results, if this occurred by a pinocytic mechanism.

Once pinocytosis is stimulated the inducing agent as well as other constituents of the external environment are enclosed within a cytoplasmic vesicle. This is the locus into which hydrolytic enzymes are secreted, presumably via Golgi vesicles (11). The secondary lysosome or digestive body which is then

formed is in a state of continuous flux. New elements from the environment and endogenous hydrolases may be continually added, depending upon the rate of pinocytosis, membrane fusion, and enzyme synthesis. This is illustrated in the rapid formation of new phase-lucent vacuoles in the peri-Golgi region. The subsequent fate of lysosomal contents is less well understood. This depends on the "digestibility" of granule contents and possibly alterations in the permeability of the lysosomal membrane. This membrane although derived primarily from the limiting cell membrane may have its properties altered because of its new position in the cytoplasm. Another possibility stems from observations that macroanions inhibit the activity of certain hydrolytic enzymes (12) and suggests that some of the components of digestive bodies may influence the fate of other segregated macromolecules.

What role does pinocytosis play in the economy of the mammalian cell? This may depend in large measure on the type of cell involved in the process. Whereas the endothelial and intestinal cells may employ this mechanism for the transcellular transport of luminal contents (13, 14), the macrophage presumably employs pinocytosis for other purposes. These at present encompass a possible nutritional role and the processing and degradation of "foreign" molecules. In addition, there is evidence that some element of the pinocytic mechanism stimulates the macrophage to produce larger amounts of digestive or lysosomal enzymes (6). Such a mechanism could very well be of considerable importance in the response of the host to antigenic stimuli and to its adaptive response to the inflammatory milieu. In this regard there are a number of seemingly unrelated observations concerning the physiological effects of macroanions. These include, the production of interferon (15), increased resistance to both viral and bacterial infections (16, 17), adjuvant effects in antibody formation (18, 19), enhanced bone resorbtion (20, 21), and the stimulation of cell growth (22). Certain of these influences may reflect a stimulatory role of macroanions on reticuloendothelial elements of the host, perhaps on the basis of enchanced pinocytosis. Some of the more potent inducers, such as the acid mucopolysaccharides, are widely distributed components of connective tissues. The release of such agents at inflammatory sites could conceivably serve as a stimulus for the activation and maturation of blood-born mononuclear phagocytes. Obviously the action of macroanions to stimulate pinocytosis may be influenced by binding to more cationic elements of the environment. This has been noted in experiments employing higher concentration of NBCS in the medium.

The majority of inducers employed in this article are considered to be nonspecific in nature and depend for their activity upon some physicochemical interaction with the macrophage-limiting membrane. However, other agents currently under study are more selective and will be considered in subsequent communications.

SUMMARY

The pinocytosis-inducing effect of a number of molecular species was studied in cultures of mouse macrophages. Agents were added to a basal medium containing 1% NBCS–No. 199 and allowed to interact with cells for 150 min. Vesicle counts were then performed and compared to control cells in the basal medium.

Certain proteins, i.e. albumin and fetuin, with isoelectric points of five and below were found to be potent stimulators of vesicle formation. Basic proteins including lysozyme, histone, and protamine had little influence at sublethal concentrations. The pinocytosis-stimulating activity of bovine plasma albumin could be markedly depressed by removal of bound fatty acids. The addition of either oleic or linoleic acid to de-fatted albumin restored its inducing properties to initial levels. The activity of fetuin could be abolished by either mild acid hydrolysis or neuraminidase digestion. Both procedures removed the majority of the sialic acid content of fetuin.

The D and L isomers of polyglutamic acid were found to produce a marked increase in pinosome production. In contrast, poly-DL-lysine was not effective.

Neutral and basic amino acids were without significant effect on pinocytosis, whereas aspartic and glutamic acids were stimulatory. The amides of glutamic and aspartic acid did not induce pinocytosis. The unnatural D isomers of glutamic, aspartic, leucine, and phenylalanine inhibited pinocytosis. The inhibition by D-glutamic acid could be reversed with the L isomer.

A number of acid mucopolysaccharides, including heparin, hyaluronic acid, and chondroitin sulfate, were excellent inducers. High molecular weight dextran was without significant stimulatory effect whereas dextran sulfate was very active.

Both desoxyribonucleic acid and ribonucleic acid enhanced pinosome formation.

A number of low molecular weight anions including *N*-acetylneuraminic acid were found to enhance vesicle formation.

In general, anionic molecules were better inducers than either neutral or cationic species. The minimum effective dose of macroanions was a function of molecular weight and their activity appeared unrelated to specific chemical groupings.

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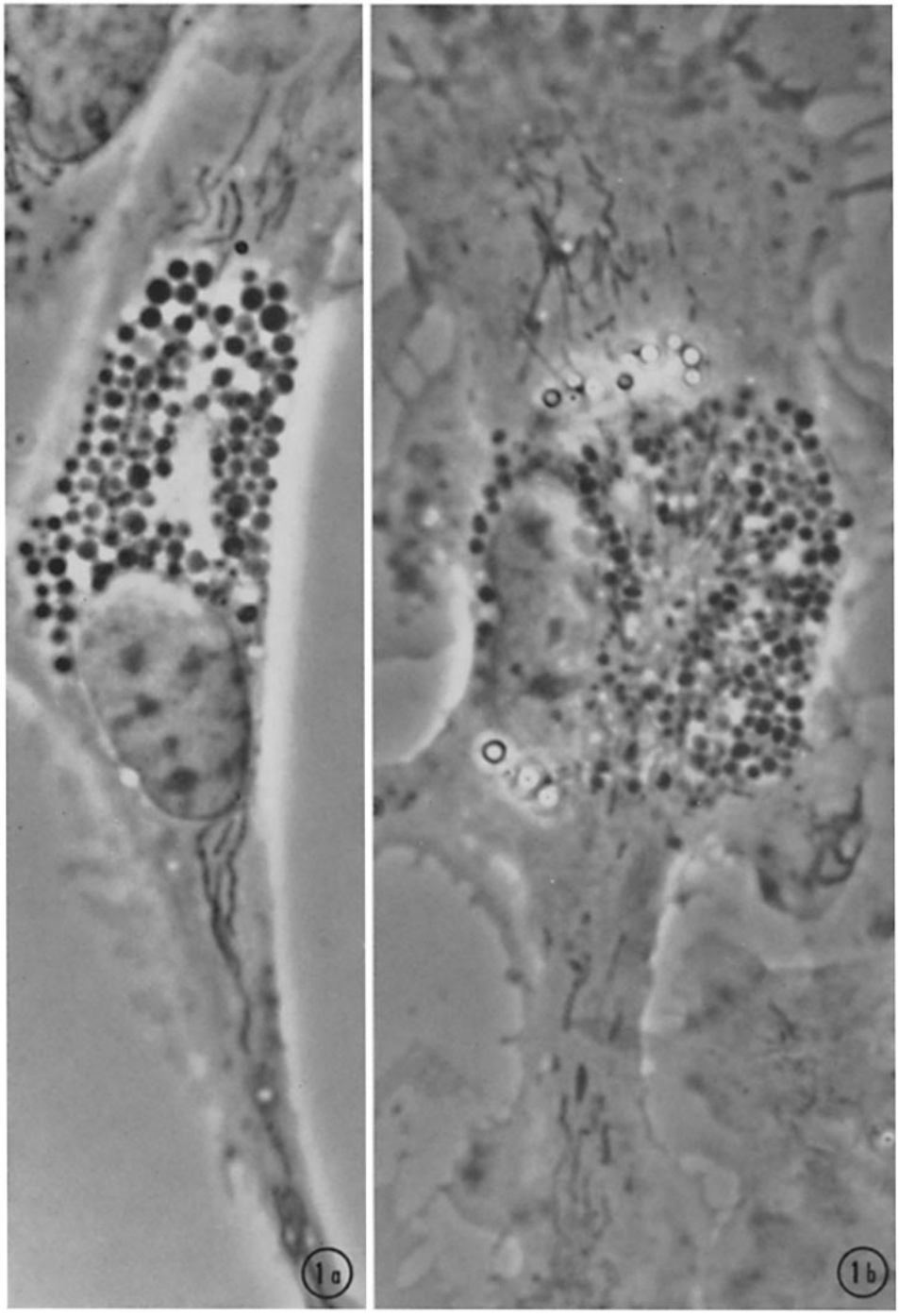
PLATES

PLATE 19

FIGS. 1 *a* and 1 *b*. Control cells fixed in 1.25% buffered glutaraldehyde. Phase contrast, × 2500.

FIG. 1 *a*. A cell cultivated for 24 hr in 50% NBCS medium, washed with No. 199, and resuspended in 1% NBCS prior to fixation. A few phase-lucent pinosomes are visible in both the upper and lower pseudopods. The centrosphere is filled with phase-dense granules as well as a few lucent vacuoles.

FIG. 1 *b*. A cell cultivated in 50% NBCS medium for 48 hr and treated in the same manner as Fig. 1 *a*. This cell is somewhat larger than the one shown in Fig. 1 *a* but has essentially the same cytoplasmic architecture. The granule-free region in the juxtanuclear area is the site of the Golgi apparatus. Cells cultivated for both 24 or 48 hr in the serum-rich medium have been employed for these studies.



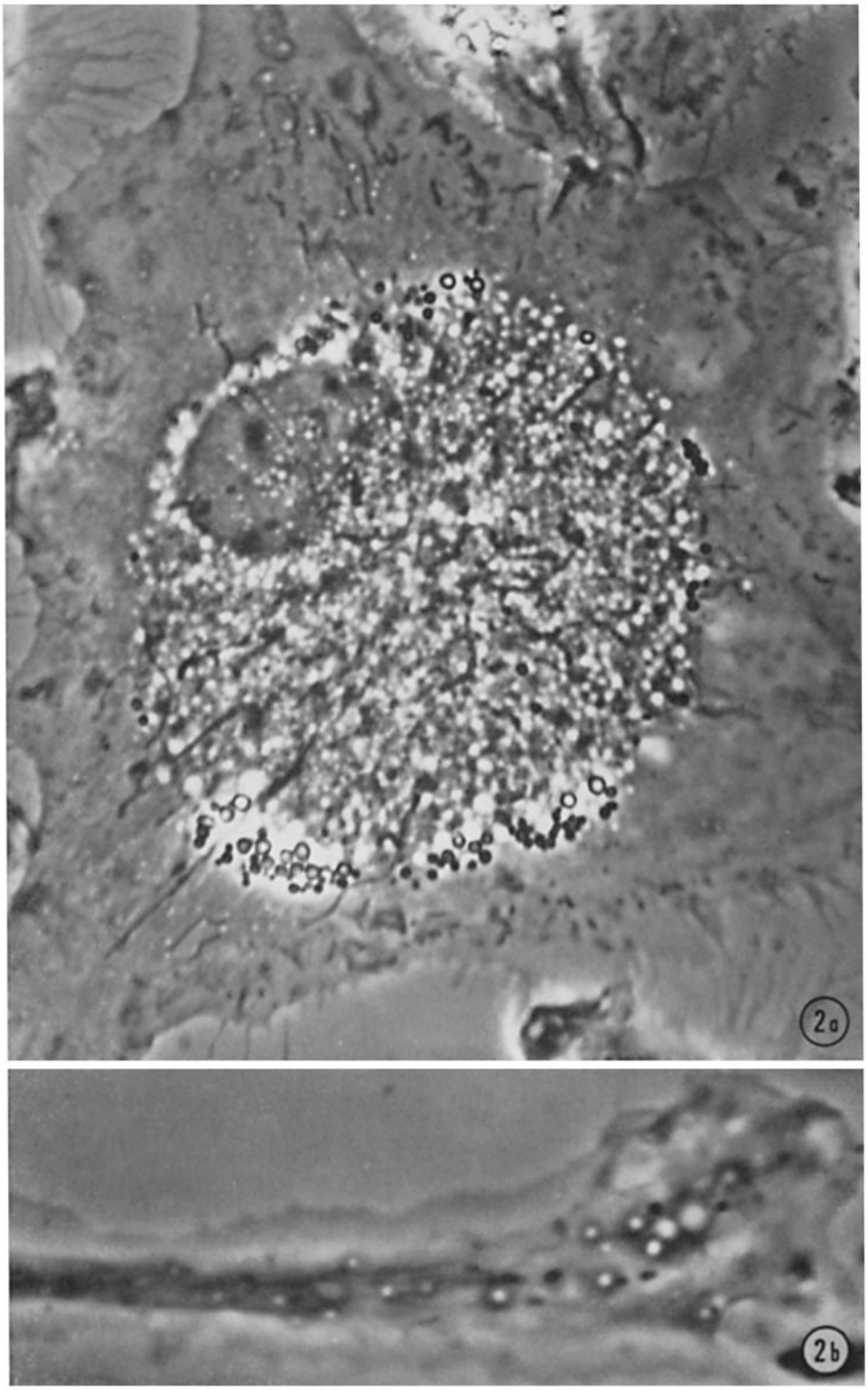
(Cohn and Parks: Pinocytosis)

PLATE 20

FIGS. 2 *a* and 2 *b*. Examples of cells exposed to 5 mg/ml poly-L-glutamic acid for 120 min prior to fixation.

FIG. 2 *a*. A portion of the cell body illustrating the repopulation of the centrosphere with new phase-lucent pinosomes. Occasional phase-dense granules are seen and the highly refractile lipid droplets have been displaced to the periphery of the centrosphere. Numerous, small pinosomes are present in the peripheral cytoplasm. Phase contrast, × 2800.

FIG. 2 *b*. A pseudopod from a poly-L-glutamic acid–stimulated cell. Pinosomes of different size were arising from the distal, ruffled membrane. Other vesicles, more proximally situated, are seen among the mitochondria. Phase contrast, × 2800.



(Cohn and Parks: Pinocytosis)

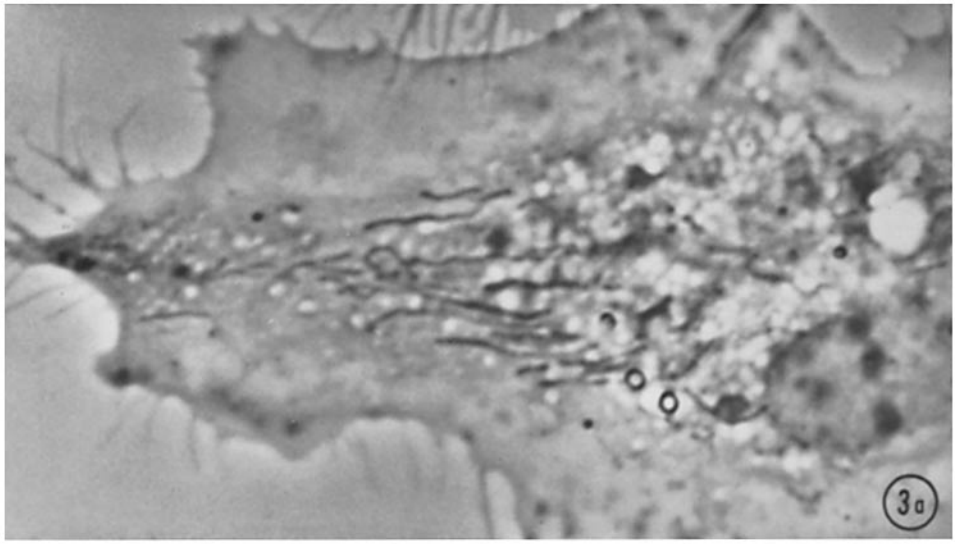
PLATE 21

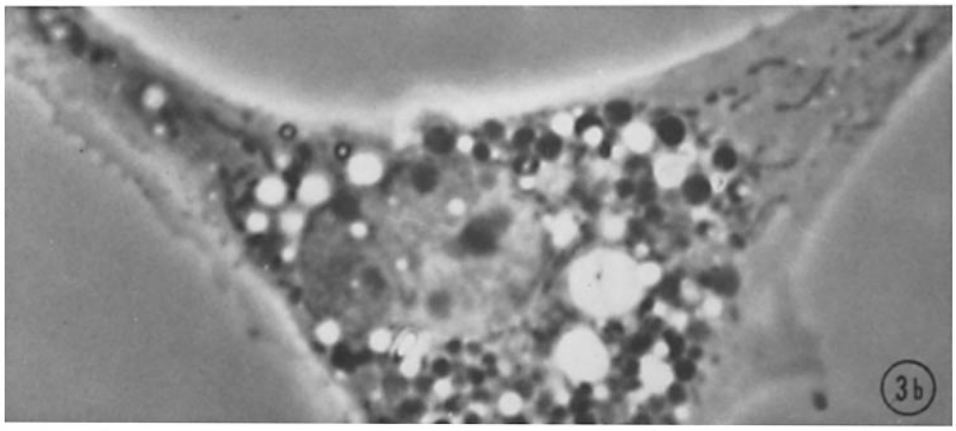
FIG. 3 *a*. A cell exposed to 20 μg/ml of hyaluronic acid for 150 min prior to fixation. The cell has a flattened appearance and numerous small pinosomes are seen. The centrosphere is filled with small, lucent vacuoles and its margins are somewhat indistinct. Phase contrast, × 2500.

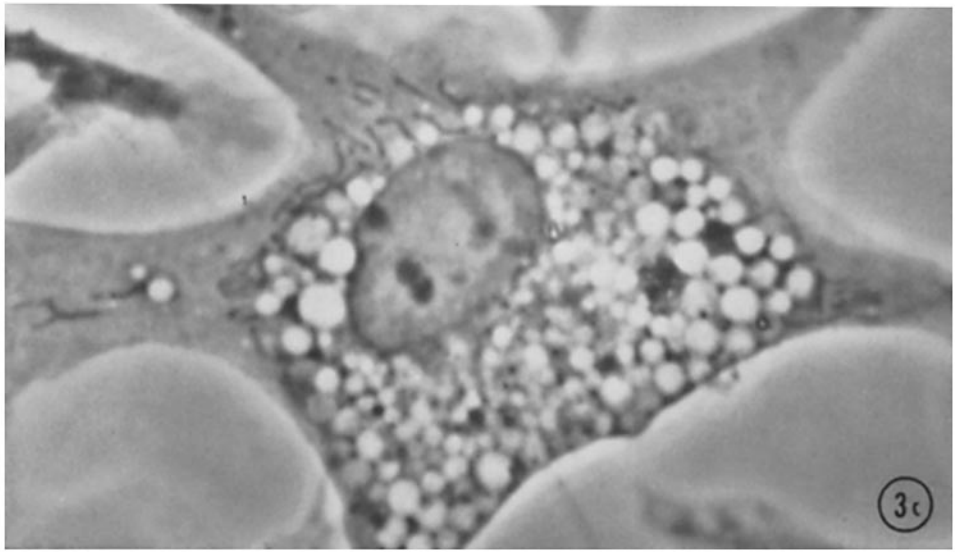
FIGS. 3 *b* and 3 *c*. Cells exposed to 5 mg/ml of fetuin. Phase contrast, × 2500.

FIG. 3 *b*. A cell fixed 30 min after exposure to the inducer. Peripheral pinosomes of all sizes are apparent. The centrosphere contains a mixture of new phase-lucent vacuoles and preexisting dense granules.

FIG. 3 *c*. A similarly treated sample fixed 120 min after exposure to fetuin. The centrosphere is now filled with phase-lucent vacuoles as the result of continued pinocytic activity.







(Cohn and Parks: Pinocytosis)