THE UPTAKE, STORAGE, AND INTRACELLULAR HYDROLYSIS OF CARBOHYDRATES BY MACROPHAGES*

By ZANVIL A. COHN, M.D., AND BARBARA A. EHRENREICH, PH.D.

(From The Rockefeller University, New York 10021)

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Recent studies have outlined some of the relationships which exist between various members of the vacuolar system of macrophages (1–5). In response to environmental stimuli, plasma membrane is interiorized as a pinocytic vesicle which then migrates to the peri-Golgi zone of the cytoplasm. Here it acquires lysosomal enzymes, presumably by way of Golgi vesicles, and is converted into a secondary lysosome or digestive body. This is the locus for the intracellular digestion of exogenous molecules, a process which in the case of proteins may be to the level of amino acids (6, 7).

Many questions concerning the macrophage lysosome remain to be answered. These include the permeability of its limiting membrane, the diversity of its hydrolytic enzymes, the fate of nondigestible molecules stored within it, and the role of extracellular hydrolases taken up by pinocytosis. Certain of these problems have been approached under in vitro conditions and through the use of selected digestible and nondigestible molecules. This article will deal with the mechanism of uptake, storage, and hydrolysis of oligosaccharides added to the culture medium of mouse peritoneal macrophages.

Materials and Methods

Macrophage Cultures.—

Leighton tubes: Morphological studies were conducted with unstimulated mouse peritoneal macrophages cultivated on flying cover slips in medium 199 containing varying concentrations of newborn calf serum (NBCS). Components of the medium were obtained from either Microbiological Associates (Bethesda, Md.) or Grand Island Biologicals (Grand Island, N.Y.). The details of this procedure have been reported previously (1). Additions to the medium will be described under Results.

T flask cultures: Mass cultures of homogeneous macrophage populations were prepared in 30 cm^2 T flasks and incubated in medium 199 containing NBCS. Cell suspensions were obtained immediately after the removal of lymphocytes at time zero (T_0) or after 24–48 hr of incubation at 37°C. The preparation and harvesting of cells have been described (1).

Fixation, Microscopy, and Photography—Cells maintained on cover slips were fixed in the

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following manner: The incubation medium was removed from the Leighton tube with a fine-tipped Pasteur pipette and 1.0 ml of ice cold 1.25% glutaraldehyde in phosphate buffer (pH 7.5) added directly over the cover slip. After fixation at 4°C for 10 min the fixative was removed and the tube rinsed twice with distilled water. The cover slip was then immediately inverted over a drop of distilled water and rimmed with a mixture of paraffin and vaseline (1:1). Observations on fixed cells were made with the oil immersion phase contrast objective of the Zeiss Ultraphot II microscope. Photographs of typical cells were prepared with Ansco Versapan 4 × 5 film.

Cells for electron microscopy were prepared according to a previously described method (5). Fixation was accomplished with 1.25% buffered glutaraldehyde for 10 min followed by 15 min fixation in 1% OsO_4. We are indebted to Dr. James G. Hirsch of The Rockefeller University for taking the electron micrographs.

Chemicals.—All the carbohydrates employed in this study were highly purified products obtained from commercial sources.

Sucrose: ^{14}C-UL was obtained from the New England Nuclear Corporation (Boston, Mass.) at an activity of 340 mc/mM.

A purified preparation of *yeast invertase* (Melibiase-free) was obtained from Nutritional Biochemical Corp. (New York) and β-glucosidase from Mann Research Laboratories (New York) at an activity of 1000 units/mg.

Measurement of Radioactivity.—The assay of radioactivity was performed in a Nuclear-Chicago, Mark I scintillation spectrometer. Aliquots of cell suspensions were prepared in Bray's solution and corrected for background and quenching. The results are expressed as disintegrations per minute (dpm). Preparation of the samples will be found under Results.

Enzyme Assays.—The assay of acid phosphatase, β-glucuronidase, and cathepsin have been described (1). Cell suspensions were frozen and thawed six times in a dry ice-alcohol bath before assay. The results are expressed as total activity per flask.

The estimation of the glycosidases in both macrophages and NBCS was performed with synthetic nitrophenyl derivatives. These were obtained from Calbiochem (Los Angeles, Calif.) as *O*-nitrophenyl-β-galactoside, α-glucoside, and β-glucoside respectively. Substrates were prepared in water at 6.0 mg/ml. The reaction mixture contained 0.5 ml enzyme, 0.5 ml substrate, and 1.0 ml 0.1 M acetate buffer, pH 5.0. Incubation took place at 37°C for 4 hr and the reaction was stopped with 1.0 ml of 10% trichloroacetic acid (TCA). After 30 min at 0°C, aliquots of the filtrate, made up to 2.0 ml with water, were employed. Color was developed with 5.0 ml of a reagent containing 200 ml 0.2 M glycine, pH 10.5, and 75 ml 0.5 N NaOH. Color was read immediately at 420 mμ against appropriate blanks. The amount of nitrophenol liberated was calculated from a standard curve which was linear between 0.05–0.35 optical density. The results are expressed as micrograms of nitrophenol liberated per hour per flask.

Hydrolysis of Disaccharides by Macrophage Lysates.—The ability of macrophage enzymes to degrade disaccharides was evaluated in the following manner: Preparations of macrophages which were frozen and thawed six times in a dry ice-alcohol bath were prepared. A reaction mixture consisting of 100 μg macrophage protein, 0.015 M disaccharide in acetate buffer, pH 5.5, was made up to a total volume of 1.0 ml. This was incubated at 37°C for 8 hr and the reaction stopped by freezing the sample at −20°C.

Identification of the substrate and hydrolysis products was performed by means of paper chromatography. Aliquots of the reaction mixture (10–50 μl) were spotted on Whatman No. 1 paper. The chromatogram was placed in a solvent of *n*-butanol:pyridine:water (3:2:1.5) and developed with three ascending runs, each of which required 12 hr at room temperature. The spots were visualized by the silver nitrate method of Trevelyan. This procedure gave adequate separation of the disaccharides and their monosaccharide hydrolysis products. No attempt was made to quantitate the extent of hydrolysis.

RESULTS

General Considerations.—Prior studies on the induction of pinocytic vesicle formation in mouse macrophages indicated that sucrose, an uncharged molecule, had a slight stimulatory effect when added to the macrophages in 1% NBCS medium (8). The unusual feature of this response was that numerous, large, phase-lucent pinocytic vacuoles accumulated in the perinuclear region and persisted for long periods without undergoing the usual transition to the smaller phase-dense granules. This effect of sucrose was examined in more detail and the influence of other carbohydrates was evaluated.

A. Sucrose.—

Morphological observations: The addition of sucrose at concentrations ranging from 0.009–0.03 M to cultivated macrophages resulted in a typical sequence of morphological alterations. Within 2–3 hr after the addition of the higher concentrations, large phase-lucent vacuoles accumulated in the centrosphere region. The number of these structures increased with prolonged cultivation without any obvious injurious effect to the cells. Macrophages exposed to 0.03 M sucrose could be maintained in culture for at least 5 days in a medium containing 20–50% NBCS. In general, the size and number of the vacuoles increased with an elevation in the NBCS concentration of the medium. The presence of sucrose did not interfere with (*a*) the initial spreading of the cells on the glass surface, (*b*) the migration of mitochondria into the pseudopods, nor with (*c*) the formation of pinocytic vesicles. An example of a cell exposed to sucrose (0.029 M) in No. 199–20% NBCS from To-T24 is shown in Fig. 6 *b*. None of the usual phase-dense granules are present and instead a uniform population of phase-lucent vacuoles exists about the nucleus. Other than the large size and decreased density of these vacuoles the cell is otherwise quite normal in appearance.

Electron microscopy of sucrose-vacuolated cells: Examination of vacuolated macrophages with the electron microscope did not show abnormalities in the rough surfaced endoplasmic reticulum, Golgi apparatus, or mitochondria.The major changes were releated to the absence of electron-dense granules and the presence of the sucrose-induced vacuoles. These vacuoles, although relatively electron lucent did contain small amounts of amorphous material and occasional vesicles. What was not apparent from light microscopy were the occasional connections between adjacent vacuoles. Examples of this form of limited vacuolar fusion are illustrated in Fig. 6 *a*. In most cases only a small channel connected the vacuoles. This union may facilitate the mixing of pinocytosed molecules in the sucrose vacuoles.

Factors influencing the sucrose-induced vacuolation: The mechanism by which the sugar was taken up formed the basis of the second group of experiments.

Macrophages were cultivated in Leighton tubes for 24 hr in a medium containing No. 199 and 20% NBCS. At this time the cells contained typical phase-dense granules in the perinuclear region. The cells were then washed in medium 199 and resuspended in fresh 20% NBCS medium. Sets of tubes were then pretreated for 20 min in the presence of (*a*) CO_2-nitrogen, (*b*) 4°C, (*c*) 700 μg/ml DL-parafluorophenylalanine, and (*d*) 10 μg/ml of puromycin. Controls were maintained at 37°C without further additives. After the preincubation period sucrose was added to a final concentration of 0.029 M and the cultures maintained for a period of 3 hr. At this time, the cells were fixed with glutaraldehyde and examined with the phase-contrast microscope. These inhibitors markedly depress the formation of pinocytic vesicles (9).

Under these conditions a 3 hr exposure to sucrose resulted in the extensive formation of phase-lucent vacuoles in over 95% of the control cells. In contrast, cells incubated anaerobically, at reduced ambient temperature or in the presence of inhibitors of protein synthesis, contained few or no typical vacuoles. At the most, 10% of the cells contained an occasional vacuole. It appeared, therefore, that agents which inhibited pinocytic activity also blocked the formation of sucrose-induced vacuoles. These results argue against the uptake of sucrose by diffusion through the plasma membrane, and suggest pinocytic uptake and subsequent storage.

The influence of invertase on sucrose-vacuolated macrophages: The absence of significant sucrase activity in mammalian cells made it likely that the sucrose-induced vacuoles represented the storage of a poorly digested product. If this were the case, then the uptake of the deficient enzyme might be expected to result in the intravacuolar hydrolysis of sucrose.

Macrophages were cultivated in 20% NBCS medium for 24 hr in the presence of 10 mg/ml of sucrose. The cells were then carefully washed free of sucrose with medium 199 and fresh 20% NBCS medium was added to the tubes. 500 μg/ml of yeast invertase was added to one set whereas the other served as control. Preliminary experiments had suggested this concentration of enzyme. The cells were then incubated at 37°C, fixed at times up to 3 hr, and examined under phase contrast.

Sucrose vacuolated cells which were incubated in 20% NBCS medium alone retained their phase-lucent vacuoles for 3 hr in the absence of the sugar (Fig. 7 *a*). In contrast, cells exposed to invertase illustrated striking morphological alterations. Within 1 hr after the addition of the enzyme to the medium, the number and size of the vacuoles were reduced, (Fig. 7 *b*). After 90 and 150 min of incubation, the cells were devoid of large vacuoles and small dense bodies were present in the cytoplasm (Figs. 7 *c*, *d*). This marked shrinkage in vacuole size occurred without obvious damage to the cells at invertase concentrations up to 5 mg/ml. No significant difference in the rate of invertase action could be observed between 0.5–5.0 mg/ml of enzyme.

Factors influencing the effect of invertase: The time course of invertase action is illustrated in Fig. 1 *a*. By 2 hr more than 90% of the cells had lost their large phase-lucent vacuoles as described in the previous section.

Cells were cultivated in 20% NBCS medium with 10 mg/ml of sucrose for 24 hrs, washed, and exposed to 1.0 mg of invertase in 20% NBCS medium. Ficoll, a polysucrose with a molecular weight of 400,000 was also used to load cells at a concentration of 10 mg/ml. The inhibitors employed in Fig. 1 *b* were present 20 min prior to the addition of invertase and for the subsequent 2 hr incubation with the enzyme. 2,4-Dinitrophenol was presented at a final concentration of 10^{-5} M and puromycin at 7.0 μg/ml.

To rule out nonspecific effects of invertase, cells were loaded with Ficoll, a sucrose polymer which is not appreciably degraded by the enzyme. This

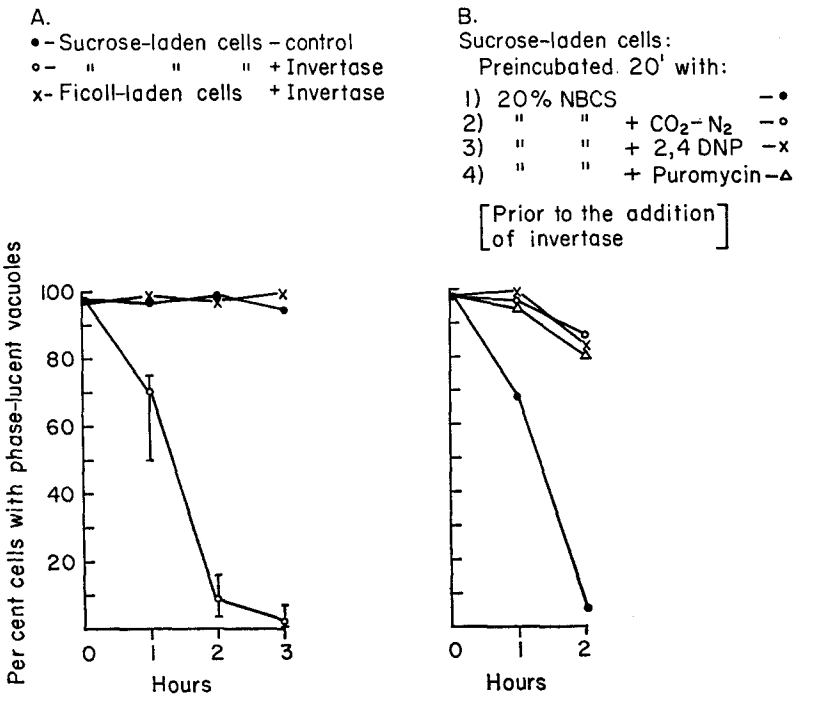

FIG. 1. A. The influence of invertase on the phase-lucent vacuoles of sucrose and Ficoll-laden macrophages. B. The inhibition of the invertase effect by conditions which block pinocytic activity.

material produced vacuoles which were identical to those of sucrose. As shown in Fig. 1 *a* the addition of invertase had no effect on the Ficoll-vacuolated cells.

It seemed reasonable from this information that invertase was taken up by the macrophages, distributed within sucrose containing vacuoles, and hydrolyzed the disaccharide in this locus. Additional experiments illustrated in Fig. 1 *b* indicate that the effect of invertase is effectively blocked by inhibiting the pinocytic activity of sucrose-laden macrophages. It seemed,

therefore, that the uptake of both the substrate (sucrose) and enzyme occurred by pinocytosis.

The rate of invertase uptake by macrophages: The rate at which effective concentrations of invertase were taken up by the cell was then studied.

Macrophages were exposed to 0.029 M sucrose for 24 hr in 20% NBCS medium. The cells were washed free of sucrose and resuspended in fresh 20% NBCS medium. At 0 time, 750 μg/ml of invertase was added to all tubes and incubation was carried out at 37°C. At intervals between 5–120 min, sets of tubes were washed free of invertase with two 4-ml aliquots of 20% medium and incubated in enzyme free medium. All incubations were continued for 120 min after the invertase had been removed from the system. This was the period required for the disappearance of vacuoles in 95% of cells maintained in the continued presence of the enzyme. Cells maintained for 4 hr in 20% NBCS medium in the absence of invertase were fully vacuolated.

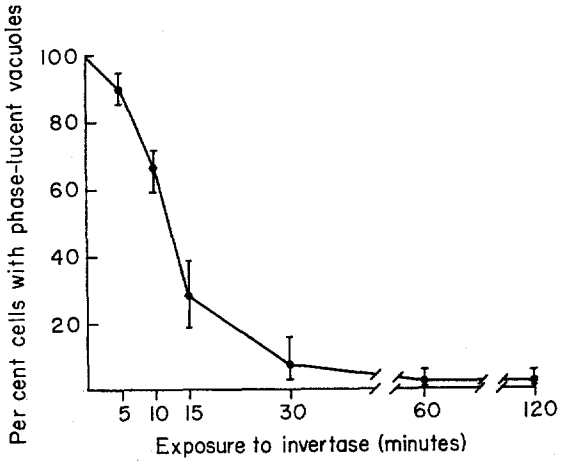

FIG. 2. The uptake of invertase by sucrose-laden macrophages.

The results of such experiments are shown in Fig. 2. It is apparent that the exposure of sucrose-laden cells to invertase for as short a period as 15 min resulted in the subsequent disappearance of vacuoles in approximately 70% of the cells. This indicated that sufficient enzyme was taken up at this interval to alter the majority of the cytoplasmic vacuoles at later intervals. After 30 min of exposure, the reaction reached completion and was not significantly different at 60 and 120 min.

The uptake, release, and intracellular hydrolysis of sucrose-^{14}C: The morphological observations strongly suggested that sucrose was contained within the phase-lucent vacuoles and was susceptible to hydrolysis by invertase. More definitive biochemical information was obtained through the use of labeled sucrose in mass cultures of mouse macrophages.

T flasks were prepared in the usual manner and incubated in 20% NBCS No. 199 at 37°C. 2 hr after cultivation was started, 10 μc/ml of sucrose-^{14}C-UL in the presence of 10 mg/ml of carrier sucrose was added to the flasks and incubation continued for an additional 22 hr. At this time, the radioactive medium was carefully removed and the cell sheet was successively washed with 12 ml of No. 199, 12 ml 20% NBCS, and 12 ml of No. 199. These three washes were sufficient to remove all extracellular isotope. The washed cells were then resuspended in 20% NBCS medium. At this point, one-half the flasks were exposed to 500 μg/ml of invertase and the rest served as controls. Flasks were then harvested from T_0-T_3 in the following manner. The incubation medium was aspirated and stored at −20°C for assay. The cell sheet was then rinsed four times with 15 ml of warm saline and 3 ml of saline were added to cover the cells. They were then removed from the glass surface by six cycles of freezing and thawing and stored at −20°C.

1 ml aliquots were precipitated with 10% TCA (final concentration) in the presence of 2 mg of bovine serum albumin. The TCA pellet was washed with 5% TCA and the residue dried at room temperature. The dried pellet was then dissolved in formic acid for radioassay. Aliquots of the medium, TCA supernatant fluid, and TCA precipitate were then added to Bray's solution and counted in the scintillation spectrometer. Other aliquots of the cell suspension were assayed for protein and hydrolytic enzymes.

In other experiments, cells were exposed to different environmental conditions, including inhibitors, and then exposed to labeled sucrose. After 24 hr cultivation in 20% NBCS, the cells were washed with medium 199. This was then replaced with (*a*) 10% NBCS,% (*b*) 20 NBCS, (*c*) 50% NBCS, (*d*) 50% NBCS containing 1.0 mg/ml *p*-fluorophenylalanine, and (*e*) 50% NBCS containing 5×10^{-4} M 2,4-dinitrophenol. After a 1 hr preincubation period, 2.0 μc/ml of sucrose-^{14}C were added and the flasks incubated for an additional 6 hr. They were then processed as described in the previous section.

The uptake of sucrose during the first 22 hr was relatively extensive. All of the radioactivity contained in the harvested macrophages was acid soluble and none was present in the acid insoluble compartment. Chromatography of an ether-washed TCA extract revealed a single radioactive spot which corresponded to unlabeled sucrose. Assuming the equivalent uptake of carrier and labeled sucrose, it was calculated that the cells contained approximately 0.8 μg sucrose/μg cell protein.

Table I illustrates the influence of serum concentration and selected inhibitors on the accumulation of sucrose-^{14}C during a 6 hr incubation. As the newborn calf serum concentration of the medium was elevated, more sucrose accumulated in the macrophages. When *p*-fluorophenylalanine or 2,4-dinitrophenol were added to 50% NBCS, there was a significant inhibition of uptake. These data are in keeping with the postulate that sucrose is taken up by a pinocytic mechanism and complement the previously mentioned experiments on the vacuolization of macrophages (Fig. 1).

Cells which were pulsed with sucrose-^{14}C were employed for the studies illustrated in Fig. 3 (lower). The macrophages which were placed in 20% NBCS medium alone did not lose any of their intracellular sucrose for a period of 3 hr. When invertase was added to the medium, a prompt fall in cell radioactivity occurred with the majority released by 3 hr. All of the isotope lost from the cells could be recovered in the incubation medium. Chromatographic

examination of the excreted products revealed the presence of only labeled glucose and fructose. This suggested that intravacuolar sucrose was being hydrolyzed by invertase and the majority of the glucose and the fructose residues were rapidly excreted into the extracellular fluid. This change in the distribution of isotope occurred without any loss of cell protein or acid hydrolases (Fig. 3, upper).

The effect of sucrose on the content of macrophage lysosomal enzymes: The effect of sucrose on the in vitro production of macrophage acid hydrolases was next examined. A typical experiment is illustrated in Fig. 4. It is apparent that sucrose increases the cell content of protein, acid phosphatase, β-glucuronidase, and cathepsin. This is a dose-dependent response with a maximum reached between 10–12 mg/ml. The accumulation of enzymes continues above control levels for at least 48 hr of incubation but was not studied at later time points.

TABLE I

*The Uptake of Sucrose-^{14}C by Cultivated Mouse Macrophages**

Test media	Uptake of sucrose-^{14}C dpm/ml cell suspension
10% NBCS	1200
20% NBCS	2000
50% NBCS	2940
50% NBCS + 1 mg/ml D,L *p*-fluorophenylalanine	1100
50% NBCS + 10^{-4} M 2,4 dinitrophenol	200

* 60 min preincubation with test media, then 6 hr incubation with 2 μc/ml sucrose ^{-14}C.

The mechanism underlying this stimulation is still unclear but may reflect increased endocytic activity.

The cytochemical distribution of acid phosphatase in sucrose-vacuolated cells: The distribution of acid phosphatase activity was examined in control and sucrose vacuolated cells.

Macrophages were cultivated in 20% NBCS medium in the presence or absence of 0.029 M sucrose for 22 hr at 37°C. The cells were then fixed with glutaraldehyde (1.25%) for 10 min at 4°C and stained for acid phosphatase activity using β-glycerophosphate as substrate (1). Incubation in substrate took place at 37°C for 20 min. The cover slips were then mounted in distilled water and viewed with either bright-field or phase-contrast optics.

Fig. 8 illustrates the appearance of typical cells in the preparation. The majority of the sucrose vacuolated cells (Fig. 8 *b*) demonstrated a more intense staining pattern than seen in control cells (Fig. 8 *a*). This was associated with larger, perinuclear organelles which corresponded to the phase-lucent vacuoles. A small proportion of cells showed a patchy distribution of reaction

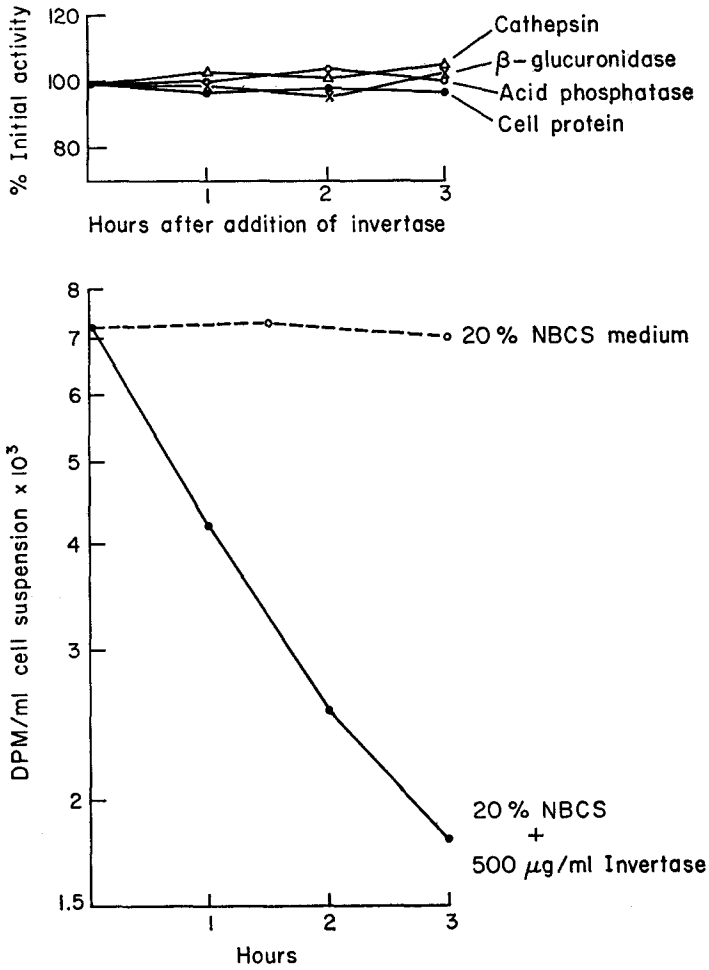

FIG. 3. (Upper) The level of macrophage acid hydrolases and protein during the course of invertase action. (Lower) The effect of invertase and 20% NBCS on the content of sucrose-^{14}C-laden macrophages.

product in which many of the large vacuoles were unreactive. This is illustrated in Fig. 8 *c, d*. It was the general impression, however, that most of the sucrose-filled vacuoles had acquired enzyme during their residence in the perinuclear region and can be considered to be secondary lysosomes. This impression was enforced when the Gomori procedure was employed at the electron microscope level. In this case almost every large vacuole contained reaction product.

B. Other Neutral Carbohydrates.—

In view of the experience with sucrose, the effects of other carbohydrates were examined. Table II illustrates the morphological response of macrophages after exposure to various monosaccharides for 24 hr of cultivation. None of the

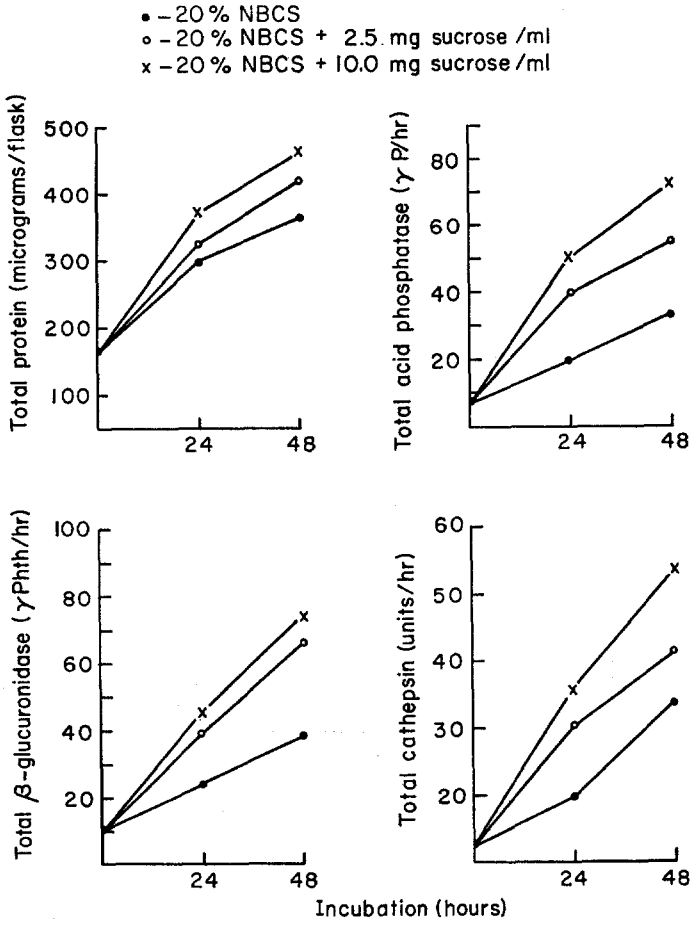

FIG. 4. The level of acid hydrolases and protein in macrophages exposed to sucrose.

sugars (C5–C7), irrespective of whether or not they were metabolizable by the cells, produced any vacuolization at concentrations of 10–20 mg/ml. Cells exposed to these agents were morphologically identical with macrophages cultivated in serum alone.

The only monosaccharide which produced a striking morphological effect was 2-deoxyglucose. This sugar analogue is actively phosphorylated by

hexokinase and accumulates intracellularly as 2-deoxyglucose-6-PO_4. When added at the beginning of cultivation (T1-T24), extensive cell death occurred at the higher concentrations. The surviving population, however, exhibited short pleomorphic mitochondria and contained few dense granules and peripheral pinosomes. The addition of 2-deoxyglucose after 24 hr of cultivation resulted in the cessation of pinocytic activity and a gradual reduction in size of preexisting secondary lysosomes.

The morphological effect of oligosaccharides: The lack of phase-lucent vacuole

TABLE II

The Influence of Monosaccharides on the Morphology of Macrophages

Sugar	Concentration	Exposure	Morphology
	mg/ml	*hr*	
Ribose	10–20	T_1–T_{24}	No phase-lucent vacuoles present in cytoplasm. Normal complement of dense granules, identical with controls in 20% NBCS-199.
Arabinose	"	"	
Xylose	"	"	
Glucose	"	"	
Fructose	"	"	
Galactose	"	"	
Sorbose	"	"	
Sedoheptulose	"	"	
2-deoxyglucose	10	T_1–T_{24}	40% cell death, 50% small round cells.
	5	"	10% spread with short plemorphic mitochondria inhibition of pinocytic vesicle formation.
	2.5	"	No phase-lucent vacuole formation.
	1.0	"	
	5.0	T_{24}–T_{48}	Cessation of pinocytosis, shortening of mitochondria.
	2.5	"	Reduction in size of preexisting dense granules.

production by monosaccharides, with molecular weights up to 220, prompted additional experiments with larger molecules. The morphological response to a variety of di-, tri-, and tetrasaccharides is shown in Table III and presents information on the configuration of the sugar, concentrations tested, and the vacuolar response. The typical morphological response to certain of these agents is illustrated in Fig. 9.

Some of the glucosides differed only in terms of linkage and optical configuration. The α-glucoside, trehalose, which was linked in the 1-1 position produced vacuolization whereas neither maltose or isomaltose, both α-glucosides, produced vacuole formation. This was of interest since trehalose is not

degraded by α-glucosidase. In contrast, both the β-glucosides, cellobiose and gentiobiose, resulted in vacuolization identical with that of sucrose and turanose.

Neither of the galactosides, whether in the α or β configuration gave a vacuolar response at concentrations up to 20 mg/ml; whereas raffinose and stachyose, tri- and tetrasaccharides respectively, yielded a positive response.

The response of cellobiose-laden cells to β-glucosidase: The response of sucrose-

TABLE III

*The Effect of Di-, Tri-, and Tetrasaccharides on Macrophage Morphology**

Carbohydrate	Configuration‡	Concentration	Morphology
		mg/ml	
Trehalose	α 1–1 G-G	3–15	> 95% cells with phase-lucent vacuoles
Maltose	α 1–4 G-G	1–20	Normal, phase-dense granules
Isomaltose	α 1–6 G-G	1–20	" "
Cellobiose	β 1–4 G-G	3–20	>95% cells with phase-lucent vacuoles
Gentiobiose	β 1–6 G-G	"	" "
Sucrose	α 1–2 G-F	3–20	>95% of cells with phase-lucent vacuoles
Turanose	α 1–3 G-F	"	" "
Melibiose	α 1–6 Gal-G	5–20	Normal phase-dense granules
Lactose	β 1–4 Gal-G	"	" "
Raffinose	α 1–6 Gal-G-F α 1–2	5–15	>95% cells with phase-lucent vacuoles
Stachyose	α 1–6 G-Gal-Gal-F α 1–6 α 1–2	"	" "

* Sugars added to cells at T_1 and incubated at 37°C for 24 hr in 20% NBCS medium. Fixed with glutaraldehyde and scored under oil immersion phase-contrast optics.

‡ G, glucose; F, fructose; Gal, galactose.

vacuolated cells to invertase suggested a similar experiment with other disaccharides. Cellobiose was selected for this purpose since a reasonably active β-glucosidase was commercially available.

Macrophages were cultivated in Leighton tubes for 24 hr in 20% NBCS medium containing either 10 mg/ml of sucrose or cellobiose. At this time the cells contained well-defined phase-lucent vacuoles in the perinuclear region, and the groups were morphologically undistinguishable. The incubation medium was removed, the cell sheet washed with No. 199 and fresh 20% NBCS medium added to the tubes. At T_0, invertase and β-glucosidase were added to each set at previously defined concentrations. Cover slips were then fixed at intervals up to 3 hr and the number of vacuolated cells scored by phase-contrast microscopy.

The data of a typical experiment are illustrated in Fig. 5. Results with sucrose-laden cells are shown on the left side and indicate that the addition of β-glucosidase had no effect on the size of the vacuoles, whereas invertase produced a prompt shrinkage. The opposite was true with cellobiose-laden cells in which the addition of β-glucosidase produced the loss of vacuoles. From this result as well as previously described experiments it seems tht the vacuole-shrinking capacity of a pinocytized enzyme is substrate specific.

The glycosidase activity of macrophages and calf serum: From the previous results it appeared that certain carbohydrates with at least the molecular

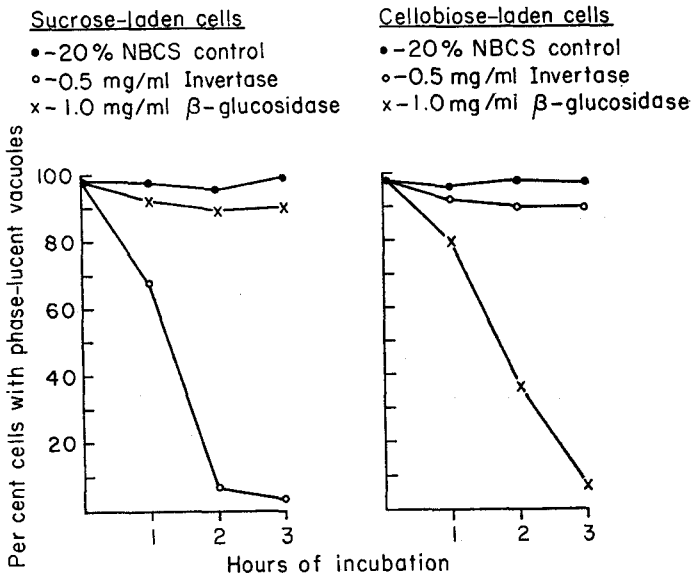

FIG. 5. The influence of β-glucosidase and invertase on the phase-lucent vacuoles of cellobiose and sucrose-laden macrophages.

weight of disaccharides were capable of producing long-lasting, phase-lucent vacuoles in macrophages. This was not true with all disaccharides and appeared to be related in part to the configuration of the carbohydrate linkage. The lack of activity of certain sugars might then be related to their hydrolysis by either serum or cells whereas the active agents would remain intact. The question was examined by assaying both serum and cells for their hexosidase activity on three synthetic substrates as shown in Table IV.

No β-glucosidase activity was discernible in macrophage extracts obtained either prior to or after cultivation, nor in calf serum. There was, however, appreciable enzyme activity against the α-nitrophenyl glucopyranoside substrate. Most of the α-glucosidase was present in the serum and small quantities

were found in the cells. The level of macrophage α-glucosidase increased with the length of cultivation and was twofold above initial levels after 48 hr. Considerably more β-galactosidase activity was present in the macrophages, increased with time of cultivation, and was absent from the specimens of calf serum tested.

TABLE IV

Activity of Hexosidases in Macrophages and Newborn Calf Serum

Source of Enzyme	Cultivation in vitro	β-Glucosidase μg nitrophenol/hr	α-Glucosidase μg nitrophenol/hr	β-Galactosidase μg nitrophenol/hr
	hr			
Macrophage	T_0	<2	3	23
Macrophage	T_{24}	<2	5	48
Macrophage	T_{48}	<2	7	76
Serum				
0.5 ml 1%	—	<2	56	<2
0.5 ml 20%	—	<2	163	<2

TABLE V

The Digestion of Disaccharides by Macrophage Extracts

Disaccharide	Configuration*	Phase-lucent‡ vacuole formation	Hydrolysis by macrophage extracts§	Hydrolysis Products
Trehalose	α 1-1 G-G	+	0	—
Maltose	α 1-4 G-G	0	+	G
Isomaltose	α 1-6 G-G	0	+	G
Cellobiose	β 1-4 G-G	+	0	—
Gentiobiose	β 1-6 G-G	+	0	—
Sucrose	α 1-2 G-F	+	0	—
Turanose	α 1-3 G-F	+	0	—
Melibiose	α 1-6 GAL-G	0	+	GAL, G
Lactose	β 1-4 GAL-G	0	+	GAL, G

* G, glucose; F, fructose; GAL, galactose.

‡ +, vacuole formation; 0, no vacuoles.

§ +, hydrolyzed; 0, not hydrolyzed.

This information correlated well with the vacuole-producing effect of the disaccharides and suggested that the presence of the hexosidase either in serum or cells precluded vacuole formation by the substrate.

The hydrolysis of disaccharides by macrophage lysates: A more direct method of demonstrating the ability of macrophages to degrade the natural disaccharides was next explored. Macrophage lysates were incubated with the

disaccharides and after 8 hr the reaction mixture was examined by paper chromatography. The results are outlined in Table V. It is clear that all of the disaccharides which produced vacuolization were not hydrolyzed by macrophage extracts. In contrast, those agents which did not vacuolize the macrophage were readily degraded by the extract. Although these results are qualitative in nature, they do indicate that the presence or absence of the macrophage hydrolases is correlated with morphological response to the disaccharides.

DISCUSSION

The exposure of an actively pinocytizing cell to relatively low concentrations of sucrose results in a rapid and extensive change in its lysosomes. Sucrose and other nonutilizable disaccharides appear to be taken up by a pinocytic mechanism and transported into the perinuclear region of the cell. Here, pinocytic vesicles fuse with preexisting secondary lysosomes, mixing their contents, with the ultimate formation of a large, phase-lucent vacuole. The stability of the vacuole probably depends upon the intravacuolar concentration of sucrose and the permeability of the lysosomal membrane to the disaccharide. It seems reasonable to speculate that the presence of a relatively impermeable, osmotically active and nondigestible molecule within the lysosome alters the transport of water and results in a more hydrated organelle. Such a scheme is also in keeping with the structural alterations which result from the intravacuolar hydrolysis of sucrose by invertase. In this instance, a pinocytosed enzyme is rapidly distributed to the sucrose laden lysosomes. Intralysosomal hydrolysis of sucrose then takes place with the formation of glucose and fructose residues and their escape from the vacuole. The resulting decrease in the osmolarity of the lysosomal contents may then lead to the loss of water and the rapid shrinkage of the vacuole. It is not clear at this time whether the loss of water and low molecular weight hydrolysis products is an active or passive process.

The results with other mono- and oligosaccharides may be explained on a similar basis. None of the monosaccharides, whether or not they can be metabolized by the macrophage, are able to produce vacuolization of the lysosomes. Molecules of this size are presumably able to permeate both the plasma and lysosomal membrane and thereby never reach sufficiently high concentrations within the lysosome to produce osmotic effects. The uptake of monosaccharides is unrelated to an endocytic mechanism in mammalian cells but can occur by this means in protozoa (10).

When one employs disaccharides with molecular weights in the neighborhood of 360, membrane permeability becomes a limiting factor and vacuolization can take place. These molecules appear to be taken up by a pinocytic mechanism and transported in bulk within a membrane-bounded vesicle. Their subsequent influence on the lysosome is then critically dependent upon

the ability of the cell to mobilize specific hydrolases. Those agents which cannot be rapidly hydrolyzed have effects similar to sucrose whereas others that can be split by either macrophage or serum enzymes are degraded to permeable subunits. In all cases studied thus far the ability of a disaccharide to become stored in the lysosome is correlated with the absence of the specific macrophage glycosidase. More detailed observations on the influence of peptides will be described in a subsequent article.

Many other larger carbohydrates are also pinocytized by cultured macrophages and stored within lysosomes. Studies with dextran, dextran sulfate, inulin, and Ficoll have been performed and indicate that these larger molecules are maintained within the cell for longer periods of time. In the case of dextran sulfate, metachromatic-staining granules are found within the cell in unchanged numbers for at least 5 days after the removal of the agents from the medium. Such cells have the general appearance of the tissue histocytes seen in a variety of inborn errors of metabolism in which a macromolecular product is retained and stored within membrane-bounded cytoplasmic structures. Certain of these disease states are associated with a deficiency of specific lysosomal hydrolases and may result from the endocytic or autophagic segregation of a nondigestible product. The use of actively endocytic cells such as blood monocytes and macrophages may offer advantage in the study of the presence of lysosomal enzymes and their deficiencies in disease states. Through the use of appropriately selected substrates it may be possible to screen cells for the storage of these materials by virtue of their ability to alter the morphology of the lysosomes. An example from these experiments would be the vacuolization and storage of a β-glucoside (cellobiose) and the absence of any detectable β-glucosidase activity in mouse macrophage extracts. In man, a readily available source of cells exists in the blood monocyte, and simple methods are available for their isolation and culture (17).

A number of related observations have been reported in intact animals and in other cell systems (16). It has long been known that hypertonic sucrose produces vacuolization of hepatic and renal cells (11, 12). More recently these vacuoles have been shown to be lysosomal in nature (13), to contain labeled sucrose (14), while their formation is blocked by the prior uptake of invertase (15).

SUMMARY

The exposure of cultivated mouse macrophages to sucrose (0.009–0.03 M) leads to the formation of large phase- and electron-lucent, acid phosphatase–positive vacuoles in the perinuclear region. The vacuolization process and the uptake of sucrose-^{14}C is blocked by inhibitors of pinocytosis and stimulated by calf serum in the medium. These results suggest the uptake of sucrose by pinocytosis and its subsequent segregation and storage in secondary lysosomes.

The addition of sucrose also increases the total content of three macrophage lysosomal hydrolases.

The addition of invertase to the environment of sucrose-laden macrophages leads to the prompt shrinkage of the sucrose-containing lysosomes. This is accompanied by the intracellular hydrolysis of sucrose to fructose and glucose residues which are promptly excreted into the medium. The uptake of invertase, as indicated by the shrinkage of sucrose-containing vacuoles, is blocked by inhibitors of pinocytosis. No effect was noted when invertase was added to macrophages laden with Ficoll, a polysucrose which is not hydrolyzed by the enzyme.

The influence of other carbohydrates was then investigated. Monosaccharides with molecular weights up to 220 did not produce vacuolization. However, a certain number of di-, tri-, and tetrasaccharides produced vacuolization identical with that of sucrose. Each of the disaccharides which produced vacuolization was resistant to the complement of macrophage hexosidases, whereas those that were ineffective were degraded by either macrophage or serum enzymes. The addition of β-glucosidase to cellobiose-laden macrophages resulted in the shrinkage of vacuoles but did not alter the vacuoles of sucrose containing cells.

The ability of small, neutral carbohydrates to produce lysosomal swelling is dependent upon both molecular weight and their resistance to lysosomal hydrolases.

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FIG. 6. Macrophages exposed to 0.02 M sucrose. *a*. Electron micrograph of a cell cultivated in sucrose for 24 hr. The membrane-bounded vacuoles are less dense than the typical secondary lysosome but do contain an amorphous material as well as occasional vesicles. Some of the vacuoles are in close contact but do not actually fuse. × 12,000. *b*. Macrophage cultivated for 72 hr in sucrose. The cytoplasm is filled with phase-lucent vacuoles with occasional highly refractile lipid droplets. Phase-dense secondary lysosomes are not present. Phase-contrast. × 2,200.

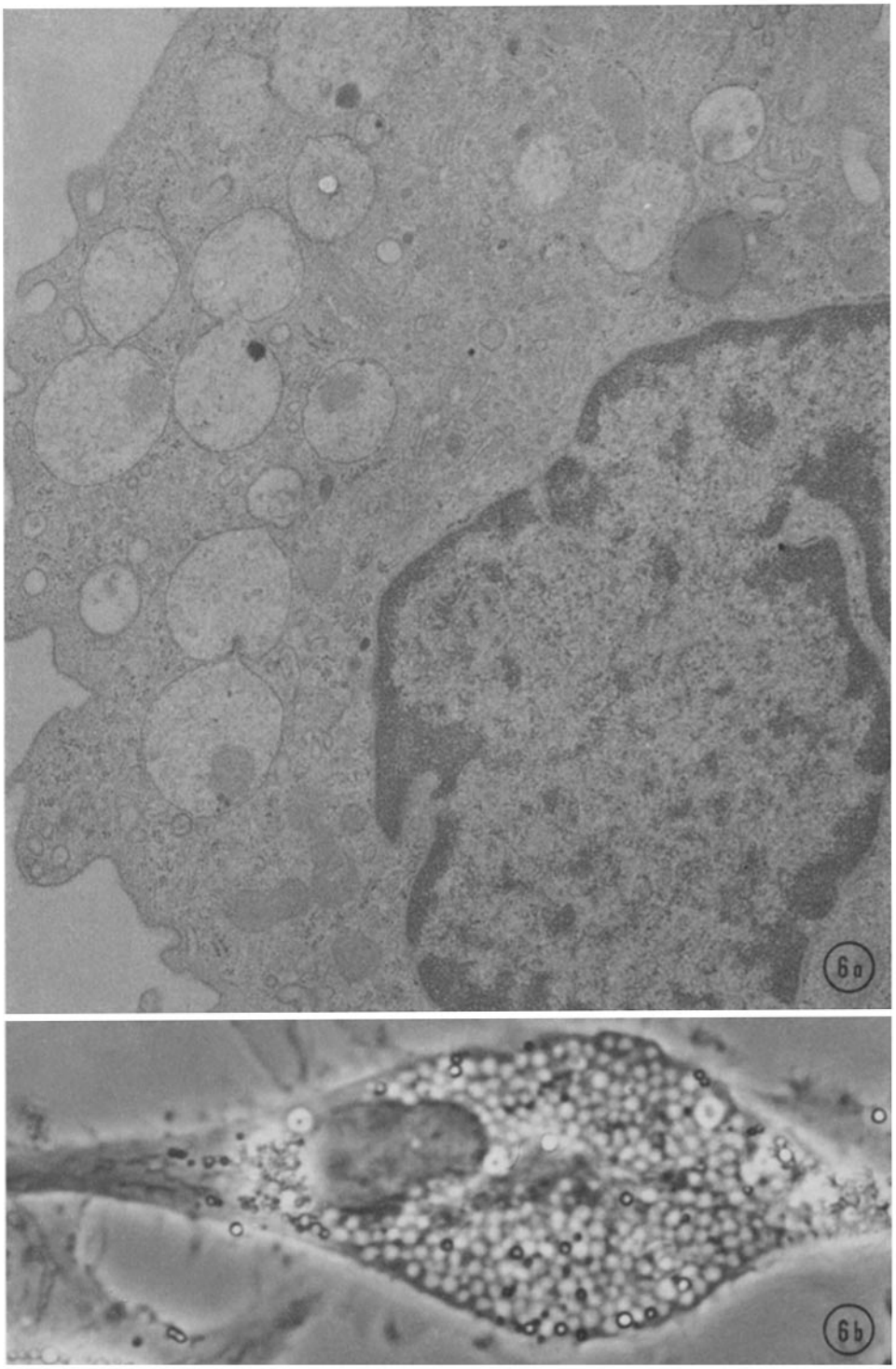

6a
6b

FIG. 7. The effect of invertase on the morphology of sucrose vacuolated macrophages. Phase-contrast. × 2500.

a. Immediately after the addition of 500 μg/ml of invertase. The cells had been cultivated for 24 hr with 0.02 M sucrose, washed, and placed in fresh 20% NBCS medium. *b*. 30 min after the addition of invertase there is a decrease in the number and perhaps the size of the vacuoles. *c*. 75 min after the addition of invertase most of the phase-lucent vacuoles have shrunk. *d*. By 120 min all the large vacuoles have disappeared leaving a residue of tiny dense granules.

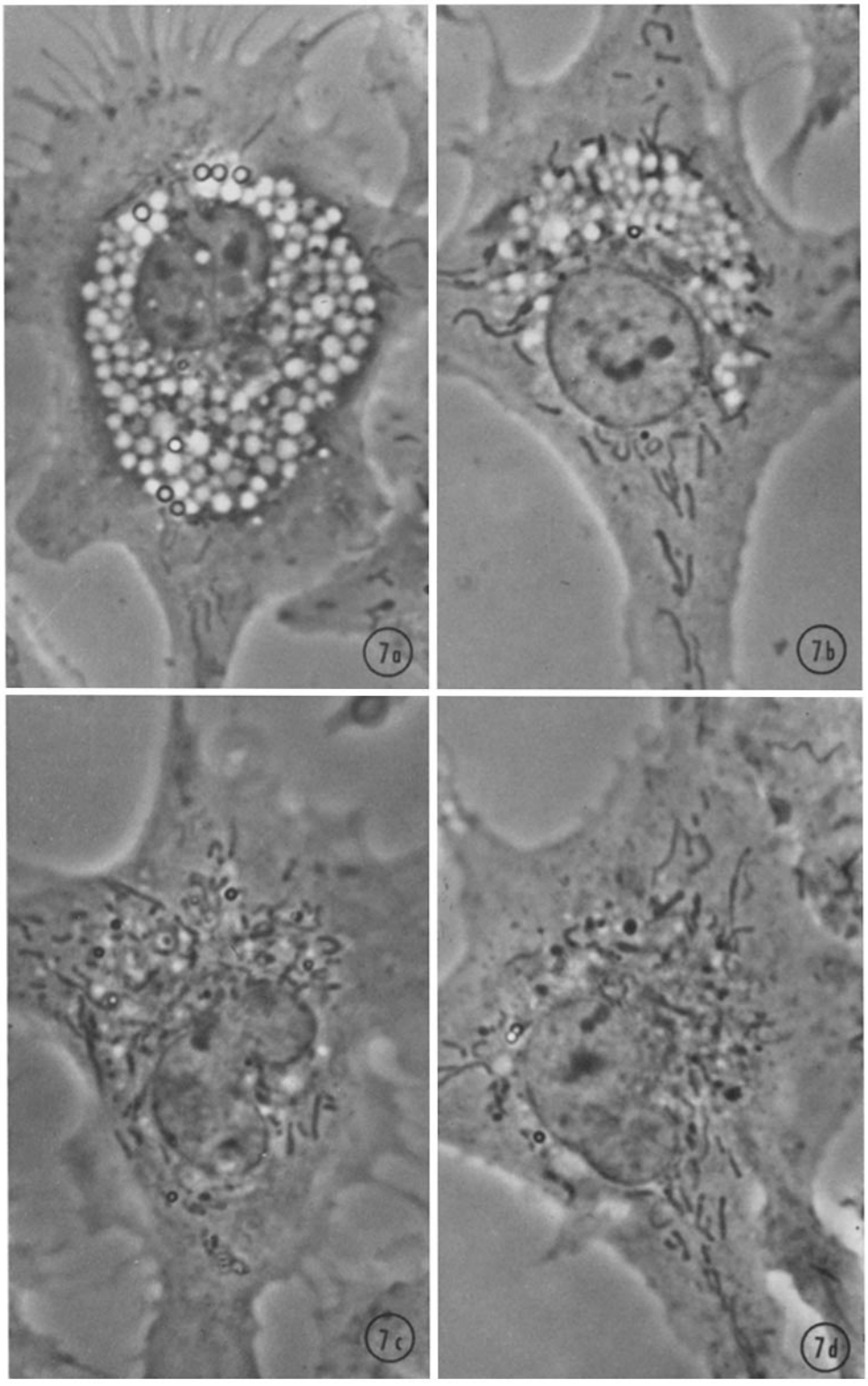

7a
7b
7c
7d

FIG. 8. Acid phosphatase stains of cultivated macrophages. Phase-contrast. × 2500.

a. Control cell cultivated in 20% NBCS medium for 24 hr. A moderate number of small granules exhibit reaction product. *b*. Cell cultivated in 20% NBCS containing 0.02 M sucrose. Many more positive granules are present and these are larger than the control. *c, d*. Same as *b* except to illustrate the patchy reaction produced in a small number of sucrose vacuolated cells.

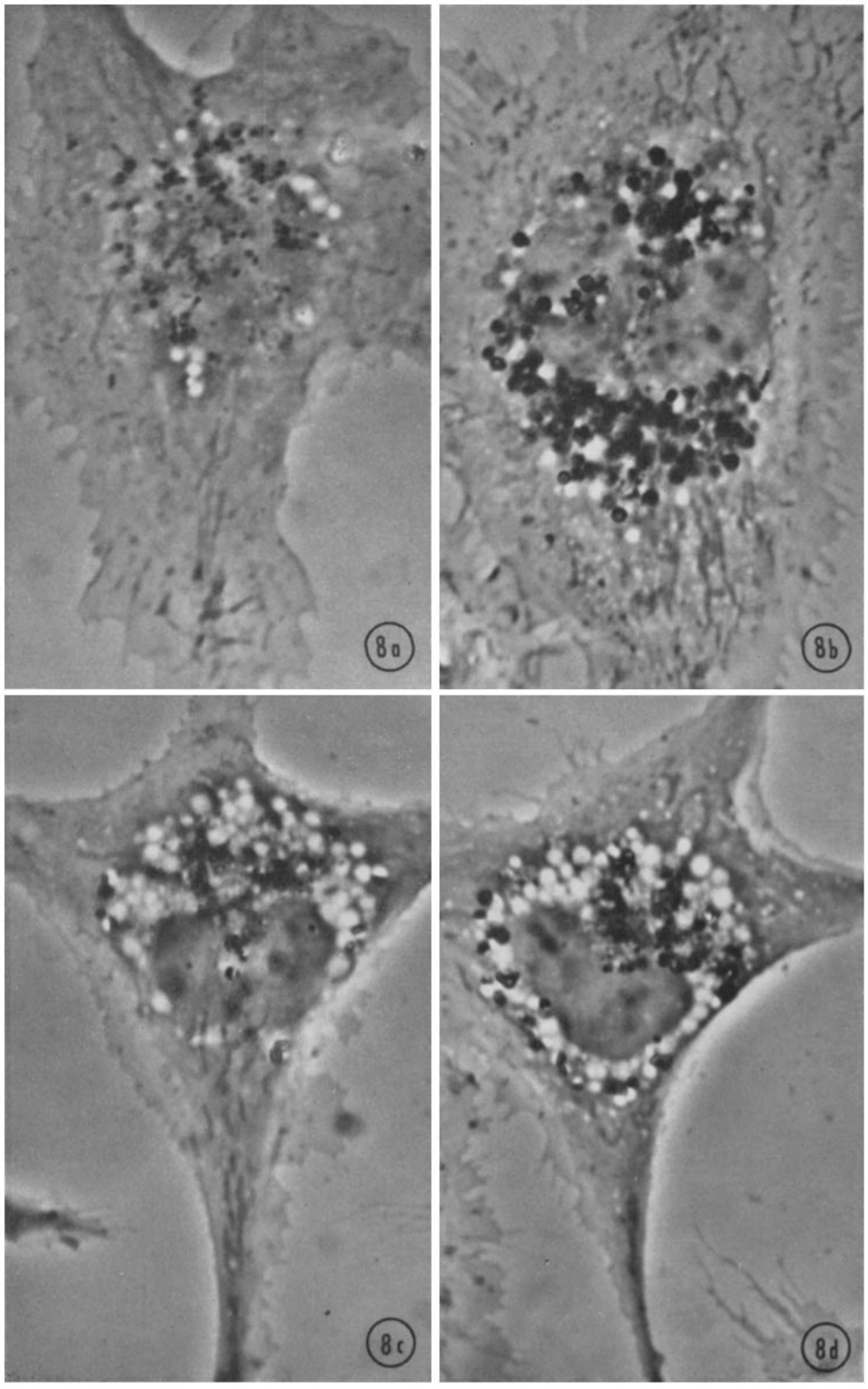
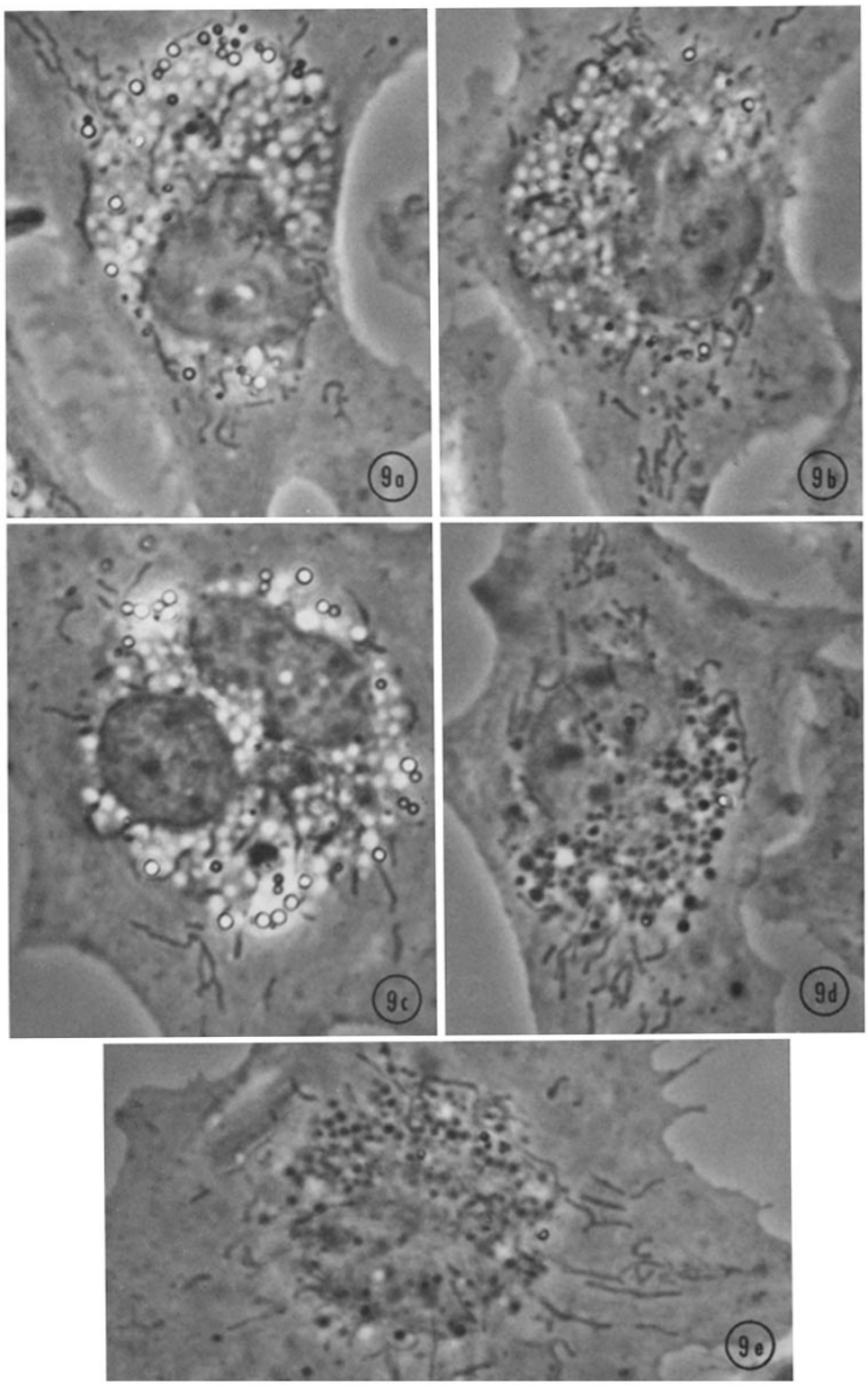

8a
8b
8c
8d

FIG. 9. The morphology of cells exposed to 0.03 M concentrations of disaccharides for 24 hr. Phase-contrast. × 2500.

a. Cellobiose; *b*, trehalose; *c*, turanose; *d*, maltose; *e*, lactose. Compounds *a–c* produce typical vacuolization of the cytoplasm whereas *d* and *e* did not alter the appearance of the secondary lysosomes.


9a
9b
9c
9d
9e