EFFECTS OF CONCANAVALIN A ON MOUSE PERITONEAL MACROPHAGES

II. Metabolism of Endocytized Proteins and Reversibility of the Effects by Mannose*

By PAUL J. EDELSON‡ AND ZANVIL A. COHN

(From The Rockefeller University, New York 10021)

In the preceding paper (8) we described the vesiculation which develops in mouse peritoneal macrophages shortly after their exposure to concanavalin A (Con A).[1] We showed that these vesicles are pinosomes which are generated as a result of the interaction of the macrophage plasma membrane with the saccharide binding sites of the Con A molecule. Once formed, these vesicles persist in the macrophage cytoplasm for hours, and show no microscopic evidence of fusion with either primary or secondary lysosomes.

In this paper, we have studied the capacity of the Con A-induced vesicles to fuse with lysosomes by measuring the degradation rates of horseradish peroxidase (HRP), or [125I]bovine albumin ([125I]BSA) which have been incorporated into the Con A vesicles. In addition, we have examined the effect that mannose, a sugar capable of competing for the Con A saccharide binding site, has on the fate of previously formed Con A-pinosomes, as judged by fluorescence and electron microscopy, and by its effects on the rates of degradation of exogenous proteins.

Materials and Methods

Techniques for cultivation of mouse peritoneal macrophages, and for light and electron microscopic study of the cells were as described in the preceding paper (8).

Preparation of Con A-Fluorescein Conjugate. Con A was conjugated to fluorescein isothiocyanate (FITC) using Rinderknecht's general technique (22) as described by Nicolson (17). FITC adsorbed to cellite was purchased from Calbiochem, San Diego, Calif. 50 mg of FITC-cellite and 100 mg Con A were mixed in 1 ml of 0.3 M NaCl-0.1 M α-methyl-mannose-0.5 M sodium bicarbonate buffer, pH 8.8, and agitated for 3 min at room temperature. The cellite was centrifuged away and the supernate, containing the conjugate and free FITC, was loaded on a 30 cm x 2 cm column of Sephadex G-25

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[1] *Abbreviations used in this paper:* [125I]BSA, radio-iodinated bovine serum albumin; Con A, concanavalin A; Con A-FITC, Con A-fluorescein isothiocyanate conjugate; HRP, horseradish peroxidase; αMM, α-methyl-D-mannose.

(Pharmacia Fine Chemicals, Inc., Piscataway, N. J.), equilibrated with 0.1 M sodium phosphate buffer, pH 7.4, and eluted with the same buffer. The conjugate was collected in the exclusion volume of the column. Fluorescein content was estimated by the absorbance of the conjugate at 490 nm, in the presence of NaOH, and protein content by the absorbance at 280 nm. The ratio OD_{490}/OD_{280} = 1.009/1.008 for a 1:10 dilution of the original conjugate. Hemagglutinating activity for trypsinized sheep erythrocytes, and for rabbit erythrocytes as determined by the method of Person and Markowitz (18) was unchanged for the conjugate as compared with native Con A. The conjugate was shielded from light and stored at −20°C, until used.

Fluorescence Microscopy. Cells were exposed, for various periods of time, at either room temperature or 4°C, to dilutions of FITC-Con A conjugate. Cover slips were then washed in phosphate-buffered saline PBS, fixed for 20 min in a mixture of 100 ml 96% ethanol:5 ml glacial acetic acid (30), rinsed in distilled water, mounted in glycerol (Buffered Glycerol Mounting Medium, Progressive Laboratories, Baltimore, Md.), and refrigerated in the dark until examined.

Preparations were examined with a Zeiss microscope (Carl Zeiss, Inc., New York) generously made available to us by Dr. John Zabriskie, The Rockefeller University, equipped with incident fluorescence, with a mercury light source, using a B 3 12 primary filter, and an OG 49 secondary filter. Photographs were taken on Kodak 35 mm Tri-X film, using exposure times of 2-4 min (Eastman Kodak Co., Rochester, N.Y.).

Cathepsin D Assay. Cathepsin D was assayed in cell lysates in 0.05% Triton X-100 as described in (5). Denatured beef hemoglobin prepared from beef hemoglobin, Type I (Sigma Chemical Co., St. Louis, Mo.) was used as substrate, and the reaction was carried out for 200 min at pH 3.6 in a large excess of substrate. The reaction was stopped, and the undegraded protein precipitated, with 3.5% trichloroacetic acid (TCA) on ice. The unprecipitated protein was then assayed by the Lowry technique (8).

Spectrophotometric Assay for HRP. HRP activity was measured using the assay of Steinman and Cohn (24) employing *o*-dianisidine as the oxygen acceptor. The assay was carried out in 0.1 M sodium phosphate buffer, pH 5.0 containing 0.003% hydrogen peroxide and 0.083 mg/ml 0-dianisidine (Sigma Chemical Co., St. Louis, Mo.) previously prepared as a 1 g% (wt/vol) solution in absolute methanol. Change in absorbance at 460 nm with time was measured on a Gilford recording spectrophotometer (model no. 240, Gilford Instrument Laboratories, Oberlin, Ohio), and the slope of the initial linear graph was converted to a measure of enzyme activity with a standard curve prepared at the same time. Cell lysates for HRP activity were prepared in 0.05% Triton X-100 (Rohm and Haas, Philadelphia, Penn.).

Radio-Iodination of Bovine Albumin. Bovine albumin (crystallized bovine plasma albumin, Metrix Division, Armour Pharmaceutical Co., Chicago, Ill.) was dissolved at a concentration of 25 mg/ml in 0.2 M NaH_2PO_4, pH 7.2, and 2 ml. of this solution was mixed with lactoperoxidase coupled to Sepharose beads (donated by Dr. S. Silverstein, The Rockefeller University) in the presence of 2 mCi of ^{125}I (carrier-free ^{125}I for protein iodination, spec act approx. 17 Ci/Mg; New England Nuclear, Boston, Mass.) and a 1:200 dilution of 0.03% H_2O_2 and incubated for 5-15 min at room temperature. The insolubilized enzyme was separated by centrifugation and the preparation was dialyzed extensively in the cold against 0.9% NaCl. Over 99% of the radioactivity in the final dialyzed preparation was precipitable with cold 10% TCA.

Cell samples assayed for radioactivity after exposure to [^{125}I]BSA were prepared in 0.1% Triton X-100, and precipitated on ice with 10% cold TCA. The precipitates were collected on filter papers (0.45 μ pore size, Millipore Corp., Beford, Mass.) which were washed with iced 5% TCA, and placed in plastic tubes for counting in a gamma-ray spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.).

Preparation of Con A-HRP Complexes. 160 mg of HRP and 210 mg of Con A were dissolved in 25 ml of NaCl, 1 M, and the solution was mixed overnight at 4°C. The mixture was then placed on a Sephadex G-200 column (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) previously equilibrated with 1 M NaCl and the protein which eluted in the void volume was collected and concentrated to approximately 2 ml by vacuum dialysis against culture mediums. The HRP activity in this solution could be quantitatively removed by adsorption to high molecular weight Dextran. Uncomplexed HRP, similarly treated, was not removed from solution.

Results

Metabolism of Proteins Pinocytised During Exposure to Con A. As indicated
in the preceding paper (8), various proteins can be included in the incubation me-
dium of cells exposed to Con A, and are interiorized in the Con A-induced pino-
somes. We therefore incubated macrophages with Con A and a test protein,
either HRP (1 mg/ml) or [125I]BSA (diluted 1:10 in BSA), for 2 h at 37°C. The cells
were washed and then returned for an additional 30 min of incubation in 199-FCS.
Cells were removed at this time and subsequently, washed several times, lysed in
0.05% Triton X-100, and either assayed for HRP activity or for TCA-precipitable
radioactivity, as appropriate.

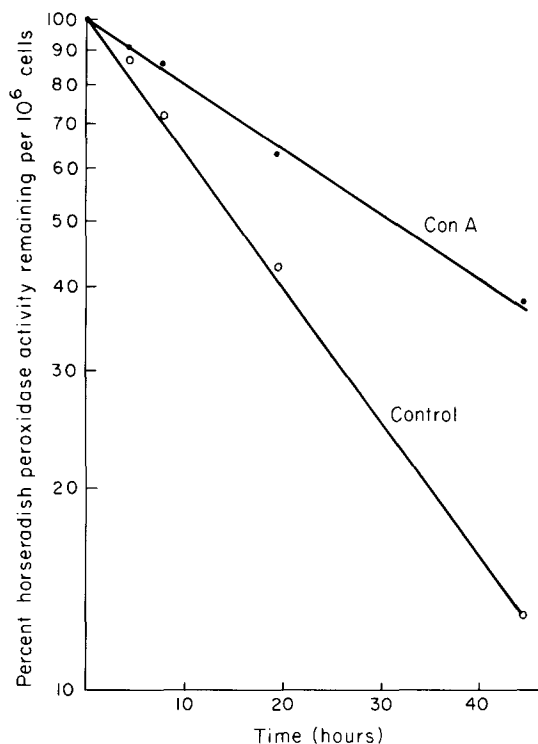As shown in Fig. 1, HRP activity in control cultures decays exponentially over

FIG. 1. Decay of HRP activity in macrophages simultaneously exposed to Con A (50 μgm/ml)
(●), or in control macrophages (O).

the 40-h sampling period, with a half-time of approximately 14 h. Similar
cultures which were exposed to Con A simultaneous with their exposure to HRP
also show an exponential decay of enzyme activity, but with a half-life of
approximately 37 h.

The same relationship between protein half-lives in control and Con A-treated
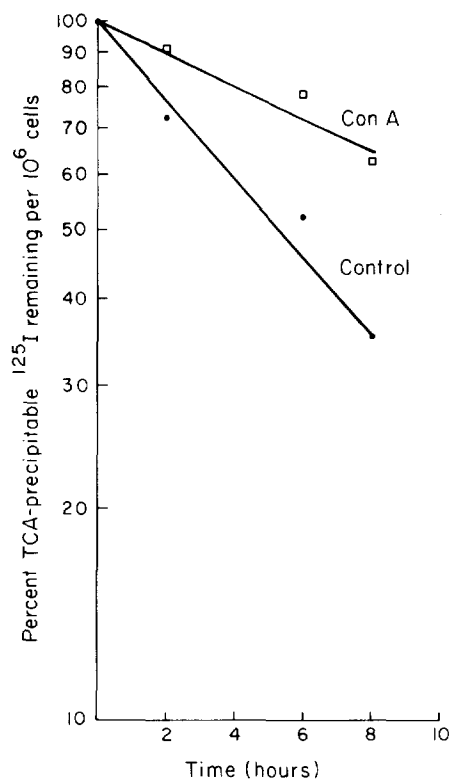cells is demonstrated using [125I]BSA as the test protein (Fig. 2). In control

Fig. 2. Decrease in TCA precipitable [125I] in macrophages ingesting [125I]BSA alone (●), or [125I]BSA and Con A (50 μgm/ml) (□).

cultures, [125I]BSA decayed exponentially with a half-life of 5.4 h, as compared with Con A-treated macrophages, in which the half-life of [125I]BSA is increased to 14.8 h.

To rule out the possibility that Con A prolonged the half-life of endocytized proteins by inhibiting intralysosomal proteases, we examined the effect of Con A on cathepsin D activity. Two varieties of experiments were carried out. In one, Con A, in a final concentration of 1,000 μg/ml, was added to a lysate of peritoneal macrophages prepared in 0.05% Triton X-100. In addition, macrophages were incubated with Con A (50 μg/ml) for 1 h at 37°C, rinsed, and then lysed in Triton. Aliquots of each of these lysates, and of a lysate of control peritoneal macrophages not exposed to Con A, were assayed for protein and cathepsin D activity. As indicated in Table I, Con A had no effect in either circumstance on the cathespin D activity of the cell lysates.

Steinman and Cohn (25) have shown that complexes of HRP-anti-HRP are endocytosed approximately 4,000 times as rapidly, and degraded more slowly, than is HRP alone. Because HRP can form complexes with Con A, we examined the uptake and degradation of preformed HRP-Con A complexes. Complexes were diluted in 199-FCS and administered to macrophage cultures for 2 h. The cultures were then washed, postincubated for 30 min, and then sampled at various times, lysed, and assayed for HRP activity. Comparison cultures

receiving uncomplexed HRP were similarly treated. As shown in Table II, complexes were neither ingested more efficiently, nor degraded more slowly, than was native HRP.

Effect of Mannose or Galactose on Preformed Con A Vesicles. Sugars which can be bound by Con A, including αMM, can inhibit the generation of Con A pinosomes when included in the culture medium while the cells are exposed to Con A (8). It was therefore of interest to study the effect of postincubating macrophages in various sugars on the fate of previously formed Con A-induced pinosomes.

When Con A-treated macrophages are postincubated in 199-FCS supplemented with galactose (100 mM) there is no change in the phase-contrast appearance of the cells (Fig. 3 *a*). However, incubation in medium supplemented with mannose (100 mM) dramatically reduces the number and size of phase-lucent pinosomes (Fig. 3 *b*), resulting in nearly normal appearing cells within 4-6 h of incubation.

The effect of postincubating macrophages in mannose can be studied in more detail by using a Con A-fluorescein isothiocyanate conjugate. Cells were exposed to the conjugate for 30 min on ice, washed extensively, and either fixed immediately or incubated at 37°C before fixation. Cells fixed without warming showed a uniform fluorescent outlining which, by phase microscopy, was seen to be localized to the plasma membrane. After warming for 15 min, many cells showed rings of fluorescence in their cytoplasm, and continued to show some plasma membrane fluorescence as well. After 1 h at 37°C, the cells showed

TABLE I

Effect of Con A on Cathepsin D Activity of Mouse Peritoneal Macrophages

Condition	Cathepsin D activity (% untreated control lysate)
Pretreatment with Con A	107.4
Addition of Con A to cell lysate	92.9

TABLE II

Uptake and Degradation of HRP Administered to Mouse Peritoneal Macrophages as HRP-Con A Complexes

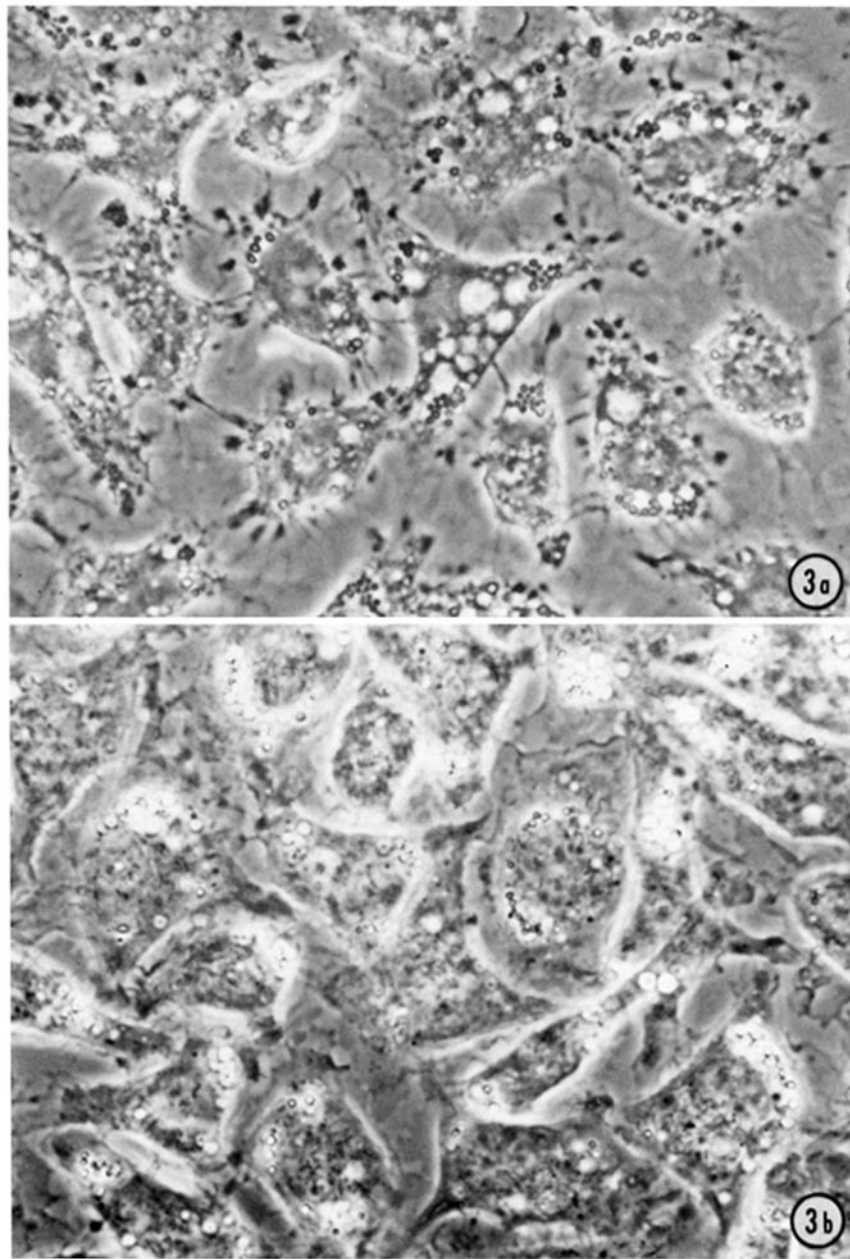|	Administered load	Fractional uptake	T ½
	mg HRP/ml	*% load/h*	*h*
HRP	0.125	0.0036	10.5
HRP-Con A complexes	0.134	0.0023	9.0

FIG. 3.   Effect of postincubation in sugar-supplemented 199 media on preformed Con A vesicles. (a) Phase contrast photomicrograph of cells exposed for 6 h to medium supplemented with galactose (100 mM). (b) Similar cells exposed for 6 h to mannose (100 mM). × 1,000.

extensive cytoplasmic fluorescence associated with the perimeters of the phase-lucent vesicles. The remainder of the cell, including the plasma membrane, was free of fluorescence.

When cells which had been exposed to Con A-FITC and then warmed for 1 h

were postincubated in 199-FCS, or 199-FCS supplemented with galactose (0.1 M), the pattern of cytoplasmic ring fluorescence was unaltered for up to 30 h. However, as shown in Table III, when cells were postincubated in mannose-supplemented medium, there was a decrease in cytoplasmic fluorescent rings, and a concomitant increase in smaller vesicles which were uniformly stained with fluorescent dye (Fig. 4).

Effects of Mannose and Galactose on Metabolism of Endocytized Proteins. The results obtained by phase-contrast and fluorescent microscopy suggested that the persistence of Con A-induced pinosomes could be specifically aborted by further incubation of the cell monolayers in mannose. We next studied the ability of mannose to affect the rate of decay of proteins which had previously been endocytized in Con A pinosomes.

Cells were prepared exactly as they were for the studies of protein metabolism

TABLE III

Distribution of Fluorescence in "Open" or "Filled" Vesicles, as Defined in Text, in Cells Exposed to Con A-FITC for 60 Min at 37°C, Washed, and Postincubated in 199-FCS Supplemented as Indicated

	Postincubation time	No. open vesicles	No. filled vesicles	Total vesicles
	h	%	%	
	0	30 (100)	0 (0)	30
Galactose (100 mM)	12	32 (84.2)	6 (15.8)	38
	30	40 (88.0)	5 (12.0)	45
	48	10 (66.0)	5 (34.0)	15
Mannose (100 mM)	4	21 (38.8)	54 (60.2)	75
	12	14 (21.8)	40 (28.2)	64
	30	2 (2.0)	95 (98.0)	97
	48	5 (4.8)	98 (95.2)	103

described above. Certain monolayers were exposed to Con A (50 μg/ml) during the period of exposure to either HRP (1 mg/ml) or to [125I]BSA (diluted as described above). Control monolayers were exposed to the same proteins in the absence of Con A. After 2 h, the cells were washed, reincubated for 30 min in fresh medium from which the test protein had been omitted, and then, at various times, washed, lysed in Triton X-100, and assayed for either peroxidase activity or radioiodine. However, at 8 h after the initial sampling, in the case of HRP, or at 3.5 h after the initial sampling, in the case of [125I]BSA, the remaining monolayers were transferred to fresh 199-FCS supplemented with either mannose (100 mM) or galactose (100 mM), according to the following plan. Certain Con A-treated monolayers were postincubated in galactose, while other monolayers were transferred to mannose-containing medium. All control cells were transferred to mannose-supplemented medium. Sampling was continued for several hours more.
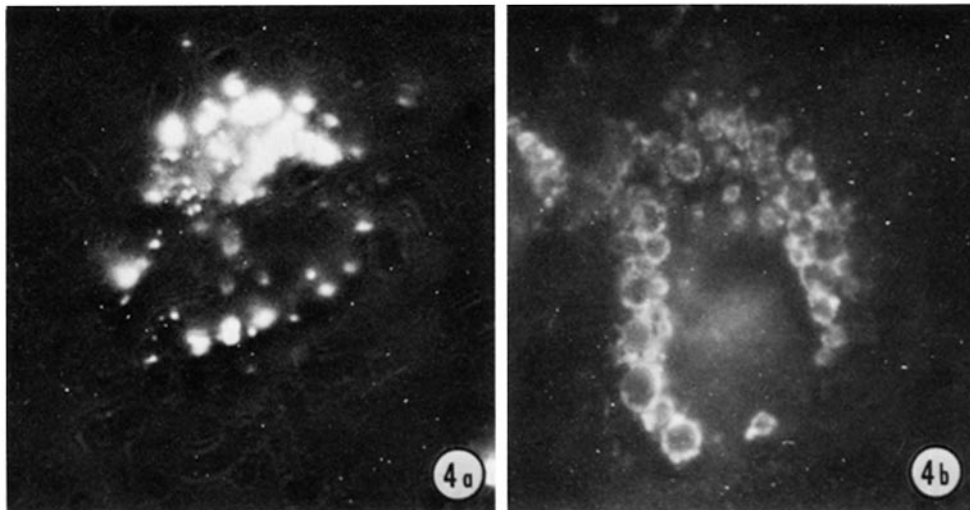
FIG. 4. Effect of postincubation in sugars on the pattern of fluorescence in macrophages previously exposed to Con A-FITC. (a) Similar cells exposed for 6 h to mannose (100 mM). (b) Cells postincubated for 6 h in galactose (100 mM). × 1,000.

As shown in Fig. 5 and Fig. 6, postincubation in galactose had no effect on the rate of metabolism of either protein endocytised in Con A vesicles. However, postincubation of similar cells in mannose markedly increased the rate of decay of either protein, in both cases restoring them to control values. Mannose had no effect on the rate of metabolism of either protein in control monolayers.

Effect of Mannose or Galactose on Formation of Phagolysosomes. As shown in the previous paper (8), Con A-induced pinosomes show morphologic evidence of impaired fusion with primary or secondary lysosomes to form phagolysosomes. We therefore examined the effect of mannose or galactose incubation on this inhibition of fusion.

Macrophages which had been preloaded with gold were exposed sequentially, on ice, to Con A (1 mg/ml), followed by HRP (1 mg/ml), incubated at 37°C for 30 min in FC 199-FCS, and then transferred to fresh medium, or fresh medium supplemented with mannose (50 mM) or galactose (50 mM). The cells were reincubated for 2 h, fixed, stained for HRP (8), and processed for electron microscopy. Fig. 7 shows a macrophage postincubated in galactose. Gold particles are present in many secondary lysosomes, while a dense reaction product, indicative of peroxidase activity, and by inference, of the presence of Con A, can be seen on the inner surface of several large vacuoles. These Con A vacuoles are, however, free of any gold particles. In Fig. 8, a similar cell is shown after incubation in mannose. In this cell, gold is clearly evident within the peroxidase-positive vacuoles. Table IV summarizes the distribution of gold in control, galactose, or mannose-treated macrophages.

Discussion

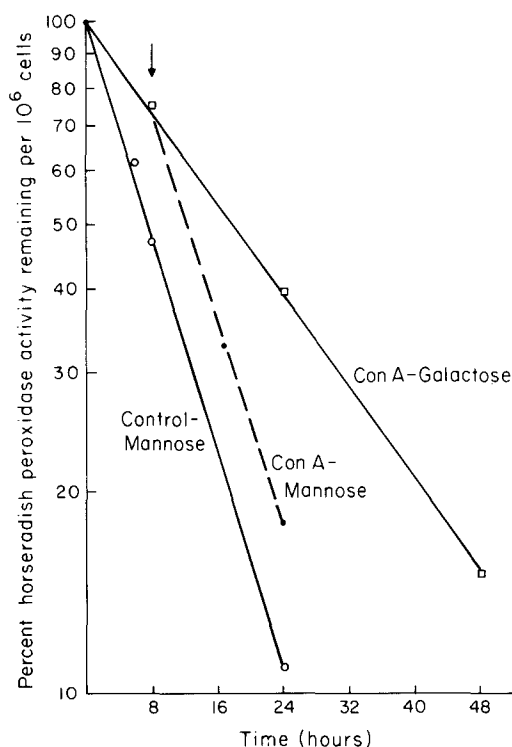In the preceding paper (8) we showed that Con A is a potent stimulator of

FIG. 5. Decay of HRP activity in Con A-treated cells, postincubated in galactose (100 mM)
(□—□), Con A-treated cells postincubated in mannose (100 mM) (●---●), or control cells
postincubated in mannose (O—O). All cells were initially postincubated in 199-FCS, then
shifted to medium supplemented with the indicated sugar at 10 h (arrow).

pinocytosis in the cultured mouse peritoneal macrophage. These pinosomes were
remarkable for their ability to persist for long periods of time in the macrophages.
Morphologic studies suggested that the pinosomes' unusual persistence could be
explained by their very reduced rate of fusion with preexisting primary or
secondary macrophage lysosomes.

In this paper, we have taken another approach to the question of the fusion of
Con A pinosomes, by measuring the half-lives of two exogenous proteins, HRP
and [125I]BSA, taken up into the Con A pinosomes.

As lysosomal enzymes are probably responsible for the bulk of this intra-
cellular degradation (15), these measurements can be sensitive to the fail-
ure of lysosomal enzymes to gain access to the substrates being studied. We
have measured the decay rates of HRP, measured enzymatically, and of
[125I]BSA, both comparable to previously reported values, using similar tech-
niques (24, 9). When these proteins were administered to the cells in the presence
of Con A, their half-times were prolonged, to approximately 34 h for HRP, and
12-14 h for [125I]BSA. These rates could be returned to normal by postincubating
the cells in mannose, but not in galactose. Mannose had no effect on the decay
rate of these proteins in control cells. In in vitro experiments the direct addition
of Con A did not inhibit the cathepsin D activity of cell lysates at concentrations
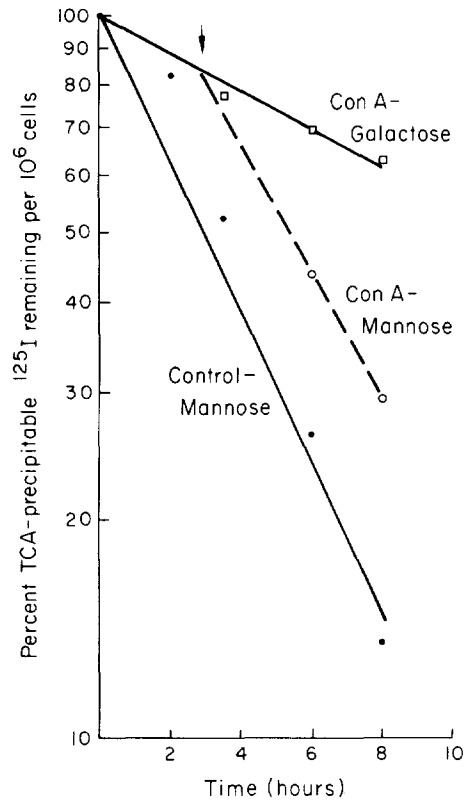up to 1,000 μgm/ml. In addition, the cathepsin D activity of lysates of cells which

Fig. 6. Decrease in TCA-precipitable [125I] in cells allowed to pinocytize [125I]BSA, in the presence of Con A, and then postincubated in galactose (100 mM) (□—□), or in mannose (100 mM) (O---O), or in control cells postincubated in mannose (100 mM) (●—●). Cells were initially postincubated in unsupplemented 199-FCS, then shifted to medium supplemented with the indicated sugar at 3 h (arrow).

had previously been treated with Con A was also normal. Since cathepsin D appears to play a major role in the degradation of various endocytized proteins in the macrophage (3), direct enzyme inhibition by Con A seems unlikely to account for our results on the increased intracellular survival of these proteins.

There is, however, an additional potential source of error in the experiments involving HRP. Horseradish peroxidase is a glycoprotein (23) which can be complexed by its sugar residues to Con A (P. J. Edelson, unpublished observation). Since antibody-HRP complexes are endocytized more rapidly and degraded more slowly than is native HRP (25), it is conceivable that the results we obtained are related to the formation of Con A-HRP complexes. This is clearly ruled out by the results of our measurements of uptake and degradation of preformed Con A-HRP complexes. The complexes are endocytized and degraded by the macrophages at rates identical to those of the native HRP. Concerns regarding artifacts due to complex formation are irrelevant in the case [125I]BSA, for which protein Con A also prolonged its half-time for degradation.

These results, taken together with our previous microscopic observations, indicate that Con A-induced pinosomes are markedly impaired in their ability to
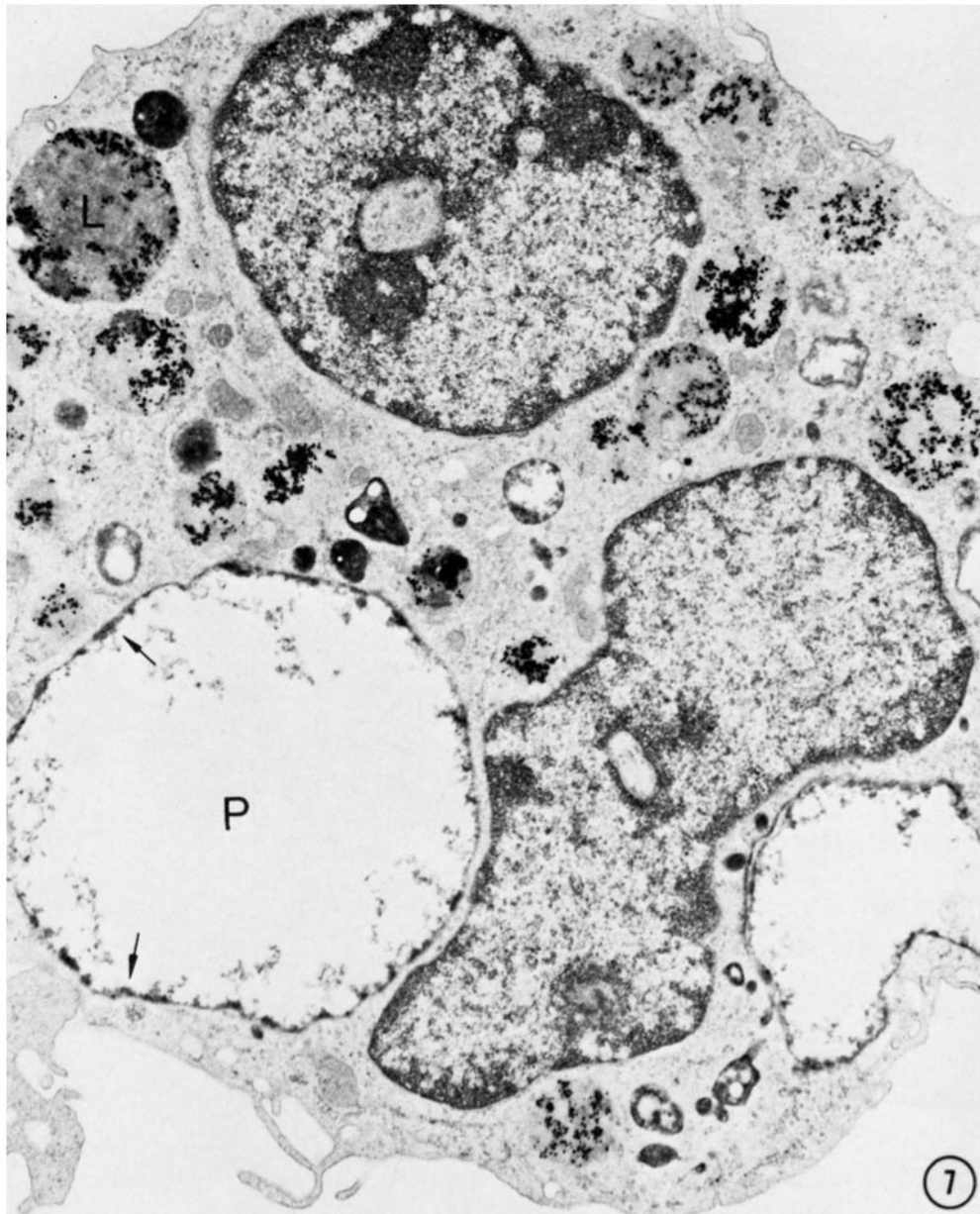
Fig. 7. Electron micrograph of macrophage preincubated in gold, then exposed sequentially to Con A and HRP at 4°C, warmed, and then transferred to 199-FCS supplemented with galactose (100 mM). Gold is seen in dense bodies, or secondary lysosomes (L), while peroxidase reaction product (arrows) after staining with diamino-benzidine, outlines Con A pinosomes (P). Note that pinosomes are free of gold. × 14,900.

fuse with lysosomes. We then examined the ability of mannose, a sugar able to compete for the Con A saccharide binding site (10), to reverse this inhibitory effect.

There are three major phenomena which we have taken as symptomatic of the
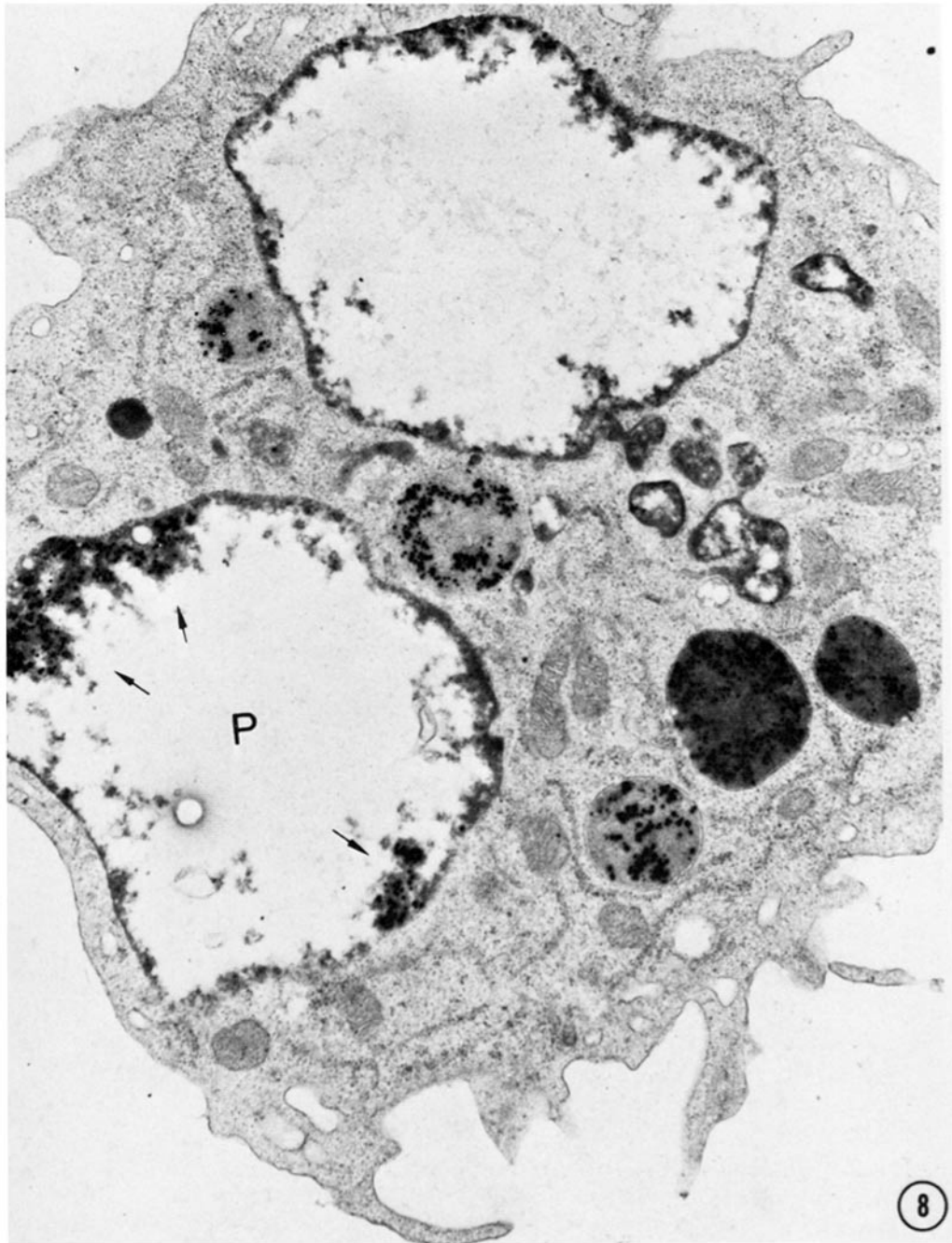
FIG. 8.   Cells treated as in preceding figure, except that postincubation medium contained
mannose (100 mM) in place of galactose. Con A pinosomes (P) now show gold particles (arrows)
presumably delivered from secondary lysosomes following phagolysosome formation. × 15,400.

TABLE IV

Numbers of Cytoplasmic Vesicles Reactive for Horseradish
Peroxidase in Which Gold Particles are Present, or Absent,
following Postincubation of the Macrophages for 2 h in 199-FCS,
or 199-FCS Supplemented with the Indicated Sugar

Postincubation sugar	No. of vesicles containing gold	No. of vesicles free of gold	Total
None	16	84	100
Mannose (100 mM)	67	33	100
Galactose (100 mM)	25	75	100

impaired fusion of Con A pinosomes with macrophage lysosomes. For each, we have examined the effects of incubation of the cells in mannose. Galactose was substituted for mannose as a control sugar not able to compete for the Con A saccharide binding site.

First, mannose diminishes the ability of Con A pinosomes to persist in the cells. The pinosomes persist for more than 24 h in galactose-supplemented medium, while they are almost entirely gone after 6 h of incubation in mannose. In addition, in experiments using a fluoresceinated Con A conjugate, it appears that Con A is normally associated with the membrane bounding the induced pinosomes, and that mannose, but not galactose, can alter this distribution to one where the Con A conjugate is more uniformly associated with the vesicle interior.

Second, mannose can restore the degradation rates for HRP, or [125I]BSA, to normal in Con A-treated cells, without any effect on the intrinsic rate of degradation of these proteins in cells not exposed to Con A. Galactose has no such effect on Con A-treated cells.

Third, electron microscopic studies have allowed us to directly examine the fusion of secondary lysosomes, marked by preadministered gold particles, and Con A-induced vesicles, labeled with HRP. In this study, approximately 25% of Con A vesicles contained gold particles, indicating a low spontaneous rate of phagolysosome formation. Galactose had little effect on this rate of fusion. Mannose, however, increased by nearly threefold the proportion of Con A vesicles which contained gold. Thus, by all three criteria, the behavior of Con A vesicles, the rates of degradation of HRP and [125I]BSA, and the electron microscopic observations of phagolysosome formation, mannose is specifically effective in reversing the inhibitory effects of Con A on phagolysosomal fusion.

In addition, our observations using Con A-FITC strongly suggest that the ability of Con A to inhibit the fusion of certain membranes depends upon a direct interaction of the lectin with the membrane. Recently, several examples have been reported of Con A directly affecting certain plasma membrane characteristics.

First, Con A binding sites are themselves mobile (4, 28). Their native distribu-

tion on most cells is probably a randomly disperse one (16). In normal cells, these sites may (28) or may not (17) cluster upon the addition of Con A. In transformed cells, they frequently do cluster (17). Their tendency to cluster in the presence of Con A may also be increased by infecting the cells with certain nontransforming viruses (21). Spontaneous clustering is seen after proteolytic treatment (16). In addition, when murine lymphocytes are exposed to Con A at 4°C, and then warmed to 37°C, the Con A molecules are organized into the typical membrane "patches" and "caps" (26), but when similar cells are directly exposed to Con A at 37°C, Con A inhibits patching and capping induced either by various antibodies, or by Con A itself (31).

In human erythrocyte ghosts, the Con A binding sites appear to be associated with mobile 80 Å membrane-intercalated particles (20) present in the interior of nearly all cell membranes (6). However, in intact nucleated cells, this association has not been demonstrable (19).

Con A, at certain doses, may directly affect the fluidity of the plasma membrane, as suggested by experiments in which a spin label was inserted into murine lymphocyte plasma membrane, and its mobility measured after exposure of the cells to increasing concentrations of Con A for short periods of time (2).

We are not yet in a position to complete an argument linking the effects of Con A on membrane fluidity, or the mobility of intramembranous components, not necessarily the same thing, with its effects on macrophage pinocytic activity or the inhibition of fusion of pinosomes and lysosomes. However, we can raise the possibility that those two areas are linked, perhaps through a requirement for the rearrangement of certain membrane components in order for both endocytosis, and phagolysosome formation to occur.

Con A may also affect the transport properties of the plasma membrane. Isselbacher (12), for example, has reported that mouse fibroblasts, both SV40 transformed and nontransformed, show a reduction in amino acid uptake after exposure to from 50–300 μg/ml Con A. This may represent a direct effect on amino acid influx, or efflux, although changes in intracellular pool size, for example, might also be involved.

In an earlier study, Inbar, et al. (11), had also reported that Con A had an inhibitory effect on the steady-state accumulation of several amino acids in transformed, but not in normal, hamster cells in culture, and a similar effect on the accumulation of D-glucose, and D-galactose, but not L-fucose, both in transformed and in normal cells. However, a modest increase in the transport of 2-amino-isobutyric acid in rat lymphocytes exposed to Con A has been reported by Van den Berg and Betel (29).

Con A seems also to affect the transport of electrolytes across a model bilayer composed of sheep erythrocyte lipids into which the major membrane glycoprotein from human erythrocytes had been dissolved (27). In this case, the addition of Con A (500 μg/ml) increased the membrane conductance nearly 8 times. It was not clear from this report, however, that binding the lectin to the glycoprotein was necessary for this enhancement, nor even that this effect might not be reproduced by the addition of other proteins to the lipid bilayer.

Although the explanation of these various experiments must be tentative, it is conceivable that Con A binding may influence some membrane transport

properties, and, in fact, it is possible that such an alteration is responsible for maintaining the rather large size of certain of the Con A-induced pinosomes which we have described.

It is quite consistent with our interpretation of these experiments as depending on the continuing direct contact of Con A with the vesicle membrane that treatment of vesiculated cells with mannose is effective in reversing the effects of Con A on the pinosomes. Although in outline these reversal experiments are reminiscent of similar observations made on sucrose-induced vacuoles by Cohn and Ehrenreich (7), it should be pointed out that in that case, reversal was probably more directly related to the degradation, of the sugar by exogenously provided invertase, with the subsequent diffusion of the glucose and fructose produced, and collapse of the osmotic potential maintained across the phagolyso-somal membrane. In our case, it appears that Con A-FITC is simply removed from its intimate contact with the inner surface of the pinosomes, and that degradation of the Con A, while it presumably occurs following the fusion of these pinosomes with lysosomes, has little to do with the initial aspects of the reversal. Thus, it seems that simply by binding to a glycoprotein component of the plasma membrane, and later the pinosome membrane, Con A can profoundly regulate several membrane functions, including some involving events on the other face of the membrane.

Failure of phagolysosome formation is an important characteristic of the infection of mouse peritoneal macrophages by *M. tuberculosis*, as Armstrong and D'Arcy Hart have shown (1). A similar situation applies to infection of mouse macrophages with *Toxoplasma gondii*, as demonstrated by Jones and Hirsch (13). In each of these cases, the living parasite seems capable of actively inhibiting fusion of its enclosing phagosome with the cell's complement of lysosomes. Dead parasites no longer have this inhibitory ability. Observations consistent with impaired phagolysosome formation have also been reported for *Chlorella* symbiotically harbored within *Paramecium bursaria* (14), as well as in several reports cited by Armstrong and D'Arcy Hart. Con A, then, seems to have potential for modelling certain parasitic or symbiotic states, and we are currently attempting experiments to exploit this potential. In addition, our experiments raise the possibility that microbial inhibition of phagolysosome formation is related to the production of a microbial material capable of interacting with components of the inner phagosome surface, and perhaps more specifically, with sugar residues exposed on that surface. One might then expect that incubation of successfully parasitized cells in medium supplemented with the correct sugar would allow phagolysosome formation to proceed normally, and perhaps, would abort the parasitic state.

Finally, the formation of Con A pinosomes may be a convenient technique for the isolation of "endocytic" plasma membrane free of lysosomal membrane contributions. This might allow a more precise comparison of the properties of the endocytic membrane with the whole plasma membrane.

Summary

The half-time for the degradation of horseradish peroxidase (HRP) is increased

from 14 h to 37 h in Con A-treated cells, while the half-time for the degradation of [^{125}I]BSA is increased from 5.4 h to 14.8 h. This supports prior microscopic observations which suggested that Con A pinosomes showed a marked impairment in their ability to form phagolysosomes. Artifacts due to anomalous behavior of HRP-Con A complexes, or to inhibition of lysosomal hydrolases by Con A, could be excluded.

These indications of impaired phagolysosome formation, as well as those described in the preceding paper, could be reversed by postincubation of the cells in mannose, but not in galactose. This reversal is accompanied by a dissociation of Con A-FITC from the inner surface of the pinosome membrane, into the vesicle contents. These observations may be relevant to the ability of Con A to affect several membrane characteristics, and are also of interest in relation to the impaired formation of phagolysosomes which has been described in certain in vitro parasitic infections of macrophages or other cells.

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