

Rockefeller University

Digital Commons @ RU

---

Publications

Steinman Laboratory Archive

---

1982

## **Intralysosomal accumulation of polyanions. I. Fusion of pinocytic and phagocytic vacuoles with secondary lysosomes**

Margaret Carol Kielian

Ralph M. Steinman

Zanvil A. Cohn

Follow this and additional works at: <https://digitalcommons.rockefeller.edu/steinman-publications>

---

# Intralysosomal Accumulation of Polyanions. I. Fusion of Pinocytic and Phagocytic Vacuoles with Secondary Lysosomes

MARGARET C. KIELIAN, RALPH M. STEINMAN, and ZANVIL A. COHN

*The Rockefeller University, New York 10021. Dr. Kielian's present address is the Section of Cell Biology, Yale University School of Medicine, New Haven, Connecticut 06510.*

**ABSTRACT** The long-term exposure of macrophages to low concentrations of a number of polyanions leads to their accumulation in high concentration within secondary lysosomes. This was associated with enlargement of the lysosomes, the presence of membranous whorls, and intense toluidine blue staining of the organelles at pH 1.0. After the ingestion of a particulate load by these cells, newly formed phagocytic vacuoles failed to fuse with polyanion-laden lysosomes. The lack of fusion was evident in both fluorescence and electron micrographic studies which followed the transfer of acridine orange or Thorotrast from 2° lysosomes to phagosomes. Agents that inhibited phagosome-lysosome (P-L) fusion included molecules containing high densities of sulfate, sulfonate, or carboxylate residues. Dextran sulfate (DS) in  $\mu\text{g/ml}$  quantities was an excellent inhibitor, whereas nonsulfated dextran (D) was without effect at 1,000-fold higher concentrations.

In contrast to their effects on P-L fusion, polyanions failed to influence the fusion of pinocytic vesicles with 2° lysosomes. The uptake, intravacuolar distribution, and intralysosomal digestion of fluid-phase pinocytic markers were unaltered in lysosomes containing either D or DS. Furthermore, subcellular fractionation studies showed that the fluid-phase pinocytic marker HRP was efficiently transferred from pinosomes to large, dense 2° lysosomes containing DS.

Previous studies from this laboratory have characterized a fluorescent vital dye method for the quantitative study of phagosome-lysosome (P-L) fusion, evaluated certain basic properties controlling the fusion of these organelles (1), and described the ability of macrophage activation (2) and phorbol myristate acetate to accelerate both the rate and extent of fusion (3). In this article we extend these observations to a group of agents which when stored within secondary lysosomes markedly inhibit the ability of these organelles to fuse with phagocytic vacuoles. These agents are in many ways similar to a set of polyanions that were shown previously to enhance vesicle formation in macrophages (4), complex with lipoproteins and stimulate their uptake (5, 6), and inhibit the fusibility of lysosomes (7, 8). We will, in addition, document the selective nature of this defect and the continuing fusion of pinocytic vesicles with the polyanion-containing lysosomes.

## MATERIALS AND METHODS

### *Cells and Culture Conditions*

Resident peritoneal macrophages were obtained from female Nelson-Collins

strain mice from the Rockefeller colony and cultured as previously described (1), with or without the addition of polyanions to the culture medium.

### *Fluorescence Assay of P-L Fusion*

P-L fusion in mouse macrophages was assayed by monitoring the transfer of a fluorescent vital dye from lysosomes to phagocytic vacuoles as previously described (1). Briefly, cover slip cultures were labeled for 20 min with 5  $\mu\text{g}$  of acridine orange (AO) per milliliter and washed, and a dilute suspension of serum-opsonized, heat-killed yeast particles was centrifuged onto the cell monolayer at 4°C. Unbound yeast was washed away, and the cultures were rapidly warmed to 37°C to synchronize particle ingestion. Viable cells were examined by fluorescence microscopy at various times after an initial 10-min ingestion period. The presence of orange-stained intracellular yeast was considered positive for P-L fusion.

### *Electron Microscope Evaluation of Endocytic Vesicle Fusion with Lysosomes*

Colloidal thorium dioxide (Thorotrast) was used as a marker for secondary lysosomes in P-L fusion studies as previously described (1). Cells were washed 2–4 h after plating, exposed to a 1:100 dilution (vol/vol) of colloid in medium for 12 h, washed four times with medium, and then cultured 4 d in medium alone or containing various inhibitors. Cells were fixed 1 h after particle ingestion and

processed for electron microscopy. Stereology was used to evaluate the extent of fusion in specimens labeled with this particulate marker (1).

In some experiments, soluble horseradish peroxidase (HRP) or Thorotrast was used as a pinocytic marker for electron microscopy. HRP activity was visualized by the Graham and Karnovsky diaminobenzidine method (9). For HRP-labeled samples, thin sections were examined without uranyl acetate and lead citrate staining.

### Sulfate Determinations

The ester sulfate in various compounds was assayed by the method of Terho and Hartiala (10) after hydrolysis in 0.5 N HCl 60 min at 100°C. Samples were evaporated to dryness to remove the HCl before assay. Free sulfate was assayed in nonhydrolyzed samples.

### Toluidine Blue Staining

Toluidine blue staining (11) was done on fixed cover slip preparations at 0.1% dye in 0.1 M acetate buffer pH 4.0 or in distilled H<sub>2</sub>O adjusted to pH 1.0 with HCl. After 30 min of staining at room temperature, cover slips were washed in buffer at the same pH.

### Subcellular Fractionation

4-d cultures of control, dextran-treated, or dextran sulfate-treated cells were washed (on ice) three times with phosphate-buffered saline and once with 0.3 M sucrose containing 2 mM EDTA and 5 mM HEPES, pH 7.4. Two 60-mm dishes of each cell type ( $3-4 \times 10^7$  cells) were then scraped into 1 ml of the buffered sucrose and homogenized in a 7-ml Dounce homogenizer (Kontes Co., Vineland, NJ) using a B pestle. Two to five strokes resulted in 85–95% cell breakage, as assessed by phase microscopy. This whole-cell homogenate was then layered over a 12-ml linear metrizamide gradient (15–40 or 20–40% in a HEPES-saline buffer) (12) and centrifuged at 10,000 g for 90 min (4°C) using an HB-4 swinging bucket rotor and a Sorvall centrifuge (DuPont Co., Wilmington, DE). 0.55-ml fractions were collected and assayed as follows:

### AO

Cells were labeled with AO (5 µg/ml for 20 min, 37°C) before homogenization. Fractions were assayed by adding 100-µl aliquots to 2 ml of 95% ethanol and reading fluorescence in an MPF-44 fluorometer in ratio mode, excitation 490 nm with a slit of 3 nm, emission 520 nm, with a slit of 6 nm.

### HRP Activity

HRP was assayed using *O*-dianisidine (Sigma Chemical Co., St. Louis, MO) as previously described (13), with or without the addition of 0.1% Triton X-100 (Triton) to an aliquot of the fraction.

### 5'-Nucleotidase (5'NTase)

5'NTase was assayed as described by Edelson and Cohn (14) using adenosine-2-[<sup>3</sup>H]-5'-monophosphate (ammonium salt; Amersham-Searle Corp., Arlington Hts., IL) as substrate.

### N-Acetyl Glucosaminidase (NAGase)

NAGase activity was measured using the assay of Peters et al. (15) using 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glycopyranoside (Koch-Light Laboratories, Colnbrook, Buckinghamshire, England) as substrate.

HRP, 5'NTase, and NAGase assays were not affected by 5 mg/ml dextran (D) or dextran sulfate (DS) in the reaction mixture.

### <sup>3</sup>H-DS

DS (500,000 mol wt) was tritium-labeled by New England Nuclear (Boston, MA) using the Wilzbach (16) method. Its purification and characterization are described in the accompanying article. Cell fractions containing <sup>3</sup>H-DS were assayed by liquid scintillation counting, using 100 µl of the fraction to 3 ml of Hydrofluor (National Diagnostics, Somerville, NJ).

### Isopycnic Centrifugation

Lysosomal fractions were harvested with a bent Pasteur pipette from the velocity gradients and diluted to <10% metrizamide with HEPES-saline buffer

containing 1 mM EDTA. These fractions were layered over sucrose step gradients of 50, 40, 30, and 20% sucrose. All sucrose gradients were wt/wt percentages and contained 20 mM potassium phosphate buffer and 1 mM EDTA (pH 7.4). Step gradients were centrifuged at 100,000 g, using the SW-41 rotor and an L5-65 ultracentrifuge (Beckman Instruments Inc., Palo Alto, CA), for 60 min at 4°C.

### Protein Determinations

Protein was determined by the method of Lowry et al. (17) using crystalline bovine serum albumin as standard.

## RESULTS

The effect of polyanion treatment on P-L fusion was assessed by following the transfer of AO, a fluorescent vital dye, or Thorotrast, an electron-dense colloid, into yeast-containing phagocytic vacuoles (see Materials and Methods for details). Macrophages were cultured for several days in medium containing polyanions or nonsulfated dextran, and the effects on P-L fusion were then assayed in polyanion-free medium.

### Polyanions That Inhibit P-L Fusion

Fig. 1 contrasts the rate of P-L fusion using the AO assay in 4-d cultures treated with D, a 500,000 mol wt glucose polymer, or DS, its sulfated derivative containing 17% sulfur. Cells cultured for 4 d with 30 mg/ml D showed the usual rapid fusion rate of 4-d cells (1), resulting in a ~85% positive staining within 1 h. In contrast, DS at a concentration three orders of magnitude lower (10 µg/ml) markedly inhibited P-L fusion in treated cells. Particle uptake was similar in the two cell types, and most particles ingested by DS-treated cells were unstained even several hours after ingestion.

Fig. 2a and b are fluorescence micrographs of fusion assays in D- or DS-treated cells 60 min after particle ingestion. Dextran cells contain many positively stained particles as well as numerous AO-stained lysosomes. Abundant lysosomal staining is also seen in DS-treated cells, but the dye has not been transferred to yeast-containing phagosomes. By fluorescence microscopy, both cell populations show enlarged AO-stained lysosomes. By phase microscopy (Fig. 2c), D cells contain numerous large, phase-lucent vacuoles, similar to those in macrophages cultured in medium containing sucrose or newborn calf serum (18, 19). The vacuoles in D-treated cells presumably are due to the high concentration of endocytosed, nondigestible polymer. DS-treated cells (Fig. 2d) have similar large vacuoles, even though the concentration of nondigestible DS is much lower.

The presence of DS in the large vacuoles of treated cells was evaluated by staining with toluidine blue, a cationic dye. As

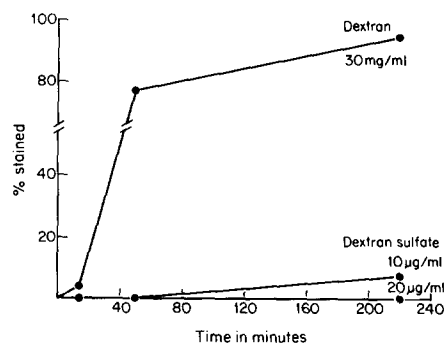


FIGURE 1 Effect of dextran (D) or dextran sulfate (DS) on P-L fusion. Cells were cultured for 4 d with 500,000 mol wt D or DS at the indicated concentrations. Cells were given fresh medium each day, and assayed on day 4 in the AO assay.

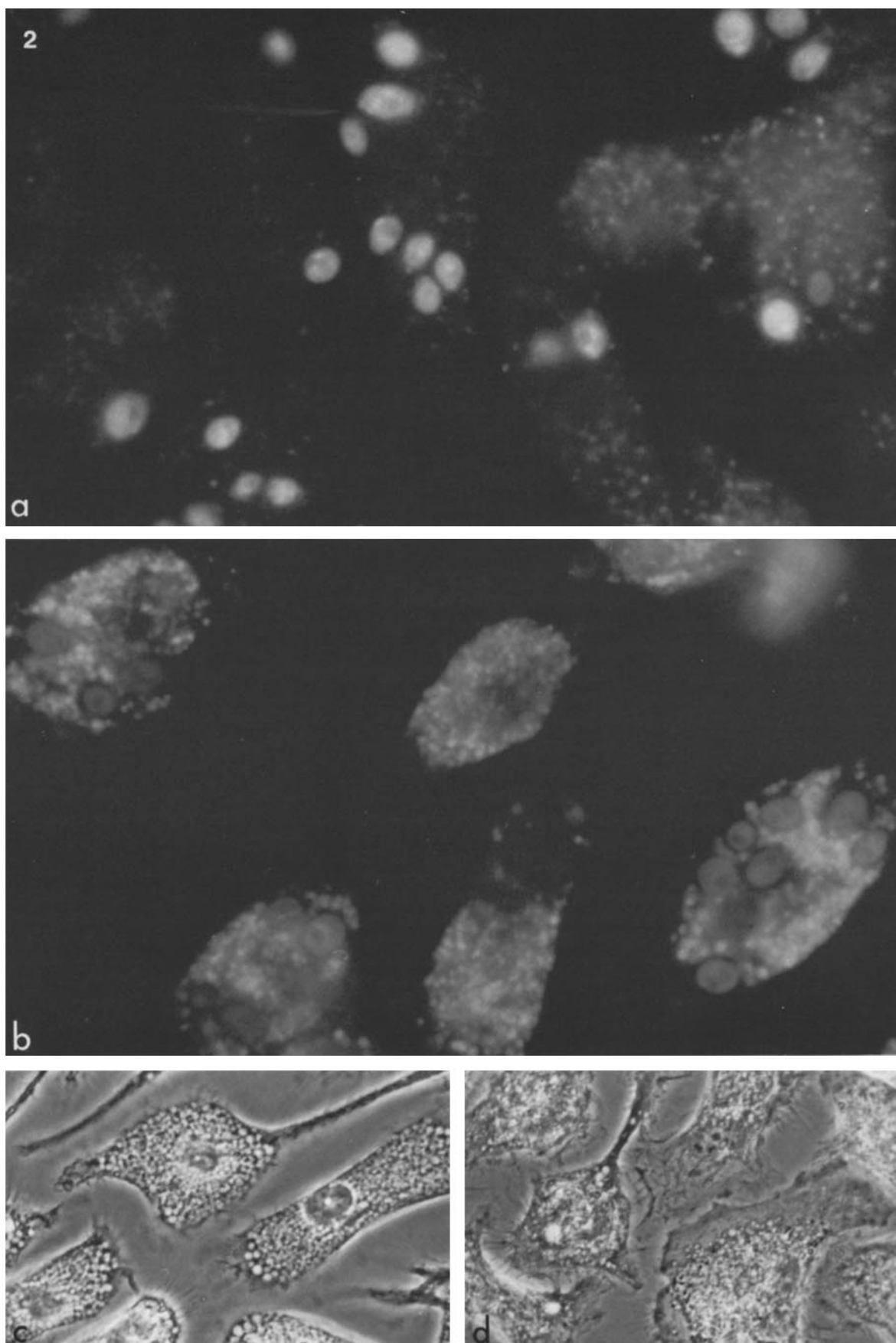


FIGURE 2 Fluorescence and phase microscopy of D- and DS-treated cells. Cells were cultured as in Fig. 1, using 10  $\mu$ g/ml DS and 30 mg/ml D. (a and b) AO-stained D- (a) or DS- (b) treated cells ~60 min after yeast ingestion. Both cell types have abundant AO-stained lysosomes.  $\times 1,900$ . (c and d) Phase micrograph of D- (c) or DS- (d) treated cells, showing numerous large vacuoles.  $\times 950$ .

shown in Fig. 3, when DS-treated cells were fixed and exposed to toluidine blue at pH 1.0, the dye heavily stained cytoplasmic granules. Under these staining conditions, only sulfate groups or polyphosphates remain unprotonated and interact with toluidine blue (11). The majority of cytoplasmic vacuoles in DS cells appear to be positively stained, implying that most of the large secondary lysosomes of DS cells contain significant polyanion. When stained at pH 4, DS cells showed both nuclear and cytoplasmic staining, while control or D cells showed only nuclear staining.

Various other polyanionic compounds were examined for their effects on P-L fusion. Table I lists those found to inhibit fusion, together with their molecular weights and the lowest concentration which caused inhibition when present in the medium during a 3- to 4-d culture period. The only inhibitory agents found were polyanions. Sulfate, sulfonate, or carboxyl

groups could cause inhibition, as could low molecular weight (i.e., suramin) or high molecular weight substances. The most potent inhibitor found was DS (500,000 mol). Lower molecular weight DS and suramin only caused inhibition at much higher concentrations, although their sulfate or sulfonate contents were comparable to that of 500,000 mol wt DS. This could reflect the stimulation of pinocytosis by DS or its binding to lipoproteins, both of which appear to be higher for the longer chain polymers (4, 20). Chondroitin sulfate was inhibitory only at very high concentrations, and has a lower sulfate content than either suramin or DS.

All of these inhibitors caused the formation of large cytoplasmic vacuoles, similar to D and DS in Fig. 2. Again, for all of these inhibitors, toluidine blue staining showed a concentration of anionic groups in cytoplasmic granules.

### Electron Microscope Evaluation of Fusion

Results obtained by the fluorescence assay of P-L fusion were correlated with electron microscope evaluations. Cultures were prelabeled with Thorotrast and then cultured in medium containing inhibitors for 4 d. Prelabeling of cells with Thorotrast before exposure to inhibitors ensures equivalent labeling of the different cell populations. Table II compares results from the two assay systems for suramin, DS, and control cells. Marked inhibition of fusion was found by electron microscopy as well as by the fluorescence assay. DS again appeared to be a more potent inhibitor than suramin.

### Ultrastructural Modifications in Polyanion-treated Cells

Fig. 4a is a representative electron micrograph of a cell from a 4-d, DS-treated culture. The cell cytoplasm contains many large vacuoles which show a fine fibrillar material. This may

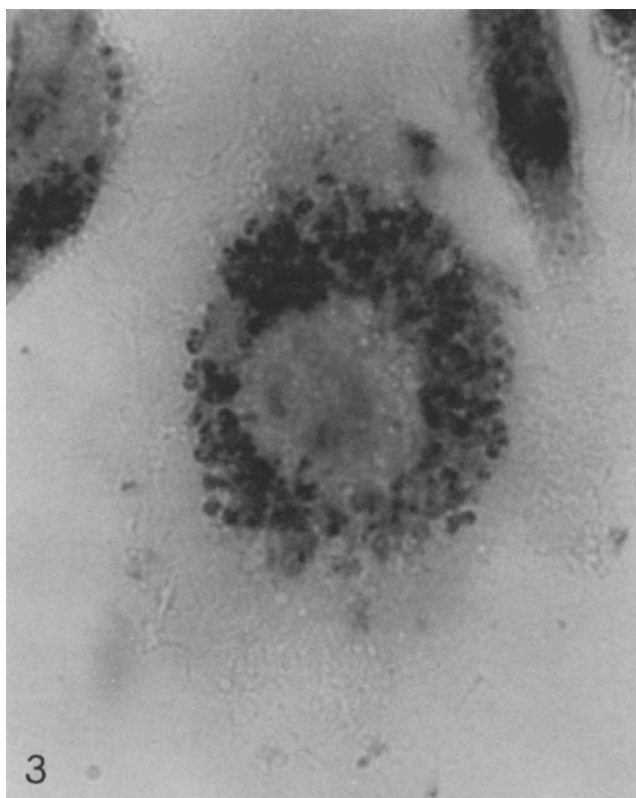


FIGURE 3 Bright-field micrograph of toluidine blue-stained cells. Macrophages cultured for 4 d in 10  $\mu$ g/ml DS, fixed, and stained with toluidine blue at pH 1. Note absence of nuclear staining but heavily stained cytoplasmic granules.  $\times$  1,600.

TABLE II  
EM Assay of P-L Fusion in Polyanion-treated Cells

Assay conditions	% Fusion after 1 h	
	AO assay	Thorotrast-EM assay*
Cultured for 4 d in:		
Medium alone	85 $\pm$ 13.6 (5)‡	37.3 $\pm$ 4.9 (3)
Medium + 200 $\mu$ g/ml suramin	0 (5)	10.4 (1)
Medium + 20 $\mu$ g/ml DS	0 (5)	2.3 (1)

\* For each determination, 30-60 vacuoles were evaluated by stereology, and total line crossing of 600-1,500 was obtained.

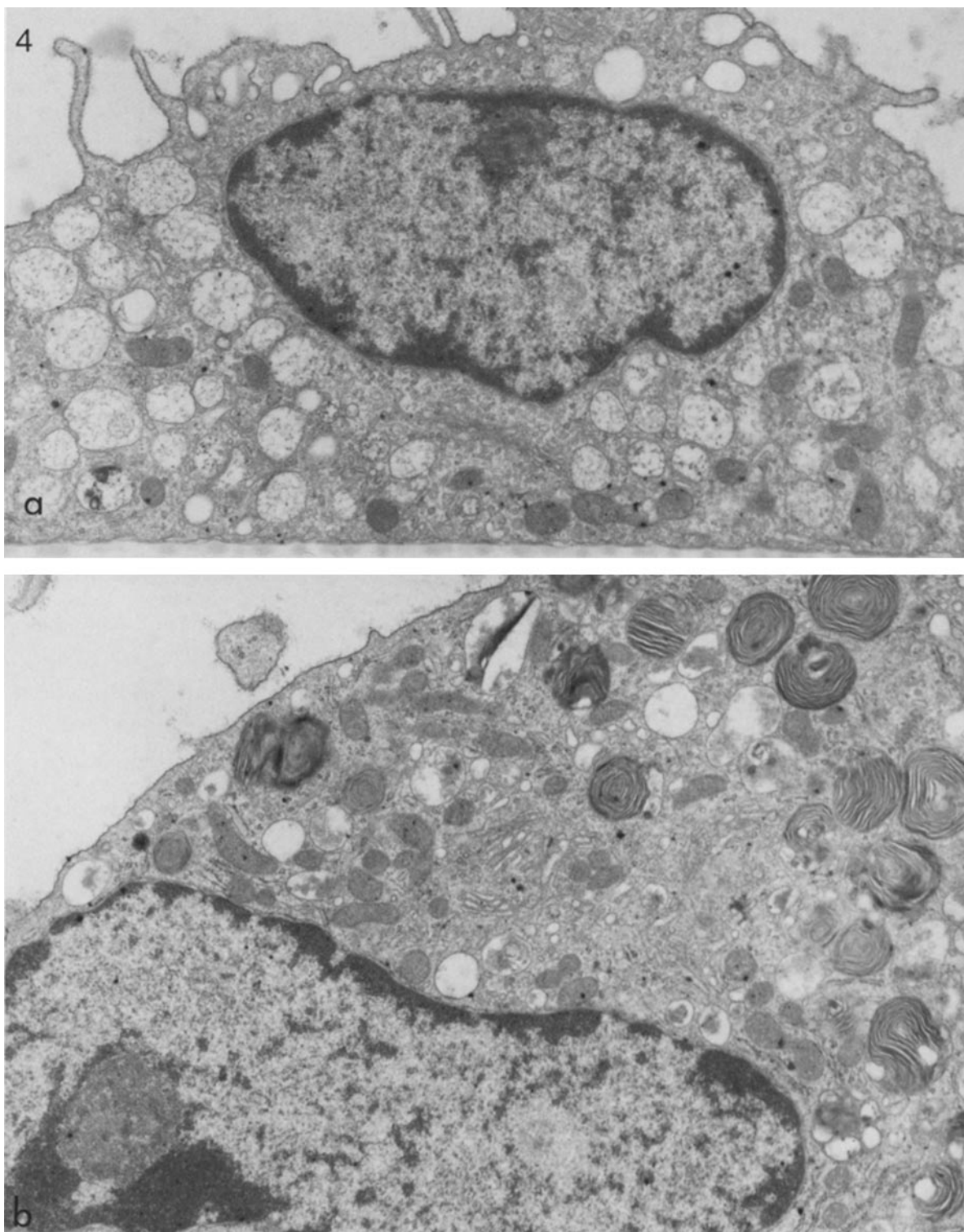
‡ The number of experiments is given in parentheses.

TABLE I  
Compounds That Inhibit P-L Fusion

Compound	Molecular weight	Inhibitory concentration*	% Sulfur as ester sulfate	
			Reported	Experimental
Suramin	1,429	200 $\mu$ g/ml ( $1.4 \times 10^{-4}$ M)	13.5‡	—
DS	500,000	10 $\mu$ g/ml ( $2 \times 10^{-8}$ M)	17	15.7
	40,000	500 $\mu$ g/ml ( $1.25 \times 10^{-5}$ M)	—	13.8
	8,000	500 $\mu$ g/ml ( $6.25 \times 10^{-5}$ M)	—	12.2
Poly-D-glutamate	35,000	0.5-1 mg/ml ( $1.4 \times 10^{-6}$ M)	—	—
Heparin	—	5 mg/ml	—	—
Chondroitin sulfate C	—	10 mg/ml	—	3.9

\* Defined as the lowest concentration which maintained >99% inhibition by AO assay 1 h after particle ingestion in cells treated 3-4 d with polyanion.

‡ Sulfur as sulfonate.



**FIGURE 4** Electron micrographs of polyanion-treated macrophages. (a) Cell treated with 10  $\mu\text{g/ml}$  DS as in Fig. 1. Note numerous large secondary lysosomes containing a fibrillar material.  $\times 19,200$ . (b) Cell treated with 100  $\mu\text{g/ml}$  suramin as in Fig. 1. Again, large secondary lysosomes are seen and many contain whorls of membranous material (myelin figures).  $\times 14,014$ .

represent endocytosed DS or other material brought into the cell due to increased pinocytosis. When secondary lysosomes were prelabeled with Thorotrast as in Table II, Thorotrast label was found in the large vacuoles which appear to be the enlarged secondary lysosomes of the DS-inhibited cells, visualized in fluorescence and phase microscopy in Fig. 2. The majority of these large vacuoles appeared to stain with both toluidine blue

and AO, implying the presence of DS and their acidic, presumably lysosomal, nature. Other organelles appear unaltered and the cells are well-spread and healthy under these culture conditions.

Fig. 4b shows the ultrastructure of 4-d cultures of suramin-treated cells. Again, large vacuoles, some containing fibrillar material, are seen in the cytoplasm. In many of the vacuoles,

however, striking whorls of membranous material have accumulated. This was occasionally seen in cells treated with other polyanions, but the myelin figures in suramin cells were larger and much more numerous. These myelin figures appear similar to those described in alveolar macrophages that have ingested surfactant (21).

### Effects on Pinosome-Lysosome Fusion

There was a clear effect of polyanion treatment on phagosome-lysosome fusion. Several approaches were used to evaluate whether this inhibition extended to pinosome-lysosome fusion as well. First, HRP was used as fluid-phase pinocytic marker, and its uptake and degradation by control, D-treated, or DS-treated macrophages were evaluated. As shown in Table III, the uptake of 1 mg/ml HRP was similar in all three cell types, on a cell protein basis. When cells were given HRP in a 1-h pulse and its subsequent degradation was followed, the half-life of its enzymatic activity ranged from 6.4 to 8.7 h, within the general range of that seen in previous studies (13). Using HRP degradation as an indirect measure of pinosome-lysosome fusion, no significant difference was seen in polyanion-treated cells. This could mean that there is a population of primary lysosomes and/or secondary lysosomes that have not yet taken up very much DS and that are able to fuse and degrade pinocytosed HRP.

Although by biochemical assay (see Materials and Methods) HRP activity was found to be unaffected by D or DS, histochemical staining for HRP showed nonspecific staining in D-treated cells, and we were also concerned that DS could inhibit HRP staining. Thus, HRP was not used as a morphological pinocytic marker. To assess heterogeneity in the lysosome population of these cells, Thorotrast was used instead of HRP as a pinocytic marker for electron microscopy. After 4 d of culture in medium with or without D or DS, cells were exposed to Thorotrast in medium for 1 h and fixed for EM. The distribution of pinocytosed Thorotrast is shown in DS-treated cells (Fig. 5). Many of the large cytoplasmic vacuoles in the DS-treated cells have been labeled with the pinocytic marker. There did not appear to be preferential labeling of small vacuoles that might contain less DC. Thorotrast also labeled the large vacuoles in D-treated cells. About 250 vacuoles of each cell type were evaluated for the presence of Thorotrast. A range of 28–90% of the total vacuole population within a cell was labeled by Thorotrast. An average of about 50% of the D

cell vacuoles and 60% of the DS vacuoles contained discernible Thorotrast.

### Characterization of Pinosome-Lysosome Fusion by Subcellular Fractionation

Finally, subcellular fractionation was used to monitor the transfer of pinocytosed HRP to a lysosomal fraction obtained from D or DS cells. These studies made use of the increased size and density of the secondary lysosomes from these cells. The increased density of D-filled lysosomes had been previously noted in subcellular fractionation of liver from D-injected rats (22).

Whole-cell homogenates from either cell type were layered over linear metrizamide gradients and centrifuged at 10,000 *g* for 90 min, and gradient fractions were assayed as described in Materials and Methods. The lysosomal marker NAGase sedimented in a band halfway down the gradient (Fig. 6*a* and *b*). NAGase activity at the top of the gradient showed little latency, while that in the lysosomal band was increased four to five times by Triton. Most of the plasma membrane marker 5'NTase remained at the top of the gradient. In another experiment, the lysosomal bands from D and DS velocity gradients were collected with a bent Pasteur pipette, diluted in HEPES-saline (12), and layered over 20–50% sucrose step gradients. After centrifugation at 100,000 *g* for 60 min, the D lysosomes banded at the 40–50% sucrose interface, while the DS lysosomes pelleted below the 50% sucrose step. Thus, by isopycnic sedimentation in sucrose, the D lysosomes have an apparent density between 1.179 and 1.232, whereas the DS lysosomes have an apparent density >1.232.

For comparison, velocity gradient sedimentation was also performed on untreated cells, using 15–40% metrizamide gradients. As shown in Fig. 6*c*, untreated lysosomes are much less dense and far more heterogeneous. Most of the NAGase activity does not enter the gradient under these centrifugation conditions, although the NAGase activity in the homogenate was increased two to three times by Triton. When cells were prelabeled with AO, AO-fluorescence comigrated with the NAGase peak (Fig. 6*c*). Similar AO-labeling of D and DS cells also showed that >90% of the AO-fluorescence comigrated with the peak of NAGase activity seen in the middle of the gradients.

Since unmodified lysosomes thus sediment in a different position on these gradients than D- or DS-filled lysosomes, velocity sedimentation was used to assay for the transfer of HRP to dense D or DS lysosomes. Fig. 7 shows the distribution of HRP after 1 h of pinocytic uptake. The HRP activity closely parallels the distribution of NAGase activity for both D and DS cells. DS cells were cultured in medium containing <sup>3</sup>H-DS, and the tritium label comigrates with both the NAGase and HRP activity. By these three approaches, pinosome-lysosome fusion appears unaffected by the presence of DS in secondary lysosomes.

### DISCUSSION

Other investigators have found that intralysosomal accumulation of polyanions inhibits subsequent P-L fusion. Suramin (7) and a sulfatide from *Mycobacterium tuberculosis* (23) were the polyanions first studied. In several later reports from D'Arcy Hart and his colleagues, inhibition by poly-D-glutamate (8) and an oxidized amylose (24, 25) is discussed.

Some controversy does exist in the literature concerning P-L

TABLE III  
Uptake and Degradation of HRP by Macrophage Cultures

Culture conditions*	Uptake‡	<i>t</i> <sub>1/2</sub> of Inactivation of cell-associated HRP activity§
	ng HRP/100 µg cell protein/2 h	h
4-d Cultures	125	8.5
4-d D-treated cultures	111	8.7
4-d DS-treated cultures	143	6.5

\* Standard culture conditions with the addition of 10–30 mg/ml D or 10 µg/ml DS where indicated.

‡ 1 mg/ml HRP in medium was given to triplicate macrophage cultures for 30 min and 2 h. Average data calculated for 2-h uptake.

§ Macrophage cultures were exposed to 1 mg/ml HRP for 1 h, washed four times with minimum essential medium, and recultured in HRP-free medium. At 0.5, 4, 8, and 24 h, triplicate cultures were washed, lysed in 0.1% Triton, and assayed for enzymatic activity.



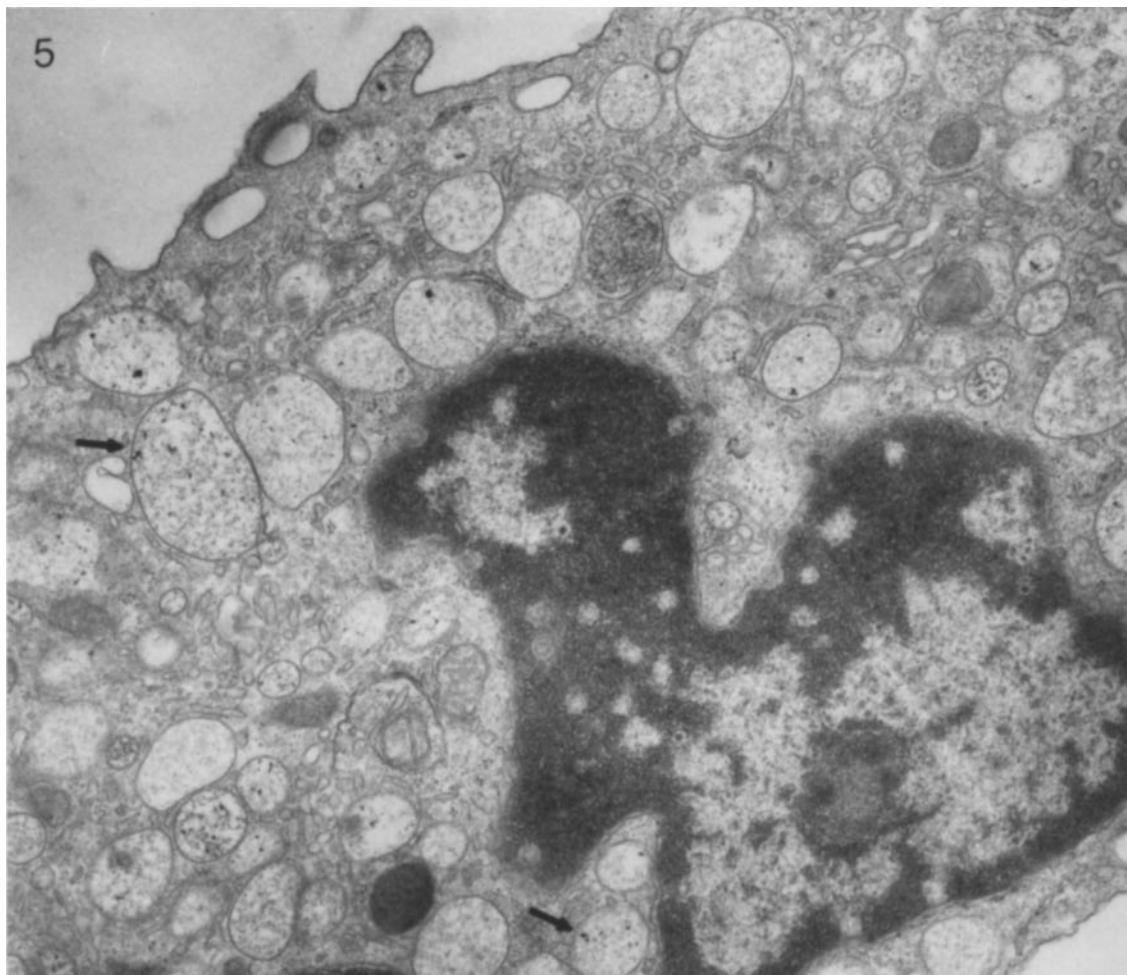


FIGURE 5 The intravacuolar distribution of Thorotrast in DS-treated cells. Cells were cultured for 4 d in  $20 \mu\text{g}/\text{ml}$   $^3\text{H}$ -DS, washed, and exposed to a 1:40 dilution of Thorotrast in medium (vol:vol) for 1 h. Cells were then washed and processed for EM. Electron-dense Thorotrast is seen within the large DS-filled secondary lysosomes (arrows).  $\times 19,500$ .

fusion inhibition by polyanions. Pesanti (26) did not find inhibition of P-L fusion in suramin-treated cells as assayed by electron microscopy. Goren (27) also could not demonstrate inhibition by electron microscopy and raised doubts about the use of AO in fusion assays of polyanion-containing lysosomes which might bind the dye to ionic interactions. Results reported here confirm the inhibition of P-L fusion by suramin and DS using a sensitive EM assay and corroborate the inhibition observed by the AO assay.

The percent fusion as determined by the two assays is not strictly comparable since the lysosomal markers are evaluated in whole-cell preparations for AO and in thin sections using stereological analysis for Thorotrast. It is important that marked inhibition is observed in both assays, however, since AO sequestration is potentially sensitive to changes in the pH of any subcellular compartment (1).

#### *Effects on Pinosome-Lysosome Fusion*

By three different experimental approaches, pinosome-lysosome fusion was not altered in polyanion-treated cells. As previously described (2), inhibition by concanavalin A (Con A) also differs between these two vesicle types. Pinosome-lysosome fusion is inhibited by Con A bound to the luminal membrane of the pinocytic vesicles (28), whereas membrane or

particle-bound Con A does not inhibit phagosome-lysosome fusion. Con A acts from within the lumen of the pinosome, while polyanions appear to act from within the secondary lysosome. Since pinosome fusion is unaffected by polyanions, pinocytic vesicles containing DS can continue to fuse with secondary lysosomes and deliver more polyanion to this compartment. Inhibition of P-L fusion by polyanion does not appear to require the inhibitor on the plasma membrane or in the phagocytic vacuole, as judged from the time course of inhibition and from the maintenance of inhibition in the absence of extracellular polyanion.

The reason for the striking difference in the fusion of the two types of endocytic vacuoles is not clear. Both pinocytosis and phagocytosis require plasma membrane fusion to form the endocytic vacuole. While fluid-phase pinocytosis is a constitutive cell process without known exogenous determinants, phagocytosis requires circumferential receptor-ligand interactions, as in Fc receptor-mediated phagocytosis (29). The triggering of phagocytosis by receptor-binding involves elements of the actomyosin cytoskeleton, whereas this may not occur in pinocytosis. Pinocytic uptake may require clathrin-membrane interactions in coated vesicle formation (30). This difference during vesicle formation may be preserved in the interiorized vesicle, resulting in different cytoplasmically disposed vesicle faces. Studies to date from this laboratory imply that both



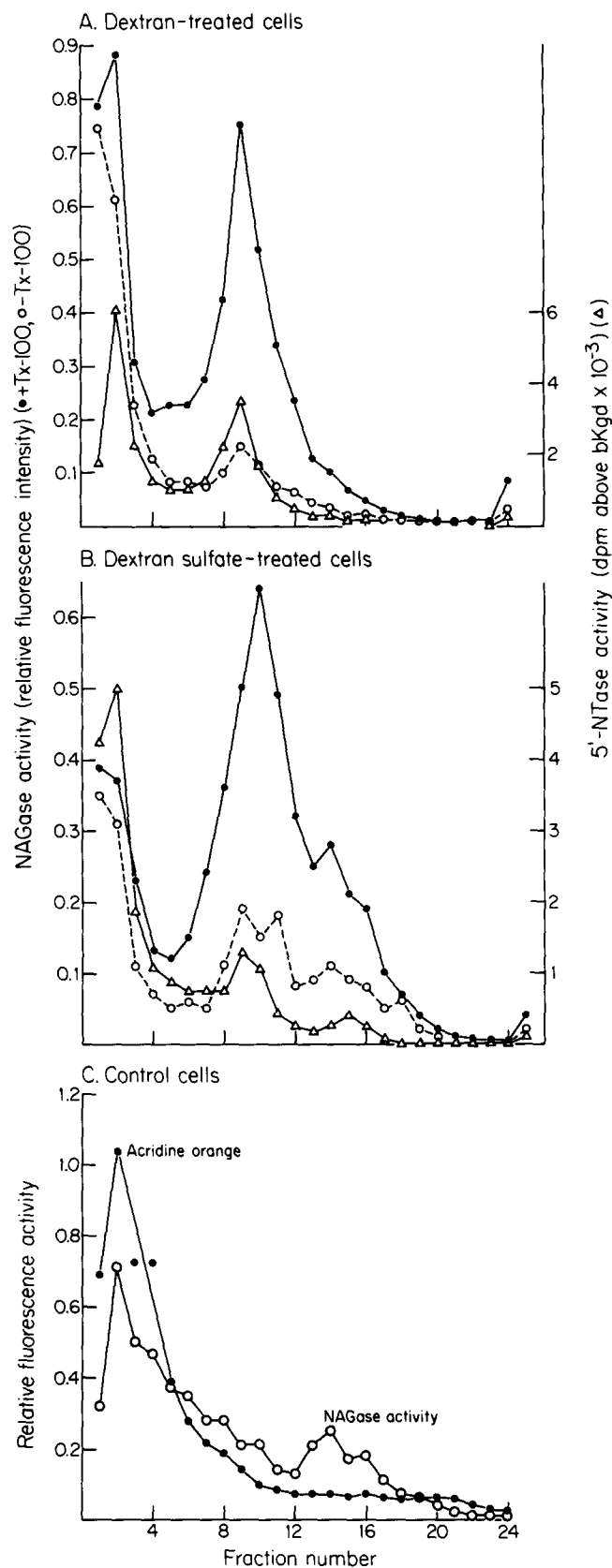


FIGURE 6 Subcellular fractionation of untreated and D- or DS-treated cells. Sedimentation from left to right. Fractions 1 and 2 are load volume. 5'-NTase was assayed in the presence of 0.05% Triton, and recovery ranged from 60 to 75%. Background was  $\sim 400$  dpm. NAGase was assayed with and without 0.1% Triton and showed twofold to threefold latency in the cell homogenates. (a) 4-d D cultures, 30 mg of D/ml of 15–40% metrizamide gradient. (b) 4-d

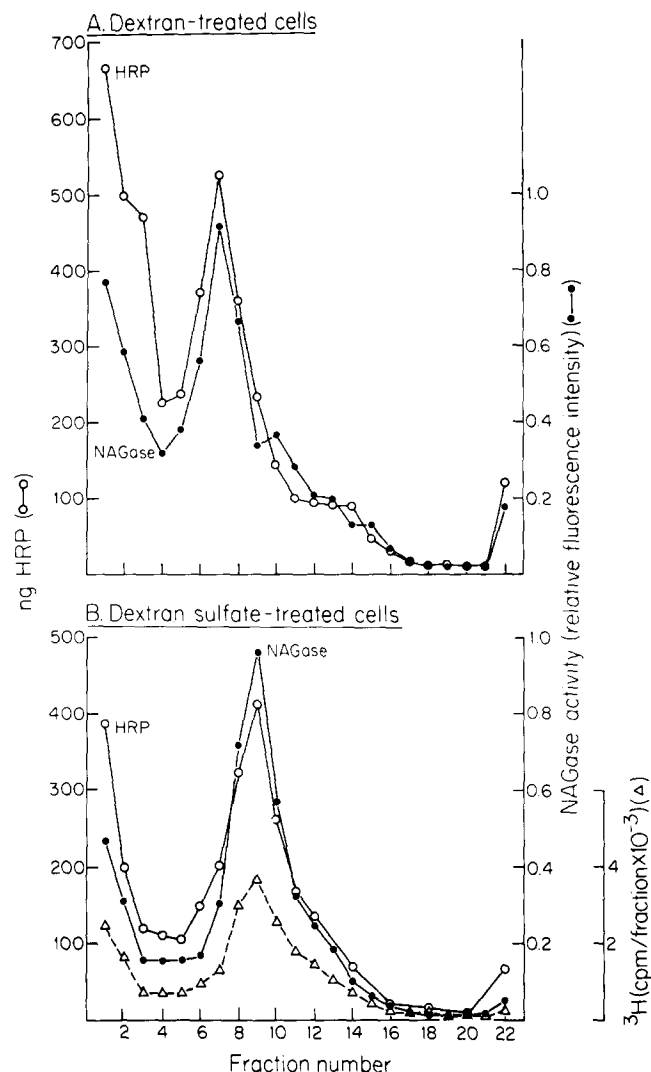


FIGURE 7 Transfer of a pinocytic marker into the lysosomal fraction of D- or DS-treated cells. Both sets of cells were labeled for 60 min with 1 mg/ml, HRP, washed, homogenized, and run on gradients as described in Materials and Methods. Sedimentation from left to right. Fractions 1 and 2 are load volume. Both enzymes were assayed in the presence of Triton. Latency of HRP was 2- to 2.5-fold in each homogenate. Recovery of enzymes and label ranged from 75–129%. (a) 4-d D cultures, 10 mg of D/ml of 15–40% metrizamide gradient. (b) 4-d DS cultures, 7.5  $\mu$ g of cold DS/ml and 2.5  $\mu$ g of  $^3$ H-DS/ml of 20–40% metrizamide gradient.

phagocytosis and pinocytosis cause the internalization of representative samples of plasma membrane polypeptides (31, 32). The composition of cytoplasmically disposed polypeptides is not, however, defined for either vesicle type.

Pinocytosis and phagocytosis are also affected differently by saturated fatty acid enrichment of membrane phospholipids (33). Both processes are inhibited in cultures supplemented with *trans*-18:1 or -19:0, but the activation energy is only increased for phagocytosis. This may mean that heterogeneity exists within the lipid phase of the membrane and that pinocytosis occurs within relatively unsaturated domains. Again,

DS cultures, 10  $\mu$ g of DS/ml of 20–40% metrizamide gradient. (c) 2- or 4-d untreated cells. 15–40% metrizamide gradients showing the distribution of AO label and NAGase activity in two separate experiments. NAGase activity in the fractions was assayed in the presence of 0.1% Triton.

differences in lipid microdomains could influence fusion of the two different vesicle types.

Vesicle size is another variable that theoretically could influence fusion (34). Average pinosome diameter of resident macrophages was estimated to be  $\sim 0.2 \mu\text{m}$  (35), whereas the diameter of the yeast test particles used in this study of P-L fusion is  $\sim 3.8 \mu\text{m}$  (1). This large difference in vesicle size could be a factor in the ability of these vesicles to fuse with DS-filled lysosomes or to be inhibited by membrane-bound Con A. While the reason for the difference in vesicle fusion is not understood, it is evident from this discussion that already many distinctions between phagosomes and pinosomes exist and that further functional differences are not unexpected.

### Effects of Polyanions on Endocytosis and Lysosome Function

The polyanions used in these studies have several known effects on endocytosis in macrophages. First, several of these compounds were observed to cause a rapid increase in vesicle formation upon addition to macrophage cultures (4). After 4 d of treatment with DS, however, HRP uptake was not appreciably increased when HRP was pinocytosed in polyanion-free medium (Table III). Secondly, DS appears to interact with lipoproteins in culture medium to increase their uptake by macrophages (6). DS and sulfated glycosaminoglycans such as heparin are known to form both soluble and insoluble complexes with lipoproteins (5, 6, 36). Increased uptake of lipoproteins by a receptor-mediated type of pinocytosis and/or stimulation of bulk fluid-phase pinocytosis by polyanion could explain the membrane whorls and enlarged lysosomes in polyanion-treated cells. Some of the increase in lysosomal size may also be due to osmotic swelling, since the nondigestible charged polymer could carry in counterions.

The extensive myelin figures seen in suramin cells are reflected in an increased phospholipid/protein ratio and an increase in the proportion of sphingomyelin from 1 to 6 mol % phospholipid (Kielian and Cohn, unpublished observations).

This could be due to decreased metabolism of phospholipids by suramin-containing lysosomes. For example, suramin has been found to inhibit several of the enzymes necessary for the metabolism of glycosaminoglycans, leading to their intralysosomal accumulation similar to the situation in mucopolysaccharidosis (37). The polyanions discussed here thus may also act within the lysosome to alter the normal metabolism of cellular substrates or of exogenous macromolecules such as lipoproteins.

The following paper examines the uptake characteristics of polyanion inhibition. The role of pinocytosed lipoproteins in inhibition and the effects of polyanions on lysosome function and membrane fluidity are further defined therein.

We thank Richard Huntley for expert technical assistance, Judy Adams for assistance with microscopy and photographic work, and Grace Silvestri and Betty Broyles for preparing the manuscript.

This work was supported by grant AI 07012 from the National Institutes of Health.

Received for publication 17 August 1981, and in revised form 13 January 1982.

### REFERENCES

- Kielian, M. C., and Z. A. Cohn. 1980. Phagosome-lysosome fusion. Characterization of intracellular membrane fusion in mouse macrophages. *J. Cell Biol.* 85:754-765.
- Kielian, M. C., and Z. A. Cohn. 1981. Modulation of phagosome-lysosome fusion in mouse macrophages. *J. Exp. Med.* 153:1015-1020.
- Kielian, M. C., and Z. A. Cohn. 1981. Phorbol myristate acetate stimulates phagosome-lysosome fusion in mouse macrophages. *J. Exp. Med.* 154:101-111.
- Cohn, Z. A., and E. Parks. 1967. The regulation of pinocytosis in mouse macrophages. II. Factors inducing vesicle formation. *J. Exp. Med.* 125:213-232.
- Nishida, T., and U. Cogan. 1970. Nature of the interaction of dextran sulfate with low density lipoproteins of plasma. *J. Biol. Chem.* 245:4689-4697.
- Basu, S. K., M. S. Brown, Y. K. Ho, and J. L. Goldstein. 1979. Degradation of low density lipoprotein-dextran sulfate complexes associated with deposition of cholesteryl esters in mouse macrophages. *J. Biol. Chem.* 254:7141-7146.
- D'Arcy Hart, P., and M. R. Young. 1975. Interference with normal phagosome-lysosome fusion in macrophages, using ingested yeast cells and suramin. *Nature (Lond.)* 256:47-49.
- D'Arcy Hart, P., and M. R. Young. 1979. The effect of inhibitors and enhancers of phagosome-lysosome fusion in cultured macrophages on the phagosome membranes of ingested yeasts. *Exp. Cell Res.* 118:365-375.
- Graham, R. C., Jr., and M. J. Karnovsky. 1966. The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. *J. Histochem. Cytochem.* 14:291-302.
- Terho, T. T., and K. Hartiala. 1971. Method for determination of the sulfate content of glycosaminoglycans. *Anal. Biochem.* 41:471-476.
- Chayen, J., L. Bitensky, and R. G. Butcher. 1973. Practical Histochemistry. J. Wiley & Sons, Inc., New York.
- Munthe-Kaas, A. C., and P. O. Seglen. 1974. The use of metrizamide as a gradient medium for isopycnic separation of rat liver cells. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 43:252-256.
- Steinman, R. M., and Z. A. Cohn. 1972. The interaction of soluble horseradish peroxidase with mouse peritoneal macrophages in vitro. *J. Cell Biol.* 55:186-204.
- Edelson, P. J., and Z. A. Cohn. 1976. 5'-Nucleotidase activity of mouse peritoneal macrophages. I. Synthesis and degradation in resident and inflammatory populations. *J. Exp. Med.* 144:1581-1595.
- Peters, T. J., M. Müller, and C. de Duve. 1972. Lysosomes of the arterial wall. I. Isolation of subcellular fractionation of cells from normal rabbit aorta. *J. Exp. Med.* 136:1117-1139.
- Wilzbach, K. E. 1957. Tritium-labeling by exposure of organic compounds to tritium gas. *J. Am. Chem. Soc.* 79:1013.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
- Cohn, Z. A., and B. A. Ehrenreich. 1969. The uptake, storage, and intracellular hydrolysis of carbohydrates by macrophages. *J. Exp. Med.* 129:201-225.
- Cohn, Z. A., and B. Benson. 1965. The *in vitro* differentiation of mononuclear phagocytes. II. The influence of serum on granule formation, hydrolase production and pinocytosis. *J. Exp. Med.* 121:835-848.
- Burstein, M., H. R. Scholnick, and R. Morfin. 1970. Rapid method for the isolation of lipoproteins from human serum by precipitation with polyanions. *J. Lipid Res.* 11:583-595.
- Nichols, B. A. 1976. Normal rabbit alveolar macrophages. I. The phagocytosis of tubular myelin. *J. Exp. Med.* 144:906-919.
- Beaufay, H. 1969. Isolation and lysosomes. In *Lysosomes in Biology and Pathology*. Vol. 2. J. T. Dingle and H. B. Fell, editors. 537-539.
- Goren, M. B., P. D'Arcy Hart, M. R. Young, and J. A. Armstrong. 1976. Prevention of phagosome-lysosome fusion in cultured macrophages by sulfatides of *Mycobacterium tuberculosis*. *Proc. Natl. Acad. Sci. U. S. A.* 73:2510-2514.
- Geisow, M. J., G. H. Beaven, P. D'Arcy Hart, and M. R. Young. 1980. Site of action of a polyanion inhibitor of phagosome-lysosome fusion in cultured macrophages. *Exp. Cell Res.* 126:159-165.
- Geisow, M. J., P. D'Arcy Hart, and M. R. Young. 1981. Temporal changes of lysosome and phagosome pH during phagolysosome formation in macrophages: studies by fluorescence spectroscopy. *J. Cell Biol.* 89:645-652.
- Pesanti, E. L. 1978. Suramin effects on macrophage phagolysosome formation and antimicrobial activity. *Infect. Immun.* 20:503.
- Goren, M. B., C. L. Swendsen, and J. Henson. 1980. Factors modifying the fusion of phagosomes and lysosomes: art, fact, and artefact. In *Mononuclear Phagocytes: Functional Aspects*. R. van Furth, editor. Martinus Nijhoff, The Hague. 999-1038.
- Edelson, P. J., and Z. A. Cohn. 1974. Effects of concanavalin A on mouse peritoneal macrophages. I. Stimulation of endocytic activity and inhibition of phagolysosome formation. *J. Exp. Med.* 140:1364-1386.
- Silverstein, S. C., R. M. Steinman, and Z. A. Cohn. 1977. Endocytosis. *Annu. Rev. Biochem.* 46:669-722.
- Anderson, R. G. W., M. S. Brown, and J. L. Goldstein. 1977. Role of the coated endocytic vesicle in the uptake of receptor-bound low density lipoprotein in human fibroblasts. *Cell* 10:351-364.
- Muller, W. A., R. M. Steinman, and Z. A. Cohn. 1980. The membrane proteins of the vacuolar system. I. Analysis by a novel method of intralysosomal iodination. *J. Cell Biol.* 86:292-303.
- Mellman, I. S., R. M. Steinman, J. C. Unkeless, and Z. A. Cohn. 1980. Selective iodination and polypeptide composition of pinocytic vesicles. *J. Cell Biol.* 86:712-722.
- Mahoney, E. M., A. L. Hamill, W. A. Scott, and Z. A. Cohn. 1977. Response of endocytosis to altered fatty acyl composition of macrophage phospholipids. *Proc. Natl. Acad. Sci. U. S. A.* 74:4895-4899.
- Poste, G., and A. C. Allison. 1970. Membrane fusion. *Biochim. Biophys. Acta* 300:421-465.
- Steinman, R. M., S. E. Brodie, and Z. A. Cohn. 1976. Membrane flow during pinocytosis. A stereologic analysis. *J. Cell Biol.* 68:665-687.
- Cornwell, D., and F. Kruger. 1961. Molecular complexes in the isolation and characterization of plasma lipoproteins. *J. Lipid Res.* 2:110-134.
- Constantopoulos, G., S. Rees, B. G. Cragg, J. A. Barranger, and R. O. Brady. 1980. Experimental animal model for mucopolysaccharidosis: suramin-induced glycosaminoglycan and sphingolipid accumulation in the rat. *Proc. Natl. Acad. Sci. U. S. A.* 77:3700-3704.