Intralysosomal Accumulation of Polyanions. II. Polyanion Internalization and Its Influence on Lysosomal pH and Membrane Fluidity

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ABSTRACT Dextran sulfate (DS) was previously shown to inhibit phagosome-lysosome (P-L) fusion whereas dextran (D) of equivalent size was ineffective. The uptake and interiorization of DS were examined with a tritiated product over the course of 4 d in culture. The exposure of macrophages to 20 μg/ml of ^{3}H-DS led to linear uptake for 4 d, at which time fusion was inhibited. Macrophage interiorization of ^{3}H-DS was greatly increased by forming insoluble complexes with either serum lipoproteins or purified human low density lipoproteins (LDL). Under these conditions fusion was inhibited within 4 h. The uptake of large quantities of acetylated LDL in the absence of DS was not associated with the inhibition of fusion. Lipoproteins therefore served as the DS carriers and were not themselves inhibitory.

The intralysosomal pH of control and D-treated macrophages was 4.76 (±0.06) and 4.68 (±0.02), respectively. Storage of DS was associated with a decreased pH to 4.36 (±0.14). Increasing the intralysosomal pH with either NH_4Cl or chloroquine failed to modify inhibited P-L fusion. Hydrogen ion concentration was therefore not an important factor in DS inhibition.

Secondary lysosomes were isolated from D- and DS-loaded cells and exhibited excellent latency. These lysosomes were exposed to the membrane probes, α- and β-parinaric acid, and compared in fluorescence polarization measurements. The results with the β isomer consistently indicated that the membranes of DS lysosomes were more rigid than the D samples. It is suggested that high intralysosomal concentrations of DS interact directly with either lipid and/or polypeptide moieties of the luminal face of the membrane, thereby decreasing its fluidity and fusibility.

Our preceding paper (Kielian et al. *J. Cell Biol.* 93:866–874), described the striking inhibition of phagosome-lysosome (P-L) fusion which occurred with the intralysosomal storage of highly anionic macromolecules. Inhibition of P-L fusion resulted from the presence of anionic groups rather than the macromolecular nature of the saccharide backbone. In this context, dextran sulfate (DS) was an excellent inhibitor, whereas dextran (D) was ineffective.

To examine this phenomenon in more detail we wished to understand (*a*) the factors governing the interiorization of the polyanion and (*b*) its influence on the lysosomal limiting membrane and its enclosed contents. In this paper we report on the significant amplification of polyanion interiorization by serum lipoproteins and their influence on intralysosomal pH and membrane fluidity.

MATERIALS AND METHODS

Details of cell culture and assays of phagosome-lysosome fusion are the same as those described in the preceding paper.

Purification and Acetylation of Low Density Lipoprotein (LDL)

The purified LDL was the kind gift of Dr. Mary Rifkin (Rockefeller University) and was purified from human serum (1). First, a lipoprotein fraction was obtained by ultracentrifugation of plasma adjusted to a density of 1.255 by the addition of solid KBr. This fraction was then separated into very low density lipoprotein (VLDL), LDL, and high density lipoprotein (HDL) with an agarose column.

Purified LDL was acetylated by the method of Fraenkel-Conrat (2) and as described by Basu et al. (3). The purity of the LDL fractions and the acetylation of LDL were monitored by cellulose acetate electrophoresis. One major oil red O

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staining band was seen in the preparations before and after acetylation. This LDL band had a more anodal migration after acetylation.

Oil red O staining of macrophages treated with LDL was done on fixed cover slip preparations at 0.5% oil red O in 60% triethylphosphate, 10 min at room temperature, followed by washing in H_2O (4).

Synthesis and Purification of Labeled DS

DS (500,000 mol wt) was tritium-labeled by New England Nuclear (Boston, MA) using the Wilzbach (5) method. 500 mg of DS was exposed to 15 Ci tritium gas for 2 wk at 76-mm pressure and room temperature. Labile tritium was removed with water. The labeled material was purified by precipitating the aqueous solution with 2 vol of 95% ethanol and 1% (final concentration) potassium acetate as described by Tourtellotte and Dziewiatkowski (6). The precipitate was washed twice with absolute ethanol, twice with anhydrous ether, and dried *in vacuo*. This material was redissolved in H_2O and reprecipitated three times to a constant specific activity of 73.7 μCi/mg DS. The carbohydrate content of a weighed sample was determined by the anthrone assay (7) using DS standards, sulfate content was determined by the method of Terho and Hartiala (8), and the molecular weight estimated by gel filtration on a Sepharose 2B column in 0.5 M NaCl. Labeled DS showed one peak on gel filtration with an average molecular weight of 300,000, and contained 10% sulfur as ester sulfate, as compared with 12.6% for untreated DS, 12.8% for reprecipitated, unlabeled DS, and 17% as the manufacturer's estimate for 500,000 mol wt DS. Radioactivity was determined by counting in Hydrofluor (National Diagnostics, Somerville, NJ); counting efficiency was calculated using a tritiated toluene standard. Both 500,000 mol wt D and DS were from Pharmacia Fine Chemicals (Piscataway, NJ).

Uptake of ^{3}H-DS by Macrophages

Uptake studies of ^{3}H-DS were performed on cover slip cultures of macrophages in wells of tissue culture dishes (Costar, Data Packaging, Cambridge, MA), with triplicates for each time point. Time points were taken by aspirating labeled medium, washing wells once with cold phosphate-buffered saline (PBS), and washing cover slips sequentially in three beakers of cold 0.3% NaCl. Cover slips were then placed in scintillation vials, cells were solubilized in 200 μl of 2% SDS, 3 ml of Hydrofluor was added, and radioactivity was determined by liquid scintillation counting.

^{3}H-DS serum complexes were made as in the procedure of Cornwell and Kruger (9) for the precipitation of LDL from serum by DS. To 1 vol of serum was added 1 mg/ml ^{3}H-DS (or unlabeled DS in some studies) and $CaCl_2$ to 0.1 M. After mixing and overnight incubation at 4°C, the resulting precipitate was pelleted by centrifugation in an International centrifuge, (International Equipment Co., Needham, Heights, MA) at 2,000 rpm for 20 min, 4°C. The pellet was washed twice in ice-cold distilled water without resuspending, and resuspended to various concentrations in 5% fetal calf serum/minimum essential medium (FCS/MEM). Complexes were sonicated to give a colloidal suspension that could be readily ingested by the cells. Under these conditions, ~35% of the added ^{3}H-DS was precipitated as complex. The usual LDL band seen in human serum after cellulose acetate electrophoresis was absent after DS-precipitation.

Purified LDL-DS complexes were formed by adding 2 mg/ml DS and 0.1 M $CaCl_2$ to purified LDL fractions in 0.15 M NaCl. Precipitates were processed as above, and then resuspended by sonicating in 5% FCS/MEM at twice the original volume, such that the maximum possible concentration of DS would be 1 mg/ml. The LDL column fractions were usually concentrated to the approximate original serum volume before precipitation.

Uptake studies of DS complex were performed as for soluble DS, except that culture dishes were rocked on a rotating platform (Nutator; Clay Adams, Parsippany, NJ) at 37°C during the uptake period. This helped to keep the complexes from sticking nonspecifically to the cells and cover slips.

Quantitation of Intralysosomal pH

The technique of Okuma and Poole (10) was used to measure lysosomal pH. 3-d cultures of untreated cells, or cells treated with 10 mg/ml D or 10 μg/ml DS, were exposed for 24 h to these media with or without added fluorescinated dextran (Sigma Chemical Co., St. Louis, MO) at 0.5 mg/ml. Cells were then washed in PBS, mounted in a cover slip holder which fit into a fluorescence cuvette, and scanned in PBS plus 1 mg/ml glucose at 37°C. Cells were allowed to equilibrate for 5–10 min before scanning. An MPF-44 fluorometer (Perkin-Elmer Corp., Norwalk, CT) with a water-jacketed cuvette holder was used. Excitation spectra of cells or standards were scanned from 400 to 500 nm, and emission was measured at 519 nm with 5-nm slits on both monochrometers. The ratio of the emission at 495 nm to that at 450 nm was plotted as a function of the pH of the standards, and this standard curve was used to determine the pH of the probe within the cells. DS or D at 5 mg/ml PBS did not alter the excitation spectrum of the fluorescinated probe.

Preparation of Samples for Fluorescence Polarization

Fluorescence polarization was performed on lysosomal fractions isolated from D- or DS-treated cells, using metrizamide gradient centrifugation as described in the preceding paper. Since metrizamide was found to quench probe fluorescence, the lysosomal band removed with a bent Pasteur pipette from the gradient was diluted to <10% metrizamide by the addition of 125 mM NaCl containing 1 mM EDTA and 10 mM HEPES, pH 7.4. This was layered over 6 ml of 20% sucrose (wt/vol) containing 1 mM EDTA + 10 mM potassium phosphate buffer, pH 7.4. The lysosomal fraction was washed by sedimentation through this step gradient, centrifuging at 5,000 *g* for 35 min, 4°C, in the HB-4 rotor of a Sorvall centrifuge (DuPont Co., Wilmington, DE). Pelleted lysosomes were then resuspended in polarization buffer as described in the figure legends. *N*-Acetyl glucosaminidase (NAGase) activity in fractions and homogenates was assayed as described in the preceding paper.

Two isomers of parinaric (octadecatetraenoic) acid (PnA) were used for these studies, α (9, 11, 13, 15-*cis*,-*trans*,-*trans*,-*cis*) and β (9, 11, 13, 15 all -*trans*). Both were obtained from Molecular Probes (Roseville, MN) and stored as stock solutions under N_2 at −20°C, in $CHCl_3$ plus 0.1% butylated hydroxytoluene. Before use, an aliquot of the stock solution was dried under N_2 and redissolved in ethanol with the addition of BHT to a 1:1 molar ratio with PnA. 2-μl aliquots of the ethanolic stock were added to 1-ml samples of lysosomes in polarization buffer in 1-cm pathlength fluorescence cuvettes. Samples were stirred during probe equilibration and subsequent measurements.

After measurements, aliquots of samples and of the original cell homogenates Owere extracted (11) and the amount of phospholipid was determined (12). Under these conditions, 7–15% of the total cell phospholipid was recovered in the lysosome fraction. The PnA to phospholipid ratio in these experiments ranged from 1:35 to 1:65, below the ratio at which probe-mediated membrane perturbations appear to occur (13).

Fluorescence Measurements

Fluorescence measurements were made using an MPF-44 fluorometer with an excitation wavelength of 320 nm for β-PnA and 325 nm for α-PnA, slit 10 nm. Emission was measured at 420 nm, slit 18 nm, for both probes, using the 390-nm cutoff filter to decrease background fluorescence and light-scattering. Two Polaroid HNP'B polarizers were mounted in the fluorometer, and an automatic rotator (C. N. Wood Mfg. Co., Newtown, PA) was used. Temperature scans were performed using a water-jacketed cuvette holder connected to a circulating water bath and electronic temperature programmer (Neslab Instruments, Inc., Portsmouth, NH). Heating or cooling scans were performed at ≤0.5°C/min. For each sample, temperature, and polarizer orientation, three 30-s readings of fluorescence intensity were collected for the sample and an identical unlabeled lysosome preparation. Sample temperature was monitored using a thermocouple (Yellow Springs Instruments, Yellow Springs, OH) immersed in a cuvette containing polarization buffer.

Polarization (P) was determined using the formula $P = (Ivv - Ivh\,[G])/(Ivv + Ivh\,[G])$ where Ivv and Ivh were the measured fluorescence intensities parallel and perpendicular to the vertically polarized excitation light. The factor G (Ihv/Ihh) corrects for instrumental anisotropy as described previously (14, 15).

Heating or cooling scans of the samples produced equivalent results where performed and fluorescence intensity was decreased by ~40% after the experiment. Absorbance of unlabeled samples was usually $A_{320} \cong 0.6$, and background fluorescence was ~5–10% at 5°C, and was subtracted before P calculation.

To decrease probe oxidation, polarization buffers were vigorously deoxygenated with N_2 before use, and cuvettes containing samples were flushed with N_2 and capped after PnA addition. Absorption spectra of stock solutions were recorded on a Perkin-Elmer 557 scanning spectrophotometer. Spectra were sharp and similar to those previously published (16); probe degradation in stock solutions was negligible.

RESULTS

It appeared from the time course of P-L fusion inhibition by polyanions and from staining with toluidine blue that these compounds were gradually endocytosed over the course of days and stored in secondary lysosomes. To examine this process, tritium-labeled DS was prepared and purified as described in Materials and Methods. The ^{3}H-DS had a somewhat lower molecular weight (300,000) as estimated by gel filtration, and gave inhibition comparable to that of unlabeled DS after 3–4 d of culture at 20 μg/ml.

Uptake and Inhibition by Soluble DS

The uptake of ^{3}H-DS at this inhibitory concentration was followed for a 4-d culture period. As shown in Fig. 1, the uptake of polyanion was linear on a per-cell basis for the entire culture period. An initial boost is seen in uptake; this increase may represent a stimulation of vesicle formation by polyanion as has been previously described (17). The *inset* in Fig. 1 correlates the uptake of 20 μg/ml ^{3}H-DS with its effect on fusion. Complete inhibition of P-L fusion by the AO assay is observed after 3–4 d of uptake.

In another experiment, the amount of uptake at 76 h was normalized to cell protein and resulted in 4.21×10^2 cpm/μg cell protein. These uptake data are analyzed further in the Discussion.

Uptake and Inhibition by DS-serum Complexes

Two questions about polyanion inhibition were next considered. First, could inhibition be mediated rapidly or were several days of uptake required to mediate inhibition? Secondly, was the polyanion directly causing inhibition, or was it increasing the uptake of other molecules that were responsible for the effect?

To approach these questions, a system was devised to cause the rapid internalization of large quantities of polyanion. Taking advantage of the known interaction of DS with lipoproteins (9, 18), complexes of DS and serum lipoproteins were prepared by adding DS and $CaCl_2$ to human serum (see Materials and Methods). The addition of this colloidal DS-lipoprotein suspension to macrophages resulted in the rapid association of large amounts of complex with cells (Fig. 2*a*). Even when the complex was kept in suspension by rotation of the culture dish during the uptake at 37°C, background cover-slip counts were high and it was difficult to wash away all extracellular complex, as monitored by phase microscopy. Thus, the uptake data for ^{3}H-DS complex are much less accurate than for soluble DS. The uptake curve in Fig. 2 does give an estimate of the large amount of ^{3}H-DS taken up as complex, and the rapidity with which it becomes cell-associated.

Under these uptake conditions, inhibition could be achieved within 3–4 h of uptake (Fig. 2*b*). A 10-fold higher concentration of soluble DS did not cause inhibition within 5 h. The fine particulate suspension of lipoprotein-DS complex apparently enabled cells to take up large quantities more rapidly. Prolonged incubation is thus not a requirement for inhibition.

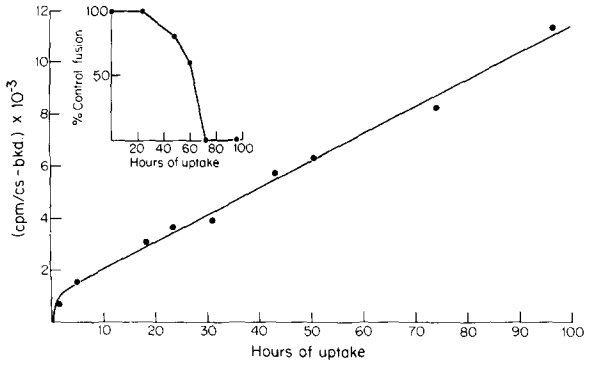

FIGURE 1 Uptake of soluble ^{3}H-DS. Shown are the average cpm of triplicate cover slip (cs) cultures plated at 5×10^5 peritoneal cells/cs. Explanted cells were adhered for 2 h, washed, and placed into medium containing 20 μg/ml ^{3}H-DS. Cells were given fresh medium each day. Background is for cells exposed to ^{3}H-DS for 2 h on ice and was 200 cpm/cs. *Inset* shows the time course of fusion inhibition by 20 μg/ml ^{3}H-DS-treated cells.

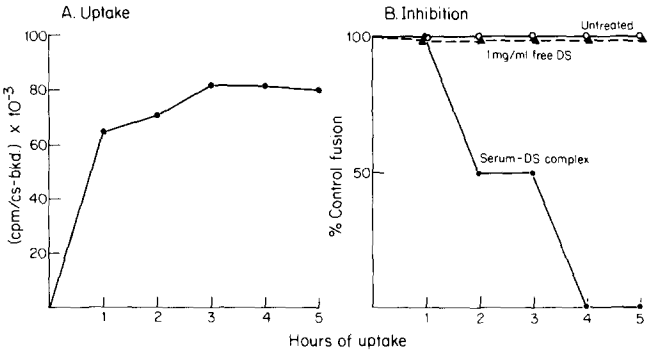

FIGURE 2 Uptake and fusion inhibition by ^{3}H-DS-serum complexes. (*a*) Uptake of 100 μg/ml ^{3}H-DS as serum complex. Shown are the average cpm of triplicate cs cultures plated at 5×10^5 peritoneal cells/cs, and cultured for 4 d in normal medium. On day 4, cover slips were exposed to serum complex at 37°C with shaking. Background is for cells exposed to complex for 1 h on ice and was 12×10^3 cpm/cs. (*b*). Time course of inhibition of fusion in 4-d cultures exposed as in *a* to similar unlabeled DS-serum complex, to 1 mg/ml soluble cold DS in medium, or to medium alone. Fusion was assayed ~1 h after particle ingestion for each cell type and time point.

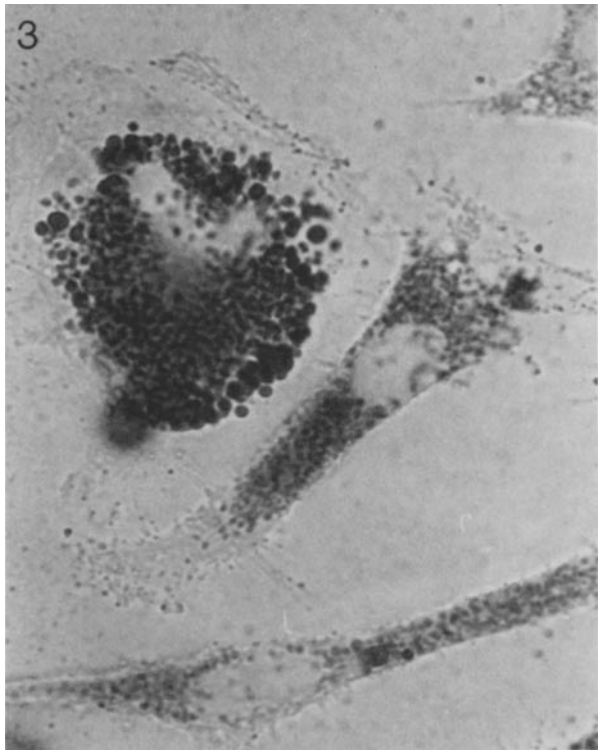

FIGURE 3 Bright-field micrograph of oil red O-stained cells. 24-h macrophage cultures were exposed to 1 mg/ml acetyl LDL in medium with 15% serum for 24 h. Cells were then fixed and stained with oil red O. X 1,680.

Uptake of Purified LDL

Since the usual DS-treated cells are cultured in serum-containing medium, and since DS has been reported to increase

the uptake of LDL by macrophages (19), we then asked whether LDL was mediating the inhibition. Mouse peritoneal macrophages do not have a receptor for LDL but have been shown to avidly take up acetylated LDL (20). Acetylation of LDL was therefore used as a means of introducing large quantities of the lipoprotein into macrophages in the absence of polyanions.

When macrophages were exposed to 1 mg/ml acetyl LDL for 24 h of culture, cells took up large amounts of the acetylated lipoprotein (Fig. 3) and stained heavily with oil red O. When P-L fusion in these cells was compared with that in untreated cultures, however, no inhibition was seen (Table I). Purified LDL did cause inhibition when it was complexed with DS, and, again, 4 h of uptake was sufficient for inhibition (Table I).

Cells exposed to acetyl LDL stained even more heavily with oil red O than those exposed to LDL-DS complexes, implying that the amount of LDL accumulation was not limiting in the acetyl LDL-treated cells. Thus, inhibition appears to be mediated by the polyanionic component of the DS-LDL complex.

Electron Microscopy of DS-lipoprotein Uptake

The morphology of complex uptake was evaluated and compared with the morphology of cells treated with soluble polyanion as described in the previous paper. As shown in Fig. 4*a*, 30 min after exposure to complex, macrophages have amorphous complex on the cell surface and some of it has been internalized into large cytoplasmic vacuoles. Complex is internalized pinocytically (see *inset*, Fig. 4*a*) as larger aggregates.

After longer periods of uptake (Fig. 4*b*), the cytoplasm has become filled with vacuoles containing the complex suspension, and many vacuoles show evidence of lysosomal digestion of the complex. These vacuoles now contain lipid droplets and whorls of membranous material. Complex uptake thus displays morphological characteristics similar to those of both the fibrillar material seen in the secondary lysosomes of DS-treated cells and the myelin figures prevalent in suramin cells.

Intralysosomal pH in D and DS Cells

Highly acidic DS might affect the maintenance of intralysosomal pH. Lysosomal pH in D and DS cells was therefore compared and its role in the inhibitory process evaluated.

Treated cells were cultured in D- or DS-containing medium for 3 d and then cultured for 24 h in these media plus 500 μg/ml fluoresceinated dextran. These culture conditions were used to ensure that the fluorescent probe was labeling the treated lysosome population. The standard curve in Fig. 5 shows the relationship between probe fluorescence and pH. As previously reported (10), both the change in 495/450 ratio and the quantum yield of the probe decrease below pH ~4.6, so that the estimated intralysosomal pH is the upper limit of the average intralysosomal pH. Marked on the standard curve are the determined intralysosomal pH values for the three cell types. The average values (five to seven determinations) and standard deviations were: control, 4.76 ± 0.06; D-treated, 4.68 ± 0.02; and DS-treated, 4.36 ± 0.14.

The pH in DS cells was 0.4–0.3 pH unit lower than that in control or D cells. This small difference in intralysosomal pH seemed unlikely to explain the inhibition of P-L fusion, although, as already discussed, any very acidic lysosomes are difficult to detect in these measurements. Taking advantage of the known effects of weak bases on intralysosomal pH (10), ammonium chloride was used to transiently raise the pH of lysosomes in the three cell types. Cells were given particles to ingest and, after a 15-min ingestion period, were put into media containing 5 or 10 mM NH_4Cl for 40 min. After a 10-min wash-out period in NH_4Cl-free media, cells were stained with AO and P-L fusion was evaluated. No effect of treatment with NH_4Cl on control fusion or on DS-inhibition was observed. Cells were spread and showed good lysosomal staining.

It appears unlikely that lysosomal pH is involved in DS-inhibition, since raising the lysosomal pH in cells which had previously ingested particles did not reverse the inhibition of DS-containing lysosomes. A similar experiment using 25 or 100 mM chloroquine also showed no effect on DS inhibition.

TABLE I

Effect of LDL Uptake on P-L Fusion

Treatment of 2-d cells	% Control fusion 60 min after yeast ingestion
Untreated	100
24-h exposure to 1 mg/ml acetyl LDL	100
4-h exposure to LDL-DS complex*	0–5

* Complex was formed by adding 2 mg/ml DS and 0.1 M $CaCl_2$ to 5 ml of the LDL fraction, purified as described in Materials and Methods. Column fraction was from 30 ml of human serum. Both acetyl LDL and LDL-DS complex were diluted in medium with 15% serum.

Effects of Polyanions on Lysosomal Membrane Fluidity

DS is known to interact with phospholipids and proteins, with or without calcium or other "bridging" cations (21, 22). Intralysosomal DS might therefore interact with these components on the luminal surface of the lysosome membrane. This interaction could be extensive enough to modify the lateral mobility of the membrane, affecting both protein mobility and lipid fluidity. Fluorescence polarization of α- and β-PnA in DS- and D-containing lysosomes was used to estimate the relative difference in membrane fluidity between inhibited and non-inhibited lysosomes. The differential partitioning of α- vs. β-PnA into solid- and fluid-phase lipid (23) made comparison of the two probes of interest.

Fig. 6*A* shows a plot of fluorescence polarization vs. temperature for β-PnA in DS and D lysosomal fractions. For both samples, a gradual decrease in P is observed with increasing temperature (T), corresponding to a more fluid probe environment (13, 24). This decrease is relatively linear vs. T, and no phase transitions are observed. At each temperature, however, P was higher in DS than D lysosomes. This difference ranged from about 0.01 to 0.025 and similar results were observed in several separate experiments. The difference was greater than the small experimental variation observed when identical samples were analyzed, or when decreasing concentrations of PnA/phospholipid were analyzed in the same lysosome preparation (data not shown).

The data in Fig. 6*A* were obtained from samples in 1 mM EDTA, to prevent vesicle aggregation. Some DS interactions are mediated by divalent cations (21, 22) so that P vs. T was also analyzed using samples prepared in buffer containing 1 mM $CaCl_2$. These results are shown in Fig. 6*B*. P was ~0.02 higher in DS than D samples throughout the temperature scan. In both samples, ΔP vs. T decreased above ~30°C, so that in the presence of $CaCl_2$ a small deflection in the curve was

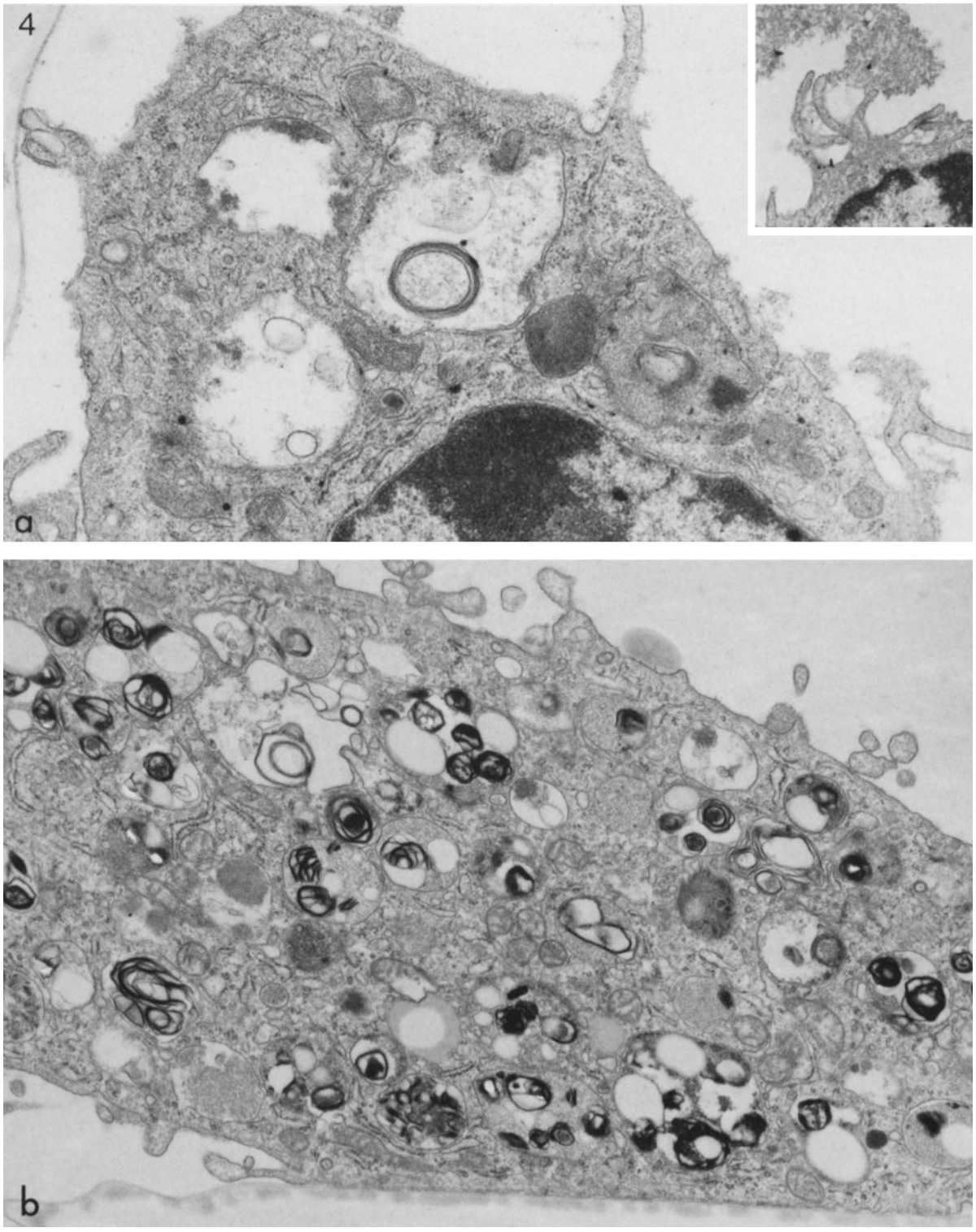

FIGURE 4 Electron microscopic appearance of cells given DS-serum complexes to ingest. 4-d macrophage cultures in 35-mm dishes were given 2 ml of 50% complex (see Materials and Methods) for the indicated times before washing, fixation, and EM processing. (*a*) Cells exposed to complex for 30 min. Amorphous complex is seen in large intracytoplasmic vacuoles as well as adsorbed to the cell surface. × 27,300. *Inset* shows cell apparently ingesting larger aggregate of complex. × 14,625. (*b*) Cells exposed to complex for 3.5 h. Some apparent lysosomal digestion of complexes has occurred. Vacuoles now contain amorphous complex and also lipid droplets and whorls of membranous material. × 17,875.

observed. The difference in P of β-PnA in DS and D samples was not, however, strikingly increased in the presence of $CaCl_2$.

β-PnA partitions preferentially into solid-phase lipid, while α-PnA partitions more equally into coexisting fluid- and solid-phase lipid (23). Fig. 7 shows P vs. T when α-PnA was used as the fluorescence probe. For both samples, the observed P is less for α-PnA than β-PnA, especially at low temperature (compare with Fig. 6 *A*). Using α-PnA, no significant difference

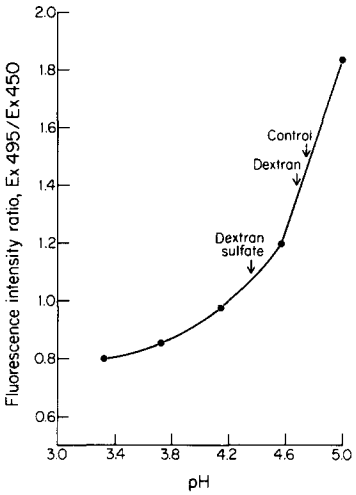

FIGURE 5 The pH of secondary lysosomes as estimated by a fluorescent probe. Shown is a standard curve relating the excitation spectrum of fluorescinated dextran to various pHs. All solutions contained 5 μg/ml fluorescinated dextran in 50 mM NaCl and 10 mM acetate buffer, at the indicated pH. The determined intralysosomal pH for each cell type is marked. Values are the mean of five to seven determinations for each cell type. Background fluorescence of unlabeled cells was subtracted in each case.

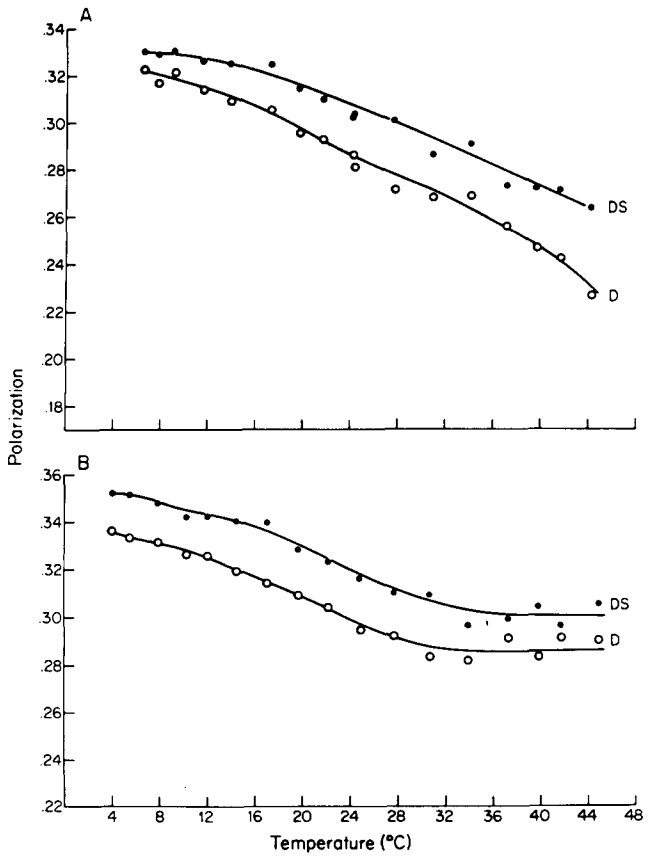

FIGURE 6 Temperature dependence of the polarization (P) of β-PnA in lysosomal fractions from D- (●) or DS-treated (○) cells. Each point represents the mean of three determinations at that temperature. (*A*) An experiment in which the polarization buffer was 10% sucrose, 1 mM EDTA, and 10 mM phosphate buffer, pH 7.4. (*B*) An experiment in which the polarization buffer was 10% sucrose, 1 mM $CaCl_2$, and 10 mM HEPES buffer, pH 7.4.

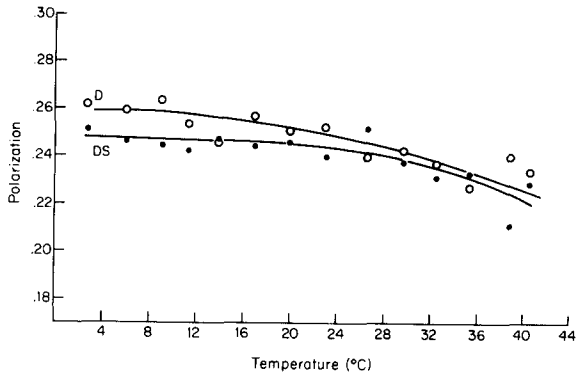

FIGURE 7 Temperature dependence of the polarization (P) of α-PnA in lysosomal fractions from D- (●) or DS-treated (○) cells. Polarization buffer as in Fig. 6 *A*. Each point represents the mean of three determinations at that temperature.

was seen between D and DS samples, and, at low temperature, P in D samples was even slightly higher than in DS samples.

The polarization buffers used in the experiments in Figs. 6 and 7 contained 10% sucrose. This buffer was used to preserve latency and the lysosomal contant of DS. When NAGase activity in D and DS samples was measured at the start of a polarization experiment, it was increased four to five times by Triton X-100. It is not known whether this enzyme latency was preserved after the heating scan to 50°C, but cooling scans, where performed, were superimposable on the heating scans.

In one experiment, D and DS samples were analyzed using PBS plus 1 mM EDTA as polarization buffer. When β-PnA was used as a probe in this system, the results were similar to those seen in Fig. 6*A* (data not shown).

DISCUSSION

Uptake of DS and DS-LDL Complexes

Two types of polyanion uptake have been described in this work. First, soluble ^{3}H-DS was gradually pinocytosed by macrophages at a linear rate over a 4-d culture period. This uptake may be in the form of soluble DS-lipoprotein complexes, since DS appears to interact with lipoproteins in culture medium to increase their uptake by macrophages (19). In the second type of uptake, DS was very rapidly endocytosed when given as an insoluble complex with serum lipoproteins. When added to serum in lipoprotein purification, DS precipitates mainly LDL and VLDL (9, 18). Complexes of DS with purified LDL similarly mediated rapid uptake, intralysosomal storage, and P-L fusion inhibition. Increased uptake of lipoprotein may explain the myelin figures observed in polyanion-treated cells in the preceding paper, since similar structures are seen in cells given DS-LDL complexes. In the inhibition of P-L fusion, however, the lipoprotein seems to act merely as a DS carrier and has no effect when endocytosed in large quantities in the absence of DS. It thus appears that the long culture time required for DS inhibition correlates with the establishment of a high intralysosomal DS concentration, whereas conditions that cause a rapid influx of polyanion result in rapid inhibition.

Effect of DS Uptake on Lysosomal pH

From the uptake of soluble DS at 76 h of culture, an average of 4.206×10^2 cpm were cell-associated per μg of protein, or 3.365×10^{-2} cpm/cell (using the figure of 80 μg of protein/10^6 cells [25, 26]). At the specific activity in this experiment of

3.801×10^4 cpm/μg, this is 8.85×10^{-13} g of DS/cell, of which 10% is sulfur or 8.85×10^{-14} g of S/cell. Thus, each cell contains 2.76×10^{-15} mol S/cell or that amount of anionic equivalents (for each S, one OSO_3^-). Assuming a volume for the secondary lysosome compartment in normal macrophages of 10 μm^3 (25) or 10^{-14} liter, this is a concentration of 0.276 mol/liter. The volume of the DS-loaded lysosomes could be 10-fold greater than this estimate and still result in an anion concentration of 0.028 mole/liter. These calculations suggest that concentration of DS and negative charge estimated to be within lysosomes is very high.

The accumulation of DS within lysosomes was found to lower the intralysosomal pH at least 0.3–0.4 pH unit. If protons accumulate as counterions via a Donnan equilibrium, there is sufficient negative charge in these lysosomes to explain the pH difference seen. There is good evidence that an energy-requiring proton pump is involved in the maintenance of low lysosomal pH (10, 27), and it is not clear how polyanions could shift this active mechanism to an equilibrium at a new, decreased pH. It seems unlikely that both a proton pump and a Donnan-type equilibrium could be operating simultaneously, since a Donnan equilibrium implies that protons are free to diffuse both in and out of the lysosome, while an active proton pump implies that the membrane is relatively impermeable to protons.

A study of lysosomal pH in macrophages treated with oxidized amylose, another polyanion, reported that lysosomal pH appeared to be increased to about pH 5.0 in these cells (28). This discrepancy could be due to the fact that carboxyl groups are not as strongly acidic as sulfate groups.

Implications of the High Intralysosomal Concentration of DS

The surprisingly high concentration of DS that accumulates in secondary lysosomes of inhibited cells may imply that the charged sulfates interact with the lysosomal membrane, and that very extensive interaction is necessary before fusion is modified. The inhibitor need not be polymeric, however, as suramin can also cause inhibition. Since suramin contains six sulfonate groups per molecule, if an ionic interaction occurs it still has the potential for cross-linking. It is not known whether a "monovalent" anion could cause inhibition. Uptake studies have only been done for the 500,000 mol wt DS, but the high concentration of chondroitin sulfate necessary for inhibition correlates with its correspondingly lower sulfate content. Presumably, comparable sulfate concentrations in secondary lysosomes mediate inhibition by chondroitin sulfate and DS. Poly-D-glutamate also causes inhibition, and the D-polymer, not being digested by L-amino acid specific hydrolases, can accumulate in high concentrations. Larger amounts of poly-D-glutamate may be required to inhibit since the carboxyl groups are not as strongly anionic as sulfate or sulfonate.

From studies of lipoprotein-DS interactions, it is known that DS forms both soluble and insoluble LDL complexes (21, 29). In the absence of divalent metal ions, DS interacts primarily with positively charged groups of the lipoprotein. Decreasing the positive charges on the lipoprotein by acetylation decreased this interaction, while hydrolysis of the phospholipids by phospholipase C had little effect (21, 29). In the presence of divalent metal ions, both positive and negative charges were involved as the cation acted as a "bridge" (21). Studies of the interaction of phosphatidyl choline with DS similarly show direct interaction of the sulfate and the choline nitrogen, and calcium cross-linking of the phosphate groups with sulfate groups (22).

Fluorescence Polarization Studies

The increased size and density of DS- and D-treated lysosomes enabled their rapid purification on metrizamide gradients. α- and β-PnA as probes then enabled the biophysical comparison of two functionally different intracellular organelle populations which exhibited latency. Thus, interactions between high concentrations of DS in the lysosomal contents and lipid and/or polypeptides components of the luminal surface of the lysosomal membrane should be maintained.

The results from β-PnA suggest that DS lysosomal membranes are a more rigid environment for the probe than comparable D membranes. The free fatty acid probes used for these studies have a quantum yield in lipid much greater than that in an aqueous environment. Thus, any probe not in membrane is essentially invisible (16). For β-PnA, the ratio of the partition coefficient in solid-phase lipid to fluid phase is ~3, while for α-PnA the ratio is ~0.6 (23). Thus, β-PnA is especially sensitive to differences in solid-phase lipid between D and DS lysosomes. The difference in polarization of β-PnA in D and DS lysosomes could conceivably be due to differences in the fluorescence lifetime of the probe (16) or unknown contaminants in the fractions.

While not completely conclusive, the β-PnA results are reasonable evidence that the membrane of DS lysosomes is more rigid than that of D lysosomes, which exhibit normal fusion capability.

The comparable content of fatty acid, phospholipid, and cholesterol in whole-cell extracts argues against fluidity changes which are based upon chemical alteration of the membrane per se (Kielian and Cohn, unpublished observation). This is in keeping with the normal fusion of pinocytic vesicles with DS lysosomes and the resulting rapid normalization of membrane contents. We favor the idea of direct interaction of the polyanion with the luminal face of the lysosomal membrane as a reasonable explanation of these results.

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