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Characterization of Phagosome-Lysosome Fusion in Macrophages

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CHARACTERIZATION OF PHAGOSOME-LYSOSOME FUSION IN MACROPHAGES

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by

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PREFACE

During my career as a graduate student, I have enjoyed the advice and encouragement of many members of the University community. Bill Bowers and his group were my first lab at Rockefeller and have remained friends and colleagues since. I also thank Brian Poole for advice and equipment for measuring lysosomal pH, Micky Rifkin for gifts of purified LDL, Bernie Gilula and Ellie Morales for instruction in freeze-fracture, and John Gregory for advice on the purification of ^{3}H-dextran sulfate.

All of the members of the Zanvil Cohn laboratory have contributed to making it an exciting and enjoyable place to work. Special thanks to Richard Huntley for technical assistance during this work, and to Judy Adams for skillful help with electron microscopy and photography. Carl Nathan was generous with both help and his much-used spectrofluorometer. Bill Scott and the members of his lab have been generous sources of much equipment and advice throughout this work. I am especially grateful to Ralph Steinman for finding time to discuss data and suggest new experimental approaches.

Marie Grossi brought order out of chaos with her superlative typing of this manuscript.

Finally, my thanks and appreciation to my advisor, Zanvil Cohn. I feel very lucky to have had an advisor with his insight into problems, breadth of knowledge, and understanding of people. I appreciate both the independence and the constant support I received while working with him.

SUMMARY

Several approaches were used to study the determinants of phagosome-lysosome (P-L) fusion in intact mouse macrophages. Lysosomes were labeled with the fluorescent vital dye acridine orange and the rate and extent of their fusion with yeast-containing phagosomes was monitored by fluorescence microscopy. Fusion was also assayed by electron microscopy, using horseradish peroxidase or thorium dioxide as markers for secondary lysosomes. Good agreement was found with results obtained from vitally-stained cells, thin section samples using an enzymatic marker, and thorium dioxide-labeled samples evaluated by stereology.

The rate of fusion as assayed by fluorescence was not affected by the number of particles ingested, serum concentration, or prior uptake of digestible or non-digestible substances. Fusion was also not affected by enzymatic modification or concanavalin A crosslinking of the plasma membrane or by coating the phagocytic particle with concanavalin A or immune serum. Pretreatment of cells with 10^{-6} - 10^{-4} M colchicine, or treatment immediately after ingestion with 1 - 10 μg/ml cytochalasin did not alter P-L fusion; implying that the cytoskeleton does not control fusion in a rate-limiting way.

With the fluorescence assay it was possible to observe the rate of fusion separate from and uninfluenced by the phagocytic rate. Both the rate and extent of fusion were dramatically increased after several days in culture and similar changes were found using the EM assays. Fusion was strongly affected by incubation temperature, having a Q_{10} of 2.5. No detectable fusion occurred below 15°C, and this inhibition was rapidly reversed when cells were returned to 37°C. Fusion was strikingly elevated in 5 hr cultures of activated macrophages from immune-boosted mice. A lower enhancement was seen in cells activated by proteose-peptone, a non-specific inflammatory agent.

Treatment with the tumor promoter, phorbol myristate acetate (PMA) caused a striking increase in the rate and extent of P-L fusion. Exposure of cells to phorbol, free myristate, or the monoesters of PMA did not reproduce this effect. Macrophages required from 2 - 3 hr of pretreatment to express maximal P-L fusion and this was maintained for at least 20 hr when cells were returned to PMA-free medium.

Catalase, superoxide dismutase, indomethacin and hydrocortisone, agents which are known to block the effect of PMA on H_2O_2, O_2^-, prostaglandin or plasminogen activator, did not affect the stimulation of P-L fusion by PMA. The effect of PMA was blocked by 10 μg/ml puromycin or 5 μg/ml cycloheximide when cells were preincubated for 2 hr with the protein synthesis inhibitor and exposure to the inhibitor maintained during incubation with PMA and subsequent assay. Similar treatments with puromycin or cycloheximide had no effect on the high rate of fusion in four day cells.

Labeled PMA was rapidly taken up by macrophages, with a plateau of uptake at about 3 hr. When cells were returned to PMA-free medium, cell-associated label was rapidly released, returning to background levels within 1 hr. The released label was found to be a metabolite of PMA by thin layer chromatography. This product migrated between the monoester, phorbol-12-myristate, and free phorbol. Rapid metabolism of PMA was also observed by a macrophage cell line, J774, and to a lesser extent by primary rat embryo fibroblasts.

P-L fusion was markedly inhibited by several days of culture in various polyanionic compounds such as dextran sulfate (DS), which were endocytosed and concentrated in secondary lysosomes. Polyanions did not inhibit pinosome-lysosome fusion, as assayed by the intracellular degradation of a pinocytic marker, by electron microscopy, and by monitoring the transfer of a pinocytic marker to a lysosome-enriched fraction obtained from polyanion-treated cells.

The inhibition of P-L fusion did not appear to be due to alterations in lysosomal pH, to changes in membrane structure as assayed by freeze-fracture analysis or to alterations in the cell's phospholipid composition.

A tritium-labeled DS was purified and characterized. Soluble ^{3}H-DS was endocytosed by cells at a linear rate for up to 100 hr of culture, and caused P-L fusion inhibition after 3 - 4 d of uptake. More rapid uptake of DS occurred when it was presented to cells in the form of a DS-low density lipoprotein (LDL) complex. Under these conditions, fusion was inhibited after 3 - 4 hr of uptake. Macrophages endocytosed large amounts of LDL after acetylation of the lipoprotein, but LDL by itself did not affect fusion. The inhibition of P-L fusion by polyanions thus may be due to an interaction of the polyanion with the lysosomal membrane.

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LIST OF ABBREVIATIONS

AO	acridine orange
CS	coverslip
D	dextran, 500 K molecular weight
DS	dextran sulfate, 500 K molecular weight
FCS	fetal calf serum
HRP	horseradish peroxidase
IMP	intramembrane particle
LDL	low density lipoprotein
MEM	Dulbecco's modified Eagle's medium
NAGase	N-acetyl glucosaminidase
NC	Nelson-Collins
NCS	newborn calf serum
5'NTase	5'nucleotidase
P	phorbol
PA	phorbol-13-acetate
PBS	phosphate buffered saline (plus divalent cations)
P-L fusion	phagosome-lysosome fusion
PM	phorbol-12-myristate
PMA	phorbol-12-myristate-13-acetate
SDS	sodium dodecyl sulfate
TLC	thin layer chromatography
Tx-100	triton X-100
VLDL	very low density lipoprotein
HDL	high density lipoprotein

1. INTRODUCTION. MEMBRANE FUSION

Membrane fusion has been extensively studied in several model systems which will be discussed first. Several of the morphological and biochemical observations on intracellular membrane fusion will then be presented.

A. Artificial Membranes

Studies on artificial membranes suggest possible mechanisms which could cause fusion of biological membranes. In many systems, however, it is difficult to evaluate if membrane fusion, rather than lipid-lipid exchange or endocytosis of liposomes by cells, is actually occurring. The direct extrapolation of observations on simple liposomes to complex biological membranes may also not be warranted.

The fusion of liposomes composed of acidic phospholipids such as phosphatidyl serine has been extensively studied by Papahadjopoulos and his colleagues (see [1, 2] for review). These vesicles are ordinarily refractory to fusion either above or below the transition temperature. Papahadjopoulos postulates that liposome fusion begins with charge neutralization (caused by either Mg^{2+} or Ca^{2+}) followed by aggregation of vesicles and close apposition of the bilayers. Only Ca^{2+} is thought to be able to induce trans complexes or "bridges" between the phospholipid head groups, leading to the elimination of water from the bilayer complex. An isothermic phase change of the acyl chains from a fluid to a crystalline state, as detected by differential scanning calorimetry, is thought to be a key event in the actual fusion of the apposed bilayers. Localized heat from acyl chain crystallization may provide energy for fusion at the boundaries of crystalline domains. The triggering of exocytosis by an influx of calcium and the distribution of negatively charged

phospholipids on the cytosolic side of the plasma and intracellular vesicle membranes are proposed as evidence that similar mechanisms could be acting *in vivo* during exocytosis.

The fusion of liposomes with planar lipid bilayers has also been characterized. In one example of this type of study ([3], [4]), fusion was assayed both by the incorporation of a vesicular membrane marker into the planar bilayer and by the transfer of vesicle content across the planar bilayer. The incorporation of voltage dependent anion channels in the vesicle membrane enabled the assay of fusion by monitoring sudden changes in the conductance of the planar bilayer. Fusion required an osmotic gradient across the planar bilayer, with the vesicle-containing side hyperosmotic. Fusion could be greatly potentiated by micromolar calcium when a calcium-binding protein, purified from synaptic membranes, was incorporated into the planar bilayer.

B. Virus-Membrane Fusion

The role of proteins in membrane fusion has been best studied in several virus systems, using virus-induced cell-cell fusion, virus penetration, hemolysis, or virus-liposome fusion as assays.

For paramyxoviruses such as Sendai and SV5 ([5], [6]), the two membrane glycoproteins of the virus have different roles in the fusion process. The HN glycoprotein has the hemagglutinating and neuraminidase activity and appears to be necessary for virus-cell binding. The F glycoprotein appears responsible for the fusion activity of the virus. Thus, inactive F_o is activated by proteolytic cleavage, resulting in two disulfide-linked subunits of active F, which now mediates infection and cell-cell fusion. Antibodies to F block fusion but not hemagglutination ([7]), and purified F reconstituted into membranes will cause hemolysis when wheat germ agglutinin is present to mediate liposome-red blood cell binding ([8]).

In some host cells infected with herpes simplex virus, cell fusion depends on the levels of two viral glycoproteins (9). One glycoprotein, B_2, appears to promote cell-cell fusion, while the level of the other, C_2, correlates with suppression of fusion. C_2 can suppress fusion even in the presence of the fusion promoter B_2.

Another interesting mechanism of virus-membrane fusion was first characterized using Semliki Forest virus (10,11) and some aspects have since been extended to other viruses (12). Semliki Forest virus binds to cell surface receptors and is endocytosed. Upon exposure to the low pH of lysosomes or other intracellular vacuoles, fusion between the virus and vacuole membranes is induced, releasing the nucleocapsid into the cytoplasm. This low pH-induced fusion was also observed when virus-liposome fusion was assayed (13). Again, the viral spike protein is required, although it is not clear which of the virus spike polypeptides are mediating fusion. In the liposome fusion assay, cholesterol is strictly required, as opposed to the situation in the simple liposome systems already discussed. Also in contrast to the simple liposome systems, virus-liposome fusion occurred with high efficiency in the absence of divalent cations. Fusion was maximal at temperatures above $30^{\circ}C$ but occurred to a lesser extent (25% of maximal) even at $0^{\circ}C$.

C. Exocytosis -- An Example of Intracellular Membrane Fusion

One of the best-studied examples of the fusion of biological membranes is the fusion of intracellular vesicles with the plasma membrane which occurs during exocytosis. Several different cell types have been utilized in these studies and the similarities and differences between them are of interest.

Exocytosis in several protozoa has been studied extensively, using both morphology and genetics to define the stages and pathways which occur. In *Tetrahymena*, the orderly surface topography of cilia and secondary meridians made possible the correlation of mucocyst "docking" position

with specific sites on the plasma membrane where exocytosis occurs (14, 15). A rosette of large intramembrane particles (IMPs) is found at this site. An anulus of smaller IMPs rims the region on the mucocyst where fusion appears to initiate. Fusion is postulated to occur between these two IMP-depleted regions, which probably correspond to lipid-enriched, protein-depleted areas. A calcium ion flux appears to be the trigger for exocytosis.

A similar exocytotic process occurs during trichocyst secretion in *Paramecium tetrauralia* (15,16). Exocytosis-deficient mutants have been analyzed by freeze-fracture, trichocyst microinjection and sexual conjugation. This dissection enabled the separation of various parts of the exocytotic pathway. A similar docking site and vesicle annulus are seen. Complementation of the various mutants suggests that a cytoplasmic factor may also be involved. The rosettes may be the site of a calcium ATPase activity (16) which triggers fusion.

Membrane specializations also may determine the sites of synaptic vesicle fusion in the neuromuscular junction. A ridge, flanked on either side by two rows of large IMPs, characterizes the active zone, an area where vesicles are more likely to fuse (17,18,19). Quick freezing and freeze-fracture techniques have been used to capture the early stages of vesicle fusion upon nerve stimulation. Vesicles appear most likely to fuse in an area 70 nm from the center of the active zone. Fusion appears to be random, with both active zones and individual vesicles behaving independently of one another. Since calcium is known to trigger actual vesicle fusion, the ordered IMPs could be calcium channels which mediate its influx. The small size of the vesicles made it impossible to visualize the initial site of fusion in freeze-fracture preparations.

Degranulation in mast cells is a concerted, rapid, exocytotic event involving first vesicle-plasma membrane fusion and then vesicle-vesicle fusion (20,21,22). Sensitized cells, which have cytophilic IgE bound to Fc receptors on their surfaces, are stimulated to degranulate by the cross-linking of surface IgE. Concanavalin A (Con A) or anti-IgE cross-

linking will serve as triggers, as will the calcium ionophore A23187. Con A covalently coupled to a bead will induce localized degranulation at the site of contact (21).

Freeze-fracture studies of resting mast cells reveal no apparent specializations on the plasma or vesicle membranes (20,21,23). During exocytosis, extensive areas of contact between vesicle and plasma membrane are seen in fixed and glycerinated specimens (20,23). In these studies, areas of membrane fusion again appear depleted of particles. Also, in thin sections, ferritin-coupled ligands did not bind to fusing areas of the plasma membrane (20), implying that fusion occurred between protein-depleted areas. In quick-frozen specimens (22), however, IMP clearing and extensive contact of vesicle and plasma membrane are not seen. Narrow pores between membranes are the first signs of fusion observed. IMP segregation and membrane contact are likely pre-fusion events, but may be limited to a small domain.

A morphological study of phagocytizing polymorphonuclear leukocytes (24) revealed no symmetrical arrangements of IMPs such as those seen in protozoa or in the neuromuscular junction. IMP-free regions were seen in newly formed phagolysosomes or on the plasma membrane when exocytosis was triggered by A23187. Whether these areas would be observed in quick-frozen specimens is not known.

These *in vitro* and *in vivo* systems demonstrate both the similarities and differences which exist in several fusion events. It is of interest to compare phagosome-lysosome fusion to these systems, although the triggers and molecular mechanisms involved may not be the same. It is evident from the virus-liposome fusion systems that biological membrane fusion is likely to be controlled by protein as well as lipid-lipid interactions, and that calcium is not likely to be the universal trigger for membrane fusion. The various studies do suggest that the control and specificity of membrane fusion could be maintained by various mechanisms: the controlled migration of vesicles to certain regions of the cell, the presence of specific proteins in the membrane, the ability of a membrane domain to generate a flux of calcium or a pH change, or the movement of

membrane proteins within the plane of the membrane to generate a localized protein-depleted area. Several of these control mechanisms can be evaluated, albeit imperfectly, for their role in controlling phagosome-lysosome fusion.

2. INTRODUCTION. PHAGOSOME-LYSOSOME FUSION

A. Assay Systems for Phagosome-Lysosome Fusion

Phagosome-lysosome (P-L) fusion has usually been assayed by electron microscopy, using electron-dense secondary lysosome markers such as ferritin, colloidal gold or thorium dioxide, or staining for enzymes such as acid phosphatase or exogenously-fed peroxidase. Several other assays have also been developed:

The functional consequences of the fusion of phagosomes with lysosomes are the basis for two types of assays. First, the digestion of a particle such as radiolabeled bacteria by lysosomal hydrolases was used as an indirect measure of P-L fusion (25). P-L fusion was also measured indirectly in experiments where yeast particles labeled with pH indicator dyes were endocytosed by polymorphonuclear leukocytes, and the pH change detected assumed to reflect fusion with lysosomes (26).

More direct measurement of P-L fusion was made possible by the isolation of phagocytic vacuoles, first described by Wetzel and Korn (27) for *Acanthamoeba*, a free-living, phagocytic amoeba. This procedure is based on the floatation of polystyrene latex-containing phagocytic vacuoles in a sucrose step gradient. This rational was used to assay P-L fusion in macrophages by monitoring the transfer of acid phosphatase to polyvinyltoluene bead (28) or paraffin oil emulsion (29)-containing phagosome fractions, similarly isolated on gradients. These assays require large numbers of cells and make detailed rate studies difficult. While they allow quantitation of fusion, they do not assess heterogeneity of the response. P-L fusion in these systems is characteristically very rapid and no further transfer of acid phosphatase is observed fifteen minutes after particle ingestion.

Oates and Touster (30,31,32) have developed an *in vitro* fusion system using *Acanthamoeba*. Two populations of phagolysosome-rich cell homogenates, labeled with yeast or red blood cells, were mixed and the proportion of hybrid vacuoles containing both particles was evaluated by electron and light microscopy. They conclude that fusion is essentially random with respect to vacuole content, that vacuoles can participate in more than one fusion event, and that a fraction of the vacuoles is resistant to fusion. In this system, the rate of fusion is accelerated by cyclic AMP or phosphodiesterase inhibitors.

Again, this assay requires large numbers of cells, and its assessment by light and electron microscopy is difficult. Although modifications of the assay (31) were found to prevent vacuole lysis, the assay does not appear suitable for evaluating the role of factors which may influence fusion in the intact cell.

Hart and Young (33) have taken advantage of the localization of acridine orange to lysosomes in vitally stained cells to develop a phagosome-lysosome fusion assay using fluorescence microscopy. The staining pattern of viable yeast cells was observed after fusion with acridine-labeled lysosomes in live macrophages. The system has been used to study several polyanionic compounds which appear to inhibit P-L fusion (33,34), including a sulfated glycolipid purified from *Mycobacterium tuberculosis* (35). We have adopted and modified this fluorescence microscopic assay to monitor the rate and extent of P-L fusion.

B. The Biology of Phagosome-Lysosome Fusion

The involvement of P-L fusion in the survival of intracellular parasites has been studied in macrophages and some other cell types (for review see 36). Most of these studies used electron microscopic tracers to follow lysosome fusion with parasite-containing vacuoles (37,38); several have used fluorescent vital dyes such as AO (39) or quinacrine (40) to assay for fusion.

Several patterns of intracellular parasite survival have been observed. *Trypanosoma cruzi* enters macrophages in a phagocytic vacuole, escapes from the vacuole and multiplies free in the cytoplasm (41). *Mycobacteria* show two pathways of intracellular survival. *M. lepraemurium* survives and multiplies within phagolysosomes, while *M. tuberculosis* inhibits the fusion of lysosomes with its phagocytic vacuole. Amastigotes of *Leishmania* are similar to *M. lepraemurium* while *Chlamydia, Rickettsia and Toxoplasma* inhibit P-L fusion. The inhibition is observed only with live parasites and is reversed if the live organisms are first pre-coated with specific antibody which is in itself not cytotoxic. Symbiotic algae of paramecium can similarly inhibit P-L fusion and again, only living organisms have this effect (42).

For *M. tuberculosis*, antibody pretreatment reverses the inhibition of fusion but the bacteria are nevertheless observed to multiply within phagolysosomes. As mentioned in the previous section, a sulfated glycolipid purified from this bacterium appears to inhibit P-L fusion in macrophages pretreated with the sulfatide for several days (35). Another proposed mechanism for the inhibition comes from the observation that inhibitory *Mcyobacteria* cause an increase in the cAMP content of the infected cells (43). However, the increase was observed even with antibody-coated parasites and is thus unlikely to be the cause of P-L fusion inhibition. Finally, the ammonia content of *M. tuberculosis* culture supernatants was reported to be the cause of the inhibition of P-L fusion in macrophages exposed to the supernatants for several hours before fusion assay (44). It is difficult to interpret this result in light of the antibody effect on inhibition. Also, the AO assay of fusion is itself affected by ammonia or other weak bases (45).

The mechanism of P-L fusion inhibition by *Toxoplasma gondii* is also not understood, but in this case may be required for the survival of the parasite. Some recent work suggests a mechanism for the reversal of inhibition by antibody. Studies by Murray et al. (46,47) demonstrated the toxoplasmacidal activity of products of oxygen reduction, OH. and $^1O_2$. These oxygen intermediates are the presumed products of O_2^-

and H_2O_2 interaction. Macrophage resistance to *T. gondii* appeared to correlate with their ability to generate oxidative metabolites. Also, Wilson et al. (39) demonstrated that resident macrophages produced toxic oxygen metabolites upon phagocytosis of antibody-coated but not unopsonized *Toxoplasma*. The reversal of inhibition by antibody may be an indirect effect of its ability to trigger oxygen metabolites which kill *T. gondii*.

PART ONE. THE ASSAY SYSTEM

I. MATERIALS AND METHODS

A. Cells and Cell Culture

Primary cultures of peritoneal macrophages were prepared from resident cells of female or male Nelson-Collins strain (NC) or Swiss Webster mice (22-30 g) (48). Cells were routinely cultured in 15-20% fetal calf serum (heat-inactivated 56°C) in Dulbecco's modified Eagle's medium (MEM) containing 100 units/ml penicillin and 100 µg/ml streptomycin, and were given fresh medium at least every other day. Cells were plated at densities of 4×10^5 peritoneal cells per 12 mm glass coverslip for fluorescence studies, or at $6 - 10 \times 10^6$ peritoneal cells per 35 mm dish and at $1.5 - 2 \times 10^7$ per 60 mm dish.

$P388D_1$, a macrophage-like cell line, was obtained from Dr. J. Unkeless and maintained in spinner culture in 10% FCS/MEM (49).

Culture dishes with 16 mm wells were obtained from Costar, Cambridge, Massachusetts.

B. Particle Preparation for Fusion Studies

Fresh baker's yeast was processed by the method of Lachman and Hobart (50). This procedure consists of autoclaving, reduction by mercaptoethanol, alkylation with iodoacetamide, and extensive washing in PBS. The resulting preparation could be stored indefinitely in PBS without Ca^{2+} and Mg^{2+} plus 0.02% NaN_3.

To opsonize particles with complement components, 300 µl of a 5% suspension of yeast in veronal buffer was mixed with 300 µl of mouse serum (fresh or stored at -70°C), incubated 30 min at 37°C and washed

several times in cold veronal buffer. Preparations were used within 3 days of opsonization.

Latex particles of 0.50, 1.10, 2.02, 3.14, 4.27, 5.7 and 8.0 μ in diameter were obtained from Dow Chemical Co. and Duke Scientific Co., washed 5 times in PBS and stored at 4^{o} until used.

C. Fluorescence Assay of Phagosome-Lysosome Fusion

Monolayer cultures on 12 mm glass coverslips were labeled for 20 min at 37^{o}C in 15% FCS/MEM by adding acridine orange (AO) stock solution to a final concentration of 5 μg/ml. A sterile stock solution of 100 μg AO/ml in PBS without Ca^{2+} and Mg^{2+} could be stored in the dark at 4^{o}C for 3-4 weeks. Each coverslip was then placed in a 16 mm diameter well of a plastic culture dish containing 1 ml of prewarmed medium, and incubated 10 min at 37^{o}C, to reduce background fluorescence and decrease photodamage. The medium was then aspirated and 1 ml of a cold 0.004% (v/v) suspension of opsonized yeast was added per well. The yeast suspension was centrifuged onto the cell monolayer by placing the culture dish in a centrifuge carrier designed for microtiter plates (Cooke Laboratory Products) and centrifuging in an International centrifuge at 1200 rpm for 2 min at 4^{o}C. The yeast particles bound to the macrophage complement receptor, and any unbound particles were removed by washing with cold MEM. Cold 5% FCS/MEM was added, the cultures were rapidly warmed to 37^{o}C by placing in a water bath in a 37^{o}C incubator, and relatively synchronous ingestion of the bound yeast resulted. The usual number of yeast particles bound and ingested per cell was 2. Cells were examined by fluorescence microscopy at various time points after an initial 10 min ingestion period. The presence of orange-stained intracellular yeast was considered positive for P-L fusion.

To examine live cell preparations, acridine orange-labeled cells were prepared by inverting coverslips over a drop of ice-cold PBS, blotting, and rimming with nail polish. Specimens were then examined

with a Zeiss photomicroscope III, using a mercury lamp adjusted for epi-illumination, BG-12 filter and fluorescein dichroic mirror for excitation and a 53 barrier filter. Black and white pictures were taken with Kodak Tri-X film, color pictures were taken with Kodak Ektachrome film, ASA 200.

D. Electron Microscopy

Monolayers were fixed for 30 min at room temperature in 2.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.4. After 3 saline washes, HRP was visualized in labeled cultures by the Graham and Karnovsky diaminobenzidine method (51). Cultures were then post-fixed in 1% osmium tetroxide in 0.1 M cacodylate pH 7.4 60 min on ice, and stained with 0.25% uranyl acetate in 0.1 M sodium acetate buffer 30 min at room temperature. Specimens were dehydrated in graded alcohols and embedded in Epon. Thin sections were examined in a Siemens Elmiskop II with or without uranyl acetate and lead citrate staining.

Scanning electron microscopy was kindly performed by Dr. Gilla Kaplan. Monolayer cultures on 12 mm coverslips were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. Specimens were dehydrated in alcohol, transferred to amyl acetate and critical point dried (Sorval C.P.D. System) in liquid CO_2. The cells were gold-coated (Edwards 5150 sputter coater) and viewed with an ETEC autoscan.

E. Electron Microscopic Evaluation of P-L Fusion

Two markers for secondary lysosomes were used, horseradish peroxidase (HRP) and colloidal thorium dioxide (thorotrast). Cultures were pulsed with 2 mg/ml HRP in medium for 2 - 3 hr, washed 4 times with MEM, and cultured 20 - 60 min longer in medium without HRP. Cells were then given yeast or latex particles and fixed 1 hr after ingestion.

Specimens were processed for EM, and thin sections examined without uranyl acetate and lead citrate staining. Phagocytic vacuoles were evaluated for the presence of a diffuse "rim" of electron-dense HRP reaction product.

Non-digestible thorotrast is a useful marker since cell populations can be labeled at identical times in culture, when pinocytic uptake of the marker is similar. Cells were washed as usual 2 hr after plating, exposed to a 1 to 100 dilution (vol/vol) of colloid in medium for 12 hr, washed 4 times with MEM and then cultured for a total of 1 - 4 days. Cells were fixed 1 hr after particle ingestion.

Stereology was used to evaluate the extent of fusion in specimens labeled with this particulate marker. Random samples were obtained from monolayer cultures fixed and processed as described above. At the propylene oxide step, the monolayer floated off the plastic dish and could then be further washed and embedded as a pellet in Epon. Random sections through this pellet were selected, and micrographs of most cells on a section were taken at the same final magnification. These negatives were projected through a 3X enlarger onto a grid of 1 cm squares. Counts were made, for both horizontal and vertical lines, of the number of times a line crossed the membrane of the phagocytic vacuole versus the number of times the line crossed thorotrast in the vacuole. Thorotrast was present as discrete particles and distributed linearly around the rim of the vacuole. Counts were expressed as percent of total possible crossings which were thorotrast positive.

For each determination, from 25 - 60 vacuoles were evaluated, and total line crossings of from 600 - 1500 were obtained.

F. Other Techniques and Reagents

Acridine Orange Quantitation

Coverslip cultures were pulsed with 5 µg/ml AO in FCS/MEM for 20 min, washed, and extracted in 95% ethanol. Fluorescence of samples was

compared to standards in the same solvent, using an MPF-44 fluorometer (Perkin-Elmer Corp., Norwalk, Conn.) with an excitation wavelength of 490 nm and emission wavelength of 520 nm. Values were related to the amount of cell protein on parallel coverslips, using the fluorescamine protein assay (52) with bovine serum albumin as a standard. Acridine standards in solvent with or without unlabeled cells were comparable.

Reagents

Fetal calf serum (FCS), newborn calf serum (NCS), and Dulbecco's modified Eagle's medium were obtained from the Grand Island Biological Co. Diaminobenzidine was from Sigma Chemical Co., and acridine orange came from the National Biological Stains Department of Allied Chemical Co. Fluorescamine was obtained from Roche Diagnostics, and colloidal thorium dioxide (Thorotrast) from Fellows Testagar Div., Fellows Mfgr. Co., Inc.

II. THE FLUORESCENCE ASSAY OF FUSION

The general method of Hart and Young (33) was employed to evaluate the extent of P-L fusion at the light microscope level. This utilizes the fluorescent, vital dye acridine orange, a weak base which is selectively concentrated in the acid environment of lysosomes (53,54,55). The structure of acridine orange is shown in Figure 1. Pre-stained, viable cells are exposed to phagocytizable particles and the transfer of lysosomal fluorescence into phagocytic vacuoles evaluated by fluorescence microscopy. Most of the following experiments utilize non-viable yeast particles which are fully permeable to the dye and fluoresce uniformly when AO is transferred into the vacuole. To compare rates of P-L fusion in different cell populations the experimental variables involved were analyzed.

A. The Influence of Phagocytic Rate and Load

In order to study the rate of P-L fusion it was necessary to dissociate this process from phagocytosis. This was examined by means of scanning electron microscopy. Opsonized yeast was centrifuged onto one day macrophage monolayers at $4^{o}C$ and either fixed immediately (Figure 2a) or after a 10 min incubation at $37^{o}C$ (Figure 2b). At $4^{o}C$, an average of 1.4 particles were bound to the cell surface, while after 10 min at $37^{o}C$, 94% of the particles had been ingested. Thus, phagocytosis was essentially complete at the first time point of the fusion assay and additional particle uptake would not complicate long-term assays. Similar results were obtained by phase and fluorescence microscopy.

Using this assay system, the effects of phagocytic load could be examined on an individual cell basis. Figure 3 shows the percent of yeast particles which were positively stained as a function of the

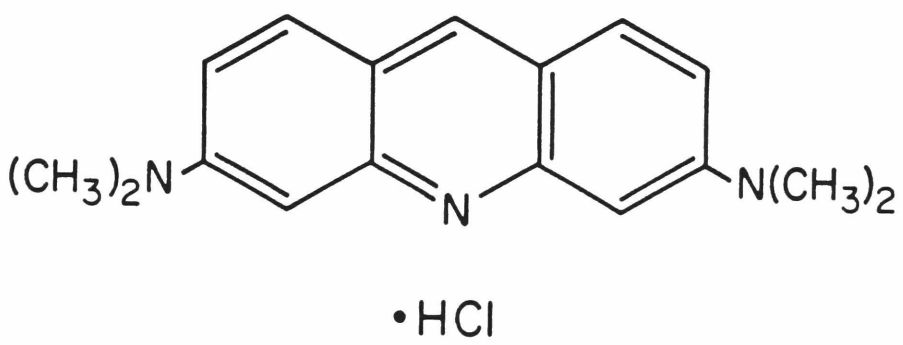


Figure 1. The structure of acridine orange [3,6-bis-(dimethylamino)acridine hydrochloride].

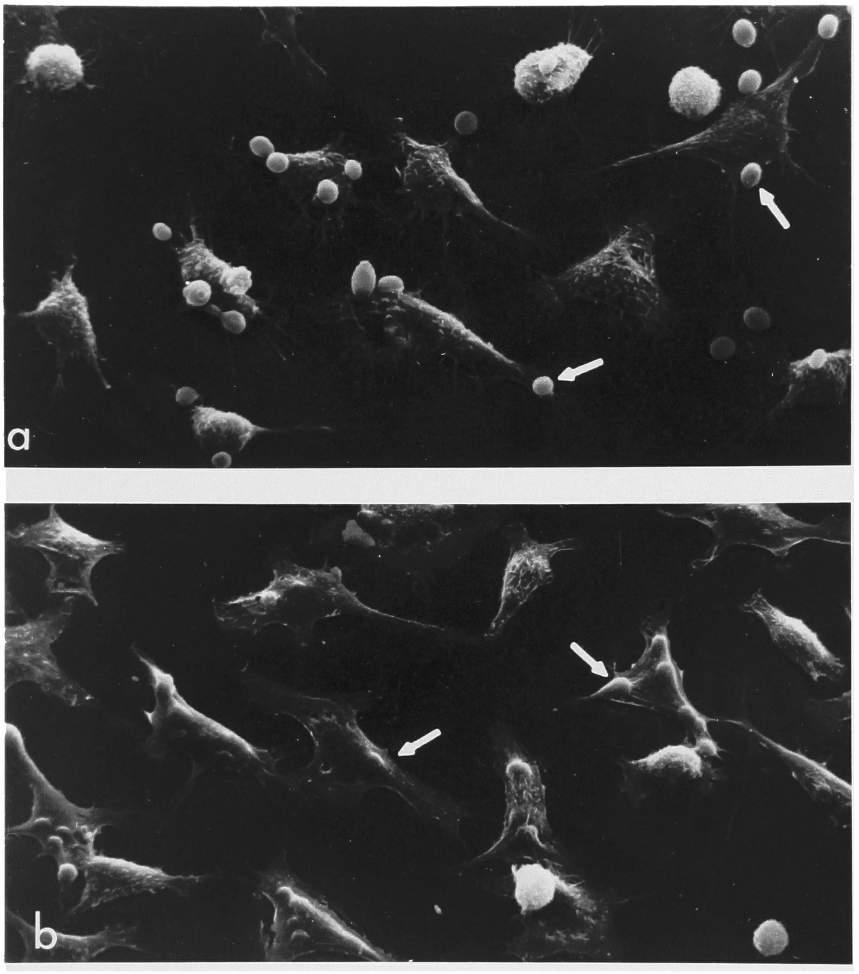


Figure 2. Scanning EM of cells before and after phagocytic pulse. Performed by Dr. Gilla Kaplan.

2A. Twenty-four hr monolayer cultures were exposed to opsonized yeast and fixed immediately without a 37°C incubation. Almost all yeast particles (arrows) are extracellular and associated with either the cell body or a cytoplasmic process. The average number of particles per cell is 1.4. X 1080.

2B. As in 2a., except that cells were incubated 10 min at 37°C. Cells have respread and 94% of the particles had been ingested at this time point. The outlines of phagocytosed yeast (arrows) can be seen. X 540.

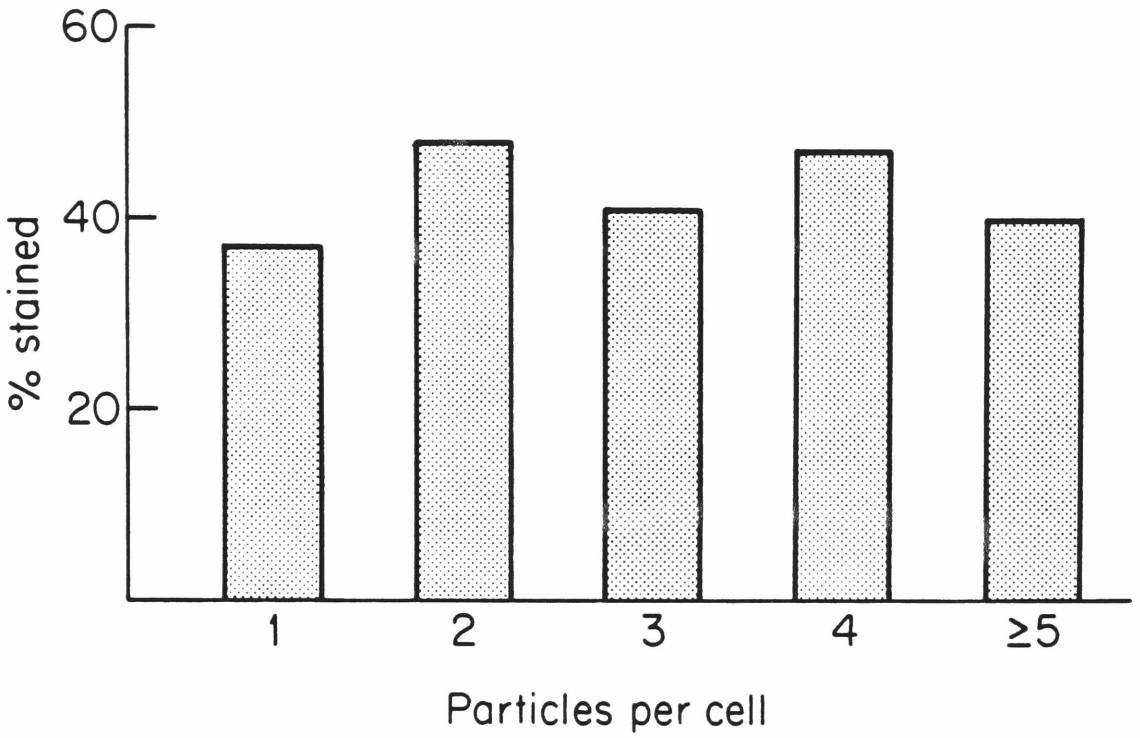


Figure 3. Effect of the number of interiorized particles on the rate of P-L fusion. A fusion assay of a 2 day culture, 60 min after the start of ingestion. The percent of intracellular particles stained is plotted as a function of the number of particles each cell has ingested.

number of particles ingested per cell. At 60 min after ingestion, the percent of stained particles was not affected by the amount of uptake, and similar results were observed at 30 min. Thus, the extent of P-L fusion is independent of the number of particles ingested, and there is enough acridine concentrated by a cell to stain at least 5 particles. Therefore, variations in the number of particles ingested do not affect the subsequent results.

B. Choice of Phagocytic Particle

The particles used for this assay were heat-treated, reduced and alkylated yeast which were opsonized with fresh mouse serum. These particles are very uniform in binding, uptake and fluorescent staining characteristics. Since the particles are permeable to the dye but do not concentrate it, their color in the cytoplasm is an indication of the dye concentration in the phagocytic vacuole and therefore the extent of P-L fusion. The concentration of the dye is higher for orange-stained vacuoles than for green-stained vacuoles as is known from the metachromatic properties of acridine orange (56,57). Thus, although it cannot be concluded that no fusion has occurred in green-stained vacuoles, the relative amount is less, and orange is the end point counted as positive for fusion. This end point is easily distinguished and is stable for hours in viable cells, enabling long-term experiments. Staining the macrophages before or after particle ingestion gives the same time course or fusion rate, implying that the phagocytic vacuole itself is not acidic until its fusion with lysosomes.

Several other particles were tested in the fluorescence assay and proved less suitable for this type of analysis. Live yeast did not appear freely permeable to AO, but showed instead a rim of fluorescence which was more difficult to distinguish. Viable yeast cells are also capable of delaying the fusion process, as has been previously noted (33). Antibody-coated red blood cells were not appropriate particles -- in part related to their rapid intracellular digestion. Finally,

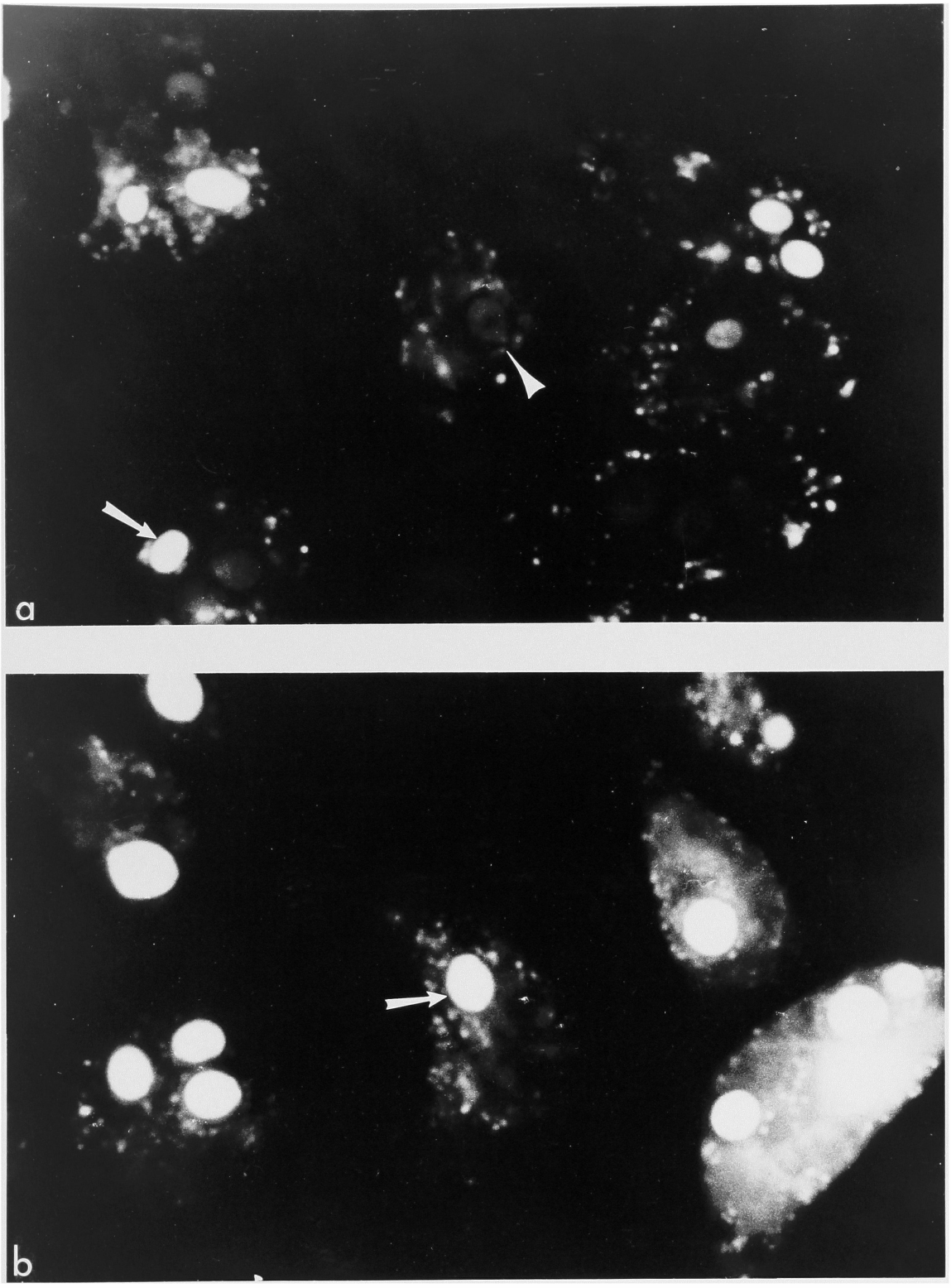
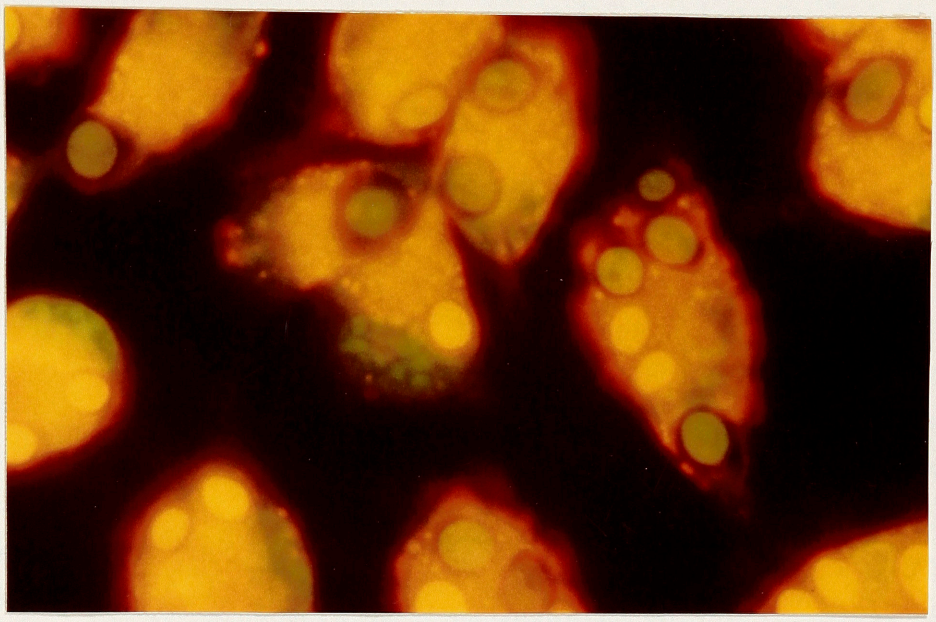


Figure 4. The appearance of acridine orange stained cells in one and four day cultures.

4A. One day cells 160 min. after yeast ingestion. Both positively- (arrow) and negatively- (arrowhead) stained yeast are seen several hours after ingestion, as well as abundant lysosomal staining. X 2000.

4B. Four day cells 40 min. after ingestion. All ingested particles are brightly stained (arrow) and lysosomal staining is seen in cells containing several positively-stained yeast. X 2130.



4C. Color print of four day cells 30 min. after ingestion. Some positively-stained (orange) yeast are seen, while other particles (green) are still largely unstained. Punctate orange lysosomal staining is plentiful. X 1600.

polystyrene latex spheres proved unsuitable because they bound AO non-specifically in the extracellular milieu.

Figures 4a-c are representative fluorescence pictures of one and four day old cultures after ingestion of opsonized yeast. Pinpoint lysosomal staining can be seen, with the absence of appreciable nuclear or cytoplasmic background. Positive yeast appear very brightly fluorescent, while negative yeast are much more faintly stained and show a greenish fluorescence under the microscope. In one day cultures numerous negative particles are seen 160 min after ingestion, while in four day cultures almost all particles are positive after 40 min.

C. Effect of Culture Time on P-L Fusion

This difference in fusion rate was examined in greater detail. These results are presented in Figure 5 which shows the fusion rate in cells cultured *in vitro* for 5 - 96 hr. At early times after culture, the majority of yeast particles failed to fuse even several hours after ingestion and a plateau was reached at about 60 min. If these cells were cultured for an additional 24 hr both the rate and extent of fusion increased and this trend continued progressively up to 96 hr of culture. In four day cells, the initial rate of fusion was about eightfold higher than at 5 hr and resulted in the positive staining of 90% of the particles 60 min after ingestion. If one day cells with intracellular particles were cultured overnight, 90% of the particles then were positively stained, showing that P-L fusion was continuing at this slow rate.

These striking differences in the rate of P-L fusion might be the result of the amount of AO concentrated by the cells. For this reason, quantitative studies on the uptake of AO by one and four day cells were conducted and are presented in Table I. No significant difference in dye uptake was noted between parallel coverslip cultures or when the data were normalized for cell protein, and this mechanism could not explain the progressive change in fusion rates.

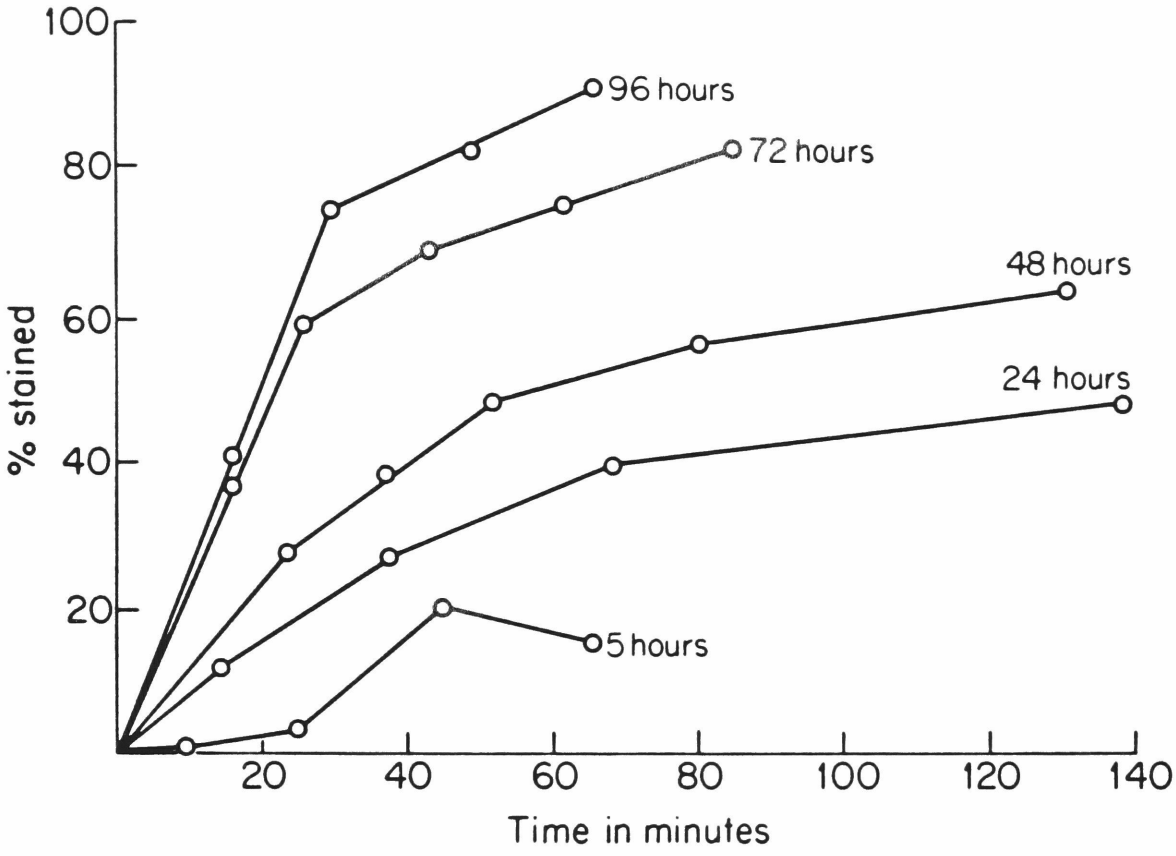


Figure 5. Effect of *in vitro* cultivation on the rate and extent of P-L fusion. The percent of intracellular particles stained is plotted vs. time after particle ingestion. Time in culture is shown in hours. Average results from three separate experiments.

Table I

COMPARISON OF ACRIDINE ORANGE UPTAKE BY 1 AND 4 DAY CULTURES

Sample	pmoles AO/μg protein*
1 day cultures	49.3 ± 16 (6)
4 day cultures	54.3 ± 15 (6)

*Mean and standard deviation of six separate experiments

The rate of P-L fusion was also determined in the phagocytic, macrophage-like cell line $P388D_1$ (58,49). These cells replicate in culture as opposed to the non-dividing freshly explanted peritoneal macrophage. When $P388D_1$ cells growing in spinner culture were plated on coverslips and examined 1 and 24 hr thereafter, the rate and extent of P-L fusion was rapid and similar to that of 4 day cultures of peritoneal macrophages.

D. Correlation with EM Assays

We next felt that it was important to show a correlation of these results with other assay systems and thus followed the fusion of phagosomes with lysosomes which had been prelabeled with electron dense or enzymatic markers. Cultures were pulsed with 2 mg/ml horseradish peroxidase (HRP) for 3 hr, chased 60 min with normal medium, given test particles to ingest and fixed 1 hr after the phagocytic pulse. Figure 6a shows an example of P-L fusion in these cells after ingestion of 3.14 μ latex spheres. Electron dense reaction product from the diaminobenzidine staining is seen in secondary lysosomes and also in a rim around the particles. Figure 6b shows a similar assay in cells which were labeled for 12 hr with colloidal thorium dioxide, and then cultured 4 days. Labeling of secondary lysosomes and of a yeast-containing vacuole is seen. Stereology (see Materials and Methods) was used to compare the extent of fusion in specimens labeled with this particulate marker.

Results from all of these assays for P-L fusion are presented in Table II. In each assay system, fusion is greater after the macrophages had been cultured for four days. Differences between one and four day cultures are about twofold for all assays. Thus, these three assay systems are in relative agreement, in spite of the use of different markers, stereological analysis, and thin sections as compared to whole cell preparations.

We noticed in the course of these studies that lysosome fusion with latex phagosomes, as evaluated by transfer of HRP, occurred to a much

Figure 6. Electron microscopic evaluation of P-L fusion using electron-dense lysosomal markers.

6A. Two day cells pulsed with HRP, and given 3.14 μ latex spheres. Note reaction product in secondary lysosomes (Ly), one of which has partially fused with a latex-containing phagosome (arrow). All latex vacuoles have a rim of reaction product. Much of the latex was dissolved by the propylene oxide. X 6807.

6B. Four day cells prelabeled with thorotrast and given opsonized yeast. Thorotrast is seen in secondary lysosomes (Ly), and distributed linearly around an ingested yeast (Y). X 15,000.

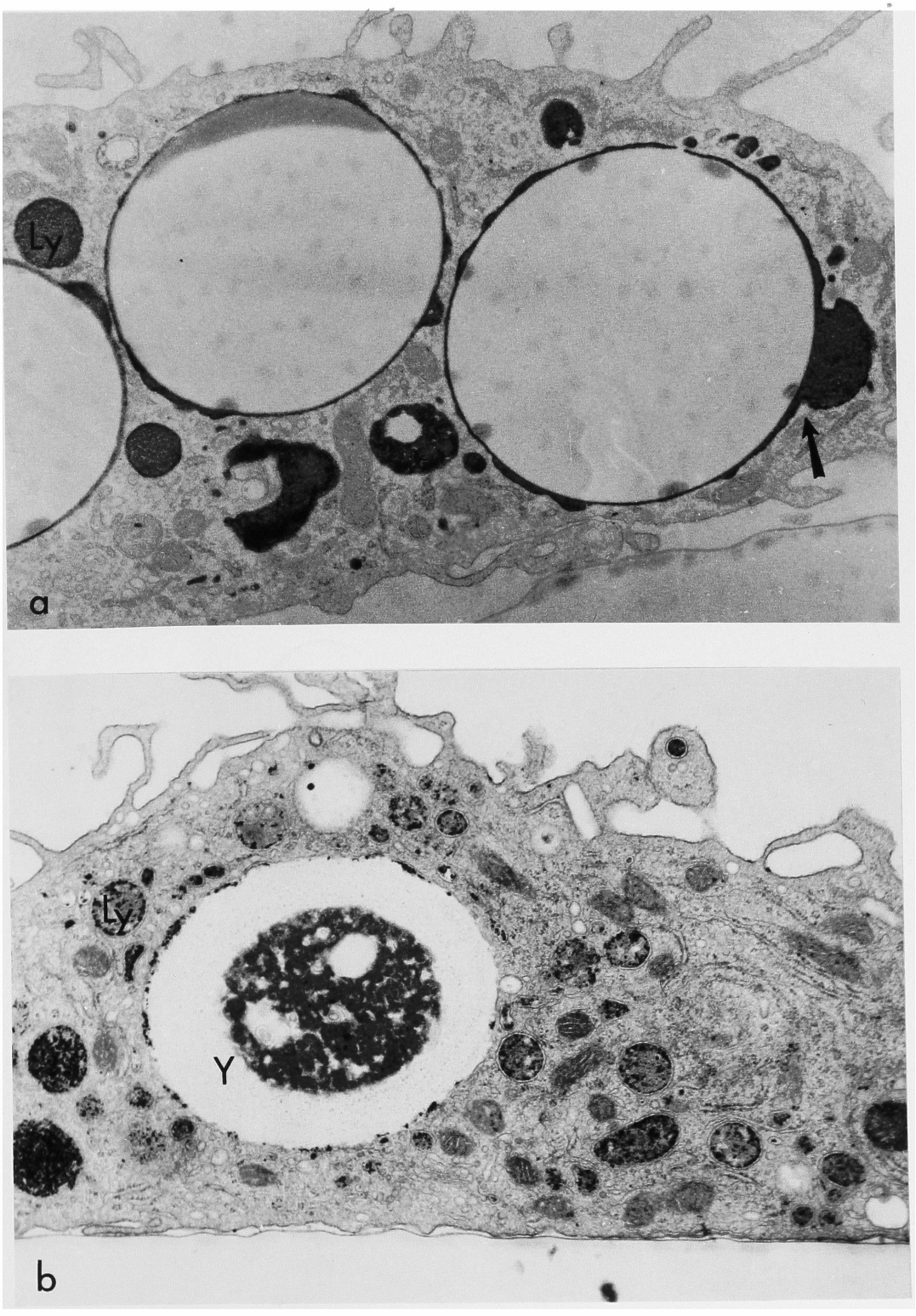

Ly
a
Ly
Y
b

Table II

COMPARISON OF VARIOUS ASSAY SYSTEMS FOR PHAGOSOME-LYSOSOME FUSION

	Fusion After 1 Hour*		
Assay Conditions	Acridine Assay	HRP-EM Assay	Thorotrast-EM Assay
Cultured 1 day	37% ± 5.8† (4)	25.9% ± 6.2 (3)	16.9% (2)
Cultured 1-2 days; 0.5-3.14 μm latex particles	-	85.5% ± 7.6 (5)	-
Cultured 4 days	85% ± 13.6 (5)	57.5% (2)	37.3% ± 4.9 (3)

*The number of experiments is given in parentheses.

†Percent and standard deviation.

higher extent than with yeast phagosomes. When this was evaluated in one day cultures (Table II), latex phagosomes showed more fusion than yeast-containing vacuoles in either one or four day cultures. This result does not appear to be due to the smaller size of 1.1 μ latex particles, since in another experiment, latex of 0.5, 1.1, 2.02, 3.14, 4.27, 5.7 and 8 μ in diameter showed from 80 to 90% fusion within one hour after ingestion.

E. Effect of Lysosome Size on the Rate of P-L Fusion

The increased rate of P-L fusion in cultured macrophages might also reflect modifications in the size of the secondary lysosomal compartment. This possibility was examined by utilizing two previously described methods for increasing the size of the lysosome and the relative intracytoplasmic volume of the lysosomal pool. For this purpose cells were cultured in either 40% newborn calf serum (NCS) or in medium containing 0.03 M sucrose. Newborn calf serum contains antibodies which bind to the macrophage surface and increase pinocytic uptake (59), while sucrose increases the lysosomal pool since macrophages do not have invertase to hydrolyze this disaccharide (60).

Forty-eight hours after cultivation in 40% NCS AO stained cells illustrate the increase in lysosome size (Figure 7). When such cells were employed for studies of lysosomal fusion (Figure 8B) there was no alteration in either the rate or extent of P-L fusion. Similar results were obtained with sucrose laden cells (Figure 8A). In addition, cells preloaded with either 1.1 μ latex or *Micrococcus lysodeikticus* showed no change in fusion rate. Thus, prior uptake of digestible or non-digestible agents of either a soluble or particulate nature did not influence fusion with lysosomes.

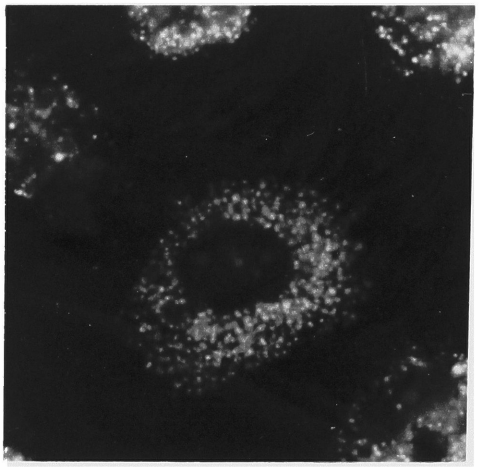


Figure 7. Cells cultured 2 days in 40% newborn calf serum before acridine orange staining. The size of the AO-stained lysosomes is increased as compared to the cells cultured in normal medium (see Figure 4). Cells grown in 0.3 M sucrose show a similar increase. X 1200.

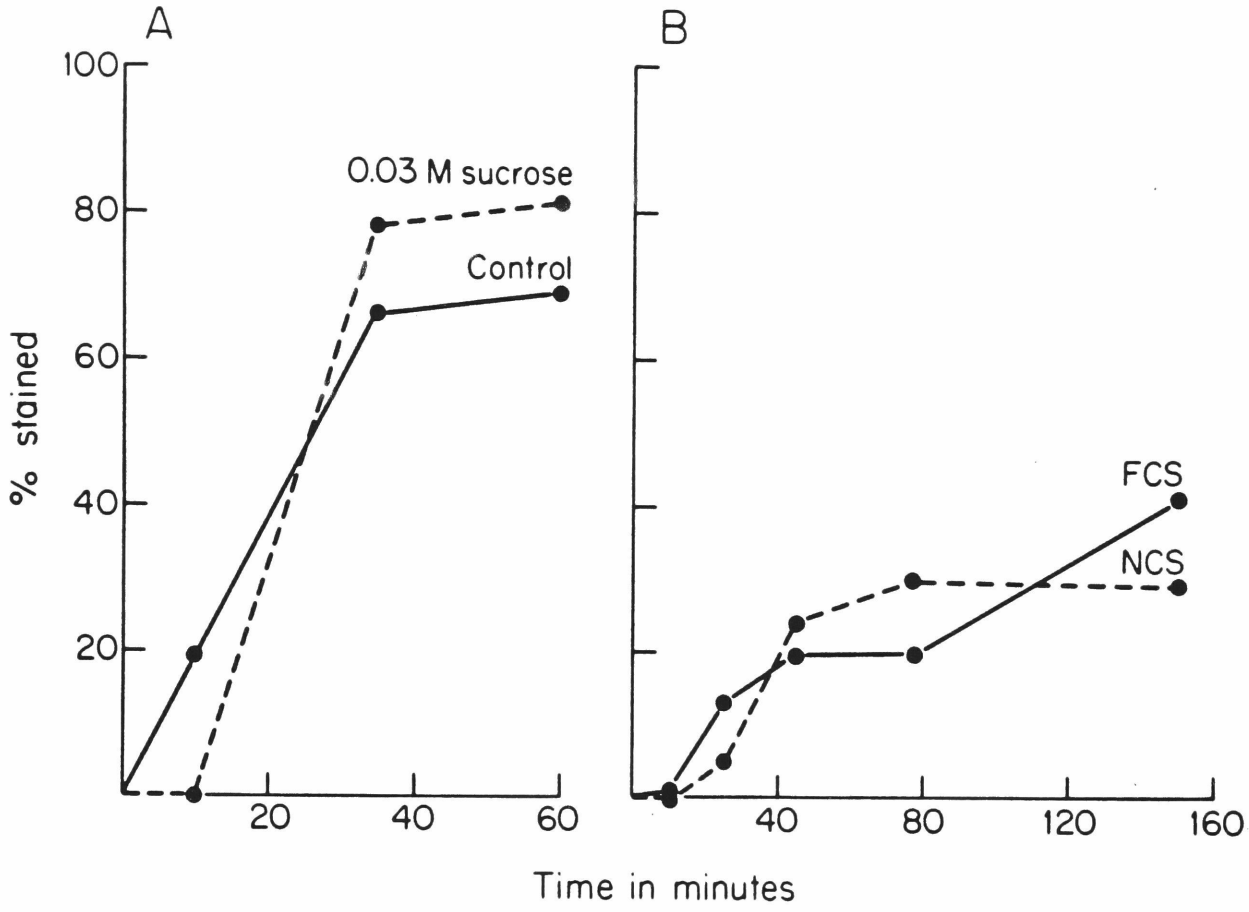


Figure 8. Effect of increased lysosome size on the rate of P-L fusion.

8A. Rate of P-L fusion in 3 day cells cultured in medium with or without 0.03M sucrose.

8B. Rate of P-L fusion in 2 day cells cultured in 20% fetal calf serum (FCS) or 40% newborn calf serum (NCS).

III. DISCUSSION

A. General Technique

These studies employed an opsonized yeast preparation as the test particle. This particle was well suited for fluorescence studies and its relatively slow fusion rate enabled the investigation of variables such as temperature which required the separation of ingestion and fusion. It is interesting that latex spheres showed a more rapid fusion rate in EM studies, making them impractical for several types of experiments. This more rapid fusion may be an intrinsic property of the polystyrene latex. Alternatively, the yeast cell surface may have some inhibitory activity (33), interacting with the membrane of the phagocytic vacuole and modifying its subsequent fusion with lysosomes.

Mouse peritoneal macrophages were useful for these studies since these cells have specific membrane receptors and phagocytize avidly. Also, many properties of macrophage plasma membrane and lysosomes have been characterized, as well as the fate in macrophages of several intracellular parasites which can inhibit P-L fusion (36). Stable macrophage-like cell lines such as $P388D_1$ are available, and show similar P-L fusion in the fluorescence assay.

The assay described is a vital dye method which can be employed at the level of the fluorescence microscope and enables the assay of P-L fusion in the natural milieu of the cytoplasm. Many vital dyes are segregated within secondary lysosomes and serve as useful markers to trace the presence of lysosomes and their fusion with endocytic vacuoles (61,62). The present technique, which is based on the acridine orange method of Hart and Young (33), has been previously utilized in the examination of the inhibitory influence of microorganisms and their products on the fusion process (35).

The fluorescence assay has many advantages but can only be used under certain conditions. Thus, agents which modify the intralysosomal pH or which cause quenching of AO fluorescence interfere with the assay. For example, ammonium chloride, chloroquine or treatment with 2-deoxy-glucose and sodium azide all cause an increase in intralysosomal pH (63) and also caused a visible decrease in lysosomal staining by AO. Therefore, it is important that the use of thin sections and markers other than vital dyes gives comparable information. The effect on fusion of lysosome uptake of fluorescence or EM markers is not known.

B. Effects of Particle Uptake

It is useful to consider the size of the vacuolar compartments involved in phagosome-lysosome fusion in order to approximate the degree of degranulation and the extent of P-L fusion. From stereological analysis of the secondary lysosome compartment in mouse macrophages (64), the average secondary lysosome volume was 0.0074 μM^3, there were an estimated 1,100 lysosomes per cell, and thus the total volume of the secondary lysosome compartment was about 10 μM^3, in an average cell volume of 395 μM^3. Acridine uptake was about 50 pmoles/μg cell protein or 4×10^{-15} mole/cell, using the figure of 80 μg protein/10^6 macrophages (64,65). If the amount of acridine free in the cytoplasm or bound to nucleic acid is considered to be negligible, this gives an estimate of 3.6×10^{-18} mole AO/lysosome.

From light micrographs, the diameter of the yeast used in the experiments was estimated to be about 3.8 μ. Assuming that the yeast are spherical, their volume is then (by $v = \pi D^3/6$) about 30 μM^3. At least 5 particles or about 150 μM^3 total volume can be positively stained by the amount of AO sequestered by one cell. Since the volume of the total lysosome compartment is considerably less than that of the yeast phagocytic vacuole, AO is diluted upon P-L fusion, although still sufficiently concentrated to stain the yeast orange. Even in the case of high

particle uptake, in which the particle volume is estimated to be at least 15 times that of the total secondary lysosome volume, fusion can occur with all particles and considerable lysosomal staining is still observed. This is similar to results obtained from quantitating the transfer of acid phosphatase to latex phagolysosomes (28), by which Pesanti and Axline demonstrated that even at the maximum particle dose, only 35% of the total acid phosphatase was found in the latex phagolysosome fraction. Thus, total degranulation does not occur although the volume of the phagosomal compartment is much greater than that of the lysosomal compartment.

This may mean that there exists in the cytoplasm a subpopulation of lysosomes which are more available or more likely to fuse. Also, it is possible that primary lysosomes are fusing with phagocytic vacuoles and either transferring content and/or adding membrane components to the pool which is in flux with the plasma membrane (66). The extent of primary lysosome fusion in these experiments is not known.

C. Effect of Lysosome Size

Increasing the surface area of vesicle membrane available for fusion might be expected to have several effects. First, it might greatly increase the random contacts of phagosomes with lysosomes, thus increasing the opportunities for fusion to occur. Conversely, theoretical considerations of charge repulsion between two membranes would argue that close contact can only be made by microvesicles with a low ($< 0.1\ \mu$) radius of curvature (67). If charge repulsion affects P-L fusion, an increase in lysosome size might thus be expected to decrease the probability of fusion.

Several experiments examined the effect of loading lysosomes with sucrose, serum, bacteria or latex on their subsequent fusion with phagocytic vacuoles. In these experiments, only a part of the lysosome population would be influenced, although in the case of sucrose or newborn

calf serum treatment this proportion is probably quite high (59,60). None of these treatments altered the rate or extent of P-L fusion. Although obviously these results are not conclusive, they do suggest that the rate-limiting step in P-L fusion is not random vesicle contact but represents a more selective event.

D. Effects of *In Vitro* Cultivation

All assays of P-L fusion showed a progressive increase in fusion rate after several days of cell culture. The expression of this macrophage activity can thus vary, similar to other previously studied processes. These include the specific activity of plasma membrane ectoenzymes and lysosomal acid hydrolases, as well as pinocytic uptake and phagocytosis (68). These processes and P-L fusion are influenced by *in vitro* cultivation and/or cell activation. The fact that particles such as latex, yeast, and parasites show different fusion rates may imply that a post-endocytic modification of the phagocytic vacuole occurs which alters its recognition and fusion with lysosomes.

PART TWO. BASIC DETERMINANTS OF PHAGOSOSOME-LYSOSOME FUSION

IV. MATERIALS AND METHODS

A. Activated Cell Populations

Proteose-peptone elicited macrophages were harvested from NC mice 3 days after intraperitoneal injection of 1 ml sterile proteose-peptone solution (1% w/v in distilled H_2O).

In vivo activated macrophages (69,47) were kindly supplied by Drs. N. Nogueira and H. Murray. NC mice were primed with an ip injection of 5 x 10^6 live culture forms of *Trypanosoma cruzi* and boosted ip 4 weeks later with 5 x 10^6 heat-killed *T. cruzi*. Cells were harvested 60 hr after boosting. NC mice were also chronically infected with *Toxoplasma gondii*, challenged ip with 5 x 10^6 heat-killed toxoplasma, and peritoneal cells were harvested 3 days later.

B. Covalent Coupling of Concanavalin A to Latex Beads

Concanavalin A (Con A) (Sigma Chemical Co., St. Louis, Mo.) was directly coupled to carboxylate-modified 0.860 μ latex beads (Dow Chemical Co., Indianapolis, Ind.) using a protocol similar to that described by Rutishauser and Edelman (70) for coupling to nylon fibers. 150 λ of latex (10% solids) was washed 3 times in 0.15 M NaCl, and resuspended in 1 ml of 0.15 M NaCl containing 5 mg Con A and 1.6 x 10^6 cpm of ^{3}H-labeled Con A (56 Ci/mmol) (Amersham, Arlington Hts., Il.). 25 mg of the water-soluble carbodiimide 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-P-toluene sulfonate (CMC) (Pierce Chemical Co., Rockford, Il.) was then added, and the bead suspension stirred at room temperature for 30 min. The beads were then washed 4 times with PBS and could be stored at -20°C in 50% glycerol-PBS for months without noticeable change in binding activity.

Small samples of beads were assayed for labeled Con A by liquid scintillation counting in Aquasol. Under the above conditions, about 20% of the added Con A was adsorbed to the beads when CMC was not added. This adsorbed Con A could be removed by washing the beads once in 0.3 M NaCl, 10 mM Tris pH 8.6 plus 0.1% sodium dodecyl sulphate and 0.05% Nonidet P-40. After washing, beads treated with CMC bound 17.6% of the added label or 880 μg Con A per 150 λ of original latex stock. When CMC was not added, 0.07% remained bound after washing.

Con A-coupled beads showed binding activity by their ability to agglutinate yeast into very large mixed aggregates.

C. Preparation of Anti-yeast Antibodies

New Zealand white rabbits were injected in the marginal ear vein 2 - 3 times weekly for 5 weeks with a suspension of whole *Saccharomyces cerivisiae* which had been autoclaved and processed as previously described (45). Each injection was 1 ml, and contained progressively increasing doses of yeast from 2×10^6 - 5×10^7 cells/ml of 0.9% NaCl. One week after the last injection, heat-inactivated sera gave a tube agglutination titer of 1:1024, and this sera was used to opsonize yeast particles.

D. Temperature Studies

After an initial phagocytosis step at 37°C, cultures were maintained at temperatures from 15° - 30°C in a water-jacketed chamber (71) gassed with 5% CO_2/95% air. Temperature was controlled by using a constant-temperature circulating water bath and cultures were kept at 2°C in an ice bath.

V. DETERMINANTS OF FUSION

A. Plasma Membrane Modification

Several intracellular parasites (36) and the plant lectin concanavalin A (72) inhibit the fusion of endocytic vacuoles with lysosomes. These agents, present on the luminal surface of the vacuole presumably influence the cytosolic face, inhibiting fusion by an as yet unknown mechanism. Several classes of cell surface perturbants were therefore evaluated for their ability to modify the intracellular fate of plasma membrane derived phagosomes.

Three day macrophage cultures were treated with a variety of proteolytic enzymes or with neuraminidase under conditions which are known to degrade protease-sensitive exteriorly disposed polypeptides and receptors (41). The pretreated cells showed excellent cell viability and phagocytic indices. As shown in Table III, P-L fusion was in all cases similar to that seen in untreated cultures.

B. Particle Surface Modification

Fusion inhibition caused by several intracellular parasites can be reversed by coating the parasite with specific immune serum (36). We tested whether the rate of lysosomal fusion with yeast-containing vacuoles could be increased by opsonization with immune serum. Yeast was coated for 30 min at 37^{o}C with either heat-inactivated whole anti-yeast antiserum (see Materials and Methods), or normal mouse serum, washed, and sonicated to break up clumps. As shown in Table III, one day cultures showed the usual slow fusion rate after ingesting either particle preparation.

Table III

FACTORS WHICH DO NOT INFLUENCE P-L FUSION

		Time After Particle Ingestion	% Positive Staining with AO	
			Control	Treated
1. Enzyme* pretreatment of the cell surface	20 U/ml neuraminidase 37° 30'	40'	48	53
	112 U/ml trypsin 37° 10'	40'	65	71
	100 µg/ml pronase 37° 30'	120'	64	76
	100 µg/ml chymotrypsin 37° 30'	120'	64	61
2. Particle surface treatment	whole rabbit α yeast antiserum *vs.* normal mouse serum 37° 30'	140'	25	29
			% HRP stained by EM assay	
			Control	Treated
	Con A-coupled latex *vs.* untreated latex	60'	> 95%	> 95%
3. Cell surface crosslinking	Con A bound to cell surface 50 µg/ml 30' 4°C *vs.* untreated cells Latex as test particle	60'	> 95%	> 95%

*_Vibrio cholera_ neuraminidase was from Calbiochem, 3x crystallized trypsin was from Worthington, and pronase and chymotrypsin were from Sigma.

Concanavalin A (Con A) has been previously shown to inhibit pinosome-lysosome fusion in macrophages when added to the culture medium or prebound to the cell surface in the cold (72). The effect of Con A on phagosome-lysosome fusion was evaluated using latex beads covalently coupled to Con A or unmodified latex given to Con A-treated cells. Fusion was evaluated by electron microscopy, using horseradish peroxidase (HRP) as a lysosomal marker and was in both cases similar to controls (Table III).

C. Cytoskeletal Drugs

Various elements of the cytoskeleton are thought to be involved in the movement or anchoring of vesicles in the cytoplasm and thus could control the rate of fusion of these organelles. Drugs which affect the cytoskeleton were used to evaluate this involvement. Figure 9A shows that pretreatment with colchicine has no effect on P-L fusion. AO-stained lysosomes in colchicine-treated cells were dispersed in the peripheral cytoplasm instead of being concentrated in the perinuclear region. This alteration of organelle polarization is a characteristic of microtubule depolymerization (73).

Since cytochalasin treatment blocks phagocytosis (74), acridine-labeled cells were treated with cytochalasin B 10 min after the start of particle ingestion but before extensive fusion. Figure 9B shows that this treatment did not affect fusion rate, although the cells showed the typical rounding and membrane blebbing associated with microfilament disruption (74). Similar results were obtained with cytochalasin D.

D. Temperature

Phagocytosis and pinocytosis require membrane fusion to form the endocytic vacuole and both processes show a strong temperature dependence. The effect of temperature on P-L fusion was examined, taking advantage of

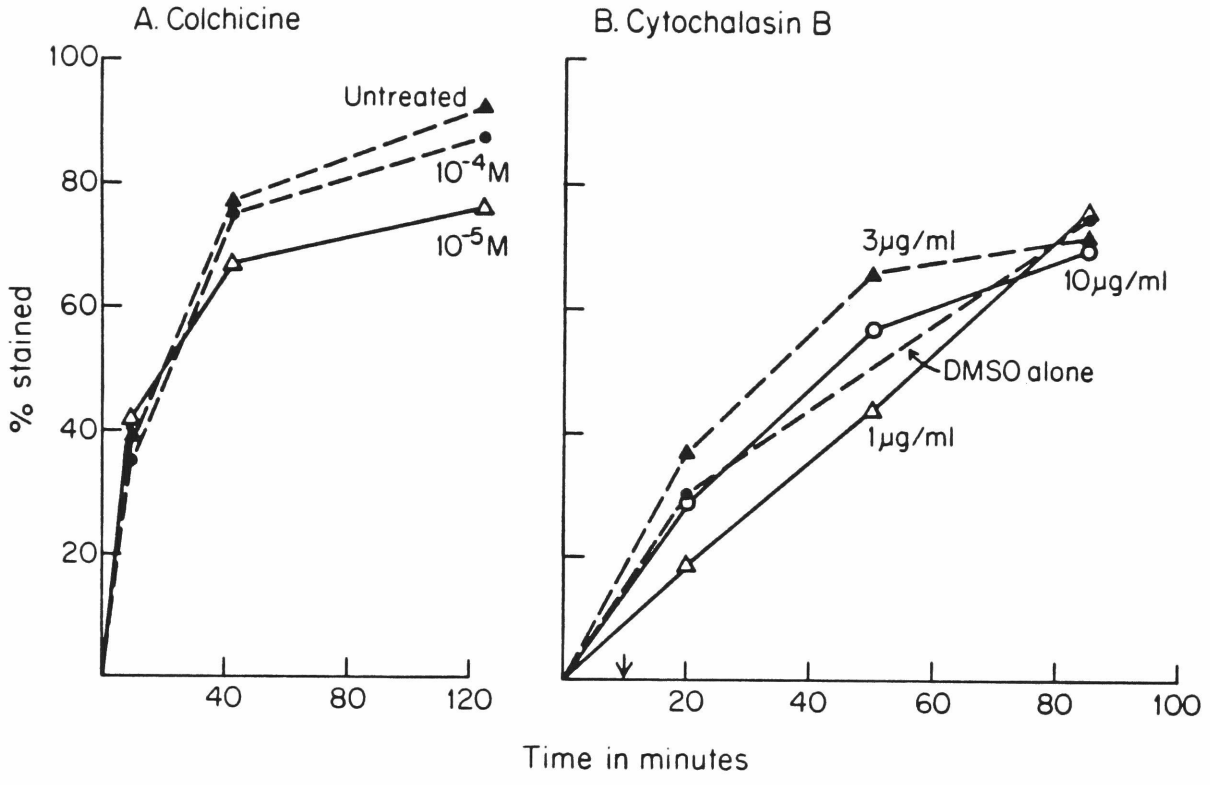


Figure 9. Effect of cytoskeletal drugs on P-L fusion.

9A. Rate of P-L fusion in 4 day cells pretreated for 2 hr with 0, 10^{-5} or 10^{-4} M colchicine. Assay was performed in the continuous presence of the drug.

9B. Rate of P-L fusion in 3 day cells treated with 0 - 10 µg/ml cytochalasin B. Arrow on x-axis marks the time of cytochalasin addition. 10 µg/ml is 2.1 x 10^{-5} M cytochalasin B.

the observation that in early cultures fusion continues for several hours after ingestion is complete. Acridine-labeled 2 day cells were pulsed with opsonized yeast and after 10 min of phagocytosis at 37°C were shifted to various temperatures. Figure 10 shows the effect of these incubation temperatures on subsequent P-L fusion. A strong temperature dependence was observed. Fusion continued but with decreasing rates for temperatures from 37°C to 20°C. At 15°C no further fusion was observed and the inhibition appeared as great as that seen at 2°C. Figure 11 shows that the inhibition at 2°C is readily reversible. Fusion resumes when these cells are returned to 37°C and the rate is similar to that in control cells until a similar extent of fusion is reached.

The log of the initial rate of fusion for temperatures from 20° - 37°C was plotted against the reciprocal of the incubation temperature in degrees Kelvin, giving a linear plot with a correlation coefficient of 0.97 (see inset Figure 10). From this Arrhenius plot, an energy of activation E_{act} for P-L fusion was obtained of 16.4 kcal/mole, with a Q_{10} of 2.5. This is comparable to the E_{act} for pinocytosis, 18 - 25 kcal/mole (71,75) and considerably less than that seen for phagocytosis, 54 kcal/mole (71). P-L fusion as detected by this assay has a threshold temperature below which no fusion occurs. In this sense it is similar to phagocytosis which has a cut-off temperature between 18 - 21°C (76) and contrasts with pinocytosis which is linear from 2° - 27°C (75).

E. Macrophage Activation

Activated macrophages have altered size, spreading, endocytic, microbicidal and secretory properties (68). There is also evidence that the state of activation differs between macrophages involved in a nonspecific inflammation and those activated by sensitized lymphocytes or lymphokines. P-L fusion was assayed in several types of *in vivo* activated populations (Figure 12) and was found to be considerably elevated compared to the low levels seen in resident cells soon after plating. The most striking increases were seen in microbicidal cells from

T. gondii or *T. cruzi*-infected and challenged mice. A smaller increase was observed in inflammatory cells elicited by proteose-peptone, a non-specific irritant.

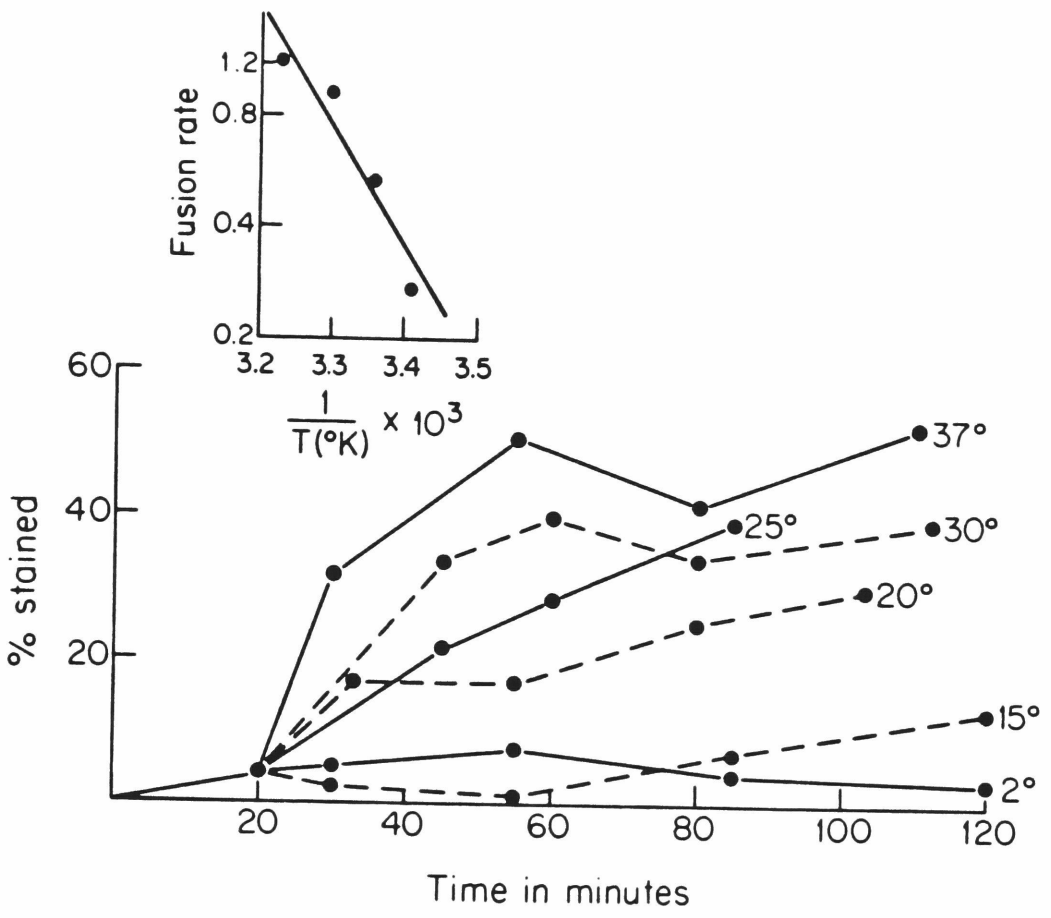


Figure 10. Effect of incubation temperature on fusion rate. After 10 min of phagocytosis at 37°C, 2 day cultures were shifted to the indicated temperatures and the effect on subsequent fusion was monitored. The abscissa gives the time since the start of the phagocytic pulse. Insert shows an Arrhenius plot of the initial rates for temperatures from 20 - 37°C. The slope defines E_{act}.

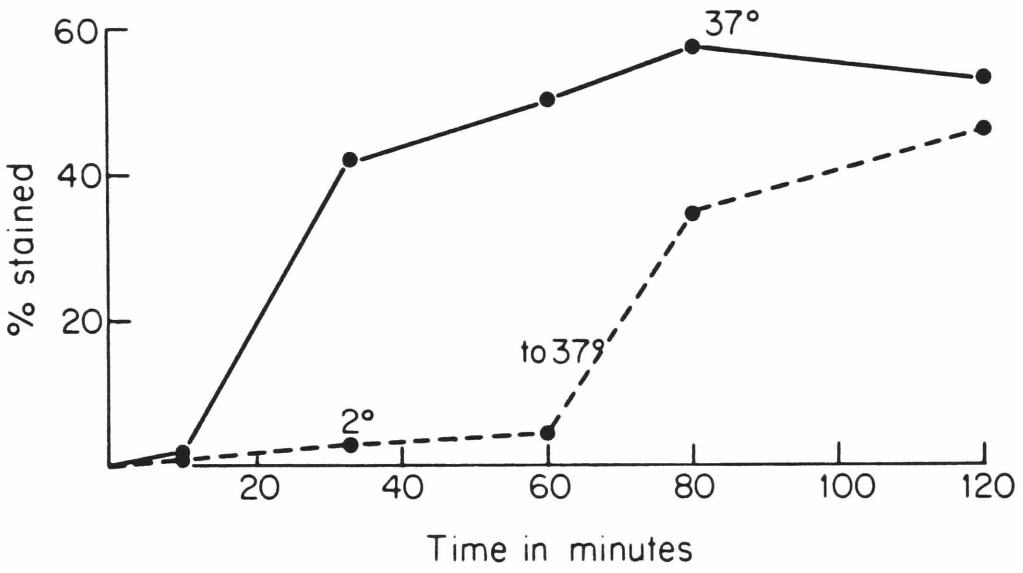


Figure 11. Reversibility of low-temperature inhibition of P-L fusion. Incubation temperature was down-shifted as in Figure 10. At 60 min after phagocytosis, 2°C cultures were shifted back to 37°C.

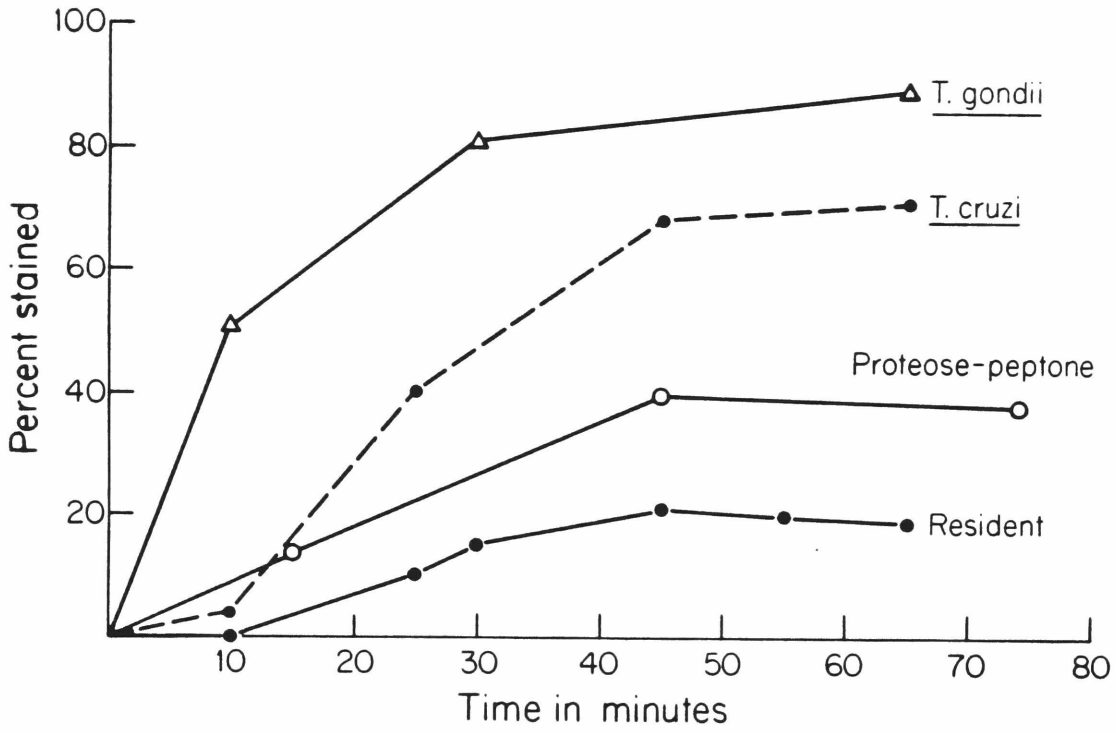


Figure 12. Effect of *in vivo* activation on the rate and extent of P-L fusion. Shown are the results of 3 separate experiments in which *T. gondii* (Δ), *T. cruzi* (•--•), and proteose-peptone (o) cells were assayed 5 - 6 hr after plating. Results were normalized to the average values for fusion in resident cells (•——•) plated 5 - 6 hr. See Materials and Methods for immunization protocols.

VI. DISCUSSION

A. Trans-membrane Modification of Fusion

Since the phagocytic vacuole is derived from the plasma membrane, it was postulated that some modification of this structure might result in organelles which would exhibit reduced fusion with lysosomes. Neither enzymatic digestion nor cell surface crosslinking by the lectin Con A had any influence on P-L fusion. Furthermore, opsonization with immune serum or Con A-coating of the phagocytic particle itself did not modify P-L fusion. The presumed trans-plasma membrane inhibition of fusion caused by live parasites (36) or by Con A in pinocytic vesicles (72) is thus far without counterpart in this system.

The striking difference in the effect of Con A on the fusability of pinocytic and phagocytic vacuoles is another example of the distinction between these forms of endocytosis (76). Pinocytosis is a constitutive cell process in which extracellular fluid and any plasma membrane-adsorbed ligands are interiorized. It is linear with temperature from 2^{o} - 37^{o}C, has an energy of activation of 18 - 25 kcal/mole (71,76), and is little affected by cytoskeletal drugs (76). In contrast, phagocytosis is induced by the interaction of large particulates with the plasma membrane and results in their uptake while excluding most extracellular fluid. It is inhibited by cytochalasin and temperatures below 18 - 21^{o}C, and has an energy of activation of 54 kcal/mole (71). In terms of their fusion with lysosomes, both phagocytic vacuoles and pinocytic vacuoles (77) appear not to fuse at temperatures below about 20^{o}C, while only pinosome-lysosome fusion is inhibited by Con A.

B. Role of the Cytoskeleton in Vesicle Fusion

Macrophage microtubules have been postulated to control non-Brownian saltatory movement of organelles (78) and thus could influence vesicle contact and fusion. Two hours pretreatment of macrophages with 10^{-6} M colchicine has been shown to abolish identifiable microtubules in electron microscopic samples of treated macrophages (28). Our detailed studies of P-L fusion show that its rate and extent are not affected by 10^{-6} - 10^{-4} M colchicine. Thus, similar to Bhisey and Freed (78), and Pesanti and Axline (28), we conclude that microtubules do not seem necessary to "direct" P-L fusion.

During phagocytosis, a filamentous network enriched in actin, myosin, and actin-binding protein (79,80) assembles beneath the plasma membrane at the site of ingestion, excluding lysosomes and other organelles (81). Treatment of macrophages with cytochalasins B and D was used to evaluate the role of this network in the control of P-L fusion rate. Within 10 min after the addition of 1×10^{-5} M cytochalasin B, organized microfilaments in macrophages are disrupted and phagocytosis is inhibited (74). *In vitro*, this compound prevents the gelling of actin by actin-binding protein (82,83). Within the time limits of our experimental system, these drugs did not affect P-L fusion, implying that the actomyosin system does not control fusion in any rate-limiting way.

C. Effect of Temperature

The effect of temperature on membrane fusion has probably been best studied in liposome systems. Calcium-induced fusion of phosphatidyl serine vesicles, for example (2), is apparently dependent on the phase change from fluid to solid state. Thus vesicle fusion is most likely to occur at a temperature where the acyl chains will be fluid before addition of divalent cation, and crystalline after addition. Fusion and this cation-controlled phase change are both inhibited at temperatures below the transition temperature.

Macrophage membrane phenomena such as phagocytosis and pinocytosis are strongly affected by ambient temperature. Similarly, P-L fusion was dependent on temperature, with an E_{act} similar to that found for pinocytosis and a cut-off temperature below which no fusion was detectable.

The E_{act} was considerably lower than that observed for phagocytosis, which probably reflects other requirements as well as alterations in membrane fluidity. These include the circumferential attachment of ligand to receptor (76) which then induces the aggregation of contractile proteins and the subsequent engulfment of the particle. In contrast, it is not clear whether pinocytosis requires any involvement of the actomyosin system (76).

D. Fusion in Activated Macrophages

Parasites such as live *Toxoplasma* are known to inhibit P-L fusion in resident but not activated macrophages (36). These studies demonstrate that the rate and extent of P-L fusion are greatly increased in activated cells when an inert particle such as killed yeast is considered. The increase in fusion correlates with the activation state of the macrophages, being highest in microbicidal cells and somewhat less in inflammatory cells. Inflammatory cells show increased size and spreading, stimulation of glucose oxidation and oxygen consumption, alterations of plasma membrane ectoenzyme levels, increased endocytic activity, and enhanced secretion of neutral proteases (68). Microbicidal cells express these properties and in addition display enhanced production of H_2O_2 and increased tumor cell and parasite killing (68). Enhanced P-L fusion is another trait of activated cells acquired during exposure to the humoral and cellular effectors of the activation process. It may be a consequence of the aforementioned metabolic and membrane alterations in these cells.

PART THREE. ENHANCEMENT OF FUSION BY PHORBOL MYRISTATE ACETATE

VII. INTRODUCTION

Phorbol myristate acetate, a fatty acid diester of a tetracyclic diterpene alcohol, is the most active of several compounds purified from croton oil (84). Its structure is shown in Figure 13. PMA is a cocarcinogen which potentiates tumor formation by suboptimal doses of initiators and induces an intense inflammatory reaction in mammalian skin (84,85). Continued applications of promotor are required to produce tumors in the mouse skin carcinogenesis assay (85). Many activities of PMA on cultured animal cells have been described (for review, see 86,87). These fall under two general headings: first, that PMA causes changes which mimic or accentuate the transformed phenotype, such as loss of cell-surface fibronectin or induction of plasminogen activator. Secondly, PMA appears to modify cell differentiation, for example, inhibiting myogenesis.

When added to explanted macrophages PMA produces dramatic changes in cell shape, spreading, endocytosis and the release of neutral proteinases and oxygen metabolites (65,88-90). These changes resemble some of the metabolic and structural correlates of macrophage activation, a process which results in enhanced phagosome-lysosome (P-L) fusion (91). Therefore, a detailed study of the effects of PMA on macrophage membrane fusion was carried out. PMA in submicrogram doses markedly stimulated both the rate and extent of fusion of phagosomes with pre-existing secondary lysosomes. The association of PMA with macrophages, the longevity of its effect on fusion, and the fate of macrophage-associated PMA were also examined.

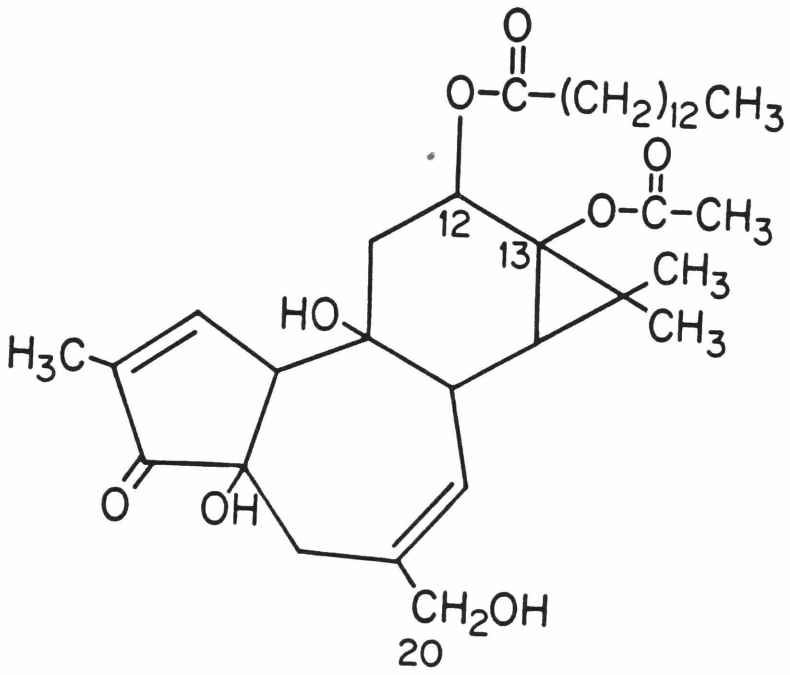


Figure 13. The structure of phorbol myristate acetate.

VIII. MATERIALS AND METHODS

A. Cells and Culture Conditions

J774 cells were maintained in spinner culture and were kindly provided by Dr. J. Unkeless. These cells were plated at a density of 1.3×10^6/35 mm culture dish for 2 hr before being used in PMA studies. Primary rat embryo fibroblasts were from Microbiological Associates and were generously provided by Dr. R. Steinman as aliquots frozen in medium with 20% FCS and 5% DMSO. Cells were thawed, washed, plated in 35 mm dishes, cultured overnight and used the next day at densities just below confluence.

B. Association of ^{3}H-PMA with Macrophages

Uptake and efflux studies were performed with [20-^{3}H]-PMA at a specific activity of $4 - 5 \times 10^6$ cpm/μg. PMA in DMSO was added to 15% FCS/MEM at a final concentration of 0.1 μg/ml PMA and a maximum of 0.5% DMSO. Coverslip cultures in Costar tissue-culture trays were exposed to 0.5 - 1.0 ml of PMA-containing medium for the required times. This medium was then removed, coverslips washed once in PBS containing 1% FCS, and then sequentially in 3 beakers of ice-cold PBS. Coverslips were added to scintillation vials, cells solubilized in 200 λ 2% SDS, and counted in 3 ml Hydrofluor. Under these conditions, coverslips alone or cell cultures at 2°C (time zero) bound from 100 - 400 cpm. This protocol was also used to follow the efflux of ^{3}H-PMA from prelabeled macrophages.

C. Metabolism of ^{3}H-PMA

One ml of ^{3}H-PMA in 15% FCS/MEM was added per 35 mm dish of cells, at a concentration of 0.1 μg PMA/ml and specific activity of 2 x 10^{7} cpm/μg. After being pulsed with PMA, cells were washed 3 x with 5% FCS/PBS, scraped into normal saline, and extracted with 3 x 1 volume ethyl acetate (92). Medium containing label released from cells was similarly extracted. For most samples, 30 μg each of unlabeled PMA, phorbol-12-myristate (PM), phorbol-13-acetate (PA), and phorbol (P) were added as standards just before extraction. Greater than 90% of the original radioactivity was recovered in the pooled ethyl acetate washes. Extracts were concentrated under N_2 and chromatographed on silica gel 60 plates using two developments in dichloromethane:acetone (3:1 v/v). Internal standards were detected by I_2 or vanillin:H_2SO_4 (93). Both sets of standards showed the same migration. ^{3}H-PMA gave a single radioactive peak which corresponded to unlabeled PMA. Similar results were obtained using water-saturated diethyl ether or chloroform:acetone (3:1) as solvents, although the CH_2Cl_2:acetone system gave better separation of PA and PM.

Plates were sectioned into 1 cm strips, scraped, and fractions counted in 0.5 ml H_2O plus 3 ml Hydrofluor.

D. Reagents

[20-^{3}H] phorbol-12-myristate-13-acetate was obtained from New England Nuclear (Boston, Mass.). Phorbol myristate acetate was from Consolidated Midland Corporation (Brewster, N.Y.). Phorbol, phorbol-12-myristate and phorbol-13-acetate were from Dr. P. Borchert at Chemical Carcinogenesis Co. (Eden Prairie, Minn.). All phorbol derivatives were stored as stock solutions at -70°C in the dark. Precoated silica gel 60 plates (0.25 mm) were from EM Laboratories

(Elmsford, N.Y.) and were prerun in ethyl ether before use. Superoxide dismutase (2900 U/mg, bovine blood), catalase (beef liver, 32,000 U/mg), Indomethacin, Hydrocortisone-21-phosphate (disodium salt), and Puromycin dihydrochloride were from Sigma Chemical Co. (St. Louis, Mo.). Cycloheximide was from Boehringer Mannheim (W. Germany).

IX. THE EFFECTS OF PMA ON MACROPHAGE MEMBRANE FUSION

A. The Stimulation of Phagosome-Lysosome Fusion by PMA

The addition of PMA to macrophage cultures led to a stimulation of P-L fusion within the first 15 min after particle ingestion (Figure 14). This occurred with doses from 0.1 - 1.0 μg PMA/ml (1.6×10^{-7} - 1.6×10^{-6} M) and led to an increase in both the rate and extent of fusion, comparable to that observed previously in activated populations (91) or long-term cultures (45). Neither the DMSO carrier alone nor 0.001 μg PMA/ml had a demonstrable effect on fusion. The addition of the non-esterfied phorbol alcohol (P), or the monoesters phorbol-12-myristate (PM) or phorbol-13-acetate (PA) at concentrations of 1.6×10^{-7} - 1.6×10^{-6} M were also without effect on fusion.

The dose-response determinations reported above were all performed in cells exposed to PMA for a 2 hr pre-incubation period. This was required because of the pronounced lag between the addition of PMA and the expression of enhanced fusion (Figure 15). These results contrast with the almost instantaneous effects of PMA on the spreading of macrophages (88) and the release of H_2O_2 (65).

The Longevity of PMA-Stimulation Cells pretreated with PMA as presented in Figure 15 exhibit enhanced fusion throughout the period of assay. Since some of the other effects of PMA on macrophage physiology are short-lived, it was of interest to examine wash-out experiments carried out for longer time periods after pretreatment. Figure 16 shows that the removal of PMA after 2 hr pretreatment results in a sustained enhancement of fusion for at least 20 hr of additional culture in PMA-free medium. It appears therefore that after a relatively long induction period, the presence of PMA is not required to maintain a maximum fusion response.

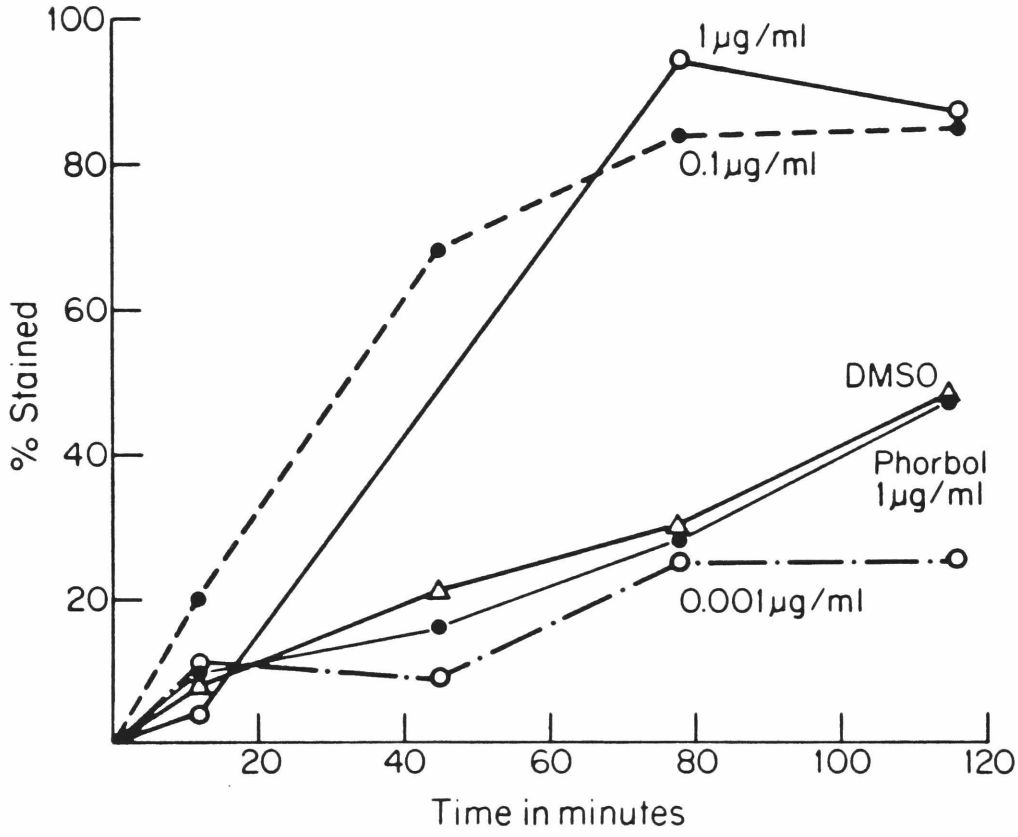


Figure 14. The effect of pretreatment with PMA on P-L fusion. Rate of P-L fusion in cells cultured for 2 days, and pulsed for 2 hr with either PMA, phorbol, or 0.33% DMSO (highest concentration used for PMA addition).

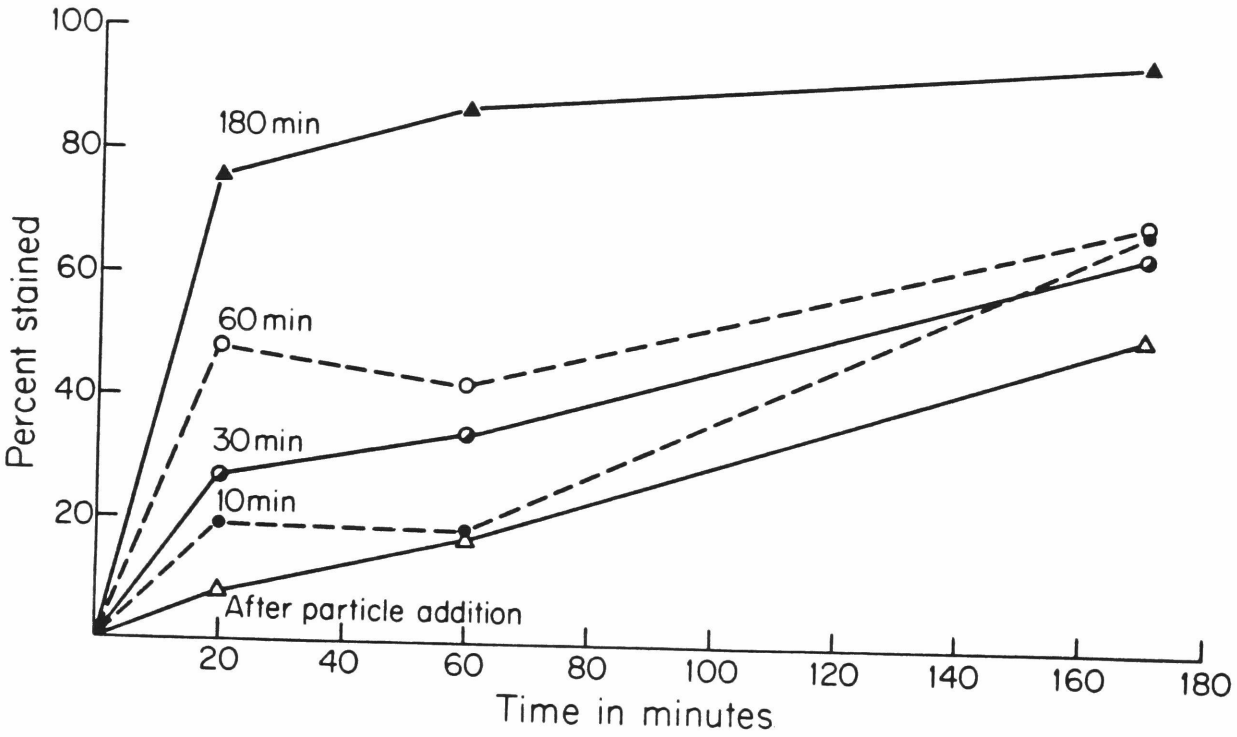


Figure 15. Time of PMA pretreatment necessary for enhancement of P-L fusion. Two day cultures were pulsed with 1 μg/ml PMA for the indicated times before particle addition and fusion assay. One set of coverslips (Δ) was given PMA after particle addition but just prior to being warmed to 37°C, and maintained in PMA-containing medium.

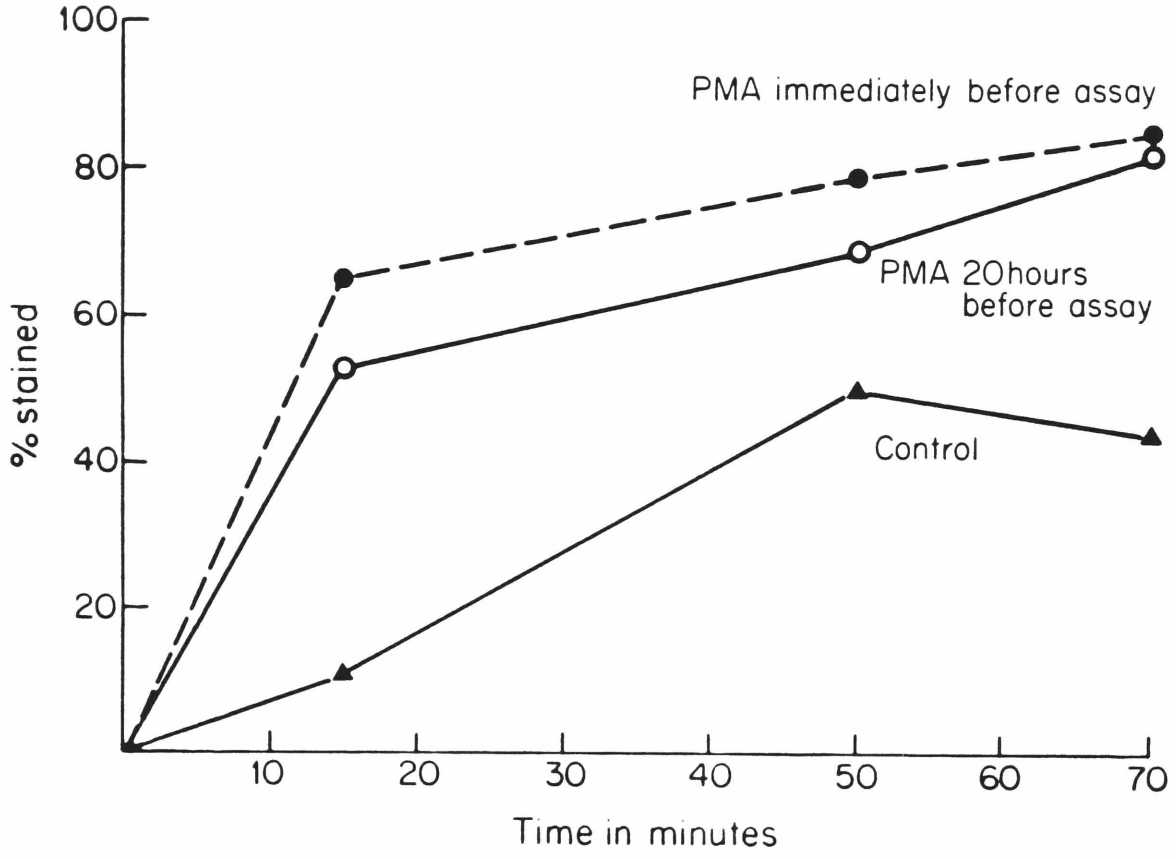


Figure 16. Maintenance of PMA effect after treatment. Cultures were pulsed for 2 hr with 0.1 μg/ml of PMA either immediately (0---0) or 20 hr prior to assay (0——0), washed, and returned to PMA-free medium. Controls received 0.03% DMSO (Δ). All 3 cell populations have been in culture a total of 48 hr.

B. The Association of ^{3}H-PMA with Macrophages

The kinetics of PMA stimulated fusion raised many questions concerning the association and fate of PMA in macrophages. For this reason the tritiated compound was obtained and used to examine the rates of uptake and release of PMA by cultured macrophages.

The association of ^{3}H-PMA with macrophage monolayers showed a rapid and linear increase until 2 - 4 hr (Figure 17A). No significant increase above this plateau occurred with continued incubation up to 24 hr. Thus the time course of the uptake of PMA paralleled the time course of its effect on fusion.

After a pulse of labeled PMA, cell-associated label was rapidly released into the medium and returned to background levels within 1 hr (Figure 17B), while the effect on fusion continued for at least 20 hr (Figure 16). Thus the continued physical association of PMA with macrophages is not necessary to maintain the enhanced fusion rate.

Prior uptake and release of PMA by macrophages did not alter the subsequent uptake of additional PMA. Label was released somewhat more slowly in medium without serum. Lower serum concentrations were used in the following chromatography experiments, both to facilitate extraction and to enable the comparison of released and cell-associated label. Results were qualitatively the same for 1 - 15% serum concentrations.

C. Metabolism of PMA

Metabolism of PMA by Macrophages Ninety minutes after a pulse of ^{3}H-PMA, cells and medium were extracted in ethyl acetate and analyzed by thin layer chromatography (see Materials and Methods). Label associated with cells at the start of the ninety minute incubation comigrated with the PMA standard (data not shown). Label which was still cell-associated at the time of extraction chromatographed as shown in

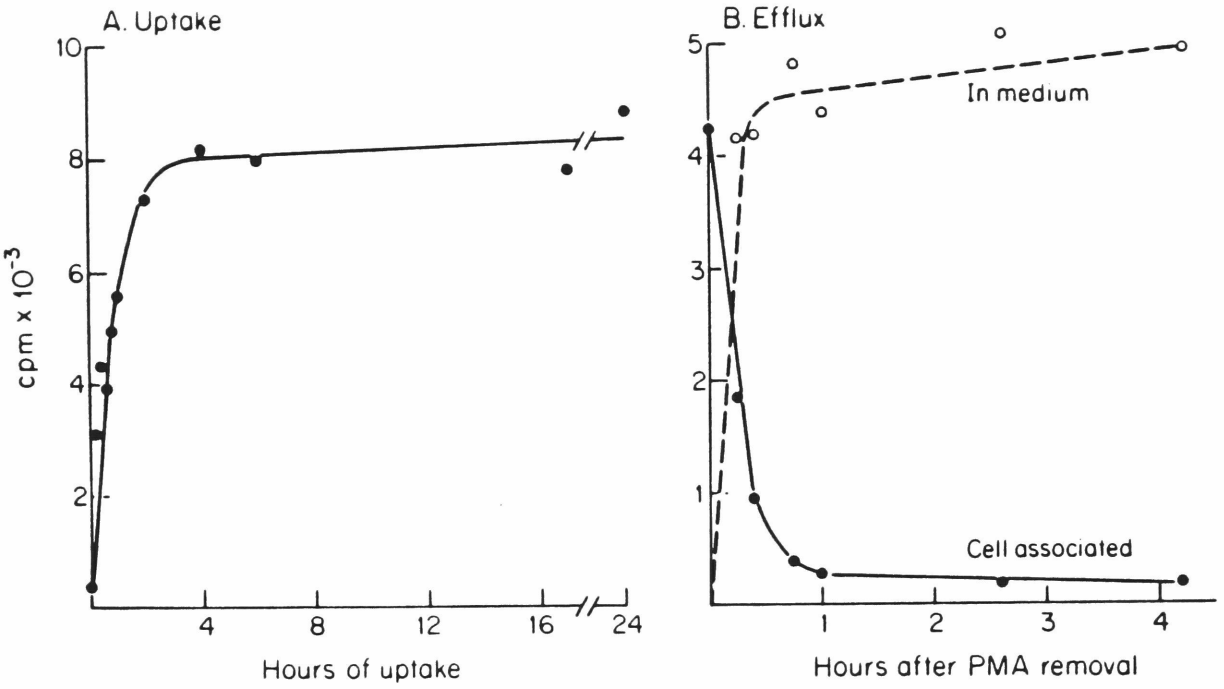


Figure 17. Uptake and efflux of ^{3}H-PMA in macrophage cultures.

A. Association of ^{3}H-PMA with macrophages. Two day cultures were exposed to medium containing 0.1 µg/ml PMA at 37°C for the indicated times. Points are the average cpm for triplicate coverslips at 5 x 10^{5} peritoneal cells per coverslip. Maximum incorporation was 1.7% of the added label.

B. Efflux of ^{3}H-PMA from macrophages. One day cells were exposed to 0.1 µg/ml PMA for 3 hr, washed, and returned to culture (time 0) in 15% FCS/MEM. At various times thereafter, triplicate coverslip cultures were assayed for cell-associated and released label. Incorporation at time 0 was 2.2% of the added label.

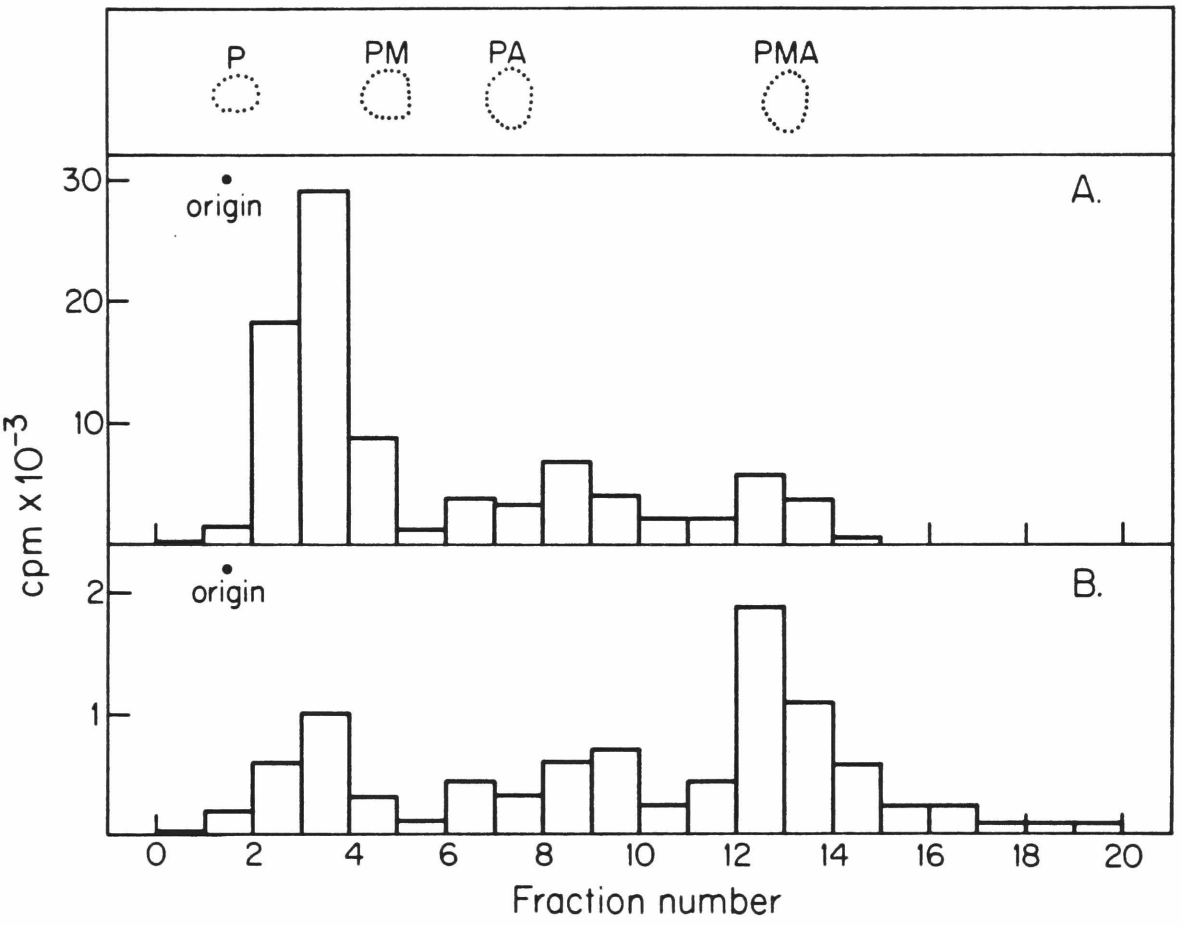


Figure 18. Thin layer chromatography of released (A) and cell-associated (B) label from primary macrophage cultures treated with 0.1 μg/ml ^{3}H-PMA. Two 35 mm dishes of 24 hr macrophages were treated with PMA for 4 hr, washed, and recultured in 2% FCS/MEM without PMA for 90 min. Cells and medium were then extracted, chromatographed and analyzed as described in Materials and Methods. Top: positions of standards included on the same TLC. Solvent front was at 19 cm for both developments in CH_2Cl_2:acetone.

Figure 18B. The major peak of the material comigrated with the PMA standard, while some label ran as a more polar material between unesterified P and PM.

Label which was released from macrophages (Figure 18A) was almost exclusively the polar metabolite and little if any unmodified PMA remained. In contrast, PMA incubated in medium without cells comigrated with the PMA standard after extraction and chromatography. Thus macrophages appear to metabolize and de-esterify PMA to a more polar product, with an R_f value not identical to either of the monoesters and close but not identical to that of the parent alcohol. Since many oxidation products of PMA are possible (94), the metabolite could be an oxidized form of phorbol or the monoester.

When the released label was extracted, concentrated, resuspended in fresh medium and added to macrophages, little or no rebinding of the metabolite occurred. This implies that PMA remains cell-associated only when present as the intact molecule.

Metabolism of PMA by Other Cell Types We wished to compare the metabolism of PMA by the highly endocytic mouse macrophage to that of other cell types. Accordingly, release and chromatography studies were performed for J774 cells, a macrophage-like murine cell line originally derived from a reticulum cell sarcoma (95), and for primary rat embryo fibroblasts. As shown in Figure 19 A and B, J774 cells rapidly released labeled PMA after a pulse, and most of the label in the medium again migrated between phorbol and phorbol myristate. Label which was still cell-associated at the time of extraction mainly appeared to be unmodified PMA. Thus, the rapid release of metabolized PMA by J774 cells is similar to that of freshly explanted macrophages.

The results of a similar experiment with rat embryo fibroblasts were somewhat different from those with macrophages. The release of PMA was slower than for J774, and 37% remained cell-associated at 90 min. Cell-associated label (Figure 19 D) again was almost exclusively unmodified PMA. Released label (Figure 19 C) showed greater heterogeneity with three radioactive peaks, one of which comigrated with PMA standard, one

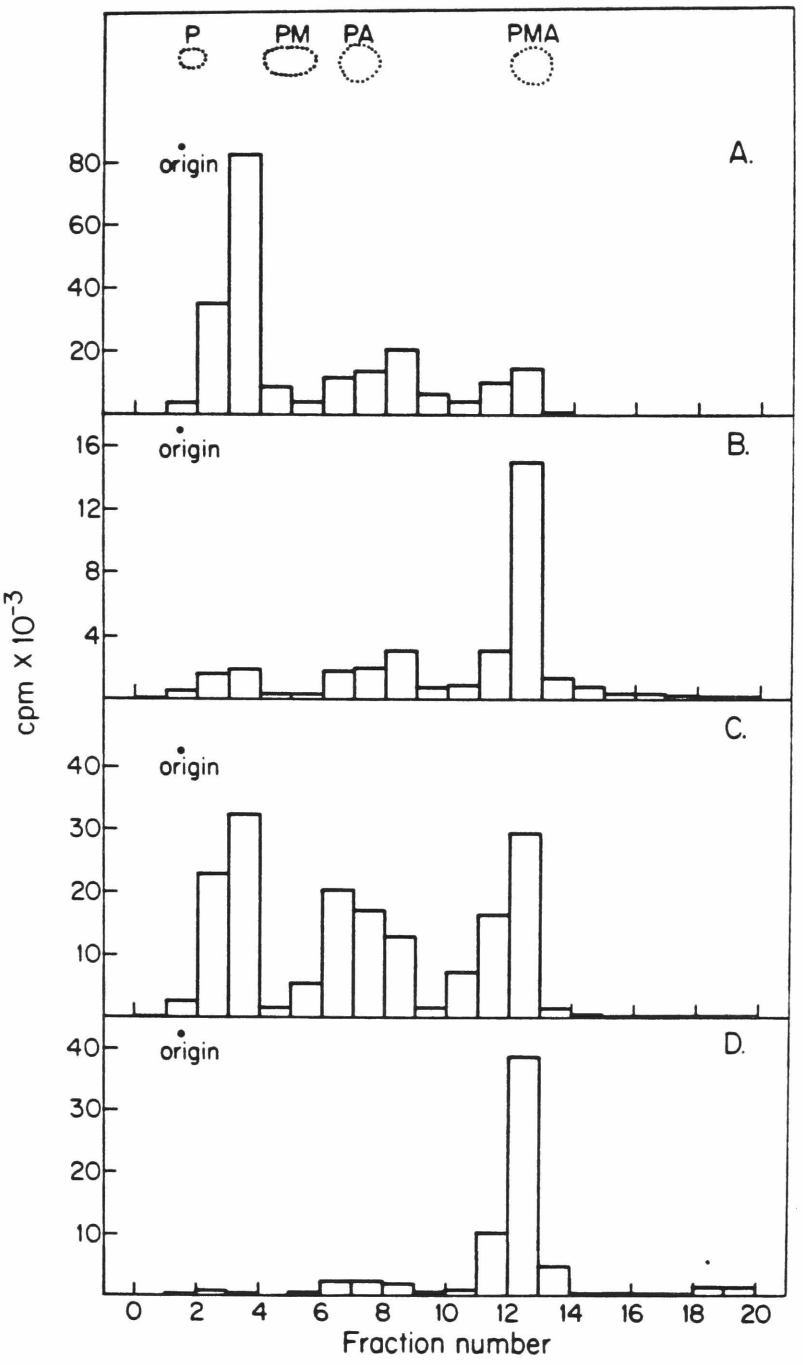


Figure 19. Thin layer chromatography of released (A) and cell-associated (B) label from J774 cultures and released (C) and cell-associated (D) label from rat embryo fibroblast cultures treated with 0.1 μg/ml ^{3}H-PMA. Two 35 mm dishes of each cell type were treated with PMA for 2 hr, washed, and recultured in 1% FCS/MEM without PMA for 90 min. Cells and medium were then extracted and chromatographed. Top: positions of standards included on the same TLC. Solvent front was at 19 cm for both developments in CH_2Cl_2:acetone.

which ran as the usual metabolite seen with the other cell types, and a third broad intermediate peak which migrated as phorbol acetate.

D. Fusion versus Other Effects of PMA on Macrophages

Fusion and the Other Metabolic Consequences of PMA PMA is known to trigger the release of oxygen intermediates by macrophages (65,90), and to stimulate the production of prostaglandins (96,97). The enhancement of P-L fusion by PMA was not blocked by concurrent treatment throughout the assay with superoxide dismutase (1350 U/ml) or catalase (15,000 U/ml). These conditions inhibit microbicidal and tumoricidal activities in macrophages by hydrolyzing O_2^- or H_2O_2. Preincubation of macrophages with 10^{-5} M Indomethacin for 1 hr and its presence during PMA stimulation block prostaglandin production (98) but were without effect on the increase in fusion.

The increased pinocytic rate seen in PMA-treated macrophages (88) can be reversed by 10^{-5} M colchicine, while enhanced fusion was unaffected by treatment of macrophages with colchicine at 10^{-5} M. Finally, the induction of plasminogen activator secretion by PMA is inhibited by glucocorticoids (89), while the fusion effect was not reduced by 10^{-5} M Hydrocortisone, a concentration which shows potent inhibition of the secretion of this enzyme (99). These studies imply that the effect of PMA on fusion can be dissociated from several of its other effects on macrophages.

PMA-stimulated P-L Fusion Requires Protein Synthesis The pronounced lag phase of PMA stimulation, its rapid de-esterification and release from the macrophage and its prolonged influence when PMA was removed from the medium suggested that the molecule had induced the formation of a relatively stable product. Since blocking several of the known effects of PMA on macrophages did not affect the enhancement of P-L fusion, it was of interest to know if the enhancement was mediated by a protein product. For this purpose the inhibitors cycloheximide

and puromycin were employed at concentrations which inhibit ^{3}H-leucine incorporation by 90% (89) and the re-expression of surface receptors (100). The results are presented in Figure 20. One day after explanting macrophages the fusion rate is normally low (Figure 20A) and PMA showed a dramatic stimulatory effect. Pretreating the macrophages with inhibitors almost completely ablated this effect. Similar results were obtained two days after explant. However, four days after *in vitro* incubation the fusion rate of untreated cells had already reached maximum levels (45) and the addition of PMA had little influence. Under these conditions neither puromycin nor cycloheximide influenced the rate of fusion over a 5 hr exposure (Figure 20B).

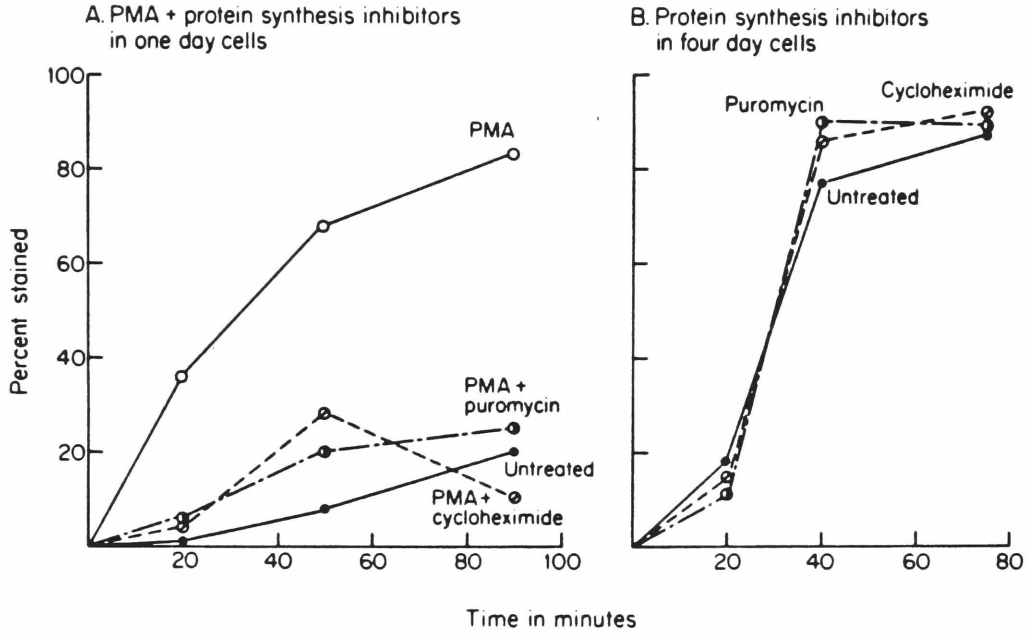


Figure 20A. The effect of protein synthesis inhibitors on the enhancement of P-L fusion by PMA. One day cultures were incubated for 2 hr in normal medium or medium containing 10 µg/ml puromycin or 5 µg/ml cycloheximide. PMA (0.2 µg/ml) was then added for 5 hr to all except untreated cultures. Fusion was assayed as usual except that medium contained protein synthesis inhibitors where indicated.

20B. The effect of protein synthesis inhibitors on P-L fusion in four day cells. Cells were preincubated for 5 hr in medium alone or containing 10 µg/ml puromycin or 5 µg/ml cycloheximide. These concentrations were included during the fusion assay where indicated.

X. DISCUSSION

A. The Sequence of PMA's Effects on Macrophages

In macrophages, the following sequence of events occurs after PMA treatment. Within the first 10 - 20 min after the addition of PMA to resident macrophage cultures, the cells spread on the substrate (88) and within the first 30 min a burst of prostaglandin and thromboxane release is observed (96,97). Another rapid effect of PMA is the dramatically increased release of products of the reduction of molecular oxygen such as H_2O_2 (65) and O_2^- (90). This release is much greater when activated macrophages are treated, and unelicited cells show only a small increase in release. Within 1 hr of PMA treatment, stimulation of fluid phase pinocytosis and topological rearrangement of lysosomes into a radial pattern along microtubules occurs (101). Again, these two effects are much more pronounced in elicited rather than resident cells.

In contrast to these rapid effects the induction of plasminogen activator in resident macrophages is much slower, has a lag period of several hours, and continues for at least 24 hr in culture after induction (89). In these aspects, PMA's effect on fusion resembles the induction of plasminogen activator and is also expressed in unelicited cells. The requirement for the continued presence of PMA in these other macrophage responses has not been well-characterized. There is some evidence that the effect of PMA on fusion is dissociable from other effects since it is not blocked by catalase, superoxide dismutase, indomethacin, colchicine, or hydrocortisone.

B. Uptake and Metabolism of ^{3}H-PMA

PMA is a very lipophilic molecule and is known to interact with phosphatidyl choline monolayers, changing the monolayer surface pressure by physical intercalation, while having no apparent effect on model membrane permeability or fluidity (102). Using (20-^{3}H)-PMA, the association of PMA with macrophages was examined to see if a physical intercalation might explain the effect on the ability of the membranes to fuse.

The results reported here indicate that mouse macrophages rapidly take up ^{3}H-PMA from the culture medium, as has been described for several other cell types in culture (92,103). Linear uptake levels off after about 3 hr, at which time about 2% of the added label is cell-associated, or about 6.4×10^{-12} mole PMA/1 $\times 10^{6}$ peritoneal cells plated. When cells are returned to PMA-free medium, this label is rapidly released. Cell-associated label appears to be unmodified PMA, while the released form migrates as a more polar compound in TLC. All of the label partitions into the organic phase during extraction, implying that the ^{3}H is still on the phorbol skeleton, and the compound migrates between phorbol and phorbol myristate. As others have reported (104,105), we also found that PA migrated faster than PM, in spite of the shorter length of its acyl chain.

The release of metabolized PMA suggests that the plateau of uptake does not represent a saturation of specific binding sites for PMA. Rather, an equilibrium seems to be established between the uptake of native PMA and release of the metabolized form. The macrophage cell line J774 showed similar rapid kinetics of PMA uptake and metabolism, producing a form which migrated in the same position as that from primary macrophages. Primary rat embryo fibroblasts had somewhat slower kinetics of release, and much of the label released was either unmodified PMA or ran at the same position as phorbol acetate. Thus, results with rat fibroblasts are more similar to results in the literature in which either no metabolism of PMA was seen (92,103,104,106) or in which

phorbol acetate was the only metabolite seen (104,105). Human cells have been reported not to metabolize PMA (104,107), and it would be interesting to know if this were true for human macrophages as well.

The effect of PMA on P-L fusion is maintained for hours after the release of PMA from treated cells, and thus this effect is not dependent on the physical association of PMA with membranes (102) or continued binding to a specific receptor (92,108). The time course of the PMA effect and its inhibition by puromycin and cycloheximide suggest that PMA is inducing the synthesis of a protein or proteins which mediate the effect.

C. Comparison to Other Modulators of P-L Fusion

P-L fusion is inhibited by several intracellular parasites (36), by lysosomal uptake of polyanions (33), and by decreased temperature (45). Fusion is dramatically increased by time in culture (45), by *in vivo* macrophage activation (91), and by PMA pretreatment, while it is insensitive to a great number of other variables. Thus PMA pretreatment is one of a very limited number of experimental conditions which are able to modulate fusion. PMA may be inducing protein(s) in early cultures which are already present at higher levels in activated and four day cultures. This product could be an integral membrane protein of lysosomal or plasma membrane, which directly influences fusion of its surrounding lipid domain. Another possibility is that a cytosolic or loosely associated membrane protein could be acting between the phagosome and lysosome membranes to affect their fusion. The lack of inhibition by puromycin and cycloheximide in four day cultures implies that if such molecules are involved, their turnover rate is slow. Slow turnover of a protein mediator would also explain the stability of the increased fusion rate for 20 hr after the removal of PMA.

PART FOUR. INHIBITION OF FUSION BY POLYANIONS

XI. INTRODUCTION

Thus far, these studies describe the enhancement of P-L fusion by *in vivo* activation, PMA treatment, and time in culture. Fusion was markedly inhibited by low temperature. In considering other possible inhibitors of P-L fusion, we noted reports in the literature which dealt with inhibition by two polyanionic compounds, suramin (33), a sulfonated compound which is used as a drug in treating African trypanosomiasis, and a sulfated glycolipid purified from *M. tuberculosis* (35). Suramin's effect on P-L fusion in macrophages was therefore evaluated. In addition, other polyanions whose effects on pinocytosis in macrophages had previously been studied (109) were assessed for their inhibition of P-L fusion. This section details the compounds and conditions which inhibited P-L fusion and the characterization of the requirements for inhibition. In the course of these studies, other investigators also reported on inhibition of P-L fusion by dextran sulfate (34) and poly-D-glutamate (34,110).

XII. MATERIALS AND METHODS

A. Purification and Acetylation of LDL

The purified LDL was the kind gift of Dr. M. Rifkin and was purified from human serum (111). First, a lipoprotein fraction was obtained by ultracentrifugation of plasma adjusted to a density of 1.225 by the addition of solid KBr. This fraction was then separated into VLDL, LDL and HDL with an agarose column.

Purified LDL was acetylated by the method of Fraenkel-Conrat (112) and as described by Basu et al. (113). The purity of the LDL fractions and the acetylation of LDL were monitored by cellulose acetate electrophoresis. A Beckman microzone electrophoresis system was used, and the manufacturer's recommended procedure followed. Samples were spotted in the B slot of the apparatus, electrophoresed 25 min at a constant voltage of 300 v, fixed, and stained for 90 min at 37°C in 0.8% oil red O in 70% aqueous methanol.

LDL-dextran sulfate complexes were formed by adding 2 mg/ml DS and 0.1 M $CaCl_2$ to purified LDL fractions in 0.15 M NaCl. After overnight incubation at 4°C, the resulting precipitate was pelleted by centrifugation in an International centrifuge at 2000 rpm for 20 min, 4°C. The pellet was washed twice in ice-cold distilled water without resuspending, and then resuspended by sonicating in 5% FCS/MEM at twice the original volume, such that the maximum concentration of DS was 1 mg/ml. The LDL column fractions were usually concentrated to the approximate original serum volume before precipitation.

B. Synthesis and Purification of Labeled Dextran Sulfate

Dextran sulfate (DS) (500 K molecular weight) was tritium-labeled by New England Nuclear using the Wilzbach (114) method. 500 mg 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 volumes 95% ethanol and 1% (final concentration) potassium acetate as described by Tourtellotte and Dziewiatkowski (115). 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 3 times to a constant specific activity of 73.7 u Ci/mg dextran sulfate. The carbohydrate content of a weighed sample was determined by the anthrone assay (116) using DS standards, sulfate content was determined by the method of Terho and Hartiala (117), and the molecular weight estimated by gel filtration on a sepharose 2B column in 0.5 M NaCl. Labeled DS showed 1 peak on gel filtration with an average molecular weight of 300 K, and contained 10% sulfur as ester sulfate, as compared to 12.6% for untreated DS, 12.8% for reprecipitated, unlabeled DS, and 17% as the manufacturer's estimate for 500 K MW DS. Radioactivity was determined by counting in Hydrofluor; counting efficiency was calculated using a tritiated toluene standard. Both 500 K molecular weight dextran and dextran sulfate were from Pharmacia.

C. Uptake Studies with ^{3}H-DS

Uptake studies of both soluble DS and DS-serum complexes were performed on coverslip cultures of macrophages in wells of Costar dishes, with triplicates for each time point. For soluble DS uptake, time points were taken by aspirating labeled medium, washing wells once with cold PBS, and washing coverslips sequentially in 3 beakers of cold 0.9% NaCl. Coverslips were placed in scintillation vials, 200 λ of 2% SDS added, followed by 3 ml of Hydrofluor.

^{3}H-DS-serum complexes were made similar to the procedure of Cornwell and Kruger (118) for the precipitation of LDL from serum by DS. To a volume 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, the precipitate was washed as described in section A, and resuspended to various concentrations in 5% FCS/MEM. Complexes were sonicated to give a colloidal suspension which could be readily ingested by the cells. Under these conditions, about 35% of the added ^{3}H-DS was precipitated as complex.

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, N.J.) at 37°C during the uptake period. This helped to keep the complexes from sticking non-specifically to the cells and coverslip.

D. Freeze-fracture Analysis

Four day cultures in 60 mm dishes were used for freeze-fracture analysis. Cultures were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.4, 30 min at room temperature, washed 3 times in buffer and scraped off the dish in buffer. Cells were resuspended in 25% glycerol in 0.1 M cacodylate, kept overnight at 4°C, and aliquots of the settled cell suspension were layered onto small metal discs which had been previously coated with a film of 1% poly-L-lysine and allowed to dry. Cells between two discs were rapidly frozen in the liquid phase of partially solidified chlorofluoromethane (Freon 22, E. I. duPont de Nemours & Co., Wilmington, Del.). Freeze-fracturing was performed with a Balzers apparatus model BA360 (Balzers High Vacuum Corp., Santa Ana, Ca.) with a stage temperature of -115°C and carbon and platinum shadowing. Replicas were cleaned with bleach, picked up on uncoated grids, and examined in a Siemens Elmiskop II. Freeze fracture electron micrographs are mounted with the shadow direction from bottom to top.

E. Subcellular Fractionation

Four day cultures of control, D or DS-treated cells were washed (on ice) 3 times with PBS, 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 ($3\text{-}4\times10^7$ cells) were then scraped into the buffered sucrose and homogenized in a 7 ml Dounce homogenizer (Kontes Co., Vineland, N.J.) using a B pestle. Two - five strokes resulted in 85 - 95% cell breakage. This whole cell homogenate was then layered over a 12 ml linear metrizamide gradient (15-40 or 20-40% in a Hepes or Hepes-saline buffer (119)), and centrifuged at 10,000 x g for 90 min (4^oC) using an HB-4 swinging bucket rotor and a Sorvall centrifuge. 0.5 ml fractions were collected and assayed as follows:

Acridine Orange Cells were labeled with AO (5 μg/ml 20 min, 37^oC) before homogenization. Fractions were assayed by adding 100 λ aliquots to 2 ml 95% ethanol and reading fluorescence in an MPF-44 fluorometer, excitation 490 nm slit 3 nm, emission 520 nm slit 6 nm.

HRP Activity HRP was assayed as described in G with or without addition of 0.1% Tx-100 to an aliquot of the fraction.

5'-nucleotidase 5'NTase was assayed as described by Edelson and Cohn (120) using adenosine-2-[^{3}H]-5' monophosphate (ammonium salt, Amersham-Searle Corp., Arlington Hts., Ill.) as substrate.

N-acetyl Glucosaminidase NAGase activity was measured using the assay of Peters et al. (121) 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 D or DS in reaction mixture.

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 solutions were wt/wt percentages and contained 10 mM potassium phosphate buffer and 1 mM EDTA (pH 7.4). Step gradients were centrifuged at 100 K x g using the SW-41 rotor and a Beckman L5-65 ultracentrifuge for 60 min at 4°C.

F. Quantitation of Intralysosomal pH

The technique of Okuma and Poole (63) was used to measure lysosomal pH. Three day cultures of untreated cells, or cells treated with 10 mg/ml D or 10 µg/ml DS were exposed for 24 hr to these media with or without added fluorescinated dextran (Sigma) at 0.5 mg/ml. Cells were then washed in PBS, mounted in a coverslip holder which fit into a fluorescence cuvette, and scanned in PBS plus 1 mg/ml glucose at 37°C. Cells were allowed to equilibrate 5 - 10' before scanning. An MPF-44 fluorometer with a water-jacketed cuvette holder was used. Excitation spectra of cells or standards were scanned from 400 - 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 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.

G. Other Assays and Procedures

Phospholipid Composition Macrophages cultured under various conditions were washed 3 times with PBS and once with 0.9% NaCl, scraped into 0.9% NaCl and extracted on ice by the method of Bligh and Dyer (122) as modified by Ames (123), using redistilled solvents. Phospholipid classes were separated by two-dimensional thin layer chromatography as described by Mahoney et al. (124). Lipid-containing regions were visualized by I_2 staining, the gel was scraped from the plate, and phosphate was determined by the method of Bartlett (125) using KH_2PO_4 as standard.

Sulfate Determinations The ester sulfate in various compounds was assayed by the method of Terho and Hartiala (117) 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 non-hydrolyzed samples.

HRP Assay Peroxidase activity was assayed using O-dianisidine (Sigma Chemical Co.) as previously described (126).

Histochemistry Toluidine blue staining (127) was done on fixed coverslip preparations at 0.1% dye in 0.1 M acetate buffer pH 4.0 or in distilled H_2O adjusted to pH 1.0 with HCl. After 30 min of staining at room temperature, coverslips were washed in buffer at the same pH. Oil red O staining (127) was done on fixed coverslip preparations at 0.5% oil red O in 60% triethylphosphate 10 min at room temperature, followed by washing in H_2O.

Protein Determinations Protein was determined by the method of Lowry (128) using crystalline bovine serum albumin as standard.

XIII. POLYANION EFFECTS ON MACROPHAGES

A. Polyanions Which Inhibit P-L Fusion

Figure 21 contrasts the rate of P-L fusion in 4 d cultures treated with dextran (D), a 5 x 10^5 molecular weight glucose polymer, or dextran sulfate (DS), its sulfated derivative containing 17% sulphur. Cells cultured 4 d with 30 mg/ml dextran showed the usual rapid fusion rate resulting in about 85% positive staining within 1 hr. In contrast, sulfated dextran at a concentration 3 orders of magnitude lower (10 μg/ml) markedly inhibited P-L fusion in treated cells. Most particles ingested by DS-treated cells were unstained even several hours after ingestion. Particle uptake was similar in the two cell types.

Figures 22A and B are fluorescence micrographs of fusion assays in D or DS-treated cells about 60 min after particle ingestion. Dextran cells contain many positively-stained particles as well as many AO-stained lysosomes. Abundant lysosomal staining is seen in DS-treated cells, but this fluorescence has not been transferred to yeast-containing phagosomes. By fluorescence microscopy, both cell populations show enlarged AO-stained lysosomes. By phase microscopy (Fig. 22C), D cells contain numerous large, phase-lucent vacuoles, similar to macrophages cultured in medium containing sucrose or newborn calf serum (59,60). The vacuoles in D-treated cells presumably are due to the high concentration of endocytosed, non-digestible polymer. DS-treated cells (Fig. 22D) have similar large vacuoles, even though the concentration of non-digestible DS is much lower. These vacuoles may result in addition from a stimulation of pinocytotic uptake by polyanion as has previously been described (109), or by increased uptake of serum components (129). The latter possibility is considered in more detail in a later section.

The presence of DS in the large vacuoles of treated cells was evaluated by staining with toluidine blue, a cationic dye. As shown in Figure 23,

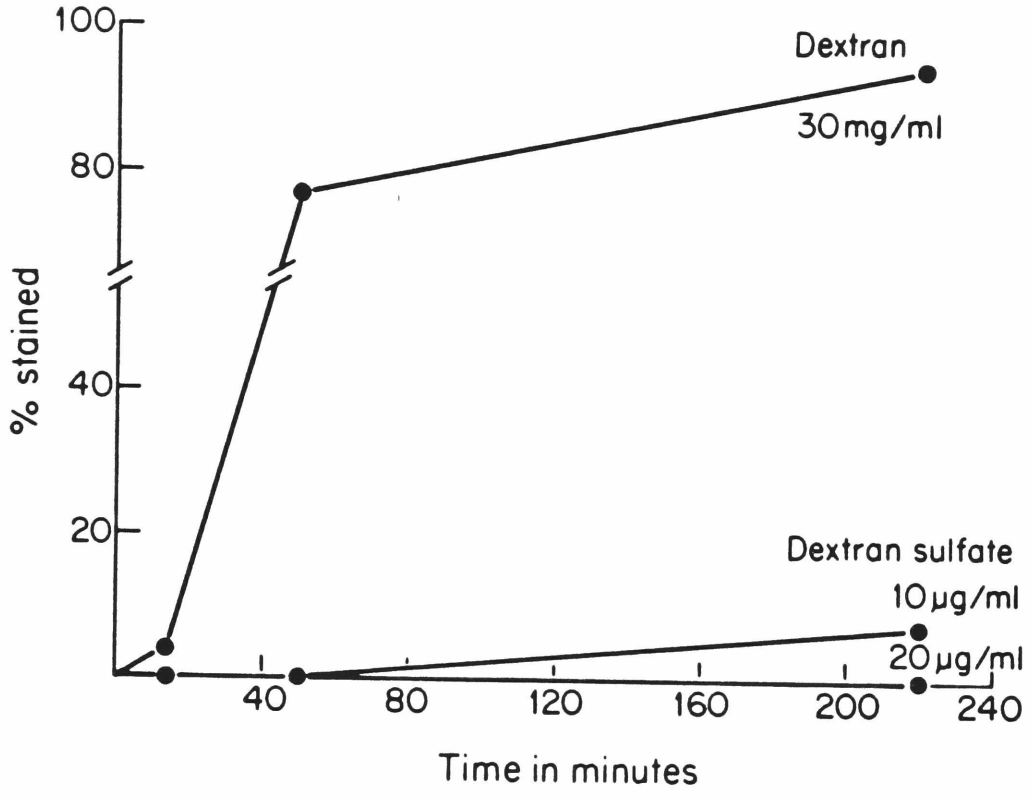
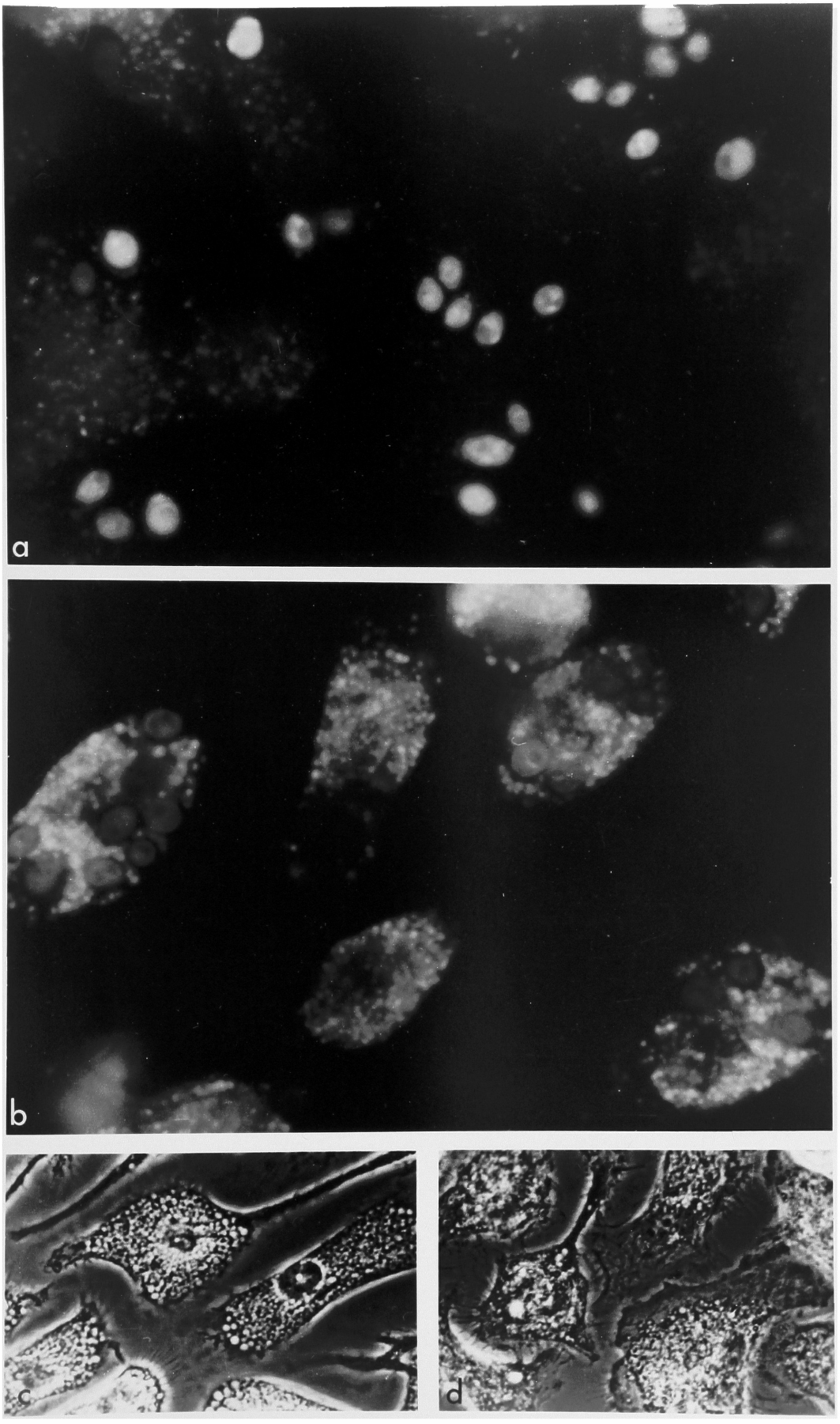


Figure 21. Effect of dextran or dextran sulfate on P-L fusion. Cells were cultured four days with 500,000 molecular weight D or DS at the indicated concentrations. Cells were given fresh medium each day, and assayed on day 4.

Figure 22. Fluorescence and phase microscopy of dextran and dextran sulfate-treated cells. Cells were cultured as in Fig. 21, using 10 μg/ml DS and 30 mg/ml D.

22A&B. Acridine orange stained D (a) or DS (b)-treated cells ∿ 60 min. after yeast ingestion. Both cell types have abundant AO-stained lysosomes. X 1900.

22C&D. Phase micrograph of D (c) or DS (d)-treated cells, showing numerous large vacuoles. X 950.


a
b
c
d

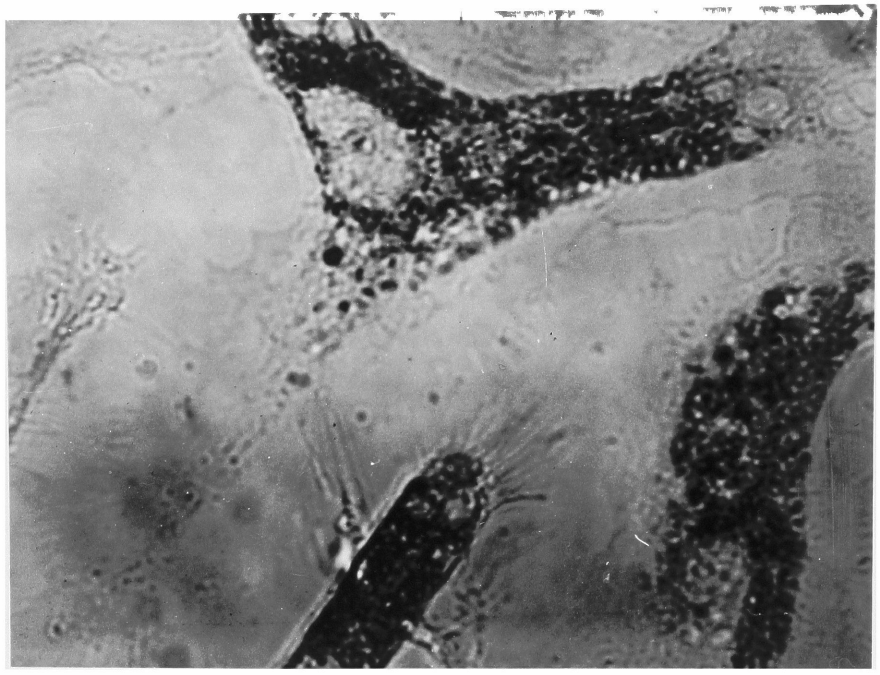


Figure 23. Bright field micrograph of toluidine blue-stained cells. Macrophages cultured 4 d in 10 µg/ml DS, fixed and stained with toluidine blue at pH1. Note absence of nuclear staining and heavily stained cytoplasmic granules. X 1660.

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 (127). 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 IV lists those which were 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 dextran sulfate ($5x10^5$ MW). Lower molecular weight DS only caused inhibition at much higher concentrations (500 μg/ml) although its sulfate content was comparable. This again may reflect the stimulation of pinocytosis by DS, which appeared to be higher for the longer chain polymer (109). Chondroitin sulfate was inhibitory only at very high concentrations, and had a lower sulfate content than Suramin or DS.

All of these inhibitors caused the formation of large cytoplasmic vacuoles, similar to D and DS in Figure 22. Again, for all of these inhibitors, toluidine blue staining showed a concentration of anionic groups in cytoplasmic granules.

Electron Microscopic Evaluation of Fusion Results obtained by the fluorescence assay of P-L fusion were correlated with electron microscopic evaluations. Cultures were prelabeled with thorotrast and then cultured in medium containing inhibitors for 4 days. Prelabeling cells with thorotrast before exposure to inhibitors ensures equivalent labeling of the different cell populations. Table V compares results from the two assay systems for Suramin, dextran sulfate and control cells. Marked inhibition of fusion was found by electron microscopy as well as by fluorescence. Dextran sulfate again appeared to be a more potent inhibitor than suramin.

Table IV

COMPOUNDS WHICH INHIBIT PHAGOSOME-LYSOSOME FUSION

Compound	Molecular Weight	Inhibitory Concentration*	% Sulphur as Ester Sulfate	
			Reported	Experimental
Suramin	1,429	200 μg/ml (1.4×10^{-4} M)	13.5‡	--
Dextran Sulfate	500,000	10 μg/ml (2×10^{-8} M)	17	15.7
	40,000	500 μg/ml (1.25×10^{-5} M)	--	13.8
	8,000	500 μg/ml (6.25×10^{-5} M)	--	12.2
Poly-D-glutamate	35,000	0-5-1 mg/ml (1.4×10^{-5} 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 one hour after particle ingestion in cells treated 3 - 4 d with polyanion.

‡% sulphur as sulfonate.

Table V

Assay Conditions	% Fusion after 1 Hour	
	Acridine Assay	Thorotrast-EM Assay*
Cultured 4 days in:		
Medium alone	85 ± 13.6 (5)‡	37.3 ± 4.9 (3)
Medium + 200 µg/ml suramin	0 (5)	10.4 (1)
Medium + 20 µg/ml dextran sulphate	0 (5)	2.3 (1)

*For each determination, from 30 - 60 vacuoles were evaluated by stereology, and total line crossing of from 600 - 1500 were obtained.

‡The number of experiments is given in parentheses.

Ultrastructural Modifications in Polyanion-treated Cells Figure 24A is a representative electron micrograph of a cell from a four-day, dextran sulfate-treated culture. The cell cytoplasm contains many enlarged secondary lysosomes which show a fine fibrillar material. This may represent endocytosed DS or other material brought into secondary lysosomes due to increased pinocytosis. Other organelles appear unaltered, and the cells are well-spread and healthy under these culture conditions.

Figure 24B 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 are similar to those which have been described in alevolar macrophages which have ingested surfactant (130).

B. Effects on Pinosome-Lysosome Fusion

There was a clear effect of polyanion treatment on phagosome-lysosome fusion. Several approaches were used to evaluate if this inhibition extended to pinosome-lysosome fusion as well. First, HRP was used as a fluid-phase pinocytic marker and its uptake and degradation by control, D or DS-treated macrophages was evaluated. As shown in Table VI, 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 hr pulse and its subsequent degradation followed, the $t_{1/2}$ of its enzymatic activity ranged from 6.4 to 8.7 hr, within the general range of that seen in previous studies (126). 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 which have not yet taken up very much DS which are able to fuse and degrade pinocytosed HRP. To evaluate heterogeneity in the lysosome population of these cells, thorotrast was

Table VI

UPTAKE AND DEGRADATION OF HRP BY MACROPHAGE CULTURES

Culture Conditions*	Uptake‡ (ng HRP/100 µg cell protein/2 hr)	$t_{1/2}$ of Inactivation of Cell-associated HRP Activity†
4 d cultures	125	8.5 hr
4 d dextran-treated cultures	111	8.7 hr
4 d dextran sulfate-treated cultures	143	6.4 hr.

*Standard culture conditions with the addition of 30 - 10 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 hr. Average data calculated for 2 hr uptake.

†Macrophage cultures were exposed to 1 mg/ml HRP for 1 hr, washed 4 times with MEM, and recultured in HRP-free medium. At 30 min, 4, 8, and 24 hr, triplicate cultures were washed, lysed in 0.1% TX-100, and assayed for enzymatic activity.

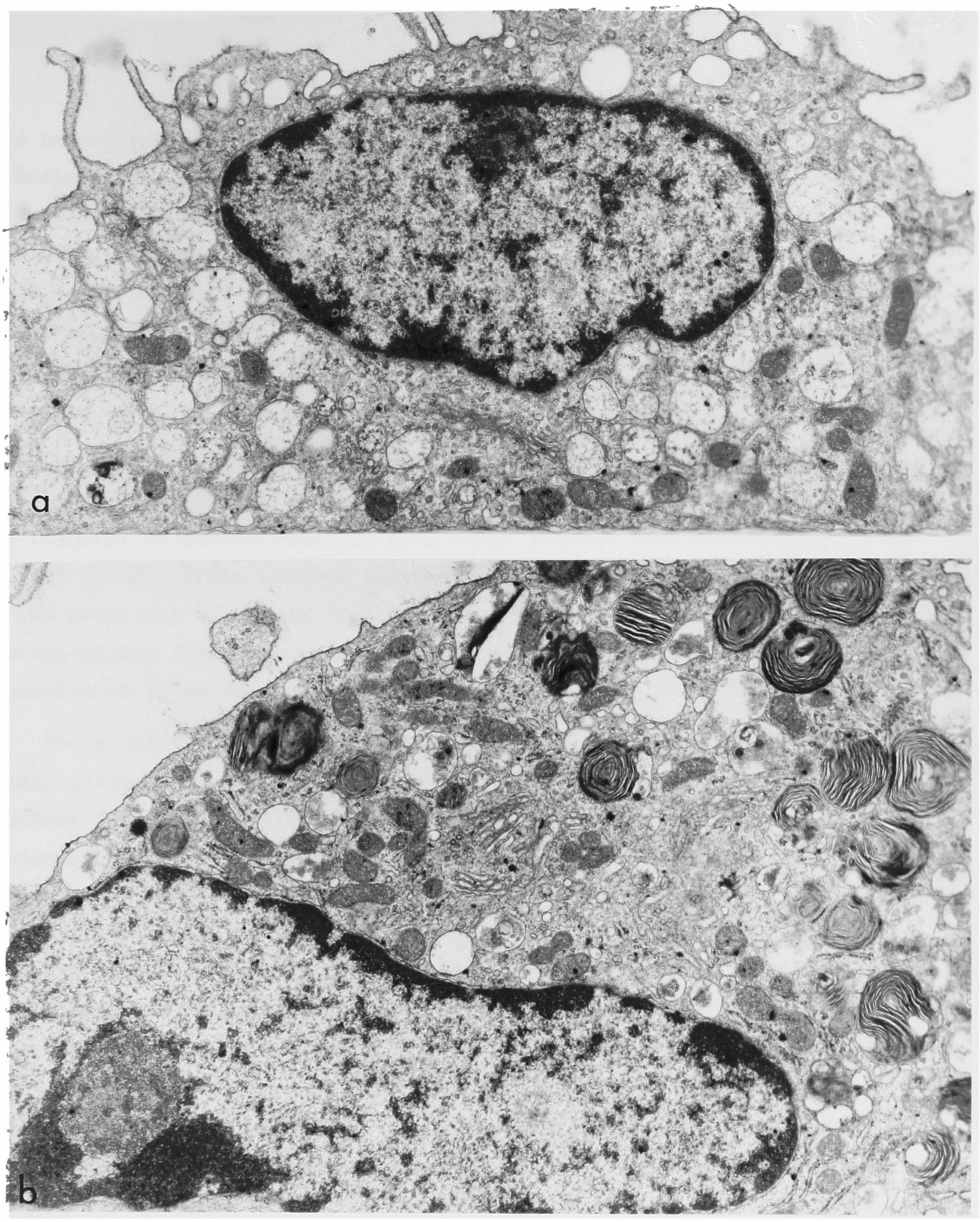


Figure 24. Electron micrographs of polyanion-treated macrophages.
A. Cell treated with 10 µg/ml DS as in Fig. 21. Note numerous large secondary lysosomes containing a fibrillar material. X 19,200.
B. Cell treated with 100 µg/ml Suramin as in Fig. 21. Again, large secondary lysosomes are seen and many contain whorls of membranous material (myelin figures). X 14,014.

used as a pinocytic marker for electron microscopy. After 4 d culture in medium or medium containing D or DS, cells were exposed to thorotrast in medium for 1 hr and fixed for EM. The distribution of pinocytosed thorotrast is shown in DS-treated cells (Fig. 25). Most 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 smaller vacuoles which might contain less DS. Thorotrast also labeled the large vacuoles in D-treated cells.

Characterization of Pinosome-Lysosome Fusion by Subcellular Fractionation Finally, subcellular fractionation was used to monitor the transfer of pinocytosed HRP to a large granule 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 dextran-filled lysosomes had been previously noted in subcellular fractionation of liver from dextran-injected rats (131).

Whole cell homogenates from either cell type were layered over linear metrizamide gradients, centrifuged at 10,000 x g for 90 min, and gradient fractions assayed as described in Materials and Methods. The lysosomal marker N-acetyl glucosaminidase (NAGase) sedimented in a band halfway down the gradient (Fig. 26). NAGase activity at the top of the gradient showed little latency, while that in the lysosomal band was 4-5-fold increased by TX-100. Most of the plasma membrane marker 5' nucleotidase (5'-NTase) remained at the top of the gradient. In another experiment, the lysosomal bands from D and DS gradients were collected with a bent Pasteur pipette, diluted in Hepes-saline, and layered over 20-50% sucrose step gradients. After centrifugation at 100 K x 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 the D lysosomes have an apparent density between 1.179 and 1.232, while the DS lysosomes have an apparent density greater than 1.232.

For comparison, velocity gradient sedimentation was performed on untreated cells, using 15-40% metrizamide gradients. As shown in Figure 27, untreated lysosomes are much less dense and far more heterogeneous.

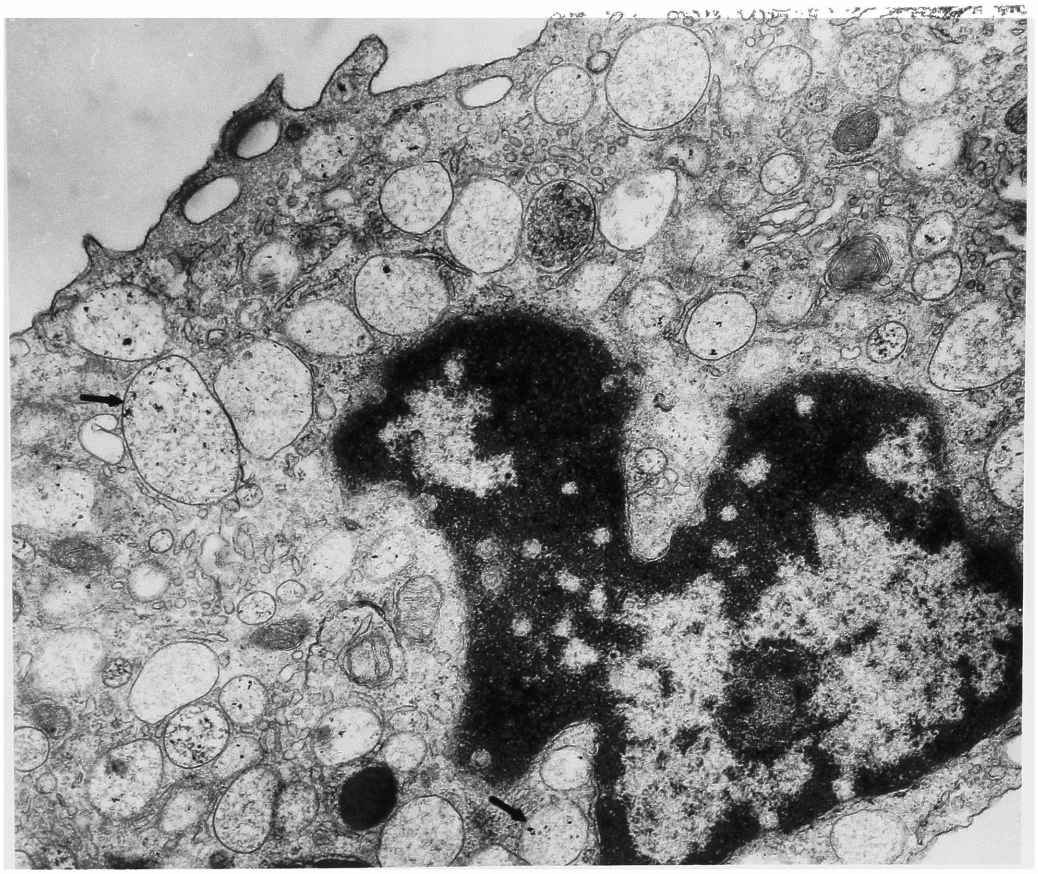


Figure 25. The intravacuolar distribution of thorotrast in dextran sulfate-treated cells. Cells were cultured 4 d in 20 μg/ml ^{3}H-DS, washed, and exposed to a 1:40 dilution of thorotrast in medium (v/v) for 1 hr. Cells were then washed and processed for EM. Electron-dense thorotrast is seen within the large DS-filled secondary lysosomes (arrows). X 19,500.

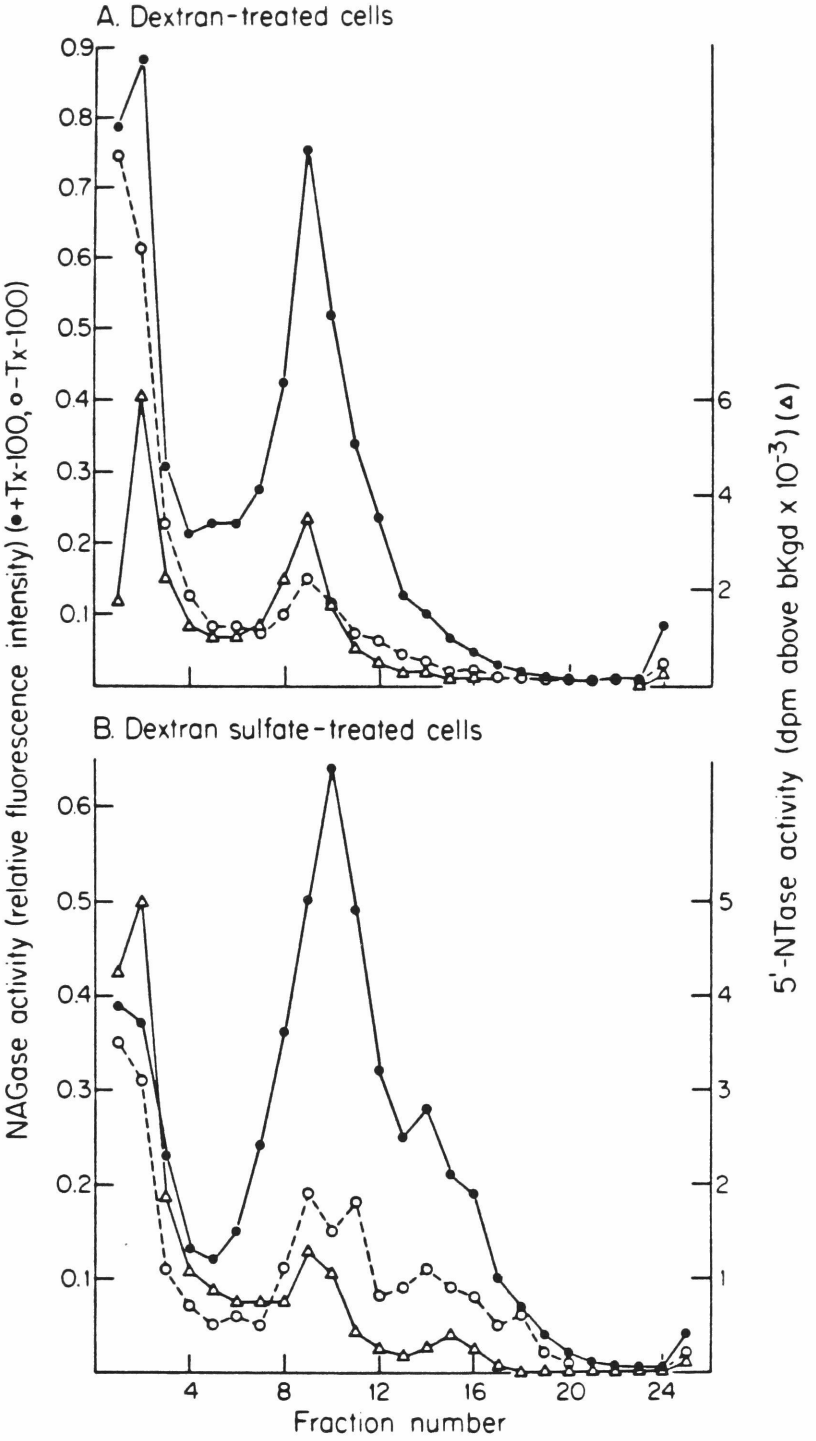


Figure 26. Separation of lysosome-enriched fractions from D or DS-treated cells. Sedimentation from left to right. Fractions 1 and 2 are load volume. 5'NTase was assayed in the presence of 0.05% Tx-100, and recovery ranged from 60 - 75%. Background was ∿ 400 dpm. NAGase was assayed with and without 0.1% Tx-100.

26A. Four day D cultures, 30 mg D/ml. 15-40% metrizamide gradient. Threefold latency of NAGase in the cell homogenate, and fivefold latency in fraction 9.

26B. Four day DS cultures, 10 μg DS/ml. 20-40% metrizamide gradient. Twofold latency of NAGase in the cell homogenate, and 4.3-fold latency in fraction 10.

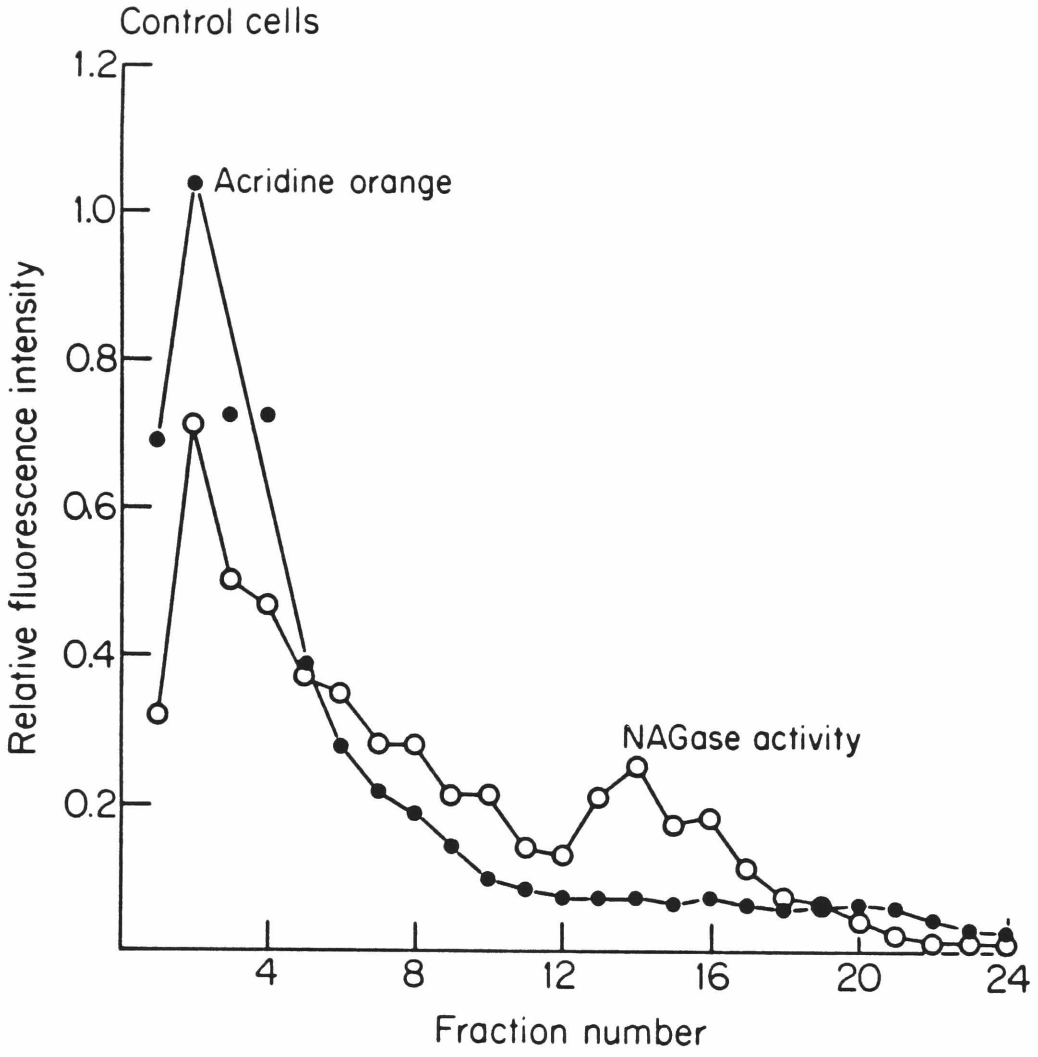


Figure 27. Subcellular fractionation of untreated cells. 15-40% metrizamide gradients showing the distribution of acridine label and NAGase activity in two separate experiments. Fractions 1 and 2 are load volume. Sedimentation from left to right. Twofold latency of NAGase activity in the homogenate. NAGase activity in the fractions assayed in the presence of 0.1% Tx-100.

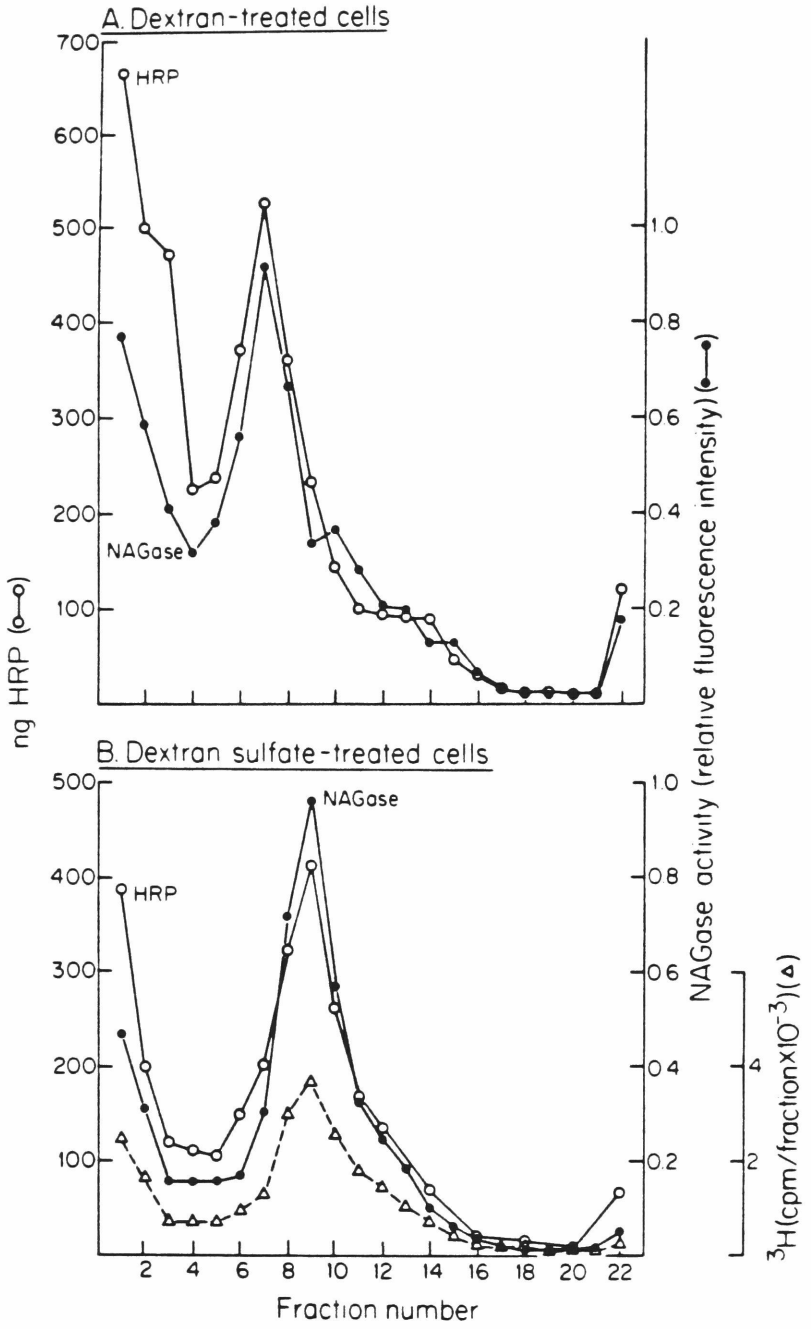


Figure 28. Transfer of a pinocytic marker into lysosomal fraction of D or DS-treated cells. Both sets of cells were labeled 60 min with 1 mg/ml HRP, washed, homogenized and run on gradients as described in Materials and Methods. Sedimentation from left to right. Fractions 1 and 2 are load volume. Both enzymes were assayed in the presence of TX-100. Latency of HRP was 2 to 2.5-fold in each homogenate.

28A. Four day D cultures, 10 mg D/ml. 15-40% metrizamide gradient.

28B. Four day DS cultures, 7.5 µg cold DS/ml and 2.5 µg ^{3}H-DS/ml. 20-40% metrizamide gradient.

Most of the NAGase activity does not enter the gradient under these centrifugation conditions. When cells were prelabeled with AO, AO-fluorescence comigrated with the NAGase peak (Fig. 27). Similar AO-labeling of D and DS cells also showed that 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. Figure 28 shows the distribution of HRP after 1 hr 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 ^{3}H-labeled 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 dextran sulfate in secondary lysosomes.

C. Other Properties of Polyanion-containing Lysosomes

In considering the possible mechanism of polyanion inhibition of P-L fusion, information derived from other membrane fusion systems was considered (reviewed in Introduction 1). From the fusion of Semliki Forest virus with liposomes, pH was known to be important in some membrane fusion events. Studies of exocytosis by freeze-fracture revealed the importance of membrane organization in fusion. The fusion of artificial membranes had been used to evaluate the role of lipid composition in fusion. These studies suggested several characteristics of D and DS cells for comparison as possible mediators of polyanion inhibition. The intralysosomal pH, freeze-fracture characterization and phospholipid analysis of D and DS cells will be discussed in turn.

Intralysosomal pH in D and DS Cells The technique of Okuma and Poole was used to measure the internal pH of secondary lysosomes in control and treated cells. Treated cells were cultured in D or DS-containing

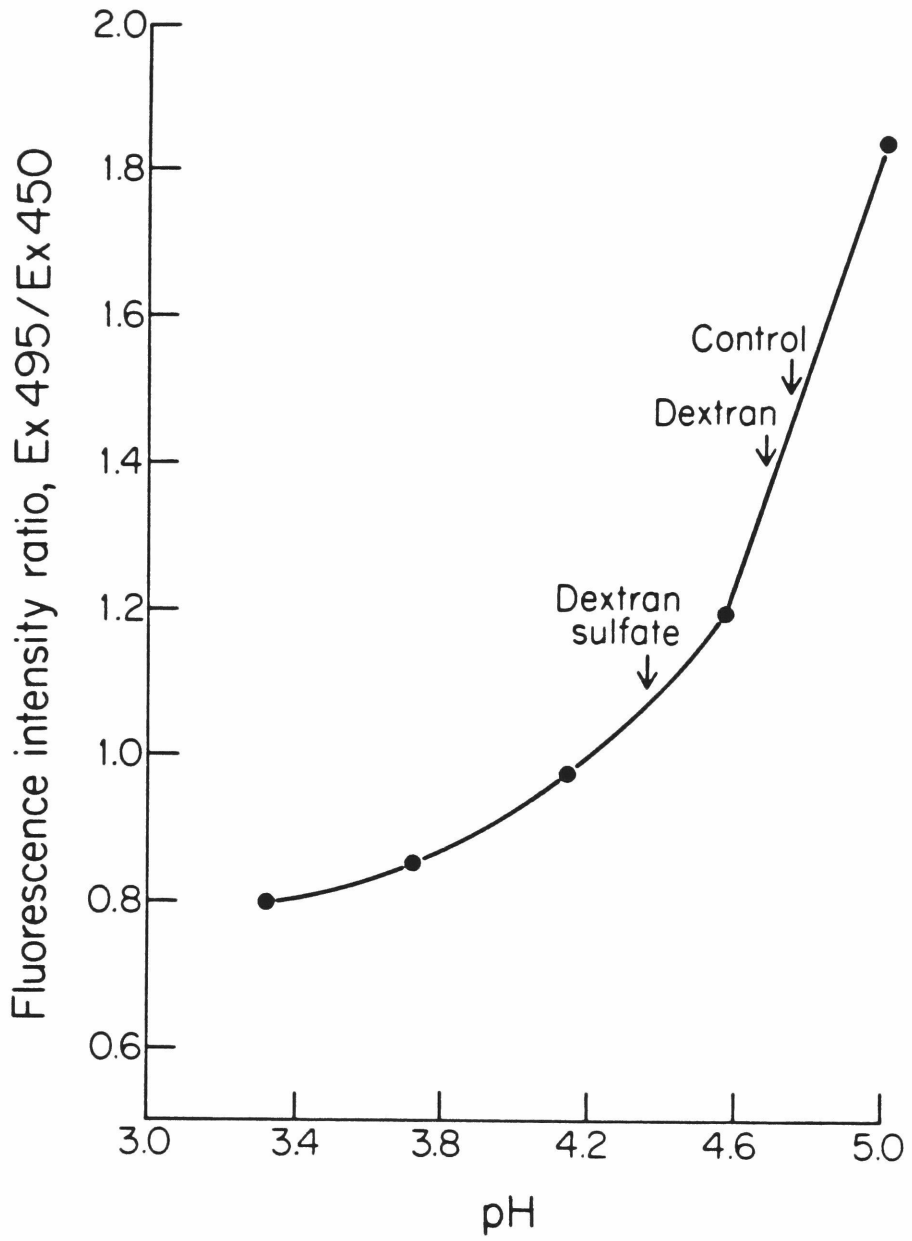


Figure 29. The pH of secondary lysosomes as estimated by a fluorescent probe. Shown is a standard curve relating the excitation spectrum of fluorescinated dextran to various pHs. All solutions contained 5 μg/ml fluorescinated dextran in 50 mM NaCl and 10 mM acetate buffer, at the indicated pH. The determined intralysosomal pH for each cell type is marked. Values are the mean of 5-7 determinations for each cell type. Background fluorescence of unlabeled cells was subtracted in each case.

medium for three days and then cultured 24 hr in these media plus 500 μg/ml fluorescinated dextran. These culture conditions were used to ensure that the fluorescent probe was labeling the treated lysosome population. The standard curve in Figure 29 shows the relationship between probe fluorescence and pH. As previously reported (63), 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 (5-7 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. Free DS or D (5 mg/ml) did not affect the excitation spectrum of the probe in solution. 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 (63), ammonium chloride was used to raise the pH of lysosomes in the three cell types. Cells were given particles to ingest, and after a 15 min ingestion period put into media containing 5 or 10 mM NH_4Cl for 40 min. After 10 min in NH_4Cl free media, cells were stained with AO and P-L fusion 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 the lysosomal pH is involved in DS-inhibition.

Freeze-fracture Analysis of D and DS Cells Freeze-fracture was used to evaluate possible differences in the intramembrane particle (IMP) distribution in the large lysosomes of D and DS cells. The increased size of these vacuoles made their identification and comparison easier. As shown in Figures 30 and 31, numerous IMPs are seen in the large vacuole membranes, but their density, size, and state of aggregation appear similar in the two cell types. No striking differences in membrane structure, as evaluated by freeze-fracture analysis, were seen between D and DS cells.

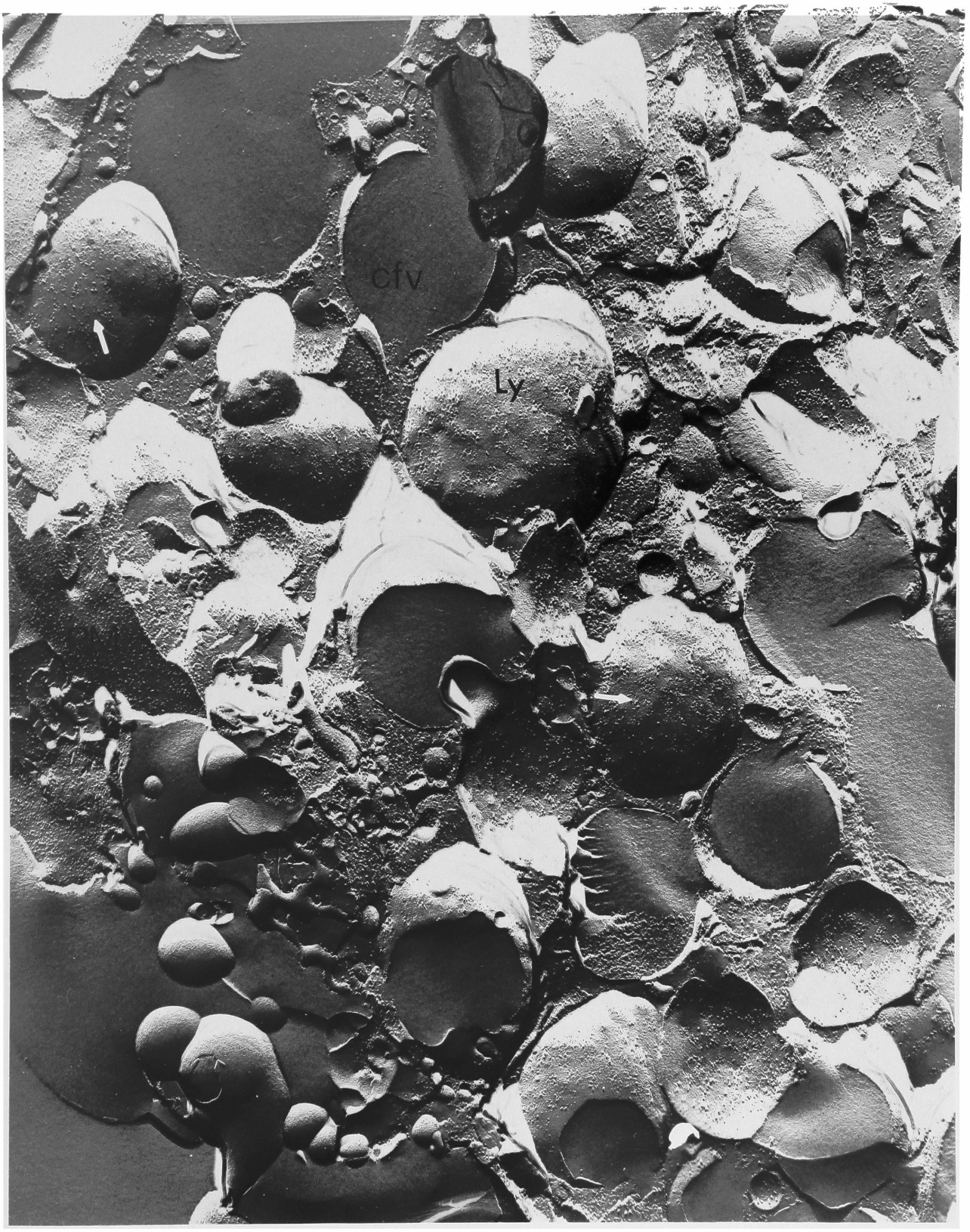


Figure 30. Freeze-fracture appearance of a four day cell treated with 30 mg/ml dextran. IMPs of the large secondary lysosomes (Ly) are somewhat aggregated (arrows). PM, plasma membrane. CFV, cross-fractured vacuole. X 30,000.

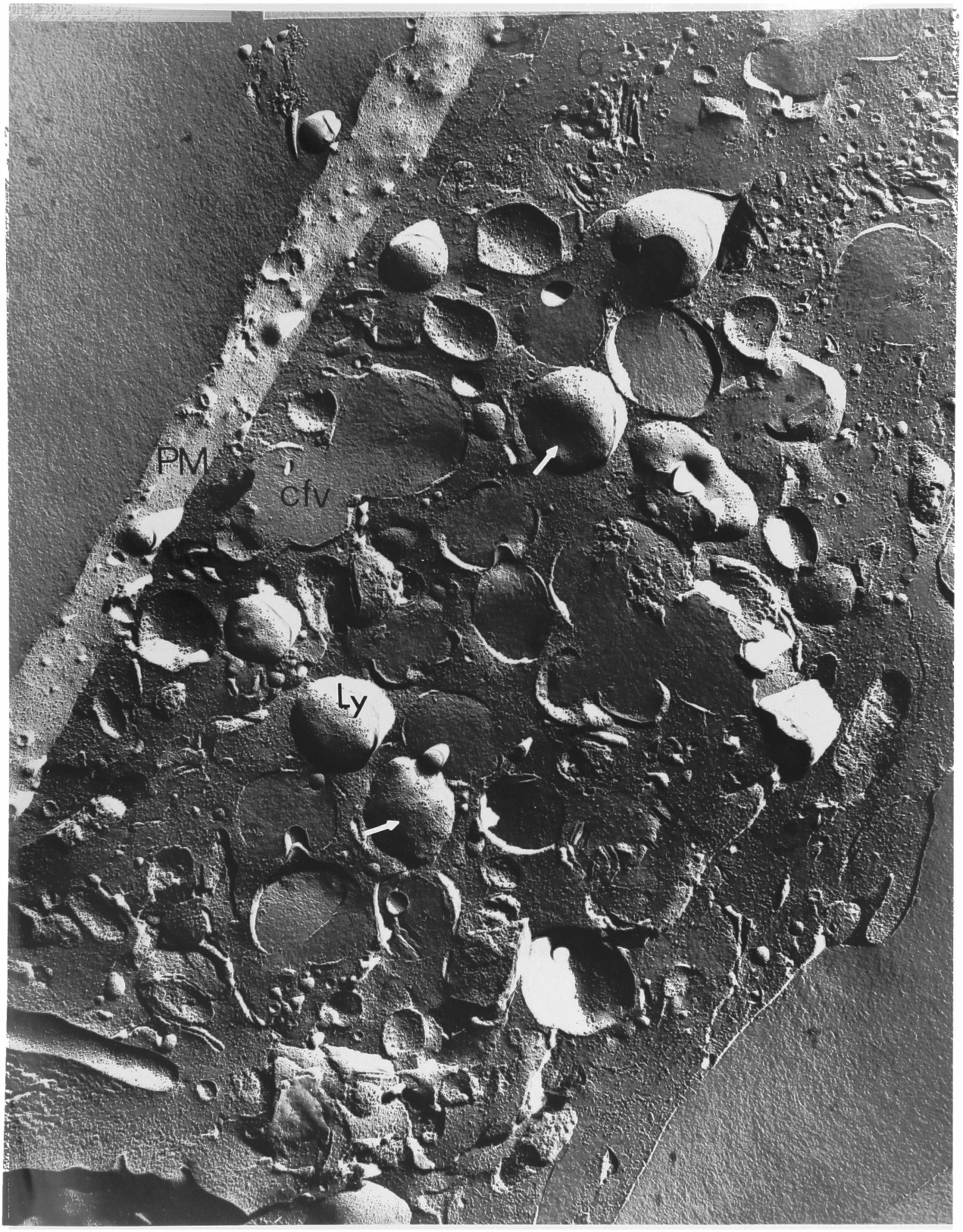


Figure 31. Freeze-fracture appearance of a four day cell treated with 10 µg/ml dextran sulfate. Again, IMPs of the large secondary lysosomes (Ly) are somewhat aggregated (arrows) but appear similar to those in Fig. 30. PM, plasma membrane. CFV, cross-fractured vacuole. G, golgi-region. X 30,000.

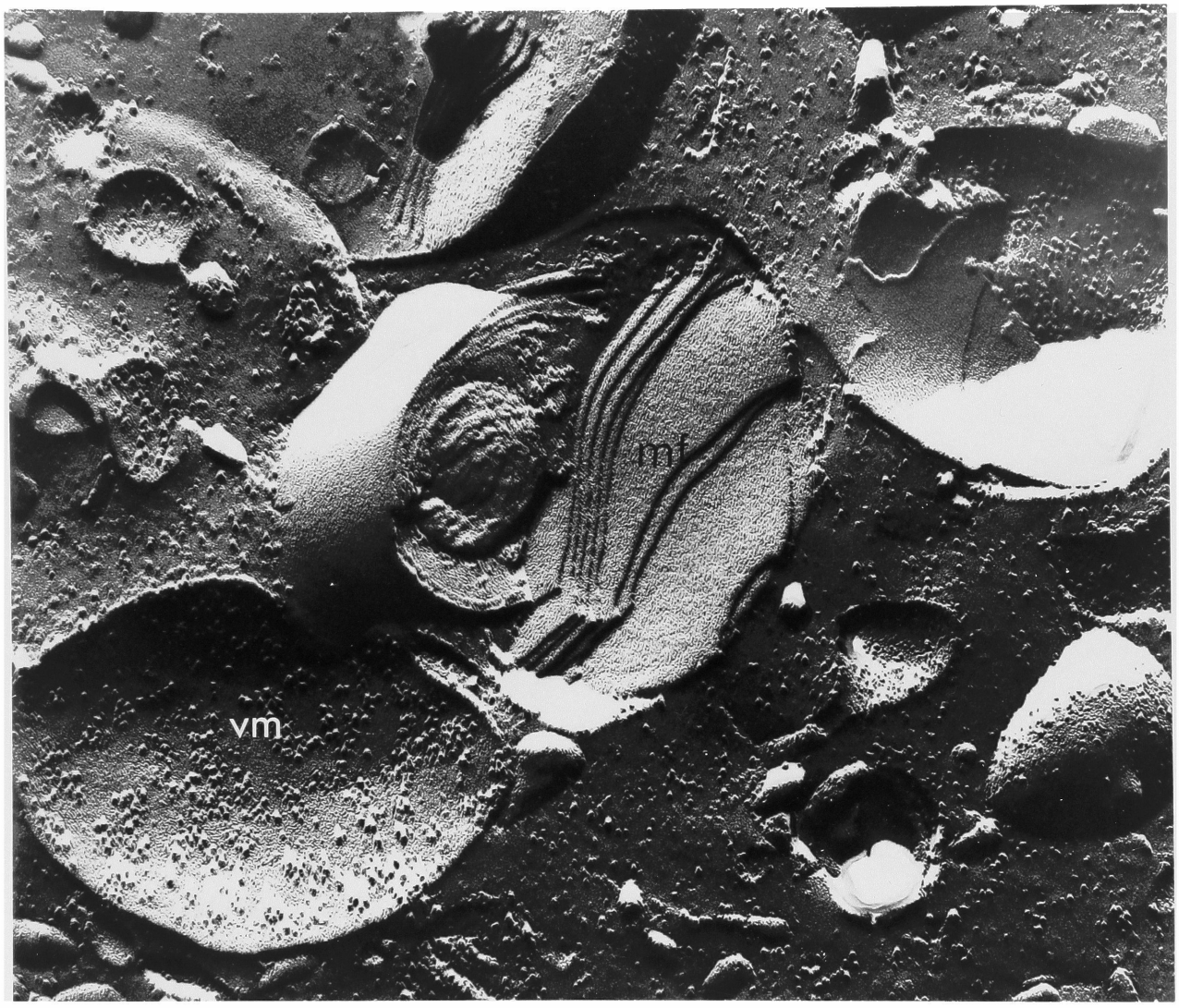


Figure 32. Freeze-fracture appearance of myelin figure in four day DS-culture. The large IMPs of the vacuole membrane (VM) are not seen in the myelin figure membrane (MF). X 78,000.

Table VII

PHOSPHOLIPID COMPOSITION OF MACROPHAGES

Culture Conditions‡	Phospholipid*					
	SM	PS	PI	PC	PE	DPG+PG
	Mol % Lipid Phosphorus					
1 day cells	1.0	7.7	5.9	51.3	31.5	2.6
4 day cells	1.8	8.5	5.7	44.7	33.5	5.8
suramin treated 4 day cells	6.1	5.0	8.1	52.1	26.5	2.3
dextran-treated 4 day cells	< 1	10.5	5.7	42	35.5	6.2
dextran sulfate-treated 4 day cells	< 1	8.4	4.9	40	37.4	5.1

*SM, sphingomyelin; PS, phosphatidyserine; PI, phosphatidylinositol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; DPG, diphosphatidylglycerol (cardiolipin); PG, phosphatidylglycerol.

‡Standard culture conditions with the addition of 200 μg/ml suramin, 30 mg/ml D, or 10 μg/ml DS where indicated.

Figure 32 shows the freeze-fracture appearance of a myelin figure seen in a DS-treated cell preparation. It's interesting that the IMP content of the whorls of membranous material appears greatly decreased compared to the surrounding vacuole membrane. This may represent the more rapid digestion of protein components over lipid components in this membrane.

Phospholipid Analysis of Polyanion-treated Cells The interaction of polyanions with membranes in treated cells might cause alterations in the lipid composition which could affect subsequent membrane fusion. The phospholipid compositions of total cell lipids from various cultures were compared (Table VII). Phospholipid compositions were similar in one and four d cultures which have different fusion rates. Phospholipid compositions were also similar in D and DS-treated cultures which display opposing patterns of P-L fusion. Although these data are for total cell lipid, the large amount of membrane in secondary lysosomes of these cells makes it likely that a difference in the lysosomal pool would be reflected in the total cell lipid. The phospholipid composition of Suramin-treated cells was altered in that the mole % sphingomyelin was increased from the usual 1-1.8% to 6%. This increase probably reflects the amount of lipid in the previously described "myelin figures" which were very plentiful in suramin-treated cells. Sphingomyelin is one of the phospholipids most resistant to deacylation by lysosomal enzymes from rat liver (132) and thus may be relatively increased within suramin-treated lysosomes.

D. Uptake Requirements for Inhibition

It appeared from the time course of inhibition by polyanions and from the staining with toluidine blue that these compounds were gradually endocytosed 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 K) as

estimated by gel filtration, and gave comparable inhibition to 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 four day culture period. As shown in Figure 33, 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 pinocytosis by polyanion as has been previously described. The inset in Figure 33 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 hr 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 section.

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 environmental molecules which 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 (118,133), 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 cell-association of large amounts of complex (Fig. 34A). Even when the complex was kept in suspension by rotation of the culture dish during the uptake at 37°C, background coverslip 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. 34 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.

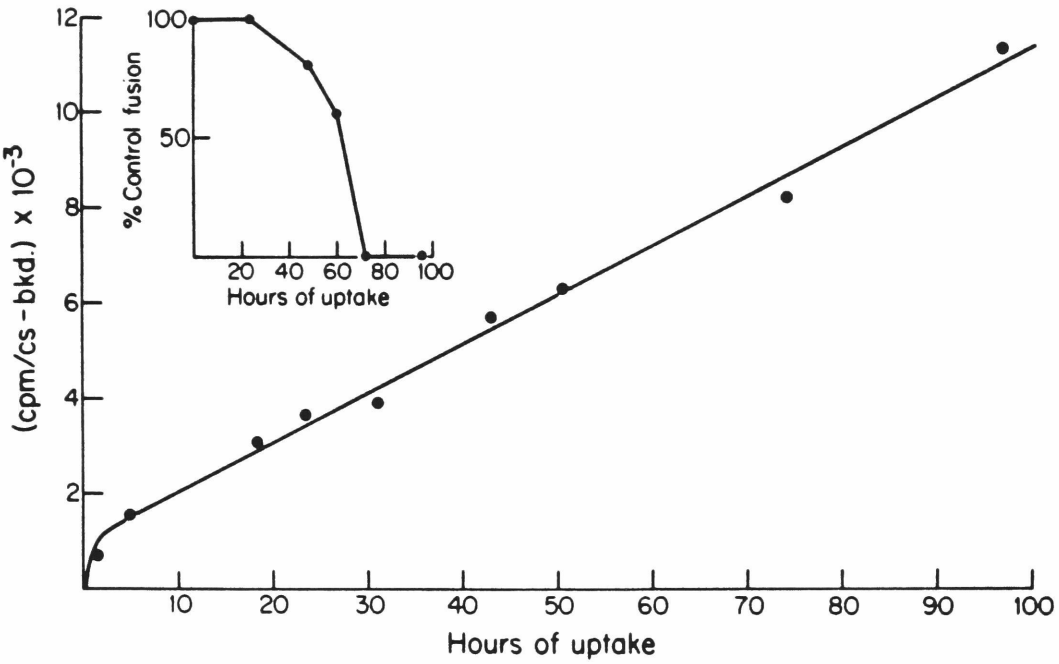


Figure 33. Uptake of soluble ^{3}H-dextran sulfate. Shown are the average cpm of triplicate coverslip (cs) cultures plated at 5 x 10^{5} peritoneal cells/cs. Explanted cells were adhered for 2 hr, washed and placed into medium containing 20 µg/ml ^{3}H-DS. Cells were given fresh medium each day. Background is for cells exposed to ^{3}H-DS 2 hr on ice and was 200 cpm/l. Inset shows the time course of fusion inhibition by 20 µg/ml ^{3}H-DS-treated cells.

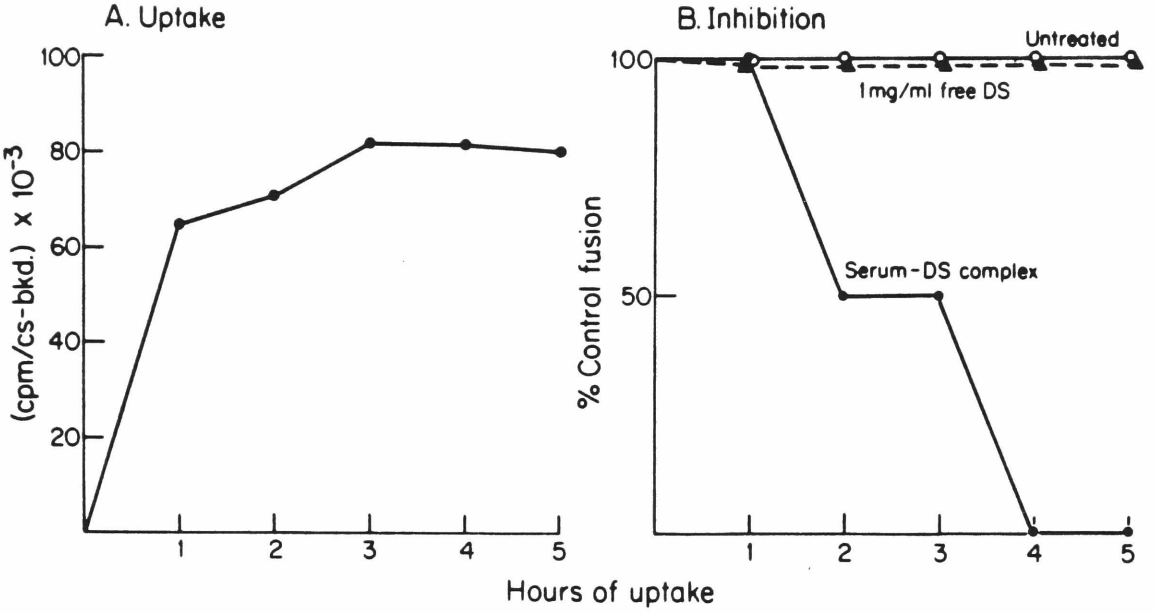


Figure 34. Uptake and fusion-inhibition by ^{3}H-DS-serum complexes.

34A. Uptake of 100 µg/ml ^{3}H-DS as serum complex. Shown are the average cpm of triplicate cs cultures plated at 5 x 10^{5} peritoneal cells/cs, and cultured 4 d in normal medium. On day 4, coverslips were exposed to serum complex at 37°C with shaking. Background is for cells exposed to complex 1 hr on ice and was 12 x 10^{3} cpm/cs.

34B. 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 hr after particle ingestion for each cell type and time point.

Under these uptake conditions, inhibition could be achieved within 3-4 hr of uptake (Fig. 34B). A tenfold higher concentration of soluble DS (Fig. 34B) did not cause inhibition within 5 hr. The fine particulate suspension of lipoprotein-DS complex enabled cells to take up large quantities more rapidly. Prolonged incubation is not a requirement for inhibition.

Uptake of Purified LDL Since the usual DS-treated cells are cultured in serum-containing medium and DS has been reported to increase the uptake of LDL by macrophages (129), we then asked if LDL was mediating the inhibition. Macrophages do not have a receptor for LDL but have been shown to avidly take up acetylated LDL (134). Figure 35 shows the electrophoretic mobility of purified human LDL before and after acetylation. One major oil red O staining band was seen in the preparations before and after acetylation. The LDL band seen in lane c was absent from human serum after dextran sulfate precipitation. This LDL band had a more anodal migration after acetylation (lanes a and b).

When macrophages were exposed to 1 mg/ml acetyl LDL for 24 hr of culture, cells took up large amounts of the acetylated lipoprotein (Fig. 36) and stained heavily with oil red O. When P-L fusion in these cells was compared to untreated cultures, however, no inhibition was seen (Table VIII). Purified LDL did cause inhibition when it was complexed with DS, and again, 4 hr of uptake was sufficient for inhibition (Table VIII). 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 to the morphology of cells treated with soluble polyanion (Fig. 24). As shown in Figure 37A, 30 min after exposure to complex, macrophages have amorphous complex on the cell surface and some has been internalized into large cytoplasmic vacuoles. Complex is probably internalized pinocytically in both the fluid phase and adsorbed to the cell membrane. In addition (see inset Fig. 37A) larger aggregates appear to be phagocytized by the cells.

After longer periods of uptake (Fig. 37B), the cytoplasm has become filled with vacuoles containing the complex suspension and many vacuoles

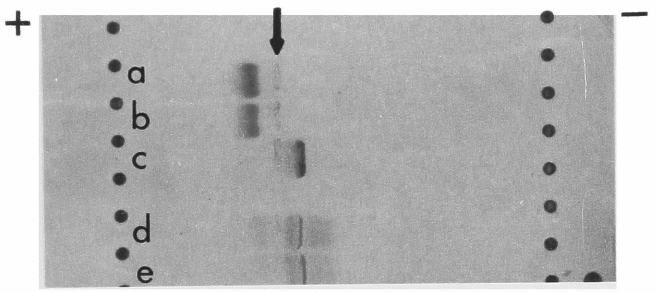


Figure 35. Lanes a & b - 1.0 λ of purified LDL preparation after acetylation. Lane c - 1.0 λ of purified LDL fraction from 30 ml human serum. Lanes e & f - 1.0 λ whole human serum. Arrow marks application point. In lanes e & f, order is (from left to right) albumin, α_1 or HDL, α_2 or VLDL, β or LDL, γ globulins.

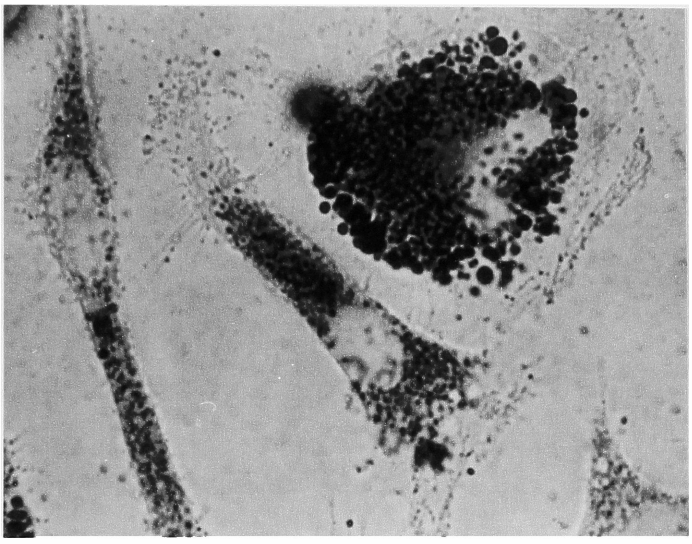


Figure 36. Bright field micrograph of oil red O stained cells. 24 hr macrophage cultures were exposed to 1 mg/ml acetyl LDL in medium with serum for 24 hr. Cells were then fixed and stained with oil red O. X 1680.

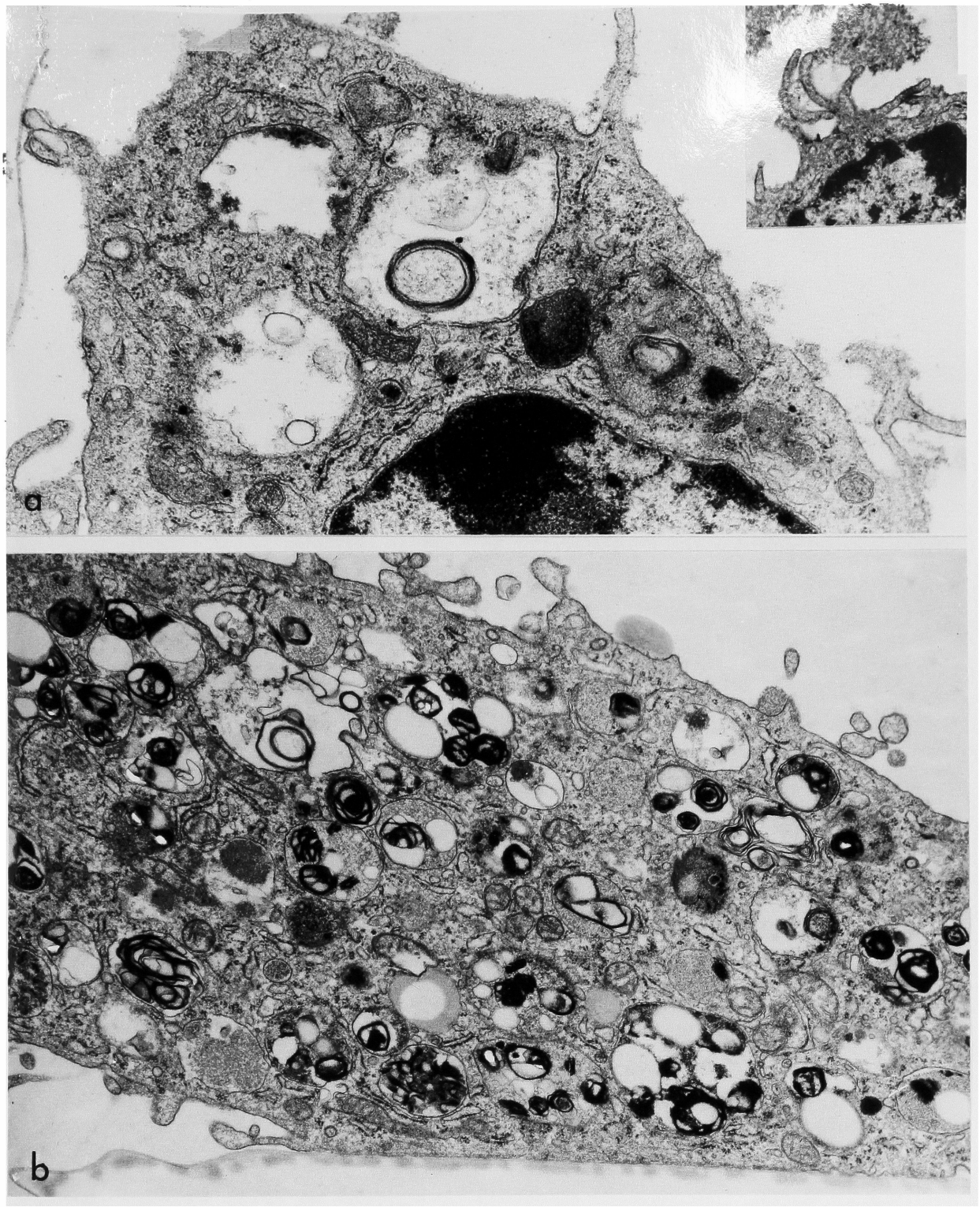


Figure 37. Electron microscopic appearance of cells given DS-serum complexes to ingest. Four day macrophage cultures in 35 mm dishes were given 2 ml of 50% complex (see Materials and Methods) for the indicated times before washing, fixation, and EM processing.

37A. Cells exposed to complex for 30 min. Amorphous complex is seen in large intracytoplasmic vacuoles as well as adsorbed to the cell surface. X 27,300. Inset shows cell apparently ingesting larger aggregate of complex. X 14,625.

37B. Cell exposed to complex for 3.5 hr. Some apparent lysosomal digestion of complexes has occurred. Vacuoles now contain amorphous complex, and also lipid droplets and whorls of membranous material. X 17,875.

Table VIII

THE EFFECT OF LDL UPTAKE ON PHAGOSOME-LYSOSOME FUSION

Treatment of 2 Day Cells	% Control Fusion 60 Min After Yeast Ingestion
Untreated	100
24 hr exposure to 1 mg/ml acetyl LDL	100
4 hr 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.

show evidence of lysosomal digestion of the complex. These vacuoles now contain lipid droplets and whorls of membranous material. Complex uptake thus appears similar to both the fibrillar material seen in the secondary lysosomes of DS-treated cells (Fig. 24A) and to the myelin figures prevalent in suramin cells (Fig. 24B).

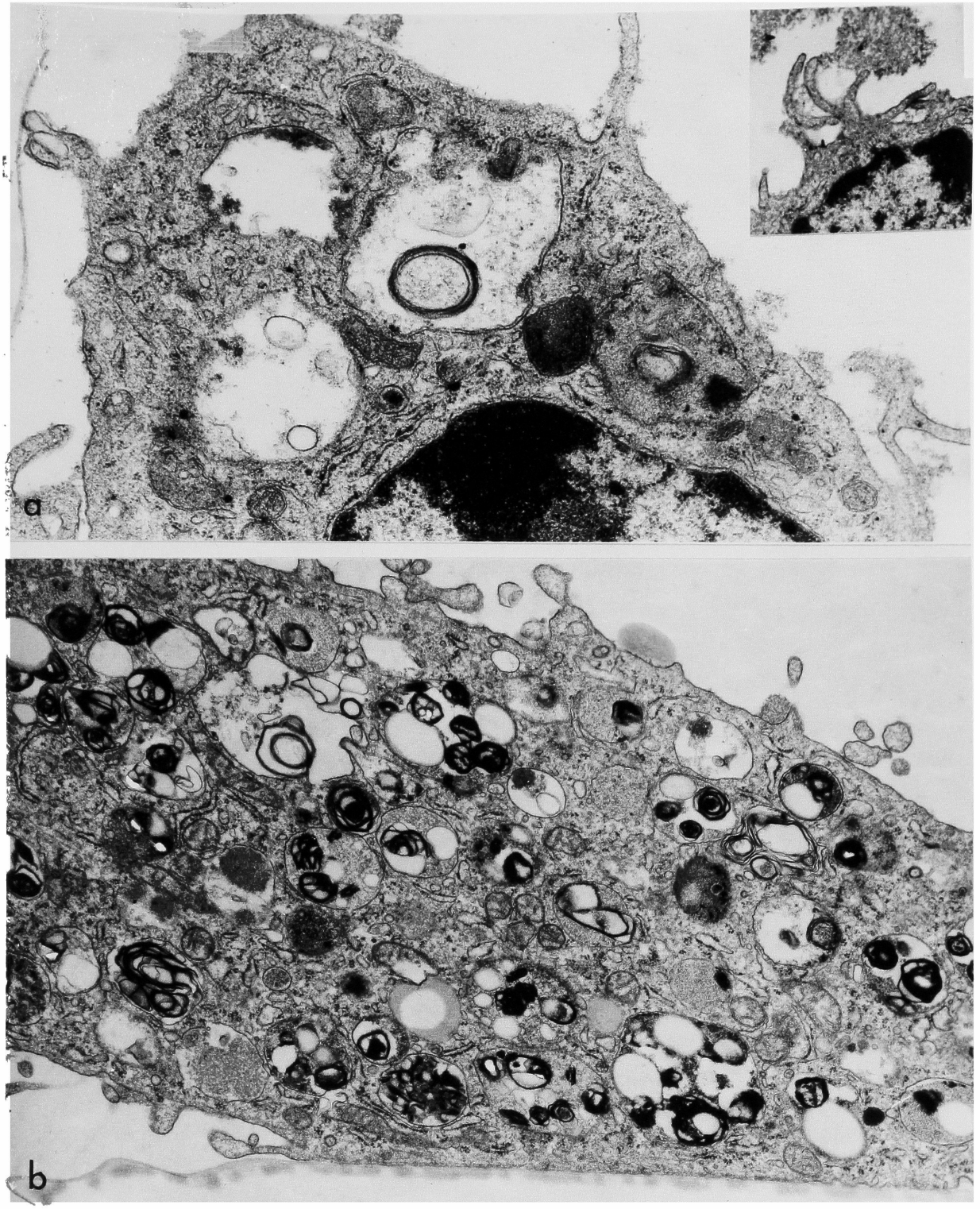


Figure 37. Electron microscopic appearance of cells given DS-serum complexes to ingest. Four day macrophage cultures in 35 mm dishes were given 2 ml of 50% complex (see Materials and Methods) for the indicated times before washing, fixation, and EM processing.

37A. Cells exposed to complex for 30 min. Amorphous complex is seen in large intracytoplasmic vacuoles as well as adsorbed to the cell surface. X 27,300. Inset shows cell apparently ingesting larger aggregate of complex. X 14,625.

37B. Cell exposed to complex for 3.5 hr. Some apparent lysosomal digestion of complexes has occurred. Vacuoles now contain amorphous complex, and also lipid droplets and whorls of membranous material. X 17,875.

XIV. DISCUSSION

A. Other Studies on Polyanion Inhibition

As previously discussed, other investigators had reported on the inhibition of P-L fusion by polyanions. Suramin (32) and a sulfatide from *M. tuberculosis* (35) were the polyanions first studied. In several later reports from Hart and his colleagues, inhibition by poly-D-glutamate was discussed. This polyanion was found to be inhibitory at concentrations of 100 μg/ml after 5 d of culture (34). Also, treatment of these inhibited cells with chloroquine was reported to reverse the inhibition. It is difficult to evaluate this reversal process, since most of the data were obtained from the fluorescence assay (34,110). The AO-assay is itself affected by weak bases such as chloroquine which compete out the fluorescent marker.

It is thus of note that when reversal was assayed by electron-microscopy of ferritin-labeled preparations, only about 10% of the total phagosome profiles contained the marker (34). In one experiment, we evaluated P-L fusion in 1 d cells treated with 30 μg/ml chloroquine for 40 min, using HRP as a secondary lysosome marker. No significant increase in the usual low rate of P-L fusion was observed.

Some controversy does exist in the literature concerning inhibition by polyanions. Pesanti (135) did not find inhibition of P-L fusion in suramin-treated cells as assayed by electron microscopy. Goren (136) also could not demonstrate inhibition by electron microscopy, and raised doubts about the use of AO in fusion assays of polyanion-treated cells which might bind the dye by ionic interactions. Results reported here confirm inhibition by Suramin and DS using a sensitive EM assay and corroborate the inhibition observed by the AO assay.

B. Effects on Pinosome-Lysosome Fusion

By three different experimental approaches, pinosome-lysosome fusion was not altered in polyanion-treated cells. As discussed in Part Two of this thesis, inhibition by Con A also differs between these two vesicle types. In the latter case, Con A is acting from within the lumen of the pinosome, while polyanions appear to act from within the secondary lysosome. Since pinosome fusion is unaffected, pinocytic vesicles containing soluble DS or DS-LDL complexes 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 (76). The triggering of phagocytosis by receptor-binding may involve elements of the actomyosin cytoskeleton, whereas this may not occur in pinocytosis. Pinocytic uptake may require clathrin-membrane interactions in coated vesicle formation (137). These presumably trans-membrane interactions are one apparent difference in the two types of endocytosis. 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 phagocytosis and pinocytosis cause the internalization of representative samples of plasma membrane polypeptides (138,139). 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 (71). Both processes

are inhibited in cultures supplemented with trans-18:1 or 19:0, but the Eact 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, differences in lipid microdomains could influence fusion of the two different vesicle types.

Vesicle size is another variable which theoretically could influence fusion (67). Average pinosome size of resident macrophages was estimated to be ∿ 0.2 μm (64), while the diameter of the yeast test particles used in this study of P-L fusion is ∿3.8 μm. 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.

C. Uptake of DS and DS-LDL Complexes

The uptake of soluble DS was linear for 4 d of culture. When used in lipoprotein purification, DS precipitates mainly LDL and VLDL (118,133). This interaction appears to be mediated both by protein and lipid components of the lipoprotein (140,141). DS also appears to interact with lipoproteins in culture medium to increase their uptake by macrophages (129), acting apparently at a different receptor than that which mediates acetyl-LDL uptake. In the inhibition of P-L fusion, the lipoprotein seems to act merely as a DS carrier and has no effect when endocytosed in large quantities in the absence of DS. Increased uptake of lipoprotein may explain the myelin figures observed in polyanion-treated cells since similar structures are seen in cells given DS-LDL complexes. Alternatively, suramin has been found to inhibit several of the enzymes necessary for the metabolism of glycosaminoglycans, and leads to their intralysosomal accumulation, similar to the situation in mucopolysaccharidoses (142). The altered lysosomes in polyanion-treated

cells could thus be a consequence of increased uptake of exogenous substrate, decreased lysosomal digestion of cellular substrates, or both.

The Amount of DS in the Lysosomal Pool From the uptake studies of soluble DS, at 76 hr of culture an average of 4.206×10^{2} cpm were cell-associated per μg protein, or 3.365×10^{-2} cpm/cell (using the figure of 80 μg protein/10^{6} cells [64,65]). At the specific activity in this experiment of 3.801×10^{4} cpm/μg, this is 8.85×10^{-13} g DS/cell, of which 10% is sulphur or 8.85×10^{-14} g S/cell. Thus, each cell contains 2.76×10^{-15} mole S/cell or that amount of anionic equivalents (for each S, one OSO_3^-). Assuming a volume for the secondary lysosome compartment of 10 μM^3 (64) or 10^{-14} ℓ, this is a concentration of 0.276 mole/ℓ. Using these calculations, and making several major assumptions, this amount of negative charge could generate a pH of less than 1 (or ∿ 0.56). The assumptions are: (1) That the lysosomal pool in DS cells is the same size as that of untreated macrophages (although a tenfold increase would still result in a pH of 1.56). (2) That DS stored in lysosomes still contains the same number of sulfate residues. We do know that little wash-out of the tritium-label occurs after several days of culture. (3) That each of the resultant negative charges in the lysosome results in the accumulation of a proton as counterion via a Donnan equilibrium. The pKa of the SO_4^- groups is reported to be less than 2 (143).

There does appear to be sufficient negative charge in these lysosomes to explain the pH difference seen. The cell may compensate for the large excess of negative charge and maintain the intralysosomal pH close to the usual value of ∿ 4.7. The pH of DS lysosomes could be lower than our estimate due to the intrinsic limitations of the pH assay as previously discussed (63).

The surprisingly high concentration of DS which 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 6 sulfonate groups per molecule, if an ionic interaction occurs

it still has the potential for cross-linking. It is not known whether a "monovalent" polyanion could cause inhibition. Uptake studies have only been done for the 500,000 MW 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. All of the polyanions which inhibited P-L fuison were also found to cause precipitation when added to serum with calcium. The supernate from this precipitation no longer contained the LDL band upon electrophoresis (Kielian and Cohn, unpublished observations).

D. The Mechanism of Polyanion Inhibition

In conclusion, it appears that lysosomal pH, total cell lipid composition, and membrane structure as assayed by freeze-fracture do not show differences between D-treated and DS-inhibited cells.

From studies of lipoprotein-DS interactions, it is known that DS forms both soluble and insoluble LDL complexes (140, 144). Several types of interactions are involved. 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 (140,144). In the presence of divalent metal ions, both positive and negative charges were involved as the cation acted as a "bridge" (140). 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 (141).

Given the known proclivity of DS to interact with phospholipids and proteins, with or without calcium or other "bridging" cations, it is likely that DS interacts with membrane components of the lysosome. This interaction could be extensive enough to modify the lateral mobility of the membrane, affecting both protein mobility and observed lipid fluidity. Using the lysosome-enriched fractions from D and DS cells, prepared as described in this thesis, we are currently exploring these interactions. α- and β-parinaric acid are fluorescent tetraene fatty acids which resemble naturally-occurring fatty acids of the plasma membrane (145,146). Using these two probes, fluorescence polarization measurements of D and DS lysosomes will give an estimate of any relative differences in the rigidity of the lysosomal membranes. The differential partitioning of α- vs. β-parinric acid into solid and fluid phase lipid (147) makes comparison of the two probes of interest. These current studies will enable the biophysical comparison of two functionally different intracellular membranes.

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