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Endocytosis of liposomes by macrophages: binding, acidification and leakage of liposomes monitored by a new fluorescence assay

David L. Daleke [1,*], Keelung Hong [1] and Demetrios Papahadjopoulos [1,2]

[1] *Cancer Research Institute and* [2] *Department of Pharmacology, University of California, San Francisco, CA (U.S.A.)*

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The interaction of liposomes with macrophage cells was monitored by a new fluorescence method (Hong, K., Straubinger, R.M. and Papahadjopoulos, D., J. Cell Biol. 103 (1986) 56a) that allows for the simultaneous monitoring of binding, endocytosis, acidification and leakage. Profound differences in uptake, cell surface-induced leakage and leakage subsequent to endocytosis were measured in liposomes of varying composition. Pyranine (1-hydroxypyrene-3,6,8-trisulfonic acid, HPTS), a highly fluorescent, water-soluble, pH sensitive dye, was encapsulated at high concentration into the lumen of large unilamellar vesicles. HPTS exhibits two major fluorescence excitation maxima (403 and 450 nm) which have a complementary pH dependence in the range 5–9: the peak at 403 nm is maximal at low pH values while the peak at 450 nm is maximal at high pH values. The intra- and extracellular distribution of liposomes and their approximate pH was observed by fluorescence microscopy using appropriate excitation and barrier filters. The uptake of liposomal contents by cells and their subsequent exposure to acidified endosomes or secondary lysosomes was monitored by spectrofluorometry via alterations in the fluorescence excitation maxima. The concentration of dye associated with cells was determined by measuring fluorescence at a pH independent point (413 nm). The average pH of cell-associated dye was determined by normalizing peak fluorescene intensities (403 nm and 450 nm) to fluorescence at 413 nm and comparing these ratios to a standard curve. HPTS-containing liposomes bound to and were acidified by a cultured murine macrophage cell line (J774) with a $t_{1/2}$ of 15–20 min. The acidification of liposomes exhibited biphasic kinetics and 50–80% of the liposomes reached an average pH lower than 6 within 2 h. A liposomal lipid marker exhibited a rate of uptake similar to HPTS, however the lipid component selectively accumulated in the cell; after an initial rapid release of liposome contents, 2.5-fold more lipid marker than liposomal contents remained associated with the cells after 5 h. Coating haptenated liposomes with antibody protected liposomes from the initial release. The leakage of liposomal contents was monitored by co-encapsulating HPTS and *p*-xylene-bis-pyridinium bromide, a fluorescence quencher, into liposomes. The time course of dilution of liposome contents, detected as an increase in HPTS fluorescence, was coincident with the acidification of HPTS. The rate and extent of uptake of neutral and negatively charged liposomes was similar; however, liposomes opsonized with antibody were incorporated at a higher rate (2.9-fold) and to a greater extent (3.4-fold). In addition, the rate and extent of incorporation of liposome encapsulated HPTS was dependent on temperature and the metabolic state of the cell, consistent with uptake of liposomes by endocytosis. The use of HPTS allowed accurate and simultaneous quantitation of liposome uptake, acidification, cell-induced leakage of liposomes, and regurgitation of liposome contents. In addition to cell-surface induced leakage, liposomes leaked extensively during endocytosis coincident with acidification; half of the cell-induced dilution of liposome contents was accounted for by leakage at the cell surface, while the remainder occurred coincident with acidification. Liposome contents labeled the aqueous space of endosomes and lysosomes and were regurgitated rapidly as liposomal lipid accumulated selectively. Opsonization of liposomes, to induce Fc-mediated endocytosis, afforded protection to the initial dilution of liposome contents, but not the rate of leakage, after endocytosis. Implications of these studies for the use of liposomes as drug delivery vehicles are discussed.

Abbreviations: HPTS, 1-hydroxypyrene-3,6,8-trisulfonic acid; PC, egg phosphatidylcholine; PS, bovine brain phosphatidylserine; [^{3}H]DPPC, di-[9,10-^{3}H]palmitoylphosphatidylcholine; DNP-PE, *N*-dinitrophenyl phosphatidylethanolamine; DPX, *p*-xylene-bis-pyridinium bromide; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; FD, fluorescein isothiocyanate conjugated dextran; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; λ_{ex}, excitation wavelength; λ_{em}, emission wavelength.

* Correspondence: D.L. Daleke (present address): Department of Chemistry, Indiana University, Bloomington, IN 47405, U.S.A.

Introduction

The endocytotic capabilities of macrophages have been exploited in the design of systems for the enhanced delivery of drugs, activating agents, and antigens. Liposomes provide an ideal vehicle for the delivery of macromolecules to macrophages. Once injected into the circulatory system, liposomes are removed rapidly by macrophages of the reticuloendothelial system [1–3] resulting in specific, passive delivery of liposomes and their contents. Liposome encapsulated macrophage activating agents such as *N*-acetyl-muramyl-L-alanyl-D-isoglutamine (muramyl dipeptide) and a lipophilic derivative of muramyl dipeptide induce significant tumoricidal activity in vitro [4] or in vivo [5–7]. Other lymphokines encapsulated in liposomes similarly induce an in vivo tumoricidal response [8]. Antibiotics encapsulated in liposomes are more effective than unecapsulated drug in the treatment of several intracellular infections of macrophages [9–11]. Finally, liposomes are effective adjuvants for vaccine production [12,13].

The use of liposomes as a delivery system offers several advantages over unencapsulated compounds. Liposomes act as a depot, increasing the effective time of administration of drugs [14,15] and reducing nonspecific toxicity [10,11,16–18]. Encapsulation of labile compounds in liposomes offers protection against their clearance from the bloodstream or degradation before delivery to the target site. Furthermore, by conjugation to appropriate ligands, liposomes can be targeted to specific cells [19–22].

However, the general use of liposomes as a drug delivery system has been hindered by several problems. Among these are leakage of liposomal contents mediated by serum proteins [2,23–26] and cells [27–29], slow extravisation of liposomes from the bloodstream to target cells [30,31] and, if the target cells are not macrophages, clearance of liposomes from the vasculature by cells of the reticuloendothelial system [1–3]. Some of these problems may be circumvented by utilizing the natural targeting to cells of the reticuloendothelial system [5,32] or by developing liposomes which avoid uptake by macrophages [33–35]. Nonetheless, clearance of liposomes from the vasculature by macrophages of the reticuloendothelial system remains a major factor affecting their in vivo disposition to various tissues. A further understanding of the mechanisms by which macrophages recognize and incorporate liposomes and a simple method to measure liposome-cell interactions will aid in the design of liposomes for in vivo delivery.

Previous work has shown that the primary mode by which liposomes are incorporated into cells is endocytosis [36,37] via the coated pit pathway [37]. Once endocytosed, liposomes come in contact with low pH compartments [37] within the cell, presumably endosomes and lysosomes [38,39]. The location of liposomes within cells and the kinetics of liposome uptake are difficult to measure accurately. Commonly used pH-dependent fluorescent probes in the study of liposome-cell interactions are membrane permeant (carboxyfluorescein, [37]), are quenched at low pH, precluding direct pH measurement (fluorescein and calcein, [40]), or have a pH dependence outside the physiological range [41].

Recently an assay utilizing the fluorophore pyranine (1-hydroxypyrene-3,6,8-trisulfonic acid, HPTS), has been developed to accurately measure the pH of liposomes endocytosed by CV-1 fibroblasts [42,43]. HPTS is a highly water-soluble, pH-dependent fluorophore with properties well suited to pH measurement. The fluorophore is readily encapsulated in liposomes, is non-permeant, and responds to intraliposomal changes in pH [44–46]. The excitation spectrum of HPTS exhibits two major peaks, one which is maximal at low pH (403 nm) and one which is maximal at high pH (450 nm), as observed at its broad emission maximum centered at 510 nm. A pH-independent isosbestic point at 413 nm permits the normalization of fluorescence intensities to the total amount of HPTS.

The use of HPTS has made it possible to simultaneously and directly measure liposome binding, leakage of liposomes induced by cells, the average pH of endocytosed liposomes, and, by fluorescence microscopy, the intracellular location of the dye. In combination with a fluorescence quenching agent, liposome-encapsulated HPTS is used to monitor the leakage of liposome contents within endosomal compartments. This fluorophore uniquely provides simultaneous quantitation of parameters associated with the endocytosis of liposomes.

Materials and Methods

Materials. Egg phosphatidylcholine (PC), bovine brain phosphatidylserine (PS), and *N*-dinitrophenyl-phosphatidylethanolamine (DNP-PE) were purchased from Avanti Polar Lipids. Fluorescein isothiocyanate dextran (FD, mol. wt. 20000) and cholesterol were obtained from Sigma Chemical Co. Cholesterol was recrystalized once from methanol before use. 1-Hydroxypyrene-3,6,8-trisulfonic acid (trisodium salt, HPTS) and *p*-xylene-bis-pyridinium bromide (DPX) were obtained from Molecular Probes. [^{3}H]Dipalmitoyl-phosphatidylcholine ([^{3}H]DPPC, approx. 5 Ci $\cdot$ μmol^{-1}) was obtained from Amersham Corp., and purified (> 98%) by CM-52 cellulose chromatography [47]. Anti-dinitrophenol antibody was kindly provided by Dr. T. Heath. Protein concentration was determined by the method of Lowry et al. [48]. The scintillation cocktail Liquiscint was obtained from National Diagnostics. All other chemicals were reagent grade.

Cell culture. The macrophage-like cell lines J774, RAW 264.7, and P388D1 were maintained in mono-

layer culture in Dulbecco's modified Eagle's medium supplemented with 3 $g \cdot l^{-1}$ glucose and 10% calf serum (DMEM) and incubated under 7% humidified CO_2. Cells were plated at a concentration of 10^6 cells per 9.6 cm^2 plastic culture dish 24 h prior to use.

Liposome preparation. Lipid mixtures (PC/cholesterol (3 : 1); PC/PS/cholesterol (2 : 1 : 1), PC/PS/DNP-PE/cholesterol (2 : 1 : 0.04 : 1), in some experiments containing [^{3}H]DPPC (1.9 $\mu Ci \cdot \mu mol^{-1}$ lipid)) were prepared in chloroform and the solvent was removed under reduced pressure. Solutions of HPTS (35 mM) in 2.5 mM Hepes, 75 mM NaCl, 50 μM EDTA (pH 7.4) or HPTS (35 mM) + DPX (50 mM) in 2.5 mM Hepes, 50 μM EDTA (pH 7.4; adjusted to approx. 300 mOsm with 1 M NaCl) were added and liposomes were prepared by the reverse-phase evaporation procedure [49]. Liposomes were extruded through 0.4 μm, then 0.2 μm polycarbonate filters [50] and unencapsulated material was separated from liposomes by gel filtration on a Sephadex G-75 column (1 × 15 cm) equilibrated with 5 mM Hepes, 150 mM NaCl, pH 7.4 (HBS). Phospholipid concentration was determined by the method of Bartlett [51]. The liposomes averaged 165 nm in diameter as measured by laser light scattering and entrapped an aqueous volume of 2.9 $\mu l \cdot \mu mol^{-1}$ lipid. The liposomes were 44% unilamellar and 56% bilamellar as calculated from the measured trapped volume and theoretical trapped volumes of 165 nm diameter, uni- and bi-lamellar liposomes (4.3 $\mu l \cdot \mu mol^{-1}$ lipid and 1.88 $\mu l \cdot \mu mol^{-1}$ lipid, respectively). This corresponded to $1.6 \cdot 10^9$ liposomes $\cdot$ $nmol^{-1}$ phospholipid.

Multilamellar liposomes were used for the calibration of quenching by DPX. Solutions of HPTS (35 mM) with increasing DPX concentration (0–50 mM) were constructed by mixing appropriate volumes of the HPTS and the HPTS/DPX solutions described above. In addition, solutions of decreasing HPTS/DPX concentration were made by diluting the HPTS (35 mM) + DPX (50 mM) solution with HBS. Lipid mixtures (PC/cholesterol, 3 : 1) were prepared as described above. The HPTS/DPX solutions were added, vortexed for 5 min and extruded three times through a 0.2 μm polycarbonate filter. Unencapsulated material was separated from liposomes by gel filtration as described above.

Liposome-cell incubations. DMEM was removed from cells and the cells were washed twice with 2 ml 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4 (PBS) supplemented with 0.4 mM calcium, 0.4 mM magnesium, and 5 mM glucose (PBS-CMG). Liposomes were diluted to 80 μM phospholipid in PBS-CMG and 0.5 ml was added to each culture dish (approx. $(2–3) \cdot 10^6$ cells). In experiments with HPTS/DPX liposomes, 1 ml of 100 μM phospholipid was added to each culture dish. In experiments with DNP-PE-containing liposomes, anti-DNP antibody was diluted to a final concentration of 50 nM in the liposome suspension. Cells were incubated with liposomes at 37°C in a humidified incubator or on ice. To effect energy depletion, cells were incubated with NaN_3 (5 mM) + deoxyglucose (50 mM) in PBS-CM for 30 min at 37°C prior to exposure to liposomes.

Fluorescence microscopy. After incubation with HPTS-containing liposomes, cells were washed twice with 2 ml PBS-CMG and viewed in PBS-CMG with a Leitz fluorescence microscope equipped with epifluorescence, a Zeiss 25 × phase contrast water immersion objective, and a Nikon camera with an automatic exposure meter. Cells were viewed by phase contrast or epifluorescence with two excitation filter sets; either with a set that produces excitation in the range 350–410 nm, and allows observation of fluorescence emission above 455 nm with a long wave pass dichroic mirror and barrier filter, or with a set that produces excitation in the range 450–490 nm, and allows observation of fluorescence emission at wavelengths greater than 515 nm with a long wave pass dichroic mirror and barrier filter. Fluorescence micrographs were automatically corrected for intensity with an exposure meter. Exposure times were typically 1 s for phase contrast, 1 min for λ_{ex} 350–410 nm fluorescence, and 5 min for λ_{ex} 450–490 nm fluorescence.

Fluorometry. After incubation with liposomes for various times, cells were washed twice with PBS-CMG and incubated for 5 min at 37°C with 1.5 ml PBS containing 3 mM EDTA. Cells were dislodged and diluted to $(2.5–5) \cdot 10^5$ cells/ml in PBS. Corrected fluorescence excitation spectra (λ_{ex} 395–465 nm, 1.8 nm bandwidth) were measured at 510 nm emission with a wide (4.5 nm) emission bandwidth using a SPEX Fluorolog 2 fluorometer outfitted with a stirred, temperature controlled cuvette (20°C). For calibration of HPTS quenching by DPX as described above, liposomes were diluted to 5 μM in PBS, and fluorescence was measured as described above. In some experiments, Triton X-100 (0.1%) was added and fluorescence was remeasured after 1 min. Spectra were smoothed using a 13 point smoothing routine [52], peak heights (λ_{ex} 403, 413, 450 nm) were measured and the fluorescence excitation ratios 403/413 nm and 450/413 nm were calculated. Fluorescence units are expressed as photon counts per second per 10^6 cells.

Pinocytosis of fluorescein dextran and free HPTS. DMEM was removed from cells, replaced with media containing 0.1 $mg \cdot ml^{-1}$ FD or 100 μM HPTS and cells were incubated for 24 h at 37°C. HPTS-treated cells were washed with PBS-CMG, resupplied with fresh media and incubated for 5 h at 37°C. Cells were washed with PBS-CMG, dislodged as described above, counted, and resuspended in PBS ($(2.5–5) \cdot 10^5$ cells/ml). HPTS fluorescence was measured as described above. Fluorescence excitation spectra (λ_{ex} 425–500 nm) of FD treated cells were obtained at λ_{em} 520 nm and the pH-depen-

dent fluorescence ratio λ_{ex} 495/450 was calculated [39]. Average pH values were calculated from standard curves of HPTS or FD generated from solubilized (0.1% Triton X-100) samples.

Scintillation counting. Samples were prepared for scintillation counting by dissolving in Liquiscint scintillation fluid and radioactivity was measured in a Beckman LS-3801 scintillation counter. The number of liposomes associated with cells was calculated from the specific activity of liposomal lipid (3.8 Ci [^{3}H]DPPC/mol phospholipid) and the number of liposomes per mole of phospholipid ($1.6 \cdot 10^9$ liposomes/nmol phospholipid).

Results

Properties of HPTS in relation to endocytosis

When incubated with cells, liposomes bind to the plasma membrane and, depending on the cell type and liposome composition, a portion is taken up into the cell where the local pH within endosomal and lysosomal compartments is expected to be low [36,37,53]. The spectral shifts of HPTS with changes in pH make it a powerful new marker of the fate of liposome contents following endocytosis [42,43]. The excitation spectrum of HPTS measured at λ_{em} 510 nm exhibits two complementary pH-dependent peaks (λ_{ex} 403 nm and 450 nm) and a pH-independent point at λ_{ex} 413 nm that can be used to normalize measurements for changes in dye content (Fig. 1A). The fluorescence excitation ratio 450/413 nm (Fig. 1B, squares) is linear over a greater range of pH and shows a greater magnitude of change in the range pH 6–8 than the 403/413 nm ratio (Fig. 1B, circles). The 450/413 nm ratio becomes insensitive to pH below pH 6.0.

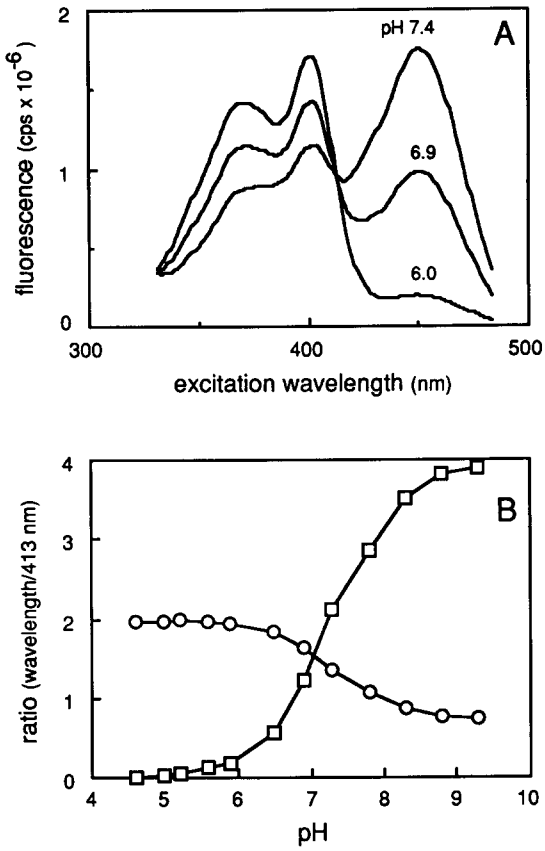

Fig. 1. The pH-dependent properties of HPTS. (A) Excitation spectra of HPTS. HPTS (1 μM) in PBS was adjusted to various pH values (as noted) and excitation spectra (1.8 nm bandwidth) were measured at λ_{em} 510 nm with a 4.5 nm bandwidth. Fluorescence is expressed as photon counts per sec (cps). (B) Fluorescence emission intensities at λ_{ex} 403 nm and λ_{ex} 450 nm normalized to intensity at λ_{ex} 413 nm; 403/413 nm (○), 450/413 nm (□).

Fluorescence microscopy

Fluorescence micrographs of J774 cells treated with HPTS-containing PC/PS/cholesterol liposomes from a typical experiment are shown in Fig. 2. Cells were treated with liposomes for 1 h at 37°C, washed twice in PBS-CMG and were viewed by phase contrast and fluorescence microscopy using λ_{ex} 350–410 nm and λ_{ex} 450–490 nm filters. A phase contrast light micrograph of a typical field of cells is shown in Fig. 2a. When viewed with the λ_{ex} 350–410 nm filter, resulting in excitation of the relatively pH-insensitive region, the cells fluoresce brightly wherever HPTS is located (Fig. 2b). Under excitation with longer wavelengths (λ_{ex} 450–490 nm) the pH-sensitive 450 nm peak is excited, thus fluorescence results only from dye that is at high pH (pH 7.4, Fig. 2c). Therefore, fluorescence apparent under both excitation filters is from HPTS at high pH and fluorescence with the λ_{ex} 350–410 nm filter only is from HPTS at low pH. At early times (1 h), the short λ_{ex} fluorescence (λ_{ex} 350–410 nm, Fig. 2b) is on the cell periphery as well as in punctate intracellular compartments under short wavelength excitation, however the pattern is mostly peripheral under long wavelength illumination (λ_{ex} 450–490 nm, Fig. 2c). At later times (4 h) both the pattern and the intensity of fluorescence changes; the peripheral pattern is diminished and the fluorescence is in perinuclear vacuoles under both short (Fig. 2e) and long (Fig. 2f) wavelength excitation, though the intensity of fluorescence under long wavelength excitation is diminished. The corresponding phase contrast micrograph is shown in Fig. 2d. This indicates that at early times liposomes bind to the cell surface, where they are at a high pH, and with continued incubation they accumulate in low pH intracellular compartments. At all times fluorescence with λ_{ex} 450–490 nm illumination was less intense than with λ_{ex} 350–410 nm; the micrographs in Figs. 2c and 2f were exposed 5 times longer than the micrographs in Figs. 2b and 2e. In separate experiments, the pattern of fluorescence observed after treatment with acridine orange, a lysosomotropic agent [54], is similar to that observed with HPTS

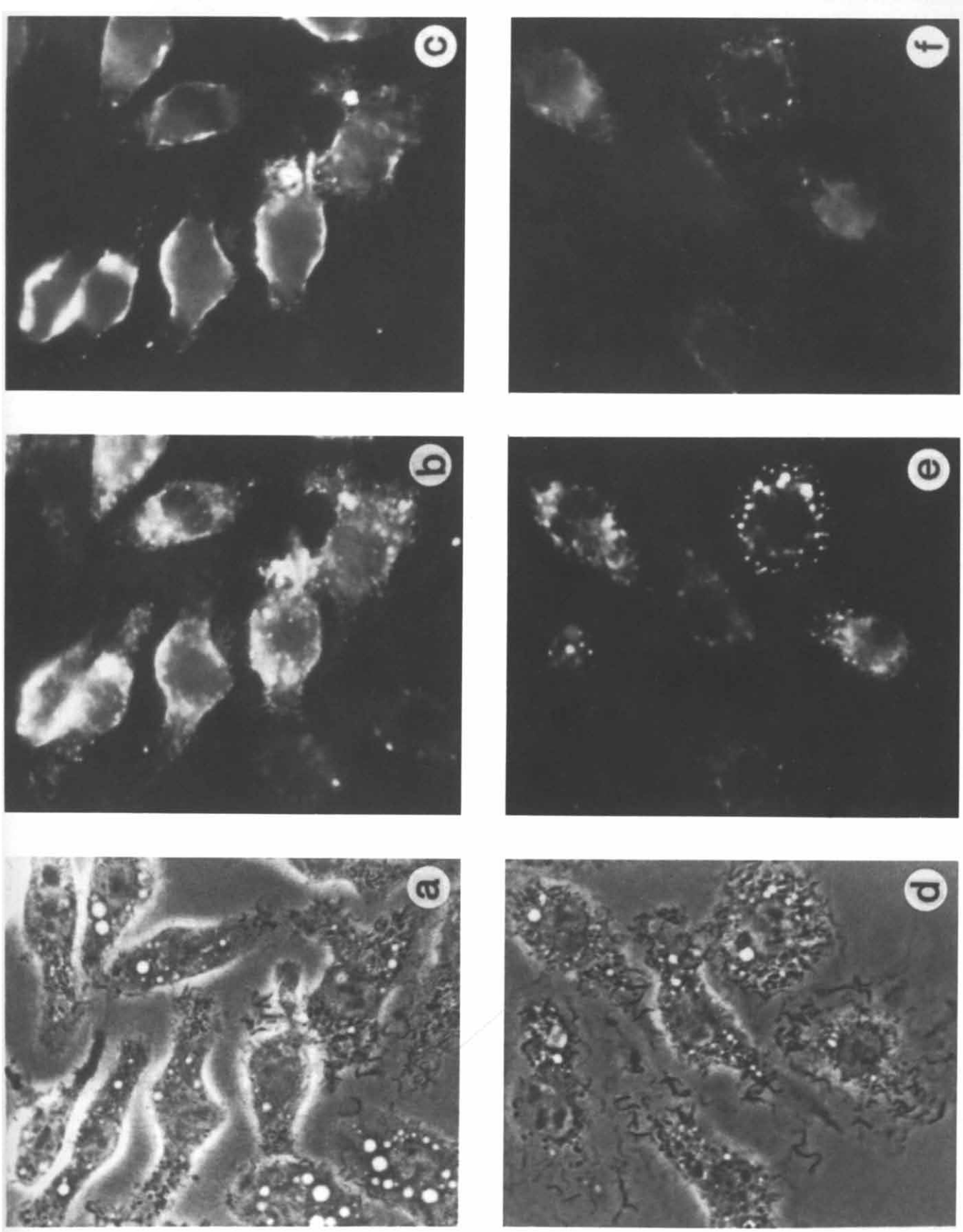

Fig. 2. Fluorescence micrographs of J774 macrophages treated with HPTS-containing PC/PS/cholesterol (2:1:1) liposomes. Cells were treated with liposomes for 1 h (a–c) or for 4 h (d–f) at 37 °C, washed, and viewed with a water immersion objective by: phase contrast (a, d), or epifluorescence with λ_{ex} 350–410 (b, e) or λ_{ex} 450–490 (c, f) filters.

indicating that these probes accumulate in similar intracellular compartments (not shown).

The uptake of liposomes by macrophages is energy- and temperature-dependent. Fluorescence patterns observed after incubating HPTS-containing liposomes with J774 cells subsequent to azide (5 mM) + deoxyglucose (50 mM) pretreatment (30 min) or by incubation at 4°C is peripheral and bright under both excitation filter sets (not shown), indicating that the liposomes are on the cell surface at a high pH.

Quantitation of HPTS incorporation by spectrofluorometry

The fraction of dye taken up by endocytosis can be calculated using the 450/413 nm ratio. Because endosomal and lysosomal compartments are acidic (pH < 6), the measured 450/413 nm ratio will lie between the ratio at pH 7.4 (approx. 2.0) and at pH 6 (approx. 0.2). The pK_a of HPTS varied between 7.0 and 7.5, depending on the presence of protein, detergent, buffer strength and ionic strength of the medium (Table I). Isotonic concentrations of salt slightly increased pK_a, while 15 mM phosphate increased pK_a by half a pH unit. Detergent (0.1% Triton X-100) and protein (0.1% albumin) decreased the pK_a. A decrease in pK_a would cause an increase in the observed 450/413 ratio and result in an apparent increase in pH. Changing salt, buffer, and protein concentrations during endocytosis may alter the apparent pK_a of HPTS. Thus, a direct measure of the lowest 450/413 nm ratio obtainable for HPTS pinocytosed by J774 cells was made. Cells were incubated with free HPTS (100 μM) in DMEM for 24 h at 37°C, washed and reincubated in fresh DMEM for 5 h at 37°C to concentrate HPTS in lysosomal compartments. The 450/413 nm ratio obtained ($ratio_{low}$), 0.5, corresponds to an average pH of 6.5 (Fig. 1B). Other macrophage cell lines yielded lower 450/413 nm ratios of 0.20 (RAW 264.7) and 0.28 (P388D1), corresponding to lower average pH values of 6 (RAW 264.7) and 6.1 (P388D1). Measurements of endosomal/lysosomal pH using pinocytosed FD (Table II) gave similar values for RAW 264.7 cells (5.76 ± 0.13) and P388D1 cells (5.88 ± 0.14), but a lower value for J774 cells (5.59 ± 0.20) compared with HPTS measurements. Some shift in the pK_a of HPTS may have occurred or HPTS may be accumulating in another compartment with a higher pH (see Discussion). The HPTS $ratio_{low}$ for J774 cells was used to calculate the fraction of HPTS endocytosed in liposome experiments:

$$\text{fraction endocytosed} = \frac{(\text{ratio}_{\text{pH 7.4}} - \text{ratio}_{\text{measured}})}{(\text{ratio}_{\text{pH 7.4}} - \text{ratio}_{\text{low}})} \quad (1)$$

where $ratio_{measured}$ is the 450/413 nm ratio of liposome-treated cells and $ratio_{pH\ 7.4}$ is the 450/413 nm ratio of liposomes in PBS.

The uptake of a variety of different liposomes (PS/PC/cholesterol (1 : 2 : 1), PC/cholesterol (3 : 1) or PC/PS/DNP-PE/cholesterol (2 : 1 : 0.04 : 1) liposomes in the presence or absence of anti-DNP antibody) by J774 macrophages was measured during continuous incubation at 37°C. HPTS uptake was measured directly as fluorescence intensity at λ_{ex} 413 nm. The rate and extent of incorporation of HPTS into cells was similar for negative (PS-containing) and neutral (PC) liposomes (Fig. 3A); approx. 0.26 and 0.44%, respectively, of the HPTS dose became associated with the cells within 2 h. Coating DNP-PE-containing liposomes with antibody resulted in an 8-fold increase in the amount of HPTS incorporated and a 5-fold increase in the rate of uptake compared with DNP-PE-containing liposomes not exposed to antibody. HPTS encapsulated in antibody coated liposomes was taken up at a greater rate (2.5-fold) and to a greater extent (4-fold) than in negative (PS-containing) or neutral (PC) liposomes.

The fluorescence excitation spectrum of HPTS was altered during the time-course of incubation of cells with vesicles. The peak at 450 nm progressively decreased while the peak at 403 nm increased. The normalized 450 nm values (450/413 nm ratio) from J774 cells exposed to PC, PS-containing, or DNP-PE-containing liposomes (in the presence or absence of anti-DNP antibody) are shown in Fig. 3B. A decrease in the 450/413 nm ratio is indicative of a decrease in pH. Assuming that HPTS is in one of two compartments, outside the cell at pH 7.4 (high pH) or within endosomes or lysosomes at low pH, then the fraction of dye endocytosed can be calculated using eqn. (1). Approx. 60% of the PC or PS-containing liposomes associated with the cells within 30 min were at low pH (Fig. 3C).

TABLE I

pK_a values for HPTS in aqueous solutions

Conditions	pK_a
water	7.01
0.15 M NaCl	7.09
PBS + 0.1% Triton X-100	7.32
PBS + 0.1% albumin	7.40
PBS	7.52

TABLE II

Average pH of HPTS or fluorescein dextran (FD) pinocytosed by macrophages

Macrophage	HPTS	FD
J774	6.5	5.59 ± 0.20
P388D1	6.1	5.88 ± 0.14
RAW 264.7	6.0	5.76 ± 0.13

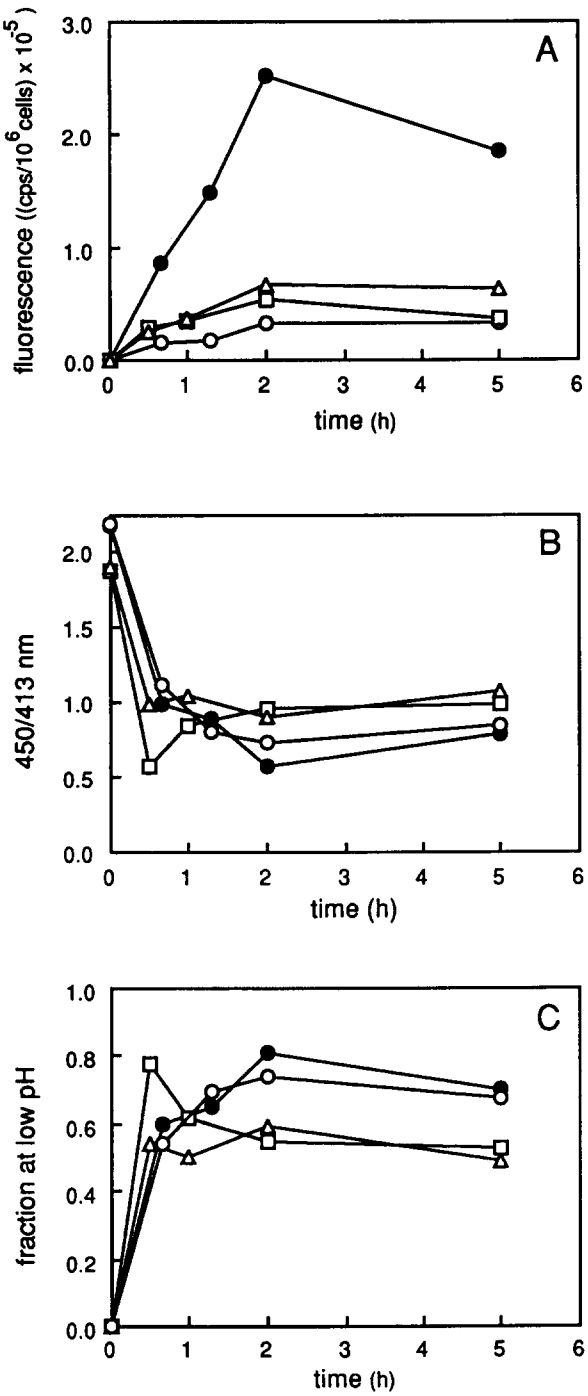

Fig. 3. Time-course of uptake of HPTS-containing liposomes by J774 macrophages. Cells were treated with PC/cholesterol (3 : 1) liposomes (□), PC/PS/cholesterol (2 : 1 : 1) liposomes (△), or PC/PS/DNP-PE/cholesterol (2 : 1 : 0.04 : 1) liposomes in the presence (●) or absence (○) of anti-DNP antibody. Fluorescence at λ_{ex} 413 and 450 nm was measured, the ratio 450/413 nm was calculated, and the amount of HPTS incorporated into the cells was calculated using equation (1): (A) Fluorescence intensity at λ_{ex} 413 nm (photon cps/(10^6 cells)), (B) 450/413 nm ratio, and (C) fraction of HPTS endocytosed (at low pH).

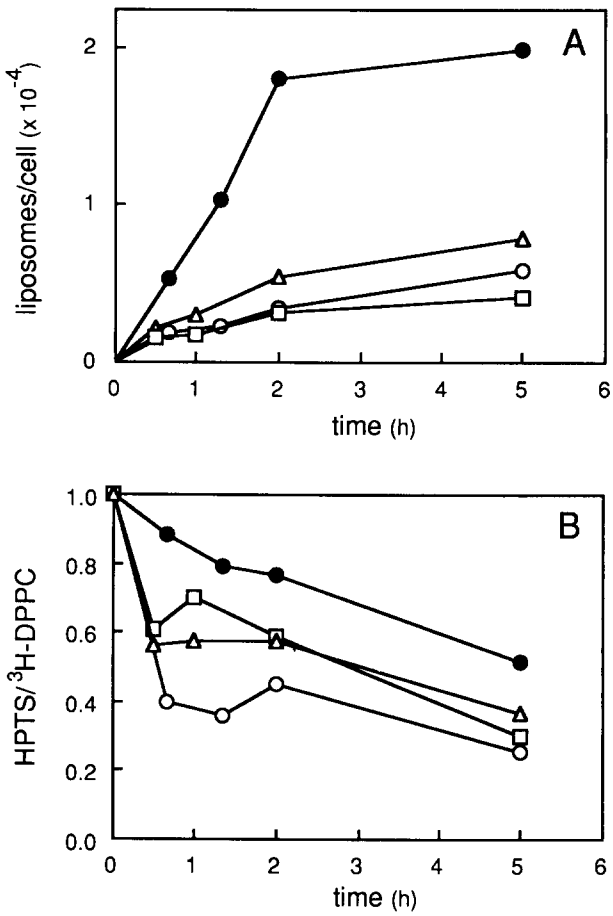

Fig. 4. Time-course of uptake of [^{3}H]DPPC containing liposomes by J774 macrophages. Liposomes from the experiment described in Fig. 3 also contained (3.5–4)·10^3 cpm [^{3}H]DPPC per nmole phospholipid. Cells were treated with PC/cholesterol (3 : 1) liposomes (□) PC/PS/cholesterol (2 : 1 : 1) liposomes (△), or PC/PS/DNP-PE/cholesterol (2 : 1 : 0.04 : 1) liposomes in the presence (●) or absence (○) of anti-DNP antibody, as described in Fig. 3. Radioactivity of aliquots of samples was determined after fluorescence measurements: (A) number of liposomes per 10^6 cells, (B) ratio of HPTS/[^{3}H]DPPC (photon cps/[^{3}H]DPPC cpm) normalized to the ratio in the absence of cells. Starting ratios: PC/cholesterol liposomes, 22.4; PC/PS/cholesterol liposomes, 18.5; and PC/PS/cholesterol/DNP-PE liposomes, 18.1.

DNP-PE-containing liposomes, in the presence or absence of antibody, were acidified to a greater extent (80%), though the rate of acidification of HPTS was similar for all liposomes.

Comparison of uptake of lipid and aqueous contents

The uptake of liposomal lipid was measured using [^{3}H]DPPC as a tracer (Fig. 4, Table III). [^{3}H]DPPC in PC and PS-containing liposomes was incorporated into cells at approximately the same initial rate; however, PS-containing liposomes were incorporated to a greater extent than PC liposomes. These observations are consistent with measurements of liposome uptake by J774 cells by Stevenson et al. [55]. The initial rate of incorporation of radiolabelled lipid from DNP-PE-containing

liposomes was similar to that observed for PC and PS-containing liposomes; however, the rate increased in the presence of antibody. The extent of incorporation of radiolabel from antibody-treated DNP-PE-containing liposomes was approx. 3.4-fold greater than for non-antibody coated DNP-PE-containing liposomes. Compared with HPTS uptake (Fig. 3A), [^{3}H]DPPC was incorporated at similar relative rates and extents (Fig. 4A); however, the absolute amount of incorporation of liposomal lipid and contents markers was not equivalent. As shown in Fig. 4B, the ratio of liposomal contents to liposomal lipid (HPTS/[^{3}H]DPPC) decreased during the course of incubation for all types of liposomes, indicating selective accumulation of liposomal lipid with respect to contents. Within the first hour of incubation cells treated with PC, PS-containing, or DNP-PE-containing liposomes (in the absence of antibody) accumulated 40–60% more lipid than contents label. Upon further incubation cells continued to selectively accumulate liposomal lipid at a slower rate (11–18% · h^{-1}), as evinced by the decrease in the HPTS/[^{3}H]DPPC ratio. The initial loss of liposomal contents within the first hour of incubation was not observed with DNP-PE-containing liposomes in the presence of anti-DNP antibody; the rate of selective lipid accumulation was 12% · h^{-1}, similar to the second rate observed with PC, PS-containing, or DNP-PE-containing liposomes.

Quantitation of liposome leakage

Cell-induced dilution of liposomal contents was detected by co-encapsulating HPTS and a non-fluorescent quencher, DPX, into liposomes. The quenching of HPTS with DPX was calibrated by coencapsulating 35 mM HPTS with increasing concentrations of DPX (0–50 mM). The amount of fluorescence (413 nm) observed was compared to the fluorescence in the presence of 0.1% Triton X-100 (defined as 100%). As shown in Fig. 5 (inset), 50 mM DPX was required to achieve greater than 99% quenching of 35 mM HPTS. This concentration of HPTS and DPX was used for subsequent studies of liposome leakage. To calibrate the effects of liposome leakage and dye/quencher dilution, a solution of 35 mM HPTS + 50 mM DPX was diluted with buffer and encapsulated in liposomes. Increasing dilution of the HPTS/DPX solution resulted in an increase in fluorescence (Fig. 5), however a significant increase in fluorescence was observed only at high dilutions; a 43% increase in fluorescence corresponded to greater than 90% dilution of the dye/quencher mixture. This curve was used in subsequent studies to correlate the increase in fluorescence of HPTS/DPX-containing liposomes induced by cells to the amount of liposomal contents that have been diluted.

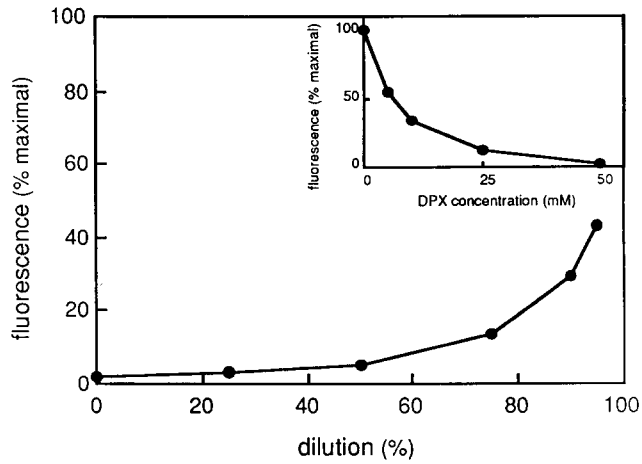

Fig. 5. Quenching of HPTS by DPX. Fluorescence of multilamellar liposomes (PC/cholesterol (3:1)) containing 35 mM HPTS+0–50 mM DPX (inset) or dilutions of a 35 mM HPTS+50 mM DPX solution with buffer was measured in the absence and presence of 0.1% Triton X-100. Data are expressed as maximal fluorescence = $\frac{\text{fluorescence in the absence of detergent}}{\text{fluorescence in the presence of detergent}} \times 100$.

Cells were treated with PC/PS/cholesterol (2:1:1) liposomes containing 35 mM HPTS + 50 mM DPX at 37°C. Fig. 6 shows phase contrast (a and c) and fluorescence (b and d) micrographs of cells treated for 1 h (a and b) or 4 h (c and d). Punctate fluorescence was observed with illumination at λ_{ex} 350–410 nm at both short (1 h, Fig. 6b) and long (4 h, Fig. 6d) incubation times. Illumination at λ_{ex} 450–490 nm resulted in fluorescence too dim for photography, indicating that almost all of the dequenched dye was at low pH (not shown). Thus, endocytosed liposomes had leaked their contents but the dye remained within low pH compartments and was not present in the cell cytoplasm.

The uptake of HPTS/DPX-containing liposomes by cells and their subsequent leakage was measured by fluorometry. Cells were treated with PC/PS/cholesterol liposomes containing HPTS or HPTS/DPX at 37°C, washed, and fluorescence was measured as above. The samples were treated with 0.1% Triton X-100 and the

TABLE III

Rate and extent of uptake of [^{3}H]DPPC-containing liposomes by J774 cells

Liposome [a]	Initial rate (liposomes/cell per h)	Extent at 5 h (liposomes/cell)
PC	2360	3090
PC/PS	3080	5870
PC/PS/DNP-PE	1990	4310
PC/PS/DNP-PE + antibody	5900	14700

[a] PC = PC/cholesterol (3:1); PC/PS = PC/PS/cholesterol (2:1:1); PC/PS/DNP-PE = PC/PS/DNP-PE/cholesterol (2:1:0.04:1); antibody = anti-DNP antibody. All liposomes contain approx. 3.8 Ci [^{3}H]DPPC/mol phospholipid.

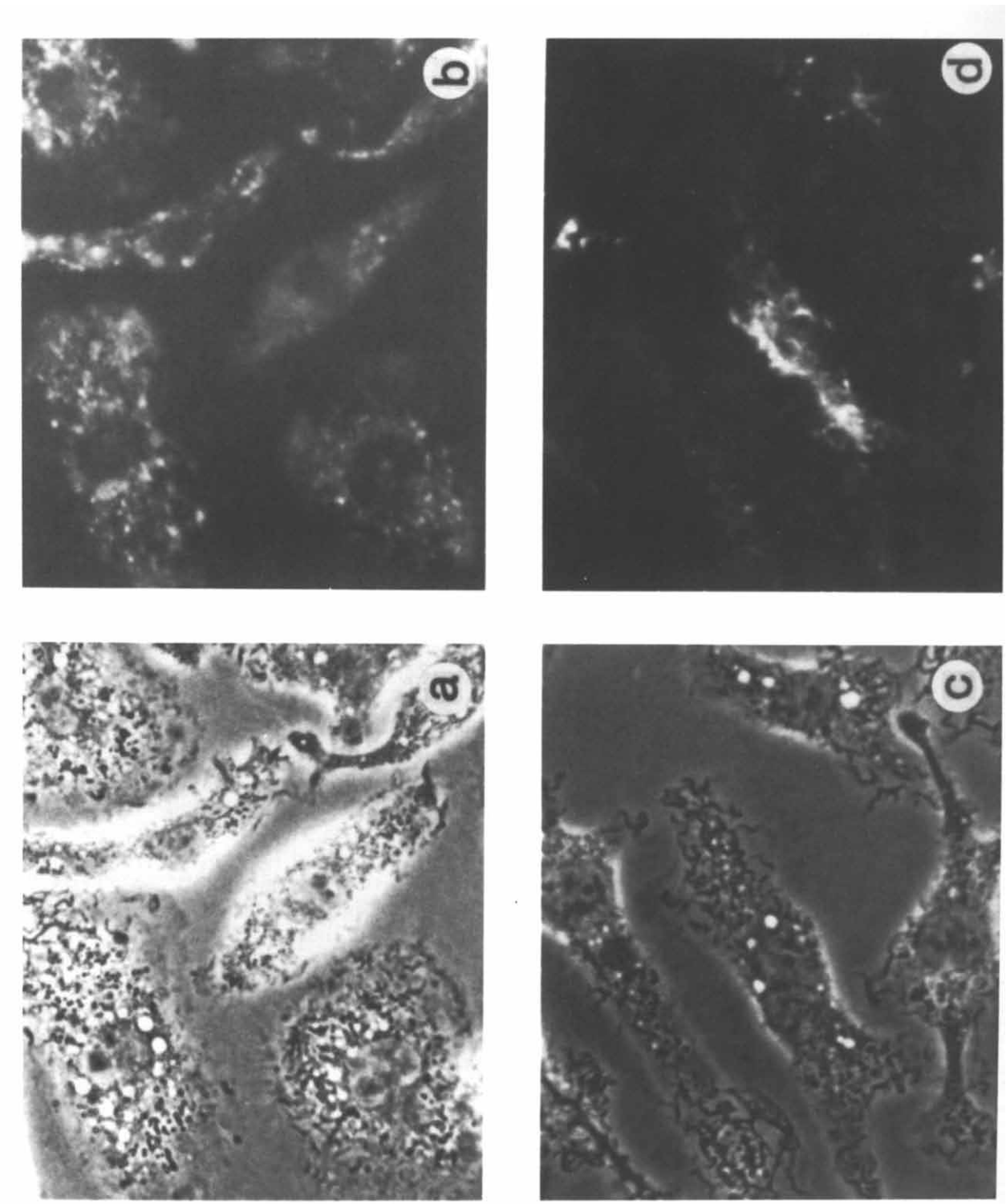

Fig. 6. Fluorescence micrographs of J774 macrophages treated with HPTS/DPX-containing PC/PS/cholesterol (2:1:1) liposomes. Cells were treated with liposomes for 1 h (a, b) or for 4 h (c, d) at 37°C, washed with PBS-CM and viewed with a water immersion objective by: phase contrast (a, c), or epifluorescence with λ_{ex} 350–410 (b, d) filters.

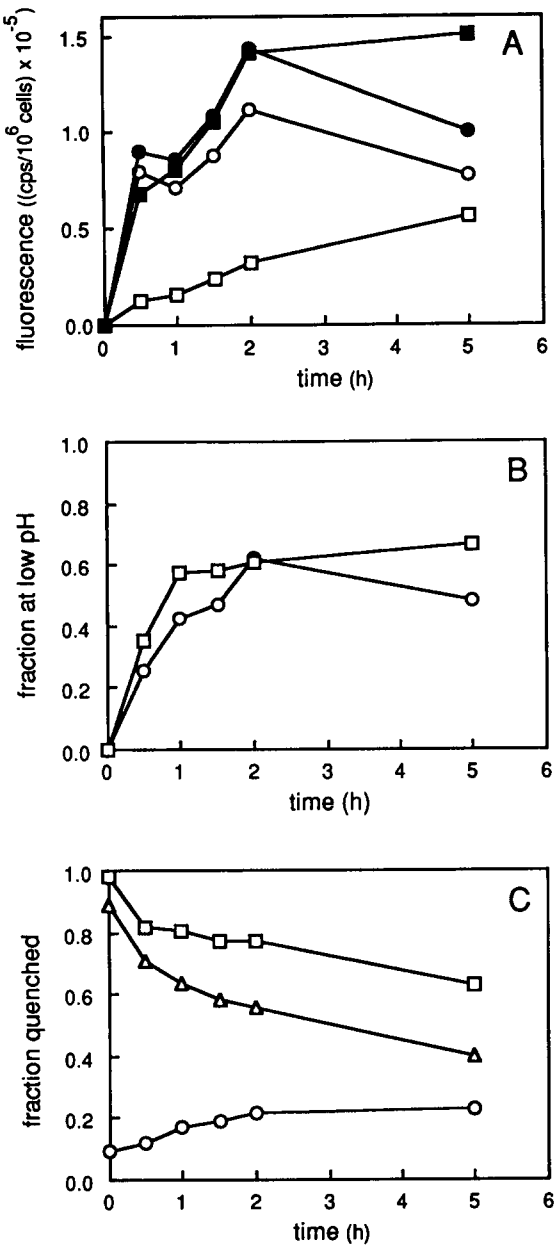

Fig. 7. Time-course of uptake of HPTS and HPTS/DPX-containing liposomes by J774 macrophages. Cells were treated with PC/PS/cholesterol (2:1:1) liposomes containing 35 mM HPTS (circular symbols) or 35 mM HPTS+50 mM DPX (square symbols). Fluorescence at λ_{ex} 413 and 450 nm was measured, the ratio 450/413 nm was calculated, and the amount of HPTS incorporated into the cells was calculated using equation (1). (A) Fluorescence intensity at λ_{ex} 413 nm (photon cps/(10^6 cells)), before (○, □) and after (●, ■) the addition of 0.1% Triton X-100, (B) fraction of HPTS endocytosed (at low pH) and (C) fraction of HPTS remaining quenched (○, □) and corrected fraction remaining quenched (fraction quenched$_{HPTS/DPX}$ − fraction quenched$_{HPTS}$, △).

fluorescence was measured again. As shown in Fig. 7A, less fluorescence was associated with cells treated with HPTS/DPX liposomes (open squares) than with HPTS liposomes (open circles). This difference was a result of quenching of the HPTS by DPX; when these samples were lysed with 0.1% Triton X-100 and remeasured, approximately equal amounts of HPTS fluorescence were observed (closed symbols, Fig. 7A). The uptake of HPTS was coincident with a decrease in the 450/413 nm ratio (not shown) and thus, acidification of HPTS (Fig. 7B). The fraction of dye incorporated into low pH compartments (60%) and the half time of acidification (roughly 30 min) was similar for liposomes containing HPTS or HPTS/DPX.

The increase in HPTS fluorescence in the presence of detergent is a measure of the quenching of the dye. For HPTS/DPX liposomes, fluorescence quenching decreased rapidly and reached a plateau at approximately 60% quenched dye within 5 h (squares, Fig. 7C), corresponding to approximately 95% dilution of the dye/quencher solution (Fig. 5). Liposomes containing HPTS alone became quenched over the same time-course when incubated with cells. The fraction of quenched HPTS rose from an initial value of 10% to approx. 20% after 5 h (circles, Fig. 7C). The net leakage (dequenching) of HPTS/DPX liposomes associated with cells was obtained after correcting for quenching of liposomes containing HPTS alone (triangles, Fig. 7C). Within 5 h, the fraction of dye that is quenched reaches a plateau at approx. 40%, corresponding to about 97% leakage of the liposomes (Fig. 5). There are two possible explanations for this result. The absolute amount of dilution of the dye/quencher mixture of liposomes internalized by J774 cells may be 97%. Alternatively, in the presence of excess liposomes a steady state may develop of liposome binding, internalization, and replacement at the surface with intact exogenous liposomes. The continual binding of intact liposomes would decrease the average value of liposome contents dilution and would increase the average liposome pH.

To eliminate interferences in measurements of liposome leakage and internalization by exogenous liposomes, the previous experiment was modified. Cells were treated with PC/PS/cholesterol liposomes containing HPTS or HPTS/DPX at 37°C for 30 min, washed with PBS-CMG to remove excess liposomes, and reincubated in PBS-CMG at 37°C in the absence of additional liposomes (Fig. 8). At the times indicated, cells were washed again, and fluorescence was measured as above (Fig. 8A, open symbols). Removing exogenous liposomes after 30 min resulted in a rapid loss of dye from the cells upon reincubation; only 25% of the HPTS or HPTS/DPX-containing liposomes remained after a 30 min reincubation. The samples were treated with 0.1% Triton X-100 and the fluorescence was remeasured (Fig. 8A, filled symbols). Detergent treatment resulted in a large increase of fluorescence at 30 min and a smaller increase at later times (Fig. 8A, square symbols). In comparison, when cells are treated continuously with liposomes the fractional increase in fluorescence in the presence of detergent is greater at all

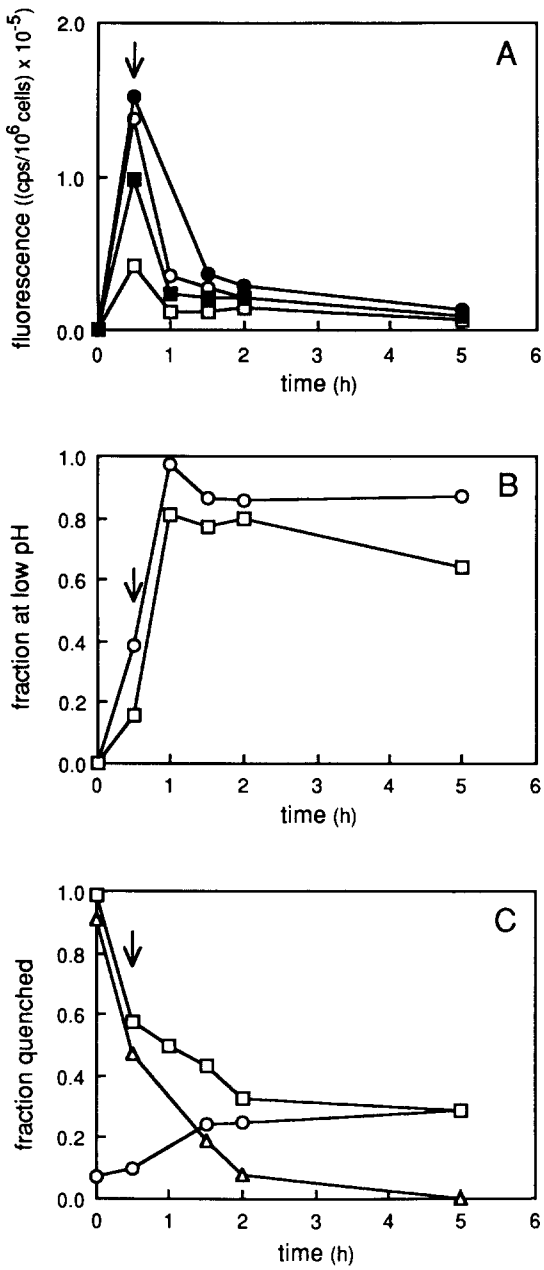

Fig. 8. Time-course of uptake of HPTS and HPTS/DPX-containing liposomes by J774 macrophages. Cells were treated with PC/PS/cholesterol (2:1:1) liposomes containing 35 mM HPTS (circular symbols) or 35 mM HPTS+50 mM DPX (square symbols) for 30 min at 37°C. Cells were washed (indicated by arrows), resuspended in PBS-CMG and incubation was continued at 37°C. Fluorescence values at λ_{ex} 413 and 450 nm were measured, the ratio 450/413 nm was calculated, and the amount of HPTS incorporated into the cells was calculated using equation (1). (A) Fluorescence intensity at 413 nm (photon cps/(10^6 cells)), before (○, □) and after (●, ■) the addition of 0.1% Triton X-100, (B) fraction of HPTS endocytosed (at low pH), (C) fraction of HPTS remaining quenched (○, □) and corrected fraction remaining quenched (fraction quenched$_{HPTS/DPX}$ − fraction quenched$_{HPTS}$, △).

time points (Fig. 7A, filled square symbols) than that observed in the absence of exogenous liposomes (Fig. 8A, filled square symbols). This indicates that under conditions of continuous exposure, cell associated liposomes have leaked less on the average. After removing exogenous liposomes, the fraction of dye remaining bound to the cell was primarily (> 80%) in a low pH environment (Fig. 8B). In comparison, when incubated continuously with liposomes the fraction of dye in a low pH environment was not greater than 60% (Fig. 7B).

Dye quenching by DPX was quantitated by comparison of fluorescence in the absence and presence of detergent (Figs. 5 and 8A). Fig. 8C shows the fractional quenching as calculated from the increase in fluorescence in the presence of 0.1% Triton X-100. Before exposure to cells, HPTS-containing liposomes were approx. 10% quenched and this fraction increased to approx. 30% during the incubation (Fig. 8C), similar to those values measured in Fig. 7C. The quenching observed in HPTS/DPX-containing liposomes decreased to about 60% during the initial 30 min incubation and continued to decrease to approx. 30% over the time-course of the experiment. The corrected quenching (triangles, Fig. 8C) indicated that after an initial rapid decrease (53% within 30 min, corresponding to 96% leakage (Fig. 5)) liposomes endocytosed by cells eventually leaked all of their contents. During continuous incubation (Fig. 7C) the maximum amount of leakage observed was 97% within 5 h. Thus, removing exogenous liposomes resulted in a greater fraction of dye at low pH and greater degree of average liposome contents dilution, consistent with continual binding of exogenous liposomes to the cell surface if not removed by washing.

Discussion

In the design of liposomes as drug carriers, all modes of liposome-cell interaction must be considered: lipid and protein exchange, adsorption to the cell surface, serum and cell-induced leakage of liposome contents, fusion with cells and endocytosis [56]. Each of these factors affect the efficiency of drug delivery and the choice of liposome components. Although initial studies indicated that liposomes fuse with the cell plasma membrane [57,58], subsequent work established that liposomes are endocytosed in coated vesicles and encounter a low pH compartment inside the cell [37]. Evidence for the low pH compartmentalization of liposomes inside cells has come from the use of cytotoxic agents [21,22] and the pH dependent fluorescent markers carboxyfluorescein and calcein [27,37,58,59]. Both carboxyfluorescein and calcein exhibit a pH dependent quenching of their fluorescence, while only the former becomes membrane-permeant at low pH values [37]. In contrast, the properties of HPTS make it a good marker for liposome endocytosis. It is useful for both microscopic (Figs. 2 and 6) and fluorometric (Figs. 3–5, 7, 8) analysis and accurately reports the pH of its environment [42–45]. It is highly water soluble, membrane imper-

meant, shows a pH dependent shift rather than quench of its fluorescence spectrum, and has an isosbestic point at 413 nm, permitting correction of the measurements to the total amount of dye present in the sample (Fig. 1). Furthermore, the properties of HPTS allow for the use of fluorescence microscopy to detect the location and approximate pH of the dye in cells. By choosing appropriate filters, illumination of HPTS in pH-independent (λ_{ex} 350–410 nm) and pH-dependent (λ_{ex} 450–490 nm) regions of the excitation spectrum can be made (Fig. 2). Thus, the binding and progressive acidification of liposomes can be continuously monitored. In addition, this change in the pattern of fluorescence is energy- and temperature-dependent (not shown), consistent with endocytosis.

The incorporation and acidification of liposomal HPTS can be quantitated by spectrofluorometry. The fraction of dye endocytosed is calculated assuming that the HPTS is present in one of two compartments of different pH: either on the external surface of the cell or in the cell cytoplasm at a high pH (7–7.4), or in intracellular endosomes and lysosomes at a low pH (< 7).

Macrophages are highly phagocytic cells and are ideal for studies of endocytosis and intracellular processing of endocytosed material. Negatively charged liposomes bind to and are endocytosed by cells to a greater extent than neutral liposomes [29,55,60,61]. However, for the macrophages used in the present work this difference is not as great as that seen with other cell lines. Negatively charged PS-containing liposomes are taken up by J774 cells to a slightly greater extent, though at a similar initial rate, than neutral PC liposomes (Fig. 3 and 4, Table III). Similarly, Stevenson et al. [55] found that negatively charged liposomes containing carboxyfluorescein were internalized slightly more efficiently than neutral liposomes by J774 cells. The number of PS-containing liposomes associated with cells at 5 h (approx. 6,000 per cell) is 2-fold greater than values obtained by Stevenson et al. [55] (approx. 3,000 per cell) for J774 cells treated for 3 h with 250 μM PS liposomes (egg PC/cholesterol/PS (7 : 2 : 1). In addition, under their conditions J774 cells bound 3-fold fewer neutral liposomes (egg PC/cholesterol). These discrepancies may be a result of incubation conditions: Stevenson et al. [55] used a 10-fold lower concentration of cells and used liposomes containing 2.5-fold less PS.

Macrophages possess Fc receptors on their surface that bind to the Fc portion of antibodies and are subsequently endocytosed via the coated pit pathway. Liposomes containing DNP-PE are endocytosed in the presence of anti-DNP antibody at a greater rate and to a greater extent than uncoated DNP-PE-containing liposomes (Fig. 3 and 4, Table III). The increased uptake of antibody coated liposomes is probably due to specific Fc receptor-mediated endocytosis [62,63]. Uncoated liposomes may bind to the cell surface and be endocytosed by a separate, perhaps nonspecific, endocytotic mechanism.

The binding of liposomes to cell surfaces induces leakage of liposome contents [27–29] that may be the result of surface protein-liposome interactions [64]. As shown in Figs. 3 and 4, cell associated liposomes rapidly lose HPTS relative to the radiolabelled lipid ([^{3}H]DPPC); the ratio of the HPTS liposome contents marker to the [^{3}H]DPPC lipid marker decreases by 50% within the first 30 min of exposure of neutral or negatively charged liposomes to cells. The initial decrease in in the HPTS/[^{3}H]DPPC ratio is absent in DNP-PE-containing liposomes coated with anti-DNP antibody, indicating that the antibody affords some protection to the disruption of liposome membranes induced by cells. This indicates that leakage of liposome contents induced by cells is a result of direct interaction of the liposome and cell plasma membrane; the presence of antibody prevents close contact of DNP-PE liposomes with the cell membrane. Indeed, the process of cell surface-induced leakage may be related to endocytosis; binding of liposomes to surface proteins or to the lipid bilayer may trigger endocytosis and initiate liposome leakage.

Degradation of liposomes after endocytosis is similar with or without bound antibody; the HPTS/[^{3}H]DPPC ratio decreases steadily and the rate of decrease is similar for neutral, negative or antibody coated liposomes. As surface-bound liposomes are endocytosed, more liposomes take their place on the surface and lose contents. If there is no efflux of liposome contents after endocytosis, the HPTS/[^{3}H]DPPC should approach a constant value indicative of surface induced leakage. However, the HPTS/[^{3}H]DPPC ratio decreases continuously (Fig. 4B), indicating that liposomes are endocytosed and liposome aqueous contents continually efflux from the cell. Liposome leakage (within endosomes) after endocytosis may continue by a mechanism similar to that which occurs on the cell surface. In addition, acidification of liposomes and cell surface proteins after endocytosis may alter protein conformation leading to increased protein–liposome interactions and perhaps penetration of proteins into the liposome bilayer. As liposomes accumulate in lysosomes, other mechanisms such as lipid transfer and lipid degradation contribute to liposome breakdown. Liposome contents released into endosomes and lysosomes become aqueous markers of these compartments, and subsequent efflux of liposomal aqueous contents from cells may result from regurgitation of the contents of early endosomes or from excretion from lysosomes [65,66].

The decrease in HPTS fluorescence associated with cells is not due to degradation of the dye; 80–90% of HPTS originally encapsulated in liposomes can be recovered from the cells and incubation medium (not

shown). In addition, the leakage of HPTS from liposomes after endocytosis is not a result of increased permeability of the dye at low pH; HPTS encapsulated in liposomes similar to those used in this work does not leak readily in the pH range 5–8 [42,67].

In order to measure independently the leakage of liposome contents, an assay based on quenching of HPTS fluorescence by the non-fluorescent molecule, DPX, was developed. DPX has been extensively used as a quencher of aminonapthalene trisulfonate in model studies of membrane fusion and leakage [68], and recently as a liposomal HPTS quencher for microscopy [69]. When coencapsulated with 35 mM HPTS, 50 mM DPX efficiently quenches HPTS fluorescence (Fig. 5). The quenching of HPTS (35 mM) by DPX (50 mM) is efficient even at relatively high dilutions (Fig. 5); greater than 80% dilution is required to achieve appreciable (20% maximal) fluorescence. Liposomes containing HPTS/DPX will fluoresce only if they have leaked more than 50% of their contents. Fluorescence microscopy of HPTS/DPX-liposome uptake demonstrates that leakage subsequent to endocytosis is greater and at a lower pH than leakage at the cells surface; fluorescence is observed only in low pH, punctate compartments within the cells, and not on the cell surface (Fig. 6), although surface bound HPTS-containing liposomes are present (compare Figs. 2b, c and 5b).

Liposome leakage, as monitored by fluorescence dequenching with HPTS/DPX, is concomitant with acidification (Fig. 7). Cells induce an initial rapid dilution of liposome contents; the quenching of cell-associated dye decreases to about 70% within 30 min (Fig. 7C), indicating a 9-fold dilution of liposomes contents (Fig. 5). This dilution represents dye lost to the environment from surface-bound liposomes or by regurgitation from the cells (50% as measured by the [^{3}H]DPPC/HPTS ratio) and leakage of dye from endocytosed liposomes, but retained within cells (remaining 40%), presumably in endosomes. Fluorescence microscopy indicates that the amount of leakage at the cell surface is small compared to leakage that occurs after endocytosis (Fig. 6). A continual slower dilution of liposome contents occurs and quenching eventually plateaus at approximately 40% (Fig. 7C). This signifies an average 5-fold dilution of the dye/quencher mixture remaining with the cells (Fig. 5). During the same time, most of the liposomes (60%) rapidly accumulate in a low pH compartment.

In the preceding experiments, excess unbound liposomes are available to bind to the cells and replace those that have become internalized. Hence, the fraction of dye at low pH and the amount of liposomes that have leaked their contents may be greater for the liposomes that are internalized. To clarify the question of leakage at the cell surface, the experiment described in Fig. 7 was repeated, except unbound exogenous liposomes were removed after a 30 min incubation (Fig. 8). Liposomes initially bound to the cell either desorb from the cell surface or are endocytosed, and are not replaced by exogenous liposomes. As a result, the total amount of dye associated with the cells decreases continuously, possibly due to liposome loss from the cell surface or regurgitation of liposome contents from cells (Fig. 8A). In addition, the fraction of dye in a low pH environment increases to greater than 80% (Fig. 8B), indicating rapid internalization of remaining liposomes. Liposomes remaining associated with, or incorporated into, cells leak all of their contents (Fig. 8C). These results are consistent with nearly complete endocytosis of those liposomes bound to the cell within 30 min followed by leakage and breakdown of liposomes within 2 h, similar to results found with Kupffer cells [70]. The rapid leakage of liposomes occurs on a time scale faster than hydrolysis of liposomal phospholipid in lysosomes [71], indicating that liposome leakage probably occurs in endosomes without lipid breakdown. Finally, if the leakage at the cell surface was much slower than leakage after endocytosis, liposomes containing HPTS/DPX would report a lower pH than liposomes with HPTS alone. As shown in Figs. 7B and 8B, the time-course of acidification of HPTS liposomes and HPTS/DPX liposomes is identical. Thus, the rate of leakage of liposomes at the cell surface is at least as great as leakage after endocytosis. However, microscopy experiments indicate that leakage at the surface is less than 50%, while leakage after endocytosis is greater than 80%. An alternative explanation is that fewer liposomes remain bound to the cell surface compared with the number endocytosed and thus contribute less to the pH measurements.

The small amount of diffuse cytoplasmic fluorescence seen in Figs. 2 and 6 may represent a pathway for the delivery of material to the cytoplasm. Average pH measurements of HPTS pinocytosed by J774 cells differs significantly (0.9 pH units higher) from measurements made with the larger fluorescent probe fluorescein isothiocyanate dextran. Other macrophage cell lines do not show significant differences between these two measurements (Table II). These results imply that J774 macrophages, and not CV-1 fibroblasts [43] or other macrophages (RAW 264.7 and P388D1), have a mechanism for accumulation of HPTS in a higher pH compartment. This compartment may be the cytoplasm and represents 10–20% of the dye remaining associated with the cell after liposome treatment. Furthermore, J774 cells treated with HPTS-containing liposomes do not retain significant amounts of the dye after removal of exogenous liposomes (Fig. 8), however CV-1 fibroblasts treated similarly retain substantial amounts of dye up to 24 h [43]. It is possible that HPTS leaks out of endosomes or lysosomes into the cytoplasm via probenecid-inhibitable organic anion channels [72] or through leaky endosome-lysosome fusion. Once in the cyto-

plasm, HPTS may be transported out of the cell via anion channels in the plasma membrane resulting in rapid loss of the dye from the cell.

The present work has several consequences for liposome-mediated delivery of macromolecules. Cell surface-induced leakage lowers the effective concentration of drug for delivery to cells. The apparent protective effect of antibody binding on cell-surface-induced leakage could indicate that in vivo opsonization of liposomes by plasma proteins may afford some protection to the cell-surface-induced leakage of liposome contents. Leakage of contents during endocytosis results in labelling of the aqueous compartments of endosomes and exposure of the drug to lysosomal enzymes. Efflux of dye from endosomes into the cytoplasm before exposure to lysosomes indicates a (minor) pathway whereby small anionic molecules may avoid degradation in lysosomes. Finally, retention of the liposomal lipid component after endocytosis indicates that lipophilic compounds may be delivered more effectively to cells than small water-soluble compounds.

In conclusion, the use of HPTS as a liposomal contents marker allows a detailed kinetic analysis of liposome binding, endocytosis and leakage by macrophages. It is readily applied to fluorescence microscopy and can be easily quantitated by spectrofluorometry. In conjunction with liposomal lipid markers or fluorescence quenchers, the dye reveals information regarding the selective uptake of liposomal components and the leakage of liposomal contents from liposomes and subsequently from macrophages. Ongoing work is directed towards understanding the mechanisms that trigger liposome endocytosis and requirements for the recognition of liposomes by cells, with emphasis on liposomes of lipid compositions that show decreased in vivo uptake by macrophages [33–35].

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