International Journal of Pharmaceutics, 45 (1988) 169–177
Elsevier

IJP 01547

Mechanism of Nile red transfer from o/w emulsions as carriers for passive drug targeting to peritoneal macrophages in vitro

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(Received 19 June 1987)
(Modified version received 11 January 1988)
(Accepted 22 January 1988)

Key words: Emulsion drug carrier; Lipid transfer; Phagocytosis; Collision complex

Summary

The uptake of the lipid-soluble fluorescent dye Nile red by plated mouse peritoneal macrophages at 37°C from 5% sesame oil emulsions containing various surface active agents was not significantly affected by sodium azide, cytochalasin B, and pretreatment of the macrophages by glutaraldehyde, albeit somewhat reduced at 4°C. These findings supported that the lipid transfer was not due to phagocytosis of oil droplets by the cells. The uptake rate was proportional to Nile red concentration when the emulsion concentration was maintained constant. However, when the concentration of emulsion as well as Nile red varied proportionally, the plot of uptake rate against the Nile red concentration resembled that of a saturable kinetic process. An inverse relationship was observed between the Nile red transfer rate and the partition coefficient of Nile red between the oil used in the emulsion preparations and a phosphate buffer. This observation was made using a series of binary mixtures of triacetin and trioctanoin as the oil phase of emulsions. Finally, the rate of Nile red was at least 10-fold smaller when the emulsion was physically separated from the macrophage monolayer by a polycarbonate membrane with 0.1 μm porosity. The average diameter of emulsion droplets was 0.2 μm when determined by photon correlation spectroscopy. These observations together with non-endocytic uptake described above support that the Nile red transfer to macrophage monolayers is collision-mediated rather than via a sequential desorption–diffusion–partition process.

Introduction

As part of the host defense mechanism, the mononuclear phagocytic cell system, commonly known as reticuloendothelial system (RES), takes up blood-borne foreign particulates such as invading pathogens or endogenous debris such as senescent erythrocytes. This natural process offers a potentially powerful method for targeted drug delivery to the RES by means of colloidal particulate drug carriers. The concept as applied in liposomal suspensions has been referred to as passive (Poste, 1983) or natural (Weinstein, 1983) drug targeting.

Development of liposomes as a drug carrier can be a formidable, if not impossible, task because of uncertain long-term physical stability (Crommelin et al., 1986). In contrast, pharmaceutical technol-

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ogy for various fat emulsions has been available in recent years, as shown by several commercial products for total parenteral nutrition. These oil-in-water (o/w) emulsions usually contain phosphatidylcholines from either egg or soybean at a concentration ranging from 1.2 to 2.5% (Pelham, 1981; Hansrani et al., 1983). The lipid molecules are presumably located at the oil/water interface with the polar choline phosphate group facing the bulk aqueous phase and the acyl moieties partitioning into the oil phase. Thus the interface of oil droplets in an o/w emulsion system should be very similar to that of liposomes.

The initial and obligatory event involved in the phagocytic process is the contact and adhesion of particles to the cell surface (Ueda et al., 1981). The step, if viewed as a surface recognition phenomenon (Van Oss, 1978), may not differentiate oil droplets in an emulsion from liposomes. Uptake of liposomes by macrophages particularly by Kupffer cells of the liver, has been extensively studied in recent years (Dijkstra et al., 1985), and a similar endocytic process has been suspected for o/w emulsions. Indeed there has been some supportive evidence in the literature that intravenously administered certain emulsions are also cleared by the RES (DiLuzio, 1972; Koga et al., 1975; Laval-Jeantet et al., 1982; Davis and Hansrani, 1982; Bucala et al., 1983; Nugent, 1984; Mizushima, 1985). Some of earlier in vitro studies indicated the importance of opsonization in the phagocytosis of oil droplets by macrophages (Mason et al., 1973; Cox and Stossel, 1976). Factors in serum which coat particle surfaces in such a way that the particles are more readily ingested by phagocytes have been designated as opsonins (Griffin, 1977, 1982). In short, it is well established that oil droplets in an emulsion system are phagocytosed by macrophages, especially when they are properly opsonized. What is not clear, however, is whether the transfer of substances incorporated in the oil droplets to macrophages occurs exclusively along with the phagocytosis of the droplets. The present study aims to elucidate the mechanism of transfer of a lipophilic substance, Nile red, from various o/w emulsions to plated peritoneal macrophages harvested from thioglycollate-treated mice.

Materials and Methods

Preparation and characterization of emulsions

A stock solution of Nile red (Molecular Probes) in sesame oil, NF (Norda) was prepared by filtering a saturated solution using a 0.22 μm Millex-GS filter (Millipore). Nile red concentration was determined spectrofluorometrically (see below) and adjusted to 0.5 mg/ml. Aqueous phases used in the emulsion preparations all contained 2.0% glycerin, USP. In addition, they contained various surface active agents; 1.2% Infusol (American Lecithin) for negatively charged emulsions, 0.25% Tween 80 (ICI Americas) and 0.25% Arlacel 60 (ICI Americas) for emulsions with no surface charge, and 0.25% Tween 85 (ICI Americas) and 0.25% Arquad 2HT-75 (Armak Chemicals) for positively charged emulsions. Into 19.0 ml of aqueous phase, 1.0 ml of the Nile red stock solution in sesame oil was injected using a syringe while emulsification was taking place in a Polytron homogenizer (Brinkman). This primary emulsion was further emulsified in a sonicator (Branson Model 350 Sonifier) equipped with a probe of 12 mm diameter for 5 min at power setting 5 and 50% duty cycle. Emulsification was conveniently carried out in a rosette flask placed in an ice bath, always immediately prior to an uptake study.

ζ-Potential, as a measure of surface charge, was determined on PenKem Model 501 Laser Zee Meter. Emulsions were diluted 1000-fold in a cell incubation medium in which Nile red uptake was studied (see below). Size of oil droplets was determined with a Nicomp Model 200 Laser Particle Sizer after 1000-fold dilution in the medium. Gaussian analysis was made in relative area mode. The concentration of Nile red in an emulsion was determined after 1000-fold dilution in calcium- and magnesium-free phosphate buffered saline (CMF). Aliquots of this diluted emulsion, usually 0.05 ml, were combined with 0.4 ml of 0.05% Triton X-100 (Sigma) containing 0.01% bovine serum albumin (BSA, Sigma). Fluorescence intensity was measured as described below.

Spectrofluorometric analysis of Nile red

Fluorescence intensity was measured with an SLM/Aminco Model 500 PFS Spectrofluorometer

with excitation and emission at 488 and 530 nm, respectively. Bandpasses were set at 2 and 20 nm for excitation and emission, respectively. High voltages on the sample and reference channels were set at 985 and 515 mV, respectively. Calibration curves were obtained each day of the experiment using a series of standard Nile red solutions in heptane (Burdick and Jackson) of known concentrations. When samples were in aqueous media, 0.8 ml was extracted with 3.0 ml heptane. An aqueous stock solution of Nile red was prepared by filtering a supersaturated solution in the medium used in the uptake studies (see below) which also contained 0.2% bovine serum albumin. This solution was further diluted to a series of standard solutions with varying concentrations of Nile red but constant Triton X-100 and BSA concentrations at 0.05% and 0.01%, respectively. Calibration curves were obtained from fluorescence intensity measured in the heptane layer after extraction. Nile red detection level was in the order of 0.1 ng/ml.

Uptake of Nile red by macrophages from emulsions

Thioglycollate-elicited mouse peritoneal macrophages were obtained as follows. Female CF-1 mice weighing 15–17 g (Charles River Labs) were stimulated in vivo by injection of 1.0 ml fluid thioglycollate medium, USP (BBL Microbiology Systems) into the peritoneal cavity. On day 3, mice were sacrificed by cervical dislocation. Peritoneal exudates were obtained by lavage with 10 ml of 0.02% EDTA in CMF. Exudate was placed in a 50 ml polyethylene centrifuge tube (Corning) and centrifuged for 10 min at 200 *g*. Cell pellets were resuspended in RPMI 1640 (Gibco) supplemented with 50 U/ml penicillin (Gibco), 50 U/ml streptomycin (Gibco), 1.0 mM HEPES (Sigma), and 10% fetal bovine serum (Hyclone). This medium without the fetal bovine serum is hereafter referred to as RPMI. Normally 5×10^6 cells were obtained from each mouse, and the cell concentration in the final suspension was adjusted to approximately 7×10^5 cells/ml. Cell concentration was determined by means of a hemacytometer.

To each well of 24-well tissue culture plate (Corning) 1.0 ml of the final cell suspension was added. Cells were incubated for 60 min at 37°C under 5% CO_2 atmosphere (Queue Model 2220/2221 CO_2 Incubator), and then washed with CMF to remove non-adherent cells. In separate preliminary experiments, approximately $88 \pm 10\%$ ($n = 3$) of the cells recovered at this stage was found to be positive for immunological staining with rat monoclonal anti-mouse macrophage IgG (Sera Labs, MAS 0346). Cells were further incubated overnight in fresh RPMI with serum.

Transfer of Nile red from oil droplets of o/w emulsions to plated macrophages was studied as follows. After adhered cells were washed with CMF, 1.0 ml of emulsion samples diluted (commonly 500-fold) in RPMI was added to each well at time zero. The Nile red concentration ranged from 7.5 to 50 ng/ml in the incubation with the macrophages. Studies seldom lasted longer than 60 min. Using a longer incubation or higher emulsion concentrations resulted in cell detachment. At a given time interval, the wells were drained off and immediately rinsed vigorously in 4 consecutive liters of wash solutions consisting of 2 liters of 0.01% BSA in CMF and 2 liters of CMF. Wash solutions were all at 4°C to inhibit further uptake of Nile red. A similar procedure was reported (Swanson et al., 1985). A statistically significant number of wells was selected at random from the culture plates for Lowry protein assay. Any loss of protein observed over time was considered as an indication of toxicity to the cells. Viability of the cells was routinely tested by exclusion of a 4% Trypan blue solution in CMF.

To each of the wells set aside for protein assay, 0.4 ml of 0.5 N NaOH containing 0.5% sodium deoxycholate (Sigma) was added. The cell layer in each well was scraped off with a rubber policeman and an aliquot of 0.2 ml was subject to Lowry assay. Each well contained approximately 10 μg of cellular proteins. A series of protein standard solutions for calibration curves were prepared in 0.5 N NaOH containing 0.5% sodium deoxycholate from a BSA stock solution at 1.0 mg/ml. Each of the wells set aside for Nile red assay was treated with 0.4 ml of 0.05% Triton X-100 containing 0.01% BSA to permeabilize cells. Wells were scraped with a rubber policeman. The resulting solution of cell debris was extracted with 3.0 ml of heptane and the fluorescence intensity was measured as

described earlier. Accumulation of Nile red for each well was expressed in terms of ng Nile red/mg protein. Each time point reported is a mean of duplicate or triplicate determinations. Background fluorescence from cells alone was always negligible.

Modified uptake studies

Effects of various inhibitors of cellular uptake were studied as follows. Immediately before monitoring Nile red uptake, plated macrophages were preincubated at 37°C for 10 min with 10 mM sodium azide (Sigma) or 5 μg/ml cytochalasin B (Adrich). Cells were washed with CMF and added was 1.0 ml of emulsion diluted in RPMI which also contained 10 mM sodium azide or 5 μg/ml cytochalasin B. In addition to these inhibitory tests, glutaraldehyde fixed cells were also tested for Nile red uptake. Plated macrophages were treated with 0.5% glutaraldehyde (Sigma) in CMF for 5 min at room temperature (RT), washed with and incubated in 0.2 M glycine in CMF for 5 min at RT. Finally the cells were washed with CMF and incubated in RPMI containing emulsions.

Nile red transfer from emulsions which were physically separated from the plated macrophages by means of microporous membranes was studied as follows. Six-well tissue culture plates were prepared by attaching a silicone gasket of 25 mm o.d. and 1.0 mm thickness (Millipore) to the bottom of the well with medical adhesive (Dow 891). A glass tube also 25 mm o.d. was cut into 2.0 cm length. At one end of the glass tube, 0.1-μm polycarbonate membrane with 25 mm diameter (Nuclepore) was attached with the medical adhesive. The glass tube, with the membrane end down on the silicone gasket, formed the upper chamber. The plates and glass tube with the membrane were allowed to dry overnight. The culture wells thus prepared were sterilized with UV irradation from a 4 W light for 30 min. Cells harvested from mice as described above were allowed to adhere within the area of the silicone gasket at the bottom of the well, to which 0.4 ml of RPMI was added. The upper chamber containing 1.0 ml of emulsion diluted 1000-fold in RPMI was gently overlaid avoiding entrapment of air bubbles. At appropriate time intervals, chambers were removed from the wells. The cells in the wells were washed and processed as described earlier for Nile red determination.

Partition coefficient of Nile red between oil and water

Oil phases were composed of mixtures of triacetin (Union Carbide) and trioctanoin (Eastman) with varying composition. Vials containing 5.0 ml of 0.01 M-phosphate buffered saline at pH 7.4 and 0.5 ml of 0.9 ng/ml Nile red in a given oil mixture were shaken at RT for 24 h on a wrist action shaker. The aqueous phase obtained upon standing was filtered through a 0.22 μm Millex-GS syringe filter. The filtrate was clear to the naked eye. The concentration of Nile red in both aqueous and oil phases was measured following the procedure described earlier.

Results and Discussion

Transfer of a lipophilic substance incorporated in oil droplets of an o/w emulsion to plated macrophages can occur via one of the following 3 limiting mechanisms. The cellular uptake can be exclusively phagocytic. That is, the appearance rate for an entrapped substance in the cells can be identical to that of oil. One can a priori envision this process dominating when the oil droplets are properly opsonized and the receptor-mediated endocytosis is not saturated. Transfer of Oil red O from a diisodecyl phthalate emulsion in the presence of *E. coli* lipopolysaccharide (Cox et al., 1976) to macrophages occurs presumably through the phagocytosis of the droplets.

Alternatively, the entrapped substance can be transferred to the acceptor cells via rate-determining dissociation, convection through the aqueous medium, and association with the cell membrane. Such a process should be analogous to transfer between liposomes of certain types of phospholipids (Nichols and Pagano, 1981), cholesterol (McLean and Phillips, 1981; Bar, 1986), or long-chain fatty acids (Storch and Kleinfeld, 1986). Another non-phagocytic transfer process entails partition of the entrapped substance at the time of collision between donor and acceptor. Such a collision-

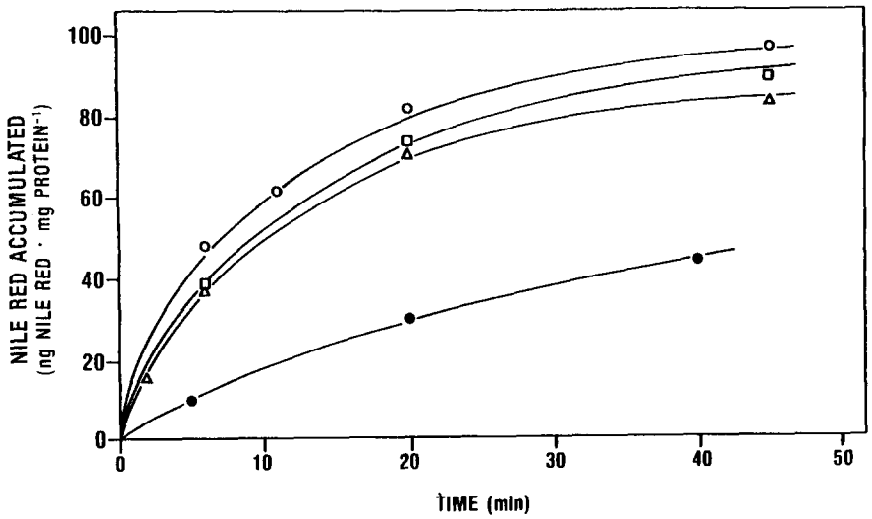

Fig. 1. Uptake of Nile red by mouse peritoneal macrophages from a Nile red-containing o/w emulsion. The cell monolayer was incubated in a medium containing 39 ng Nile red · 2.0 μl emulsion^{-1} · ml RPMI 1640 medium^{-1} at 37 °C (○), 4 °C (●), in the presence of 5 μg/ml cytochalasin B (□) at 37 °C and 10 mM sodium azide (△) at 37 °C.

mediated transfer was recently reported for exchange of lipid molecules from unilamellar vesicles as the donor to brush border vesicles as the acceptor (Mütsch et al., 1986).

The present study aims to elucidate the transfer mechanism involved in the appearance of Nile red in plated macrophages in the absence of any potential opsonins. As shown in Fig. 1, the accumulation curve was generally curvilinear under most of the experimental conditions studied over a 50 min period. In each well, plated cells were incubated with 2.0 μl of an emulsion containing 30 ~ 60 ng Nile red, in total 1.0 ml RPMI. Total cellular protein recovered from a confluent cell layer usually amounted to 13.5 μg. Thus, the amount of Nile red accumulated in the cells over a 50 min period in the absence of any inhibitors accounts for about 2 ~ 5% of the initial Nile red present in the medium. At 4 °C, the uptake was significantly reduced. However, within experimental error, there was no significant difference in the accumulation curve regardless of whether the study was conducted in the presence of a metabolic inhibitor sodium azide or an inhibitor of phagocytosis cytochalasin B.

Quantitative information derived not only from Fig. 1 but also from similar experiments with emulsions with difference surface charge is listed in Table 1. The slope of the straight line between data points 15 and 25 min was arbitrarily chosen as the transfer rate in the table. Sodium azide at 10 mM reduced the rate by 8%, a condition under which phagocytosis is effectively inhibited (Ueda et al., 1981). Similarly, cytochalasin B at 5 μg/ml reduced the transfer rate by only 4%. Still in another experiment, pretreatment of the cells with

TABLE 1

Nile red (NR) transfer rate (ng NR · mg protein^{-1} · h^{-1}) from sesame oil emulsions to plated mouse peritoneal macrophages at 37 °C under various experimental conditions

Effect of surface charge at total 38 ng NR per well		
ζ-Potential (mV)	Transfer rate	Ratio
−23	53	1.0
+1	42	0.8
+9	51	1.0
Effect of inhibitors at total 54 ng NR per well (see Fig. 1)		
Inhibitor	Transfer rate	% Inhibition
None	150	0
Azide	138	8
Cytochalasin B	144	4
4 °C	70	53

0.5% glutaraldehyde reduced the rate only about 6% (raw data not reported).

In contrast, as much as 50% of the transfer rate was inhibited at 4°C. Thus, inhibitors of cellular metabolism and phagocytosis were only slightly effective in preventing the Nile red transfer from oil droplets to the cells at 37°C. Furthermore, incapacitating the cells by cross-linking the free amino groups and perhaps other nucleophiles in the cellular membrane did not inhibit the lipid transfer. From these observations it is proposed that the Nile red transfer to the macrophages is not through phagocytosis. Data obtained at 4°C are also consistent with the notion that there was significant accumulation of Nile red in the cells. At 4°C, little phagocytic activity is usually observed (Silverstein et al., 1978; Besterman and Low, 1983). Great care was taken in this study to establish the physical stability and homogeneity of the emulsions under experimental conditions. In every case the particle size did not change significantly from 0.2 μm diameter. It was also established that the Nile red concentration in the RPMI medium remained constant at 37°C throughout the experiment. It, therefore, rules out other possible explanations based on uncertain system stability such as change in size (Pratten and Lloyd, 1986) in deriving the above conclusion. Finally, lack of surface charge effect on the transfer rate is also consistent with a non-phagocytic mechanism of transfer. Had the process been primarily phagocytic, difference in surface charge would have influenced the uptake rate (Davis and Hansrani, 1985).

As shown in Fig. 2, the Nile red transfer rate was proportional to the initial Nile red concentration when the number of oil droplets in the system was maintained constant. The experiments were conveniently carried out using a series of emulsions diluted in a blank emulsion containing no Nile red. On the other hand, when not only Nile red concentration but also the number of oil droplets varied, the transfer rate was steadily smaller than expected from the proportionality as the number of oil droplets increased. This observation cannot be explained by the mechanism of rate-determining Nile red desorption from oil droplets. The conclusion was derived from the following deduction. Although the present experiment was not run at a constant Nile red concentration, the results imply that the transfer rate would have been much smaller at a high oil concentration, had the Nile red concentration been held constant. If the desorption of Nile red from

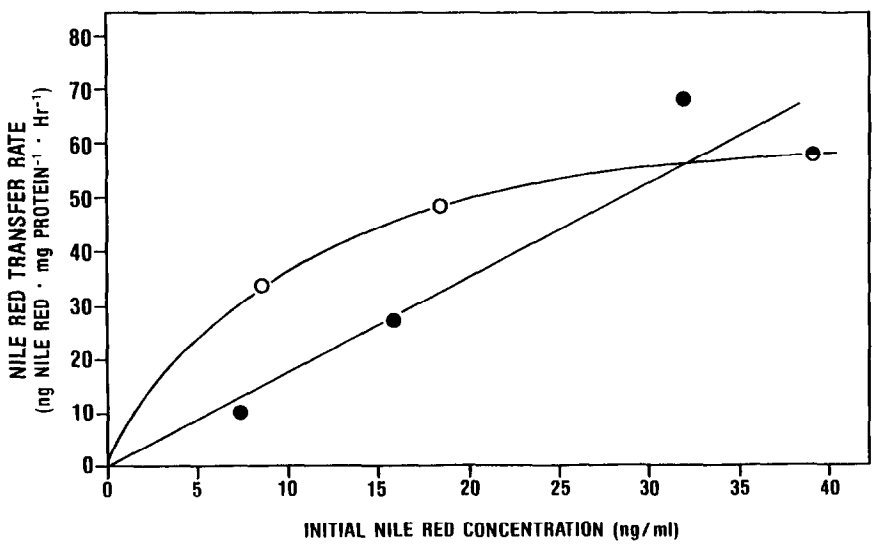

Fig. 2. Rate of Nile red transfer from an o/w emulsion to mouse peritoneal macrophages at 37°C as a function of initial Nile red concentration at a constant emulsion concentration (●) and at emulsion concentrations proportional to that of Nile red (○).

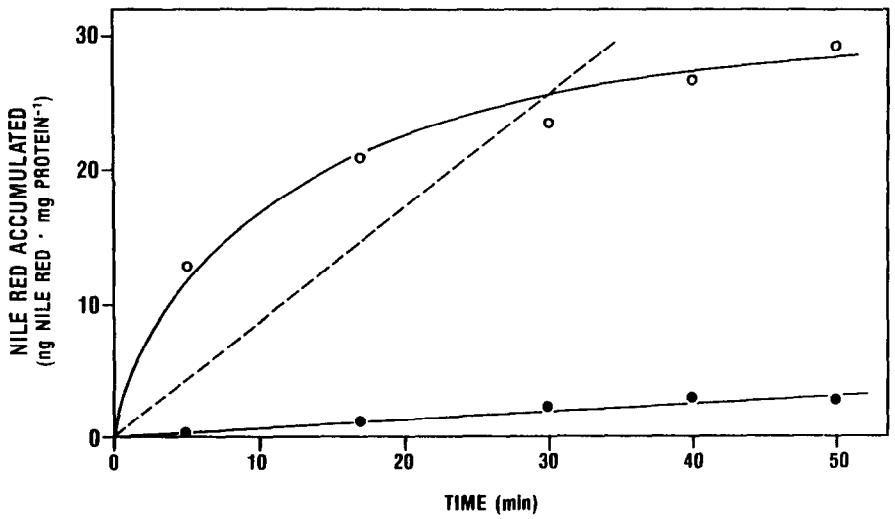

Fig. 3. Uptake of Nile red by mouse peritoneal macrophages from a Nile red-containing o/w emulsion at 37°C. The cell monolayer was incubated in a medium containing 15 ng Nile red · μl emulsion^{-1} · ml RPMI 1640 medium^{-1}; spontaneous transfer (○) and transfer through a 0.1 μm polycarbonate membrane (●). Dotted line represents the appearance of Nile red in the cell-free acceptor chamber which was separated by the membrane from a Nile red solution in 0.2% bovine serum albumin in the donor chamber.

the surface oil droplet into the bulk aqueous phase had been rate-limiting, the overall transfer rate would have been independent of the oil concentration (i.e., the number of oil droplets). It is because the rate would be proportional to the product of the surface concentration of Nile red and total interfacial area, which must be constant under the above hypothetical experimental conditions. A similar kinetic analysis was presented for lecithin and cholesterol exchanges between liposomes and lipoprotein particles (Jonas and Maine, 1979).

One possible explanation for the saturable pro-

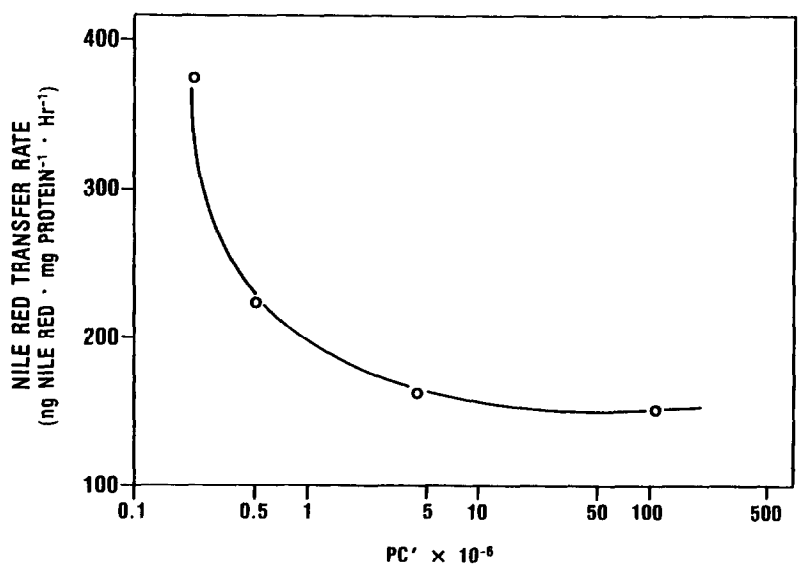

Fig. 4. Rate of Nile red transfer at 37°C from oil droplets in an emulsion to plated macrophages as a function of the apparent partition coefficient (PC′) of Nile red between the oil used in the emulsion preparation and an aqueous buffer. Oils used were mixtures of triacetin and trioctanion at various compositions.

cess of Nile red shown in Fig. 2 is a collision complex model in which the residence time of an oil droplet attached onto the macrophage monolayer subsequent to collision with the cell layer exceeds the time required for Nile red to partition to the cell surface. In this model, up to a certain concentration of donor particle relative to the acceptor concentration the transfer rate would be proportional to the donor particle concentration, although the concentration of a lipid marker is kept at a constant value. As the number of donor particles increases, however, more of the acceptor surface would be occupied by the donor particles from which the lipid molecules have already departed. A saturable kinetic process of particle adherence onto mouse peritoneal macrophages was thoroughly analyzed in the literature (Ueda et al., 1981).

Collision-mediated transfer of Nile red from oil droplets of an o/w emulsion to plated mouse peritoneal macrophages was further tested by physically separating the donor particles from the cell layer by means of a polycarbonate membrane of 0.1 μm porosity. As shown in Fig. 3, Nile red accumulation in the macrophages was minimal when the emulsion was added to a chamber which was separated from the cell layer. In a separate experiment, it was demonstrated that the Nile red appearance in the receiving well was at least an order of magnitude faster when the same amount of Nile red solubilized in 0.2% bovine serum albumin was placed in the donor chamber. That is, Nile red molecules in a solution, most likely as complexed to bovine serum albumin, were able to equilibrate rapidly between the chambers which were separated by the polycarbonate membrane. Once again, had the desorption–convection–adsorption mechanism prevailed, the transfer rate would not have been affected by the presence of the porous membrane.

Although preceding data are all consistent with a view that the Nile red transfer occurs via a collision complex, it remains uncertain if diffusion within the complex in aqueous environment is significant. As shown in Fig. 4, the transfer rate was critically dependent upon the partition coefficient of Nile red between an oil used in emulsion preparation and an aqueous medium; the higher partition coefficient, the slower the transfer rate. A wide range of partition coefficient was conveniently obtained using a series of binary mixtures of triacetin and trioctanoin, with varying composition ranging from 25% to 75% (v/v) triacetin. If one considers the free energy of partitioning as the driving force for molecular transfer to another phase, diffusion in an aqueous environment with a collision complex is supported by the results shown in Fig. 4.

In conclusion, the present study supports that the transfer of Nile red from various o/w emulsions to plated mouse peritoneal macrophages occurs via a collision complex mechanism. Although other lipophilic substances, when incorporated into o/w emulsions, may behave differently, this study does suggest that non-specific transfer to circulating cells or adipose tissue can occur in vivo prior to phagocytosis by the RES. Such a possibility should be seriously assessed before an o/w emulsion system is proposed as a drug carrier for passive or natural targeting to the RES.

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