Spectrofluorometric studies of the lipid probe, nile red

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Abstract We found that the dye nile red, 9-diethylamino-5H-benzo[α]phenoxazine-5-one, can be applied as a fluorescent vital stain for the detection of intracellular lipid droplets by fluorescence microscopy and flow cytofluorometry (*J. Cell. Biol.* 1985. **100:** 965–973). To understand the selectivity of the staining, we examined the fluorescence properties of nile red in the presence of organic solvents and model lipid systems. Nile red was found to be both very soluble and strongly fluorescent in organic solvents. The excitation and emission spectra of nile red shifted to shorter wavelengths with decreasing solvent polarity. However, the fluorescence of nile red was quenched in aqueous medium. Nile red was observed to fluoresce intensely in the presence of aqueous suspensions of phosphatidylcholine vesicles (excitation maximum: 549 nm; emission maximum: 628 nm). When neutral lipids such as triacylglycerols or cholesteryl esters were incorporated with phosphatidylcholine to form microemulsions, nile red fluorescence emission maxima shifted to shorter wavelengths. Serum lipoproteins also induced nile red fluorescence and produced spectral blue shifts. Nile red fluorescence was not observed in the presence of either immunoglobulin G or gelatin. These results demonstrate that nile red fluorescence accompanied by a spectral blue shift reflects the presence of nile red in a hydrophobic lipid environment and account for the selective detection of neutral lipid by the dye. Nile red thus serves as an excellent fluorescent lipid probe. —**Greenspan, P., and S. D. Fowler.** Spectrofluorometric studies of the lipid probe, nile red. *J. Lipid Res.* 1985. **26:** 781–789.

Supplementary key words 9-diethylamino-5H-benzo[α]phenoxazine-5-one • neutral lipids • fluorescent hydrophobic probe • lipid histochemistry • lipoproteins • membranes • phospholipids

Nile red is a red phenoxazone dye that is present in trace amounts in commercial preparations of the lipid stain nile blue (1). Nile red is responsible for the metachromatic coloring of tissue lipids by staining procedures that utilize the nile blue dye (2). We have recently synthesized nile red and found that it can be used on living cells as a fluorescent stain for the detection of intracellular lipid droplets by fluorescence microscopy (3). An example of the application of nile red as a lipid stain is illustrated in **Fig. 1**. Stained cultured macrophages previously incubated with acetylated low density lipoprotein to induce intracellular lipid droplets, as described by Goldstein, Brown and colleagues (4, 5), are shown in Fig. 1a; stained control macrophages are shown in Fig. 1b for comparison. Numerous large fluorescent bodies are evident in the cells incubated with acetylated low density lipoprotein, thus demonstrating that the nile red staining reveals cytoplasmic lipid droplets. We have also successfully employed nile red staining of these same cells to quantitate by flow cytofluorometry the degree of cellular lipid overloading (3).

We found that the best selectivity for cytoplasmic lipid droplet staining is obtained when nile red-treated cells are examined for yellow-gold fluorescence (528 nm) rather than for red fluorescence (>610 nm) (3). With the latter conditions, a diffuse staining of the entire cell is observed; while with the former, a more selective staining of lipid droplets is obtained. To understand these observations, we conducted spectrofluorometric studies on the interaction of nile red with various lipid models including phospholipid vesicles, serum lipoproteins, hepatic microsomes, and isolated adipocytes. Our investigations, which we present here, demonstrate that nile red acts as a hydrophobic probe in which the fluorescence maxima exhibit a blue-shift proportional to the hydrophobicity of the environment. This, and the preferential solubility of the dye in lipid, account for the selective staining of the intracellular lipid droplets by nile red. Our studies also suggest that the unique fluorochromic properties of nile red could be of great use to lipid chemists as a tool to investigate the hydrophobic domains of lipoproteins and cell membranes.

A preliminary report of this work has been presented previously (6).

MATERIALS AND METHODS

Chemicals

Nile blue chloride (CI 51180, dye content approximately 95%) was purchased from Sigma Chemical Co. Phosphatidylcholine (egg lecithin) was obtained from Lipid Products, Surrey, England; the other lipids were purchased from Sigma Chemical Co. Metrizamide was purchased from Nyegaard and Co., Oslo, Norway. Organic solvents were reagent grade and were obtained either from Fisher Scientific or Sigma Chemical Co. Bovine serum albumin

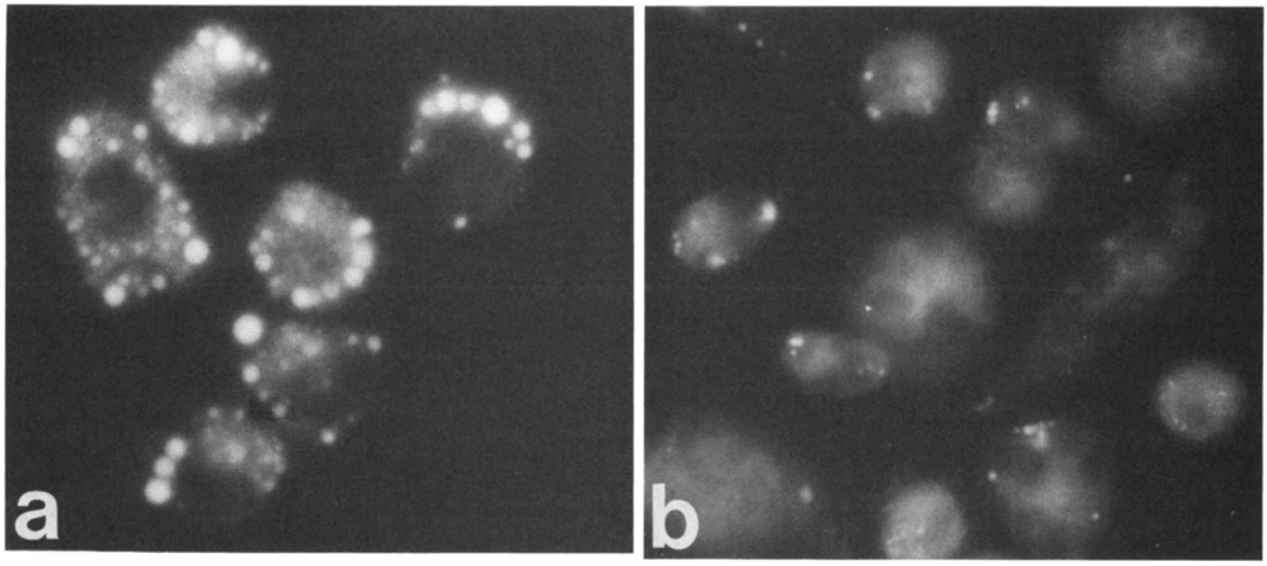

Fig. 1. Nile red fluorescence of lipid-loaded mouse peritoneal macrophages. (a) Cultured macrophages previously incubated 40 hr with acetylated low density lipoprotein (50 μg protein/ml) in 10% fetal calf serum to induce production of cytoplasmic lipid droplets (4, 5). (b) Control cultured macrophages incubated a similar period in the presence of 10% fetal calf serum only. Prior to staining, the cells were fixed with 1.5% glutaraldehyde in phosphate-buffered saline and washed with buffered saline. Nile red concentration: 100 ng/ml. Excitation wavelength, 450–500 nm; emission wavelength, >528 nm. Magnification, ×1040.

(essentially fatty acid-free) was purchased from Sigma Chemical Co. and further purified from fatty acids by the method of Chen (7).

Preparation of nile red

Nile red[1] was prepared from nile blue by dissolving 0.92 g of the latter in 500 ml of 0.5% H_2SO_4 and boiling it under reflux for 2 hr (**Fig. 2**) (8). After cooling, nile red was separated by repeated extraction into equal volumes of xylene until only a faint pink color appeared in the organic phase. The organic solvent was removed by flash evaporation, leaving a dark purple residue (0.18 g). The latter was identified as nile red on the basis of ultraviolet and infrared spectra (9, 10). The compound exhibited a sharp melting point (192–193°C). The preparation appeared to be >98% pure based on high pressure liquid chromatography in three solvents: acetonitrile–water 90:10 (v/v); acetonitrile–tetrahydrofuran 80:20 (v/v); and a linear gradient of acetonitrile–0.1% acetic acid in water (25:75 (v/v) initial condition to 75:25 (v/v) final condition) on a μBondapak C18 column (Waters Associates, Inc.).

Preparation of lipid vesicles and microemulsions

Unilamellar vesicles of phosphatidylcholine (egg lecithin or dimyristoylphosphatidylcholine), or phosphatidylcholine (egg lecithin) and trioleoylglycerol (molar ratio 66:1) were prepared by sonication at 45°C for 12 min as described by Brecher et al. (11). Phosphatidylcholine (egg lecithin)/cholesterol (molar ratio 1:0.8) vesicles were prepared by sonication at 45°C for 1 hr. These preparations were centrifuged for 15 min at 20,000 rpm in a Beckman Ti-75 rotor (Beckman Instruments, Inc.) to remove any titanium particles produced by the sonication.

Microemulsions of phosphatidylcholine and trioleoylglycerol (molar ratio 1:1) were prepared by a 1-hr sonication at 45°C as described by Burrier and Brecher (12). The resultant mixture was centrifuged for 1 hr at 40,000 rpm in a Beckman Ti-75 rotor. The top 1–2 ml of solution was removed and the remaining infranatant was recentrifuged for 16 hr at 40,000 rpm. The upper 1 ml of the resulting supernatant was used as our microemulsion preparation. The dimyristoylphosphatidylcholine and cholesteryl linoleate (molar ratio 1:1) microemulsion was prepared as described by Ginsburg, Small, and Atkinson (13) with minor modifications, and was generously provided to us by Dr. John Parks, Bowman Gray School of Medicine, Winston-Salem, NC.

Isolation of lipoproteins

Lipoproteins were isolated from human plasma by sequential ultracentrifugation in KBr (14). The lipoproteins were exhaustively dialyzed against saline containing 0.01% EDTA, pH 7.4. The purity of the preparation was confirmed by cellulose acetate gel electrophoresis.

Isolation of adipocytes

Adipocytes were kindly provided by Dr. Donald O.

[1]In view of its heterocyclic structure, we handled nile red as a carcinogen, though no evidence exists that such is the case.

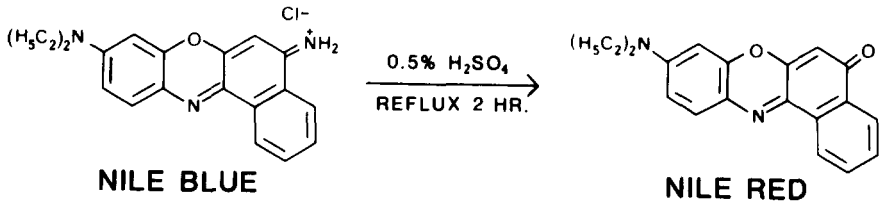

Fig. 2. Oxidation of nile blue to form nile red. After boiling nile blue in dilute H_2SO_4, the nile red produced is extracted into xylene. Nile blue, 5-amino-9-diethylamino-benzo[α]phenoxazine, is a basic dye, the chloride and sulfate salts of which are blue while the free base is red. Nile red, 9-diethylamino-5H-benzo[α]phenoxazine-5-one, is a neutral dye, red in color, and intensely fluorescent in the visible range.

Allen, Department of Pharmacology, School of Medicine, University of South Carolina. The cells were obtained from epididymal fat pads of adult rats by collagenase enzymic digestion as described by Lech and Calvert (15).

Preparation of microsomal membranes

A microsomal (P) fraction was prepared from a rat liver homogenate as described by de Duve et al (16). The preparation was diluted to 1 mg of protein/ml in 100 mM Na carbonate, pH 11.5, incubated at 0°C for 1 hr, and then centrifuged 1 hr at 50,000 rpm in a Beckman Ti-75 rotor. The pellet was resuspended with a Dounce homogenizer in 0.85% NaCl (pH 7.4). The carbonate treatment effectively strips ribosomes and peripheral proteins from the membranes (17).

Fluorescence spectroscopy

Excitation and emission fluorescence spectra were determined with a Perkin-Elmer 650-40 fluorescence spectrophotometer (Perkin-Elmer Corp) equipped with a 150 watt xenon lamp, concave grating monochromators, and a red sensitive Hamamatsu R928 photomultiplier tube. Except as indicated, spectra were recorded at room temperature with both slits set at 5 nm. The spectra presented are partially corrected by use of a ratio quantum counter system (ratio mode). They are shown plotted as wavelength versus normalized fluorescence intensity in which the y-axis scale units have been adjusted to place maximum peak heights at 70% of full chart scale. The relative fluorescence intensity of nile red in the presence of the various samples was obtained after subtraction of both the autofluorescence of the samples and the fluorescence intensity of nile red alone in buffer.

Assay for nile red

The content of nile red in aqueous solutions could be determined by extraction of the dye into 3 ml of iso-amylacetate with vigorous vortexing followed by brief centrifugation. The iso-amylacetate solvent is particularly useful if the aqueous sample contains protein. The fluorescence intensity of nile red in iso-amylacetate was measured using 10-nm slit widths at excitation and emission wavelength settings of 517 and 584 nm, respectively. A 10-nM solution of nile red in iso-amylacetate was employed as a standard. The fluorescence was found to be linearly proportional to nile red content up to a concentration of 15 μM. Nile red concentrations in organic solvents were similarly measured following evaporation of the solvent and dissolving the dye in 3 ml of iso-amylacetate.

Other assays

Protein was determined by the method of Lowry et al. (18) using bovine serum albumin as the standard. Phospholipids were quantitatively extracted by the method of Bligh and Dyer (19); they were ashed and the total phosphate was measured according to Ames and Dubin (20).

RESULTS

Physical properties of nile red

Nile red is a benzophenoxazone dye (Fig. 2); it is sometimes referred to in older literature as nile blue A-oxazone. **Table 1** lists some properties of nile red helpful for its identification and use. The dye dissolves in a wide range of organic solvents, but negligibly in water. It is also substantially less soluble in heptane and higher alkanes than in other solvents such as xylene, chloroform, or acetone. The partition coefficients of nile red in several organic solvents relative to water were found to be approximately 200 at 4°C. An exception was n-heptane for which the partition coefficient was found to be 58. Similar ratios were obtained when the solvent partitions were performed at room temperature.

Nile red is a dye that exhibits solvatochromism (9), its absorption band varies in spectral position, shape and intensity with the nature of the solvent (24). The dye is also highly fluorescent in organic solutions.[2] The fluorescence intensity of nile red approaches that of rhodamine B (Table 1).

[2]A variety of phenoxazone dyes have been examined for their fluorescence, and, of those studied, nile red was found to be the most intensely fluorescent (25).

TABLE 1. Physical properties of nile red

Molecular formula[a]	$C_{20}H_{18}N_2O_2$, m.w. = 318
Melting point	192–193°C
Solubility	1 mg/ml acetone 62 μg/ml n-heptane <1 μg/ml water
Partition coefficient (4°C)	xylene/water 210 chloroform/water 196 iso-amylacetate/water 198 n-heptane/water 58
Absorption maxima[b] ($HCCl_3$ solvent)	ultraviolet, 264 nm; visible, 538 nm
Infrared maxima ($HCCl_3$ solvent)[c]	1000, 1105, 1265, 1305, 1550, 1580, 1612 (C=O), 1710 cm^{-1}
Fluorescence intensity relative to rhodamine B ($HCCl_3$ solvent)[d]	0.36

[a]For reviews of the chemistry of phenoxazone dyes see refs. 21 and 22. For separation of nile red from nile blue by thin-layer chromatography, see ref. 23.

[b]Solvent dependent, visible absorption maxima range from 487 nm (isooctane) to 564 nm (formamide) (9, 23).

[c]Similar to data of Stužka et al. (10).

[d]Solutions (0.5 μM) measured at the excitation/emission maxima of nile red and rhodamine B, which were 543/595 nm and 554/579 nm, respectively.

Solvent effects on the fluorescence spectra of nile red

The emission spectra of many fluorescent compounds, especially those containing polar substituents on the aromatic rings, are known to be sensitive to the chemical and physical properties of solvents (26). As demonstrated in **Fig. 3**, nile red is a fluorophore of this kind. **Table 2** summarizes the fluorescence properties of nile red in eight different solvents. Both the excitation and emission maxima shift over a span of 100 nm, the colors ranging from golden yellow to deep red. The red shift of the maxima parallels increases in the dielectric constant of the solvent as expected for general solvent effects on fluorescence spectra (26). The relative fluorescence intensity of the dye is equal in all the nonpolar solvents examined. The fluorescence intensity of nile red in water, however, is 40-fold less.

Examination of the fluorescence spectra of nile red in organic solvents (Fig. 3) shows alterations in the shape of the spectral curves. This suggests the presence of specific solvent effects, such as hydrogen bonding or some other interaction. Confirming this notion, addition of small amounts of ethanol (0.1–1.0%) to a heptane solution of nile red resulted in red shifts in the fluorescence emission maxima of up to 50 nm (data not shown).

Nile red fluorescence in the presence of lipid

In view of likely complex interactions of nile red with organic solvents, we chose a more empirical approach to understand selective staining of lipids by nile red. We began by examining the fluorescence of the dye in the presence of aqueous suspensions of lipids. Similar to the fluorescence of nile red in various solvents, the excitation and emission spectra were different for various lipid preparations (**Fig. 4**, **Table 3**). For example, the fluorescence spectra of nile red in the presence of phosphatidylcholine (egg lecithin) vesicles are sharp and symmetrical, with excitation and emission maxima of 549 nm and 628 nm, respectively (Fig. 4a). In contrast, the fluorescence spectra of nile red in the presence of a phosphatidylcholine–trioleoylglycerol 1:1 microemulsion are shifted toward the blue region of the spectrum; the emission maximum of the microemulsion is at 576 nm, although the emission spectrum exhibits a broad shoulder that extends almost to the nile red maximum associated with phosphatidylcholine vesicles alone.

Fig. 4b shows the interaction of nile red with a microemulsion composed of cholesteryl linoleate and dimyristoylphosphatidylcholine. The fluorescence spectra of nile red in the presence of dimyristoylphosphatidylcholine vesicles alone is nearly identical to that recorded for egg lecithin vesicles (Fig. 4a). At 25°C, a small but significant blue shift is seen in the nile red fluorescence maximum in the presence of the dimyristoylphosphatidylcholine–cholesteryl linoleate 1:1 microemulsion, indicative of an interaction between the dye and the liquid crystalline cholesteryl linoleate. This interaction can be enhanced by heating the sample to 46°C. At this temperature, the cholesteryl linoleate core is in an isotropic liquid, disordered state (28). The fluorescence spectrum of nile red is then markedly shifted toward the blue region in a

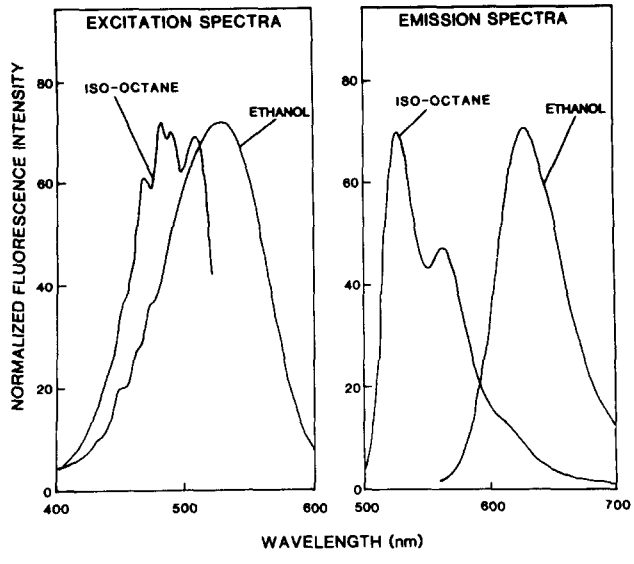

Fig. 3. Excitation and emission fluorescence spectra of nile red in isooctane and ethanol. A 1 μg/ml solution of nile red in each solvent was used. The excitation and emission spectra of nile red were recorded at their corresponding emission or excitation maxima.

TABLE 2. Fluorescence properties of nile red in various solvents

Solvent	Dielectric Constant[a]	Excitation Maximum	Emission Maximum	Relative Fluorescence Intensity
		nm	*nm*	
Water	78.5	591	657	18
Ethanol	24.3	559	629	355
Acetone	20.7	536	608	687
Chloroform	4.8	543	595	748
iso-Amylacetate	—	517	584	690
Xylene	2.4	523	565	685
n-Dodecane	2.0	492	531	739
n-Heptane	—	484	529	585

Nile red was analyzed at a concentration of 1 μg/ml in all solvents. Excitation and emission maxima were determined and the relative fluorescence intensity in each solvent was measured at the corresponding excitation and emission maxima.

[a] Derived from (27).

manner nearly identical to the spectral shift observed for the nile red fluorescence of the phosphatidylcholine–trioleoylglycerol 1:1 microemulsion. The temperature-dependent blue shift in the fluorescence spectra of nile red was not observed with dimyristoylphosphatidylcholine vesicles alone nor with the lipid preparations listed in Table 3.

As indicated in Table 3, the fluorescence properties of nile red in the presence of lipid suspensions are dependent on the concentration of nile red employed: *a*) the emission maxima vary depending on the amount of nile red used, and *b*) the relative fluorescence intensities observed in the presence of all the lipid preparations are dependent on the nile red concentration. We found that, at the concentrations of lipid used in this study, the fluorescence obtained increased linearly with dye concentration up to 0.5 μg of nile red/ml.

Nile red fluorescence in the presence of proteins

Nile red also fluoresces when combined with serum lipoproteins or proteins bearing hydrophobic domains. As shown in **Table 4**, the fluorescence of nile red bound to the neutral lipid-rich lipoproteins is strong. The excitation and emission maxima of nile red fluorescence in the presence of both very low density and low density lipoproteins are shifted to shorter wavelengths compared to nile red fluorescence in the presence of high density lipoprotein. Defatted albumin also induces nile red fluorescence, but the intensity is one-tenth that attained with very low density lipoprotein. In contrast, immunoglobulin G and gelatin, two hydrophilic proteins, did not induce nile red fluorescence. At the highest concentrations of nile red used (1.66 μg/ml), distinct differences in the fluorescence intensity of nile red with the different proteins are observed. The very low density lipoproteins, carrying the greatest mass of lipid, produce the most intense nile red fluorescence.

Fluorescence of nile red-stained adipocytes and microsomal membranes

As a final test of the fluorochromic differences attainable with nile red coloring, we compared nile red staining of two distinctly different biological systems: adipose tissue cells laden with neutral lipids, especially triacylglycerols, and microsomal membranes in which phospholipid is the principal lipid class present. The fluorescence spectra of the nile red-stained preparations are shown in **Fig. 5**. Nile red excitation and emission fluorescence maxima

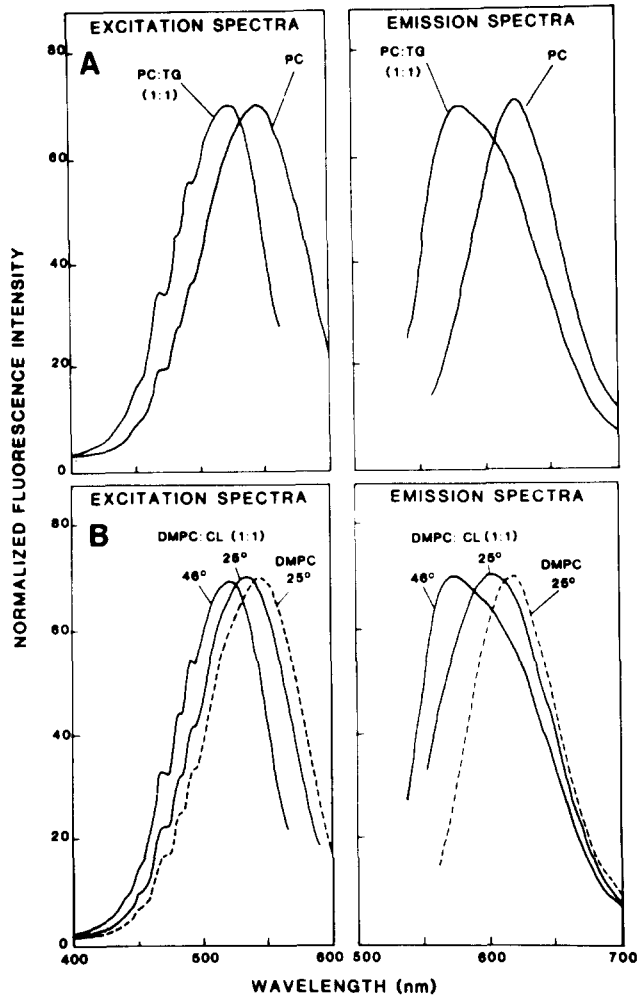

Fig. 4. Excitation and emission fluorescence spectra of nile red in the presence of phospholipid vesicles or phospholipid–neutral lipid microemulsions. (A) Nile red fluorescence spectra in the presence of phosphatidylcholine (egg lecithin) (PC) vesicles or phosphatidylcholine–trioleoylglycerol (TG) 1:1 (molar ratio) microemulsion. (B) Nile red fluorescence spectra in the presence of dimyristoylphosphatidylcholine (DMPC) vesicles or dimyristoylphosphatidylcholine cholesteryl linoleate (CL) 1:1 (molar ratio) microemulsion. The lipid suspensions were diluted to a concentration of 250 nmol of phosphorus/ml in phosphate-buffered saline. Five μl of an acetone solution of nile red (100 μg/ml) was mixed with 3 ml of the lipid suspension and the fluorescence spectra were recorded. The excitation and emission spectra were measured at their corresponding emission or excitation maxima.

TABLE 3. Fluorescence of nile red in the presence of aqueous suspensions of lipid

	0.166 μg Nile Red/ml		1.66 μg Nile Red/ml	
	Excitation, Emission Maxima	Relative Fluorescence Intensity	Excitation, Emission Maxima	Relative Fluorescence Intensity
	nm		*nm*	
Phosphatidylcholine vesicles	549,628	19	548,633	225
Phosphatidylcholine-trioleoylglycerol 66:1 vesicles	549,629	23	548,633	207
Phosphatidylcholine-trioleoylglycerol 1:1 microemulsion	529,576	23	534,623	210
Phosphatidylcholine-cholesterol 1:0.8 vesicles	549,605	22	550,621	206

Aqueous suspensions of lipid were prepared as described in Methods. The phosphatidylcholine was egg lecithin. Each preparation was diluted with phosphate-buffered saline to give a final concentration of 250 nmol of phosphorus/ml. Five μl of an acetone solution of nile red (0.1 mg/ml or 1 mg/ml) were mixed with 3 ml of a lipid suspension and the fluorescence spectra were recorded. The relative fluorescence intensity of nile red in the presence of each model lipid was measured at the corresponding excitation and emission maximum.

were recorded at 521 nm and 582 nm, respectively, for stained adipocytes (Fig. 5a). In contrast, the maxima occur at 540 and 624 nm, respectively, for nile red-stained microsomes (Fig. 5b). The spectra of the latter are similar to those observed for phosphatidylcholine vesicles colored with nile red whereas the former are similar to phosphatidylcholine–trioleoylglycerol 1:1 microemulsions treated with nile red (Fig. 4a).

DISCUSSION

Nile red exhibits spectral properties similar to the fluorescent probes TNS (2-*p*-toluidinylnaphthalene-6-sulfonate) (29) and PRODAN (6-propionyl-2-[dimethylamino] naphthalene) (30): *a*) quenched fluorescence in aqueous media, and *b*) blue shift of the fluorescence spectrum with decreasing polarity of the solvent environment. Both TNS and PRODAN have been widely used as hydrophobic probes. Our studies show that nile red can be similarly used as a hydrophobic probe for the detection of neutral lipids or mixtures of lipids and proteins that create a hydrophobic environment. We found that nile red will fluoresce intensely in the presence of phospholipid vesicles and that a blue shift in the fluorescence spectra will occur if neutral lipids such as cholesteryl esters or triacylglycerols are incorporated with the phospholipid to form microemulsions. Serum lipoproteins also induce nile red fluorescence, especially very low density lipoprotein and low density lipoprotein which also cause a spectral

TABLE 4. Fluorescence of nile red in the presence of proteins

	0.166 μg Nile Red/ml		1.66 μg Nile Red/ml	
Protein	Excitation, Emission Maxima	Relative Fluorescence Intensity	Excitation, Emission Maxima	Relative Fluorescence Intensity
	nm		*nm*	
Very low density lipoprotein[a]	530,576	33	526,586	424
Low density lipoprotein	531,584	33	532,603	286
High density lipoprotein	543,610	27	543,619	165
Albumin	553,620	3	568,640	40
Gelatin	–	–	593,657	1
Immunoglobulin G	–	–	–	–
Hepatic microsomal membranes	540,624	25	537,632	162

With the exception of hepatic microsomal membranes, proteins were diluted to a concentration of 100 μg/ml with phosphate-buffered saline. Hepatic microsomal membranes were diluted to the same concentration with phosphate-buffered saline containing 12% metrizamide. Five μl of an acetone solution of nile red (0.1 mg/ml or 1 mg/ml) were mixed with 3 ml of the protein solution and the fluorescence spectra were recorded. The relative fluorescence intensity of nile red in the presence of each protein was measured at the corresponding excitation and emission maxima.

[a]Isolated as lipoproteins of density < 1.019 g/ml, this preparation may also contain some intermediate density lipoprotein.

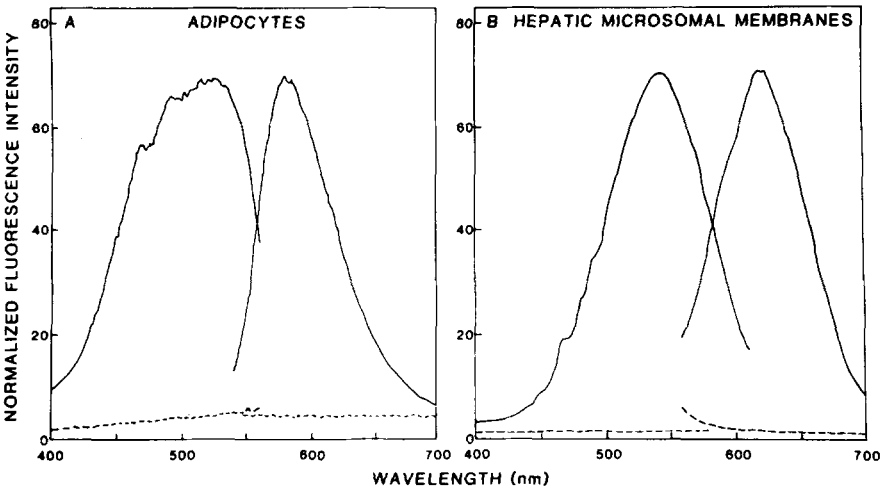

Fig. 5. Excitation and emission fluorescence spectra of nile red-stained rat adipocytes and hepatic microsomal membranes. (A) Aliquots of adipocytes, to which nile red was added to obtain a final concentration of 10 μg/ml, were placed in a 96-well black cell culture plate (Dynatech Corp.). The cells were allowed to float to the solution surface. The excitation (left) and emission (right) spectra were then recorded by measuring surface fluorescence using 10-nm slit width settings with a Perkin-Elmer TLC scanning attachment. The dashed lines represent the nearly superimposable fluorescence spectra of adipocytes in the absence of nile red and of an aqueous solution of nile red alone. (B) Hepatic microsomal membranes were diluted to a concentration of 100 μg protein/ml in phosphate-buffered saline containing 12% metrizamide (to slow sedimentation of the vesicles). Five μl of an acetone solution of nile red (100 μg/ml) was mixed with 3 ml of microsomal membranes and the excitation (left) and emission (right) fluorescence spectra were recorded using 5-nm slit width settings. The dashed lines represent the fluorescence of microsomal membranes in the presence of metrizamide and in the absence of nile red. All excitation and emission spectra were determined at their corresponding emission or excitation maxima.

blue shift. The selectivity of the spectral shifts, even in complex systems, is illustrated by the difference in fluorescence spectra of nile red-stained adipocyte (triacylglycerol-rich) compared to microsomal membranes (phospholipid-rich) (Fig. 5).

It is important to recognize that nile red fluorescence is an indication of lipid hydrophobicity and physical state and not just chemical structure. Thus, if the temperature is raised to 46°C, microemulsions containing cholesteryl ester produce blue shifts in the nile red spectra comparable to those obtained with microemulsions containing triacylglycerol. Nile red will even fluoresce in the presence of cholesterol crystals (unpublished observations) and cholesterol incorporated into phosphatidylcholine vesicles induces a blue shift of the nile red fluorescence. Nile red fluorescence is also induced with oleic acid in aqueous medium, the fluorescence spectra at acid pH appearing at shorter wavelength (approximately 30 nm) than the fluorescence spectra of the mixture at alkaline pH (unpublished observation).

The emission and excitation maxima of nile red in lipid suspensions and in various proteins appear to be dependent upon the concentration of the dye. The emission maxima at the lower concentration of the dye tend to be of a shorter wavelength than that observed in the presence of higher concentrations. In the presence of low density lipoproteins or phosphatidylcholine–trioleoylglycerol 1:1 microemulsions, this effect was most pronounced. The physical structure of the particles with which the nile red interacts may explain in part the shifts seen. At low concentrations of nile red, both the core hydrophobic and coat amphipathic components could be interacting with nile red, giving an emission maximum of approximately 570 nm. With increasing concentrations of nile red, the hydrophobic core may become saturated and the excess nile red could then only interact with the coat phospholipids or hydrophobic proteins. The emission maximum should then shift toward that observed with phosphatidylcholine vesicles and microsomal membranes (620–630 nm), just as is observed (Tables 3 and 4). Other observations support this notion. *a*) The solubility of nile red in n-heptane was found to be approximately 1/16 of that in acetone (Table 1). Thus, nile red could more easily attain saturation in an extremely nonpolar environment. *b*) Patton et al. (31) have described limited solubilities of hydrophobic molecules in liquid fat. *c*) Comparisons of the nile red fluorescence intensities of high density lipoproteins under spectral conditions that preferentially detect strongly hydrophobic domains suggest that nile red saturation of core lipids does occur (3). From the above discussion it is clear that the dye concentration dependence of nile red fluorescence must be carefully considered when interpreting nile red staining of lipoproteins, membranes, or whole cells.

In conclusion, the results of the studies described here provide a rational basis to explain the selective fluorescent staining of cellular lipids by nile red. When the fluorescence of nile red-stained cells is examined at wavelengths of 580 nm or less, the fluorescence of nile red interacting with extremely hydrophobic environments (i.e., neutral lipid droplets) is maximized while the fluorescence of nile red interacting with cellular membranes is minimized (Fig. 1). This is not the case when the fluorescence is detected at emission wavelengths greater than 590 nm. Under the latter conditions, the interaction of nile red with phospholipid-rich intracellular membranes becomes a more important determinant of cellular fluorescence. By proper selection of spectral conditions, nile red should be a powerful fluorescent reagent to examine intracellular lipid loading in various pathologic conditions such as atherosclerosis, genetic lipid storage diseases, and drug-induced phospholipidoses.

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REFERENCES

1. Conn, H. J. 1953. Biological Stains. Sixth Edition. Williams and Wilkins Co., Baltimore.
2. Adams, C. W. M., and O. B. Bayliss. 1975. Lipid histochemistry. *In* Techniques of Biochemical and Biophysical Morphology. Vol. 2. D. Glick and R. M. Rosenbaum, editors. Wiley Interscience, New York. 99–156.
3. Greenspan, P., E. P. Mayer, and S. D. Fowler. 1985. Nile red: a selective fluorescent stain for intracellular lipid droplets. *J. Cell Biol.* **100:** 965–973.
4. Goldstein, J. L., Y. K. Ho, S. K. Basu, and M. S. Brown. 1979. Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. *Proc. Natl. Acad. Sci. USA.* **76:** 333–337.
5. Brown, M. S., J. L. Goldstein, M. Krieger, Y. K. Ho, and R. G. W. Anderson. 1979. Reversible accumulation of cholesteryl esters in macrophages incubated with acetylated lipoproteins. *J. Cell Biol.* **82:** 597–613.
6. Greenspan, P., E. P. Mayer, and S. D. Fowler. 1984. Use of nile red in fluorescence microscopy and flow cytometer analysis of intracellular lipid loading. *J. Cell Biol.* **99:** 58a.
7. Chen, R. F. 1967. Removal of fatty acids from serum albumin by charcoal treatment. *J. Biol. Chem.* **242:** 173–181.
8. Thorpe, J. F. 1907. A reaction of certain coloring matters of the oxazine series. *J. Chem. Soc.* **91:** 324–336.
9. Davis, M. M., and H. B. Hetzer. 1966. Titrimetric and equilibrium studies using indicators related to nile blue A. *Anal. Chem.* **38:** 451–461.
10. Stužka, V., V. Šimánek and Z. Stránský. 1967. Infra-red spectroscopy of benzo(a)phenoxazines. *Spectrochim. Acta.* **23:** 2175–2183.
11. Brecher, P., J. Chobanian, D. M. Small, and A. V. Chobanian. 1976. The use of phospholipid vesicles for in vitro studies on cholesterol ester hydrolysis. *J. Lipid Res.* **17:** 239–247.
12. Burrier, R. E., and P. Brecher. 1983. Hydrolysis of triolein in phospholipid vesicles and microemulsions by a purified rat liver acid lipase. *J. Biol. Chem.* **258:** 12043–12050.
13. Ginsburg, G. S., D. M. Small, and D. Atkinson. 1982. Microemulsions of phospholipids and cholesterol esters. Protein-free models of low density lipoprotein. *J. Biol. Chem.* **257:** 8216–8227.
14. Havel, R. J., H. A. Eder, and J. H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34:** 1345–1353.
15. Lech, J. J., and D. N. Calvert. 1966. Protein content and osmotic behavior of isolated fat cells. *J. Lipid Res.* **7:** 561–564.
16. de Duve, C., B. C. Pressman, R. Gianetto, R. Wattiaux, and F. Appelmans. 1955. Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat liver tissue. *Biochem. J.* **60:** 604–617.
17. Fujiki, Y., A. L. Hubbard, S. Fowler, and P. B. Lazarow. 1982. Isolation of intracellular membranes by means of sodium carbonate treatment: application to endoplasmic reticulum. *J. Cell Biol.* **93:** 97–102.
18. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
19. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37:** 911–917.
20. Ames, B. N., and D. T. Dubin. 1960. The role of polyamines in the neutralization of bacteriophage deoxyribonucleic acid. *J. Biol. Chem.* **235:** 769–775.
21. Schäfer, W. 1964. The chemistry of the phenoxazones. *In* Progress in Organic Chemistry. Vol. 6. Sir J. Cook and W. Carruthers, editors. Butterworths, London. 135–163.
22. Barton, Sir D. H. R., and W. D. Ollis. 1979. Comprehensive Organic Chemistry. Vol. 4. Pergamon Press, New York. 1068–1078.
23. Dunnigan, M. G. 1968. Chromatographic separation and photometric analysis of the components of nile blue sulphate. *Stain Technol.* **43:** 243–248.
24. Hantzsch, A. 1922. Über die Halochromie und "Solvatochromie" des Dibenzalacetons und einfacherer Ketone, sowie ihrer Ketochloride. *Chem. Ber.* **55:** 953–979.
25. Stuzhka, V., A. P. Golovina, I. P. Alimarin, and N. B. Zorov. 1970. Luminescence and structure of phenoxazine dyes. *Khimiya.* **25:** 74–78.
26. Lakowicz, J. R. 1983. Principles of Fluorescence Spectroscopy. Plenum Press, New York and London.
27. Weast, R. C., editor. 1977. CRC Handbook of Chemistry and Physics. CRC Press Inc., Cleveland.
28. Small, D. M. 1970. The physical state of lipids of biological

importance: cholesteryl esters, cholesterol, and triglycerides. *In* Surface Chemistry of Biological Systems. M. Blank, editor. Plenum Press, New York, 55–83.

29. McClure, W. O., and G. M. Edelman. 1966. Fluorescent probes for conformational states of proteins. I. Mechanism of fluorescence of 2-*p*-toluidinylnaphthalene-6-sulfonate, a hydrophobic probe. *Biochemistry.* **5:** 1908–1919.
30. Weber, G., and F. J. Farris. 1979. Synthesis and spectral properties of a hydrophobic fluorescent probe: 6-propionyl-2-(dimethylamino) naphthalene. *Biochemistry.* **18:** 3075–3078.
31. Patton, J. S., B. Stone, C. Papa, R. Abramowitz, and S. H. Yalkowsky. 1984. Solubility of fatty acids and other hydrophobic molecules in liquid trioleoylglycerol. *J. Lipid Res.* **25:** 189–197.