Use of nile red for the rapid in situ quantitation of lipids on thin-layer chromatograms

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Abstract We describe the use of the fluorescent dye nile red, 9-diethylamino-5H-benzo[α]phenoxazine-5-one, as a general-purpose reagent for the rapid detection and quantitation of a wide variety of lipids and other hydrophobic compounds separated by thin-layer chromatography. After samples are applied to silica gel plates and chromatographed, the plate is briefly dipped into a nile red solution (8 μg/ml of methanol–water 80:20, v/v). Background fluorescence of nile red dye adsorbed to the silica gel is then preferentially destroyed by dipping the plate in a dilute aqueous solution of bleach. After drying, lipid bands are visualized under ultraviolet light. Reflectance fluorometry (Ex: 580 nm; Em: 640 nm) is utilized for in situ quantitative analysis of the fluorescence of the lipids on the nile red-stained plate. Neutral lipids, phospholipids, sphingolipids, and fatty acids can be examined, although the nile red fluorescence intensity varies significantly among the lipid classes. Also, staining is stronger for unsaturated lipids than for saturated lipids. The lower detection limit of the assay is 25–100 ng for cholesterol, cholesteryl esters, triacylglycerols, and phospholipids.—**Fowler, S. D., W. J. Brown, J. Warfel, and P. Greenspan.** Use of nile red for the rapid in situ quantitation of lipids on thin-layer chromatograms. *J. Lipid Res.* 1987. **28:** 1225–1232.

Supplementary key words cholesterol • 9-diethylamino-5H-benzo-[α]phenoxazine-5-one • fatty acids • fluorometry • neutral lipids • phospholipids • sphingolipids

The microassay of lipids by fluorometric scanning of thin-layer chromatograms has several analytical advantages. The technique combines the speed, convenience, and versatility of thin-layer chromatography (TLC) with the great sensitivity and specificity of fluorometry. A number of general procedures have been described for the fluorometric quantitation of lipids separated by TLC. These include treating chromatograms with fluorescent dyes such as 1-anilino-8-naphthalene (1, 2), 2',7'-dichlorofluorescein (3), rhodamine (4, 5), primuline (6), or 6-*p*-toluidino-2-naphthalenesulfonic acid (7). Other methods involve formation of fluorescent reaction products by heating lipids in the presence of ammonium hydrogen salts (8–11), strong acids (12, 13), or silicon tetrachloride (14). These latter procedures have the same disadvantages as acid charring for densitometry, i. e., destruction of the lipid samples and difficulty in controlling the reaction process. On the other hand, most of the fluorescent dyes proposed fade rapidly (15), and only rhodamine has been popular. The use of rhodamine for the fluorometric TLC microassay of lipids, however, requires a preliminary hydrogenation of samples with platinum oxide catalyst to saturate fatty acid moieties before chromatographic separation and quantitative analysis is carried out (4).

We found that the fluorescent dye, nile red, can be used as a sensitive stain for the detection of lipids and other hydrophobic compounds such as drugs separated by TLC. Furthermore, the fluorescence produced by nile red staining can be measured for in situ quantitation of lipid mass. Nile red is a hydrophobic probe and has several useful characteristics (16, 17): *a*) it is intensely fluorescent; *b*) the fluorescence appears in organic solvents, but is quenched in water; *c*) the fluorescence is relatively photostable and fades slowly; and *d*) the dye preferentially dissolves in hydrophobic compounds such as lipids.

In this report we describe studies carried out to optimize nile red staining to produce a general-purpose fluorometric TLC microassay for lipids. The method can be employed for a variety of lipid classes and can be utilized over a great range of concentrations. No preparative procedure is required before the lipid sample is applied. The nile red staining is stable and the fluorescence produced is highly reproducible. The method permits rapid quantitative analysis of complex lipid mixtures. A preliminary report of this work has been presented previously (18).

Abbreviation: TLC, thin-layer chromatography.

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MATERIALS AND METHODS

Chemicals

Nile red (9-diethylamino-5H-benzo[α]phenoxazine-5-one) was prepared as described (17), or obtained from Eastman Kodak, Inc. Silica gel TLC plates (type 60 A, K6) were purchased from Whatman Chemical Separation, Inc. Lipids were purchased from Sigma Chemical Company. Chloroform was HPLC grade and other organic solvents were reagent grade; all were obtained from Fisher Scientific, Inc. Clorox (5.25% (v/v) sodium hypochlorite solution) was manufactured by The Clorox Co., Oakland, CA, and was purchased locally.

Chromatography and nile red staining of lipids

Silica gel TLC plates (20 × 20 cm) were prewashed in chloroform–methanol 2:1 (v/v) prior to use. Twenty lanes for lipid samples were prepared by etching parallel lines 5 mm apart from the bottom to the top of the plate. Each sample lane was separated by a spacer lane 2.5 mm wide. Narrow sorbent lanes are required to prevent expansion of sample spot diameter during chromatography (19). After etching, the plate was dipped in methanol–water 50:50 (v/v) to remove loose silica gel particles; the plate was activated at 100°C for 45 min in a vacuum oven. The lipid samples were then applied to the individual lanes. (Amounts usually did not exceed 20 μg per zone in order to avoid distortion of the spot shape and distance of migration.) The mixtures were separated by chromatography in a suitable solvent. For neutral lipids, we used hexane–diethyl ether–acetic acid 90:20:1.5 (v/v/v) (solvent A); phospholipids were separated using chloroform–methanol–acetic acid–water 50:30:8:4 (v/v/v/v) (solvent B). The developed plate was dried by heating at 100°C for 5 min. To stain for lipids, the plate was dipped for a few seconds in a tank (Dipping Chamber, 200 × 200 × 5 mm, Cat. No. 4960-410, Whatman Ltd., Maidstone, Kent, England) of nile red solution (8 μg of nile red/ml of methanol–water 80:20, v/v, freshly prepared) followed by drying at 100°C for 5 min. Background staining of silica gel with nile red was removed by briefly dipping the plate in a dilute solution of bleach (1/2,500 dilution of Clorox in distilled water). The plate was then thoroughly dried by heating in a vacuum oven for 30 min at 100°C. Chromatographed lipids appear as intense red fluorescence bands when viewed with a short wavelength ultraviolet lamp.

For qualitative work involving only detection and identification of hydrophobic compounds, a very quick procedure to reveal the compounds may be employed. Spray the chromatographed plate with nile red (40 μg/ml of acetone), dry briefly, then mist with distilled water; view plate under short wavelength ultraviolet irradiation.

Analysis of nile red stained lipids

Quantitation of lipid mass following TLC and nile red staining was determined by measuring nile red fluorescence intensity of individual bands with a Perkin-Elmer model 650-40 fluorescence spectrophotometer (Perkin-Elmer Corp.) equipped with a TLC scanning attachment. The instrument was set so that the irradiation area of the TLC plate was 6 mm × 1 mm, sufficient to cover the width of the sample lanes. Excitation and emission wavelengths were set at 580 and 640 nm, respectively, with 15-nm slit widths. The instrument was blanked against a portion of the plate below the origin to correct for any background fluorescence. The lanes were scanned at a rate of 60 mm/min to determine relative fluorescence intensities of individual bands; integration of peaks in the fluorescence scan was carried out by a SpectraPhysics model 4270 integrator (SpectraPhysics Corp). In all instances, calibration standards of the lipids to be analyzed (ranging from 50 ng to 10 μg) were also included on the same plate. To diminish day-to-day variability in nile red staining, we routinely set the recorder range so that the fluorescence of the calibration standard having the greatest mass (usually 10 μg of trioleoylglycerol) was set at 90% of full scale (maximum fluorescence).

Excitation and emission fluorescence spectra of the nile red-stained lipids on TLC plates were obtained as described previously (17).

Cultured skin fibroblasts

Human skin fibroblasts were cultured in modified Eagle's medium containing antibiotics and 10% (v/v) fetal calf serum (20). At approximately 50% confluence, the cells were placed in culture medium containing 2 mg/ml lipoprotein-deficient serum (21). After 24 hr of incubation, the culture medium was changed to medium containing 25 μg of protein/ml of human low density lipoprotein (22). Control cultures were incubated in lipoprotein-deficient serum in the absence of low density lipoprotein. After 48 hr of incubation, the culture medium was removed and the cells were washed with phosphate-buffered saline containing 2 mg/ml albumin and then with phosphate-buffered saline alone. The cells were then collected by trypsinization.

Lipid composition of cultured skin fibroblasts

The lipid composition of cultured skin fibroblasts was determined by the method outlined above following extraction of the lipids into organic solvent. For this purpose, cells trypsinized from culture dishes were washed in phosphate-buffered saline by centrifugation and then burst by brief sonication. Lipids in the cellular homogenate were extracted into chloroform (23). An aliquot of the chloroform phase corresponding to 75 μg of cell pro-

tein was transferred to a silica gel TLC plate.[2] Neutral lipids were chromatographed in a solvent consisting of hexane–diethyl ether–acetic acid 146:50:4 (v/v/v) (solvent C). This solvent was run to approximately 5 cm from the top of the plate. The plate was then air-dried and rechromatographed with hexane to separate the nonpolar lipids from cholesteryl ester. We included standards for both triacylglycerols and cholesteryl esters which reflected the ratio (2:1, oleate/stearate or palmitate) of unsaturated to saturated fatty acids found in these neutral lipids in mammalian cells (24, 25).

RESULTS

The appearance of a nile red-stained TLC plate viewed under ultraviolet light is shown in **Fig. 1.** Before decoloring by bleach treatment, fluorescent red lipid bands are visible, but are masked by a strong background red fluorescence of nile red adsorbed onto the silica gel. Following brief dipping in dilute bleach, the background fluorescence is destroyed, while the fluorescence of the dye dissolved in the lipid bands remains strong. Both neutral and phospholipids are stained.

In previous studies, the fluorescence spectra of nile red was observed to vary both with solvent hydrophobicity and in the presence of aqueous suspensions of lipid microemulsions of varying composition (17). Spectral shifts do not occur, however, for nile red-stained lipid bands on TLC plates. As shown in **Fig. 2** the fluorescence excitation and emission spectra of nile red are identical regardless of the lipid type stained. Fluorescence excitation maxima occur at 580 nm and emission maxima occur near 640 nm for cholesteryl oleate, trioleoylglycerol, and sphingomyelin. Even the background staining of silica gel by the dye produced similar excitation and emission maxima, although the fluorescence intensity was much less. The same nile red fluorescence emission color for all stained lipid classes separated on a TLC plate is fortunate, since it allows rapid quantitation of the mass of each lipid band by simple measurement at one wavelength pair.

In order to utilize nile red fluorescence intensity as a measure of lipid mass, parameters of the staining procedure were examined and optimized. **Fig. 3** shows that in situ lipid fluorescence increases proportionately to the concentration of nile red used for staining, up to 12 μg of dye/ml. At higher dye concentrations, fluorescence of the lipid bands decreased. The decrease in fluorescence at high nile red concentrations was consistently observed in several experiments. While these studies suggest that the staining procedure should employ 12 μg of nile red/ml for maximum sensitivity, we found that the background fluorescence of the plate varied significantly even after bleaching. Consequently, we elected to use 8 μg of nile red/ml, a concentration of dye for which the problem of variable background fluorescence was substantially reduced. **Fig. 4** illustrates the effect of bleach concentration on decoloring a nile red-stained TLC plate. At a dilution of bleach of 1/2,500, a near perfect balance of nile red-stained lipid band fluorescence and diminished plate background fluorescence is reached. With more concentrated bleach solutions, fluorescence of the lipid bands is greatly reduced, while at too low bleach concentrations the background plate fluorescence is not sufficiently uniform to permit quantitative analysis. In this one instance, the effect of bleach (at a 1/2,500 dilution) on the fluorescence of the triacylglycerol was much more pronounced than that observed for cholesteryl ester.

The change in intensity of nile red fluorescence with increasing lipid mass for both neutral and phospholipids is shown in **Fig. 5**. The relation is not linear, but rather is curvilinear with a plateau indicating an upper limit to the amount of nile red fluorescence that can be obtained under the conditions used. This upper limit varies depending on the lipid class. For amounts of lipid less than 0.6

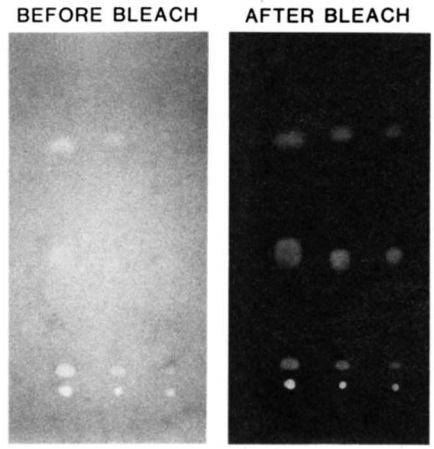

Fig. 1. Appearance of nile red-stained TLC plate and its decolorization by bleach. Lipid mixtures were spotted on a TLC plate at three concentrations (80, 20, and 5 μg of each lipid type) and chromatographed in solvent A to separate the lipids into bands. The plate was then dipped in nile red (8 μg/ml), dried, and photographed under ultraviolet light (left photograph). The same plate was then dipped in bleach solution (1/2,500 dilution of Clorox), dried, and photographed again (right photograph). Fluorescence appears white against dark background. Lipid bands in order of increasing mobility to the top are: sphingomyelin, cholesterol, trioleoylglycerol, and cholesteryl oleate.

[2]We minimized the volume of chloroform extract that is concentrated by evaporation and applied to the TLC plate. We found impurities in some lots of chloroform which, when concentrated, will stain with nile red and interfere with the lipid analysis. The presence of such impurities in chloroform can be monitored by nile red staining after spotting a blank lane on a TLC plate with an aliquot of the solvent previously concentrated by evaporation. These impurities can appear even in redistilled chloroform.

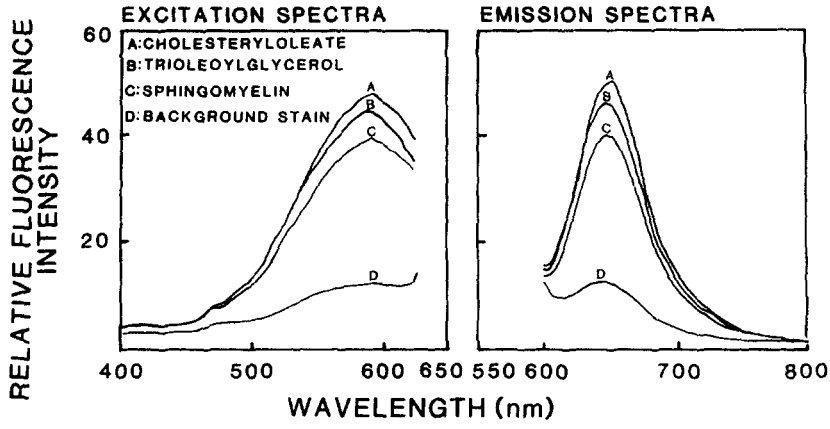

Fig. 2. Excitation and emission fluorescence spectra of nile red-stained lipids. A mixture of 20 μg of each lipid type was spotted on a TLC plate and chromatographed in solvent A. In this study, the plate was dipped in nile red solution (15 μg/ml), then decolorized in bleach (1/2,000 dilution of Clorox), and dried. Excitation and emission spectra of nile red-stained lipids were recorded at their corresponding emission or excitation maxima. Excitation and emission maxima of 580 nm and 640 nm, respectively, were chosen for subsequent fluorescence intensity measurements of stained lipids on TLC plates.

μg (Fig. 5, left), the relationship between fluorescence and the quantity of lipid present is approximately linear. With the dye and bleach concentrations that we recommend, the minimum amount of trioleoylglycerol and cholesteryl oleate detectable is about 50 ng. The minimum mass of cholesterol or sphingomyelin detectable is 100–200 ng.

An accurate estimate of lipid mass can be obtained by measurement of nile red fluorescence on TLC plates. **Fig. 6** shows the results of measurement of 18 serum samples for total cholesterol by nile red staining of TLC plates and by the enzymatic cholesterol oxidase procedure (26). The average difference between values obtained by the two methods for 18 samples was 6%; a correlation coefficient of 0.891 was calculated for the data. To study the reproducibility of nile red fluorescence values obtained, we spotted 1 μg of trioleoylglycerol 19 times on a single plate and then measured the fluorescence of each spot after staining. The standard error of the mean was found to be less than 1% of the mean fluorescence value. We also examined the plate-to-plate variability of nile red fluorescence of 2 μg of trioleoylglycerol spotted on five different plates. After standardization of the fluorometer as described in the Methods, the standards error of the mean was 5% of the mean fluorescence value observed for the five plates.

A wide variety of lipid classes, including glycolipids, can be stained by nile red (**Fig. 7**). However, the intensity of fluorescence obtained is variable. At high concentrations (2.5 μg), cholesteryl oleate, trioleoylglycerol, and oleic acid give much stronger nile red fluorescence than the other lipid compounds studied. We observed that saturation of the fatty acid moiety of the lipids significantly reduces the intensity of the nile red fluorescence produced. As seen in **Fig. 8**, when equal masses of lipid were plated, both triacylglycerol and cholesteryl ester with stearic acid as the esterified fatty acid were less fluorescent than the corresponding lipids with either oleic, linoleic, or linolenic acids as the fatty acid moiety. In agreement with these results, we observed that saturated fatty acids, such as palmitic and stearic acid, produce little nile red fluorescence (data not shown).

To test the utility of the technique for the quantitation of cellular lipids, human skin fibroblasts were incubated with low density lipoprotein and the extent of lipid accumulation was determined. The chromatogram scan tracings are shown in **Fig. 9**. When cells are incubated for 48 hr with lipoprotein-deficient serum, no measurable cholesteryl ester was found although the triacylglycerol content was 4.5 μg/mg of cell protein. When identical cul-

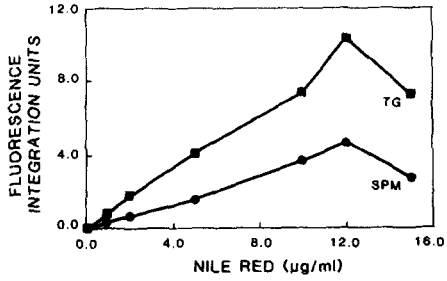

Fig. 3. Dependence of fluorescence intensity of stained lipids on nile red concentration. A mixture of trioleoylglycerol and sphingomyelin (1 μg each) was spotted on six TLC plates. The lipids were then separated by chromatography in solvent A and the plates were dipped in solutions of nile red of various concentrations. The plates were then decolorized in bleach (1/2,500 dilution), dried, and fluorescence intensity of the stained lipids was measured.

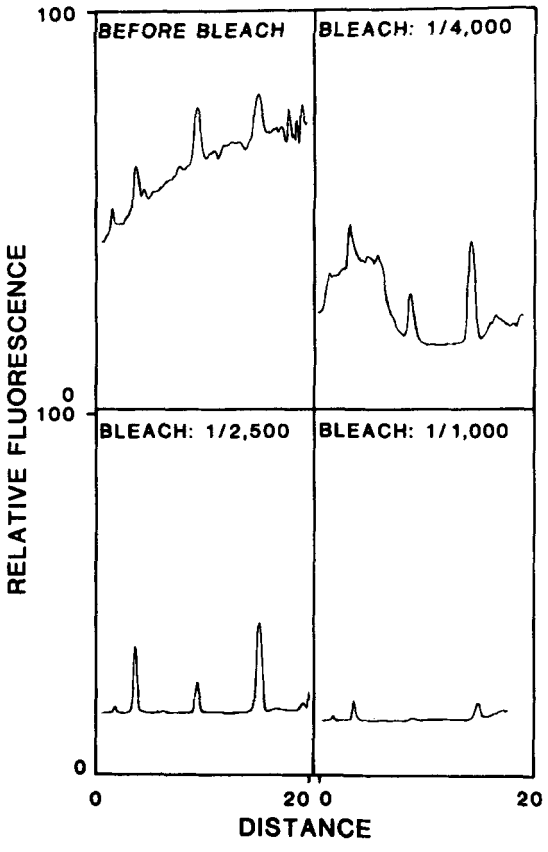

Fig. 4. Effect of bleach concentration on the fluorescence of nile red-stained lipids. A mixture of cholesterol, trioleoylglycerol, and cholesteryl oleate (0.25 μg each) was spotted on four TLC plates. The lipids were separated by chromatography in solvent A and the plates were dipped in a solution of nile red (8 μg/ml). The plates were then dipped in bleach solutions of various concentrations and dried. TLC scan tracings of the fluorescence of the stained plates are shown for the various bleach treatments. At bleach dilution of 1/2,500, peaks representing cholesterol (left), trioleoylglycerol (center), and cholesteryl oleate (right) are readily identified on the tracing.

tures are incubated for the same time with 25 μg protein/ml low density lipoprotein added, both the cholesteryl ester and the triacylglycerol contents increased to 23.0 and 8.6 μg/ml of cell protein, respectively. No significant increase in the content of unesterified cholesterol was found. A duplicate sample chromatographed in solvent B is recommended for phospholipid analysis. This experiment illustrates the applicability of the nile red staining procedure for rapidly detecting and quantitating cellular lipid accumulation.

DISCUSSION

Certain technical details of our procedure warrant additional comment. Dipping the TLC plate in a methanol–water solution of nile red produces uniform and reproducible staining of the plate. Lipids appear not to be readily extracted by the solvent during the dipping steps since we found only a small fraction of cholesterol was removed from the plate using radiolabeled tracer. The more traditional approach of spraying plates with the dye resulted both in uneven staining across the plate and in variation in amount of stain applied to separate plates. The latter was particularly a problem when analyzing plates stained on different days. Elimination of background fluorescence from nile red adsorbed to the silica gel sorbent is essential. We observed that intense sources of ultraviolet light or sunlight will preferentially fade nile red background fluorescence relative to fluorescence coming from lipid bands. However, it proved difficult to standardize the procedure for routine use. Since nile red fluorescence is quenched by water, stained plates can also

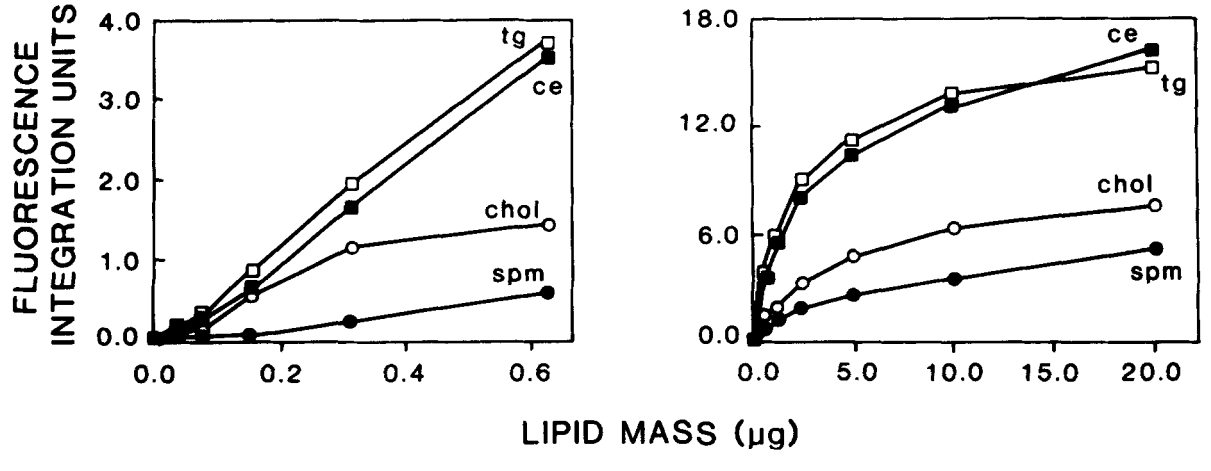

Fig. 5. Mass calibration plots of nile red-stained lipids. Indicated amounts of cholesterol, cholesteryl oleate, trioleoylglycerol, and sphingomyelin classes were spotted on a single TLC plate. After chromatography in solvent A to separate the lipids, the plate was stained with nile red and decolorized as described in Methods. The nile red fluorescence of each lipid band was then determined.

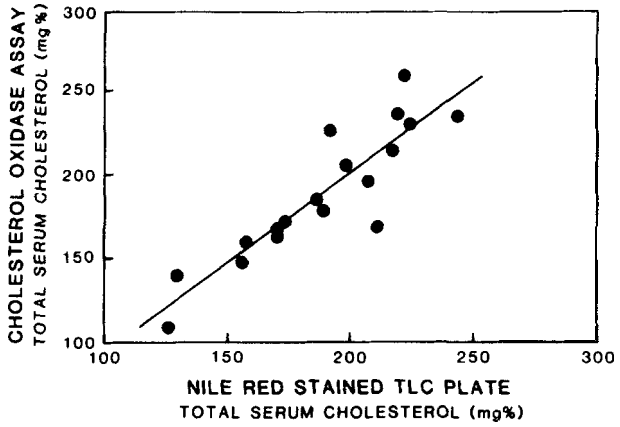

Fig. 6. Correlation of serum total cholesterol values determined by the nile red method with values obtained by a clinical procedure on the same samples. Total cholesterol on 18 serum samples was initially analyzed by the enzymatic cholesterol oxidase assay (26). Each sample (100 μl) was then saponified with alcoholic KOH, and the free cholesterol was extracted into hexane (27). A 10-μl aliquot of the hexane solution of each sample was applied to a TLC plate and the plate was chromatographed in chloroform–methanol–acetic acid 98:2:1 (v/v/v). Nile red fluorescence of the cholesterol band was then determined. Solid line shown represents best least squares fit through the data points.

be sprayed with a water mist to reduce background fluorescence. But the fluorescence reappears as the plate dries, thereby progressively affecting measurement of plate fluorescence with the fluorometer. In contrast, treatment of the nile red-stained plate with dilute bleach produces a uniform reduction of background fluorescence intensity. Dye dissolved in the lipid bands is apparently protected from oxidation by the bleach. We have utilized the method described only with Whatman silica gel 60A K-type TLC plates. Use of other types of TLC plates with our procedure will require tests for feasibility.

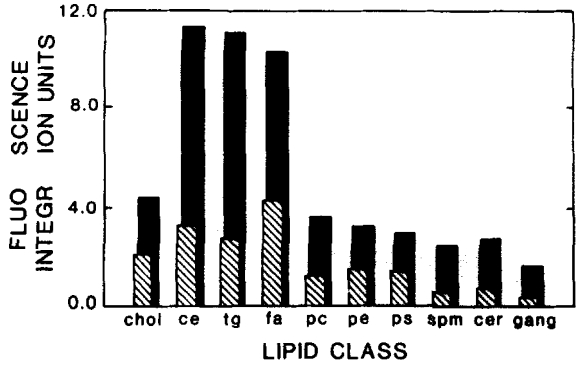

Fig. 7. Survey of lipid classes stained by nile red. Various lipids were spotted on a TLC plate in two different amounts (0.5 μg, shaded bars, and 2.5 μg, solid bars), chromatographed (neutral lipids in solvent A, phospholipids and sphingolipids in solvent B), and then measured for fluorescence after nile red staining. The lipids examined were cholesterol, cholesteryl oleate, trioleoylglycerol, oleic acid, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, sphingomyelin, cerebroside, and ganglioside, respectively.

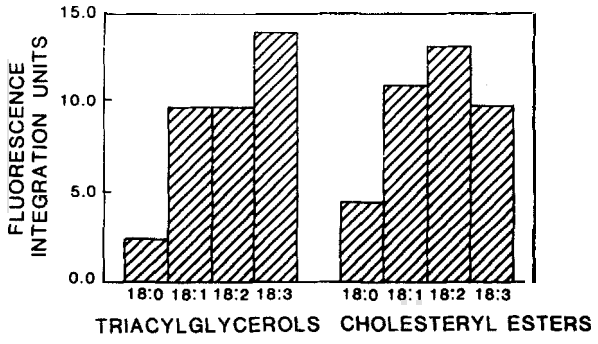

Fig. 8. Influence of saturation of fatty acid moieties of lipids on the observed nile red fluorescence. A mixture of cholesteryl esters and triacylglycerols (2.5 μg of each lipid) of varying fatty acid composition was spotted on a TLC plate and chromatographed in solvent A. After staining with nile red and decolorization, the fluorescence of the stained lipids was ascertained.

As with rhodamine staining (4, 5), mass calibration plots of nile red-stained lipids consist of a family of curvilinear graphs. The relationship between change in fluorescence and change in mass varies with each lipid class, as indicated in Fig. 5. Remarkably, the shape and relative height of the curves are similar from run to run. These curvilinear calibration plots likely reflect the sum of complex interactions involving *a*) physical properties of individual lipids that affect the dissolution of nile red in them; *b*) influence of the silica gel substrate upon which the staining occurs; and *c*) light scattering, quenching, and absorption of the emitted fluorescence depending on spot size and shape. Therefore, for accurate quantitation of an unknown mixture, it is necessary to include a range of lipid calibration standards on each plate. The values for lipid concentrations are then reproducible.

An apparent limitation of in situ TLC quantitation of lipids either by fluorometry or by densitometry of carbonized specimens is the varying effect of saturated versus unsaturated fatty acid moieties on the level of staining produced (28). Our study summarized in Fig. 8 demonstrates that nile red staining is also influenced by the extent of unsaturation of the lipid sample. Presumably, straight chain fatty acids are stearically less compatible with the presence of nile red molecules than are the bent chains of unsaturated fatty acids. However, nile red staining is not affected to the degree of that of other procedures (4, 12); neutral lipids with a full complement of saturated fatty acids do stain and the difference in nile red fluorescence intensity produced with monounsaturated compared to polyunsaturated fatty acid moieties is minimal. For the most accurate determinations, we recommend use of lipid calibration standards which reflect the fatty acid composition of the samples being quantitated. This was done for the study of lipid deposition in cultured fibroblasts shown in Fig. 9.

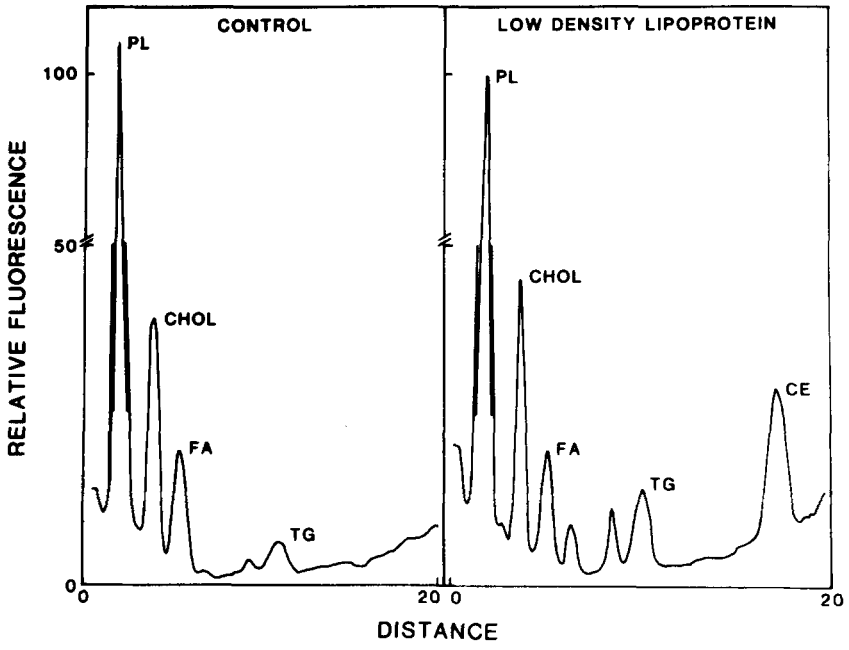

Fig. 9. Analysis of cellular lipid composition by TLC and nile red staining. Cultured skin fibroblasts were grown and incubated in the presence of lipoprotein-deficient serum alone (control) or in the same medium with the addition of low density lipoprotein (25 μg of protein/ml). Chloroform extracts, corresponding to 75 μg of cellular protein, were applied to a TLC plate. After chromatographic separation (solvent C) of the lipids, the plate was stained with nile red and decolorized. TLC scan tracings of the fluorescence of the samples are shown.

Our assay, from the spotting of the lipid samples to determining lipid mass, takes approximately 4 hr. Unlike many assays for lipids, this procedure does not require the use of acid or corrosive solutions. For most lipid classes, the minimum amount detected was approximately 100 ng of lipid, although the sensitivity of the method for neutral lipids approached 25–50 ng. The protocol is simple to apply, the assay can be applied over a wide range of lipid concentrations, and the results are highly reproducible. As indicated, fluorescence intensity is subject to the degree of unsaturation of individual lipids. Nonetheless, our assay permits the simultaneous quantitation of all lipid classes in a tissue extract by a single method, and thus it should be a valuable aid to lipid chemists.

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