Interaction of Phospholipids with Rhodamine 6G in Toluene

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Upon interaction of various glycerophospholipids with Rhodamine 6G in toluene, a typical difference spectrum with an absorption maximum at approximately 515 nm is obtained. This spectrum is obtained with phosphatidylcholine only after treatment with NaCl, which presumably weakens intra- and/or inter-molecular electrostatic binding between the negatively charged phosphate moiety and the protonated nitrogen in this molecule. Absorption at 515 nm was linear for all of the phospholipids investigated from a concentration of approximately 1.2 μM up to at least 50 μM. The highest extinction coefficient was obtained for diphosphatidylglycerol (251 mM^{-1} cm^{-1}) and all of the compounds tested, with the exception of phosphatidylethanolamine, demonstrated extinction coefficients higher than that of palmitic acid.

Thus, the absorption spectrum which results from the interaction of purified glycerophospholipids with Rhodamine 6G in organic solvent is a sensitive measure of the amount of phospholipid present.

Quantitation of phospholipids is today still most commonly performed using variations on methodology which is three decades old, i.e., charring of extracted lipids and subsequent determination of inorganic phosphate. Such procedures are relatively time-consuming and require moderate amounts of material. Some time ago we developed a sensitive assay for phospholipids based on the ability of these compounds to solubilize radioactive nickel in organic solvents.[1] Unfortunately, free fatty acids interfere with this assay and, more seriously, it is not applicable to choline-containing phospholipids.

In an initial attempt to design other sensitive assays for phospholipids, we have examined here the interaction of the phospholipids which occur most commonly in biological systems with Rhodamine 6G. The Rhodamine 6G base is a sensitive acid indicator,[2] which upon interaction with an acid forms a colored species which may be quantified spectrophotometrically. Such interaction with free fatty acids has been used as the basis for a sensitive assay procedure.[3,4] In addition, the interaction of Rhodamine 6G with biological components such as phospholipids and fatty acids may be of interest with respect to the biological effects of this compound (see below).

Experimental

Chemicals. The following lipids were purchased from Sigma Chemical Co. (St. Louis, MO, USA): L-*a*-phosphatidylcholine, L-*a*-phosphatidylethanolamine, L-*a*-phosphatidyl-L-serine, L-*a*-phosphatidylinositol, sphingomyelin, phosphatidic acid, diphosphatidylglycerol, palmitic acid and tripalmitin. Rhodamine 6G and toluene (p.a.) were obtained from Merck Chemical Co. (Darmstadt, FRG). All other chemicals were bought from common commercial sources at the highest purity available.

Methods. A Hitachi U-3200 spectrophotometer and 1 cm cuvettes were used. The Rhodamine 6G color reagent was prepared according to Andersson and McCarty[4] by first dissolving 400 mg of this substance in 40 ml 0.2 M phosphate buffer adjusted to pH 11 with NaOH. This solution was then extracted with 400 ml toluene and the toluene phase, which was yellowish-brown,[2] was decanted and stored over NaOH pellets in the dark. For making various calculations, it was assumed that the dye was quantitatively extracted into toluene by this procedure. This stock solution of Rhodamine 6G, which had a concentration of 0.1 % (w/v), was diluted to 0.005 % immediately before use. The more concentrated the solution of Rhodamine 6G, the longer it remains stable. Thus, the 0.005 % solution must be renewed every 3–4 h, whereas the 0.1 % solution remains stable for 2–3 weeks.[4]

The absorbance spectra of phospholipid samples in the presence of 0.005 % Rhodamine 6G in toluene were determined against a blank containing the same concentration of dye alone. Addition of larger amounts of Rhodamine 6G did not affect the absorption spectrum obtained. Between measurements, cuvettes were washed with spectral-grade ethanol, then rinsed with the 0.005 % Rhodamine 6G solution and rinsed again with toluene. These rinsings were important, since short-chain alcohols such as ethanol interact with Rhodamine 6G.

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Results and discussion

Fig. 1 illustrates the typical difference spectrum obtained upon interaction of various glycerophospholipids (in this case diphosphatidylglycerol) with Rhodamine 6G in toluene. (None of the lipids themselves absorb in this region of the spectrum.) Similar curves were obtained with phosphatidyl-serine, -ethanolamine and -inositol and with phosphatidic acid.

However, when phosphatidylcholine was utilized as purchased, a somewhat different absorption spectrum between 480 and 600 nm, with an absorption maximum at 535 nm, was obtained. We suspected that this finding might reflect intra- and/or inter-molecular electrostatic binding between the negatively charged phosphate group and the protonated nitrogen which is strengthened when phosphatidylcholine is dissolved in an organic solvent.[1] Therefore, the solution of phosphatidylcholine in toluene was first shaken with a 1 M solution of NaCl and subsequently combined with the Rhodamine 6R reagent mixture. After this procedure, the typical absorption spectrum shown in Fig. 1 was also obtained with phosphatidylcholine, except that the shoulder at 535–540 nm was not seen. This is the wavelength at which short-chain alcohols absorb[1] and phosphatidylcholine contains no free hydroxy group. Thus, this shoulder may be related to hydroxy moieties in phospholipids. On the basis of these observations, we decided to treat phospatidylcholine routinely with NaCl before investigating its interaction with Rhodamine 6G.

The interaction of phospho- and other lipids with Rhodamine 6G in toluene was subsequently determined on the basis of the absorption peak at 515 nm. Fig. 2 illustrates that this absorption was linear ($R \geq 0.99$ in all cases) for all of the phospholipids investigated from a concentration of approximately 1.2 μM up to at least 50 μM. In a 1 ml cuvette these concentrations correspond to 1.2–50 nmol of phospholipid. Thus, relatively small amounts of phospholipid can be determined on the basis of their interaction with Rhodamine 6G in toluene.

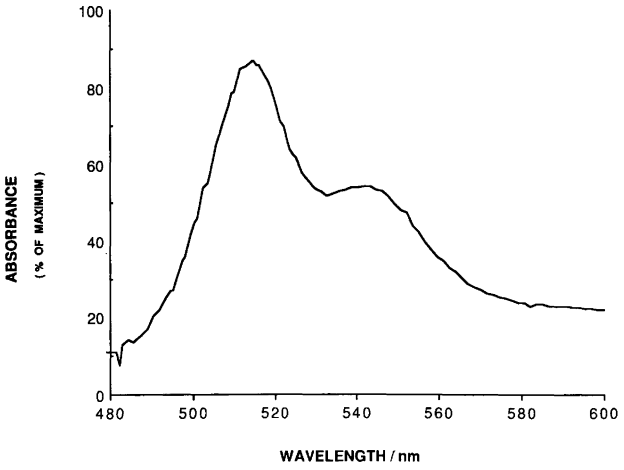

Fig. 1. Difference spectrum obtained upon interaction of diphosphatidylglycerol (3 μM) with Rhodamine 6G in toluene.

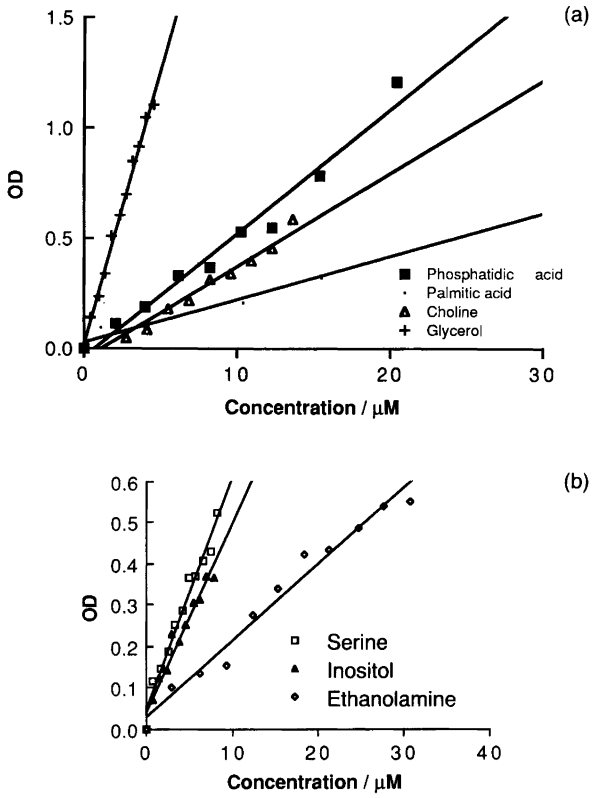

Fig. 2. Absorption of increasing amounts of various phospholipids at 515 nm upon interaction with Rhodamine 6G in toluene: (a) Diphosphatidylglycerol, phosphatidic acid, phosphatidylcholine and palmitic acid. (b) Phosphatidylserine, phosphatidylinositol and phosphatidylethanolamine.

The sensitivity of this approach is further documented in Table 1, which lists the extinction coefficients obtained for the various phospholipids tested. It can be seen that the highest value is obtained for diphosphatidylglycerol (251 $mM^{-1}\ cm^{-1}$) and that all compounds tested, with the exception of phosphatidylethanolamine, exhibit extinction coefficients higher than that of palmitic acid. Anderson and

Table 1. Extinction coefficients, at 515 nm, for the species formed by interaction between various glycerophopholipids and Rhodamine 6G in toluene (calculated from the data in Fig. 2).

Lipid	$\varepsilon_{515}/mM^{-1}\ cm^{-1}$
Diphosphatidylglycerol	251
Phosphatidylserine	56.6
Phosphatidic acid (dipalmitoyl)	55.8
Phosphatidylinositol	46.0
Phosphatidylcholine	42.5
Palmitic acid	19.6
Phosphatidylethanolamine	18.5
Tripalmitin	0

McCarty[4] reported an extinction coefficient of 27 mM^{-1} cm^{-1} for the species formed upon interaction of linolenate, linoleate or oleate with Rhodamine 6G under their assay conditions.

We have not examined the molecular nature of the interaction between phospholipids and Rhodamine 6G in any detail here. However, the fact that no absorbance is obtained with tripalmitin (Table 1) suggests that the diacylglycerol backbone is not involved in this interaction. In addition, the fact that most of the phospholipids, including phosphatidic acid, demonstrate similar extinction coefficients suggests that the phosphate moiety may be the most important group on the phospholipid in this respect. However, the findings with diphosphatidylglycerol and phosphatidylethanolamine do not fit into this generalization. On the basis of its two phosphate moieties, diphosphatidylglycerol would be predicted to have an extinction coefficient of ca. 100, but actually has a value which is 2.5 times higher than this. On the other hand, the value for phosphatidylethanolamine is too low, which may again reflect partial electrostatic binding between the negatively charged phosphate moiety and the protonated nitrogen atom (see above). In this regard it should be noted that sphingomyelin, as purchased, gave no absorption with Rhodamine 6G at all, even after being shaken with NaCl (see above).

Of interest in connection with the present work are the findings reported by Lotta and coworkers[5] concerning the interaction of 7,7,8,8-tetracyanoquinodimethane (TCNQ) with diacyl-phosphatidylcholines and -phosphatidylglycerols. On the basis of a photoacoustic Fourier transform infrared study, these investigators concluded that 'the molecular interaction between TCNQ and the phospholipids is seen in the polar headgroup region. The donated electrons are most likely located on the oxygens of the phosphate group in the polar head.'

Thus, the absorption spectrum which results from the interaction of glycerophospholipids with Rhodamine 6G in an organic solvent is a sensitive measure of the amount of phospholipid present. However, it must be remembered that many other compounds – including acids and bases in general and free fatty acids in particular – also interact with Rhodamine 6G in organic solvents to give rise to similar absorption spectra. Accordingly, this procedure can be used to quantify glycerophospholipids only after these have been purified, e.g., by thin-layer chromatography.

Finally, it is of interest to ask whether interactions between glycerophospholipids and Rhodamine 6G can explain some or all of the biological effects of this dye. Rhodamine 6G has been reported to affect both the structure and function of mitochondria and, perhaps as the result of such effects, exert a cytotoxic effect on cells.[6-10] This highly interesting question remains to be examined.

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References

1. DePierre, J. W. and Lundqvist, G. *Acta Chem. Scand., Ser. B 36* (1984) 273.
2. Palit, S. R. and Ghosh, P. *Microchem. Technol. 2* (1961) 663.
3. Chakrararty, M. M., Bhattacharyya, D. and Kundu, M. *J. Am. Oil Chem. Soc. 46* (1969) 473.
4. Anderson, M. M. and McCarty, R. E. *Anal. Biochem. 45* (1972) 260.
5. Ranganathan, S. and Hood, R. D. *Teratogen. Carcinog. Mutagen. 9* (1989) 29.
6. Ranganathan, S., Churchill, F. F. and Hood, R. D. *Toxicol. Appl. Pharmacol. 99* (1989) 81.
7. Bullough, D. A., Ceccarelli, E. A., Roise, D. and Allison, W. S. *Biochim. Biophys. Acta 975* (1989) 377.
8. Hood, R. D., Jones, C. L. and Ranganathan, S. *Teratology. 40* (1989) 143.
9. Zhang, W. W., Hood, R. D. and Smith-Sommerville, H. E. *Toxicol. Lett. 51* (1990) 35.
10. Lotta, T. I., Tulkki, A. P., Virtanen, J. A. and Kinnunen, P. K. J. *Chem. Phys. Lipids 52* (1990) 11.

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