An acetylation method for the quantification of membrane lipids, including phospholipids, polyphosphoinositides and cholesterol

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A method for the quantification of membrane lipids by acetylation with [³H]acetic anhydride is described. A standard lipid, labelled with ¹⁴C or ³²P, is added to the sample and is simultaneously acetylated. The ³H/¹⁴C or ³H/³²P ratios obtained in the acetylated lipids are proportional to the initial lipid concentration. The method has been used to quantify cholesterol, phosphatidylethanolamine, phosphatidylinositol, sphingomyelin, phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate in amounts between 2 and 10 nmol.

INTRODUCTION

Methods used to measure the amounts of phospholipids and neutral lipids in biological material usually involve digestion or saponification of the lipid, followed by colorimetric (Bartlett, 1959; Itaya & Ui, 1966; Rudel & Morris, 1973) or enzymic (Siedel *et al.*, 1985) assay. Quantification using these methods is inevitably prone to losses, owing to the many steps involved. In small amounts of membrane fractions it is usually possible to measure phosphatidylcholine (PtdCho) content using colorimetric methods, since PtdCho is the major component of most eukaryotic cell membranes. However, it is difficult to measure accurately the content of minor phospholipids and cholesterol in very small samples (less than 10 nmol of individual lipids) of membrane fractions, and we therefore sought a method to achieve this. Acetylation and quantification of diacylglycerols (DGs) using radioactive acetic anhydride has been described (Banschbach *et al.*, 1974). We have found that phospholipids bearing either an amino group, e.g. phosphatidylethanolamine (PtdEtn), or one or more hydroxy groups, e.g. PtdIns, can also be acetylated at these positions with acetic anhydride. We have investigated the conditions for acetylation of phospholipids, including the polyphosphoinositides, and for isolation of the acetylated derivatives by t.l.c. Conditions for acetylation of cholesterol have also been defined, together with separation by t.l.c. of cholesterol acetate and acetates of monoacylglycerol (MG) and DG. It should be noted that PtdCho and phosphatidic acid (PtdOH) do not have hydroxy or amino groups that can be acetylated in this way.

A dual-labelling modification was developed to ensure that the assay recorded the amount of lipid present in the original lipid sample. The principle was to add to the sample to be measured a small amount of high-specific-activity ¹⁴C- or ³²P-labelled standard lipid, and then to acetylate the lipid with [³H]acetic anhydride. The labelled lipid was isolated by t.l.c., and the ratio of ³H/¹⁴C or ³H/³²P measured and compared with ratios obtained from a set of standards treated similarly. Since the values were derived from ratios, they were unaffected by losses in work-up, by counting efficiency or by the precise value of specific activities.

EXPERIMENTAL

Materials

Pyridine, chloroform, tetrahydrofuran (THF), *NN*-dimethyl-

formamide (DMF), *NN*-di-isopropylethylamine, triethylamine, toluene and acetic anhydride were from Aldrich Chemical Co., Gillingham, Dorset, U.K., and 4-dimethylaminopyridine was from Kodak, Kirkby, Liverpool, U.K. PtdIns, sphingomyelin (Sph), PtdEtn and phosphatidylserine (PtdSer) were from Lipid Products, South Nutfield, Redhill, Surrey, U.K. PtdIns4P, PtdIns(4,5)P₂ (both 98 % pure), crude brain polyphosphoinositides, cholesterol, 1-monooleoylglycerol, 1,2-dioleoylglycerol, and 1,3-dioleoylglycerol were from Sigma Chemical Co. Poole, Dorset, U.K. Pure PtdCho was synthesized as described by Smith & Stein (1982). Precoated sheets for t.l.c. (Polygram Sil G and Sil N-HR) were from Camlab, Cambridge, U.K. [¹⁴C]Cholesterol, [*N*-methyl-¹⁴C]Sph, 1,2-dioleoyl L-3-phosphatidyl-L-[3-¹⁴C]serine, 1-palmitoyl-2-[1-¹⁴C]linoleoyl L-3-phosphatidylethanolamine, 1-palmitoyl-2-[1-¹⁴C]linoleoyl, L-3-phosphatidylinositol, all supplied at approx. 55 mCi/mmol were from Amersham International. [³H]Acetic anhydride (50 mCi/mmol) was from NEN Research Products, du Pont (U.K.), Stevenage, Herts., U.K., and was diluted with acetic anhydride in toluene to an appropriate specific radioactivity for the sensitivity required, usually 2–10 mCi/mmol. A working solution of 2–10 μmol of acetic anhydride/10 μl of toluene was convenient.

Methods

Plasma membranes were prepared from human neutrophils on a continuous Ficoll gradient [see the accompanying paper (Stein & Luzio, 1991)]. Neutral lipid extracts were prepared from the membranes by extraction with chloroform and methanol (Folch *et al.*, 1957; Bligh & Dyer, 1959). T.l.c. of phospholipids was as described by Stein & Smith (1982), total phosphorus assay as described by Bartlett (1959) and cholesterol assay as described by Rudel & Morris (1973). ³²P-labelled polyphosphoinositides were prepared by the method of Allan & Michell (1978) from fresh human erythrocytes and extracted in an acid chloroform/methanol solvent system (Hauser *et al.*, 1971). The extracts contained ³²P-labelled PtdIns4P and ³²P-labelled PtdIns(4,5)P₂. In addition, ³²P-labelled PtdOH was present, as reported by Allan & Michell (1978), but did not interfere with the assay, since it ran well ahead of the polyphosphoinositide acetates under the t.l.c. conditions used (Stein *et al.*, 1990). Bulk lipid acetate preparations to use as carriers in t.l.c. separations were prepared from 1 mg quantities of PtdIns, PtdEtn, Sph, PtdSer, cholesterol, MG and DG, and from 5 mg of a crude brain polyphospho-

Abbreviations used: PtdOH, phosphatidic acid; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdSer, phosphatidylserine; Sph, sphingomyelin; DG, diacylglycerol; MG, monoacylglycerol; THF, tetrahydrofuran; DMF, *NN*-dimethylformamide.

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Table 1. Summary of methods for acetylation and separation of lipids

Lipid	Reagents	Time and temperature	Extraction	T.l.c. solvents	T.l.c. plate
PtdEtn PtdIns Sph Cholesterol	Pyridine, THF, acetic anhydride	12 h, 37 °C	Folch <i>et al.</i> (1957)	Chloroform/methanol/15 M-NH ₃ (13:5:1, by vol.) Ethyl acetate [7% (v/v) in toluene] Chloroform/methanol/water/ acetic acid (75:25:3:8, by vol.)	Sil G
		24 h, 37 °C			
		24 h, 37 °C			
		12 h, 37 °C			
PtdSer	NaHCO ₃ , chloroform, acetic anhydride	2 min, 20 °C			
PtdIns4P	DMF containing 4- dimethylaminopyridine, NN-di-isopropylethylamine, acetic anhydride, sonicated	3 h, 37 °C	Hauser <i>et al.</i> (1971)	Chloroform/methanol/4 M-NH ₃ (6:4:1, by vol.)	Sil N-HR, oxalate-treated
PtdIns(4,5)P ₂		8 h, 37 °C			

inositide mixture using conditions given below, adapted to larger amounts of lipid and using unlabelled acetic anhydride.

Sample preparation, incubation and processing for dual-label lipid assay. A portion of the lipid extract was used for quantification. Cholesterol, DG and MG were determined either separately or in combination with PtdEtn, PtdIns and Sph (subsection *a* below). PtdSer was determined alone in a separate portion of the lipid extract (subsection *b* below). Polyphosphoinositide determination was as in subsection (*c*) below.

The sample to be quantified (i.e. the lipid extract prepared from membranes) and appropriate lipid standards (range 5–20 nmol) were prepared. To each sample was added an equal amount of radioactivity (approx. 10000–15000 c.p.m.) of the ¹⁴C- or ³²P-labelled derivative of the same lipid as that used for the standard curve. When quantifying amounts of lipid less than 10 nmol, pure PtdCho (approx. 10 µl of a solution of PtdCho in chloroform; 0.5 mg/ml) was added to prevent adsorption of lipids to the containing vessel. (Since PtdCho has no amino or hydroxy groups which can be acetylated, it acts as an inert lipid for this purpose.) All lipid samples, except PtdSer, where this is not necessary, were dried very thoroughly using a high-vacuum pump for several hours, then incubated with acetic anhydride (details are given in subsections *a*, *b* and *c*). All reactions were stopped by the addition of water to hydrolyse remaining acetic anhydride that had not reacted, followed by addition of unlabelled carrier lipid acetate to aid t.l.c. Table 1 summarizes the methods used.

(*a*) Cholesterol, PtdEtn, PtdIns and Sph. To each sample, prepared as above, was added THF (20 µl), pyridine (9 µl) and [³H]acetic anhydride in toluene (20 µl). Each sample was sealed and then incubated at 37 °C. Incubation times were chosen to give complete acetylation of the lipids under investigation (see Table 1 for incubation times for complete acetylation of standards). For complex lipid mixtures and membrane samples the standard incubation time was 36 h. The reaction was stopped with water (100 µl) to hydrolyse remaining [³H]acetic anhydride. After 15 min, chloroform/methanol (2:1, v/v; 1 ml) was added, along with sufficient (unlabelled) carrier phospholipid acetates to help in the t.l.c. separation and detection of the labelled lipids. A further addition of water (100 µl) completed the Folch extraction and resulted in separation into two phases. The upper phase was discarded and the lower phase washed several times with chloroform/methanol/water (3:48:47, by vol.; 500 µl) with 0.25 M-NaHCO₃ substituted for water in the first wash. This removed both pyridine and the remaining [³H]acetic acid (formed from hydrolysis of [³H]acetic anhydride that had not reacted)

from the lipids. The lower phase was evaporated and the acetylated lipids redissolved in a small amount of chloroform/methanol (2:1, v/v). T.l.c. separation was then performed and varied according to the lipid acetates.

(i) Cholesterol, DG and MG acetates were separated with 7% (v/v) ethyl acetate in toluene as solvent. *R_F* values are given in Table 2.

(ii) PtdEtn, PtdIns and Sph acetates were separated with chloroform/methanol/15 M-NH₃ (13:5:1, by vol.) as solvent. Development (optional) of the t.l.c. plate in the second dimension with chloroform/acetone/methanol/acetic acid/water (6:8:2:2:1, by vol.) gave improved discrimination between spots. *R_F* values are given in Table 2.

(iii) For separation of a mixture of cholesterol and glycerol acetates, together with PtdEtn, PtdIns and Sph acetates on one plate, the lipid mixture was applied two-thirds of the distance up the plate, which was developed with 7% ethyl acetate in toluene as solvent so that neutral lipid acetates were separated on the top one-third of the plate. This portion of plate was cut off and used for measurement of cholesterol MG and DG. The remaining portion of the plate with PtdEtn, PtdIns and Sph acetates at the origin was then developed in chloroform/methanol/15 M-NH₃ (13:5:1, by vol), with optional development in the second dimension with chloroform/acetone/methanol/acetic acid/water (6:8:2:2:1, by vol.) as in (ii).

(*b*) PtdSer. The sample for analysis and a set of PtdSer standards were prepared as described in subsection (*a*), except that the reaction took place in a two-phase system. To each prepared sample was added saturated NaHCO₃ (10 µl), chloroform (100 µl) and [³H]acetic anhydride (10 µmol). Incubation was at 20 °C for 15 min, and the reaction mixture was then diluted with water (100 µl), followed by addition of unlabelled PtdSer acetate as carrier. Extraction of lipids was as described above (*a*). T.l.c. separation was performed using chloroform/methanol/water/acetic acid (75:25:3:8, by vol.) as solvent. *R_F* values are given in Table 2.

(*c*) PtdIns4P and PtdIns(4,5)P₂. To each of the lipid samples of unlabelled PtdIns(4)P and/or PtdIns(4,5)P₂ (standard range 5–20 nmol) was added the same amount of radioactivity (approx. 5000–10000 c.p.m.) of ³²P-labelled polyphosphoinositides (see above for preparation). Water was thoroughly removed as follows. The samples were dried with a high-vacuum pump for several hours, then DMF (100 µl) was added to each and dried again at the pump overnight. Acetylation was performed by adding to each sample DMF (40 µl) containing approx. 200 µg of 4-dimethylaminopyridine/ml, NN-di-isopropylethylamine (20 µl) and [³H]acetic anhydride (10 µl), containing 20 µCi and

Table 2. Effect of acetylation of R_F values of lipids separated by t.l.c.

Values are means $\pm$ S.E.M., with the number of determinations in parentheses, or mean $\pm$ range when only two measurements were made; * single measurement. Acylation of PtdIns(4,5) P_2 resulted in two separate spots after t.l.c. separation (d).

Solvent	Lipid	R_F value	
		Native lipid	Acetylated lipid
(a) 7% (v/v) Ethyl acetate in toluene	Cholesterol	0.17 $\pm$ 0.01 (4)	0.68 $\pm$ 0.03 (8)
	1,2-DG	0.16 $\pm$ 0.01 (3)	0.48 $\pm$ 0.02 (6)
	1,3-DG	0.25 $\pm$ 0.01 (3)	0.55 $\pm$ 0.02 (6)
	MG	0.07*	0.24 $\pm$ 0.01 (4)
(b) Chloroform/ methanol/15 M-NH ₃ (16:5:1, by vol.)	PtdEtn	0.38 $\pm$ 0.04 (8)	0.62 $\pm$ 0.04 (8)
	PtdIns	0.18 $\pm$ 0.03 (5)	0.84 $\pm$ 0.02 (3)
	Sph	0.28 $\pm$ 0.03 (7)	0.44 $\pm$ 0.04 (6)
(c) Chloroform/ methanol/water/ acetic acid (75:25:3:8, by vol.)	PtdSer	0.45*	0.55 $\pm$ 0.02 (4)
(d) Chloroform/ methanol/4 M-NH ₃ (6:4:1, by vol.)	PtdIns4P	0.33 $\pm$ 0.02 (3)	0.50 $\pm$ 0.05 (9)
	PtdIns(4,5) P_2	0.11 $\pm$ 0.01 (3)	0.15 $\pm$ 0.01 (2)
			0.07 $\pm$ 0.01 (2)
(e) Chloroform/ methanol/water/ triethylamine (60:20:3:15, by vol.)	PtdIns4P	0.10 $\pm$ 0.02 (3)	0.36 $\pm$ 0.05 (2)
	PtdIns(4,5) P_2	0.03 $\pm$ 0.0 (4)	0.29*

2–5 μ mol of acetic anhydride. The sealed samples were sonicated for 5 min, then incubated (water rigorously excluded) at 37 °C for 8 h. The reaction was stopped by adding water (100 μ l). After hydrolysis of unchanged acetic anhydride unlabelled carrier polyphosphoinositide acetates (5–10 μ g) were added. The lipids were extracted in acid chloroform/methanol and the lipid-containing chloroform phase washed several times with acidified Folch upper phase, with both procedures as described by Hauser *et al.* (1971). This was necessary to retain the polyphosphoinositide acetates in the chloroform solution and to remove free [3 H]acetic acid from the chloroform solution. T.l.c. separation of the PtdIns4P acetates and PtdIns(4,5) P_2 acetates was on oxalate-impregnated plates of Sil N-HR (Billah & Lapetina, 1982) with chloroform/methanol/4 M-NH₃ (6:4:1, by vol.) as solvent. R_F values are given in Table 2.

Calculation of results. Areas containing lipid acetates were cut out from t.l.c. plates and 3 H, 14 C or 32 P radioactivity measured. The ratio of 3 H/ 14 C d.p.m., after correction for overspill from the 14 C channel into the 3 H channel and for quenching, was linear when plotted against nmol of phospholipid (Figs. 2a–2f). In addition to this, the specific radioactivity of the [3 H]acetic anhydride was calculated from the standards; this value was used to work out amounts of the other [3 H]acetylated phospholipids in the same lipid sample, assuming that recoveries were the same. When additional appropriate 14 C labelled derivatives were available as standards, the recoveries of each lipid assayed was separately measured.

RESULTS AND DISCUSSION

The chromatographic behaviour of lipids was changed by acetylation. Substitution of hydroxy groups (e.g. of cholesterol and PtdIns) with acetyl groups resulted in a loss in polarity; acetylation at an amino group (e.g. of PtdEtn and PtdSer) resulted in the loss of a positive charge on the nitrogen atom and also resulted in altered chromatographic mobility. These effects allowed lipid acetates to be separated from unchanged lipids by t.l.c. (Table 2).

Conditions for acetylation of some of the lipids commonly occurring in membranes were investigated. The speed of acetylation varied predictably with the groups to be acetylated and their molecular environment. Acetylation of cholesterol was complete by 12 h when incubated at 37 °C, but required 24 h when the incubation was at 20 °C (Fig. 1a). Acetylation of PtdEtn by acetic anhydride was complete after 5 h incubation at 37 °C, whereas acetylation of PtdIns was slower, passing through intermediate stages of partial acetylation, and was only complete at 24 h (Fig. 1b). Sph also was acetylated slowly, reaching completion after 24 h at 37 °C (Fig. 1b); the position of the hydroxy group, which is near the polar head group, may explain this. The choice of 37 °C as the incubation temperature was solely for convenience, as a standard laboratory incubator was used. Clearly the reaction would be completed in a shorter time if the incubation temperature were raised, but we have not investigated this. From theoretical considerations, the amino group of PtdSer should be acetylated very rapidly by acetic anhydride. Preliminary experiments established that, when the conditions for acetylation of PtdIns, PtdEtn and Sph were used for PtdSer, it decomposed and gave PtdOH as the sole non-volatile product. However, we found that, under modified conditions, PtdSer could be acetylated without decomposition. These were that excess aq. NaHCO₃ was present in a two-phase system to prevent the PtdSer from forming a mixed anhydride and subsequently undergoing a β -elimination. Under these conditions (detailed in the Experimental section), conversion of PtdSer into PtdSer acetate was very rapid (2 min at 20 °C) and was without degradative losses.

Cholesterol, PtdEtn, PtdIns, Sph and PtdSer all gave a linear relationship between the 3 H/ 14 C ratio and nmol of lipid (Figs. 2a–2e) and also were linear over the full ranges tested (2–60 nmol for PtdIns and Sph; 2–300 nmol for cholesterol, PtdEtn and PtdSer; results not shown). For comparison of quantification by the dual-label and by colorimetric assay, the lipids of neutrophil plasma membranes were analysed. The lipids were separated into polar and non-polar fractions on silicic acid columns (Stein & Smith, 1982), total phospholipid phosphorus was measured in the polar fraction (Barlett, 1959) and cholesterol was measured in

Fig. 1. Time course of lipid acetylation

(a) Cholesterol. Samples of cholesterol (100 nmol), together with added [^{14}C]cholesterol, were incubated as described in the Experimental section, but with unlabelled acetic anhydride (20 μmol in 10 μl of toluene), for various times, at 20 $^{\circ}\text{C}$ ($\square$) or 37 $^{\circ}\text{C}$ ($\blacksquare$). The products were extracted as described in the Experimental section, separated by t.l.c., and radioactivity determined in the cholesterol acetate spots. (b) PtdEtn, Sph and PtdIns. Samples (100 nmol) of PtdEtn, Sph or PtdIns, together with added [^{14}C]PtdEtn, [^{14}C]Sph, or [^{14}C]PtdIns respectively, were incubated as described in the Experimental section, but with unlabelled acetic anhydride (20 μmol in 10 μl of toluene) for various times, at 37 $^{\circ}\text{C}$. The radioactivity in the lipid acetate spots was determined as in (a). $\bullet$, PtdEtn acetate; $\circ$, PtdIns acetate; $\blacktriangle$, Sph acetate. (c) PtdIns4P and PtdIns(4,5) P_2 . Samples of PtdIns4P and PtdIns(4,5) P_2 (40 nmol) were incubated at 37 $^{\circ}\text{C}$ for various times. Conditions were as in the Experimental section and used [^3H]acetic anhydride (10 μmol in 10 μl of toluene), but did not use added ^{32}P -labelled polyphosphoinositides. The radioactivity in PtdIns4P acetate and PtdIns(4,5) P_2 acetate spots was determined as in (a). $\blacksquare$, PtdIns4P acetate; $\square$, PtdIns(4,5) P_2 acetate.

the non-polar fraction by colorimetric assay (Rudel & Morris, 1973) and by the dual-label assay. The cholesterol/phospholipid molar ratio obtained was 0.30 ± 0.28 (3) from colorimetric assay and 0.32 ± 0.05 (3) from dual-label assay. A comparison was also

Fig. 2. Lipid quantification

Representative standards for (a) cholesterol, (b) PtdEtn, (c) PtdIns, (d) Sph, (e) PtdSer, (f) PtdIns4P ($\square$) and PtdIns(4,5) P_2 ($\blacksquare$) are shown, with incubation conditions as described in the Experimental section.

Table 3. Comparison of molar ratios of neutrophil plasma-membrane phospholipids quantified by dual-label and by colorimetric assay

Plasma-membrane phospholipids were quantified by colorimetric assay or by dual-label assay as described in the Experimental section. Colorimetric results are expressed as percentage recovered lipid phosphorus (a). Each total P_i analysis were then recalculated as mol of each phospholipid/10 mol of Sph (b). Dual-label assay results (c) are also expressed as mol of phospholipid/10 mol of Sph. Values are means $\pm$ S.E.M. for three separate assays; N.D., not determined.

Phospholipid	(a) Colorimetric assay (% of total P_i)	(b) Colorimetric assay (molar ratio)	(c) Dual-label assay (molar ratio)
Sph	17.0 ± 3.7	10	10
PtdEtn	22.7 ± 2.8	15.5 ± 5.1	17.3 ± 1.1
PtdIns	11.3 ± 0.5	7.8 ± 2.6	5.9 ± 1.8
PtdSer	8.7 ± 1.8	5.8 ± 1.7	4.1 ± 0.9
PtdCho	40.3 ± 2.6	27.8 ± 9.6	N.D.

made between the content of PtdEtn, PtdIns, Sph, PtdSer and cholesterol in neutrophil plasma membranes assayed by t.l.c. followed by colorimetric assays and, alternatively, by the dual-label assay (Table 3). The values obtained by the two methods were not significantly different and were similar to previously published values for a human neutrophil plasma membrane preparation using a colorimetric assay (Smolen & Shohet, 1974).

We found that polyphosphoinositides were converted only slowly into their fully acetylated derivatives when incubated with the pyridine/acetic anhydride mixture, and a specific reagent system was therefore developed for acetylation of PtdIns4P and PtdIns(4,5) P_2 . This used the dipolar aprotic solvent DMF, in order to prevent formation of inverted micelles and the consequent exclusion of the non-polar acetic anhydride from the

reaction site. We included 4-dimethylaminopyridine as a catalyst and also used *NN*-di-isopropylethylamine, an apolar hindered base, to ion-pair with the phosphate groups of PtdIns4*P* and PtdIns(4,5)*P*₂. In this system total acetylation was achieved under conditions mild enough to avoid degradation of polyphosphoinositides. A time course of acetylation of PtdIns4*P* and PtdIns(4,5)*P*₂ (Fig. 1c) showed that acetylation of PtdIns4*P* was complete after 3 h and of PtdIns(4,5)*P*₂ after 8 h incubation at 37 °C. Acetylation of PtdIns4*P* resulted in a product which ran as a single spot on t.l.c. (*R*_F 0.5 in Table 2; solvent system *d*). Acetylation of PtdIns(4,5)*P*₂ proceeded more slowly and gave a product which ran as two adjacent spots on t.l.c. (*R*_F values 0.15 and 0.07 in Table 1d). Data from the spot with *R*_F 0.15, which comprised 50–95% of the total PtdIns(4,5)*P*₂ acetate, was used for calculations in the dual-label assay of the total PtdIns(4,5)*P*₂ acetate. A linear relationship between the ³H/³²P ratio and nmol of PtdIns4*P* and PtdIns(4,5)*P*₂ was obtained (Fig 2f). We noted that minor acetylated products of PtdIns(4,5)*P*₂ were formed if the incubation time were prolonged, one of which partly overlapped the PtdIns4*P* acetate spot. T.l.c. separation of PtdIns4*P* and PtdIns(4,5)*P*₂ acetates was also carried out with chloroform/methanol/water/triethylamine (60:20:3:15, by vol.); elution of the polyphosphoinositides in this much-lower-polarity solvent was facilitated by ion-pairing with the triethylammonium ion. This gave much greater increases in *R*_F after the polyphosphoinositides were acetylated (Table 2e), although resolution on the basis of phosphorylation status [i.e. PtdIns4*P* versus PtdIns(4,5)*P*₂] was reduced. This solvent system may be useful to observe partially acetylated states.

The dual-label method requires high-specific-radioactivity ¹⁴C- or ³²P-labelled lipid standards so that [³H]acetate incorporation into the ¹⁴C- or ³²P-labelled material is minimized. In addition, the specific radioactivity of the [³H]acetic anhydride should be high enough to give about ten times the amount of ³H radioactivity to ¹⁴C or ³²P radioactivity in the final acetylated lipid. We have measured 2–10 nmol of lipid per assay using [³H]acetic anhydride, which we diluted to a specific radioactivity of 2 mCi/mmol. In theory, the sensitivity of the method can be increased by another order of magnitude, since [³H]acetic anhydride can be obtained at 50 mCi/mmol, but this introduces further problems, since in order to provide enough acetic anhydride to drive the reaction forward, the amount of radioactivity used may be increased to unacceptable levels. For quantification of larger amounts of lipids (say 50–200 nmol), the specific radioactivity of the [³H]acetic anhydride should be correspondingly lower, to allow enough acetic anhydride to drive the reaction forward without adding unnecessary radioactivity.

The assay described here allows direct quantitative determinations of the amounts of lipid (> 2 nmol) in the initial sample. When used to measure cholesterol, the dual-label method can be

applied to samples containing salts and buffers that would interfere with an enzymic assay performed at this level of sensitivity. When the dual-label assay is used for phospholipids, the amount of a single phospholipid in a sample may be measured separately if required, rather than total phosphorus measured in every separated phospholipid and the amount of each calculated as a percentage of the total lipid phosphorus. The sensitivity of the dual-label method compares very favourably with quantification carried out by measurement of total lipid phosphorus and, moreover, requires only simple apparatus, uses precoated t.l.c. sheets, and does not need a heating and acid-digestion stage. A further advantage of the method is that phospholipids, cholesterol MG and DG can be quantified in the same acetylated sample of membrane lipids.

We have found that other lipids (cardiolipin, phosphatidylglycerol and neutrophil glycosylceramides) can be acetylated by acetic anhydride in the pyridine-containing mixture (J. M. Stein, G. A. Smith & J. P. Luzio, unpublished work). We suggest that the methodology can be extended to quantify such lipids, and also polyphosphoinositide breakdown products, including inositol phosphates.

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