Biochemical Pharmacology, Vol. 29, pp. 789–793.

0006-2952/80/0301-0789 $02.00/0

EFFECT OF CHLORPROMAZINE ON THE CYTOPLASMIC PHOSPHATIDATE PHOSPHOHYDROLASE IN RAT LIVER

HAYAO IDE and YASUO NAKAZAWA

Medical Research Institute, Tokyo Medical and Dental University, 3–10, 2-chome, Kanda-Surugadai, Chiyoda-ku, Tokyo 101, Japan

(*Received* 27 *June* 1979; *accepted* 27 *September* 1979)

Abstract—The inhibitory effect of chlorpromazine on phosphatidate phosphohydrolase (EC 3.1.3.4) activity was studied with respect to the selectivity of the molecular species of phosphatidate. Rat liver microsomes which include endogenously labeled [^{14}C]phosphatidic acid were prepared by the incubation of microsomes with *sn*-[^{14}C]glycerol-3-phosphate and used as substrate. The distribution of radioactivity among the molecular species of [^{14}C]phosphatidate remaining after incomplete hydrolysis of the substrate exhibited little difference from that of the untreated substrate. When the hydrolysis was suppressed by the addition of chlorpromazine, however, the radioactivity distributed in the monoenoic and dienoic [^{14}C]phosphatidate increased. The preference of the molecular species of phosphatidate in the inhibition was further confirmed by the experiment run with microsomes containing 2-[1-^{14}C]palmitoyl, oleoyl, linoleoyl and arachidonyl species of phosphatidate as substrate.

Amphiphilic drugs having an ionizable amine residue have been found to stimulate ^{32}P incorporation into CDP-diacylglycerol and acidic phospholipids such as phosphatidic acid and phosphatidylinositol in various tissues [1–7], while decreasing the rate of synthesis of triacylglycerol, phosphatidylcholine and phosphatidylethanolamine.

Sturton and Brindley showed that these amphiphilic cations inhibit the activity of phosphatidate phosphohydrolase *in vitro* [8], and proposed that the inhibition of phosphatidate phosphohydrolase might involve an interaction between phosphatidate and positively charged drugs.

In the present paper, the effect of chlorpromazine, one of the potent inhibitors, on the activity of cytoplasmic phosphatidate phosphohydrolase was studied using membrane-bound phosphatidate as a substrate, and the specificity of the inhibition of the substrate to fatty acyl species was investigated.

MATERIALS AND METHODS

Materials. *sn*-[^{14}C]Glycerol-3-phosphate (130 mCi/mmole), [1-^{14}C]palmitic acid (59 mCi/mmole), [1-^{14}C]oleic acid (58 mCi/mmole), [1-^{14}C]linoleic acid (60 mCi/mmole) and [1-^{14}C]arachidonic acid (56 mCi/mmole) were purchased from the Radiochemical Centre, Amersham, England. ATP and CoA were obtained from Kyowa Hakko Co., Tokyo. Phospholipase A from *Crotalus adamanteus* was purchased from Sigma Chemical Co., St. Louis, MO. Phospholipase C was prepared from culture fluid of *Bacillus cereus*. Phospholipase D was prepared from cabbage leaves according to the method of Davidson and Long [9]. All other chemicals were of reagent grade.

Male Wistar rats weighing 100–120 g were used for all experiments. They were housed two per cage and fed Clea CF-2 Laboratory Chow and water *ad lib.* prior to experiment.

Preparation of subcellular fractions and phosphatidate phosphohydrolase. The livers were homogenized in 5 vol. of 0.25 M sucrose containing 0.05 M Tris–HCl (pH 7.4) using a Potter–Elvehjem homogenizer. The homogenate was centrifuged at 1000 *g* for 10 min to sediment nuclei and cell debris, and the supernatant was next centrifuged twice at 11,700 *g* for 20 min in a Hitachi model 18PR-3 centrifuge. The resulting supernatant was centrifuged at 104,000 *g* for 1 hr in a Hitachi model 65P ultracentrifuge. The pellet was suspended in 25 ml of 0.25 M sucrose containing 0.05 M Tris–HCl (pH 7.4) and again centrifuged at 104,000 *g* for 1 hr. The pellet was finally resuspended in 5 ml of 0.25 M sucrose containing 0.05 M Tris–HCl (pH 7.4) and used as microsomal fractions. The suspension contained 22–27 mg protein per g liver.

Phosphatidate phosphohydrolase was prepared from particle-free supernatant by ammonium sulfate precipitation according to the method of Lamb and Fallon [10].

Preparation of microsomes including endogenously labeled [^{14}C]*phosphatidate.* The microsomes (1.5 mg protein) were incubated with 1.2 mM *sn*-[^{14}C]glycerol-3-phosphate, 1.2 mM ATP, 0.03 mM CoA, 60 mM potassium fluoride, 6.25 mg of fatty acid-poor bovine serum albumin (Fraction V, Pentex Inc., Kankakee, IL), 0.15 mM dithiothreitol and 3.5 mM Tris–HCl, pH 7.4. After 60 min, the reaction mixture was cooled on ice and centrifuged at 105,000 *g* for 30 min. The resulting pellets were suspended in 0.01 M Tris–HCl (pH 7.4) containing 0.25 M sucrose [11].

Preparation of microsomal membrane-bound

phosphatidate labeled with [^{14}C]*fatty acid.* 1-Saturated acyl-*sn*-glycero-3-phosphocholine was prepared by the catalytic hydrogenation [12] of 1-acyl-*sn*-glycero-3-phosphocholine derived from rat liver microsomal lecithin. Phosphatidic acid having saturated fatty acid in the 1-position and [^{14}C]fatty acid in the 2-position were prepared with slight modification of the method of Lands and Hart [13]. The incubation mixture consisted of 1.0 μmoles of 1-saturated acyl-*sn*-glycero-3-phosphocholine, 0.64 μmoles of [1-^{14}C]fatty-acyl CoA [14], rat liver microsomes (0.1 mg protein) and 4.0 ml of 0.5 M Tris–HCl (pH 7.4) in a total volume of 20 ml. The incubation was carried out at room temperature and the reaction rate was monitored with 10 mM 5,5′-dithiobis(2-nitro-benzoic acid) (0.1 ml) added in the incubation mixture. When the reaction curve reached the plateau, the mixture was cooled on ice and centrifuged at 104,000 *g* for 1 hr. Lipids were extracted from the supernatant according to the method of Folch *et al.* [15]. Phosphatidylcholine was isolated by silicic acid column chromatography and treated with phospholipase D for 3 hr at room temperature to produce 1-saturated acyl-2-[1-^{14}C]acyl-*sn*-glycerol-3-phosphate. The product was converted to sodium salt by passing through a column of Chelex resin (Na^+ form) as described by Renkonen [16].

An aliquot of microsomal suspensions corresponding to 20 mg protein was incubated at 37° with phosphatidate phosphohydrolase (15 mg protein), 2 mM $MgCl_2$, 0.25 M sucrose and 0.05 M Tris–HCl (pH 7.4) in a total volume of 3.5 ml. After 3 hr, the mixture was cooled on ice and centrifuged at 105,000 *g* for 1 hr. The pellet resuspended in 5 ml of 0.05 M Tris–HCl (pH 7.4) containing 0.25 M sucrose was further incubated with phospholipase C (7.5 mg protein) and 5 mM $MgCl_2$ at room temperature for 20 min. The mixture was cooled on ice and centrifuged at 105,000 *g* for 1 hr. The phospholipid-depleted microsomal fraction thus obtained was incubated with 120 mM potassium fluoride and 0.9 μmoles of 1-saturated acyl-2-[1-^{14}C]acyl-*sn*-glycero-3-phosphate (sodium salt) in 2.5 ml of 0.05 M Tris–HCl (pH 7.4) containing 0.25 M sucrose at room temperature. After 1 hr, 5 mg of rat liver lecithin dispersed in 1.0 ml of 0.25 M sucrose was added and incubation was continued for 20 min. The mixture was cooled on ice and centrifuged at 105,000 *g* for 1 hr. The resulting pellet was resuspended in 0.05 M Tris–HCl (pH 7.4) containing 0.25 M sucrose.

Assay of phosphatidate phosphohydrolase. Two different types of membrane-bound substrate were used. The incubation mixture contained 0.01 M Tris–HCl (pH 7.4), 2 mM $MgCl_2$ and 27.8 nmoles of endogenously labeled [^{14}C]phosphatidate in a total volume of 4.0 ml. In the case of another substrate, 5.0 nmoles of phosphatidate labeled with [1-^{14}C]fatty acid were replaced as a substrate in a total volume of 1.0 ml. The incubation was carried out at 37° for 30 min. The reaction was stopped by the addition of 5.0 ml of chloroform–methanol (2:1, v/v) and lipids were extracted according to the method of Folch *et al.* [15]. The lipid was separated on a thin layer chromatoplate (silica gel H containing potassium oxalate) developed with chloroform–methanol–acetic acid–water (65:25:5:1, by vol.) after the addition of suitable amount of carrier lipid. The area of silica gel plate corresponding to phosphatidate was scraped off into vials for measurement of the radioactivity. Chlorpromazine·HCl (2 mM) was added to the incubation mixture in the inhibition experiment.

Determination of fatty acyl species of ^{14}C-*phosphatidate.* The phosphatidate fraction was isolated from the lipid extract by thin layer chromatography. The lipid extract was separated on silica gel H plate with chloroform–methanol–28% ammonia (65:25:5, by vol.) and the area corresponding to phosphatidate was extracted with chloroform–methanol–acetic acid (50:25:5, by vol.). The extract was then evaporated to dryness and rechromatographed with chloroform–acetone–methanol–acetic acid–water (5:2:1:1:0.5, by vol.) Phosphatidate was extracted as above. Radioactive phosphatidate was converted to the dimethyl derivatives according to the method of Renkonen [16]. The dimethylphosphatidate were separated into the individual fatty acyl species on $AgNO_3$-impregnated thin layer chromatoplate developed with chloroform–methanol (49:1, v/v) after the addition of dimethylphosphatidate prepared from rat liver lecithin as a carrier. The individual bands were visualized under ultraviolet light after spraying the plates with 0.1% 2′,7′-dichlorfluorescein in methanol and scraped into separate columns (120 × 8 mm). The column was developed off with 3 ml of 90% methanol containing 1% sodium chloride and dimethylphosphatidate was separated from dye by the extraction with 4 ml of hexane after addition of 2 ml of water. A portion of dimethylphosphatidate of each fractions were subjected to transesterification with 2 ml of 0.5 N sodium methoxide to analyse the fatty acid species by gas–liquid chromatography and another portion was submitted to measurement of the radioactivity.

Other analyses. Organic phosphorus was determined by the molybdate–ascorbic acid method after digesting with 70% perchloric acid [17]. Protein was determined according to the method of Lowry *et al.* [18] with bovine serum albumin as standard. Radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer model 2002 using 10 ml of toluene–ethanol (7:3, v/v) with 0.4% (w/v) 2,5-diphenyl-oxazole and 0.01% (w/v) 2,2′-*p*-phenylene-bis(5-phenyloxazole).

RESULTS

Cytoplasmic phosphatidate phosphohydrolase activity was measured using microsomal membrane-bound ^{14}C-phosphatidate as substrate. Figure 1 shows the time course of the hydrolysis, demonstrating that about 78 per cent of membrane-bound phosphatidate was hydrolysed after the incubation for 2 hr. Prolonging the time of incubation or adding a supplementary amount of the enzyme to the incubation mixture after 3 hr had little effect on the hydrolysis of phosphatidate, suggesting that phosphatidate molecules available for the enzyme attack were almost exhausted within 2 hr.

The effect of chlorpromazine on the activity of phosphatidate phosphohydrolase is shown in Fig. 2. The addition of chlorpromazine brought about

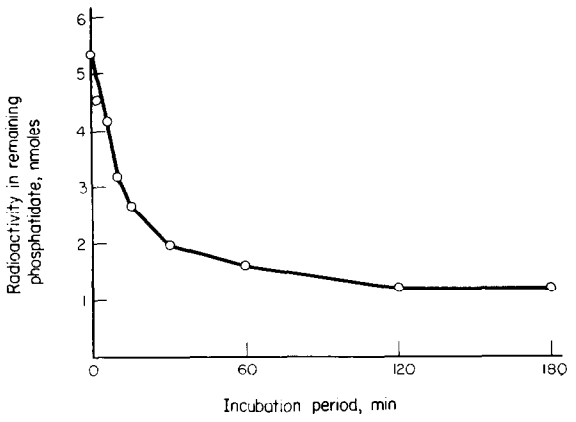

Fig. 1. Time course for the hydrolysis of [^{14}C]phosphatidate in microsomal membranes. Incubation mixture contained 1.99 nmoles of endogenously labeled phosphatidate in rat liver microsomes, phosphatidate phosphohydrolase (0.95 mg protein), 0.01 M Tris–HCl (pH 7.4) and 2.0 mM $MgCl_2$ in a final volume of 1.0 ml.

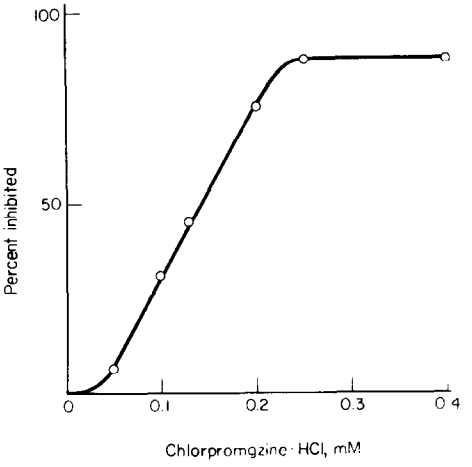

Fig. 2. Effect of varying the concentration of chlorpromazine on the activity of phosphatidate phosphohydrolase. Incubation conditions were as described in Materials and Methods.

increasing inhibition. Maximum inhibition was obtained at a chlorpromazine concentration of more than 0.25 mM and half-maximal inhibition was achieved at about 0.13 mM.

After the hydrolysis, unreacted [^{14}C]phosphatidate was separated into fatty acyl species on a $AgNO_3$-impregnated thin layer chromatoplate. The distribution of radioactivity among the fatty acyl species was not significantly different from that of unreacted phosphatidate (Table 1), indicating that phosphatidate phosphohydrolase from rat liver cytosol has little specificity towards the molecular species of microsomal phosphatidate. When the hydrolysis of phosphatidate was inhibited by 45 per cent with chlorpromazine, however, the radioactivity distributed in the monoenoic and dienoic species of phosphatidate increased slightly over those in the original substrate (Table 1). This finding suggests that chlorpromazine preferentially inhibits the hydrolysis of the monoenoic and dienoic species as compared with other species.

The possible relation between phosphatidate species and the inhibition rate of chlorpromazine led us to attempt a further study using another type of membrane-bound substrate. The substrate used was phosphatidate bearing saturated acyl radical at the 1-position and specified [1-^{14}C]fatty acyl radical at the 2-position and the radioactive phosphatidate was incorporated into the microsomes which was previously deprived of the intact phosphatidate and other phospholipids by the hydrolysis with phosphatidate phosphohydrolase and subsequent hydrolysis with phospholipase C. The microsomal preparation used as substrate in the following experiment contained radioactive phosphatidate of 1.9, 4.2, 5.1 and 4.4 nmoles per mg protein in the [1-^{14}C]palmitate, [1-^{14}C]oleate, [1-^{14}C]linoleate and [1-^{14}C]-arachidonate species, respectively, in addition to the amount corresponding to approximately 20 per cent of phosphatidate involved in the original membrane.

Figure 3 gives the time course for the hydrolysis of radioactive phosphatidate of four molecular

Table 1. Distribution of radioactivity among molecular species of microsomal [^{14}C]phosphatidate*

Molecular species	Distribution of radioactivity (%)		
	A	B	C
Saturates	7.3 ± 1.4	5.3 ± 1.3	5.0 ± 0.8
Monoenes	18.1 ± 0.7	16.6 ± 1.0	21.0 ± 1.5
Dienes	41.9 ± 2.2	43.7 ± 1.5	46.5 ± 2.1
Trienes	4.5 ± 1.0	3.2 ± 0.9	2.9 ± 0.1
Tetraenes	10.1 ± 0.7	10.5 ± 1.0	9.0 ± 0.6
Polyenes	18.1 ± 1.3	20.6 ± 1.0	15.6 ± 1.0

* Dimethylphosphatidate, which were prepared from phosphatidate, were separated into species by $AgNO_3$-TLC as described in Materials and Methods. (A) Original substrate; (B) phosphatidate remained after hydrolysis under the condition described in Materials and Methods (63 per cent of the substrate was hydrolysed); (C) phosphatidate remained after hydrolysis in the presence of 0.13 mM chlorpromazine (28 per cent of the substrate was hydrolysed). The hydrolysis carried out in (B) was inhibited by 45 per cent. The values are means ± S.E.M. from three separate incubations.

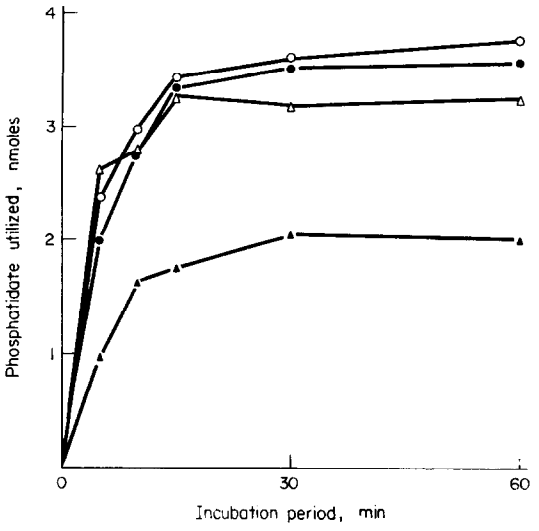

Fig. 3. Hydrolysis of [^{14}C]phosphatidate incorporated to the microsomal membrane of rat liver by phosphatidate phosphohydrolase. Incubation conditions were as described in Materials and Methods. (▲—▲), saturated; (●—●), monoenoic; (△—△), dienoic; (○—○), tetraenoic.

species prepared as described above. The monoenoic, dienoic and tetraenoic species of phosphatidate were hydrolysed at approximately the same rate (0.041, 0.034 and 0.045 nmoles/min/mg protein, respectively). However, the rate of hydrolysis of the saturated species was about one half (0.017 nmoles/min/mg protein) of the other three species, probably owing to the low content of the phosphatidate in microsomal membrane [19].

The inhibitory effect of chlorpromazine on the hydrolysis of membrane-bound [^{14}C]phosphatidate

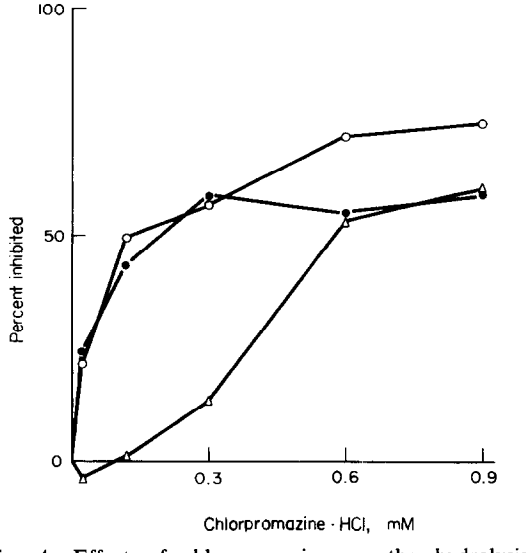

Fig. 4. Effect of chlorpromazine on the hydrolysis of [^{14}C]phosphatidate incorporated to the microsomal membranes of rat liver. Incubation conditions were as described in Materials and Methods. (○—○), monoenoic; (●—●), dienoic; (△—△), tetraenoic.

was revealed at higher rate in the monoenoic and dienoic species than tetraenoic phosphatidate, showing 50 per cent inhibition doses of 0.13, 0.20 and 0.57 mM, respectively (Fig. 4).

DISCUSSION

Phosphatidate phosphohydrolase catalyses the release of orthophosphate from *sn*-1,2-diacyl-glycero-3-phosphate to form 1,2-diacylglycerol, an intermediate reaction in *de novo* synthesis of phosphatidylcholine, phosphatidylethanolamine and triacylglycerol. The properties and subcellular distribution of this enzyme have been studied in some detail. Although the enzyme is found in mitochondria, microsomes, lysosomes and cytoplasm from various tissues, Johnston *et al.* [20] demonstrated that the cytoplasmic phosphatidate phosphohydrolase is primarily involved in diglyceride formation, while particulate enzyme play a less important role. Since the cytoplasmic enzyme has been reported to have low activity to aqueous dispersion of phosphatidate and high activity to membrane-bound substrate [21], two kinds of microsomal membrane-bound substrate were prepared and used throughout the experiment.

Since newly formed diacylglycerol was partially degraded by microsomal lipase in the enzymatic hydrolysis of phosphatidate, the rate of radioactivity distributed among the molecular species of phosphatidate remaining after the incubation was compared to that of original substrate in the experiment with the microsomes including endogenously labeled ^{14}C-phosphatidate. The results suggested that little preference of phosphatidate phosphohydrolase is exhibited towards the molecular species of phosphatidic acid. However, the inhibition of phosphatidate phosphohydrolase caused by chlorpromazine was observed to have a significant selectivity to monoenoic and dienoic species. This finding led us to attempt further experiments using microsomal membrane-bound phosphatidate bearing specified [1-^{14}C]fatty acyl radical as substrate. To prepare the membrane-bound substrate, the microsomal membranes were treated with phosphatidate phosphohydrolase and phospholipase C and then ^{14}C-phosphatidate was incorporated into the phospholipid-depleted membrane. Although the form of the radioactive phosphatidate molecule in the microsomal membrane is not elucidated, the phosphatidate were assumed to be incorporated in membrane-bound form, since they were hydrolysed at a high rate with cytoplasmic phosphatidate phosphohydrolase [21] and were not released from microsomal membrane by repeating freezing and thawing.

Phosphatidate phosphohydrolase from rat liver cytoplasm was shown in this experiment to have no selectivity towards fatty acyl species of incorporated radioactive phosphatidate. Åkesson *et al.* [22] have reported that phosphatidate phosphohydrolase does not exhibit any selectivity with respect to the fatty acid composition of its substrate and Hill *et al.* [23] have made the same observation using rat liver slices. The present results obtained in *in vitro* experiments are compatible with the conclusions of these authors.

When the hydrolysis was inhibited by the addition of chlorpromazine, monoenoic and dienoic species of phosphatidate were more susceptible to the inhibition by chlorpromazine than tetraenoic species (Fig. 4), in good agreement with the result of the experiment using endogenously labeled [^{14}C]-phosphatidate. Considering that the inhibition of phosphatidate phosphohydrolase by chlorpromazine is attributed to the interaction of the drug with phosphatidate [24], the preference of the inhibition for the molecular species of phosphatidate would be reasonably understood.

In the *de novo* synthesis of phosphoglyceride, phosphatidic acid is located in a diverging point on the pathways of synthesis of acidic and neutral phosphoglyceride. Since it has been found that phosphatidate phosphohydrolase exhibited no selectivity of molecular species in *in vivo* [22] and *in vitro* [23] experiments, the fatty acyl compositions of acidic and neutral phospholipid should be modified to the peculiar patterns by the subsequent steps. However, the inhibitory effects of chlorpromazine on the hydrolysis of phosphatidate was demonstrated in the present study to be more remarkable on monoenoic and dienoic than tetraenoic species of phosphatidate. It is likely, therefore, that the selectivity of the molecular species operates also at the step of phosphatidate phosphohydrolase by the administration of chlorpromazine, and fatty acid composition of phosphoglyceride consequently might be affected.

REFERENCES

1. I. E. Hughes and J. G. Salway, *J. Pharm. Pharmac.* **25**, 745 (1973).
2. J. Eichberg and G. Hauser, *Biochem. biophys. Res. Commun.* **60**, 1460 (1974).
3. D. Allan and R. H. Michell, *Biochem. J.* **148**, 471 (1975).
4. J. Eichberg, H. M. Shein, M. Schwartz and G. Hauser, *J. biol. Chem.* **248**, 3615 (1973).
5. G. Hauser and J. Eichberg, *J. biol. Chem.* **250**, 105 (1975).
6. N. Freinkel, C. E. Younsi and R. M. C. Dawson, *Eur. J. Biochem.* **59**, 245 (1975).
7. R. H. Michell, *Biochim. biophys. Acta* **415**, 81 (1975).
8. R. G. Sturton and D. N. Brindley, *Biochem. J.* **162**, 25 (1977).
9. F. M. Davidson and C. Long, *Biochem. J.* **69**, 458 (1958).
10. R. G. Lamb and H. J. Fallon, *Biochim. biophys. Acta* **348**, 166 (1974).
11. B. H. Holub and J. Piekarski, *Lipids* **11**, 251 (1975).
12. K. Waku and Y. Nakazawa, *J. Biochem.* **68**, 459 (1970).
13. W. E. M. Lands and P. Hart, *J. biol. Chem.* **240**, 1905 (1965).
14. W. E. M. Lands, M. L. Blank, L. J. Nutter and O. S. Privett, *Lipids* **1**, 224 (1966).
15. J. Folch, M. Lees and G. H. Sloane-Stanley, *J. biol. Chem.* **226**, 497 (1957).
16. O. Renkonen, *Biochim. biophys. Acta* **152**, 114 (1968).
17. G. Rouser, A. N. Siakotas and S. Fleischer, *Lipids* **1**, 85 (1966).
18. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
19. G. P. H. van Heusden and H. van den Bosch, *Eur. J. Biochem.* **84**, 405 (1978).
20. J. M. Johnston, G. A. Rao, P. A. Lowe and B. E. Schwarz, *Lipids* **2**, 14 (1967).
21. M. E. Smith, B. Sedgwick, D. N. Brindley and G. Hübscher, *Eur. J. Biochem.* **3**, 70 (1967).
22. B. Åkesson, J. Elovson and G. Arvidson, *Biochim. biophys. Acta* **210**, 15 (1970).
23. E. E. Hill, W. E. M. Lands and S. P. M. Slakey, *Lipids* **3**, 5 (1970).
24. M. Bowley, J. Cooling, S. L. Burditt and D. N. Brindley, *Biochem. J.* **165**, 447 (1977).