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A rapid assay for measuring the activity and the Mg^{2+} and Ca^{2+} requirements of phosphatidate phosphohydrolase in cytosolic and microsomal fractions of rat liver

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1. A rapid extraction and purification scheme was designed for the recovery of [^{3}H]diacylglycerol formed during the assay of phosphatidate phosphohydrolase. 2. The importance of removing polyvalent cations, particularly Ca^{2+}, from the phosphatidate and other reagents used in the assay of the phosphohydrolase activity was demonstrated. This was achieved mainly by treating the phosphatidate with a chelating resin and by adding 1 mM-EGTA and 1 mM-EDTA to the assays. 3. The activity of the phosphohydrolase in dialysed samples of the soluble and microsomal fractions of rat liver was very low. 4. Addition of optimum concentrations of $MgCl_2$ resulted in a 110–167-fold stimulation in activity. 5. $CaCl_2$ was also able to stimulate phosphohydrolase activity, but to a much smaller extent than $MgCl_2$. 6. Chlorpromazine, an amphiphilic cation, inhibited the reaction when it was measured in these experiments by using a mixed emulsion of phosphatidylcholine and phosphatidate at pH 7.4. 7. Microsomal fractions that were pre-incubated with albumin contained very low activities of the Mg^{2+}-dependent phosphohydrolase. When these were then incubated with the soluble fraction in the presence of oleate, the soluble phosphohydrolase attached to the microsomal membranes, and it retained its high dependency on Mg^{2+}.

INTRODUCTION

PAP activity is important in controlling the rate of synthesis of glycerolipids, especially of triacylglycerol (for reviews see [1–3]). In the liver this enzyme is subject to long-term control. Glucocorticoids [4,5], glucagon [5], cyclic AMP [4] and growth hormone [6] increase its activity. Insulin [4–6] and spermine [7] can antagonize these effects, which appear to be mediated at the level of enzyme synthesis. In addition, the stability of the PAP activity in isolated hepatocytes is increased by glucagon and cyclic GMP, and it is decreased by insulin and spermine [8]. The balance between these hormones and metabolites is thought to control the amount of PAP in the liver.

The metabolic expression of this activity is thought to be regulated by the ability of the cytosolic form of the PAP to interact with the endoplasmic reticulum on which its substrate is generated [2]. In liver [2,9,10] and adipose tissue [11,12] this seems to be mainly controlled by the accumulation of fatty acids and acyl-CoA esters on the membranes of the endoplasmic reticulum. *In vitro* at least, spermine also facilitates the association of PAP with these membranes [2,9,13,14], and it potentiates the effects of fatty acids in this respect [2,9,15]. Fatty acids therefore seem to act as feed-forward regulators that activate PAP so as to attempt to match the rate of triacylglycerol synthesis to the net fatty acid supply [2,3].

The ability of fatty acids to cause this activation appears to be under hormonal control. For example, cyclic AMP decreases the association of PAP with the membranes at low, but not at high, fatty acid concentrations [16]. This effect is paralleled by the observed rate of triacylglycerol synthesis in hepatocytes [17]. Glucagon has a similar action on the subcellular distribution of PAP, whereas insulin has the opposite effect [5]. The amphiphilic amine, chlorpromazine, also antagonizes the effect of fatty acids in that it displaces PAP from the endoplasmic-reticulum membranes [10]. In hepatocytes this displacement is paralleled by a decrease in the rate of conversion of phosphatidate into diacylglycerol and in the subsequent synthesis of triacylglycerol [10]. A limited correlation also exists between the proportion of PAP that was particulate and the rate of triacylglycerol synthesis in adipose tissue under a variety of conditions [12]. The combined observations listed above support the hypothesis that the reversible association of PAP with the membranes of the endoplasmic reticulum can be an important component in the overall regulation of glycerolipid synthesis.

To investigate this control further, we thought it essential to devise a rapid and accurate assay that could distinguish different PAP activities that are present in microsomal and soluble fractions of cells. In liver [18–20], adipose tissue [21,22], lung [23] and other tissues, the cytosolic PAP exhibits a fairly high stimulation with Mg^{2+}. By contrast, PAP activity in microsomal and in other particulate fractions is increased to a smaller extent by Mg^{2+} [21,23,24]. This observation raises the questions: (*a*) does the cytosolic PAP retain or lose its Mg^{2+} requirement when it translocates to the membranes, and (*b*) to what extent is there a Mg^{2+}-independent PAP activity in microsomal fractions that is different from the cytosolic PAP? We designed the present experiments to answer these questions.

Abbreviation used: PAP, phosphatidate phosphohydrolase (EC 3.1.3.4).

EXPERIMENTAL

Animals and materials

The sources of the rats and most materials have been described [25,26]. [1,3-^{3}H]Glycerol and [^{3}H]palmitic acid were from Amersham International, Amersham, Bucks., U.K., glycerol kinase was from Boehringer Corp. (London), Lewes, East Sussex, U.K., and silica gel 60 (70–270 mesh) was from Macherey–Nagel, Duren, West Germany. Basic aluminium oxide 90 (Brockman Activity II–III; 70–230 mesh) from Merck was purchased from BDH Chemicals, Poole, Dorset, U.K. This was heated overnight at 90 °C to dry it before use. Chelating Resin (Na^+ form; 50–100 mesh) was from Sigma (London) Chemical Co., Poole, Dorset, U.K. It was converted into the K^+ form by washing three times with 1 M-KOH and three times with a large excess of water, and it was then adjusted to pH 7.4 with acetic acid. All water that was used to prepare reagents in this work was distilled and then deionized, so that it had a resistance of more than 10 M Ω/cm. Sucrose solutions were treated with Dowex resin (Na^+ form) to remove any bivalent cations, and aqueous solutions were stored over the chelating resin.

Preparation of phosphatidate as a substrate for PAP

Unlabelled phosphatidate was prepared essentially as described by Sturton & Brindley [27], but the treatment with Chelating Resin was omitted until it was mixed with [^{3}H]phosphatidate. Phosphatidate containing [^{3}H]palmitate was also prepared by using microsomal fractions of rat liver essentially as described by Brindley & Bowley [25]. In the former preparation, however, the concentrations of [^{3}H]palmitate (500 Ci/mol) and *rac*-glycerol 3-phosphate were 0.4 mM and 5 mM respectively.

Phosphatidate labelled with [^{3}H]glycerol was synthesized by using *sn*-[^{3}H]glycerol 3-phosphate (500 Ci/mol). This was generated in a preincubation at 37 °C that contained 40 mM-[^{3}H]glycerol, 100 mM-ATP adjusted to pH 9.8 with KOH, 22 mM-$MgCl_2$ and 10 units of glycerol kinase. The reaction was monitored [28] in a parallel incubation that contained non-radioactive glycerol, and 95% conversion was obtained after 120 min. [^{3}H]Phosphatidate was then generated by taking this reaction mixture, which contained about 10 μmol of *sn*-[^{3}H]glycerol 3-phosphate, and incubating it in 25 ml with 100 mM-Tris adjusted to pH 7.4 with HCl, 1 mM-dithiothreitol, 8 mM-ATP, 50 mM-NaF, 25 μM-CoA, 0.5 mM-potassium palmitate, 0.5 mM-potassium oleate, 13.4 mM-$MgCl_2$, 75 mg of fatty-acid-poor bovine serum albumin, 50 mg of microsomal protein from rat liver and an additional 10 units of glycerol kinase. The formation of [^{3}H]lipid was monitored by determining the concentration of ^{3}H that was extracted into a chloroform phase [29]. After incubation for 2 h at 37 °C, the microsomal membranes containing [^{3}H]lipids were recovered by centrifuging at 4 °C for 80 min at 105000 *g* ($r_{av.}$ = 7.75 cm). The supernatant was then re-incubated with a further 75 mg of microsomal protein, 25 μM-CoA, 20 mM-ATP, 0.5 mM-palmitate and 0.5 mM-oleate, and the microsomal fraction was isolated, and the procedure repeated once more.

The final incubation mixture and combined microsomal pellets were then mixed with 3.75 vol. of chloroform/methanol (1:2, v/v). A two-phase system was then generated by adding 1.25 vol. of chloroform and 1.25 vol. of 2 M-KCl containing 0.2 M-H_3PO_4. The bottom phase was then washed three times with a top phase prepared from 1 litre of chloroform, 1 litre of methanol and 900 ml of 2 M-KCl containing 0.2 M-H_3PO_4. The final bottom phase was dried down, and the lipids were dissolved in chloroform that had been treated with granular $CaCl_2$ to remove ethanol. The lipid was then applied to a column containing 60 g of silica gel 60 that was prepared in ethanol-free chloroform. Neutral lipids were eluted with chloroform, and phospholipids with chloroform containing 5, 10, 15, 25 and 50% (v/v) of methanol. Fractions were analysed by t.l.c., and those that contained all of the ^{3}H in phosphatidate were combined. Phosphatidate labelled with [^{3}H]palmitate was purified in the same way.

The [^{3}H]phosphatidate that was labelled with either [^{3}H]glycerol or [^{3}H]palmitate was then mixed in chloroform with a known quantity of unlabelled phosphatidate to give a specific radioactivity of 0.4 Ci/mol. This preparation still contained some Ca^{2+} that was derived during the incubation used to generate phosphatidate from phosphatidylcholine by using phospholipase D. The [^{3}H]phosphatidate solution was then treated with 2 vol. of methanol and 0.8 vol. of 2 M-KCl containing 0.2 M-H_3PO_4 to give a one-phase system. A chloroform-rich bottom phase containing the phosphatidate was then generated by adjusting the chloroform/methanol/2 M-KCl containing 0.2 M-H_3PO_4 to 10:10:9 (by vol.). The bottom phase was then washed with methanol/0.2 M-potassium phosphate buffer, pH 7.4, containing 2 M-KCl (10:9, v/v). These washes with acid followed by phosphate buffer removed some of the contaminating Ca^{2+} and made it easier to dissolve the phosphatidate in the solvent system used with the Chelating Resin, as recommended by Renkonen [30]. Analysis of the phosphatidate before treatment with Chelating Resin showed that the molar ratio of phosphatidate:Ca^{2+} was 1:0.43, and it contained about 0.2% [^{3}H]diacylglycerol. This preparation was used in some experiments to measure PAP activity.

Alternatively, the phosphatidate was dispersed in chloroform/methanol/water (5:4:1, by vol.) at a concentration of about 3.6 mg/ml. Chelating Resin (K^+ form) was then added in the ratio of 50 ml of resin/mg of phosphatidate. The mixture was stirred, and methanol was added until the phosphatidate dissolved completely in the solvent mixture above the resin. After 30 min at room temperature, the solvent mixture was removed by filtration, the resin washed and the composition of the solvent adjusted to chloroform/methanol/2 M-KCl (10:10:9, by vol.). This produced a two-phase system in which the potassium phosphatidate was recovered in the bottom phase. The solvent was removed under reduced pressure, the phosphatidate dissolved in chloroform and its concentration determined by measurement of ^{3}H. The molar ratio of phosphatidate:Ca^{2+} in this product was in the range 1:0.05±0.01 (mean±S.D. for three preparations). Phosphatidate samples in chloroform were stored at −20 °C until use.

When required for the analysis of PAP activity, a sample of the [^{3}H]phosphatidate was dried down under a stream of N_2 with pure phosphatidylcholine prepared from egg lecithin [31]. Water or solutions of EDTA and EGTA were added as indicated, and the mixture was sonicated at 22 kHz with an amplitude of 8 μm peak-to-peak at room temperature until a uniform and

relatively clear dispersion was obtained. This process took longer with phosphatidate preparations containing relatively high Ca^{2+} concentrations in the absence of EDTA and EGTA. However, it only took 10–15 s when Ca^{2+}-depleted phosphatidate and chelators were employed. The final concentrations of phosphatidate and phosphatidylcholine were 3 mM and 2 mM respectively, and these were diluted 5-fold in the assay.

Preparation of subcellular fractions

Livers from male rats were suspended at 4 °C in 0.25 M-sucrose, adjusted to pH 7.4 with $KHCO_3$, and containing 0.2 mM-dithiothreitol. They were homogenized with five strokes up and down in a Teflon/stainless-steel homogenizer and then centrifuged for 10 min at 18000 *g* ($r_{av.}$ = 10.7 cm) at 4 °C. The resulting supernatant was then centrifuged for 80 min at 105000 *g* ($r_{av.}$ = 7.75 cm) at 4 °C to produce a microsomal pellet and a supernatant fraction. The microsomal membranes were resuspended in 0.25 M-sucrose containing 10 mg of fatty-acid-poor bovine serum albumin/ml and 0.2 M-dithiothreitol and then incubated for 10 min at 37 °C. This latter procedure was included to deplete the membranes of fatty acid and PAP activity [9]. The membrane fraction was then cooled to 4 °C, and 15 ml samples were layered over 20 ml of 0.3 M-sucrose containing 0.2 M-dithiothreitol. These tubes and samples of the supernatant fraction were then re-centrifuged at 105000 *g* for 80 min at 4 °C. The second centrifugation removed most of the remaining membranes from the supernatant fraction [9], and a final concentration of 20 mM-Hepes adjusted to pH 7.4 with KOH was added. The microsomal pellet was resuspended in 0.25 M-sucrose containing 0.2 M-dithiothreitol and 20 mM-Hepes, pH 7.4.

The microsomal and particle-free supernatant fractions were incubated alone or combined in the presence or absence of 0.75 mM-potassium oleate for 10 min at 37 °C. They were then centrifuged at 4 °C for 70 min at 110000 *g* ($r_{av.}$ = 6.43 cm), and the microsomal pellet was resuspended in 0.25 M-sucrose containing 0.2 mM-dithiothreitol and 20 mM-Hepes, pH 7.4. The subcellular fractions were stored at −20 °C, and PAP activities and protein concentrations were determined. In some experiments a final concentration of 2 mM-EDTA was added to samples of each fraction, and they were then dialysed for 16 h at 4 °C against 0.25 M-sucrose containing 0.2 mM-dithiothreitol, 20 mM-Hepes and 2 mM-EDTA adjusted to pH 7.4 with KOH, and then for 6 h against a similar medium that did not contain EDTA. Control samples were stored at 4 °C for 22 h, and these showed no significant change in PAP activity compared with samples at −20 °C.

Determination of PAP activity

This was measured by the production of [^{3}H]diacylglycerol from mixed membranes containing [^{3}H]phosphatidate and phosphatidylcholine. Each assay contained, in a final volume of 0.1 ml: 100 mM-Tris, adjusted to pH 7.4 with HCl, 1 mM-dithiothreitol, 0.2 mg of fatty-acid-poor bovine serum albumin, the mixed emulsion of 0.6 mM-[^{3}H]phosphatidate (0.4 Ci/mol) with 0.4 mM-phosphatidylcholine, and $MgCl_2$, $CaCl_2$, EGTA and EDTA if indicated. In assays where the substrate was produced in the presence of EDTA and EGTA, the incubation mixture described above was preincubated and then added to 7–200 μg of microsomal fraction or 15–45 μg of soluble protein to start the reaction, depending on the PAP activity present. When the lipids were sonicated in water alone, the reaction mixture without the substrate was added to the enzymes, and the reaction was started by adding the substrate. This was because pre-mixing the phosphatidate with Ca^{2+} or Mg^{2+} in the absence of the higher concentrations of chelators caused it to aggregate and become an ineffective substrate.

Reactions were at 37 °C for 20–60 min. The time of the incubation and the amount of protein added to the assays was adjusted so that no more than 15% of the phosphatidate was converted into diacylglycerol. This ensured that the rates of reaction were constant with time and proportional to the amount of enzyme added. The reaction was normally stopped with 2 ml of chloroform/methanol (19:1, v/v) containing 0.08% olive oil (as carrier). Dry aluminium oxide (1 g) was immediately added, and the mixture was shaken. When all of the reactions were stopped, the tubes were shaken for a further 2 min and centrifuged (bench centrifuge). Samples (1 ml) of the solvent mixture were taken, dried in counting vials, and ^{3}H was determined. In some experiments the reaction was stopped and diacylglycerol analysed as described previously [20].

Measurement of protein and Ca^{2+} concentrations

Protein was determined by the method of Bradford [32], but with PAGE Blue G-90 (BDH). Ca^{2+} concentrations in phosphatidate were determined by removing the solvent and taking up the residue in conc. HNO_3. The samples were then digested at 60–80 °C for 10 min, and then the HNO_3 was diluted to 15% (v/v) with water. The Ca^{2+} concentrations were then measured with an atomic-absorption spectrophotometer with standards of $CaCl_2$ in 15% HNO_3.

RESULTS

Validation of the extraction procedure for [^{3}H]diacylglycerol

PAP activity was determined by the formation of diacylglycerol from phosphatidate, since the measurement of P_i formation does not give a valid assay with microsomal fractions of rat liver. This is because phosphatidate can also be degraded by phospholipase-A-type activities, giving rise to glycerol phosphate, which in turn can be hydrolysed by alkaline and acid phosphatases [26]. These observations also imply that the isolation procedure for the [^{3}H]diacylglycerol should exclude as far as possible phosphatidate and any resulting fatty acid, glycerol phosphate and glycerol. The need for this also depends on whether the phosphatidate is labelled in the fatty acid or glycerol.

The aim of the new extraction was to simplify the procedure and thus to make it quicker and more accurate. Normally diacylglycerol is extracted into an organic phase, and water-soluble products are removed in an aqueous phase. The diacylglycerol is then purified by sampling the organic layer and subjecting it to t.l.c. or treatment with aluminium oxide. The later two stages were combined in the present procedure by stopping the reaction with chloroform/methanol (19:1, v/v) and then adding 1 g of dry aluminium oxide directly to the

mixture. This amount of alumina was adequate to adsorb the water from the 100 μl assay, and also most of the phosphatidate, fatty acid, glycerol phosphate and glycerol. The average recovery of [^{3}H]diacylglycerol in the solvent mixture was 92% (Table 1). The recoveries of lipids in Table 1 were almost identical with those obtained after extracting the lipids into an organic phase, followed by treatment of this with alumina [20]. This latter method gave almost complete removal of glycerol and glycerol phosphate, but this was not essential, since the recoveries of these compounds in Table 1 were already very low. Further decreases in the amounts of fatty acids and phosphatidate in the solvent mixture as reported in Table 1 could be achieved by not adding the 5% of methanol to the chloroform, but this also decreased the recovery of diacylglycerol to about 80%, and this was thought to be unacceptable.

Effects of EDTA, EGTA, Ca^{2+} and Mg^{2+} on PAP activity measured with phosphatidate containing different concentrations of Ca^{2+}

It ought to be possible to determine the activity of the Mg^{2+}-dependent PAP activity by measuring the activity in the presence of either EDTA or an optimum concentration of Mg^{2+}. The difference between these values should therefore reflect the Mg^{2+}-dependent activity [12,23,33]. This approach was therefore tested with two preparations of phosphatidate that contained different concentrations of Ca^{2+}, to see whether this cation affected the interpretation of the results. All of the following experiments were performed with samples of microsomal and particle-free supernatant fractions that were preincubated alone in the absence, or together in the presence, of 0.75 mM-oleate and then re-isolated. The treatment with oleate transferred some of the soluble PAP to the microsomal membranes [9]. Essentially similar results were obtained with the two microsomal and particle-free supernatant fractions, except that the absolute PAP activity differed. However, for the sake of simplicity only the results for the particle-free supernatant incubated without oleate and for a microsomal fraction obtained after incubation with oleate and the soluble fraction are shown in most instances, since these possessed the highest PAP activities.

Addition of 0.1–0.2 mM-EDTA to the substrate that contained 0.43 mol of Ca^{2+}/mol of phosphatidate stimulated rather than inhibited the reaction (Fig. 1*a*). This stimulation was also seen in five independent experiments with subcellular fractions from liver, and with five membrane and soluble fractions prepared from cultured hepatocytes after digitonin treatment [16,33]. Higher concentrations of EDTA inhibited the reaction. By contrast, the addition of all concentrations of EDTA inhibited the basal PAP activity with the substrates that contained only 0.05 ± 0.01 mol of Ca^{2+}/mol of phosphatidate (Fig. 1*b*).

The addition of EGTA to the substrate containing the higher Ca^{2+} concentration stimulated PAP activity (Fig. 2*a*). It had no significant effect on the activity of the substrate that contained the low Ca^{2+} concentration (Fig. 2*b*). This indicates that the higher concentration of Ca^{2+} was inhibitory and that this inhibition can be removed either by adding 1 mM-EGTA or by depleting Ca^{2+} from the phosphatidate with Chelating Resin. The effects of EDTA in the presence of 1 mM-EGTA with the phosphatidate preparation that contained the high and low concentrations of Ca^{2+} were similar to those shown in Fig. 1(*b*) (results not shown). PAP activities were decreased by EDTA, suggesting that small concentrations of cations (e.g. Mg^{2+}) that may be present in the substrate or in the subcellular fractions (particularly the microsomal fraction) might be producing some stimulation of the basal activity (Fig. 1*b*). It was therefore decided that all incubations should ideally contain 1 mM-EDTA in combination with 1 mM-EGTA.

The addition of Ca^{2+} to the incubations that contained substrates with 0.05 ± 0.01 mol of Ca^{2+}/mol of phosphatidate in the presence of 1 mM-EGTA and 1 mM-EDTA at first stimulated and then inhibited the PAP activity (Fig. 3). This stimulation was 3.1 ± 0.4- and 5.1 ± 0.6-fold (means $\pm$ ranges) respectively for particle-free supernatant and microsomal fractions in two independent experiments. If Ca^{2+} were added to the substrate that contained the high Ca^{2+} concentration in the presence of 0.1 mM-EGTA alone, only the inhibitory portion of the curve was seen (results not shown).

The effects of Mg^{2+} in microsomal and particle-free supernatant fractions obtained after incubation separately in the absence, or together in the presence, of 0.75 mM-oleate are shown in Figs. 4(*a*) and 4(*b*) respectively. The microsomal fraction that was obtained after incubation with albumin and which was then not incubated with oleate or the particle-free supernatant contained very little Mg^{2+}-dependent activity. Most of this PAP activity was located in the particle-free supernatant (Fig. 4*a*). By contrast, the microsomal fraction that had been preincubated with oleate in the presence of the particle-free supernatant fraction contained a high specific activity of Mg^{2+}-stimulated PAP (Fig. 4*b*). There was a corresponding decrease in the specific activity of the particle-free supernatant fraction, with an overall recovery of 101 ± 27% of total PAP activity (Table 2). Similar conclusions were obtained with membrane and soluble fractions prepared from

Table 1. Extraction and recovery of diacylglycerol and other compounds from the assay system used to determine PAP activity

The various compounds indicated were added to the normal assay mixture, which always contained 0.1 mM-EGTA and the mixed emulsion of phosphatidylcholine and unlabelled phosphatidate. Fatty acids and diacylglycerol were incorporated where indicated into this emulsion. The recovery in each situation was determined by the introduction of the radioactive compound as appropriate. Then 2 ml of chloroform/methanol (19:1, v/v) with 0.08% olive oil was added, followed by 1 g of dry alumina. After mixing and centrifuging, a 1 ml sample of the solvent was evaporated and radioactivity was determined. There were three independent determinations, and results are given as means $\pm$ S.D.

^{3}H-labelled compound added	Recovery in solvent (%)
Phosphatidate (60 nmol)	1.2 ± 0.4
Palmitate (2.5 μmol)	1.8 ± 0.7
Diacylglycerol (10 nmol)	92 ± 4
Glycerol (2.5 μmol)	0.46 ± 0.1
Glycerol phosphate (2.5 μmol)	0.06 ± 0.04

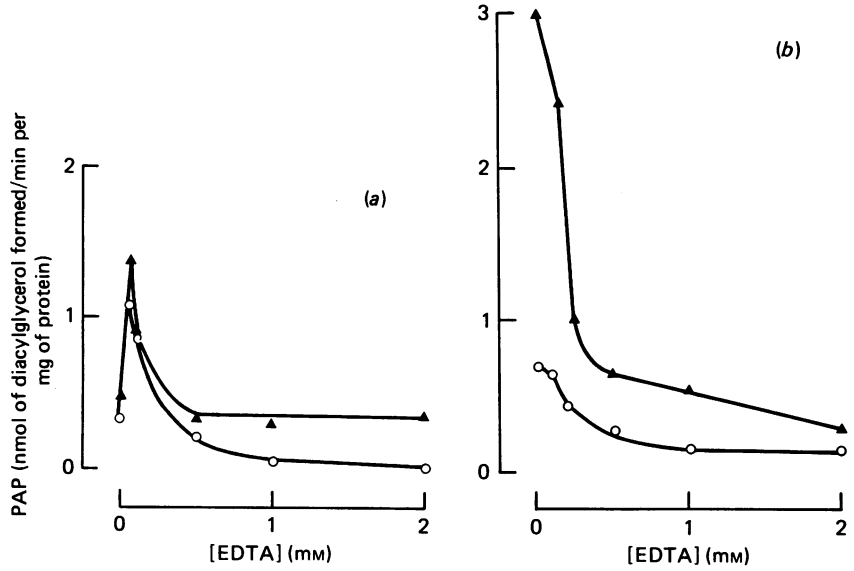

Fig. 1. Effect of EDTA on the activity of PAP

Each assay contained 0.1 mM-EGTA and phosphatidate that contained 0.43 (*a*) or 0.05 (*b*) mol of Ca^{2+}/mol. The activity of the particle-free supernatant fraction which was not incubated with oleate (○) and the microsomal fraction that was previously incubated with oleate and the soluble fraction (▲) are shown. However, similar effects of EDTA were obtained with microsomal fractions that were depleted of PAP activity by treatment with albumin and with the particle-free supernatant fraction that had been incubated with the microsomal fraction in the presence of oleate. These results were confirmed in four further independent experiments.

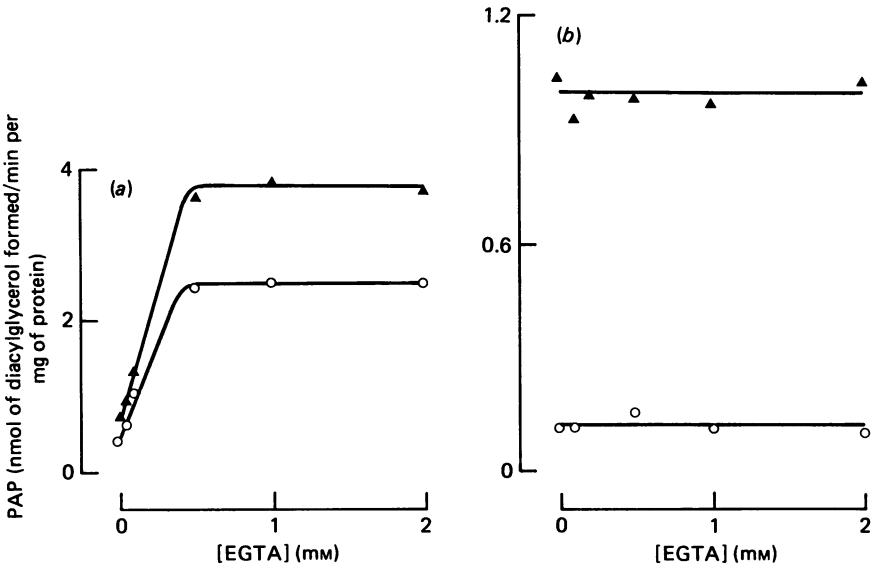

Fig. 2. Effects of EGTA on the activity of PAP

The assays contained phosphatidate that contained either 0.43 (*a*) or 0.05 (*b*) mol of Ca^{2+}/mol. PAP activity was determined in a microsomal fraction that had been preincubated with the soluble fraction in the presence of oleate (▲), and in the original soluble fraction (○), as described in Fig. 1.

isolated hepatocytes [16], which were incubated in the presence and absence of oleate (results not shown).

Phosphate buffer has also been shown to stimulate PAP activity when this is measured in the presence of 0.1 mM-EGTA [10,20]. However, this stimulation was not seen with 100 mM-potassium phosphate, pH 7.4, when 1.0 mM-EDTA and 1.0 mM-EGTA were added to the phosphatidate substrate that contained the lower concentration of Ca^{2+}. This can probably be explained by the competition of phosphate with the phosphatidate in binding polyvalent cations in the former system.

Very little PAP activity was detected in the absence of Mg^{2+}. We also checked that there was no selective loss of an Mg^{2+}-independent PAP activity from the micro-

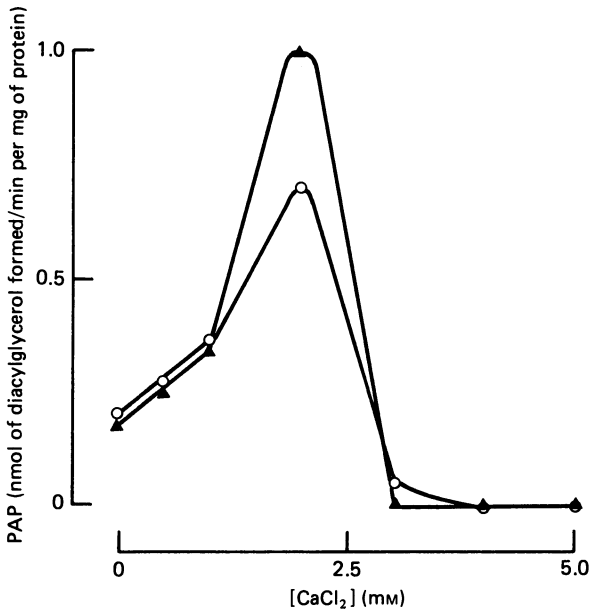

Fig. 3. Effects of Ca^{2+} on the activity of PAP

The assays included 1 mM-EGTA and 1 mM-EDTA with phosphatidate that contained 0.05 mol of Ca^{2+}/mol. PAP activity was determined in a microsomal fraction that had been preincubated with the soluble fraction in the presence of oleate (▲) and in the original soluble fraction (○). The results were reproduced in five further independent experiments.

somal membranes in the albumin-containing buffer used to wash the microsomal fraction. The extent of the Mg^{2+} stimulation of PAP activity in the particle-free supernatant fractions and the microsomal fraction after incubation with the supernatant fraction in the presence of oleate was about 43- and 34-fold respectively. Incubation of the microsomal fraction alone with oleate did not increase the Mg^{2+}-dependent PAP activity, indicating that translocation from the soluble fraction was responsible for the increase in microsomal activity.

It has been proposed that Mg^{2+}-independent PAP activity is insensitive to the effects of thiol-group reagents [20], whereas the Mg^{2+}-dependent PAP activity is inhibited [34]. The subcellular fractions were therefore incubated with *N*-ethylmaleimide to determine the extent of inhibition at 0 and 7.5 mM-Mg^{2+}. The Ca^{2+}-depleted substrate was used in the presence of 1 mM-EDTA and 1 mM-EGTA. When PAP was assayed in the microsomal and soluble fractions in the presence of 7.5 mM-Mg^{2+}, there was a 73–97% inhibition of activity after preincubation with 1.5 mM-*N*-ethylmaleimide. In assays which contained no added bivalent cation or 2 mM-$CaCl_2$, the inhibitions in the presence of *N*-ethylmaleimide were 25–99% and 51–84% respectively. Inclusion of 1 mM-dithiothreitol in the preincubation prevented these inhibitions (results not shown).

It is possible that polyvalent cations present in the subcellular fractions might have been responsible for the small activity reported in the absence of Mg^{2+} (Fig. 1*b*; Table 2). The presence of 1 mM-EDTA and 1 mM-EGTA might not have completely suppressed this activity, and therefore we decided to dialyse the fraction against 2 mM-EDTA. This resulted in a further decrease in the

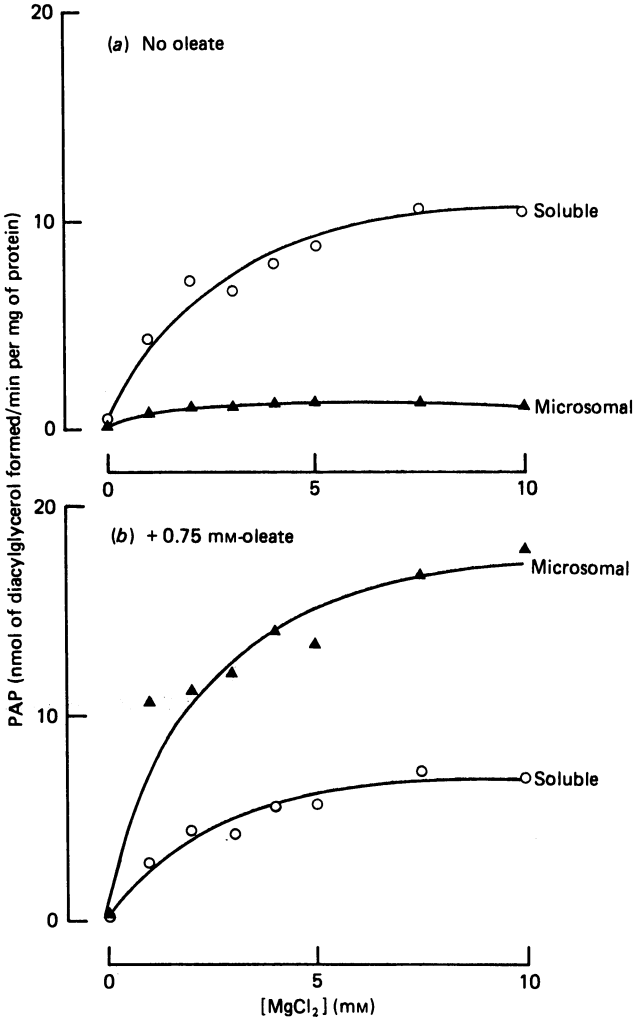

Fig. 4. Effects of Mg^{2+} on PAP activity in microsomal and soluble fractions that had been preincubated in the presence or absence of oleate

Each assay included 1 mM-EDTA and 1 mM-EGTA with phosphatidate that contained 0.05 ml of Ca^{2+}/mol. Fig. 4(*a*) shows PAP activity in soluble (○) and microsomal (▲) fractions that had not been incubated with oleate, and Fig. 4(*b*) shows the activities when the fractions had been previously incubated together with 0.75 mM-oleate. The results were confirmed in seven further independent experiments, which are partly summarized in Table 2.

basal PAP activity in the absence of Mg^{2+} in all but the microsomal fraction that was not incubated with the particle-free supernatant and oleate. The activities in the presence of 7.5 mM-$MgCl_2$ were not significantly changed in any fraction. Consequently, the stimulations produced by $MgCl_2$ for both supernatant fractions and the microsomal fraction after translocation of PAP were in the range 110–167-fold.

Effects of chlorpromazine on PAP activity

Previous work had demonstrated that chlorpromazine could stimulate PAP activity and that it might replace the requirement for Mg^{2+} [18]. We therefore examined whether this applied under the present assay conditions with phosphatidate that contained the higher or lower concentrations of Ca^{2+}. However, at pH 7.4 no significant

Table 2. Effects of EGTA, EDTA and dialysis on the stimulation of PAP activity by $MgCl_2$

The microsomal fraction was depleted of PAP by treatment with albumin, and the particle-free supernatant was centrifuged twice to remove membranes. They were then incubated separately, or together with 0.75 mM-oleate, and the fractions were re-isolated by centrifugation. In three experiments the fractions were also dialysed as indicated against 2 mM-EDTA (see the Experimental section). PAP activity was then determined as indicated in the presence or absence of $MgCl_2$. This was done with 0.1 mM-EGTA in the assay (two independent experiments) or with 1 mM-EGTA + 1 mM-EDTA present (eight independent experiments when undialysed). Results are given as means ± S.D., or ranges for the two experiments. The recoveries of PAP activity in the presence of $MgCl_2$ in fractions that were incubated together with 0.75 mM-oleate compared with those incubated separately without oleate was 78 ± 1% and 101 ± 27% when measured in the presence of 0.1 mM-EGTA or 1 mM-EGTA + 1 mM-EDTA respectively for undialysed samples. The equivalent recovery for the dialysed sample was 92 ± 12%.

Fraction	Preincubation conditions	Dialysed	Chelators in assay	PAP activity (nmol of diacylglycerol formed/min per mg of protein)		Stimulation by $MgCl_2$ (fold)
				0 mM-$MgCl_2$	7.5 mM-$MgCl_2$	
Particle-free supernatant	No oleate	No	0.1 mM-EGTA	1.4 ± 0.13	7.9 ± 0.70	5.6 ± 0.02 (2)
		No	1 mM-EGTA + 1 mM-EDTA	0.24 ± 0.1	8.0 ± 0.86	42 ± 23 (8)
		Yes	1 mM-EGTA + 1 mM-EDTA	0.07 ± 0.02	7.6 ± 1.23	110 ± 30 (3)
	0.75 mM-Oleate	No	0.1 mM-EGTA	0.71 ± 0.04	2.3 ± 0.12	3.3 ± 0.04 (2)
		No	1 mM-EGTA + 1 mM-EDTA	0.12 ± 0.08	4.65 ± 1.1	44 ± 25 (8)
		Yes	1 mM-EGTA + 1 mM-EDTA	0.03 ± 0.004	4.2 ± 2.9	167 ± 141 (3)
Microsomal	No oleate	No	0.1 mM-EGTA	0.52 ± 0.02	0.94 ± 0.07	1.8 ± 0.24 (2)
		No	1 mM-EGTA + 1 mM-EDTA	0.13 ± 0.09	1.1 ± 0.9	7.6 ± 5.3 (8)
		Yes	1 mM-EGTA + 1 mM-EDTA	0.15 ± 0.11	1.2 ± 0.6	7.5 ± 5.1 (3)
	0.75 mM-Oleate	No	0.1 mM-EGTA	3.8 ± 0.71	14.4 ± 0.74	3.9 ± 0.54 (2)
		No	1 mM-EGTA + 1 mM-EDTA	0.20 ± 0.11	16.5 ± 10.8	34 ± 17 (8)
		Yes	1 mM-EGTA + 1 mM-EDTA	0.14 ± 0.04	17.5 ± 7.6	123 ± 37 (3)

stimulation of PAP activity was obtained with chlorpromazine, and higher concentrations (0.4–1.2 mM) inhibited the reaction. It should be remembered that the phosphatidate used in the present work was added as a mixed micelle with phosphatidylcholine to increase the reaction rate and to produce a more natural substrate [19,26]. However, we reproduced the work of Bowley *et al.* [18] by using phosphatidate emulsions in the absence of phosphatidylcholine and with 20 mM-Tris/HCl, pH 6.4, containing 0.2 mM-EGTA. In this case we did confirm a 2.34 ± 0.56-fold stimulation (mean ± range in two independent experiments) for the particle-free supernatant fraction in the absence of Mg^{2+}.

DISCUSSION

A rapid extraction procedure for purifying [^{3}H]diacylglycerol from the incubation medium used to assay PAP activity was developed. This simplified the procedure, thus making the measurements more accurate. The benefits of removing polyvalent cations, especially Ca^{2+}, from the phosphatidate substrate and from the reagents that were used was demonstrated. This resulted in high PAP activities in the presence of Mg^{2+}. The addition of 1 mM-EGTA and 1 mM-EDTA to the phosphatidate when it was sonicated with phosphatidylcholine also facilitated the dispersion of the lipids.

The use of mixed membranes containing phosphatidate and phosphatidylcholine provides a very well-defined substrate, which produced higher reaction rates than are obtained with pure phosphatidate emulsions [19,23,26]. This is probably because the mixture of the zwitterionic lipid, phosphatidylcholine, with the acidic phosphatidate provides a more natural membrane with which PAP can interact. The removal of polyvalent cations from this substrate and the addition of both EDTA and EGTA also enabled us to determine the cation requirement of PAP with greater certainty.

The PAP activities that were obtained with both microsomal and soluble fractions in the absence of added bivalent cations and in the presence of EDTA and EGTA were very low (Table 2). Even so, part of this activity could be inhibited by *N*-ethylmaleimide, which indicates that it may not be caused by the Mg^{2+}-insensitive PAP [34]. This conclusion is supported by the dialysis of the samples against EDTA, which also decreased the basal activity that was measured in the absence of added bivalent cations (Table 2). This low activity was barely measurable.

A phospholipase C activity that can degrade phosphatidate has also been demonstrated in the liver [35]. This activity occurs mainly in particulate fractions that are obtained at relatively low centrifugation speeds, and it can be stimulated by Ca^{2+}. However, the rate of diacylglycerol formation that was measured in the presence of Ca^{2+} in the present experiments was partially inhibited by *N*-ethylmaleimide.

The measurement of the Mg^{2+}-dependency of PAP activities is preferably done with phosphatidate that has been treated with Chelating Resins and then by incubating dialysed fractions with a combination of EDTA and EGTA. The results in Fig. 1 show that

addition of EDTA alone to phosphatidate that was not treated with Chelating Resin and which contained 0.43 mol of Ca^{2+}/mol first stimulated and then inhibited PAP activity. It should be remembered that EDTA can also chelate Ca^{2+}, and so its effect could be a combination of this and the removal of other polyvalent cations. The removal of inhibitory concentrations of Ca^{2+} would increase PAP activity, but the addition of Ca^{2+} at certain concentrations can stimulate PAP activity (Fig. 3). This stimulation was much higher than was observed previously [18].

Substitution of EGTA for EDTA also stimulates PAP activity in the substrate that contained the higher concentrations of Ca^{2+}, but it had no significant effect with the Ca^{2+}-depleted substrate (Fig. 2). Only an inhibition of the activity was seen when EDTA was added to the Ca^{2+}-depleted substrate (Fig. 1*b*) or when 1 mM-EGTA was also present. This indicates that the incubation medium containing phosphatidate with either the higher or lower Ca^{2+} concentrations also contained a cation that could stimulate PAP activity and that this could be removed by further addition of EDTA in the presence of EGTA. This cation is probably Mg^{2+}, which could be present at relatively low concentrations in the enzyme preparations. The partial removal of this Mg^{2+} by EDTA would also explain why PAP activity was inhibited by higher concentrations of EDTA (Fig. 1), but not by EGTA (Fig. 2). Furthermore, the recovery of PAP activity measured with 2 mM-$CaCl_2$ in samples that had been dialysed against EDTA was 27–53% when compared with those that were not dialysed. By contrast, the recovery of activity measured in the presence of 7.5 mM-$MgCl_2$ was 92–105% (Table 2). We have no direct evidence for a distinct Ca^{2+}-dependent PAP, using our experimental system.

These results emphasize that phosphatidate itself is a good chelator of polyvalent cations and that the activity of PAP that can be measured is the result of a complex interaction of different cations that may be present in the substrate or in the enzyme samples. Great care was taken in the present work to exclude contaminating cations in the subcellular fractions, substrate and reagents, and to chelate any remaining cations with EDTA and EGTA. Consequently, the basal activities for dialysed samples in the presence of 1 mM-EGTA and 1 mM-EDTA, but in the absence of $MgCl_2$, were barely measurable (Table 2). It is therefore concluded that any contribution of a Mg^{2+}-independent PAP in this system can be ignored. Since the Mg^{2+}-stimulated PAP activity was not significantly altered after dialysis, this procedure is not necessary for the routine analysis of Mg^{2+}-dependent PAP activity. The stimulations of 110–167-fold that were obtained with Mg^{2+} could not be replaced by chlorpromazine in this system.

To our knowledge, no-one has previously reported such high dependencies on Mg^{2+} with subcellular fractions. The extent of the Mg^{2+} stimulation in cytosolic fractions has normally been 4–8-fold [18,21,22,24]. The Mg^{2+}-dependency of PAP in microsomal fractions is normally much lower, if it is observed at all [21,23,24]. However, partially purified PAP has an almost absolute requirement for Mg^{2+} [19,20]. We suggest three explanations for these observations: first, and most important, a failure to achieve a true basal activity, since bivalent cations were probably not completely removed from the substrate and from the subcellular fractions. Secondly, a variable amount of a true Mg^{2+}-independent PAP activity occurred in the microsomal fraction, but this needs to be rigorously characterized. Thirdly, the present work employed microsomal fractions that were specifically enriched with an Mg^{2+}-dependent PAP from the cytosolic fraction by preincubation with oleate.

Previous work with isolated hepatocytes [33] and adipocytes [12] indicated that the PAP activity which translocates to membranes when the fatty acid content of the cells increases is the Mg^{2+}-dependent form. This cation requirement was investigated in greater detail in the present work. The results demonstrate that the soluble PAP has a much higher (if not absolute) requirement for Mg^{2+} than has previously been demonstrated. This Mg^{2+}-dependency is not lost when PAP attaches to membranes of the endoplasmic reticulum.

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REFERENCES

1. Brindley, D. N. & Sturton, R. G. (1982) New Compr. Biochem. **4**, 179–213
2. Brindley, D. N. (1984) Prog. Lipid Res. **23**, 115–133
3. Brindley, D. N. (ed.) (1987) Phosphatidate Phosphohydrolase, vols. 1 and 2, CRC Press, Boca Raton, FL, in the press
4. Pittner, R. A., Fears, R. & Brindley, D. N. (1985) Biochem. J. **225**, 455–462
5. Pittner, R. A., Fears, R. & Brindley, D. N. (1985) Biochem. J. **230**, 525–534
6. Pittner, R. A., Bracken, P., Fears, R. & Brindley, D. N. (1986) FEBS Lett. **202**, 133–136
7. Pittner, R. A., Bracken, P., Fears, R. & Brindley, D. N. (1986) FEBS Lett. **207**, 42–46
8. Pittner, R. A., Fears, R. & Brindley, D. N. (1986) Biochem. J. **240**, 253–257
9. Hopewell, R., Martin-Sanz, P., Martin, A., Saxton, J. & Brindley, D. N. (1985) Biochem. J. **232**, 485–491
10. Martin, A., Hopewell, R., Martin-Sanz, P., Morgan, J. E. & Brindley, D. N. (1985) Biochim. Biophys. Acta **876**, 581–591
11. Moller, F., Wong, K. H. & Green, P. (1981) Can. J. Biochem. **59**, 9–15
12. Taylor, S. J. & Saggerson, E. D. (1986) Biochem. J. **239**, 275–284
13. Moller, F. & Hough, M. R. (1982) Biochim. Biophys. Acta **711**, 521–531
14. Jamdar, S. C. & Osborne, L. J. (1983) Biochim. Biophys. Acta **752**, 79–88
15. Martin-Sanz, P., Hopewell, R. & Brindley, D. N. (1985) FEBS Lett. **179**, 262–266
16. Butterwith, S. C., Martin, A. & Brindley, D. N. (1984) Biochem. J. **222**, 487–493
17. Pelech, S. L., Pritchard, P. H., Brindley, D. N. & Vance, D. E. (1983) Biochem. J. **26**, 129–136
18. Bowley, M., Cooling, J., Burditt, S. L. & Brindley, D. N. (1977) Biochem. J. **165**, 447–454
19. Hosaka, K., Yamashita, S. & Numa, S. (1975) J. Biochem. (Tokyo) **77**, 501–509
20. Butterwith, S. C., Hopewell, R. & Brindley, D. N. (1984) Biochem. J. **220**, 825–833
21. Jamdar, S. C. & Fallon, H. J. (1973) J. Lipid Res. **14**, 517–524
22. Lawson, N., Pollard, A. D., Jennings, R. J., Gurr, M. I. & Brindley, D. N. (1981) Biochem. J. **200**, 285–294

23. Walton, P. A. & Possmayer, F. (1985) Anal. Biochem. **151**, 479–486
24. Sturton, R. G. & Brindley, D. N. (1980) Biochim. Biophys. Acta **619**, 494–505
25. Brindley, D. N. & Bowley, M. (1975) Biochem. J. **148**, 461–469
26. Sturton, R. G. & Brindley, D. N. (1978) Biochem. J. **171**, 263–266
27. Sturton, R. G. & Brindley, D. N. (1977) Biochem. J. **162**, 25–32
28. Hohorst, H.-J. (1963) in Methods in Enzymatic Analysis (Bergmeyer, H. U., ed.), pp. 215–219, Academic Press, New York and London
29. Hajra, A. K., Seguin, E. B. & Agranoff, B. W. (1968) J. Biol. Chem. **243**, 1609–1616
30. Renkonen, O. (1969) Biochim. Biophys. Acta **152**, 114–135
31. Singleton, W. S., Gray, M. S., Brown, M. L. & White, J. L. (1965) J. Am. Oil Chem. Soc. **42**, 53–56
32. Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
33. Cascales, C., Mangiapane, E. H. & Brindley, D. N. (1984) Biochem. J. **219**, 911–916
34. Jamdar, S. C., Osborne, L. J. & Wells, G. N. (1984) Arch. Biochem. Biophys. **233**, 370–377
35. Lamb, R. G. & Schwartz, D. W. (1982) Toxicol. Appl. Pharmacol. **63**, 216–229

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