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Microsomal Phosphatidate Phosphatase in Maturing Safflower Seeds

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ABSTRACT

An assay system comprising sodium phosphatidate, phosphatidylcholine, and bovine serum albumin has been developed for the reproducible determination of phosphatidate phosphatase activity in maturing seeds of safflower (*Carthamus tinctorius* L.). The activity was detected in both membrane and soluble fractions, and the microsomal phosphatidate phosphatase was characterized. The optimum pH for Pi release was 6.7, and the activity depended on the concentration of Mg^{2+}. Phosphatidylcholine and bovine serum albumin stimulated the phosphatase reaction. This phosphatase was highly specific for phosphatidate; lysophosphatidate, and water-soluble phosphate esters did not serve as substrate. The specific activity was approximately 20 nanomoles per minute per milligram of protein, which was close to that of glycerol-phosphate acyltransferase and higher than that of diacylglycerol acyltransferase. Furthermore, the activity per seed was enough to account for the rate of triacylglycerol accumulation *in vivo*. The step of diacylglycerol formation by phosphatidate phosphatase does not appear to be rate-limiting for triacylglycerol synthesis during seed maturation.

Phosphatidate phosphatase (3-*sn*-phosphatidate phosphohydrolase, EC 3.1.3.4) is a key enzyme of glycerolipid biosynthesis, and catalyzes the hydrolysis of phosphatidate to 1,2-diacylglycerol and Pi. The diacylglycerol product is not only the direct precursor of triacylglycerol but also a substrate for the synthesis of membrane glycerolipids. Although this enzyme was first demonstrated in plants by Kates (19) in 1955, since then it has been characterized extensively with animal tissues (2). Nevertheless, since the 1970s plant phosphatidate phosphatases have been studied with seedlings of castor bean (25), broad bean (20), and mung bean (11). Douce and coworkers (4, 18) characterized the chloroplastic phosphatidate phosphatase of spinach leaves, and demonstrated that it is localized in the inner envelope. Gardiner and Roughan (9) and Frentzen *et al.* (8) suggested that phosphatidate phosphatase in chloroplasts controls the fatty acid composition of diacylgalactosylglycerol, a major component of chloroplast membranes.

Phosphatidate phosphatase in maturing oil seeds has been studied to a very minor extent (29) despite pioneering work by Barron and Stumpf (1), who have suggested operation of the phosphatidate phosphatase reaction in avocado mesocarp. It has so far been reported that phosphatidate phosphatase in maturing safflower seeds requires Mg^{2+} (10) and that the enzyme activity in groundnut varies with seed maturation (30). It is believed that the biosynthesis of triacylglycerol from glycerol 3-phosphate and acyl-CoA occurs in the ER membrane (29). In the course of an investigation of triacylglycerol formation in maturing oil-seeds, we have found that maturing safflower seeds have two types of phosphatidate phosphatase; one is membrane-bound and Mg^{2+}-dependent, the other is soluble and Mg^{2+}-dependent. The present communication deals with characterization of the microsomal, Mg^{2+}-dependent phosphatidate phosphatase in maturing safflower seeds.

In mammalian tissues, the phosphatidate phosphatase reaction is considered to be the rate-limiting step of triacylglycerol synthesis (2). We will discuss whether this is also the case for maturing safflower seeds.

To assay the activity of phosphatidate phosphatase with good reproducibility, we have developed an effective phosphatidate substrate, the preparative procedure for which is also described.

MATERIALS AND METHODS

Plant Material and Preparation of Microsomes

Maturing seeds of safflower (*Carthamus tinctorius* L.) were harvested 14 to 15 d after flowering when ^{14}C-labeled acetate was most rapidly incorporated into diacylglycerol (15), and cotyledons were homogenized in 10 mM Tris-HCl (pH 7.0) containing 0.4 M sorbitol. The homogenate was centrifuged at 5000*g* for 10 min, and then the supernatant obtained was centrifuged at 100,000*g* for 1 h. The precipitate was suspended in the same buffer and stored at −20°C. Protein was determined by the method of Lowry *et al.* (21) with BSA as standard.

Preparation of Phosphatidic Acid

Phosphatidylcholine was prepared from egg yolk and purified by silica gel column chromatography. Its fatty acid composition was 36.4% palmitic, 10.4% stearic, 35.0% oleic, 16.0% linoleic, and 2.2% arachidonic acid (by GLC). The egg yolk phosphatidylcholine (1 g) was dissolved in 20 mL diethyl ether containing 3 mg 2,6-di-*tert*-butyl-*p*-cresol, an antioxidant. To this solution was added 20 mL 0.2 M Tris-HCl buffer (pH 8.0) containing 1 mg (69 units) *Streptomyces chromofuscus* phospholipase D (Behring Diagnostics, La Jolla, CA), 20 mM $CaCl_2$, and 20 mg BSA (fatty-acid free; Armour Pharmaceutical Co., Blue Bell, PA). The mixture was stirred at room temperature for 6 h. The progress of hydrolysis was monitored by TLC with chloroform:methanol:15 N ammonium hydroxide:water (55:45:3:2, by volume) as solvent. Phospholipids were detected with the Dittmer reagent. After the complete hydrolysis (6 h), the solution was acidified with

2 N HCl. The ether layer was separated from the water layer, which was then reextracted with 20 mL diethyl ether. The ether layers were combined and washed twice with 20 mL 0.1 N HCl and then with water. After drying with anhydrous Na_2SO_4, the solvent was evaporated to 5 mL *in vacuo*. The concentrate was diluted with 50 mL ethanol, and then the pH was adjusted to 8.5 with ethanolic 0.5 N NaOH at 0°C. Sodium phosphatidate precipitate was collected by centrifugation, and dissolved in 4 mL diethyl ether. Phosphatidate was precipitated by the addition of 16 mL ethanol. This treatment was repeated, and then the precipitate was washed twice with ethanol. Phosphatidate was dissolved in 4 mL diethyl ether, and further precipitated with 8 mL acetone. After reprecipitation with acetone, the purified sodium phosphatidate was dried *in vacuo* and then dissolved in chloroform containing 2,6-di-*tert*-butyl-*p*-cresol. TLC of the product showed a single spot on a silica gel plate when developed with the solvent system described above. The yield was about 0.5 g.

Lysophosphatidic acid (ammonium salt) was prepared from egg yolk phosphatidylcholine by the procedure described earlier (16).

Assay for Phosphatidate Phosphatase Activity

Appropriate amounts of sodium phosphatidate and egg yolk phosphatidylcholine were dissolved in a small volume of diethyl ether, and BSA was added to the solution. After mixing, the ether was evaporated to dryness. Water was added to the residue, and then the mixture was sonicated for 1 min at 0°C. This emulsion of phosphatidate/phosphatidylcholine/BSA was used as substrate. The final concentrations of these components in the assay medium were given in the following paragraph. Another complex composed of phosphatidylcholine and BSA was also prepared for control experiments.

A typical assay medium contained 50 mM Tris-50 mM maleic acid-NaOH (pH 6.75), 0.1 mM EDTA (disodium salt), 2 mM $MgCl_2$, 1 mM sodium phosphatidate (0.70 mg/mL), 0.65 mM phosphatidylcholine (0.50 mg/mL), 1 mg BSA, and 75 μg microsomal protein in a total volume of 1 mL. After incubation at 28°C for 30 min, the reaction was stopped by the addition of 1 mL 1.5 M TCA. The tube was vortexed and then centrifuged. The Pi concentration of the supernatant was determined by the procedure of Chen *et al.* (7). An aliquot (1.8 mL) of the supernatant was mixed with 1.8 mL of a molybdate reagent which contained 2% (w/v) ascorbic acid and 0.5% (w/v) ammonium molybdate (tetrahydrate) in 1.2 N H_2SO_4. The mixture was incubated at 37°C for 1.5 h, and then absorbance at 820 nm was measured. TCA did not affect the color development. Control incubations were carried out for zero time, in the absence of $MgCl_2$, or in the absence of the phosphatidate substrate. Therefore, the specific activity of phosphatidate phosphatase was expressed as nanomoles of inorganic phosphate liberated from phosphatidate per minute per milligram of microsomal protein. Data are reported as the means of duplicate or triplicate assays.

RESULTS AND DISCUSSION

Intracellular Localization of Phosphatidate Phosphatase

In maturing oil seeds, the ER is the site of triacylglycerol synthesis (29). Microsomal membranes prepared from maturing safflower seeds had sufficient capacity for triacylglycerol accumulation from glycerol 3-phosphate and acyl-CoA (27). The highest specific activity of phosphatidate phosphatase in maturing safflower seeds was associated with a 100,000*g* particulate fraction (Table I), and other membrane fractions also showed high specific activities. The specific activity of the supernatant fraction was relatively low but significant in terms of total activity. Forty percent of the total activity was found in this fraction. In a separate experiment, however, the specific and total activities of the soluble phosphatidate phosphatase were significantly lower than those described here (data are not shown). The soluble activity seemed to be variable, which suggests that phosphatidate phosphatase in maturing oil seeds is present in both soluble and membrane-bound forms as also is the case with the mammalian enzyme (2). Since the substrate was an emulsion of phosphatidate/phosphatidylcholine/BSA and hence phosphatidate might not directly available to the microsomal enzyme, the soluble activity might be overestimated. Unlike phosphatidate phosphatase, all of the three acyltransferases involved in the glycerol-phosphate pathway (29) were membrane bound in safflower seeds (14, 16, 17). This difference in subcellular distribution between phosphatidate phosphatase and the acyltransferases is interesting. It seems to suggest a possible regulatory function of phosphatidate phosphatase in triacylglycerol formation (5).

Identification of Reaction Products

The reaction products of the microsomal preparation were analyzed by TLC. Figure 1 shows a chromatogram of the products separated into individual lipid classes. 1,2-Diacylglycerol was the only major product in the complete incubation system (lane 3). Endogenous nonpolar lipids were triacylglycerol, 1,2-diacylglycerol, sterols, 1,3-diacylglycerol and free fatty acids in order of content. No significant amount of

Table I. *Subcellular Distribution of Phosphatidate Phosphatase Activity in Maturing Safflower Seeds*

The seed homogenate was prepared by the same procedure as described in "Materials and Methods," and filtered through two layers of cotton cloth. The filtrate was centrifuged at 600*g* for 10 min, and then the supernatant was centrifuged at 3,000*g* for 20 min. The resulting supernatant was centrifuged at 20,000*g* for 30 min, and the 20,000*g* supernatant was further centrifuged at 100,000*g* for 1 h. Floating fat layers obtained by these centrifugation steps were combined. The particulate fractions and the combined fat layer were suspended in the homogenizing buffer. The total activity of the fat layer is underestimated because this fraction could not be completely recovered by centrifugation.

Cell Fraction[a]	Total Protein	Pi Released	
	mg 100 seeds^{-1}	*nmol min*$^{-1}$ *mg protein*$^{-1}$	*nmol min*$^{-1}$ *total protein*$^{-1}$
Homogenate	107.2	14.9	1597
600*g*, ppt.	24.1	6.0	145
3,000*g*, ppt.	10.8	17.3	186
20,000*g*, ppt.	13.7	20.2	276
100,000*g*, ppt.	6.8	23.0	158
100,000*g*, sup.	64.3	9.0	579
Fat layer	2.2	9.0	20

[a] ppt. = precipitate; sup. = supernatant.

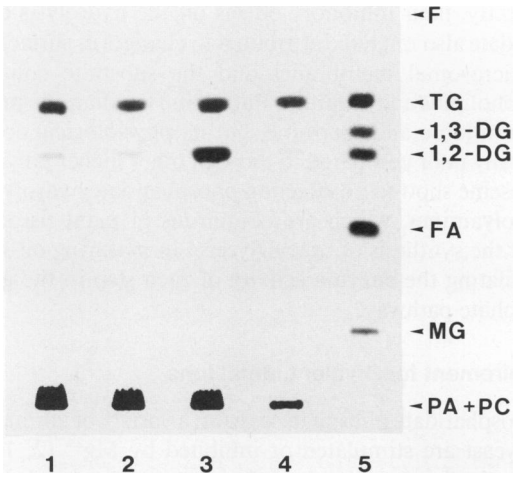

Figure 1. TLC of reaction products of the safflower microsomal preparation. The complete incubation system contained 50 mM Tris-50 mM maleic acid-NaOH (pH 6.75), 0.1 mM EDTA, 2 mM $MgCl_2$, 1 mM sodium phosphatidate (dioleoyl), 0.65 mM egg yolk phosphatidylcholine, 2 mg BSA, and 0.4 mg microsomal protein in a total volume of 2 mL. After 30 min incubation at 28°C, the reaction was terminated by the addition of 30 μL 6 N HCl and 2 mL methanol. Lipids were extracted with 3 mL chloroform, and then washed twice with methanol:water (1:1, v/v). Lipids in the chloroform layer were concentrated to dryness, and developed on a silica gel plate with chloroform:methanol:acetic acid (196:4:1, v/v/v) as solvent. Lipid classes separated were visualized by spraying 30% (w/w) sulfuric acid and then heating at 145°C. The control reactions were carried out for zero time (lane 1), in the absence of Mg^{2+} (lane 2), or in the absence of phosphatidate (lane 4). Lane 3 shows the products of the complete system, and lane 5 is the chromatogram of a mixture of authentic lipids. In this solvent system, phospholipids remained at the origin of the plate. F, solvent front; TG, triacylglycerol; 1,3-DG, 1,3-diacylglycerol; 1,2-DG, 1,2-diacylglycerol; FA, free fatty acids; MG, monoacylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine.

1,2-diacylglycerol was accumulated at zero time (lane 1), in the absence of Mg^{2+} (lane 2), or in the absence of the phosphatidate substrate (lane 4). 1,2-Diacylglycerol can also be formed from phosphatidylcholine by the backward reaction of diacylglycerol cholinephosphotransferase in maturing oil seeds, as demonstrated by Slack *et al.* (26). In the present incubation system where CMP, a cofactor of the backward reaction, was not added, phosphatidylcholine was not converted into 1,2-diacylglycerol (lane 4). Due to the absence of exogenous acyl-CoA, 1,2-diacylglycerol formed in the complete system was not further metabolized to triacylglycerol.

It should be noted that the measurement of Pi release from phosphatidate overestimates the microsomal activity of phosphatidate phosphatase from rat liver because Pi was also produced via glycerol 3-phosphate that resulted from the deacylation of phosphatidate by phospholipases (28). In safflower microsomes, however, free fatty acids did not accumulate in the incubation media (lanes 2 and 3). This indicates that phosphatidate was not deacylated at least under the conditions used here, and that Pi released reflected the net phosphatidate phosphatase activity.

General Properties of Phosphatidate Phosphatase

When the microsomal preparation from maturing safflower seeds was stored at −20°C, the phosphatidate phosphatase activity was stable for at least 3 weeks. The rate of Pi release from phosphatidate was proportional to the concentration of microsomal protein at least up to 100 μg/mL. Most other routine assays were carried out with 50 or 75 μg protein. The hydrolysis of phosphatidate proceeded at a linear rate for 80 min of incubation with a slight decline of reaction velocity after 100 min.

The optimum pH for Pi release by the phosphatase was 6.7 in Tris-maleate buffer (Fig. 2). Bis-Tris and acetate buffer gave pH profiles similar to Figure 2. No phosphatidate phosphatase activity was expressed in citrate buffer, probably due to chelation of Mg^{2+} with citrate. As shown later, Mg^{2+} is an essential cofactor of the enzyme. In contrast with this neutral phosphatidate phosphatase of maturing safflower seeds, spinach chloroplasts have an alkaline phosphatidate phosphatase, the optimum pH of which is 9.0 (18). Both germinating castor bean endosperm (24) and mung bean cotyledons (11) have two isozymes of phosphatidate phosphatase differing in pH optimum (4.25 and 6.0, and 5.0 and 7.5, respectively). The groundnut enzyme showed a broad pH optimum between 6 and 7 (30). The concentration of Tris-maleate buffer affected the reaction rate. The maximum release of Pi was observed in the range of 50 to 100 mM. The decrease in the phosphatase activity at lower concentrations can be related to changes in pH of the medium by Pi liberated. Over 100 mM, the activity gradually reduced as the concentration increased. The buffer concentration was fixed at 50 mM in other assays.

Figure 3 shows the thermolability of the phosphatidate phosphatase. The enzyme activity was stable at 30°C for at least 10 min, but was thoroughly inactivated at 55°C. Thus, the enzyme appeared to be more thermolabile than the acyltransferases (14, 16). The soluble phosphatidate phosphatase was similar to the microsomal enzyme in thermolability (K Ichihara, unpublished data).

Moller *et al.* (23) reported that diacylglycerol, one of the products of phosphatidate hydrolysis, was an inhibitor of the

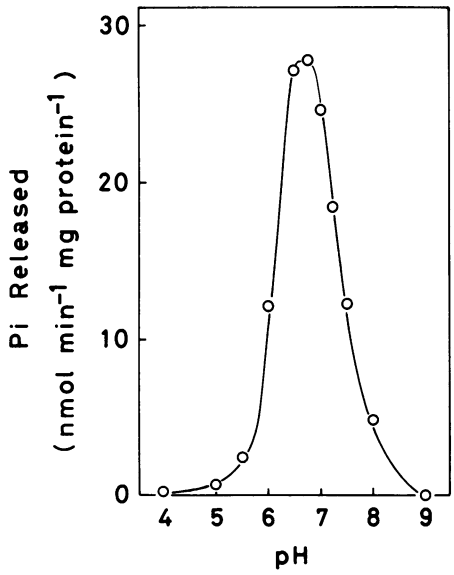

Figure 2. Pi release from phosphatidate by the microsomal phosphatidate phosphatase as a function of pH. Buffers used were acetate (pH 4.0), Tris-maleic acid-NaOH (pH 5.0–8.0), and Tris-HCl (pH 9.0). The concentration of each buffer was 50 mM.

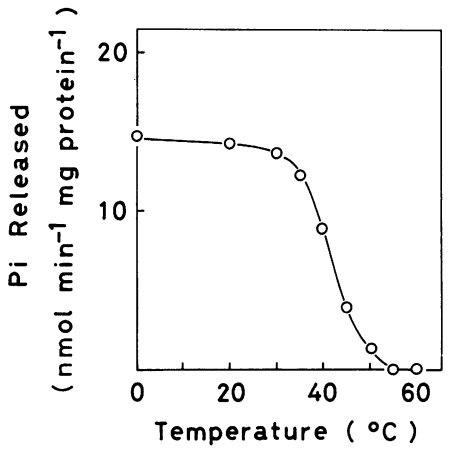

Figure 3. Thermolability of phosphatidate phosphatase. The 100,000*g* particulate fraction was preincubated in the 50 mM Tris-50 mM maleic acid-NaOH buffer (pH 6.75) containing 2 mM $MgCl_2$ at the indicated temperatures for 10 min. After the mixture was rapidly cooled to 0°C, the remaining activity was determined at 28°C in the standard incubation system as described in "Materials and Methods."

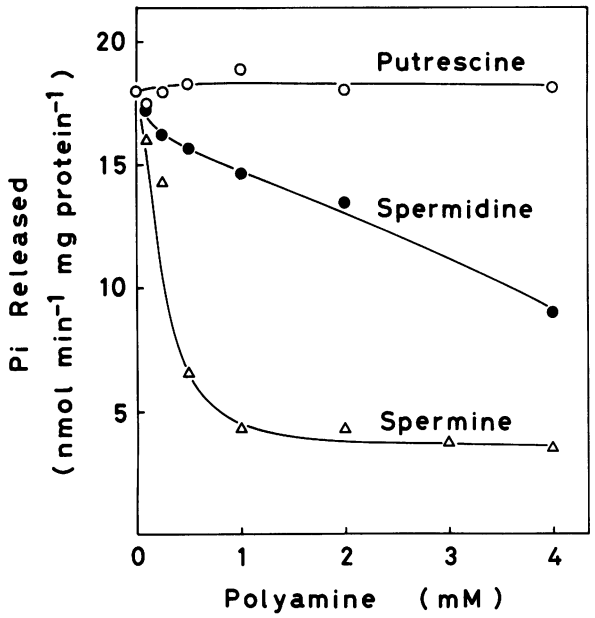

Figure 4. Effects of polyamines on phosphatidate phosphatase activity. Polyamines were added in the complete incubation medium including 2 mM $MgCl_2$, and then Pi release was determined. (○), Putrescine [$NH_2(CH_2)_4NH_2$]; (●), spermidine [$NH_2(CH_2)_3NH(CH_2)_4NH_2$]; [(△), spermine [$NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$].

reaction in rat adipocytes while the other product, Pi, was not. Pi (50 μM) did not show product inhibition for the safflower enzyme; however, the effect of diacylglycerol was not examined.

Spermidine stimulated the synthesis of phosphatidic acid (14, 16), while the reaction of phosphatidate phosphatase activated by 2 mM Mg^{2+} was inhibited by spermidine, and more strongly by spermine (Fig. 4). It is widely accepted that polyamines bind to the negatively charged phospholipid head groups or other anionic sites on membranes, thus altering the stability and fluidity of membranes. Since, as a result, they can modulate the activities of membrane-associated enzymes indirectly, their inhibitory effects on the hydrolysis of phosphatidate also might be attributed to changes in surface charge of microsomal membranes and the substrate complex of phospholipids and albumin. Putrescine is a diamine precursor of spermidine and spermine, but its physiological activity is generally poor compared to those of other higher polyamines. Putrescine showed no effect on phosphatidate hydrolysis (Fig. 4). Polyamines, which are ubiquitous in plant tissues, may affect the synthesis of triacylglycerol in maturing oil-seeds by modulating the enzyme activity of each step in the glycerol-phosphate pathway.

Requirement for Divalent Metal Ions

Phosphatidate phosphatases from a variety of animal tissues and yeast are stimulated or inhibited by Mg^{2+} (2, 12). The chloroplastic phosphatidate phosphatase of spinach leaves is inhibited by Mg^{2+} (18). Griffiths *et al.* (10) have found, in their studies on the acylation of [^{14}C]glycerol 3-phosphate by safflower microsomes, that phosphatidate phosphatase activity is stimulated by Mg^{2+} and inhibited by EDTA. Figure 5 shows the absolute requirement of the microsomal phosphatidate phosphatase of safflower seeds for Mg^{2+}. The activity depended on the concentration of this metal ion, and the maximum release of Pi was observed at 2 mM. The Mg^{2+}-independent release of Pi was below 0.8 nmol min^{-1} mg $protein^{-1}$, which was 4% or less of the Mg^{2+}-dependent release. A negligible amount of Pi (below 0.2 nmol min^{-1} mg $protein^{-1}$, 1% of the maximum rate) was released from endogenous substrates in dependence on Mg^{2+}. The Mg^{2+}-dependent release of Pi from the hydrolysis of exogenous phosphatidylcholine was an insignificant level, 0.4 nmol min^{-1} mg $protein^{-1}$ or less. Mg^{2+} cannot be replaced by spermidine for the activation of the phosphatase. In the triacylglycerol-synthesis

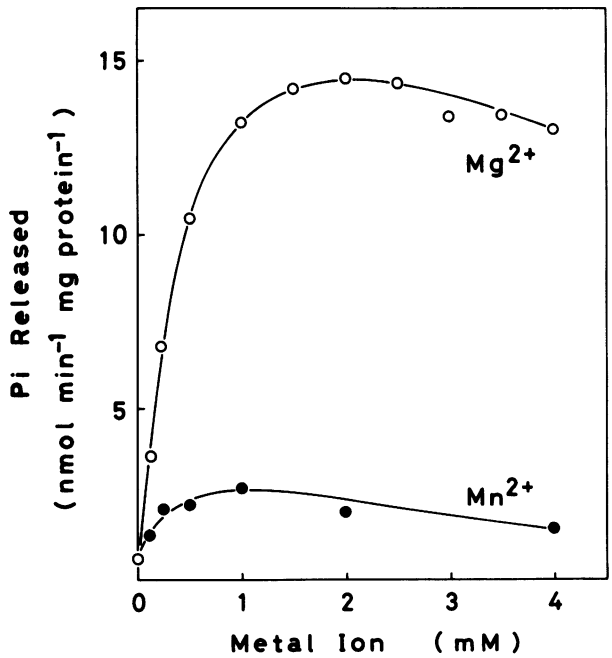

Figure 5. Dependence of phosphatidate phosphatase activity on the concentration of divalent metal ions. Control incubations were carried out for zero time or in the absence of phosphatidate. (○), Mg^{2+}; (●), Mn^{2+}.

system of safflower seeds, the acyltransferase reactions are inhibited or stimulated to a minor extent by Mg^{2+} (14, 16). Mn^{2+} slightly stimulated the phosphatase reaction (Fig. 5). Ca^{2+} showed no stimulatory effect. These profiles of the enzyme activation by divalent cations were very similar to those observed for the rat liver enzyme (13). As expected, EDTA inhibited the phosphatase reaction activated by 2 mM Mg^{2+}; the rates of Pi release were 16.6 nmol min^{-1} mg $protein^{-1}$ at 0 mM of EDTA, 5.4 at 2 mM, 1.4 at 4 mM, 1.2 at 8 mM.

Effects of Phosphatidylcholine and BSA on Phosphatidate Phosphatase Activity

The assay procedure was developed for safflower microsomal phosphatidate phosphatase, because *in situ* dephosphorylation of phosphatidate formed from [^{14}C]glycerol 3-phosphate and acyl-CoA in the microsomal membrane gave no reliable results. Although sodium phosphatidate can be dispersed in water by sonication, the emulsion formed is unstable, especially at higher concentrations. The use of detergents, such as Triton X-100 or sodium cholate, should be avoided, because they may inhibit the phosphatase activity. These forms of substrate also will differ from conditions *in vivo*. The emulsion of a complex composed of sodium phosphatidate, phosphatidylcholine, and BSA was more stable in water and more effective as substrate than a pure phosphatidate emulsion or a mixed emulsion of phosphatidate and phosphatidylcholine. It may resemble the natural form of the substrate in membranes. To find out the optimal ratio of phosphatidate/phosphatidylcholine/BSA, phosphatidate phosphatase activity was measured at various concentrations of each component. When the concentrations of phosphatidate and BSA were 1 and 2 mg/mL, respectively, 0.5 mg/mL phosphatidylcholine gave the maximum activity (Fig. 6A), which was twice as high as the activity in the absence of this phospholipid. BSA, over 0.25 mg/mL, stimulated the phosphatase activity by 40% (Fig. 6B), when the concentrations of phosphatidate and phosphatidylcholine were 0.7 and 0.5 mg/mL, respectively. At a fixed concentration of phosphatidate (0.7 mg/mL) and a constant phosphatidylcholine/BSA ratio (1:4, w/w), the concentrations of phosphatidylcholine and BSA considerably affected the effectiveness of the substrate complex (Fig. 6C). At higher concentrations, phosphatidylcholine and BSA would decrease the available concentration of phosphatidate by dilution and adsorption, respectively, while at lower concentrations stability of the substrate complex in the emulsion might decrease. The best concentration of phosphatidate was in the range of 0.5 to 1 mg/mL, when 0.5 mg/mL phosphatidylcholine and 2 mg/mL BSA were mixed with the substrate (Fig. 6D). According to these data, the most preferable combination would be 1 mM sodium phosphatidate (0.70 mg/mL), 0.65 mM phosphatidylcholine (0.50 mg/mL), and 1 mg/mL BSA. When the activity was measured at lower concentrations of phosphatidylcholine and BSA, the K_m value for phosphatidate was 70 μM.

Phosphatidylcholine stimulated the soluble phosphatidate phosphatase activity of rat adipocytes, while phosphatidylethanolamine and phosphatidylinositol inhibited it (23). These inhibitory phospholipids were not examined for the safflower enzyme. Mixed emulsions of phosphatidate and phosphatidylcholine have been used as substrates of mammalian phosphatidate phosphatases (6, 31). It is believed that the addition of phosphatidylcholine allows a better interaction between enzyme and substrate, not only by stabilizing the bilayer phase but by diminishing the negative charge density on the artificial membrane (31). Moller *et al.* (23) used BSA to prevent substrate clumping, but at higher concentrations it inhibited the hydrolysis of phosphatidate by the rat adipocyte enzyme. The microsomal phosphatidate phosphatase of maturing safflower seeds was stimulated by BSA and was not inhibited even at higher concentrations (Fig. 6B). The substrate complex developed for assay of the safflower phosphatase could be successfully utilized for assay of the enzyme from other plant and animal sources.

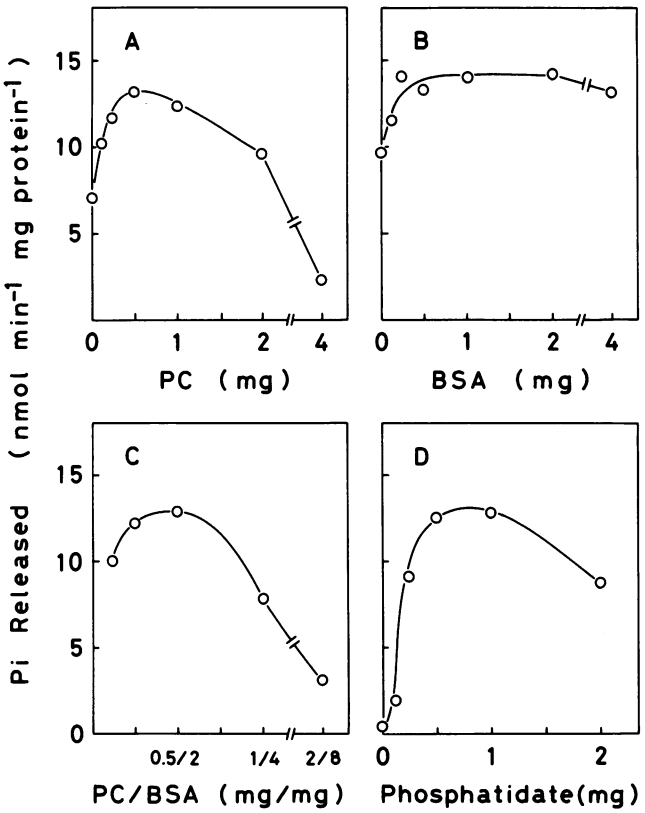

Figure 6. Effects of the concentrations of (A) phosphatidylcholine (PC), (B) BSA, (C) a mixture of phosphatidylcholine and BSA, and (D) phosphatidate on phosphatidate phosphatase activity. A, The concentrations of phosphatidate and BSA were 1 and 2 mg/mL, respectively; B, phosphatidate (0.7 mg) and phosphatidylcholine (0.5 mg) were included in 1 mL of the assay medium; C, a fixed amount of phosphatidate had been previously emulsified with different amounts of phosphatidylcholine and BSA. The ratio between phosphatidylcholine and BSA was 1:4 (w/w). The final concentration of phosphatidate was 0.7 mg/mL. D, Various amounts of sodium phosphatidate were emulsified with fixed amounts of phosphatidylcholine and BSA, the final concentrations of which were 0.5 and 2 mg/mL, respectively. Since the total volume in these assays was 1 mL, 1 mg of phosphatidylcholine and phosphatidate were equivalent to 1.3 and 1.4 mM, respectively.

Stimulation of Phosphatidate Phosphatase Activity by Various Phosphatidylcholine Species

Figure 6A has shown that phosphatidylcholine stimulates phosphatidate phosphatase activity. To reveal the influence

of the fatty acyl moieties of phosphatidylcholine upon stimulation, different molecular species of this phospholipid were emulsified with phosphatidate and BSA, and then effectiveness of the substrate preparations was determined (Table II). Among saturated phosphatidylcholine species tested, phosphatidylcholines containing short-chain acyl moieties (dihexanoyl, dioctanoyl and didecanoyl) showed no stimulatory effect. Medium-chain phosphatidylcholines (dilauroyl and dimyristoyl) were stimulative but less effective than egg yolk phosphatidylcholine. Long-chain saturated phosphatidylcholines (dipalmitoyl and distearoyl) had weak effects. Unsaturated phosphatidylcholines (dioleoyl and dilinoleoyl) markedly increased the phosphatase activity as well as egg yolk phosphatidylcholine. The low effectiveness of the palmitoyl and stearoyl species is probably due, at least partly, to difficulty in emulsification of them. However, these differences in effectiveness among phosphatidylcholine molecular species suggest the significance of hydrophobic domains in cellular membranes where the phosphatidate substrate is embedded, for expression of phosphatidate phosphatase activity. Membrane fluidity, which largely depends on both acyl-chain length and unsaturation degree of membrane lipids, will be important for interaction between phosphatidate and the phosphatase enzyme.

Table II. *Effects of the Acyl Moieties of Phosphatidylcholine on Phosphatidate Phosphatase Activity*

Phosphatidylcholines were simple-acid species, except for egg yolk phosphatidylcholine. The concentration of each species was 1 mM.

Molecular Species of Phosphatidylcholine	Pi Released
	nmol min^{-1} mg protein^{-1}
None	7.9
Hexanoyl	7.5
Octanoyl	6.3
Decanoyl	7.8
Lauroyl	10.2
Myristoyl	11.5
Palmitoyl	9.9
Stearoyl	8.6
Oleoyl	14.7
Linoleoyl	14.3
Egg yolk	14.1

Table III. *Hydrolytic Activity of Safflower Microsomes on Water-Soluble Phosphate Compounds and on Phosphatidate*

The concentration of each substrate was 1 mM. Control values were obtained at zero time or in the absence of substrates. Neither phosphatidylcholine nor BSA was included in the reaction media other than those for lysophosphatidate* and phosphatidate*.

Substrate	Pi Released	
	Mg^{2+}-dependent	Mg^{2+}-independent
	nmol min^{-1}	*mg protein^{-1}*
sn-Glycerol 3-phosphate	0.0	2.9
Glucose 6-phosphate	0.0	2.3
AMP	0.0	1.0
NADPH	0.0	12.5
Lysophosphatidate	0.0	0.4
Lysophosphatidate*	0.4	1.1
Phosphatidate*	15.7	0.5

* Emulsions with phosphatidylcholine and BSA.

Specificity for Water-Soluble Phosphate Esters

Acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) catalyzes the hydrolysis of phosphomonoesters, and is widely found in the plant and animal kingdoms. Its substrate specificity is very broad. Blank and Snyder (3) showed that the nonspecific acid phosphatase of wheat germ was capable of dephosphorylating phosphatidic acid. Therefore, care must be taken to ensure that the hydrolysis of phosphatidate is not due to acid phosphatase. Table III shows that the Pi release from phosphatidate by the safflower microsomal preparation was due to a Mg^{2+}-dependent phosphatidate phosphatase, which was specific for phosphatidate and did not attack any water-soluble phosphate compounds including lysophosphatidate under the assay conditions chosen. Mg^{2+}-dependent dephosphorylation of lysophosphatidate was detectable in the presence of phosphatidylcholine and BSA, but the rate of hydrolysis was 2.5% of that for phosphatidate.

Mg^{2+}-independent Pi release was observed for all of the water-soluble phosphate esters tested. Although phosphatidate was also hydrolyzed in the absence of exogenous Mg^{2+}, the rate of Pi release was only 3% of the Mg^{2+}-dependent release. However, it is not clear whether the Mg^{2+}-independent Pi release from phosphatidate was caused by a nonspecific acid phosphatase or by a Mg^{2+}-independent phosphatidate phosphatase. Lysophosphatidate was dephosphorylated Mg^{2+}-independently regardless of the presence of phosphatidylcholine and BSA. Monoacylglycerol has been often detected as a radioactive intermediate in labeling studies with maturing oil seeds (1). Acid phosphatase, which is intrinsically Mg^{2+}-independent and nonspecific, also may be responsible for the formation of monoacylglycerol from lysophosphatidate in maturing oil seeds. There has so far been no evidence for the existence of a lysophosphatidate-specific phosphatase. The safflower phosphatidate phosphatase was more specific for phosphatidate than the enzyme from other organisms. Partially purified phosphatidate phosphatase from yeast dephosphorylated lysophosphatidate at the rate 6 to 15% of that for phosphatidate (12). The microsomal enzymes from rat liver and lung hydrolyzed lysophosphatidate at reaction rates close to those for phosphatidate (22).

Thus, it is obvious that in maturing safflower seeds the phosphatidate intermediate of glycerolipid synthesis is metabolized to diacylglycerol by the microsomal, Mg^{2+}-dependent phosphatidate phosphatase which is strictly specific for phosphatidate, although the participation of the soluble enzyme (Table I) in diacylglycerol formation *in vivo* is not clear.

On the Rate of Triacylglycerol Synthesis

The maximum velocity of phosphatidate cleavage by the microsomal phosphatidate phosphatase of maturing safflower seeds was approximately 20 nmol min^{-1} mg protein^{-1}, and comparable to that for the acylation at position *sn*-1 of glycerol 3-phosphatate (14). This potential of the phosphati-

date phosphatase observed *in vitro* is enough to account for the rate of triacylglycerol accumulation *in vitro* (27) and *in vivo* (15, 26). The specific activities of the acylations at positions *sn*-2 and 3 of the glycerol moiety were 250 and 3 nmol min^{-1} mg microsomal $protein^{-1}$ (16, 17). Furthermore, the phosphatidic acid level in maturing safflower seeds is very low. From these data, the phosphatidate phosphatase reaction does not appear to be rate-limiting for triacylglycerol synthesis.

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