# Glycerolipid synthesis in rat adipose tissue. II. Properties and distribution of phosphatidate phosphatase

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**Abstract** The properties and subcellular distribution of phosphatidate phosphatase (EC 3.1.3.4) from adipose tissue have been investigated. The enzyme was assayed using both aqueous phosphatidate and membrane-bound phosphatidate as substrates. When measured with aqueous substrate, activity was detected in the mitochondria, the microsomes, and the soluble fraction. $Mg^{2+}$ at low concentration stimulated the phosphatidate phosphatase from soluble and microsomal fractions but had no effect on the mitochondrial phosphatidate phosphatase. At higher concentration $Mg^{2+}$ was inhibitory. In the presence of $Mg^{2+}$, the phosphatidate phosphatase from soluble and microsomal fractions was active against membrane-bound phosphatidate. No activity was demonstrated with membrane-bound substrate in the absence of $Mg^{2+}$. Mitochondria did not contain activity toward the membrane-bound substrate. The rate of utilization of aqueous phosphatidate was always higher than that of membrane-bound substrate. These results indicate that there are at least two different phosphatidate phosphatases in adipose tissue.

**Supplementary key words** aqueous phosphatidate · membrane-bound phosphatidate · $Mg^{2+}$

Phosphatidate phosphatase (EC 3.1.3.4) catalyzes the release of orthophosphate from *sn*-1,2-diacylglycerol-3-phosphate to form 1,2-diglyceride, an intermediate reaction in neutral lipid formation from *sn*-glycerol-3-P. The presence of this enzyme has been reported in several tissues including adipose tissue (1–10). The properties of this enzyme from chicken liver, rat liver, pig kidney, and erythrocytes have been studied in some detail (1, 4, 6, 7).

The subcellular distribution pattern of phosphatidate phosphatase is unusual. The enzyme is found in the mitochondria, lysosomes, microsomes, and cytosol from various tissues (1, 4). Although the enzyme is located in the different compartments of the cell, studies by Smith et al. (11) and Lamb and Fallon (12) with liver and of Johnston et al. (13) with intestine suggest that the cytoplasmic phosphatidate phosphatase is primarily involved in neutral lipid formation by these tissues while the particulate enzyme plays a less important role.

In contrast, recent evidence from this laboratory suggests that in the presence of $Mg^{2+}$, adipose tissue microsomes are more active than cytosol in utilizing membrane bound phosphatidate to form 1,2-diglyceride (10). This observation is extended further and is the basis for the present investigation of the properties of adipose tissue phosphatidate phosphatase.

## MATERIALS AND METHODS

Egg lecithin was obtained from Sigma Chemical Co., St. Louis, Mo. Phospholipase D was purchased from Boehringer Mannheim, New York. Other chemicals and the experimental animals were purchased from the sources reported previously (10).

### Preparation of substrates

Phosphatidate was prepared from egg lecithin by the action of phospholipase D (14). The purity of this preparation was determined by chromatography on silica gel G plates ($R_F$ 0.34) with the solvent system $CHCl_3$–$CH_3OH$–3 N $NH_4OH$ 130:70:16 (15). The preparation was dissolved in 5 ml of diethyl ether and dispersed in water with sonication. The diethyl ether was evaporated under nitrogen before the substrate was used. The amount of phosphatidate recovered was determined by hydroxamate formation (16).

Membrane-bound phosphatidate was prepared by incubating *sn*-[U-$^{14}$C]glycerol-3-P and palmitoyl CoA with

Abbreviations: *sn*-glycerol-3-P, *sn*-glycerol-3-phosphate.

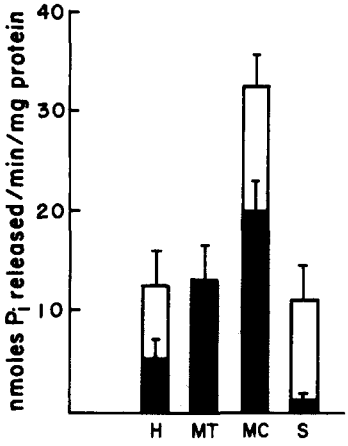


**Fig. 1.** Utilization of aqueous phosphatidate by different subcellular fractions. The mitochondrial and microsomal enzymes were assayed at pH 6.8 and the soluble enzyme at pH 7.5 in the presence □ or absence ■ of $Mg^{2+}$. MT, mitochondria; MC, microsomes; S, soluble fraction; and H, homogenate. Each value represents the mean ± SEM of assays using tissue fractions of five or six rats.

adipose tissue microsomes at 37°C for 10 min. The reaction was stopped by the addition of cold 0.05 M Tris–HCl buffer, pH 7.5, and the mixture was centrifuged at 160,000 *g* for 45 min. The resulting pellet was washed once with Tris buffer and suspended in the same buffer by gentle homogenization. This suspension was heated at 100°C for 5 min to destroy intrinsic phosphatase activity and was rehomogenized before use. For large-scale preparation of membrane-bound substrate, liver microsomes were used. Liver microsomes were incubated in the presence of Tris buffer, pH 7.5. The incubation system contained, in a final volume of 0.7 ml, 49 mM Tris, 17.5 mM KCl, 0.71 mM dithiothreitol, 0.039 mM CoA, 3.55 mM ATP, 3.44 mM $Mg^{2+}$, 3 mM *sn*-glycerol-3-P, 1.42 mM palmitate (0.54 μCi/μmole), 1.25 mg of fatty acid-poor albumin, 50 mM NaF, and 0.4–0.6 mg of microsomal protein. Incubation was at 37°C for 1 hr. The reaction was stopped by adding 0.05 M cold Tris buffer, pH 7.5, and the membrane-bound substrate was isolated by the same procedure described for the adipose tissue microsome preparations.

### Preparation of subcellular fractions

Epididymal adipose tissue obtained from two or three rats was homogenized with 3 vol of cold buffer containing 0.25 M sucrose, 1 mM Tris, pH 7.4, and 1 mM EDTA. The subcellular fractions were prepared as described previously (10).

### Phosphatidate phosphatase

The standard assay was conducted in a final volume of 1 ml containing 100 mM Tris–maleate buffer, pH 6.8 or 7.5, and 1.2 mM aqueous phosphatidate. The reaction was started by the addition of 0.1–0.2 ml of subcellular fraction and the mixture was incubated at 37°C for 30 min in a shaking water bath. The reaction was terminated by the addition of 1 ml of 10% trichloroacetic acid. Protein was collected by centrifugation, and inorganic phosphate in the protein-free supernate was estimated according to Ames and Dubin (17). The difference in the phosphate content between the experimental reaction and the reaction stopped at zero time was taken as a measure of phosphatidate phosphatase activity. The enzyme units are expressed as nmoles of inorganic phosphate released/min/mg protein.

TABLE 1. Intracellular distribution of phosphatidate phosphatase

| Fraction | $Mg^{2+}$-independent Phosphatase | | $Mg^{2+}$-dependent Phosphatase | | Protein |
|---|---|---|---|---|---|
| | % of Total | Specific Activity | % of Total | Specific Activity | % of Total |
| Fat-free homogenate | 100 | 1 | 100 | 1 | 100 |
| Mitochondria | 27 | 3 | 1 | 0.1 | 9 |
| Microsomes | 42 | 5.2 | 20 | 2.25 | 8 |
| Cytosol | 20 | 0.26 | 70 | 0.9 | 75 |

Assays were conducted with aqueous phosphatidate as described in Methods. The results are expressed both as percentage of total activity of fat-free homogenate and as relative specific activity with respect to protein, using fat-free homogenate activity equal to 1.

The soluble fraction and the homogenate were dialyzed at 4°C for 2–3 hr against several volumes of 0.02 M Tris–maleate buffer, pH 7.0, to remove endogenous inorganic phosphate and EDTA. This treatment was not necessary for mitochondria and microsomes because these fractions contained negligible amounts of free phosphates and were suspended in a buffer containing no EDTA.

Phosphatidate phosphatase was also measured by using membrane-bound radioactive phosphatidate as substrate. The reaction mixture contained 60 mM Tris buffer, pH 7.5, and 0.1–0.2 ml of a suspension of membrane-bound phosphatidate containing 120 nmoles of [$^{14}$C]phosphatidate (1–2 mg of microsomal protein). The reaction was initiated by the addition of 0.2 ml of different subcellular fractions. The contents were incubated at 37°C in a shaking water bath. Diglyceride formation was linear up to 30 min, and the reaction was terminated by the addition of 10 ml of chloroform–methanol 2:1. Lipids were extracted, washed as described by Folch, Lees, and Sloane Stanley (18), and dried under nitrogen. Lipids were dissolved in 1 ml of benzene and stored at −40°C.

The radioactive products were separated by thin-layer chromatography and identified by using authentic lipid standards. The best separation of phosphatidate was achieved with chloroform–methanol–3 N ammonium hydroxide 130:70:16 (15). Neutral lipids were separated with hexane–ether–acetic acid 146:50:4 (19). Lipids were visualized by exposure of the plates to iodine. Appropriate areas from the plates were scraped directly into scintillation vials containing 10 ml of Liquifluor and toluene mixture. The radioactivity was counted in a Beckman LS-250 liquid scintillation counter at room temperature.

The rate of phosphatidate utilization also was estimated by conducting a glycerophosphate acyltransferase assay in the absence and presence of $Mg^{2+}$ as described previously (10). The reaction mixture contained 25 mM Tris, pH 7.5, 50 mM KCl, 0.42 mM *sn*-glycerol-3-P, 0.1 $\mu$Ci of *sn*-[U-$^{14}$C]glycerol-3-P, 0.7 mM dithiothreitol, 0.065 mM palmitoyl CoA, 1.25 mg of fatty acid-poor albumin, 1 mM $Mg^{2+}$, and 0.2 ml of a suspension of mitochondria or microsomes containing 0.15–0.2 mg of protein. In the absence of $Mg^{2+}$, the major reaction product formed was phosphatidate. When $Mg^{2+}$ was included in the reaction mixture, neutral lipids were formed at the expense of phosphatidate. The difference in neutral lipid formation with or without $Mg^{2+}$ reflects phosphatidate phosphatase activity and is expressed as nmoles of neutral lipid formed/min/mg protein. In some experiments the rate of utilization of membrane-bound substrate is expressed as a ratio of diglyceride to phosphatidate.

Protein was determined by the procedure of Lowry et al. (20) with crystalline bovine albumin as the standard.

## RESULTS

### Subcellular distribution and characteristics of enzyme reaction

The subcellular distribution of phosphatidate phosphatase as measured with an aqueous dispersion of phosphatidate is shown in Table 1. In the absence of $Mg^{2+}$, 69% of phosphatase activity was present in the particulate fraction; the remaining activity was in the soluble fraction. The specific activities of the mitochondrial and microsomal enzymes were several times higher than that of the soluble enzyme (Fig. 1). Similar results have been described for liver, intestine, and adipose tissue phosphatidate phosphatase (9, 11, 13). Addition of $Mg^{2+}$ resulted in an activation of microsomal and soluble phosphatidate phosphatase. The portion of phosphatidate phosphatase that did not require $Mg^{2+}$ for its activity was designated here as $Mg^{2+}$-independent enzyme. The $Mg^{2+}$-activated portion of this enzyme was termed as $Mg^{2+}$-dependent phosphatidate phosphatase. This terminology has been used earlier by Hokin, Hokin, and Mathison (6) to distinguish two types of phosphatidate phosphatases from erythrocyte membranes.

The major portion of $Mg^{2+}$-dependent phosphatase was present in the soluble fraction and only 20% was found in the microsomal fraction. The mitochondrial fraction did not contain $Mg^{2+}$-dependent phosphatidate phosphatase. Since $Mg^{2+}$ activates phosphatidate phosphatase, the higher specific activity of the $Mg^{2+}$-independent enzyme in the particulate fractions compared with soluble fraction might have been due to the difference in the intrinsic concentration of $Mg^{2+}$ in these fractions. However, measurements of $Mg^{2+}$ in these fractions did not indicate such a possibility. The concentration of $Mg^{2+}$ was 0.66, 0.4, and 2.09 $\mu$g/mg of protein for mitochondrial, microsomal, and soluble fractions, respectively.

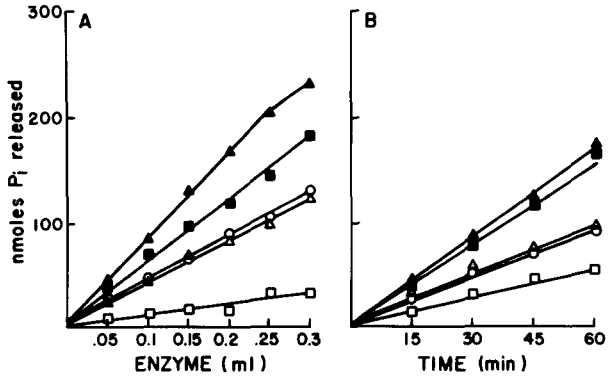


**Fig. 2.** Utilization of aqueous substrate as a function of protein concentration (*A*) and as a function of time (*B*). The mitochondrial, microsomal, and soluble fractions contained 1 mg, 0.8 mg, and 2.4 mg of protein/ml, respectively. Assays were conducted in the presence (solid symbols) or absence (open symbols) of $Mg^{2+}$. The final concentration of $Mg^{2+}$ was 1 mM for microsomal enzyme (▲) and 5 mM for soluble enzyme (■). The mitochondrial enzyme (○) was assayed only in the absence of $Mg^{2+}$.

The reaction velocity was proportional to the concentration of protein over a limited range, as shown in Fig. 2*A*. Assays performed over this concentration range were linear for 60 min (Fig. 2*B*). Since the soluble and microsomal enzymes were activated by $Mg^{2+}$, the effect of substrate concentration on the reaction velocity was studied in the presence and absence of $Mg^{2+}$. From these data a tentative Michaelis-Menten constant, $K_m$, was calculated to be $5.5 \times 10^{-4}$ M for the mitochondrial and microsomal enzyme. In the presence of 1 mM $Mg^{2+}$, the $K_m$ for microsomal enzyme decreased to $2.4 \times 10^{-4}$ M. Soluble phosphatidate phosphatase was assayed in the presence of 5 mM $Mg^{2+}$ and had a $K_m$ of $1.6 \times 10^{-4}$ M (Fig. 3).

The subcellular fractions contained phosphatase activity toward $\alpha$- and $\beta$-glycerophosphates as well as phosphatidate (Table 2). However, the rate of utilization of these substrates was substantially lower than for phosphatidate and was unaffected by the addition of $Mg^{2+}$. Moreover, the enzyme that utilized these substrates was not saturated with the substrate concentration used in the phosphatidate phosphatase assay.

The optimal pH for the mitochondrial and microsomal enzyme was 6.8 (Fig. 4). However, the activation of phosphatidate phosphatase by $Mg^{2+}$ was greater at pH 7.5 than 6.8 (Fig. 5). Since the soluble enzyme was completely $Mg^{2+}$-dependent, it was usually assayed at pH 7.5 instead of at pH 6.8. The optimal pH for the microsomal enzyme utilizing $\alpha$-glycerophosphate was 4.5.

### Effect of $Mg^{2+}$ on phosphatidate phosphatase

The effect of $Mg^{2+}$ on phosphatidate phosphatase has been studied in several tissues (1, 4, 6). In some tissues, $Mg^{2+}$ was shown to stimulate phosphatidate phosphatase,

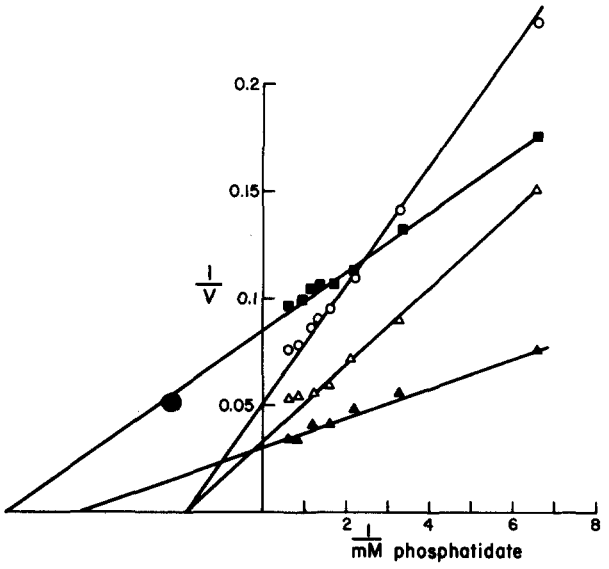


**Fig. 3.** A double reciprocal plot of release of inorganic phosphate from L-α-phosphatidate as a function of L-α-phosphatidate concentration. Incubation conditions were identical with the standard assay except for different concentrations of L-α-phosphatidate. Assays were conducted with microsomes in the presence (▲) or absence (△) of $Mg^{2+}$. Mitochondria (○) were studied without $Mg^{2+}$ and soluble fraction with $Mg^{2+}$ (■). V, nmoles of $P_i$ released/min/mg protein.

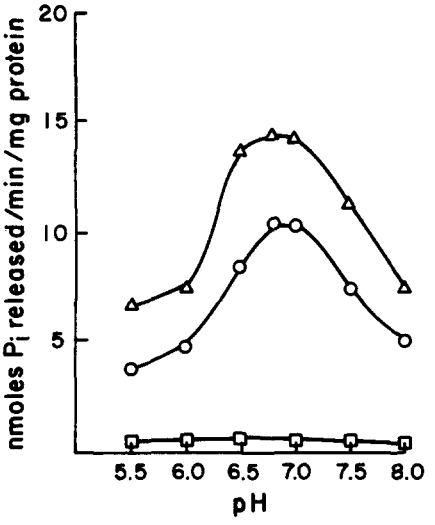


**Fig. 4.** Phosphatidate utilization as a function of the pH of the 0.2 M Tris–maleate buffer. ○, mitochondria; △, microsomes; □, soluble fraction.

while in others it was inhibitory. Fig. 5 shows the effect of $Mg^{2+}$ on phosphatidate phosphatase in adipose tissue. The concentration of $Mg^{2+}$ that produced maximum stimulation of phosphatidate phosphatase varied from 1 to 5 mM with various preparations. In the standard assay, the concentration of $Mg^{2+}$ was usually 1 mM for the microsomal enzyme and 5 mM for the soluble enzyme. At higher concentrations, $Mg^{2+}$ inhibited the phosphatidate phosphatase from the three fractions.

The effect of EDTA on phosphatidate phosphatase is shown in Fig. 6. Addition of EDTA abolished completely the $Mg^{2+}$-stimulated activity from both the soluble and microsomal fractions. However, EDTA had no effect on the mitochondrial activity or on a portion of the microsomal phosphatidate phosphatase that was not stimulated by $Mg^{2+}$. The small activity associated with the soluble fraction in the absence of $Mg^{2+}$ was diminished with the addition of EDTA. Preparation of mitochondria and microsomes in the presence of 50 mM EDTA or EGTA did not affect the $Mg^{2+}$-independent phosphatidate phosphatase from these fractions. Therefore, these studies suggest that there may be two phosphatidate phosphatases in adipose tissue, $Mg^{2+}$-dependent and $Mg^{2+}$-independent.

TABLE 2. Substrate specificity of phosphatidate phosphatase

| Substrate Concentration | Concentration of $Mg^{2+}$ | Mitochondria | Microsomes | Cytosol |
|---|---|---|---|---|
| | | *nmoles $P_i$ released/min/mg protein* | | |
| Aqueous phosphatidate, 1.2 mM | | 11 | 20 | 1.2 |
| | 1 mM | 9.5 | 38 | 5.6 |
| | 2.5 mM | 7.5 | 30 | 8.8 |
| α-Glycerophosphate, 1.2 mM | | 2.2 | 8 | 1.6 |
| | 1 mM | 3.3 | 8 | 2.0 |
| | 2.5 mM | 2.8 | 8 | 1.4 |
| 6 mM | | 6.5 | 19 | 1.6 |
| | 1 mM | 7.0 | 19 | 2.8 |
| | 2.5 mM | 7.0 | 19 | 2.0 |
| β-Glycerophosphate, 1.2 mM | | 2.3 | 4.8 | 1.6 |
| | 1 mM | 2.8 | 4.8 | 2.8 |
| | 2.5 mM | 2.5 | 4.5 | 2.8 |
| 6 mM | | 6.3 | 9.1 | 1.6 |
| | 1 mM | 7.5 | 10.1 | 2.4 |
| | 2.5 mM | 7.5 | 9.6 | 2.0 |

The standard assay was conducted in duplicate, as described in Methods, in the presence of aqueous phosphatidate or α- or β-glycerophosphates as substrates. Microsomes incubated with 25 mM and 50 mM α-glycerophosphate gave reaction rates of 25 and 33 nmoles $P_i$/min/mg protein, respectively.

## Utilization of membrane-bound substrate

In most of the earlier studies, phosphatidate phosphatase was measured in the presence of an aqueous dispersion of phosphatidate as substrate (1–9). More recently, Smith et al. (11) and Johnston et al. (13) measured this enzyme from liver and intestine by using both aqueous phosphatidate and membrane-bound phosphatidate as substrates. These workers observed that the particulate enzyme, which was most active against aqueous substrate, utilized membrane-bound substrate poorly. Conversely, the soluble enzyme was less active against aqueous phosphatidate and utilized the membrane-bound substrate efficiently. These studies were the first to establish a role for the soluble phosphatidate phosphatase in neutral lipid formation in liver and intestine. They also demonstrated the

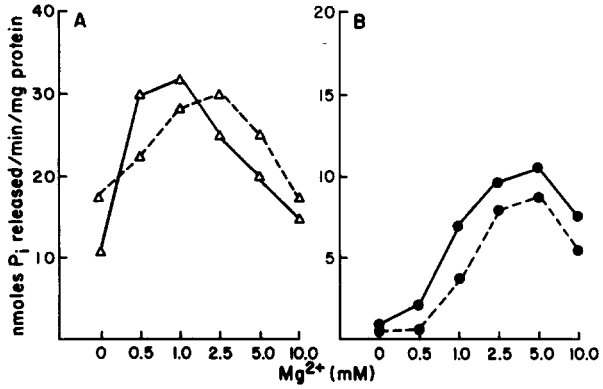


**Fig. 5.** Activation of phosphatidate phosphatase by $Mg^{2+}$ at two different pH values, 6.8 (dashed lines) and 7.5 (solid lines). *A*, microsomal enzyme; *B*, soluble enzyme.

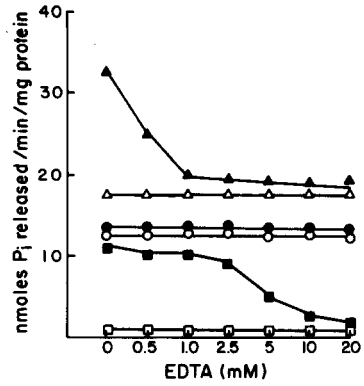


**Fig. 6.** Effect of EDTA on phosphatidate phosphatase. Assays were conducted in the presence (solid symbols) or absence (open symbols) of $Mg^{2+}$ with variable concentration of EDTA. △, ▲, microsomal enzyme; ○, ●, mitochondrial enzyme; □, ■, soluble enzyme. Concentration of $Mg^{2+}$ was 1 mM for microsomes and 5 mM for soluble enzyme.

importance of membrane-bound substrates in assays of enzymes utilizing lipid substrates.

Although the soluble enzyme is apparently important in neutral lipid formation in liver and intestine, the situation in adipose tissue seems to be different. Our earlier (10) and present studies show that in the presence of $Mg^{2+}$, microsomal enzyme from adipose tissue utilizes the membrane-bound substrate more actively than the soluble enzyme. Table 3 shows the effect of $Mg^{2+}$ on utilization of membrane-bound substrate. The membrane-bound substrate was prepared in the presence of palmitoyl CoA and *sn*-[U-$^{14}$C]glycerol-3-P under the conditions of the glycerophosphate acyltransferase assay as described previously (10). Microsomes used for the preparation of membrane-bound phosphatidate were active against this endogenous substrate. In the absence of $Mg^{2+}$, the major lipid formed with *sn*-glycerol-3-P and palmitoyl CoA was phosphatidate. Addition of $Mg^{2+}$ resulted in an increased formation of neutral lipids at the expense of phosphatidate. The activity of this enzyme was estimated from the rate of neutral lipid formation in the presence or absence of $Mg^{2+}$. The rate for microsomal enzyme was 6.65 nmoles/min/mg protein in the presence of $Mg^{2+}$ and 0.65 nmoles/min/mg protein in the absence of $Mg^{2+}$. When $Mg^{2+}$-pretreated microsomes were incubated with palmitoyl CoA and *sn*-[$^{14}$C]glycerol-3-P in the absence of additional $Mg^{2+}$, the rate of neutral lipid formation was 3.65 nmoles/min/mg protein. However, this rate was significantly lower than when $Mg^{2+}$ was present throughout the incubation (6.65 nmoles/min/mg protein). The rate of utilization of membrane substrate by the mitochondrial phosphatidate phosphatase was significantly low and was not changed by the addition of $Mg^{2+}$. Since the substrate was membrane-bound, no kinetic constants were derived from these data.

In some experiments, both membrane-bound and aqueous phosphatidate were used simultaneously to assay the particulate and soluble enzymes, as shown in Fig. 7. The membrane-bound substrate was prepared by using adipose tissue microsomes as described in Methods. In the absence of $Mg^{2+}$, the aqueous substrate was utilized at a similar rate as observed earlier. However, the membrane-bound substrate remained unused. Addition of $Mg^{2+}$ resulted in an increased utilization of both substrates by microsomal and soluble enzymes. These results indicate that $Mg^{2+}$-dependent phosphatidate phosphatase is responsible for the utilization of membrane-bound substrate, and in adipose tissue this is localized in both the microsomal and soluble fractions. The mitochondrial enzyme did not utilize membrane-bound substrate, and the rate of aqueous phosphatidate utilization was not increased in the presence of $Mg^{2+}$.

TABLE 3. Utilization of membrane-bound phosphatidate by phosphatidate phosphatase

| Subcellular Fraction | Addition of $Mg^{2+}$ | PA[a] | NL[b] | Increased NL by $Mg^{2+}$ | Ratio NL/PA |
|---|---|---|---|---|---|
| | | *nmoles lipid/min/mg protein* | | | |
| Microsomes (10)[c] | − | 5.07 ± 0.7 | 0.65 ± 0.14 | | 0.13 |
| | + | 2.12 ± 0.33 | 7.3 ± 0.55 | 6.65 | 3.4 |
| Pretreated microsomes | − | 2.1 | 3.8 | 3.15 | 1.8 |
| Mitochondria (5)[c] | − | 2.3 ± 0.3 | 0.55 ± 0.11 | | 0.23 |
| | + | 4.6 ± 0.8 | 0.72 ± 0.2 | 0.17 | 0.15 |

Membrane-bound phosphatidate was prepared by conducting the glycerophosphate acyltransferase assay in the presence of *sn*-glycerol-3-phosphate and palmitoyl CoA with or without 1 mM $Mg^{2+}$, as described in Methods. Each value represents the mean ± SEM of assays using tissue fractions from 5–10 rats. In one experiment, microsomes were pretreated with $Mg^{2+}$ at 37°C for 5 min. Excess $Mg^{2+}$ was removed by centrifuging the microsomes at 160,000 *g* for 45 min. The resulting pellet was washed once with 5 ml of Tris buffer, pH 7.5, and centrifuged again. These microsomes were then used for the assay.

[a] PA, phosphatidate.
[b] NL, neutral lipids.
[c] Number of experiments.

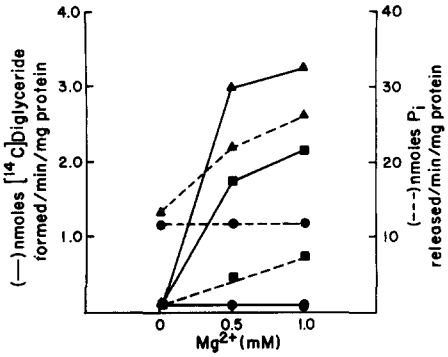


**Fig. 7.** Utilization of aqueous and membrane-bound phosphatidate. Assays were conducted with aqueous (---) and membrane-bound (——) phosphatidate in the presence or absence of $Mg^{2+}$. The assay mixture contained 60 mM Tris–maleate buffer, pH 7.5, and 1.2 μmoles of aqueous phosphatidate or 120 nmoles of membrane-bound [$^{14}$C]phosphatidate prepared by using adipose tissue microsomes. The reaction was started with 0.2 ml of different subcellular fractions containing 0.1–0.3 mg of protein. After a 20-min incubation at 37°C, the reaction was terminated. The release of $P_i$ and the formation of [$^{14}$C]diglyceride were determined as described in Methods. ●, mitochondria; ▲, microsomes; ■, soluble fraction.

Phosphatidate phosphatase from soluble and microsomal fractions of adipose tissue was also active against the membrane-bound phosphatidate obtained from liver microsomes (Table 4). Since this substrate was prepared in the presence of $Mg^{2+}$, the concentration of $Mg^{2+}$ in this preparation was sufficient to activate phosphatidate phosphatase of either the soluble or microsomal fraction, and further addition of $Mg^{2+}$ was not required. In fact, further addition of $Mg^{2+}$ was inhibitory. However, the utilization of this substrate was dependent on $Mg^{2+}$. This was evident from the impairment in the utilization of membrane-bound substrate by the soluble or microsomal phosphatidate phosphatase in the presence of EDTA.

TABLE 4. Effect of divalent cations on utilization of membrane-bound phosphatidate

| Addition | Microsomes | | | Soluble Fraction | | |
|---|---|---|---|---|---|---|
| | PA[a] | NL[b] | NL/PA | PA | NL | NL/PA |
| | *nmoles* | | | *nmoles* | | |
| None | 87 | 43.7 | 0.49 | 92 | 37 | 0.40 |
| $Mg^{2+}$ | 101 | 22 | 0.21 | 101 | 17 | 0.16 |
| $Co^{2+}$ | 128 | 10.5 | 0.08 | 88 | 7.0 | 0.07 |
| $Ni^{2+}$ | 110 | 11 | 0.10 | 106 | 7.4 | 0.07 |
| $Fe^{2+}$ | 97 | 22.4 | 0.23 | 84 | 26.6 | 0.31 |
| $Zn^{2+}$ | 107 | 10.0 | 0.09 | 97 | 10.0 | 0.10 |
| $Cu^{2+}$ | 104 | 7.1 | 0.06 | 114 | 9.2 | 0.08 |
| $Mn^{2+}$ | 103 | 9.0 | 0.08 | 107 | 10.0 | 0.09 |
| EDTA (5 mM) | 104 | 8.0 | 0.07 | 107 | 8.0 | 0.08 |

Membrane-bound substrate was prepared in the presence of [$^{14}$C]palmitate, ATP, CoA, $Mg^{2+}$, NaF, and *sn*-glycerol-3-phosphate with liver microsomes, as described in Methods. Incubation mixture contained 60 mM Tris–maleate buffer, pH 7.5, and 0.2 ml (1.0 mg of protein) of membrane-bound substrate containing 120 nmoles of [$^{14}$C]phosphatidate and 8 nmoles of $^{14}$C-labeled neutral lipids (NL/PA = 0.06). All cations were added as chloride salts at neutral pH. The final concentration of each ion was 1 mM. The reaction was started by the addition of 0.2 ml of microsomal or soluble fraction containing 0.272 mg and 0.332 mg of protein, respectively. After a 30-min incubation at 37°C, the reaction was stopped by the addition of 10 ml of chloroform–methanol 2:1. Lipids were extracted and separated by thin-layer chromatography as described in Methods.

[a] PA, phosphatidate.

[b] NL, neutral lipids.

The utilization of membrane-bound substrate by the microsomal and soluble enzymes was completely inhibited by $Zn^{2+}$, $Cu^{2+}$, $Ni^{2+}$, $Co^{2+}$, and $Mn^{2+}$. The effect of these divalent ions on the utilization of aqueous phosphatidate was also studied (Table 5). At 1 mM concentration, $Zn^{2+}$ and $Cu^{2+}$ caused more than 50% inhibition of microsomal phosphatidate phosphatase. Several of these divalent cations also abolished the stimulatory effect of $Mg^{2+}$ on microsomal and soluble phosphatidate phosphatase. Antagonism of $Mg^{2+}$ effect by these divalent cations was greater at pH 7.5 than at pH 6.8.

## DISCUSSION

In this investigation, phosphatidate phosphatase from adipose tissue was assayed in the presence of both aqueous phosphatidate and membrane-bound phosphatidate as proposed by Hübscher et al. (21). When aqueous phosphatidate was used as substrate, the presence of this enzyme was detected in the particulate and soluble fractions. The major portion of the phosphatidate phosphatase was found in the mitochondria and microsomes. The cytosol fraction contained much lower activity. Similar observations have been reported for liver and intestine (11, 13).

$Mg^{2+}$ had an effect on phosphatidate phosphatase that differed from that of several other divalent ions studied. At lower concentration, $Mg^{2+}$ stimulated the phosphatidate phosphatase from microsomal and soluble fractions. The activation of this enzyme by $Mg^{2+}$ was greater at pH 7.5

TABLE 5. Effect of divalent cations on utilization of aqueous phosphatidate

| Addition | Microsomes | | Soluble Fraction |
|---|---|---|---|
| | pH 6.8 | pH 7.5 | pH 7.5 |
| | *nmoles $P_i$ released/min/mg protein* | | |
| None | 18.2 | 14.6 | 1.2 |
| $Mg^{2+}$ | 30.2 | 32.8 | 8.8 |
| $Co^{2+}$ | 16.1 | | 1.2 |
| $Ni^{2+}$ | 18.6 | | 1.6 |
| $Mn^{2+}$ | 12.6 | | 1.9 |
| $Ca^{2+}$ | 15.6 | | 1.1 |
| $Fe^{2+}$ | 17.6 | | 1.0 |
| $Cu^{2+}$ | 8.2 | | 0.68 |
| $Zn^{2+}$ | 5.2 | | 0.43 |
| NaF (50 mM) | 2.6 | | 0.12 |
| $Mg^{2+} + Zn^{2+}$ | 4.8 | 3.8 | 0.8 |
| $Mg^{2+} + Co^{2+}$ | 30.8 | 5.9 | 2.3 |
| $Mg^{2+} + Ni^{2+}$ | 33.0 | 2.8 | 2.6 |
| $Mg^{2+} + Mn^{2+}$ | 8.6 | 2.9 | 1.8 |

Assay conditions were described in Methods. All divalent cations were added as chloride salts at pH 7.0. The final concentration of each ion was 1 mM. In some experiments, microsomes were assayed at pH 6.8 and 7.5 in the presence of $Mg^{2+}$ and different divalent cations. The soluble enzyme was assayed at pH 7.5.

than at pH 6.8. The mitochondrial enzyme was not stimulated by $Mg^{2+}$. At higher concentration, $Mg^{2+}$ inhibited the phosphatidate phosphatase from all three fractions. The stimulation of this enzyme by $Mg^{2+}$ at low concentration and the inhibition at high concentration have been reported earlier by Sedgwick and Hübscher (8) with rat liver mitochondrial enzyme and by Hokin et al. (6) with erythrocyte phosphatidate phosphatase.

In the presence of EDTA, the $Mg^{2+}$-stimulated enzyme from the microsomal and soluble fractions was completely inhibited. However, EDTA had no effect on the mitochondria and a portion of the microsomal enzyme that was not stimulated by $Mg^{2+}$. These studies indicate that there may be at least two distinct phosphatidate phosphatases in adipose tissues: $Mg^{2+}$-dependent and $Mg^{2+}$-independent. The mitochondrial fraction contained chiefly $Mg^{2+}$-independent enzyme, and the cytosol fraction contained mainly $Mg^{2+}$-dependent enzyme. Both $Mg^{2+}$-dependent and $Mg^{2+}$-independent phosphatidate phosphatases were present in the microsomal fraction. The presence of two distinct phosphatidate phosphatases in rat liver mitochondria (8) and in brain microsomes and erythrocyte membranes has been suggested earlier (6, 22). In later studies, the criterion for distinction of these enzymes was $Mg^{2+}$ dependency. According to Hokin et al. (6), $Mg^{2+}$-dependent phosphatidate phosphatase was specific for the utilization of L-$\alpha$-phosphatidate to form 1,2-diglyceride. The nonspecific substrates such as $\beta$-phosphatidate were utilized by $Mg^{2+}$-independent enzyme. $\beta$-Phosphatidate was synthesized from 1,3-diolein by the action of phosphorus oxychloride in their studies.

As shown in Table 1, the major portion of $Mg^{2+}$-dependent phosphatase measured with aqueous phosphatidate was present in the soluble fraction, and only 20% activity was found in the microsomal fraction. Such distribution could also occur if microsomes were contaminated with soluble fraction or if there were substantial leakage of microsomal enzyme into soluble fraction during the preparation of subcellular fractions, as suggested by Smith et al. (11). If it is assumed that $Mg^{2+}$-dependent phosphatase activity present in the microsomal fraction is entirely due to contamination with soluble fraction, then the rate of 12 nmoles/min/mg protein for $Mg^{2+}$-dependent phosphatase activity in the microsomal fraction indicates that the contamination of soluble fraction in microsomes is more than 100%. This is unlikely because the studies of $\alpha$-glycerophosphate dehydrogenase distribution presented earlier revealed that the contamination of soluble fraction in microsomes was not more than 4% in the present investigation (10). However, it is possible that a partial leakage of microsomal enzyme into the soluble fraction may account for the distribution of $Mg^{2+}$-dependent phosphatidate phosphatase in adipose tissue.

In the absence of $Mg^{2+}$, the rate of utilization of aqueous substrate by the phosphatidate phosphatase of particulate and soluble fractions remained detectable, but the membrane-bound substrate was not utilized under these conditions. In the presence of $Mg^{2+}$, the utilization of aqueous substrate by the microsomal and soluble enzymes was increased further along with the utilization of membrane-bound substrate. Mitochondrial phosphatidate phosphatase was not stimulated by $Mg^{2+}$ and it did not utilize membrane-bound substrate. These studies suggest that $Mg^{2+}$-dependent phosphatidate phosphatase may be responsible for the utilization of membrane-bound substrate and is localized in microsomes and the soluble fraction. This observation is different from that reported earlier for liver and for intestine by Hübscher et al. (21) and Johnston et al. (13). In their studies the membrane-bound substrate was the primary substrate of the phosphatidate phosphatase of the soluble fraction and microsomes had little or no activity with this substrate.

The rates of phosphatidate phosphatase activity calculated from the utilization of aqueous phosphatidate were quite high compared with those obtained from membrane-bound substrate. A similar type of discrepancy has been reported earlier in the studies with liver phosphatidate phosphatase (11). In adipose tissue, the rate of utilization of membrane-bound substrate by the microsomal enzyme was greater than that for the soluble enzyme. The microsomal enzyme was more active on endogenous substrate compared with exogenous substrate. The rate of utilization by the microsomal enzyme was 6.65 nmoles/min/mg protein with endogenous substrate and 3.2 nmoles/min/mg protein with exogenous substrate. With soluble phosphatidate phosphatase, the rate of utilization of membrane-bound substrate was 2.2 nmoles/min/mg protein.

To demonstrate the requirement for $Mg^{2+}$ in utilizing membrane-bound phosphatidate, the substrate should be completely free from $Mg^{2+}$. In these studies, the membrane-bound substrate was prepared in the presence of *sn*-glycerol-3-phosphate and palmitoyl CoA. Under these conditions, the utilization of this substrate by the soluble and microsomal enzymes was completely dependent on $Mg^{2+}$. However, when the substrate was prepared in the presence of *sn*-glycerol-3-phosphate, palmitate, ATP, CoA, NaF, and $Mg^{2+}$, the addition of extra $Mg^{2+}$ was not needed. These results suggest that $Mg^{2+}$ bound to this latter preparation was sufficient to activate the microsomal or soluble enzyme. It is well known that $Mg^{2+}$ is generally present in the protein-bound form in tissues (23).

Recently, Mitchell, Brindley, and Hübscher (24) also reported the partial requirement of $Mg^{2+}$ for soluble phosphatidate phosphatase from liver. However, in their studies, the membrane-bound substrate was prepared in the presence of $Mg^{2+}$, and therefore a high concentration of EDTA was added to demonstrate the requirement for $Mg^{2+}$.

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