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# Hydrolysis of long-chain alkyl phosphates and phosphatidic acid by an enzyme purified from pig brain*†

BERNARD W. AGRANOFF

*Department of Biological Chemistry and Mental Health Research Institute, University of Michigan, Ann Arbor, Michigan*

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## SUMMARY

Particulate preparations of rat and pig tissues have been found to catalyze the hydrolysis of lipid phosphates including many alcohol dihydrogen phosphates as well as phosphatidic acids. A soluble extract possessing these enzymatic activities has been prepared from a dried acetone extract of pig brain, and its properties are described. Evidence presented suggests that the enzyme catalyzing the hydrolysis of the alcohol dihydrogen phosphates studied here is identical with phosphatidic acid phosphatase.

As a result of early studies on lipid biosynthesis, Kornberg and Pricer found that acyl-CoA[1] derivatives of fatty acids reacted with α-glycerophosphate to form phosphatidic acid (1). Later, α,β-diglyceride was found to participate in a reaction with cytidine diphosphate-ethanolamine or with cytidine diphosphate-choline to form the respective phosphatides and cytidine monophosphate (2). Phosphatidic acid did not serve as substrate in this reaction. Continuity of the biosynthetic pathway for the formation of these phosphatides requires the existence of a "phosphatidic acid phosphatase." Such an enzymatic activity was described by Smith *et al.* (3) in studies with chicken-liver microsomal fraction. This preparation catalyzed the conversion of phosphatidic acid to α-β-diglyceride and inorganic phosphate and was inhibited by $Mg^{++}$ and by Tween 20,[2] a nonionic detergent used for suspension of lipid substrates. Other reports have since described the enzymatic cleavage of phosphatidic acid (4, 5, 6), and a $Mg^{++}$-dependent enzyme has been reported (7).

Since natural and synthetic phosphatidic acids are highly labile, in the present study a series of model compounds (the dihydrogen phosphates of various long-chain alcohols) were prepared. Enzymatic activity for their hydrolysis was detected in tissue preparations and was ultimately obtained in a soluble form. The soluble enzyme preparation also catalyzed the release of inorganic phosphate from phosphatidic acid prepared from egg lecithin. These activities appear to derive from the same enzyme.

### MATERIALS AND METHODS

*Preparation of Substrates.* Hexadecyl dihydrogen phosphate and octadecyl dihydrogen phosphate were synthesized by condensation of the free alcohols with diphenylphosphorochloridate followed by catalytic hydrogenation of the resulting monoalkyl diphenylester to yield the desired compound. Docosanyl dihydrogen phosphate was prepared by a similar procedure. Other mono- and dialkyl phosphates were the gift of the Victor Chemical Company, Chicago, Illinois. The DL-dipalmitoyl phosphatidic acid was synthesized by the method of Baer (9), using as starting material DL-diacetone glycerol obtained from Aldrich Chemical

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† A preliminary report of this work was presented at the Vth International Biochemical Congress, August 10–16, 1961 (Moscow).

[1] The following abbreviations are used: CoA, coenzyme A; Tris, tris(hydroxymethyl)aminomethane; TCA, trichloroacetic acid; EDTA, ethylenediamine tetraacetic acid; PHMB, p-hydroxymercuribenzoate; $P_i$, inorganic phosphate.

[2] Obtained from Atlas Powder Company, Wilmington, Delaware.



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Company, Milwaukee, Wisconsin. The final product traveled as a single spot in a chromatographic solvent containing diisobutyl ketone—acetic acid—water 40:20:3 (10) and had an ester/P ratio of 2.0:1.0. Crude egg lecithin was prepared according to Hanahan (11), but silicic acid[3] rather than alumina was used for purification. One gram of lecithin obtained by acetone precipitation was applied in petroleum ether to a column containing 31 g of silicic acid. Elution was carried out consecutively with 300 ml of chloroform, 250 ml of chloroform—methanol 4:1, and 250 ml of chloroform—methanol 1:1. The latter fraction was concentrated to a small volume and lyophilized from benzene. Six hundred milligrams of a white fluffy material was obtained that contained less than 0.05% amino nitrogen. The ester/P ratio was 2.1:1.0. This material was converted to phosphatidic acid by incubation with carrot chromoplasts (12). The product was neutralized with NaOH and was purified by repeated precipitation with acetone. The ester/P ratio was 2.0:1.0. The product was easily suspended in water as a milky emulsion (22 to 26 μmoles/ml), while the synthetic dipalmitoyl phosphatidic acid was suspended with great difficulty even in the presence of 0.5% bovine serum albumin.

The higher alcohol phosphates were neutralized with 1 N $NH_4OH$ in an aqueous suspension containing 35 to 40 μmoles of substrate per ml. The suspension was then heated, usually at 70° to 80°, until only a slight (if any) turbidity persisted. The mixture was then cooled to about 45° and was diluted with an equal volume of 1% bovine serum albumin. This final suspension could be stored indefinitely and was heated until clear just prior to use. A Vortex mixer[4] was found useful for these operations. Such mixtures showed no evidence of coagulation of the protein or degradation of the alkyl phosphate after repeated heating to 70° to 80°.

*Analytical Methods.* Lipid esters were determined by reaction with hydroxylamine (13). Phosphate was determined by the method of Fiske and Subbarow (14). Protein in particulate preparations was determined by the biuret method and in soluble fractions by ultraviolet absorption (15). Amino nitrogen was determined by the method of Lea and Rhodes (16). Spectrophotometric measurements were made in a Beckman DU spectrophotometer, unless otherwise noted.

*Gas-Liquid Chromatography of Product.* Gas-liquid chromatography was performed in an Aerograph Model A-90-ACS[5] by using a stainless steel column, 80 in. × 1/4 in. o.d., containing 9% SE-30 in acid-washed Chromosorb W,[6] 60–80 mesh.

[3] Bio-Rad Laboratories, Richmond, California.
[4] Scientific Industries, Inc., Springfield, Massachusetts.
[5] Wilkens Instrument and Research, Walnut Creek, California.

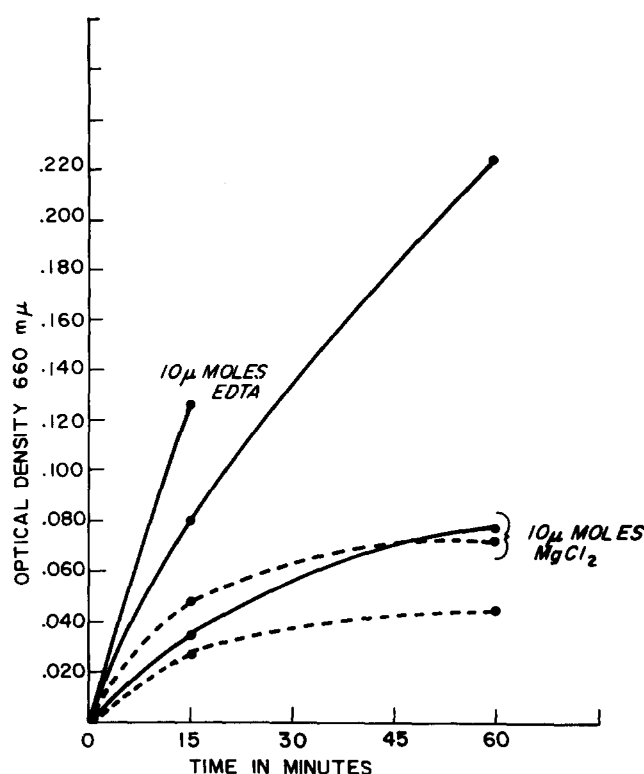


FIG. 1. Release of phosphate from hexadecyl phosphate by rat-liver homogenate. See text for incubation conditions. Solid lines represent results of incubations in presence of substrate. Dotted line represents endogenous phosphate release. Optical densities in this instance were obtained in a Coleman Universal Spectrophotometer with 5-cm microcells. Under these conditions, 1 μmole of phosphate has an optical density of 1.860.

## RESULTS

*Enzymatic Hydrolysis of Hexadecyl Phosphate.* An adult male rat was decapitated, and the liver was homogenized with a Tenbroeck homogenizer in 4 volumes of 0.25 M sucrose. Following centrifugation at 500 × *g*, the preparation was dialyzed at 2° for 24 hours and tested for activity immediately thereafter. Incubation mixtures contained 0.8 ml of enzyme, 1.4 μmoles of hexadecyl phosphate, 50 μmoles of Tris-HCl buffer, pH 8.1, and additions as noted (Fig. 1) in a total volume of 1.0 ml. Aliquots of 0.2 ml were withdrawn after specified intervals of incubation at 37° in air in a shaking water bath and analyzed for the appearance of inorganic phosphate. It can be seen that the addition of substrate results in the appearance of inorganic phosphate and also that the presence of $Mg^{++}$ appears to inhibit the hydrolysis of added substrate but that it

[6] Johns-Manville Corp., New York, N. Y.

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TABLE 1. Hydrolysis of Dodecyl Phosphate by Fractions Derived from Acetone Powder of Pig Brain*

| | Fraction | Volume | Total Protein | Total Activity | Specific Activity |
|---|---|---|---|---|---|
| | | *ml* | *mg* | *units*† | *units/mg* |
| I | Acetone powder supernatant | 273 | 2840 | 229 | 0.08 |
| II | $(NH_4)_2SO_4$, 30–65% satd. | 86 | 1316 | 190 | 0.14 |
| III | Alumina $C_\gamma$ supernatant | 129 | 845 | 140 | 0.17 |
| IV | $(NH_4)_2SO_4$, 40–60% | 60 | 321 | 106 | 0.33 |

* The incubation mixtures contained 0.4 to 1.2 ml of enzyme; 100 μmoles of Tris buffer, pH 8.1; 20 μmoles of EDTA; 4 μmoles of substrate (in 0.2 ml of 0.5% albumin); all in a total volume of 2 ml. Aliquots of 0.4 ml were added to 2 ml of 10% TCA at zero time and after 10, 20, and 30 minutes of incubation. Phosphate determinations on the filtrates were used to calculate initial rates.

† A unit is defined as the amount of enzyme that hydrolyzes 1 μmole of substrate per hour at 37°. The substrate used for the purification was dodecyl phosphate.

actually stimulates endogenous phosphate release. Homogenates of rat organs tested in this manner had the following specific activities (μmoles $P_i$ released per hour per mg protein) when the hexadecyl phosphate was the substrate: brain, 0.57; kidney, 0.55; spleen, 0.44; liver, 0.34; intestine (blendor), 0.28.

The substrate was found to be resistant to acid and alkaline hydrolysis. Refluxing in 2 N KOH or $H_2SO_4$ in 50% aqueous methanol for several days released no inorganic phosphate.

*Fractionation of Enzymatic Activity.* To obtain large amounts of tissue, pig brain was examined and found to possess enzymatic activity, although the specific activity of homogenates was lower than that observed with rat brain. Differential centrifugation (17) revealed that all of the activity was particulate and that the highest specific activity was in a fraction which sedimented at 108,000 × *g*. The activity could be released into the supernatant fraction by prior ultrasonic treatment or by treatment with the non-ionic detergent, Cutscum[7] (18, 19), but attempts at further fractionation of such "soluble" preparations resulted in sedimentation or loss of the active material. Tween 20 was found to be inhibitory (3). Acetone powders of fresh or frozen pig brain retained all of the enzymatic activity, but in a particulate form. However, about 10% of this activity could be rendered soluble by aqueous extraction of the acetone powder at room temperature, and further purification by conventional methods was possible.

One hundred grams of fresh or frozen pig brain was added in 1- to 3-cm chunks to 1000 ml of acetone at −15° and immediately homogenized in a Waring blendor for 30 seconds. The mixture was filtered with suction and washed with 1000 ml of diethyl ether at −15° and the residue was dried *in vacuo*. The powder (19 to 20 g) was dispersed in the blendor with 190 ml of 0.05 M Tris buffer, pH 8.1. An equal volume of Tris buffer was then added, and the mixture was allowed to stand at room temperature for 13 to 16 hours. The preparation was then cooled to 2° and centrifuged at 78,000 × *g* for 30 minutes. The clear supernatant fraction was brought to 30% saturation with solid ammonium sulfate (20) at 2°, and the precipitate obtained following centrifugation was discarded. The material that sedimented after further addition of ammonium sulfate to 65% saturation was immediately taken up in 60 ml of 0.05 M Tris buffer, pH 8.1. The enzymatic activity appears to be quite stable at this point, and this preparation can be stored for several days at 2° or longer in the frozen state without loss of activity. Further purification may be obtained by bringing the material to pH 5.3 with 1 N acetic acid and then adding alumina $C_\gamma$, 0.5 g per g of protein. The mixture is sedimented by centrifugation, and the supernatant material is brought to pH 8.6 with 0.5 M Tris buffer. A second ammonium sulfate fractionation is performed, and the enzymatic activity is found to be in material sedimenting between 40% and 60% saturation. The residue is dissolved in a small volume of 0.05 M Tris buffer, pH 8.1. The final product will hydrolyze 0.2 to 0.5 μmoles of dodecyl phosphate per hour per mg protein. While the specific activity is less than that obtained from microsomal fractions, the preparation represents a 4-fold purification of the soluble extract obtained from acetone powders of whole brain. This preparation is referred to as soluble enzyme (this fractionation is summarized in Table 1). Attempts at purification on DEAE cellulose were unsuccessful.

*Isolation of Product.* A tube containing 8.7 μmoles of hexadecyl phosphate suspended in albumin, 250 μmoles of Tris buffer, pH 8.1, 50 μmoles of EDTA and 3 ml of purified enzyme in a total volume of 5.0 ml was incubated at 37° for 2 hours. Total hydrolysis of the substrate was established by assay of aliquots at 20-minute intervals. The remaining mixture (4.2 ml) was shaken with 80 ml of chloroform—methanol 2:1 and filtered, and the residue was washed with an additional 20 ml of solvent. The combined extracts

[7] Obtained from Fisher Scientific Company, Fair Lawn, New Jersey.

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TABLE 2. Activity of the Soluble-Enzyme on Various Lipid Phosphates*

| Substrate | Rate of Hydrolysis |
|---|---|
| | *μmoles/hr* |
| Methyl phosphate | <0.05 |
| Ethyl phosphate | 0.14 |
| Butyl phosphate | 1.1 |
| Hexyl phosphate | 2.1 |
| Octyl phosphate | 2.3 |
| Decyl phosphate | 2.6 |
| Dodecyl phosphate | 2.4 |
| Hexadecyl phosphate | 2.2 |
| Octadecyl phosphate | 1.8 |
| Docosanyl phosphate† | 1.1 |
| Isopropyl phosphate | 0.12 |
| Isoamyl phosphate | 2.3 |
| Dibutyl phosphate | 0 |
| Dioctyl phosphate | 0 |
| Synthetic dipalmitoyl phosphatidic acid | 0 |
| Enzymatically prepared phosphatidic acid, Batch 1† | 1.6 |
| Enzymatically prepared phosphatidic acid, Batch 2† | 0.96 |

* Incubation conditions were those described in Table 1. 1.2 ml of soluble enzyme was used.
† Calculated from other experiments.

TABLE 3. Effect of $Mg^{++}$ and of Heating on Soluble Phosphatase Activity*

| Experiment | Substrate | Addition | Phosphate Released |
|---|---|---|---|
| | | | *μmoles/hr* |
| 1 | 1.4 μmoles hexadecyl phosphate | 20 μmoles $MgCl_2$ | 0.07 |
| | 1.4 μmoles hexadecyl phosphate | 20 μmoles EDTA | 0.47 |
| | 10 μmoles α-glycerophosphate | 20 μmoles $MgCl_2$ | 0.42 |
| | 10 μmoles α-glycerophosphate | 20 μmoles EDTA | 0.08 |
| 2† | 1.4 μmoles hexadecyl phosphate | 20 μmoles EDTA | 0.09 |
| | 10 μmoles α-glycerophosphate | 20 μmoles $MgCl_2$ | 0.36 |

* Incubation conditions were as indicated in Table 1, except that EDTA was replaced by $Mg^{++}$ as indicated.
† Enzyme heated to 80° for 2 minutes.

were taken to dryness, then taken up in chloroform, washed with 1 N $H_2SO_4$ twice, with 0.1 M KOH once, and then with water. The lipid phase was dried and extracted with ether, and the ether-soluble material was dried, dissolved in toluene, and subjected to gas-liquid chromatography. A single major peak corresponding to that obtained with authentic hexadecyl alcohol was observed and by integration of the curve was found to correspond to 83% to 87% of the expected amount. The material corresponding to hexadecyl alcohol was collected from the column (21) and found to have a melting point of 48° corresponding to that observed with hexadecyl alcohol.

*Substrate Specificity Studies.* The soluble enzyme was incubated with various substrates (Table 2). Maximal activity was observed with dihydrogen phosphates of alcohols having 6 to 18 carbon atoms. Synthetic dipalmitoyl phosphatidic acid was inactive, and the material produced by the action of carrot chromoplasts on egg lecithin was hydrolyzed at a somewhat lower rate than that observed with many alkyl phosphates. Dialkyl hydrogen phosphates were not hydrolyzed.

*Effect of Varying Substrate Concentration.* The rates reported in Table 2 were found to be maximal. Under the same incubation conditions, Michaelis constants were established for hexadecyl phosphate ($K_m = 8 \times 10^{-4}$ M), for octadecyl phosphate ($4.8 \times 10^{-4}$ M), for dodecyl phosphate ($6.7 \times 10^{-4}$ M), for enzymatically prepared phosphatidic acid ($8 \times 10^{-4}$ M), and for α-glycerophosphate ($1.8 \times 10^{-2}$ M). The latter was determined under identical incubation conditions except that EDTA was omitted, and 20 μmoles of $MgCl_2$ was added (see below).

*The Effect of $Mg^{++}$.* As previously noted with homogenates, $Mg^{++}$ inhibited hydrolysis of hexadecyl phosphate with the soluble enzyme (Table 3). When α-glycerophosphate was added as substrate, the opposite effect was noted; *i.e.*, $Mg^{++}$ was required for activity. If the enzyme were heated prior to incubation, the $Mg^{++}$-activated α-glycerophosphate hydrolysis was reduced by 15% while the hydrolysis of hexadecyl phosphate in the presence of EDTA was almost abolished. The effect of varying concentrations of $Mg^{++}$ on the hydrolysis of dodecyl phosphate is illustrated by Figure 2. Similar inhibition was obtained with $Ca^{++}$ and $Mn^{++}$.

*Phosphatase Activity of Brain Fractions.* In order to clarify the nature of the several activities observed, various fractions of homogenates of pig brain were reinvestigated with 3 model substrates: dodecyl phosphate, the enzymatically prepared phosphatidic acid, and α-glycerophosphate in the presence of $Mg^{++}$. The results are seen in Table 4.

*Effect of Various Agents on Enzyme Activity.* Iodoacetamide, $10^{-3}$ M, and NaF, $4 \times 10^{-2}$ M, were ineffective as inhibitors of enzymatic hydrolysis of either phosphatidic acid or dodecyl phosphate. However,

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TABLE 4. DISTRIBUTION OF PHOSPHATASE ACTIVITY AGAINST THREE SUBSTRATES IN FRACTIONS OF PIG BRAIN*

| Fraction | Total Protein | Total Activity in μmoles/hr Hydrolyzed: A. Dodecyl Phosphate | B. Phosphatidic Acid | C. α-Glycerophosphate | A/B |
|---|---|---|---|---|---|
| | *mg* | | | | |
| Dialyzed homogenate | 920 | 317 | 188 | 52† | 1.7 |
| Residue, 500 × *g* | 42 | 21 | 15 | 73 | 1.4 |
| Residue, 11,000 × *g* | 540 | 193 | 129 | 51 | 1.5 |
| Residue, 108,000 × *g* | 100 | 97 | 44 | 25 | 2.2 |
| Supernatant solution, 108,000 × *g* | 160 | 0 | 0 | 16 | ... |

* 10 g of fresh pig brain was homogenized in 4 volumes of 0.25M sucrose, and particulate fractions were obtained sequentially by differential centrifugation. Samples were assayed as in Table 1. α-Glycerophosphate incubation contained 20 μmoles of $MgCl_2$ instead of 20 μmoles of EDTA.

† The sum of the fractions consistently contained more α-glycerophosphatase activity than was found in the homogenate.

PHMB[8] at concentrations of $10^{-3}$ M consistently caused a 27% to 35% inhibition of hydrolysis of either phosphatidic acid or dodecyl phosphate by the soluble enzyme. Similar values were obtained when microsomal fraction was used as the source of enzyme. Significant but incomplete reactivation could be obtained by addition of glutathione. This slight amount of inhibition at high levels of PHMB and the only partial reactivation are not typical of a sulfhydryl enzyme site.

Activity was diminished by one-half upon the addition of *n*-octanol. Dimethylformamide and tetrahydrofuran were both inhibitory when present at a final concentration of 30% (v/v). The enzymatic hydrolysis was not altered in the presence of 0.1 M KCl or 0.1 M NaCl. Elevation of the incubation temperature from 37° to 45° caused an increase in specific activity of the microsomal fraction preparation, but not of the soluble enzyme.

*pH Optimum.* The rate of enzymatic hydrolysis of enzymatically prepared phosphatidic acid with the soluble enzyme and with the microsomal fraction is shown in Figure 3. Similar results were obtained with dodecyl phosphate.

*Partial Heat Inactivation.* In order to better establish the identity of the hydrolytic activities for

[8] Obtained from Sigma Chemical Company, St. Louis, Missouri.

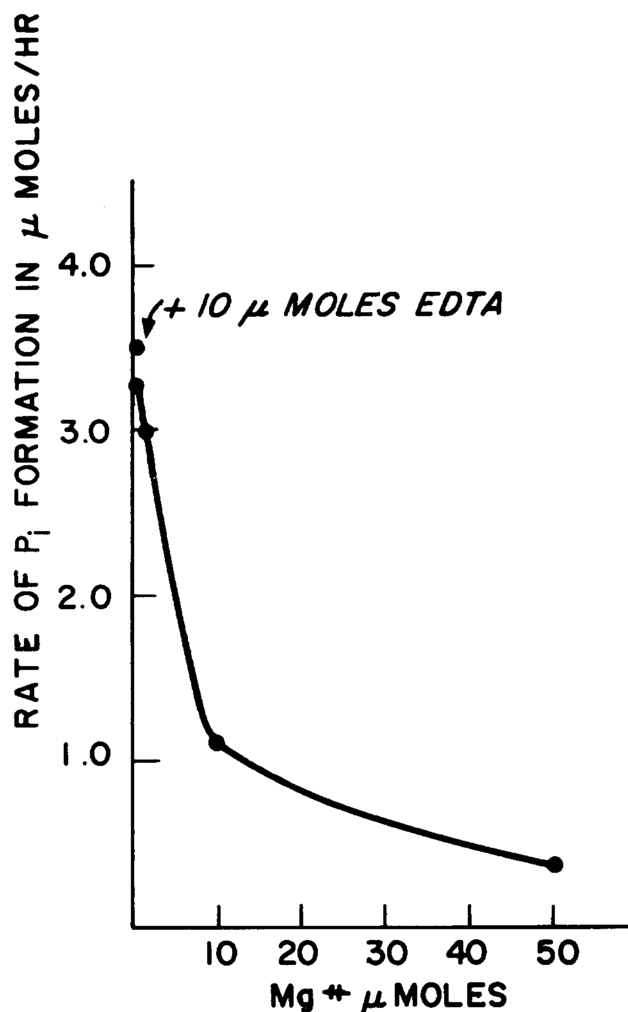


FIG. 2. Effect of $Mg^{++}$ on enzymatic hydrolysis of dodecyl phosphate. Incubation conditions were as described for Table 1, using 1.2 ml of purified enzyme.

phosphatidic acid and for the alkyl phosphates, both the microsomal fraction and the soluble enzyme were assayed following varying amounts of heating. As can be seen from Figure 4, the rate of inactivation in respect to the two substrates appears to be the same. The microsomal fraction is more heat-stable than the soluble preparation.

## DISCUSSION

The investigation of lipid phosphatases is made difficult by the insolubility of both substrate and enzyme. The present investigation was initiated with the alkyl phosphates as substrates. While only a tenth of the activity in brain homogenates was solubilized by autolysis, the fact that subsequent purification was possible favored this method over other

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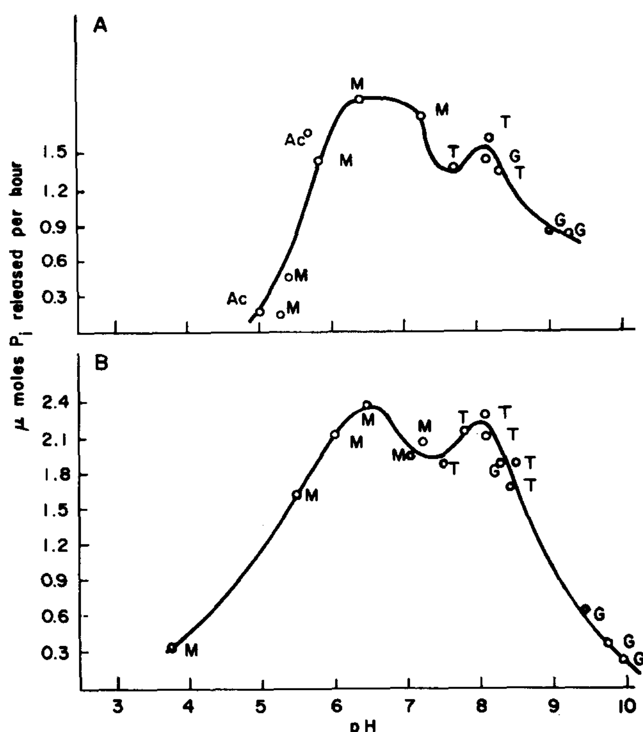


FIG. 3. pH optimum for hydrolysis of phosphatidic acid. Incubation mixtures contained 450 μmoles of buffer, 7.8 μmoles of enzymatically prepared phosphatidic acid, 30 μmoles of EDTA, and 1.5 ml of enzyme in a total volume of 3.0 ml. pH was measured with microelectrodes at the beginning and end of the incubations. Samples were withdrawn at zero time, 10, 20, and 30 minutes, and initial rates were obtained from the data graphically. (A) Soluble enzyme. (B) Washed pig-brain microsomal fraction. Buffers: Ac, K-acetate; M, K-maleate; T, Tris-HCl; G, K-glycinate.

techniques. The "purified" enzyme did not have as high a specific activity as the particulate microsomal fraction. This fact supports the hypothesis that structural integrity of the particles is required for full enzymatic activity (5). Comparison of properties of the soluble preparation with those of the microsomal fraction indicates that the hydrolytic activity is due to the same enzyme. The pH optimum, the inhibition by $Mg^{++}$, and the heating experiments also support this assumption as well as the inference that the same enzyme probably acts on both phosphatidic acid and the higher alcohol phosphates. The inhibition by $Mg^{++}$ has been previously reported (3, 4, 7) although a $Mg^{++}$-dependent phosphatidic acid phosphatase has also been reported (6). The concomitant presence of α-glycerophosphatase activity could explain such results if phosphatidic acid were degraded in preparation of the substrate or by a deacylase present during the incubation. In this study, results of incubation of phosphatidic acid preparations with $Mg^{++}$ served as an indication of the state of preservation of substrate and correlated well with ester group estimations. Both phosphatidic acid preparations in Table 2 were over 90% pure by ester group and phosphate determinations. Nevertheless, there was a variation in the activity of the enzyme on these substrates. For this reason, the relative rates of breakdown of phosphatidic acid and the affinity constants for it must be considered tentative. Furthermore, the phosphatidic acid used is undoubtedly not the natural substrate. The latter could have a considerably different fatty acid composition. An extreme case of the effect of acyl groups is the inactivity of dipalmitoyl phosphatidic acid observed here and of distearoyl phosphatidic acid (6). A factor that could determine an upper limit of chain length

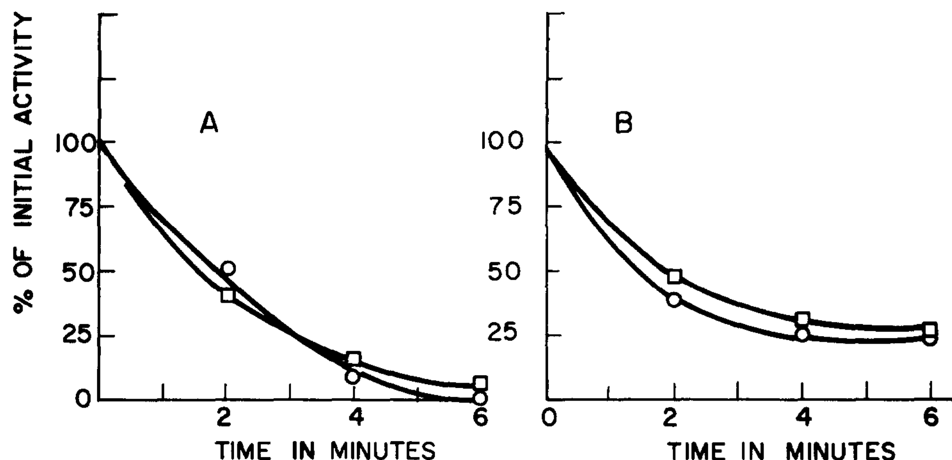


FIG. 4. Effect of heating on enzymatic activity. (A) Purified enzyme, after heating to 60° for times specified. (B) Brain microsomal fraction after heating at 70°. □, Dodecyl phosphate; ○, phosphatidic acid. Incubation conditions were those described in Table 1. Serum albumin (0.1 ml of a 1% solution) was added to incubation mixtures just before addition of TCA. This facilitated formation of a clear supernatant solution.

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for enzyme activity is the solubility of substrate. The second peak of enzyme activity observed at pH 8.1 may be due to the increased solubility of substrate, since at higher pH the substrates form visibly clearer suspensions than at pH 6–7.

The significance of fatty acid specificity of the enzyme could be great in that this phosphatase could determine the fatty acid composition of lipids derived from phosphatidic acid on the one hand and $\alpha,\beta$-diglyceride on the other. The former would include phosphatidyl inositol, phosphatidyl glycerol, and possibly triphosphoinositide and bis-phosphatidic acid. The latter category would include phosphatidyl choline, phosphatidyl ethanolamine, triglyceride, and possibly phosphatidyl serine.

The broad specificity of the enzyme suggests that it is a monoesterase for lipid phosphates. The data of Table 3 indicate that the preparation contains a contaminating heat-stable, $Mg^{++}$-dependent alkaline phosphatase.

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