Specificities of Alkaline and Acid Phosphatases in the Dephosphorylation of Phospholipids*

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ABSTRACT: The data in this paper describe the specificities of alkaline (EC 3.1.3.1) and acid (EC 3.1.3.2) phosphatases for lipid substrates with free phosphate moieties, *i.e.*, 1-acylglycerol-3-P, diacylglycerol-3-P, 1-*O*-alkylglycerol-3-P, 1-*O*-alkyl-2-acylglycerol-3-P, and 1-*O*-alkyldihydroxyacetone-P. Bacterial alkaline phosphatase removed the phosphate moiety from the acyl and *O*-alkyl substrates that do not contain aliphatic moieties in the 2 position. In contrast, the acid phosphatase from wheat germ showed no specificity in the removal of free phosphate groups from the lipid substrates. Substrates containing ether bonds reacted to a lesser extent than the corresponding analogs containing ester bonds. We have shown that alkaline phosphatase is an effective analytical tool in the lipid field.

The possible role of alkaline and acid phosphatases in lipid metabolism is discussed.

Dephosphorylation is an important step in the absolute identification of phospholipids. Generally, acidic or alkaline hydrolysis can be used successfully, but problems are encountered with poor yields and the formation of cyclic or unidentified compounds. Furthermore, the instability of *O*-alkyldihydroxyacetone-P, an intermediate in the biosynthesis of *O*-alkyl lipids, to acidic and alkaline hydrolysis made it necessary to search for other methods of dephosphorylation (Snyder *et al.*, 1970a,b; Wykle and Snyder, 1970).

Heppel and coworkers (1962) were the first to demonstrate the usefulness of alkaline phosphatase as an analytical tool in the nucleic acid field, yet its application in the area of lipid analysis has not previously been reported. Long *et al.* (1967) have used the supernatant of a homogenate of human prostate glands as a source of acid phosphatase activity to remove the phosphate moiety from 1-acylglycerol-3-P, but they did not examine its substrate specificity. Our success with alkaline phosphatase (EC 3.1.3.1) from bacteria in the identification of *O*-alkyldihydroxyacetone-P (Snyder *et al.*, 1970a; Wykle and Snyder, 1970) prompted this investigation of the specificity of alkaline and acid (EC 3.1.3.2) phosphatases for lipid substrates that contain free phosphate groups.

Methods

Source of Enzymes. The acid phosphatase and bacterial alkaline phosphatase were purchased from Worthington Biochemical Corp., Freehold, N. J. The alkaline phosphatase, isolated from an *Escherichia coli* mutant, contained 30 units/mg of protein; the acid phosphatase, isolated from wheat germ, contained 0.21 unit/mg of protein. Both activities were based on measurements using *O*-carboxyphenyl phosphate as the substrate. Phospholipase D, from cabbage, was purchased from General Biochemicals, and phospholipase A, in the form of lyophilized venom from *Crotolus adamanteus*, was purchased from Ross Allen's Reptile Institute.

Preparation and Isolation of Substrates. PHOSPHATIDYLCHOLINE, DIACYLGLYCEROL-3-P, 1-ACYLGLYCEROL-3-P, [1-^{14}C]-*O*-ALKYLDIHYDROXYACETONE-P, [1-^{14}C]*O*-ALKYLACYLGLYCEROL-3-P, and [1-^{14}C]*O*-ALKYLGLYCEROL-3-P. Phosphatidylcholine was isolated from the lipids of beef liver by a combination of DEAE-cellulose and ammonium silicate column chromatography as described by Rouser *et al.* (1966). The preparation was pure as judged by thin-layer chromatography on silica gel HRB in acidic (chloroform–methanol–acetic acid–water, 50:25:8:4, v/v) and basic (chloroform–methanol–NH_4OH, 65:35:8, v/v) solvent systems. These two solvent systems were used throughout this work as a means to check the purity of the lipid substrates. A portion of the purified phosphatidylcholine was treated with phospholipase D to prepare diacylglycerol-3-P similar to the procedure described by Long *et al.* (1967). The incubation mixture was acidified with 1 N HCl and extracted with chloroform–methanol (Bligh and Dyer, 1959). The diacylglycerol-3-P was isolated from the total lipid extract by thin-layer chromatography in the acidic solvent system described earlier.

A portion of the diacylglycerol-3-P (10 mg) was incubated with phospholipase A to produce 1-acylglycerol-3-P (Okuyama and Nojima, 1965). The resulting 1-acylglycerol-3-P was isolated by preparative chromatography in the acidic solvent system.

The [1-^{14}C]*O*-alkylacylglycerol-3-P and the [1-^{14}C]*O*-alkyldihydroxyacetone-P were synthesized from [1-^{14}C]hexadecanol, dihydroxyacetone-P, CoA, ATP, and Mg^{2+} with the use of a microsomal system from mouse preputial gland tumors and identified as previously described (Snyder *et al.*, 1970a,b; Wykle and Snyder, 1970). 1-*O*-Alkylglycerol-3-P was prepared by mild saponification of the *O*-alkylacylglycerol-P essentially according to the procedures of Dawson (1960). These lipid substrates were radioassayed by zonal scanning (Snyder and Kimble, 1965) of thin-layer chromatograms developed in the solvent systems described in the previous section. All three preparations were greater than 90% pure.

Phosphatase Incubations and Assay. The incubations with the

* From the Medical Division, Oak Ridge Associated Universities, under contract with the U. S. Atomic Energy Commission, Oak Ridge, Tennessee 37830. *Received June 24, 1970.* This work was supported in part by the American Cancer Society Grant No. E-596A.

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acid phosphatase were carried out essentially according to the procedures of Long *et al.* (1967); 6.0 units of enzyme and the substrate (see Table I for approximate quantities) in 3 ml of the 0.2 M acetate buffer (pH 5.9) were used. The incubations with the alkaline phosphatase were carried out in 0.05 M borate–borax buffer (pH 8.2) under conditions similar to those Wykle and Snyder (1970) described. All incubations were carried out for 1 hr with the same quantities of substrates as those used for the acid phosphatase experiments. Control samples containing no enzyme were incubated under identical conditions in all experiments. In one experiment, the effect of nonenzymic protein on alkaline phosphatase specificity was determined by adding 27 mg of crystalline bovine albumin (Sigma Chemical Co., St. Louis, Mo.) to each incubation vial containing *O*-alkylacylglycerol-P, diacylglycerol-P, or *O*-alkylglycerol-P as substrates. Control samples were incubated in the presence and absence of albumin. The products were extracted from the reaction mixture by the procedures of Bligh and Dyer (1959) and the upper phase was extracted a second time with chloroform.

TABLE I: Dephosphorylation of Phospholipids by Alkaline and Acid Phosphatases.

Substrate	% Dephosphorylated in 1 hr: Alkaline Phosphatase	% Dephosphorylated in 1 hr: Acid Phosphatase
Diacylglycerol-3-P (20 μg of P)	0	35
1-Alkyl-2-acylglycerol-3-P[a] (15 nmoles)	1.2	11
1-Acylglycerol-3-P (30 μg of P)	87	65
1-Alkylglycerol-3-P[a] (15 nmoles)	63	47
1-Alkyldihydroxyacetone-P[a] (30 nmoles)	49	12
Phosphatidylcholine (40 μg of P)	0	0

[a] The number 1 carbon atom of the *O*-alkyl moiety was labeled with carbon-14. The values in parentheses represent the approximate quantities of substrates added to each incubation vial. The nanomoles of ^{14}C-labeled compounds were calculated from data obtained from the enzymic systems used to synthesize these substrates.

The reaction products from the phosphatase incubations containing the ^{14}C-labeled substrates were analyzed by thin-layer radiochromatography (Snyder and Kimble, 1965). The products derived from the 1-*O*-alkyl-2-acylglycerol-3-P and the 1-*O*-alkylglycerol-3-P were separated on silica gel G layers in a solvent system of diethyl ether saturated with water, and the products derived from the *O*-alkyldihydroxyacetone-P were separated in hexane–diethyl ether–methanol–acetic acid (70:30:5:1, v/v). The extent of the reactions was determined from comparisons of products formed in the presence and absence of the enzyme. Little alteration of the substrates occurred in the control incubations in either buffer system during the 1-hr incubations.

The products derived from unlabeled phosphatidylcholine, diacylglycerol-3-P, and 1-acylglycerol-3-P were first qualitatively checked by thin-layer chromatography in the diethyl ether–water solvent system. The lipids were visualized by H_2SO_4 charring at 180° for 25 min. If no carbon deposits were seen in monoglyceride or diglyceride areas (*i.e.*, all material remained at the origin), we assumed that no reaction took place. If reaction products were seen by the qualitative check, we measured the extent of the dephosphorylation by determining the quantity of phosphorus (Rouser *et al.*, 1966) in the unreacted substrate from an aliquot of the chloroform layer of the Bligh and Dyer extract obtained at the end of an incubation.

Results and Discussion

Table I shows the extent of dephosphorylation of the substrates by the acid and alkaline phosphatases. The bacterial alkaline phosphatase removed the phosphate group from the substrates containing only one long-chain aliphatic moiety attached to the 1 position of glycerol. The acid phosphatase, however, dephosphorylated the 1-*O*-alkyl-2-acylglycerol-3-P and diacylglycerol-3-P to some extent. The dephosphorylation of these compounds was not caused by phospholipase C since phosphatidylcholine was not attacked by acid phosphatase; phospholipase A activity was also absent from the phosphatase. Schmidt and coworkers (1951) have previously noted the inability of acid phosphatase from prostate glands to attack the phosphodiester grouping of phosphoglycerides, glycerylphosphorylcholine, and glycerylphosphorylethanolamine.

Products of the control and phosphatase reactions are illustrated by the ^{14}C-zonal profile scans of thin-layer chromatograms shown in Figure 1; the substrates depicted are [1-^{14}C]-*O*-alkylglycerol-3-P (part A) and [1-^{14}C]*O*-alkyldihydroxyacetone-P (part B). The unreacted substrate remains at the origin and the dephosphorylated products, *O*-alkylglycerol and *O*-alkyldihydroxyacetone, have R_F values of approximately 0.4 in the chromatography system used. The 1-acylglycerols produced by the phosphatases had the same R_F as the *O*-alkylglycerols in the diethyl ether–water solvent system; the diacylglycerols produced by the acid phosphatase had an R_F of 0.8.

Acid phosphatase released small quantities of free fatty acids from the acyl-containing substrates except phosphatidylcholine. These data indicate that the preparation was contaminated with a lipase that reacted with the acylglycerols. The acid phosphatase also produced relatively large amounts (31%) of fatty alcohols when incubated with the *O*-alkyldihydroxyacetone-P. The instability of this keto–lipid to acidic conditions might account for these results. In contrast, the bacterial alkaline phosphatase attacked only the phosphate moiety and dephosphorylated all three lyso substrates to a greater extent than did the acid phosphatase. However, the presence of *O*-alkyl groups in the substrates decreased the extent of dephosphorylation by both phosphatase preparations. This is similar to the slow reaction rates of phospholipases observed when ether moieties are substituted for ester moieties (see review by Snyder, 1969).

Although the specific activities of the two phosphatase preparations used in these experiments differed, this did not account for the differences observed in the specificity of the alkaline phosphatase for the substrates. The effect of non-

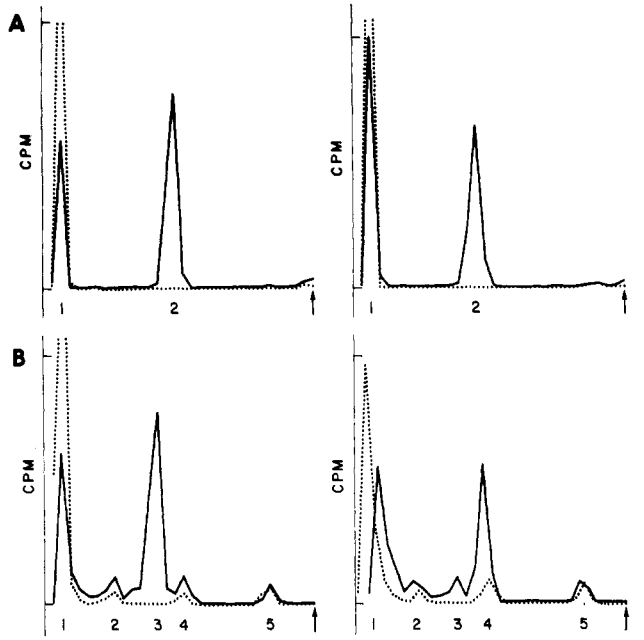
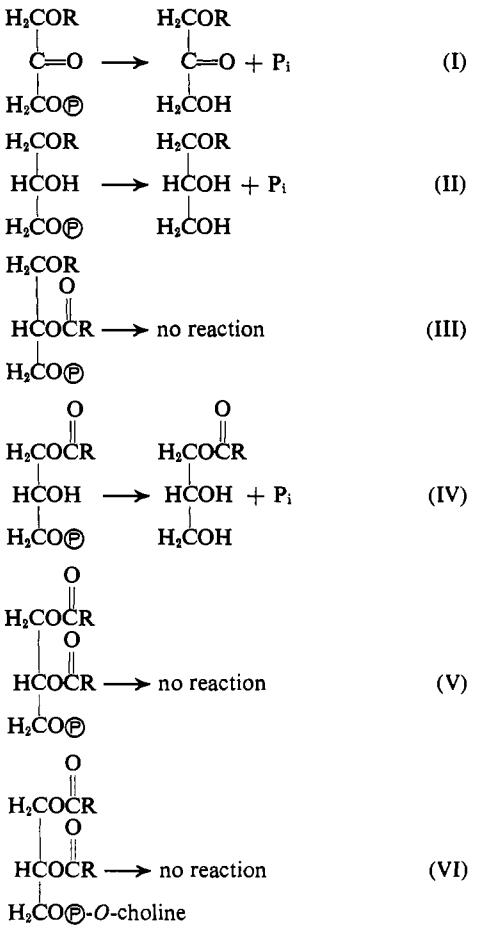


FIGURE 1: Illustration of ^{14}C-zonal profile scans obtained when [1-^{14}C]*O*-alkylglycerol-3-P (part A) or [1-^{14}C]*O*-alkyldihydroxyacetone-P (part B) was used as a substrate for alkaline phosphatase (scans on left side) and acid phosphatase (scans on right side). The dotted line represents the results from control samples and the solid line represents results from the phosphatase samples. The numbers below each peak designate unreacted substrate (1), *O*-alkylglycerol (2), *O*-alkyldihydroxyacetone (3), fatty alcohol (4), and unidentified (5). Full scale on the ordinates range from 1500 to 2200 cpm. The chromatography was done on silica gel G layers in solvent systems of diethyl ether saturated with water (part A) and hexane–diethyl ether–methanol–acetic acid (70:30:5:1, v/v) (part B).

SCHEME I



catalytic protein in this system was tested by adding bovine serum albumin to the alkaline phosphatase so that it had a protein concentration equal to that of the acid phosphatase. The added carrier albumin had no effect on substrate specificities. These results indicate that the less polar phosphatides could not be oriented in such a way at high protein–lipid ratios as to render the free phosphate moiety more accessible to the enzyme for dephosphorylation.

The data show that alkaline phosphatase has a unique specificity for dephosphorylating glycerolipids that do not contain aliphatic moieties in the 2 position. In contrast, the acid phosphatase appears to lack this selectivity in dephosphorylating lipid substrates. The enzymic reactions (I–VI) studied and the results obtained for bacterial alkaline phosphatase are depicted in Scheme I. The dephosphorylation of key lipid intermediates by alkaline and acid phosphatases suggests that these enzymes, in addition to phosphatidate hydrolase, play a significant role in the metabolism of lipids. Is the lipid composition of cells altered by abnormal levels of phosphatase activity? Certainly, the specificity of bacterial alkaline phosphatase makes it extremely useful in the identification of monophosphate esters of glycerolipids having either aliphatic moieties in the 1 position and hydroxyl or ketone groups in the 2 position.

References

Bligh, E. G., and Dyer, W. J. (1959), *Can. J. Biochem. Physiol. 37*, 911.

Dawson, R. M. C. (1960), *Biochem. J. 75*, 45.

Heppel, L. A., Harkness, D. R., and Hilmoe, R. J. (1962), *J. Biol. Chem. 237*, 841.

Long, C., Odavić, R., and Sargent, E. J. (1967), *Biochem. J. 102*, 221.

Okuyama, H., and Nojima, S. (1965), *J. Biochem. (Tokyo) 57*, 529.

Rouser, G., Siakotos, A. N., and Fleischer, S. (1966), *Lipids 1*, 85.

Schmidt, G., Cubiles, R., Zöllner, N., Hecht, L., Strickler, N., Seraidarian, K., Seraidarian, M., and Thannhauser, S. J. (1951), *J. Biol. Chem. 192*, 715.

Snyder, F. (1969), *in* Progress in the Chemistry of Fats and Other Lipids, Vol. X, Holman, R. T., Ed., Oxford, Pergamon, pp 287–335.

Snyder, F., Blank, M. L., Malone, B., and Wykle, R. L. (1970a), *J. Biol. Chem. 245*, 1800.

Snyder, F., and Kimble, H. (1965), *Anal. Biochem. 11*, 510.

Snyder, F., Malone, B., and Blank, M. L. (1970b), *J. Biol. Chem. 245*, 1790.

Wykle, R. L., and Snyder, F. (1970), *J. Biol. Chem. 245*, 3047.