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MOLECULAR STRUCTURE IN PHOSPHOLIPID ESSENTIAL TO ACTIVATE (Na^+-K^+-Mg^{2+})-DEPENDENT ATPase AND (K^+-Mg^{2+})-DEPENDENT PHOSPHATASE OF BOVINE CEREBRAL CORTEX

RYO TANAKA AND TERUFUMI SAKAMOTO

Center for Brain Research, University of Rochester, Rochester, N.Y. 14627 (U.S.A.)

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SUMMARY

1. The effects of mono- and dialkyl phosphates and related compounds were studied on (Na^+-K^+-Mg^{2+})-activated ATPase and (K^+-Mg^{2+})-activated phosphatase prepared from bovine cerebral cortex by deoxycholate treatment.

2. The ATPase was activated by either mono- or dialkyl phosphate. When both alkyl phosphates possessed ten carbon atoms in a single alkyl residue, the activation effects were highest and comparable to those of natural phospholipids. Dodecyl sulfate (in narrow ranges of concentration), long-chain fatty acids, and alcohols were slightly effective. Alkyl amines were ineffective.

3. The *p*-nitrophenyl phosphatase was stimulated by dialkyl phosphates only. Didecyl phosphate was again most active and the degree of its stimulation was the same as that of natural phospholipids. Monoalkyl phosphates, dodecyl sulfates, long-chain fatty acids, alcohols and alkyl amines were all generally inhibitory.

4. It is suggested that the essential structures needed for activation are a phosphate *plus* one or two fatty acyl residues for the ATPase and a phosphate *plus* two acyl residues for the *p*-nitrophenyl phosphatase.

INTRODUCTION

In our previous studies[1,2] it has been demonstrated that a variety of phospholipids activate (Na^+-K^+-Mg^{2+})-activated ATPase and (K^+-Mg^{2+})-activated *p*-nitrophenyl phosphatase prepared from membrane components of cerebral cortices. Phospholipids of various species and from different sources displayed a wide range of effectiveness in activation of the enzymes. Naturally, a question arises as to what the relationship is between the structure of the lipid and its effectiveness in activating the enzymes. The present investigation is an attempt to answer this question, using a variety of synthetic compounds structurally related to phospholipid, including mono- and dialkyl phosphates, dodecyl sulfate, alkyl amines, long-chain fatty acids and alcohols. The answer to this question may also give some insight into the relationship between these two enzymes which is not clearly understood as yet.

METHODS AND MATERIALS

Enzyme preparation and activity determination

The cerebral cortex of domesticated beef cattle was used as an enzyme source. The method of preparation of the enzyme and its modification were the same as previously reported[1,2], and the experiment was carried out with the preparation obtained at the end of Step 3.

The determination of ATPase and *p*-nitrophenyl phosphatase was the same as described elsewhere[1,2] except that alkyl phosphates were removed by mixing about 20 mg of acid-washed Norit "A" (decolorizing carbon) to the reaction media and by centrifuging down the charcoal at 0–4° after the reaction had been stopped by the addition of trichloroacetic acid, since in some cases alkyl phosphates interfered with the phosphate determination. The enzyme activity is expressed in terms of the amount (μmole) of hydrolyzed substrate per mg of protein in 20 min at 25°. (Na^+-K^+-Mg^{2+})-activated ATPase ((Na^+-K^+-Mg^{2+})-ATPase)-activity was assumed to be the difference between the value obtained in the presence of Na^+, K^+ and Mg^{2+} and the value obtained in the presence of Mg^{2+} only. Acetyl phosphate was determined by the methods of LIPMANN AND TUTTLE[3] after alkyl phosphate had been removed by charcoal treatment; carbamyl phosphate was determined by the method of WAHLER AND WOLLENBERGER[4]. Lipids and alkyl phosphates were dispersed in water or in 50 mM Tris buffer (pH 7.4) by sonication or homogenization with a Potter–Elvehjem-type homogenizer made of Teflon and glass.

Materials

Dialkyl phosphates were prepared by the methods of BROWN *et al.*[5] and monoalkyl phosphates by the method of NELSON AND TOY[6]. Alkyl amines and alcohols were purchased from Aldrich Chemical Co. and Eastman Kodak Co. and, when necessary, further purified before the alkyl phosphate synthesis. Lysolecithin and crude animal lecithin were obtained from Nutritional Biochemical Corp.; other lipids, acetyl phosphate and carbamyl phosphate from Sigma Chemical Co. and Applied Science Laboratories, and Duolite ES-63 from Diamond Chemicals.

RESULTS

Effects of dialkyl phosphates

Dihexyl phosphate activated both (Na^+-K^+-Mg^{2+})-ATPase and (K^+-Mg^{2+})-activated *p*-nitrophenyl phosphatase at relatively high concentrations (Fig. 1A). As the concentration of the alkyl phosphate increased, the activities of the enzymes gradually decreased. This inhibitory effect was stronger with the *p*-nitrophenyl phosphatase than with the ATPase. The highest activities of the ATPase and the *p*-nitrophenyl phosphatase were obtained in the presence of dinonyl phosphate at the concentration of 0.2 mM which was approximately half of the dihexyl phosphate concentration needed for maximum activation (Fig. 1B).

The didecyl phosphate activation of the ATPase, at its peak (0.08 mM didecyl phosphate), was approx. 10 % higher than that obtained in the presence of a natural phospholipid, phosphatidyl serine (Fig. 1C, also see Fig. 3C). The alkyl phosphate also stimulated the *p*-nitrophenyl phosphate in a manner similar to activation of the

ATPase with regard to the alkyl phosphate concentration and the degree of activation. The activating efficacy augmented as the number of carbon atoms in fatty acyl residue of the alkyl phosphate increased.

The efficacy of didodecyl phosphate in activating the ATPase was approximately half of didecyl phosphate activation (Fig. 1D). The stimulation of the *p*-nitrophenyl phosphatase was insignificant. No inorganic phosphate was liberated from the alkyl phosphate under the present incubation conditions.

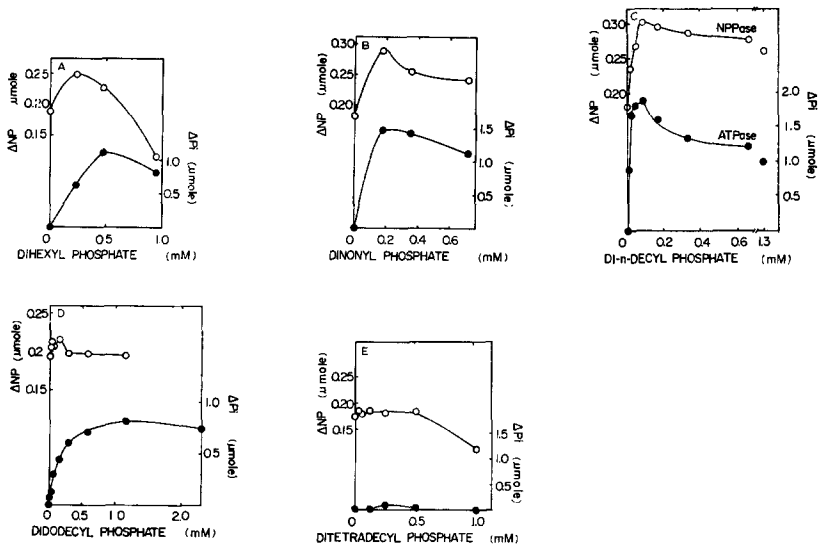

Fig. 1. Effects of dialkyl phosphates on (Na^+-K^+-Mg^{2+})-ATPase (●—●) and (K^+-Mg^{2+})-activated *p*-nitrophenyl phosphatase (○—○) A. Dihexyl phosphate. B. Dinonyl phosphate. C. Didecyl phosphate. D. Didodecyl phosphate. E. Ditetradecyl phosphate. The reaction medium for the ATPase determination contained 50 mM Tris buffer (pH 7.4), 10 mM cysteine, 3 mM $MgCl_2$, 150 mM NaCl, 10 mM KCl, 2 mM ATP, dialkyl phosphate as specified in the figure, and 0.2 ml of the enzyme preparation in a final volume of 2.0 ml. The reaction mixture for the *p*-nitrophenyl phosphatase determination contained 50 mM Tris buffer (pH 7.4), 10 mM cysteine, 3 mM $MgCl_2$, 10 mM KCl, 2 mM *p*-nitrophenyl phosphate, dialkyl phosphate as shown in the figure, and 0.2 ml of the enzyme preparation in a final volume of 2.0 ml. The enzyme was prepared by deoxycholate treatment from the membrane fraction of bovine cerebral cortex. The enzyme activity is expressed in terms of the amount (μmole) of hydrolyzed substrate per mg of protein in 20 min at 25°. NP = *p*-nitrophenyl.

Ditetradecyl phosphate showed virtually no effect on the ATPase activity, whereas the dialkyl phosphate inhibited the *p*-nitrophenyl phosphatase at high concentrations (Fig. 1E). No effects on either the ATPase or the *p*-nitrophenyl phosphatase activity were observed in the presence of dioctadecyl phosphate in the range of the tested concentrations (0.05–0.42 mM).

Effects of monoalkyl phosphates

Monohexyl phosphate activated the ATPase slightly at levels higher than 5 mM and inhibited the *p*-nitrophenyl phosphatase (Fig. 2A). The 50 % inhibition appeared at approx. 5 mM.

Though the *p*-nitrophenyl phosphatase was strongly inhibited by monoctyl phosphate (50 % inhibition at 3 mM), the ATPase was activated by the alkyl phos-

phate of over 0.6 mM and the highest activity obtained in the presence of monoctyl phosphate (5 mM) was almost 2-fold greater than that obtained in the presence of monohexyl phosphate (Fig. 2B). Inorganic phosphate was released slightly faster from 1.2 mM monoctyl phosphate by the enzyme preparation than it was from 2 mM ATP by (Na^+-K^+-Mg^{2+})-ATPase under the same incubation conditions, but the hydrolysis of the alkyl phosphate was not affected by the addition of Na^+ and K^+.

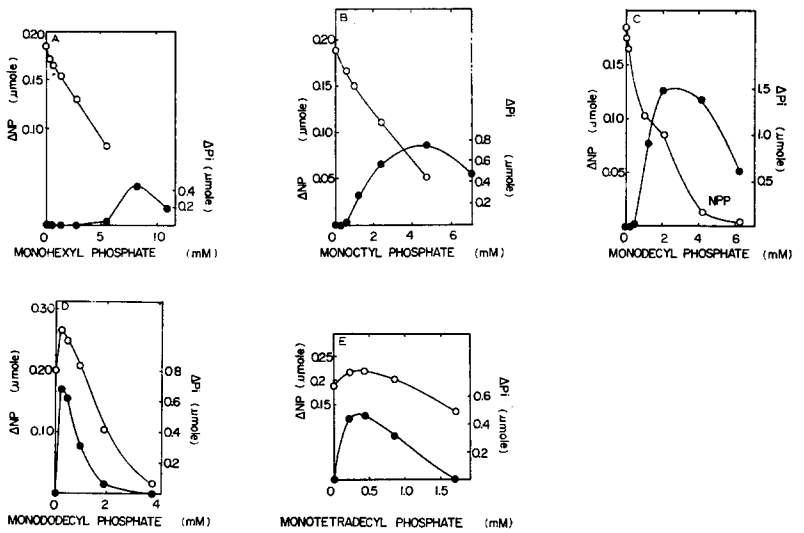

Fig. 2. Effects of monoalkyl phosphates on (Na^+-K^+-Mg^{2+})-ATPase (●—●) and (K^+-Mg^{2+})-activated *p*-nitrophenyl phosphatase (○—○). A. Monohexyl phosphate. B. Monoctyl phosphate. C. Monodecyl phosphate. D. Monododecyl phosphate. E. Monotetradecyl phosphate. The determination conditions were the same as for Fig. 1 except for the use of monoalkyl phosphates. NP = *p*-nitrophenyl.

The 50 % *p*-nitrophenyl phosphatase inhibition by monodecyl phosphate occurred at 1.8 mM (Fig. 2C). The highest ATPase activation was obtained in the presence of approx. 2 mM of the monoalkyl phosphate.

Monododecyl phosphate activation of the ATPase reached its peak at approx. 0.4 mM of the alkyl phosphate, and the highest activity was about half of the value given in the presence of monodecyl phosphate (Fig. 2D). Though the alkyl phosphate slightly stimulated the *p*-nitrophenyl phosphatase at lower concentrations, it was strongly inhibited when the concentration was higher than 1 mM.

Monotetradecyl phosphate displayed similar effects on the ATPase and the *p*-nitrophenyl phosphatase activities (Fig. 2E). The greatest activity of the ATPase obtained in the presence of the alkyl phosphate was approx. 30 % of that with monodecyl phosphate.

Monoctadecyl phosphate showed a weak activation of the ATPase and inhibitory effects on the *p*-nitrophenyl phosphatase (Table I). Extremely finely pulverized Duolite ES-63, a cation exchange resin of polystyrene phosphate, was slightly inhibitory to the *p*-nitrophenyl phosphatase.

The effects of monodecyl phosphate and didecyl phosphate on (K^+-Mg^{2+})-activated phosphatase were tested with carbamyl phosphate and acetyl phosphate,

TABLE I

EFFECTS OF LIPIDS AND RELATED COMPOUNDS

The reaction conditions were the same as for Fig. 1 except that the compounds indicated were added in place of alkyl phosphates. The activation or stimulation obtained in the presence of didecyl phosphate was set arbitrarily as 100% for the purpose of comparison. The enzyme preparation showed no activity of (Na^+-K^+-Mg^{2+})-ATPase in the absence of phospholipids, whereas the enzyme preparation displayed (K^+-Mg^{2+})-activated *p*-nitrophenyl phosphatase activity which was increased approx. 70% by the addition of the lecithin preparation.

Addition	*Concn. (mM)*	*(Na^+-K^+-Mg^{2+})-ATPase activation (%)*	*(K^+-Mg^{2+})-p-nitrophenyl phosphatase stimulation (%)*
Didecyl phosphate	0.08	100	100
Monooctadecyl phosphate	0.4	14	−1
	0.7	6	−22
Dodecyl amine	0.4	0	(Complete
	0.8	0	inhibition)
Octadecyl amine	0.4	0	−106
	0.8	0	−109
Decyl alcohol	1.0	11	—
	2.0	41	—
	4.0	34	—
Dodecyl alcohol	0.5	21	−11
	0.9	23	−33
	1.5	19	—
	2.0	4	—
Oleyl alcohol	0.5	2	−6
	0.9	5	−9
Linoleyl alcohol	0.5	13	−61
	0.9	9	−87
Linolenyl alcohol	0.5	10	−79
	0.9	6	−87
Monolein	0.7	18	—
Lysophosphatidylethanolamine	(125 μg/ml)	115	83
	(250 μg/ml)	96	71

respectively, as substrates. The relation between the concentration of the alkyl phosphates and the rate of substrate hydrolysis was identical with that obtained with *p*-nitrophenyl phosphate as a substrate.

None of the di- and monoalkyl phosphates tested stimulated Mg^{2+}-ATPase, acid and Mg^{2+}-activated *p*-nitrophenyl phosphatase. Neither dodecyl amine nor octadecyl amine showed effects on Mg^{2+}-ATPase, acid or Mg^{2+}-activated *p*-nitrophenyl phosphatase in the tested concentration range.

Effects of related compounds

Although the general pattern of dodecyl sulfate effects on the enzymes were similar to those of monoalkyl phosphates, especially of monododecyl phosphate

(Fig. 3A), the range of activating concentration and the degree of activation by the sulfate were only one-fifth ond one-half, respectively, of those by monododecyl phosphate. At concentrations higher than 0.4 mM, the alkyl sulfate inhibited Mg^{2+}-ATPase, Mg^{2+}-activated *p*-nitrophenyl phosphatase and acid *p*-nitrophenyl phosphatase. There was a total inhibition of all enzyme activities when the sulfate concentration was more than 0.9 mM.

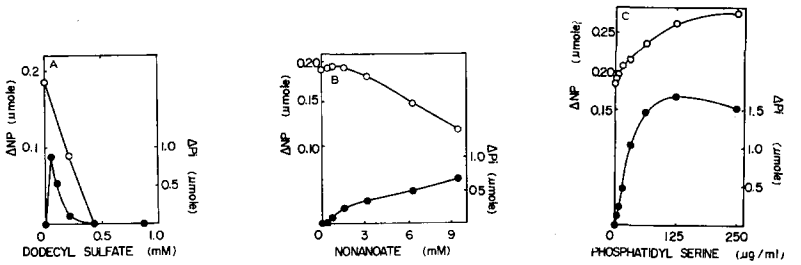

Fig. 3. Effects on (Na^+-K^+-Mg^{2+})-ATPase (●—●) and (K^+-Mg^{2+})-activated *p*-nitrophenyl phosphatase (○—○) of dodecyl sulfate (A), nonanoate (B), and phosphatidylserine (C). The incubation conditions were the same as given for Fig. 1 except for the use of the compounds indicated instead of alkyl phosphates. NP = *p*-nitrophenyl.

The effects of nonanoate on the enzymes were similar to those of monoctyl phosphate (Fig. 3B). A 3- to 5-fold higher concentration of nonanoate was needed, however, to obtain the same degree of activation or inhibition as given by the alkyl phosphate. The tridecanoate effects were almost identical with those of monotetradecyl phosphate. In order to show the similarity in pattern and degree of activation between enzyme activation by natural phospholipid and that by alkyl phosphate, the relation of (bovine) phosphatidylserine concentration to activation is illustrated in Fig. 3C which resembles those of dialkyl phosphate containing more than eight carbon atoms in a hydrocarbon chain.

The following lipids and related compounds showed no effects on the ATPase: monopalmitin, dipalmitin, diolein, triolein, trilinolein, α-glycerophosphate. Dodecyl amine and octadecyl amine were inhibitory of *p*-nitrophenyl phosphatases and did not activate the ATPase (Table I). Oleyl alcohol showed no significant effects on the enzymes, whereas linoleyl and linolenyl alcohols slightly activated the ATPase and strongly inhibited the *p*-nitrophenyl phosphatase, reminiscent of the pattern of monolein effects[2] (Table I). Dodecyl alcohol showed moderate activation and inhibition on the ATPase and the *p*-nitrophenyl phosphatase, respectively. The activating effect of decyl alcohol was higher at the concentration optimum for monodecyl phosphate than was that of dodecyl alcohol.

In addition to the lipids listed in Table I, palmitic acid, stearic acid, arachidonic acid, tripalmitin and tristearin were tested. However, since they failed to be dispersed in homogeneous suspensions, presumably due to their strongly hydrophobic property, their effects on the enzyme activities were not further analyzed.

Effects of ouabain

The low concentration of ouabain inhibited (Na^+-K^+-Mg^{2+})-ATPase activity obtained in the presence of alkyl phosphates as well as crude animal lecithin (Table II).

TABLE II

EFFECTS OF OUABAIN ON (Na^+-K^+-Mg^{2+})-ATPASE IN THE PRESENCE OF DIDECYL PHOSPHATE AND MONOTETRADECYL PHOSPHATE

The reaction conditions were the same as for Fig. 1. The activity of (Na^+-K^+-Mg^{2+})-ATPase obtained in the presence of crude animal lecithin was set arbitrarily as 100%.

Addition	*Ouabain (50 μM)*	*(Na^+-K^+-Mg^{2+})-ATPase activity (%)*
Crude animal lecithin (250 μg)	−	100
	+	9.8
Didecyl phosphate (0.6 mM)	−	88
	+	10
Monotetradecyl phosphate (0.4 mM)	−	58
	+	13

The similar effect of ouabain was also exhibited in the presence of 0.9 mM dodecyl alcohol.

DISCUSSION

Although a variety of phospholipids have been shown to activate (Na^+-K^+-Mg^{2+})-ATPase of a membrane fraction of brain[1,2], all four residues composing phospholipid (amino alcohol or choline, phosphate, fatty acid and glycerol) do not seem to be essential for activation. Since lecithin and phosphatidylethanolamine were only slightly effective in activation and phosphatidic acid belonged to one of the most active phospholipids[7], amino alcohol or choline group may not play an important role in activation of the enzyme. The phosphate group, or a negatively charged group, however, would be essential inasmuch as most of mono-, di- and triglycerides tested were ineffective. The thesis that a negatively charged group may be needed is also supported by the fact that acidic phospholipids such as phosphatidylserine and phosphatidylinositol, activated the enzyme but neutral phospholipids like phosphatidylethanolamine and lecithin did not[7]. Like a phosphate group, a fatty acyl residue may be required for activation, since lysolecithin[7] and lysophosphatidylethanolamine markedly activated the enzyme but glycerophosphate was inactive. Lecithin prepared from beef brain showed the enzyme activation to some extent, whereas synthetic dipalmitoyl lecithin with less hydrophilic character was inactive[7]. Those facts may show that the efficacy in activation is partly determined by the hydrophilic character of the lipid, which is dependent on the number of fatty acyl moieties and degree of saturation, and that the hydrocarbon chain rather than the carboxyl residue plays a central role. Thus, the compounds should consist of at least a phosphate group and a fatty acyl residue or a hydrocarbon chain in order to activate the ATPase. While the importance of glyceryl residue is not clear from the above mentioned data, compounds consisting of glyceryl and fatty acyl residues or of a glyceryl residue and a phosphate group were inactive.

In order to determine the feasibility of the above conclusion, mono- and dialkyl phosphates were tested for their efficacy in enzyme activation. The following conclusions in addition to the above would be drawn with regard to the basic structure

needed for enzyme activation. (a) A fatty alcohol is sufficient in place of a fatty acyl residue, and the hydrocarbon chain does not have to be unsaturated if the chain length is appropriate. (b) The chain length is a determinant of the efficacy of activation, probably partly deciding the hydrophilic–hydrophobic character balance and partly deciding the binding force and conformation appropriate for combination to the enzyme protein, since both mono- and dialkyl phosphates were most active when they possessed ten-carbon chains, though the optimum concentrations differed. (c) The negatively charged group required for activation does not have to be a phosphate. A sulfate or a carboxyl radical seems to be able to replace the phosphate group, though the action of alkyl sulfate and fatty acid was much weaker and more limited than that of alkyl phosphates. (d) When a phosphate group is replaced by a positively charged group, such as an amine residue, the compound is inactive.

As pointed out in an earlier report[7], (K^+-Mg^{2+})-activated *p*-nitrophenyl phosphatase differs markedly from (Na^+-K^+-Mg^{2+})-ATPase with regard to the species of phospholipids needed for their activation and the degree of activation by the same lipid species. The essential structure required for the *p*-nitrophenyl phosphatase stimulation, however, is similar to that for the ATPase activation, so far as phospholipid is concerned. The only differences are that (a) a phosphate group which is not neutralized by an attached positive group may not be as important as in the case of the ATPase, since lecithin and phosphatidylethanolamine were active in stimulation and (b) a relatively low hydrophobic character of phospholipid or the presence of double bonds in the fatty acyl sector does not seem essential for the *p*-nitrophenyl phosphatase stimulation because dipalmitoyl lecithin stimulated the enzyme but not the ATPase.

The minimum requirements of the molecular structure of the lipid-like compound activating the *p*-nitrophenyl phosphatase are suggested by the experiments using mono- and dialkyl phosphates. They are summarized as follows. (a) Two hydrocarbon chains in an alkyl phosphate molecule were needed to stimulate the *p*-nitrophenyl phosphatase, and the phosphates with single alkyl chains were generally inhibitory though the inhibition was dependent on the length of the hydrocarbon chain. As the chain elongated, monoalkyl phosphate reduced the inhibitory action, and the phosphates with twelve and fourteen carbon atoms were slightly stimulating at low concentrations only. Polystyrene phosphate, where phosphate residues were attached to a rigid styrene framework, showed little inhibition. Strongly hydrophobic character of alkyl phosphate with a long saturated chain may exclude itself from an aqueous reaction milieu or simply a too long chain and a rigid polystyrene framework would exert no inhibitory action on the enzyme even if they were bound to the protein. The inhibition of short-chain monoalkyl phosphates could be explained partly by the competition with the substrate for the active sites in the enzyme protein. (b) Dialkyl phosphate containing ten carbon atoms in the chain showed the highest stimulation of the *p*-nitrophenyl phosphatase activity. (c) A phosphate group as a negatively charged sector in alkyl phosphate is more critical for stimulation of the *p*-nitrophenyl phosphatase than for activation of the ATPase, since when it was replaced by a sulfate or a carboxyl radical the stimulation activity was lost, as shown in cases of dodecyl sulfate and fatty acids. (d) When there is an amine residue in place of a phosphate residue, the molecule is inhibitory. (e) The double bond, which determines molecular conformation and hydrophilic tendency, plays another critical role in determining the action of fatty alcohol and glyceride. As the number of double bonds with a fixed

chain length in an alcohol increases, the degree of the *p*-nitrophenyl phosphatase inhibition augments and as the number of hydrocarbon chains containing single double bonds in a glyceride increases, the extent of the inhibition decreases[2].

There are at least two possible explanations for the effects of long-chain alcohols and monolein, which activated the ATPase while inhibiting the *p*-nitrophenyl phosphates as monoalkyl phosphates did. (a) Since the structures of the alcohols and monolein, with hydrophilic residue(s) located at an end of a long hydrophobic chain, resemble that of monoalkyl phosphate and the action of the compounds would be determined mainly by the properties of their hydrocarbon chains, the effects of the alcohols and monolein are similar to those of monoalkyl phosphates. (b) The alcohols and monolein may combine with endogenous activator(s) and inhibitor(s) to make them more accessible to the enzyme proteins, or the binding of the alcohols to the enzyme proteins may make endogenous activator(s) and inhibitor(s) more accessible to the enzymes. Preliminary experiments showed that a mixture of monolein and monoctadecyl phosphate, which itself was slightly activating, activated the ATPase, and a mixture of the monoglyceride and octadecyl amine, an inhibitor, inhibited the *p*-nitrophenyl phosphatase more than could be calculated assuming that the effects of the mixtures were merely additive.

Since a low concentration of ouabain suppressed the ATPase to the same level in the presence of either crude animal lecithin, mono- or dialkyl phosphates, the alkyl phosphate-dependent portion of the present enzyme preparation may well be regarded as the same (Na^+-K^+-Mg^{2+})-ATPase as that activated by natural phospholipids.

When acetyl phosphate was used as a substrate of (K^+-Mg^{2+})-activated phosphatase, the relation of the enzyme stimulation to the concentration of the didecyl phosphate remained identical. Likewise, the monodecyl phosphate inhibition of the phosphatase followed an identical pattern when either *p*-nitrophenyl phosphate or carbamyl phosphate was used as a substrate. Consequently, the activating or inhibiting effects of alkyl phosphates on the phosphatase did not seem to be due to the combination of the alkyl phosphates with the substrate, if they combined, resulting in facilitation or obstruction of the access of substrates to the active sites, since it is unlikely that alkyl phosphates bind with an identical affinity to such structurally different substances as *p*-nitrophenyl phosphate, acetyl phosphate and carbamyl phosphate.

As the species of natural phospholipids activating the ATPase differed from those stimulating the *p*-nitrophenyl phosphatase[7], the species of alkyl phosphates and related substances activating the ATPase were shown again in the present work to be unrelated to those stimulating the *p*-nitrophenyl phosphatase. The activation patterns of the ATPase by dialkyl phosphates with varied chain lengths were independent from those of the *p*-nitrophenyl phosphatase, and monoalkyl phosphate (with less than twelve carbon atoms), dodecyl sulfate, long-chain alcohols, nonanoate and monolein activated the ATPase but inhibited the *p*-nitrophenyl phosphatase. These facts further support the previous suggestion that either the *p*-nitrophenyl phosphatase is a separate enzyme entity from the ATPase or, although the *p*-nitrophenyl phosphatase is a part of the ATPase system, the mechanism of action of the lipids on the former differs from that on the rest of the system. If the *p*-nitrophenyl phosphatase were assumed to be a part of the ATPase system, however, it would have to be further assumed that the hydrolyzing reaction of a true substrate,

which is an intermediate derived from reaction between ATP and the system, is not inhibited by the above listed compounds, whereas the reaction of exogenous substrates for phosphatase is inhibited.

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