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Mechanism of Lipid Activation of Na, K, Mg-Activated Adenosine Triphosphatase and K, Mg-Activated Phosphatase of Bovine Cerebral Cortex

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Summary. Na^+, K^+, Mg^{++}-activated adenosine triphosphatase and K^+, Mg^{++}-activated *p*-nitrophenyl phosphatase prepared from a membrane fraction of bovine cerebral cortex were studied with regard to the manner of their activation by phospholipids using phosphatidyl serine, lysolecithin, monodecyl and didecyl phosphates. The kinetic and chromatographic studies suggested the following. (1) When the enzyme proteins bind the phospholipids in a proper ratio, they attain the optimum activation. (2) The binding causes a simple conversion of the enzymes from an inactive form to a fully activated form. (3) The lipids in both micellar form and molecular dispersion activate the enzymes. (4) Of the proteins contained in the enzyme preparation, only a group of proteins possessing the ATPase and the phosphatase activities bind phospholipids, and the amount of the bound lipids is limited.

It is now well established that there is an adenosine triphosphatase (ATPase) activity associated with active ion transport, which expels Na^+ and accumulates K^+ to maintain a physiological ion distribution across the cell membrane. The membrane-bound ATPase activated by Na^+, K^+, plus Mg^{++}, is reasonably assumed to be an enzymatic manifestation of an ion transport pump itself or, at least, a component part. Although the ATPase system was believed to be composed of lipoprotein, the functional role of the lipid component was not yet completely understood.

It has been demonstrated in our previous studies (Tanaka, 1969; Tanaka & Sakamoto, 1969) that a variety of phospholipids and the related compounds activate Na^+, K^+, Mg^{++}-activated ATPase (EC 3.6.1.3) and K^+, Mg^{++}-

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activated *p*-nitrophenyl phosphatase[1] (EC 3.6.1.7) prepared by removal of the phospholipid component from a membrane fraction of cerebral cortices. Phospholipids of various species from different sources, and the alkyl compounds consisting of hydrocarbon chains of various lengths and negatively charged groups (such as phosphate, sulfate, and carboxyl), displayed a wide range of effectiveness in activation of the enzymes.

The present work is an attempt to answer, at least in part, a question as to the mechanism of the lipid activation of the ATPase and the NPPase, using natural phospholipids and alkyl phosphates. The following will be described in this report: (1) the effects of the protein-lipid ratio on the activation of the membrane enzymes; (2) the relation of the species and the amount of lipids to the K_m of the ATPase; (3) the relation of the critical micelle concentration of lipids to their concentration for optimum activation; and (4) the chromatography of the enzyme preparation and lipids on gel filtration columns.

Materials and Methods

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 (Tanaka & Strickland, 1965; Tanaka & Mitsumata, 1969), and the experiment was carried out with the preparation obtained at the end of Step 3 unless otherwise specified. At this stage, the enzyme preparation contained 0.045 μmole of chloroform-methanol extractable phosphate per mg of protein, although the intact membrane fraction contained 0.41 μmole of the phosphate. After gel filtration, the content of the lipid phosphate in the solubilized enzyme preparation remained unchanged (*cf.* Fig. 4b, upper half).

The determination of the ATPase and the NPPase was the same as described elsewhere (Tanaka & Mitsumata, 1969; Tanaka & Sakamoto, 1969). Na, K, Mg-ATPase activity was assumed to be the difference between the value obtained in the presence of Na^{++}, K^{+}, and Mg^{++} and the value obtained in the presence of Mg^{++} only. Lipid 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.

Other Determinations

The critical micelle concentrations were determined at 25 °C by measurement of surface tension using capillary rise method (Preston, 1948; Paddy, 1969). The amount of protein was determined by the method of Lowry, Rosebrough, Farr and Randall (1951) and by ultraviolet absorption (Layne, 1957). The sedimentation analysis of the enzyme

1 The abbreviations used are: Na, K, Mg-ATPase, for Na^{+}, K^{+}, Mg^{++}-activated adenosine triphosphatase; and K, Mg-NPPase for K^{+}, Mg^{++}-activated *p*-nitrophenyl phosphatase.

protein was carried out according to the method of Martin and Ames (1961). The solution in the mixing chamber contained 10% sucrose, 50 mM NaCl, 2 mM ATP, 50 mM Tris buffer, pH 7.4, and the solution in the adjacent chamber contained 3% sucrose in the same buffer containing NaCl and ATP. The centrifugation was at 200,000 × *g* for 6 h at 3 °C using a Spinco SW-40 rotor.

Monodecyl and didecyl phosphates were prepared by the methods of Nelson and Toy (1963), and of Brown, Malkin and Maliphant (1955), respectively. Palmitoyl lysolecithin, phosphatidyl serine, and crude animal lecithin were obtained from Nutritional Biochemical Corp. Phospholipids were checked for purity by thin layer chromatography (TLC) with the solvent systems of Skidmore and Entenman (1962), Skipski, Peterson and Barclay (1964), or Abood, Koyama and Thomas (1964). Palmitoyl lysolecithin and phosphatidyl serine appeared as a single spot on TLC. Crude animal lecithin preparation was found to contain at least five different components: lecithin, phosphatidyl serine, phosphatidyl ethanolamine, neutral lipid, and lyso-compounds. Bio-Gel A-15m was purchased from Calbiochem, Sepharose 4B from Pharmacia Fine Chemicals, Inc., and crystalline pyruvate kinase (Type II) from Sigma Chemical Co. (ammonium sulfate was removed by passing through a Sephadex G-25 column).

Results

When the amount of the ATPase protein was kept at 0.8 mg/ml of the reaction medium, as shown by the upper curve of Fig. 1a, the enzyme activity was enhanced as the concentration of palmitoyl lysolecithin increased and the activity reached its peak at about 0.13 mM of the lipid. When the enzyme amount was reduced to the level of 0.2 mg/ml, which was one-fourth of the previous level as given by the lower curve (Fig. 1a), its activity peak was found at the lysolecithin concentration of 0.032 mM. The concentration needed to obtain the highest activity was one-fourth of the previous case.

Fig. 1b illustrates that the same relationship was observed in the case of the NPPase. When the level of the enzyme protein was at 0.42 mg/ml as indicated by the upper curve, the peak was at 0.064 mM of the phospholipid. If the enzyme protein was fixed at the level of 0.11 mg/ml, i.e., one-fourth of the previous amount, the activity peak was at 0.016 mM of the lipid (the lower curve, Fig. 1b). The optimum lipid concentration was again one-fourth of the previous level.

Didecyl phosphate and phosphatidyl serine have a similar relationship of lipid-protein ratio to optimum activities of both the ATPase and the NPPase (Fig. 1c–f).

Fig. 2a shows the K_m of the ATPase in the presence of various amounts of crude animal lecithin. The values for lines *A*, *B*, and *C* were obtained with 16, 32, and 63 μg of the phospholipid preparation, respectively. Although the concentrations of the crude lecithin used differed, the K_m values

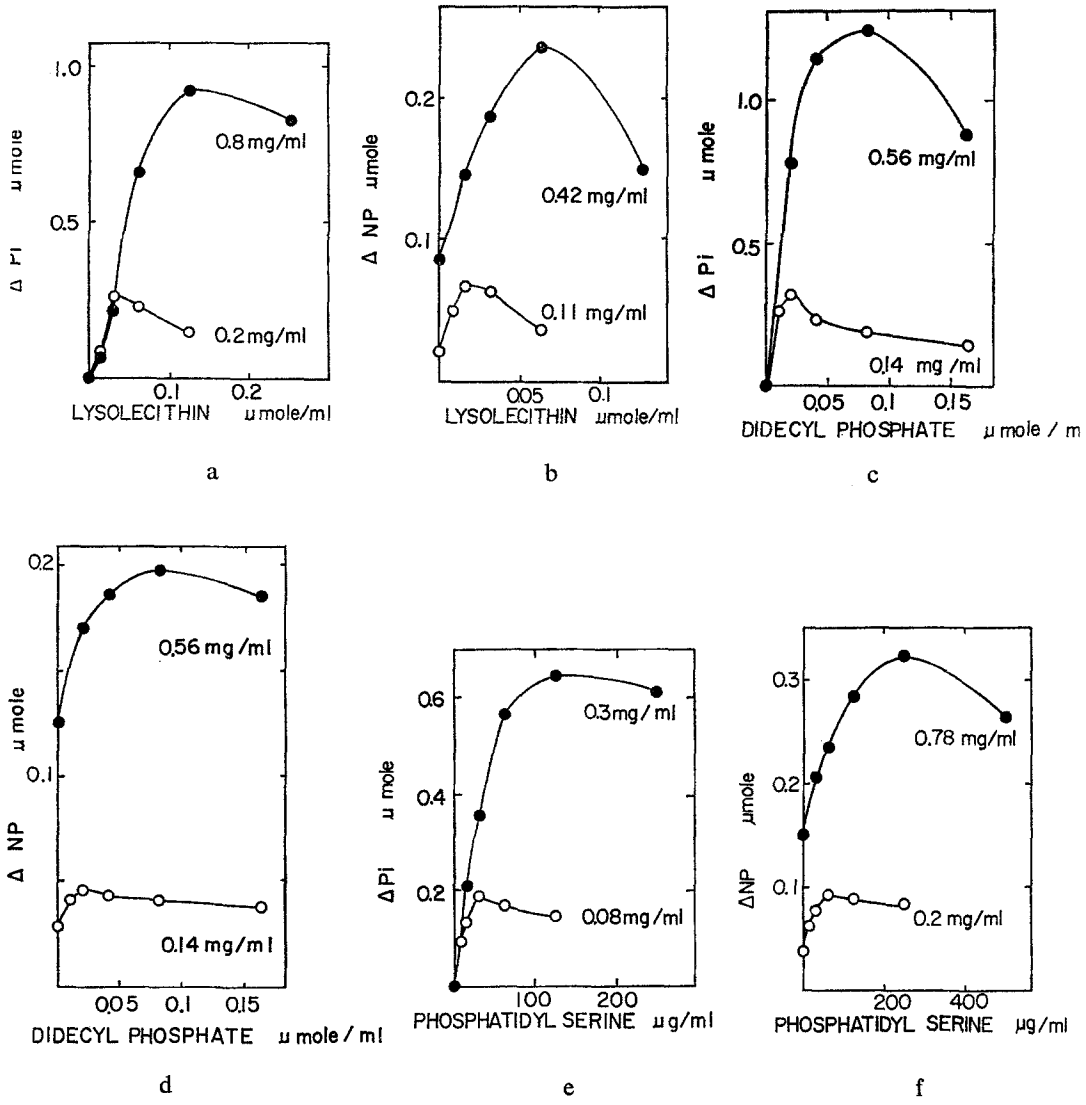


Fig. 1a–f. ATPase and NPPase activities and protein-lipid ratios. The reaction medium for the ATPase determination contained 50 mM Tris buffer, pH 7.4, 10 mM cysteine, 3 mM $MgCl_2$, 150 mM NaCl, 10 mM KCl, 2 mM ATP, lipid as specified in the figures, and the enzyme preparation in a final volume of 2.0 ml. The enzyme was prepared by deoxycholate treatment from a membrane fraction of bovine cerebral cortex. The enzyme activity is expressed in terms of the amount (μmole) of hydrolyzed substrate in 20 min at 25 °C. The reaction mixture for the NPPase determination contained 50 mM Tris buffer, pH 7.4, 10 mM cysteine, 3 mM $MgCl_2$, 10 mM KCl, 2 mM *p*-nitrophenyl phosphate, lipid as shown in the figures, and the enzyme preparation in a final volume of 2.0 ml. *NP p*-nitrophenol. The amounts of the enzyme protein used are specified in the figures. (a) ATPase with lysolecithin. (b) NPPase with lysolecithin. (c) ATPase with didecyl phosphate. (d) NPPase with didecyl phosphate. (e) ATPase with phosphatidyl serine. (f) NPPase with phosphatidyl serine

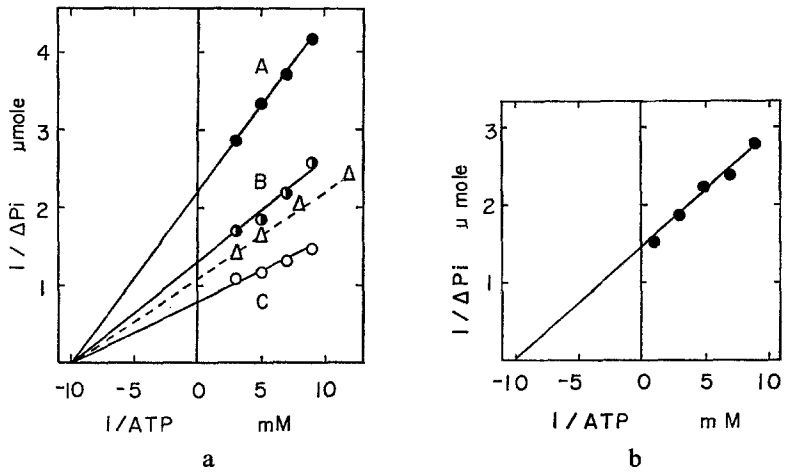


Fig. 2a and b. Double-reciprocal plots of ATPase. The determination conditions were the same as for Fig. 1 except for the use of phosphoenolpyruvate (4 mM)—pyruvate kinase (12.5 μg/ml) as an ATP-regenerating system. (a) The values for lines *A*, *B*, and *C* were determined in the presence of 16, 32, and 63 μg of crude animal lecithin, respectively and the amount of the enzyme protein was 0.9 mg. The broken line (--▵--) was obtained with the unprepared ATPase of the membrane fraction containing the original lipid component. (b) The added lipid was 125 μg of phosphatidyl serine per ml of the reaction mixture, the enzyme protein being 0.31 mg

given by extrapolation of those lines were the same, approximately 1×10^{-4} M. The broken line connecting triangles was obtained with the unprepared ATPase of the membrane fraction containing the original lipid component. The K_m of this ATPase was again identical to the value given by the enzyme protein combined with exogenous phospholipid.

The K_m of the ATPase activated by phosphatidyl serine was also the same as the value obtained in the presence of crude animal lecithin (Fig. 2b).

The relation between the surface tension and the concentration of lysolecithin in the medium for the ATPase determination (without the enzyme preparation) is shown in Fig. 3a. As the concentration of the phospholipid increased, the surface tension decreased linearly on these plots, and it remained constant beyond 0.03 mM, which indicates the critical micelle concentration of lysolecithin under these conditions. The critical micelle concentration of the lipid in the reaction mixture for the NPPase determination was almost identical with this value.

The relations between the surface tension and the concentration of monodecyl and didecyl phosphates in the medium for the NPPase determination are given in Fig. 3b and c, respectively. The critical micelle con-

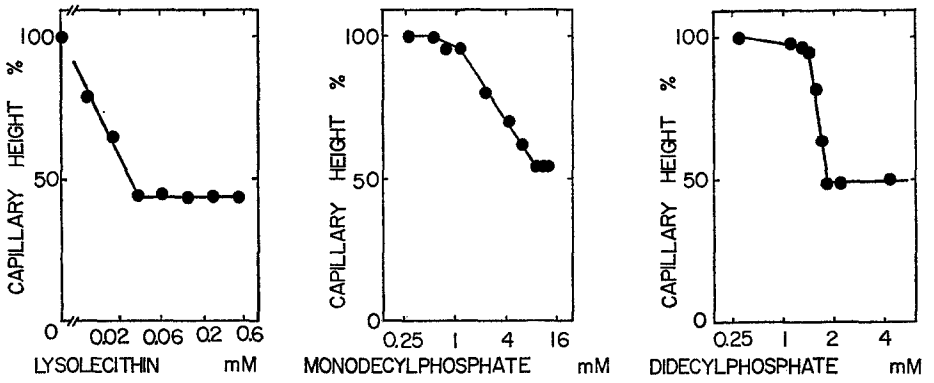


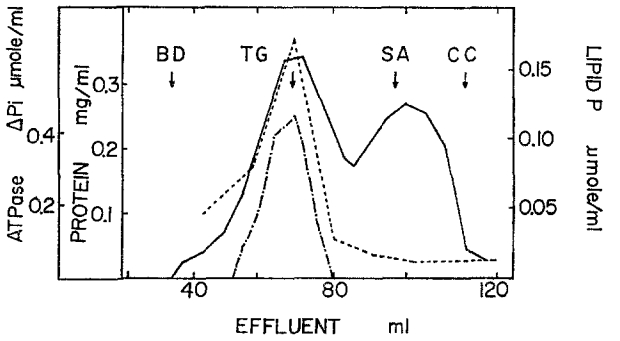
Fig. 3a–c. Lipid concentrations and surface tensions of mediums for enzyme determination. The medium was the same as for Fig. 1 except that the enzyme preparation was omitted. The capillary height in the absence of the phospholipid is arbitrarily set as 100%. (a) Lysolecithin in ATPase medium. (b) Monodecyl phosphate in NPPase medium. (c) Didecyl phosphate in NPPase medium

centrations of the mono- and dialkyl phosphates are 9.5 and 1.9 mM, respectively, under the conditions used.

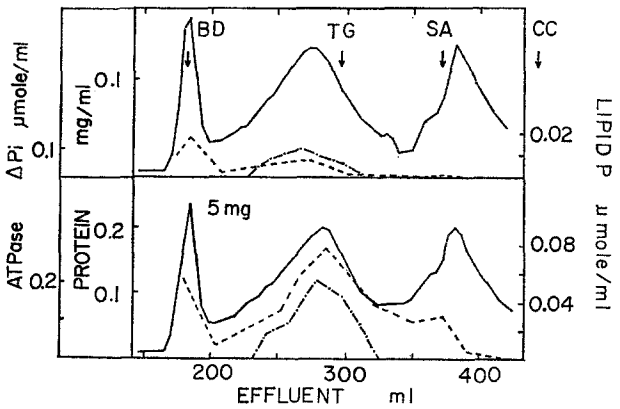
No loss of palmitoyl lysolecithin or phosphatidyl serine was detected after dialyzing in regular cellophane tubing for 4 days at room temperature (Table). Most monodecyl phosphate and a portion of didecyl phosphate, however, diffused away from the cellophane tubing under the same conditions. The fact that those alkyl phosphates dialyzed through a cellophane membrane confirmed that the alkyl phosphates exist in the form of molecular dispersion. It may be because of electrical repulsion between negative charges on the pore walls of the cellophane membrane and those on the lipid molecules that lysolecithin did not diffuse out of the dialysis tubing even though the form of molecular dispersion existed.

Table. *Dialysis of phospholipids and related compounds. The compounds were suspended in 4 ml of the reaction mixture for the ATPase determination from which the enzyme preparation and cysteine were omitted, and dialyzed with constant stirring against 1,000 ml of the same solution at room temperature for 4 days*

Compound	Initial concn. (mM)	Loss owing to diffusion (%)
Monodecyl phosphate	8.4	96
Didecyl phosphate	1.32	15
Palmitoyl lysolecithin	0.50	0
Phosphatidyl serine	0.25 mg/ml	0



a



b

Fig. 4a and b. Chromatography of ATPase preparation. The enzyme preparation obtained at the end of Step 2 was dissolved in 4 ml of water and was centrifuged at 130,000 × *g* for 60 min to remove any contaminating insoluble material. The solid line is for protein content, and the broken line for the lipid phosphate extractable with chloroform-methanol (2:1 v/v) mixture, and the interrupted line for Na, K, Mg-ATPase activity. The molecular weight standards are: *BD* Blue Dextran, *TG* bovine thyroglobulin, *SA* bovine serum albumin, and *CC* cytochrome *c*. (a) The chromatography was carried out at 4 °C on a column of Sepharose 4B (1.5 × 81 cm) equilibrated in a solution containing 50 mM Tris buffer, pH 7.4, 50 mM NaCl, and 0.04 mM lysolecithin. The eluting solvent was identical with the equilibrating solution. The amount of protein was determined by the method of Lowry *et al.* (1951). (b) A column of Bio-Gel A-15m (2.5 × 93 cm) was used in place of Sepharose. Lysolecithin was omitted from the solution, in the experiment shown in the upper half of the figure. Since Na, K, Mg-ATPase was extremely unstable after the removal of lipid, the specific activity of the enzyme was relatively low after the column chromatography without lysolecithin despite of the separation of inactive proteins. In the experiment illustrated in the lower half of the figure, 5 mg of lysolecithin was added to the enzyme preparation prior to the column chromatography; the eluting solvent contained no lysolecithin. The amount of protein was determined by ultraviolet adsorption

The chromatographic pattern of the ATPase preparation on a Sepharose 4B column (equilibrated in a solution containing 0.04 mM lysolecithin) is shown in Fig. 4a. The eluting solvent also contained 0.04 mM lysolecithin. The first peak of protein (solid line) contained the activities of both the ATPase and the NPPase. The phosphate peak of lysolecithin (broken line) overlapped the ATPase peak. The enzyme proteins in the ATPase peak bound lysolecithin while passing through the column. When lysolecithin was omitted from the eluting solvent, the position of the ATPase peak, which corresponds to the position of bovine thyroglobulin (mol. wt. 670,000), shifted to the left side of thyroglobulin, and the apparent particle weight of the proteins of the ATPase peak was estimated to be 1,400,000 (the upper half of Fig. 4b). In order to reproduce the effects lowering the apparent particle weight to the same extent as 0.04 mM lysolecithin, 2.4 mM deoxycholate in the eluting solvent was needed.

Essentially the same effect of lysolecithin on the shift in the apparent particle weight was observed when the enzyme preparation was analyzed by centrifugation on a continuous sucrose density gradient after 1.5 mg of lysolecithin had been added to approximately 8 mg of the enzyme protein prior to centrifugation.

The binding of lysolecithin to the proteins in the ATPase peak was also demonstrated when the mixture of the ATPase preparation and lysolecithin was passed through a column of Bio-Gel A-15m (equilibrated in a solution without lysolecithin) as illustrated in the lower half of Fig. 4b. When 5 mg of lysolecithin was added to the enzyme preparation prior to column chromatography, the most phospholipid appeared with the ATPase peak, which is the second peak on this column. The last small peak of phosphate extractable with the chloroform-methanol mixture was the excess unbound lysolecithin. If the amount of the added lysolecithin was increased to 20 mg, the amount of the phospholipid bound to the proteins in the ATPase peak was unchanged and only the last peak of unbound lysolecithin became larger. When 10 mg of lysolecithin only was chromatographed under the otherwise same conditions, the lipid was eluted in the position of the second lipid phosphate peak.

Didecyl phosphate was also bound to the proteins of the ATPase peak in a manner similar to that of lysolecithin.

Discussion

As the amount of the protein of Na, K, Mg-ATPase and K, Mg-NPPase preparations from bovine cerebral cortex is altered in the reaction medium,

the optimum concentration of phosphatidyl serine, lysolecithin, and didec phosphate for the enzymes activation is changed in such a manner that th ratio of moles of the lipid to the amount of the enzyme protein remair constant. Column chromatography reveals also that only a limited amour of lysolecithin is bound to a protein group showing the enzyme activit and that this amount roughly equals the amount of the lipid required fc optimum activation. Since the lipid-protein ratio for optimum activity i unrelated to the overall structure of the lipid, to the number of hydrc carbon chains in a lipid molecule, and to the cross section area of the lipi molecule per negatively charged group, it is unlikely that the enzym activation occurs because of the conformational change caused by fixatio of the enzyme protein on the surface of a micelle in which the phospholipi molecules are more or less rigidly arranged.

Both the ATPase and the NPPase are activated by lysolecithin an mono- and didecyl phosphates, and attain their maximum activities a concentrations lower than the critical micelle concentrations of the lipi and the related compounds when the amount of the enzyme protein is smal enough. Bovine phosphatidyl serine activates the enzymes also at any con centrations, as demonstrated in our previous study (Tanaka & Sakamoto 1969), and is believed to exist in a micellar state in an aqueous solution From these facts it seems that either a molecular dispersion of the lipi or a micellar form is effective in activation of the membrane enzymes Whereas Green and his co-workers (e.g., Green & Tzagoloff, 1966) hav been emphasizing the indispensability of micellar state of phospholipi in interaction with the protein extracted from heart mitochondria, the stat of phospholipid seems not important in the case of the cell membran protein of bovine cerebral cortex.

The activating effect of phospholipid may possibly be because of it ability to prevent random aggregation of the enzyme proteins, and, indeed an extremely small amount of lysolecithin reduces the apparent particl weight of a group of proteins showing the ATPase activity. The effect o lysolecithin is much stronger than that of deoxycholate, an inhibitor of th enzyme. The precise relationship between the apparent particle weight o the enzyme protein and its activity, however, remains to be seen.

In all three cases of the ATPase activation (tested by crude anima lecithin, phosphatidyl serine, and endogenous membrane lipid), the K_m values obtained are identical. Consequently, it appears as if the combinatio of phospholipid with the enzyme protein in an optimum ratio caused a simple conversion from an inactive form into a fully activated form with no further alterations, and as if there were only a single active form of the

ATPase, which was unrelated to the structures of individual molecules of the activating lipids.

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