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EFFECT OF LIPID ON PROTOHEME FERRO-LYASE

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SUMMARY

1. Protoheme ferro-lyase (EC 4.99.1.1), extracted from chicken erythrocyte stroma with sodium cholate, showed similar properties to those of duck erythrocyte stroma which were reported previously.

2. The preparation extracted with cholate showed high enzyme activity and contained lipids, while that extracted with 0.4 M KCl contained little lipid and showed little enzyme activity. Crude lipids from acetone-dried powder of chicken erythrocyte stimulated heme synthesis when they were added to the 0.4 M KCl-extracted preparation. Crude lipids from acetone-treated egg yolk cake also stimulated heme synthesis. When the lipids were previously digested with *Naja naja* phospholipase A (EC 3.1.1.4), the stimulating effect was increased in the presence of cholate and decreased in the absence of cholate.

3. The effects of purified lipids on 0.4 M KCl-extracted preparation were as follows. Acidic phospholipids, phosphatidylethanolamine and lysophospholipids were effective, while choline-containing lipids were ineffective. Palmitate was effective, whereas tripalmitin was ineffective. In the presence of cholate, choline-containing lipids were effective, acidic lipids and phosphatidylethanolamine were slightly effective or neutral, and lyso-lipids were neutral. The effects of crude lipids could be explained by those of pure phospholipids. Sonication of lipid gave stronger stimulation than manual shaking.

4. Detergency and charge of phospholipids were discussed in relation to the mechanism of protoheme ferro-lyase.

INTRODUCTION

The enzyme protoheme ferro-lyase (EC 4.99.1.1), which catalyzes the combination of Fe^{2+} and protoporphyrin to form heme, is widely distributed in nature[1–5]. The enzyme is a particulate enzyme and could be solubilized only with the aid of detergents such as cholate or Tween. We have studied the enzyme from rat liver and

duck erythrocyte stroma and suggested the participation of lipid in duck erythrocyte enzyme[6,7]. In this paper we report further studies on the role of lipids in protoheme ferro-lyase with special attention to the effect of purified phospholipids. It was found that lipid-free protoheme ferro-lyase with residual enzyme activity was markedly activated by the addition of phospholipids. The relationship between structure and activating effect of phospholipids was investigated.

MATERIALS AND METHODS

Materials

Protoporphyrin was prepared by the method of GRINSTEIN[8]. Lecithin and phosphatidylethanolamine were obtained from egg yolk lipid according to the method of LEA *et al.*[9]. Phosphatidic acid was prepared from lecithin by the treatment with cabbage phospholipase D (EC 3.1.4.4) according to KATES[10]. Lysolecithin and lysophosphatidylethanolamine were obtained by *Naja naja* venom treatment according to MATSUMOTO[11] with slight modifications. Phosphatidylmonoinositol from soybean, cardiolipin from ox heart, sphingomyelin from human erythrocyte and sphingosylphosphorylcholine were generous gifts from Kikkoman Syoyu Co. Ltd., Sumitomo Chemical Industry, Prof. YAMAKAWA of Tokyo University and Prof. TAKETOMI of Shinsyu University, respectively. Phosphatidylserine, cabbage phospholipase D and *Naja naja* venom were products of Nutritonal Biochem. Co., Boeringer and Sigma, respectively. The purity of lipids was checked by thin-layer chromatography. All other compounds were of reagent grade.

Preparation of the enzyme

The enzyme was prepared from the hemoglobin-free stroma of chicken erythrocytes according to the method previously reported[1] for duck erythrocyte stroma. Ten volumes of acetone were added to the stroma at −10°. After vigorous mixing and homogenization in the cold, the supernatant was removed by centrifugation. The residue was washed twice with cold acetone and dried. The acetone-dried powder was extracted with 15 vol. of either a solution containing 0.05 M Tris buffer (pH 8.0), 0.1 M KCl and 1% sodium cholate or a solution containing 0.05 M Tris buffer (pH 8.0) and 0.4 M KCl, for 15 hours at 4°. The extract was centrifuged for 15 min at 10 000 × *g* and the supernatant was used as the enzyme solution.

Assay of enzyme

The standard incubation mixture contained 0.1 μmole of $FeCl_3 \cdot 6H_2O$ labeled with $4 \cdot 10^5$–$5 \cdot 10^5$ counts/min $^{59}FeCl_3$, 0.1 μmole of protoporphyrin in 0.15 M $KHCO_3$, 10 μmoles of cysteine, 100 μmoles of 2-mercaptoethanol, 150 μmoles of Tris buffer (pH 8.3), 0.5 ml of enzyme preparation (1.0–1.4 mg protein) and lipid when added in a total vol. of 2.0 ml. Incubation was carried out anaerobically under N_2 in a Thunberg tube for 30 min at 37°. The reaction was started by tipping $FeCl_3$ from the side-arm, and was stopped by chilling in an ice-water bath. After the incubation, carrier hemoglobin was added and ^{59}Fe-labeled hemin was crystallized, counted and estimated as reported previously[1]. Unless otherwise stated, lipid emulsion was prepared by dispersing lipid in water with Otake sonifier at 0° for 2 min at 20 kcycles and added to the incubation mixture immediately after sonication.

TABLE I

SPECIFIC ACTIVITY OF FRACTIONS DURING PURIFICATION

150 ml of chicken erythrocytes were used. Stroma and acetone powder were extracted with 1% sodium cholate. The supernatants were used as enzyme preparation.

Fraction	*Protein (mg)*	*Units**	*Specific activity (units/mg protein)*
Hemolysate	43 428	1129	0.03
Erythrocyte stroma (cholate extract)	367	1574	4.28
Acetone powder (cholate extract)	323	950	2.94

* A unit of enzyme activity was defined as the amount of enzyme required to produce 1 nmole of hemin per 30 min under the test conditions.

Analytical methods

Lipid phosphorus was determined by the method of CHEN *et al.*[12]. Protein was measured according to LOWRY *et al.*[13].

RESULTS

Purification of the enzyme

The specific activity of the enzyme during the purification procedure is shown in Table I. By eliminating the soluble proteins, chiefly hemoglobin, a 140-fold increase of the specific activity was observed. The preparation from acetone-dried powder always showed slightly lower specific activity than that from stroma.

Properties of the cholate-extracted enzyme

When the cholate extract from acetone-dried powder was used as the enzyme, the enzyme reaction proceeded linearly with time up to 40 min. The effect of pH is given in Fig. 1. The rate of incorporation of ^{59}Fe into hemin was determined with

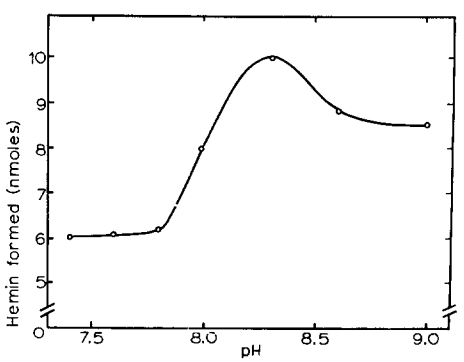

Fig. 1. Effect of pH on the protoheme ferro-lyase reaction. Assay conditions were as described in MATERIALS AND METHODS. Sodium cholate extract was used as the enzyme preparation.

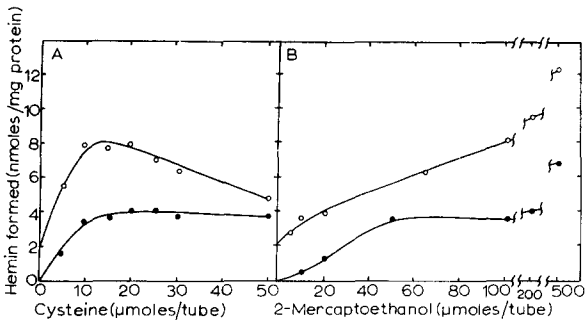

Fig. 2. Effect of cysteine and 2-mercaptoethanol on the protoheme ferro-lyase reaction. Assay conditions were the same as in Fig. 1. A. Effect of cysteine in the presence (○—○) and in the absence (●—●) of 100 μmoles of 2-mercaptoethanol. B. Effect of 2-mercaptoethanol in the presence (○—○) and absence (●—●) of 10 μmoles of cysteine.

various amounts of enzyme. Linearity between the amount of enzyme and the rate of reaction was observed at enzyme concentrations higher than 0.15 mg protein per ml incubation mixture. The Michaelis constant for Fe^{2+} was $5.5 \cdot 10^{-6}$ M and that for protoporphyrin was $2.3 \cdot 10^{-5}$ M. The effects of cysteine and 2-mercaptoethanol are shown in Fig. 2. In our present system, a decrease in heme formation was observed with aerobic conditions.

Effect of crude lipids on KCl-extracted enzyme activity

The preparation extracted with sodium cholate showed high enzyme activity and contained lipids, while the preparation extracted with 0.4 M KCl contained little lipid and little enzyme activity, as shown in Table II. The amount of protein extracted in both preparations was approximately the same. Lipids extracted with cholate were analyzed by thin-layer chromatography. The main components were lecithin and phosphatidylethanolamine. Sphingomyelin was also detected. In order to clarify the effect of lipid, crude lipids from chicken erythrocyte stroma and from egg yolk were tested for their accelerating effects on the enzymic combination of iron and protoporphyrin.

The effects of crude lipids, extracted with choloroform–methanol (1:1, v/v)

TABLE II

COMPARISON OF ENZYME PREPARATIONS EXTRACTED WITH SODIUM CHOLATE OR 0.4 M KCl

Acetone powder of chicken erythrocyte stroma was extracted with 15 vol. of 0.1 M KCl–1% sodium cholate solution or with 0.4 M KCl for 15 h at 4°. The supernatant was analyzed and assayed for the enzymic activity.

Medium	*Protein (mg/ml)*	*Phosphorus of phospholipids (μmoles/ml)*	*Activity (units/mg protein)*
0.1 M KCl *plus* 1% sodium cholate	2.8	2.07	5.38
0.4 M KCl	2.0	0.01	0.18

from acetone-dried powder of chicken erythrocyte stroma, on the 0.4 M KCl extract are shown in Table III. A 10-fold stimulation was observed in the absence of cholate, and some activation was observed in the presence of cholate. Lipids extracted directly with chloroform–methanol and those extracted with cholate and then chloroform–methanol showed almost the same pattern on thin-layer chromatography, consisting mainly of lecithin and phosphatidylethanolamine. Cholate alone had some stimulating effect.

TABLE III

EFFECT OF LIPID FROM ACETONE POWDER OF CHICKEN ERYTHROCYTE STROMA ON PROTOHEME FERRO-LYASE ACTIVITY

Lipid was prepared from acetone powder as described in the text. Lipid and sodium cholate were added to the standard incubation mixture described in the text. The amount of lipid was determined gravimetrically.

Lipid (mg)	*Sodium cholate (mg)*	*Hemin formed (nmoles/mg protein per 30 min)*
0	0	0.45
0.83	0	1.72
1.66	0	4.71
0	5.0	3.15
0.83	5.0	7.58
1.66	5.0	6.61

The lipids extracted with ethanol from acetone-treated egg yolk cake were digested with *Naja naja* venom and their degradation products were analyzed by thin-layer chromatography. The main components of the lipids before the venom treatment were lecithin and phosphatidylethanolamine. After the treatment, the amount of lecithin was decreased, phosphatidylethanolamine disappeared, lysolecithin and lysophosphatidylethanolamine were increased and fatty acids appeared as shown in Fig. 3. The effects of egg yolk lipids were altered by the venom digestion. The results are shown in Table IV. Before the digestion, lipids accelerated heme synthesis slightly, but with the addition of cholate, the activation was increased. After the treatment, the accelerating effect of lipids was increased in the absence of cholate, while in its presence the effect was decreased. This change in activating effect by phospholipase treatment suggested that the effect was due to the presence of phospholipids.

Effects of phospholipids on KCl-extracted enzyme activity

As the participitation of phospholipids in the enzymic reaction was suggested, effects of purified lipids, especially of phospholipid on the lipid-free 0.4 M KCl-extracted enzyme, were examined.

a) Acidic phosphilipids and phosphatidylethanolamine

The effects of phosphatidylethanolamine, cardiolipin, phosphatidic acid, phosphatidylinositol and phosphatidylserine are shown in Fig. 4A. The results are expressed in relative activities and the value without lipid is taken as a standard. Without lipid

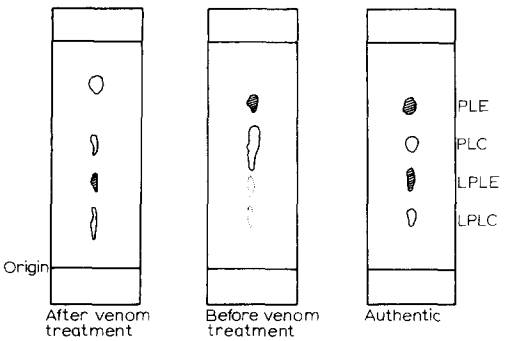

Fig. 3. Thin-layer chromatogram of lipids from egg yolk cake before and after the venom digestion on silica gel G. Solvent: chloroform–methanol–water (70:30:5, by vol.). Spots were detected with 50% H_2SO_4. Shaded areas represent ninhydrin-positive spots. PLE, phosphatidylethanolamine; PLC, lecithin; LPLE, lysophosphatidylethanolamine; LPLC, lysolecithin.

0.19 nmole of heme was formed per mg of enzyme protein under the standard conditions. With the addition of 1.73 μmoles of phosphorus of phosphatidylethanolamine, 4.2 nmoles of protoheme were synthesized. All acidic phospholipids tested and phosphatidylethanolamine strongly activated heme synthesis at low concentrations, while at higher concentrations the activating effects were decreased. The concentration of lipid that showed maximum stimulation was lower when lipids of stronger acidity were used.

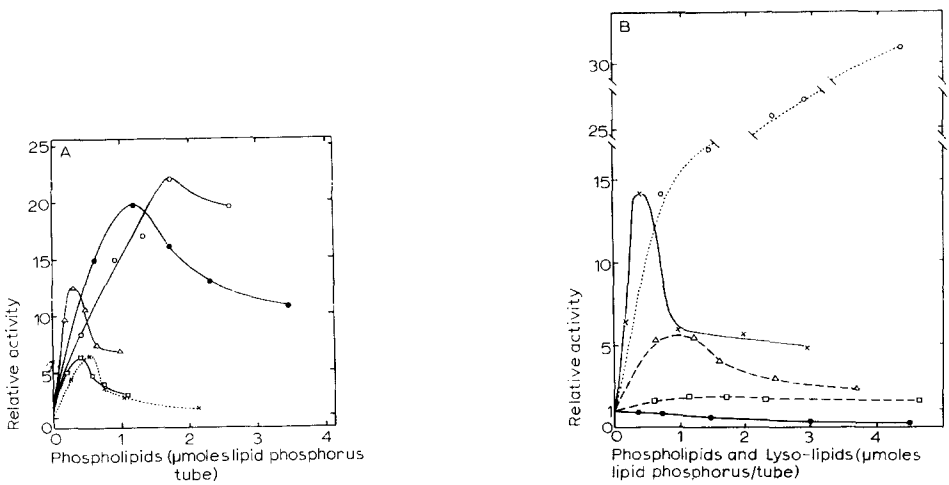

Fig. 4. A. Effect of acidic phospholipids and phosphatidylethanolamine on the protoheme ferro-lyase reaction. The results are expressed in relative activities and the value without lipid is taken as a standard. Assay conditions were as described in MATERIALS AND METHODS. 0.4 M KCl extract was used as the enzyme preparation. ○, phosphatidylethanolamine; ●, cardiolipin; △, phosphatidylserine; □, phosphatidylinositol; ×, phosphatidic acid. B. Effect of choline-containing phospholipids and lyso-phospholipids on the protoheme ferro-lyase reaction. Without lipid, 0.13 μmoles of heme synthesis was observed per mg of enzyme protein. ○, lysophosphatidylethanolamine; ×, lysolecithin; △, sphingosylphosphorylcholine; □, sphingomyelin; ●, lecithin.

b) Choline-containing phospholipids

The effects of lecithin and sphingomyelin are shown in Fig. 4B. These choline-containing lipids were ineffective in stimulating heme synthesis.

c) Lyso-phospholipids

The effects of lysolecithin, lysophosphatidylethanolamine and sphingosylphosphorylcholine are shown in Fig. 4B. Lyso-lipids were potent accelerators. Choline-containing phospholipids which were ineffective in stimulating heme synthesis showed activating effects when they were degradated to lysocompounds. As in the case of acidic phospholipids, the activating effect was decreased at higher concentrations of lyso-lipids.

d) Phospholipids in the presence of cholate

In order to investigate the high activity of cholate-extracted enzyme and the change of activating effects of crude egg yolk lipids on the 0.4 M KCl-extracted enzyme in the presence and in the absence of cholate, the effects of phospholipids in the presence of cholate were examined. The results are shown in Fig. 5. The choline-containing lipids, which were neutral in the absence of cholate, stimulated heme synthesis in the

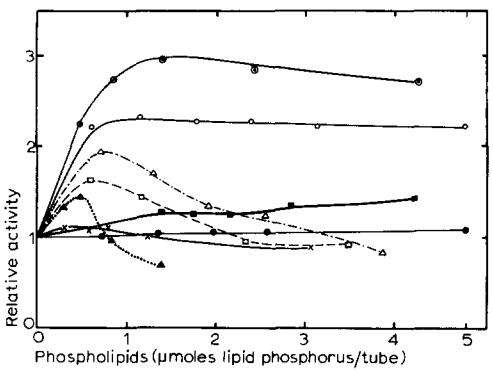

Fig. 5. Effect of phospholipids on the protoheme ferro-lyase reaction in the presence of sodium cholate. The results are expressed in relative activities and the value without lipid is taken as a standard. Assay conditions were as described in Fig. 4A except that 5 mg per tube of sodium cholate was added. Without lipid heme synthesis in the range 1.2–2.0 nmoles was observed. ⊙, lecithin; ○, sphingomyelin; △, cardiolipin; □, phosphatidylethanolamine; ▲, phosphatidylinositol; ■, lysophosphatidylethanolamine; ●, sphingosylphospholylcholine; ×, lysolecithin.

presence of cholate. The acidic phospholipids, and phosphatidylethanolamine, strong activators in the absence of cholate, were slightly stimulative or neutral in the presence of cholate. Lyso-lipids, potent stimulators in the absence of cholate, were neutral in the presence of cholate.

Effect of triglyceride and fatty acid on KCl-extracted enzyme activity

The effects of palmitate and tripalmitin, examples of natural lipids other than phospholipids, are shown in Fig. 6. Because of the difference in the solubility between palmitate and tripalmitin in the reaction medium, concentration ranges tested were

TABLE IV

CHANGE OF EFFECT OF EGG YOLK LIPID ON ENZYME ACTIVITY BY PHOSPHOLIPASE TREATMENT

Ethanol extract from acetone-treated egg yolk cake was dried and emulsified in 2 ml of 0.1 M glycylglycine buffer (pH 7.3). To the emulsion were added 1 ml of 0.1% venom in the same buffer and 0.1 ml of 0.2 M calcium acetate. The mixture was incubated for 15 h at 37° and was evaporated to dryness. All evaporation procedures were carried out under a stream of N_2. The dried material was extracted with ethanol and dried. The residue was extracted with *n*-hexane and dried. The lipid thus obtained was dissolved in ethanol, and distilled water was added to ensure a final concn. of lipid of about 5 mg per ml. The ethanol was evaporated and aqueous emulsion was added to the incubation mixture. The amount of lipid was determined gravimetrically.

	Lipid (mg)	*Venom-treated lipid (mg)*	*Sodium cholate (mg)*	*Hemin formed (nmoles/mg protein per 30 min)*
Expt. 1	0	0	0	0.20
	1.10	0	0	0.27
	0	1.26	0	1.25
Expt. 2	0	0	5.0	0.65
	0.55	0	5.0	3.14
	1.10	0	5.0	3.09
	0	0.63	5.0	1.11
	0	1.26	5.0	0.98

changed. Triglyceride was ineffective. Fatty acid was slightly effective but the effect was small compared with that of phospholipids.

Dependence of activation of enzyme on dispersion state of lipid

Lipid was made soluble by organic solvent and water was added to the solution. Organic solvent was removed by evacuation under N_2 and lipid emulsion was obtained by vigorous shaking. The effects on the enzyme activity of phosphatidylethanolamine

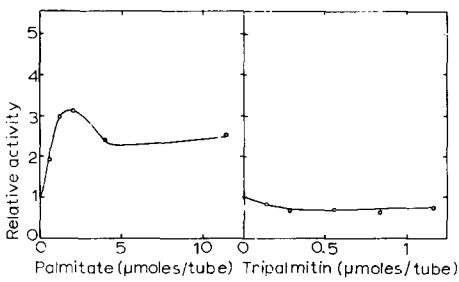

Fig. 6. Effect of palmitate and tripalmitin on the protoheme ferro-lyase reaction. The results are expressed in relative activities and the value without palmitate or tripalmitin is taken as a standard. Assay conditions were as described in Fig. 4 A.

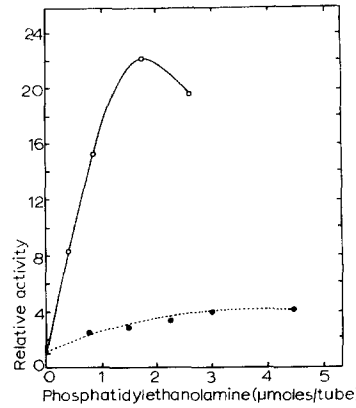

Fig. 7. Effect of phosphatidylethanolamine dispersed by sonication or manual shaking. Methods of lipid dispersion are described in the text. Assay conditions were as described in Fig. 4A. ○, sonication; ●, manual shaking.

solution obtained by this method and that by sonication were compared. The results are expressed in relative activities in Fig. 7. The values by sonication are the same as those in Fig. 4. The sonicated preparation stimulated the enzyme activity about 5 times as much as the substance prepared by shaking.

DISCUSSION

The protoheme ferro-lyase extracted with cholate from acetone-dried powder of chicken erythrocyte stroma was shown to have similar properties to those of duck erythrocyte stroma[1]. Michaelis constants for Fe^{2+} and protoporphyrin and the pH-response curve of the enzyme from chicken erythrocytes were almost the same as those from duck erythrocytes. Both enzymes are considered to be members of the enzyme system of hemoglobin synthesis. Protoheme ferro-lyase has been found in liver mitochondria[2,3] and in bacteria[4]. It may participate in the synthesis of heme enzymes. Recently heme synthesis by the enzymes of plant chloroplasts[5] was reported.

Sulfhydryl compounds stimulated the enzyme activity. PORRA AND JONES[14] reported that sulfhydryl reagent acted as reductant to maintain the iron in the ferrous state. Our results in Fig. 2 showed that both cysteine and 2-mercaptoethanol under anaerobic conditions were required to obtain the maximum activity. In addition to maintaining the iron in the ferrous state sulfhydryl reagents have some other effects, for instance they protect the sulfhydryl group of the enzyme protein[15].

The present experiments with purified phospholipids showed that acidic phospholipids, phosphatidylethanolamine and lyso-phospholipids stimulated the activity of 0.4 M KCl-extracted enzyme rather than a specific lipid. Choline-containing phospholipids were ineffective. MAZANOWSKA and her co-workers[16] reported the influences of lecithin, phosphatidylethanolamine and phosphatidic acid on the formation of zinc protoporphyrin and heme by *Rhodopseudomonas spheroides* chromatophore and mitochondria of guinea pig liver. They observed that lecithin had little effect, while phosphatidic acid exhibited a stimulative effect. The effects of these two lipids were in agreement with those of ours. Phosphatidylethanolamine stimulated the enzyme activity strongly in our system in contrast to the results of MAZANOWSKA *et al.*[16]. The discrepancy between our results and those of MAZANOWSKA *et al.*[16] might be due to different methods of lipid dispersion, different enzyme sources, or both. In dispersing lipids, they used manual shaking, while we used sonication. Stronger stimulation was observed with sonication than with manual shaking, as shown in Fig. 7.

Irrespective of the source, all the enzymes reported so far were particle bound, and some of them were solubilized with the aid of detergents. The results in Table III suggest that the high enzymic activity of cholate extract compared with the low activity of the 0.4 M KCl extract is related to the presence of lipid in the cholate-extracted preparation. Lecithin, phosphatidylethanolamine and sphingomylin, which were the main components of the cholate-extracted enzyme preparation of chicken erythrocytes could explain the high activity of the preparation. In the presence of sodium cholate, choline-containing lipids, and to a lesser degree, phosphatidylethanolamine were effective on the 0.4 M KCl-extracted enzyme. In this case sodium cholate is considered to have the following effects: it solubilizes phospholipids from acetone-dried powder, stimulates the reaction by itself and activates the enzyme working together with lecithin, sphingomyelin and phosphatidylethanolamine.

The activating effects of crude lipid on the 0.4 M KCl extract could be explained by the combination of individual pure phospholipids. The crude lipids obtained from acetone-dried powder of chicken erythrocyte stroma or acetone-treated egg yolk consisted mainly of lecithin and phosphatidylethanolamine. These crude lipids, when added to the 0.4 M KCl-extracted preparation, stimulated heme synthesis both in the presence and absence of sodium cholate. In the absence of cholate, the activating effect of crude lipids could be due to the phosphatidylethanolamine in the lipids, and in the presence of cholate the effect could be attributed to the lecithin and phosphatidylethanolamine. In the absence of cholate, the role of minor phospholipids with a strong accelerating effect, in addition to phosphatidylethanolamine, could not be excluded. Between the crude lipid from chicken erythrocyte stroma and that from egg yolk, a difference in the activating effects was observed. This could be explained by the smaller amount of phosphatidylethanolamine in egg yolk lipid compared with that in the lipid from chicken erythrocyte stroma. The change in activating effects by the venom treatment also suggests the participation of phospholipids. The venom digestion converted lecithin and phosphatidylethanolamine to the corresponding lyso-compounds and fatty acids. Before the treatment, lecithin and phosphatidylethanolamine might play a dominant role in the presence and absence of cholate, respectively. After the treatment, the effects of the lipid were similar to that of lyso-compounds, which were strong accelerators in the absence of cholate and were neutral in the presence of cholate.

Considering the stimulating effects of acidic lipids and lyso-lipids, charge and detergency seem to be important factors in the activation of the enzyme. The strong accelerating activity of acidic phospholipids suggests that the negative charge of the lipid molecule plays a role in activating the reaction. The inertness of choline-containing lipids may be attributed to the positive charge of choline which neutralized the effect of the negative charge of phosphoric acid. The stimulative effects of choline-containing lipid in the presence of cholate might be explained by the hypothesis that the negative charge of cholate diminished the inhibitory effect of positive charge of choline molecule. Effects of charge of lipids on the activities of phospholipase B (EC 3.1.1.5) and C (EC 3.1.4.3) were extensively studied by BANGHAM AND DAWSON[17,18], who showed that the activation of phospholipase by charged molecules was best explained by the electrostatic reaction between enzyme and substrate at an interface. In our system, the phosphoric acid anion of acidic phospholipids might activate the enzymic reaction by attracting Fe^{2+}. The importance of detergent effect of phospholipids is suggested by the potent stimulating influence of lyso-lipids. The accelerating effect of sodium cholate may also be due to its detergency. Detergent effects of phospholipids might be explained by their nonpolar interaction with protoporphyrin through which protoporphyrin was available for the enzyme, though we have had no evidence of this. According to PORRA AND JONES[19], a synthetic neutral detergent, Emazol, solubilized protoporphyrin at neutral pH. They observed absorption spectra of porphyrin in the presence of the detergent. With our experimental conditions, however, no sharpening of the spectra was observed in the presence of effective phospholipid. The strong activity of lysolecithin, which contained the positive charge of the choline molecule, and was expected to have little favorable charge effect, might be attributed to its detergency. WANG[20] reported that the rate of nonenzymic combination of metal ions with porphyrins was strongly solvent dependent. In order for the

porphyrin molecule to "swallow" the metal ion, most of the solvation of the metal must be peeled off. In the enzymic reaction, the hydrophobic part of the lipid molecule might play the role of a favorable solvent. The hydrophobic phosphoric acid group might help the enzyme molecule to attract the Fe^{2+} to its active center. The iron cation would be further transferred to the hydrophobic region of lipid and enzyme, stripped of solvated water and "swallowed" by the protoporphyrin molecule.

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