

## HISTOCHEMICAL STUDIES ON THE LIPID REQUIREMENT FOR THE OXIDATIVE ENZYME ACTIVITIES

### III. STUDIES ON THE EFFECTS UPON THE OXIDATIVE ENZYME ACTIVITIES OF PRE-TREATMENT WITH PHOSPHOLIPASES

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(Received for Publication June 20, 1965)

#### INTRODUCTION

As has been clarified in the previous two papers, ie. Parts I and II, phospholipids are very important substances for the manifestation of the activities of oxidative enzymes, such as cytochrome oxidase, DPNH diaphorase and so on, which are closely connected with mitochondrial structures. The Lipid requirement of the oxidative enzymes for the catalytic activities may indicate a relationship between the localization of the enzymes and the intracellular ultrastructures. Therefore, in clarifying the functions of the intracellular ultrastructures, the finding that certain oxidative enzymes require phospholipids for the catalytic activities may be important.

On the other hand, the enzymes catalyzing the hydrolysis of phospholipids are known and they are designated as phospholipases. The phospholipases are divided into four groups, such as phospholipase A, phospholipase B, phospholipase C and phospholipase D, according to the sites of the substrate molecule to be hydrolyzed.

Since phospholipids work as important factors in certain intracellular oxido-reductive reactions it may be expected that the activity of some oxidative enzymes will be altered by hydrolysis of intracellular phospholipids on pre-treatment of tissue sections with phospholipases.

Although phospholipids were proved to be important by the previous experiments, it was not known as to what parts of their molecular structures are valuable in the oxido-reductive reactions. Which parts of the phospholipid molecule are indispensable for certain oxidative enzymes may be

clarified by carrying out a comparative study on the relationship between the changes in the enzyme activity caused by pre-treatment with phospholipases and the sites of the substrate molecule to be hydrolyzed by phospholipases.

#### MATERIALS AND METHODS

Animal tissues used in the present experiments were obtained from normal adult male albino rats of Wistar strain weighing from 140~160 g. Liver, kidney, stomach, skeletal muscle and heart were used for histochemical observations. Tissue sections were prepared from tissue blocks fixed in chilled absolute acetone and embedded in soft paraffin as described in the previous papers.

Enzymes used for the pre-treatment of the tissue sections before incubation were phospholipase A, phospholipase C and phospholipase D. These enzymes were purchased from the Sigma Chemical Company.

The solution of each of the enzymes was prepared as follows:

(1) Phospholipase A

This enzyme which was extracted from *Naja Naja* and purified, was dissolved in Sørensen's phosphate buffer (pH 7.3) containing sucrose at a concentration of 0.25 M. The final concentration of the enzyme solution was 0.1%. This solution was used for pre-treatment.

(2) Phospholipase C

This enzyme which was extracted from *Cl. welchii* and purified, was dissolved in Sørensen's phosphate buffer (pH 7.3) containing sucrose at a concentration of 0.25 M. The final concentration of the solution was 0.2%. The enzyme solution thus prepared was used for pre-treatment.

(3) Phospholipase D

This enzyme prepared from cabbage was dissolved in Sørensen's phosphate buffer (pH 7.3) containing sucrose at a concentration of 0.25 M. The final concentration of the solution was 0.2%. The enzyme solution thus prepared was used for pre-treatment.

The enzymes observed with histochemical methods were as follows; cytochrome oxidase, DPNH diaphorase, TPNH diaphorase, succinic dehydrogenase, lactic dehydrogenase and malic dehydrogenase.

The pre-treatment of the tissue sections with enzyme solutions was performed as described below.

(1) The case of cytochrome oxidase

Tissue sections of 6~7 $\mu$  thickness prepared as in the previous experiments were pre-treated for two hours at 37°C with each of the phospholipase solu-

tions after dewaxing with xylene, which was removed by absolute acetone, and washing in distilled water.

After the completion of pre-treatment the tissue sections were incubated in the substrate solution.

The histochemical methods applied were the modified tetrazolium methods described in the papers of the part II.

(2) The other dehydrogenases and diaphorases

The tissue sections prepared as in the case of cytochrome oxidase were pre-treated for two hours at 37°C with each of the phospholipase solutions without dewaxing. After the completion of the pre-treatment the tissue sections were incubated in the substrate mixture for each of the dehydrogenases and diaphorases. In the cases of the dehydrogenases and diaphorases lipid was not added to the substrate mixtures.

The compositions of the substrate mixtures for each of the diaphorases and dehydrogenases were as follows ;

(1) The substrate mixture for DPNH diaphorase

|                                      |          |
|--------------------------------------|----------|
| 0.1% Nitro-blue tetrazolium          | 0.3 ml.  |
| Sørensen's phosphate buffer (pH 7.4) | 0.5 ml.  |
| DPNH                                 | 5~10 mg. |
| Distilled water                      | 0.2 ml.  |

(2) The substrate mixture for TPNH diaphorase

|                                      |          |
|--------------------------------------|----------|
| 0.1% Nitro-blue tetrazolium          | 0.4 ml.  |
| Sørensen's phosphate buffer (pH 7.4) | 0.5 ml.  |
| TPNH                                 | 5~10 mg. |
| Distilled water                      | 0.2 ml.  |

(3) The substrate mixture for succinic dehydrogenase

|                                      |              |
|--------------------------------------|--------------|
| 0.2 M Sod. succinate                 | 0.4 ml.      |
| 0.1% Nitro-blue tetrazolium          | 0.3 ml.      |
| Sørensen's phosphate buffer (pH 7.4) | 0.4 ml.      |
| 0.1% phenazine methosulfate          | 0.05~0.1 ml. |
| Distilled water                      | 0.1 ml.      |

(4) The substrate mixture for lactic dehydrogenase

|                                      |          |
|--------------------------------------|----------|
| 0.3 M Sod. lactate                   | 0.4 ml.  |
| 0.1% Nitro-blue tetrazolium          | 0.3 ml.  |
| Sørensen's phosphate buffer (pH 7.4) | 0.4 ml.  |
| DPN                                  | 5~10 mg. |

The pH of 0.3 M sod. lactate solution was adjusted to 7.0 with sod. hydroxide.

## (5) The substrate mixture for malic dehydrogenase

|                                      |          |
|--------------------------------------|----------|
| 1.0 M Sod. malate                    | 0.3 ml.  |
| 0.1% Nitro-blue tetrazolium          | 0.3 ml.  |
| Sörensen's phosphate buffer (pH 7.4) | 0.3 ml.  |
| DPN                                  | 5~10 mg. |
| Distilled water                      | 0.2 ml.  |

The pH of 1.0 M sod. malate solution was adjusted to 7.2 with 0.1 N sod. hydroxide.

## RESULTS

The activity of cytochrome oxidase was almost lost by the pre-treatment with phospholipase A and no microscopical staining was seen. Microscopically occasional faint staining in the cytoplasm of the hepatic and myocardial cells only was seen.

However, the activity of cytochrome oxidase was only slightly reduced by the pre-treatment with phospholipase C and phospholipase D. The effects of phospholipase C and phospholipase D upon the enzyme activity were not conspicuous.

The activity of succinic dehydrogenase was considerably reduced by the pre-treatment with phospholipase A. However, the degree of the reduction of the succinic dehydrogenase activity was relatively lower than that of the cytochrome oxidase activity.

Only a fair reduction was seen in the cases of pre-treatment with phospholipase C and phospholipase D. The degree of the reduction of succinic dehydrogenase activity in each of the pre-treatments with three different hydrolyzing enzymes was somewhat more conspicuous in the case of phospholipase A than in those of the other two cases. In general the reduction of succinic dehydrogenase in hepatic cells and gastric mucous membrane was considerable in the case of the pre-treatment with phospholipase A. The effects of phospholipase A upon the activity of DPNH diaphorase was considerably strong, specially in the hepatic cells, gastric mucous membrane and the epithelial cells of the proximal parts of the urinary tubules. The activity of DPNH diaphorase in the epithelial cells of collecting tubules of kidney was almost unaffected by the pre-treatments with phospholipase A.

The effects of phospholipase C and phospholipase D upon the DPNH diaphorase were weak and the reduction of the enzyme activity was slight in general. The effects of phospholipase A upon the TPNH diaphorase activity were conspicuous and the weak irregular positive staining was seen only in

the epithelial cells of collecting tubules of kidney and in gastric mucous membrane. The effects of phospholipase C and phospholipase D upon the TPNH diaphorase activity were not strong.

The effects of phospholipase A upon the activity of lactic dehydrogenase were strong and macroscopically, positive staining was almost absent. Weak irregular staining was seen under the microscope only in the epithelial cells of collecting tubules of kidney and the hepatic cells.

The effects of phospholipase C and phospholipase D upon the enzyme activity were not strong.

The effects of phospholipase A upon the activity of malic dehydrogenase were strong. Macroscopic positive staining was almost absent in any tissue sections. Only faint positive staining was seen in the cells of heart muscle and the epithelial cells of the collecting tubules of kidney.

The effects of phospholipase C and phospholipase D upon the activity of malic dehydrogenase were not serious.

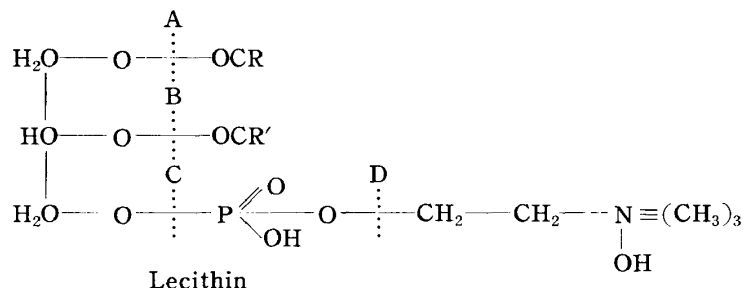
The effects of phospholipases upon the enzyme activities can be summarized as follows.

|                        | Phospholipase A | Phospholipase C | Phospholipase D |
|------------------------|-----------------|-----------------|-----------------|
| Cytochrome oxidase     | ‡‡              | ± ~ +           | ± ~ +           |
| DPNH diaphorase        | ‡‡              | +               | ± ~ +           |
| TPNH diaphorase        | ‡‡              | ± ~ +           | ± ~ +           |
| Succinic dehydrogenase | ‡‡              | +               | ± ~ +           |
| Lactic dehydrogenase   | ‡‡              | ± ~ +           | ± ~ +           |
| Malic dehydrogenase    | ‡‡              | ± ~ +           | ± ~ +           |

‡‡ Strong effect      ‡ Moderate effect  
 + Weak effect      ± Almost no effect

### DISCUSSION

As is well known, according to the classification after Zeller<sup>1)</sup> phospholipase A, phospholipase B, phospholipase C and phospholipase D hydrolyze, respectively, certain sites of phospholipids, as will be indicated below.



As an example the hydrolysis of lecithin with each of the phospholipases is illustrated above. A, B, C and D in the illustration above indicate the sites of the lecithin molecule hydrolyzed by phospholipase A, phospholipase B, phospholipase C and phospholipase D, respectively. Phospholipids, such as lecithin and cephalin are contained in great quantities in living cells and constitute the cellular membraneous structures with protein in a form of lipoprotein.<sup>2)</sup> Fatty acids which are combined to the glycerol molecule at the sites of  $\alpha$  and  $\beta$ , are almost unsaturated high fatty acids such as oleic acid. As to the substrate specificities of phospholipase A phospholipase B, according to Zeller's classification phospholipase A acts only upon the ester linkage at the site of  $\alpha$  and phospholipase B acts only upon the ester linkage at the site of  $\beta$ . However, according to the investigation by Hanahan et al<sup>3), 4)</sup> phospholipase A has been said to be able to hydrolyze the ester linkage at the site of  $\beta$  too, if the fatty acid which is combined to the  $\beta$  position is an unsaturated one. Since the fatty acids of natural phospholipids constituting the cellular membraneous structures are almost all unsaturated fatty acids, it may be reasonable to assume that on the pre-treatment of the tissue sections with phospholipase A the ester linkages not only at the  $\alpha$  position but also at the  $\beta$  position were hydrolyzed at a time.

The activity of cytochrome oxidase was greatly reduced by pre-treatment with phospholipase A, but pre-treatment with either phospholipase C or phospholipase D did not cause any serious changes in the cytochrome oxidase activity. These facts are very important and interesting for clarifying the biological functions of phospholipids. The inhibitory effect of phospholipase A upon the cytochrome oxidase activity may be reasonably assumed to be due to the hydrolysis of ester linkages at both  $\alpha$  and  $\beta$  positions of phospholipids which are indispensable for manifestation of the activity. Since the pre-treatment with phospholipase C or phospholipase D did not cause serious changes in the activity, it may be assumed that phosphoric acid, choline, cholamine and so on which are combined at the  $\alpha'$  position are not essential components of phospholipids for the enzyme activity.

The most important components of phospholipids may be fatty acids combined to  $\alpha$  and  $\beta$  positions and they play important roles in the oxidoreductive reactions in the living cells.

In the cases of diaphorases and dehydrogenases almost the same trends were recognized. That is to say, the inhibitory effects of phospholipase A upon the enzyme activities were much stronger than those of phospholipase C and phospholipase D. These histochemical findings may be naturally regarded

as to indicate that the most important components of phospholipids are the fatty acids combining with  $\alpha$  and  $\beta$  positions of glycerol.

It has been said that the most important characteristic property of phospholipids is the formation of duplicate structure with the hydrophilic radical and the lipophilic radical.<sup>2)</sup> Therefore, phospholipids are able to constitute stable intracellular membraneous structures with protein. The catalytic activities of certain oxidative enzymes may be largely dependent upon the lipophilic radical. Because, the enzyme activities were greatly reduced by pre-treatment with phospholipase A in comparing with those of phospholipase C or D.

In 1938, Wooldringe and Higginbottom<sup>5)</sup> reported that the  $\alpha$ -toxin of *Cl. welchii* inhibited the activity of succinic oxidase of mitochondria and in 1941 Macfarlane and Knight<sup>6)</sup> demonstrated that the  $\alpha$ -toxin of *Cl. welchii* was phospholipase. Thereafter, the relations between the mitochondrial oxidative enzymes and phospholipids were noticed with keen interest and various experiments have been done.

Nygaard and Sumner<sup>7)</sup> have reported that the activity of succinic dehydrogenase of mammalian tissues is greatly reduced by phospholipase A. Braganca and Quastel<sup>8), 9)</sup> have studied the inhibitory effects upon the activities of cytochrome oxidase, succinic dehydrogenase, choline dehydrogenase and some other oxidative enzymes of several kinds of snake venoms heated at 100°C for 15 minutes (proved to be phospholipase A) and found that the activities of cytochrome oxidase, succinic dehydrogenase and choline dehydrogenase was greatly reduced by snake venoms but the activity of soluble enzymes were not attacked.

These investigators have concluded that the inhibitory effects of phospholipase A upon the activity of cytochrome oxidase and so on are due to the hydrolysis of phospholipids which constitute the cellular structures and that the enzymes that are not attacked by heated venoms are apparently independent of cellular structures for their activities.

Edwards and Ball<sup>10)</sup> have studied the effects of phospholipase C upon the activity of succinic dehydrogenase and cytochrome oxidase and found that the activities of these enzymes are greatly inhibited by phospholipase C. As a result of the studies they have concluded that the inhibitive effects of phospholipase C upon the activities of succinic dehydrogenase and cytochrome oxidase are due to the hydrolysis of phospholipids upon which the enzymes are dependent for their activities.

Tookey and Ball<sup>11)</sup> have studied the inhibitive effects of cotton seed

phospholipase D upon the activity of succinic dehydrogenase and cytochrome oxidase and found that the activity of these enzymes are greatly reduced by phospholipase D. They have concluded that the inhibitive effects of phospholipase D are due to the hydrolysis of phospholipids which constitute mitochondrial structures rather than to the reaction products of the hydrolysis of phospholipids.

Therefore, although the most important components of phospholipids are fatty acids combining at the  $\alpha$  and  $\beta$  positions, it can be reasonably assumed that phospholipids constituting cellular structures must be intact for manifestation of the enzyme activity.

As a results of an investigation on the restoration of oxidative enzyme activities using artificially synthesized lecithin having saturated fatty acids at both  $\alpha$  and  $\beta$  positions, D. E. Green has demonstrated that this artificially synthesized lecithin could not restore the enzyme activity at all.<sup>2), 12)</sup> This indicates that the most important components of the phospholipid molecule are the fatty acids combining at  $\alpha$  and  $\beta$  positions, and moreover fatty acid must be in the unsaturated form. That unsaturated fatty acid is necessary for restoration may be connected with the stereochemical properties of the intracellular membraneous structures where the oxidative enzymes localize. The results of other investigators mentioned above coincide with the result of the present experiments that fatty acids of phospholipids are the most important components in the oxido-reductive reactions going on in the living cells.

#### SUMMARY

(1) Comparative studies have been made on the inhibitory effects of phaspholipase A, phospholipase C and phospholipase D upon the activities of cytochrome oxidase, DPNH diaphorase, TPNH diaphorase, succinic dehydrogenase and malic dehydrogenase.

(2) The inhibitory effect of phospholipase A was conspicuous, however those of phospholipase C and phospholipase D were weak.

(3) As a result of the compapative studies it has been clearly demonstrated that the most important components of the phospholipid molecule for the oxidative enzymes are the fatty acids combined at the  $\alpha$  and  $\beta$  positions of glycerol.



**REFERENCES**

- 1) Zeller, E. A.: The Enzymes, edited by Sumner and Myrbäck, Academic Press, Inc., 1 : 987, 1951.
- 2) Green, D. E., *et al.*: BBA Library, 1 : 325, 1963.
- 3) Hanahan, D. J.: Progr. Chem. Fats Lipids, 4 : 142, 1957.
- 4) Ansell, G. B. *et al.*: BBA Library, 3 : 154, 1964.
- 5) Wooldridge, W. R., *et al.*: Biochem. J., 32 : 1718, 1938.
- 6) Macfarlane, M. G., *et al.*: *ibid*, 35 : 884, 1941.
- 7) Nygaard, A. P., *et al.*: J. Biol. Chem., 200 : 723, 1963.
- 8) Macfarlane, M. G.: Biochem. J., 47 : XXIX, 1950.
- 9) Braganca, B. M., *et al.*: *ibid*, 53 : 88, 1953.
- 10) Edwards, S. W., *et al.*: J. Biol. Chem., 209 : 619, 1954.
- 11) Tookey, H. L., *et al.*: J. Biol. Chem., 220 : 15, 1956.
- 12) Jurtschuk, P., *et al.*: Biochem. Biophys. Res. Commun., 6 : 76, 1961.

**EXPLANATIONS OF MICROPHOTOGRAPHS**

(1) Cytochrome oxidase activity demonstrated by the modified tetrazolium method. The upper picture shows tissue sections stained ordinarily without any pre-treatment. In these tissue sections the activity of the enzyme is strong. Lower picture shows tissue sections stained after pre-treatment with phospholipase A. The activity of the enzyme is greatly diminished and almost no positive reaction was recognized under the microscope.

(2) Liver: 100×

Cytochrome oxidase activity demonstrated by the modified tetrazolium method. Tissue section was pre-treated with phospholipase C. Therefore, this picture shows the effect of phospholipase C upon the activity of cytochrome oxidase. Moderately positive reaction is recognized in the cytoplasm of hepatic cells. The inhibitory effect of phospholipase C is not so strong.

(3) Pancreas: 100×

Cytochrome oxidase activity in tissue section which was pre-treated with phospholipase C before incubation. Moderately positive reaction is recognized in pancreatic cell. The activity of the enzyme is not so seriously affected by phospholipase C.

(4) Heart: 100×

Cytochrome oxidase activity in tissue section which was pre-treated with phospholipase D before incubation. Strong positive reaction is recognized in muscle fibres.

It is evident that the activity of the enzyme was not so seriously influenced by phospholipase D.

(5) Kidney: 100×

Cytochrome oxidase activity in tissue section which was pre-treated with phospholipase D before incubation. In some area considerable reduction of the enzyme activity was recognized. However, the inhibitory effect of phospholipase D was not so serious.

(6) Liver: 100×

DPNH diaphorase activity in tissue section which was pre-treated with phospholipase A before incubation. Reduction of the activity of DPNH diaphorase was very intense and only very faint reaction was recognized under the microscope. Therefore, it is reasonable to think that the inhibitory effect of phospholipase A upon DPNH diaphorase activity is very serious. Moreover, the morphological destruction of cellular structures looks to be not so conspicuous.

(7) Liver: 100×

DPNH diaphorase activity in tissue section which was pre-treated with phospholipase C. Although reduction of the enzyme activity is recognized to some extent, fairly strong positive reaction is seen in this picture. It is evident that the inhibitory effect of phospholipase C upon DPNH diaphorase activity is not strong.

(8) Liver: 100×

DPNH diaphorase activity in this section which was pre-treated with phospholipase D. Fairly strong positive reaction is recognized in the cytoplasm of hepatic cells. The degree of reduction of the enzyme activity is low. Therefore, it is reasonable to think that the inhibitory effect of phospholipase D is not serious.

