The Enzymatic Synthesis of Plasmalogens*

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Recent work (1) has established that plasmalogenic phosphatides resemble the conventional glycerophosphatides such as phosphatidylcholine (I) very closely in structure, except that one fatty acid ester linkage is replaced in the plasmalogens by an aldehydogenic bond, presumably that of an α,β-unsaturated ether. The exact position of the aldehydogenic bond is not yet certain. It had been concluded by Rapport (2) that the aldehydogenic linkage in the choline-containing plasmalogens of beef heart is in the β position, since the remaining fatty acid ester bond is readily cleaved by lecithinase A of snake venom, described by Hanahan (3) as specific for the α′ position. However, more recent evidence of Tatthie (4) strongly suggests that lecithinase A is specific for fatty acids in the β position. This would mean that the aldehydogenic bond of the choline plasmalogens (and probably other plasmalogens as well) is in the α′ position. The structural relationship between these compounds and the well known glyceryl ethers such as batyl and chimyl alcohols would then become much more understandable. Accordingly, the structure shown in II will be assigned here to the choline plasmalogen of beef heart, but in view of the somewhat unsettled nature of the evidence to date, including claims that plasmalogens of both types may be present in nature, this assignment must be regarded as somewhat arbitrary.

It is known that the enzymatic synthesis of phosphatidylcholine (I) involves a reaction between n-α,β-diglyceride (III) and cytidine diphosphate choline (5). If a similar pathway were to lead to the biosynthesis of choline plasmalogens, presumably the last step of such a sequence would be a reaction between cytidine diphosphate choline and a compound of structure IV, which is a n-α,β-diglyceride in which one ester linkage has been replaced by an α,β-unsaturated ether bond.

Assignment of trivial names to compounds of Type IV is rather difficult. It is proposed in this paper to describe any phosphatidylcholine (II) very closely in structure, except that one fatty acid ester linkage is replaced in the plasmalogens by an aldehydogenic bond as "plasmalogenic". Thus II may be described as plasmalogenic phosphatidylcholine (or choline plasmalogen) and IV may be described as plasmalogenic diglyceride. This usage is simple, avoids an exact definition of structure at a time when this is not known with complete certainty, and is justifiable historically (6). The slight possibility of confusion arising from the description of IV as plasmalogenic diglyceride, when in fact it is not a diester of glycerol, is avoided by the explicit definition above.

The present paper will describe a method for the preparation and purification of plasmalogenic diglycerides involving the enzymatic cleavage of phosphorylcholine from the choline plasmalogen of beef heart (II) followed by chromatography of the resultant products on silicic acid. It has been found that the particulate enzymes from liver and other tissues catalyze a reaction between plasmalogenic diglycerides and cytidine diphosphate choline with the formation of choline plasmalogen. A similar reaction between plasmalogenic diglycerides and cytidine diphosphate ethanolamine has also been observed. A preliminary report of some of these results has been made (7).

MATERIALS AND METHODS

Preparation of Particulate Fraction from Rat Liver—Adult albino rats were killed by decapitation and the livers quickly removed and homogenized in a glass homogenizer, with 7 ml of ice-cold 0.25 M sucrose solution for each 3 g of fresh liver. The sediment obtained by centrifugation of this homogenate for 20 minutes at 3,000 × g at 0-4° was discarded. The particulate fraction, sedimented by a further centrifugation of 40 minutes at 30,000 × g, was taken up in 0.025 M Tris buffer at pH 8.0 and stored in the frozen state until used.

Preparation of Plasmalogonic Diglyceride—Phospholipid fractions from beef heart prepared by the method of Pangborn (8) were the generous gift of Mr. S. Rosenberg of the Sylvania Chemical Company. These fractions have been shown (9) to contain approximately 40% of phosphatidylcholine and 60% of plasmalogenic phosphatidylcholine. Gray and Macfarlane (10) have reported that the lecithinase D of Clostridium welchii cleaves phosphorylcholine from choline plasmalogens, as well as from lecithin. In confirmation of this result, we have found that prolonged incubation of such preparations with lecithinase D from Clostridium perfringens (kindly supplied by Lederle Laboratories Division, American Cyanamid Company) in the presence of lead, leads to an almost quantitative release of P-choline. A phosphorus-free lipid fraction, presumably a mixture of III + IV, could be obtained by such treatment, as shown by the following experiment.

This buffer (3 mmoles) and MgCl₂ (300 mmoles) were dissolved in 20 ml of water and to this solution the Pangborn phospholipid fraction (184 mg containing 245 μmoles of total P) was added with shaking. Lecithinase D (17 mg of lyophilized protein) was then added, followed by 20 ml of diethyl ether. The mixture was stirred with a magnetic stirrer for 24 hours at room temperature, after which the aqueous phase appeared quite translucent. The ether phase, which contained only traces of phosphorus, was transferred to a round-bottomed flask and taken

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to dryness under vacuum in a rotary evaporator. The residual oil was taken up in 5 ml of benzene and chromatographed on silicic acid. A commercial grade of silicic acid (Bio-Rad), prepared according to the methods of Hirsch and Ahrens (11) and activated by heating at 110°C for 16 hours after removal of ether, was used. Approximately 10 g of this silicic acid were packed into a column 24 cm long and 1 cm in diameter and thoroughly washed with benzene. The lipid, dissolved in benzene, was passed over the column and eluted by a gradient technique, with 250 ml of benzene in the lower mixing chamber and a mixture of equal volumes of benzene and chloroform in the upper reservoir. The fractions eluted were tested for aldehydogenic groups by the method of Wittenberg et al. (12), with myristic aldehyde as a standard, and for fatty acid esters by the procedure of Stern and Shapiro (13), with dipalmitoyl glycerol-α-benzyl ether as a standard.

The results of the chromatogram (Fig. 2) show that the plasmalogenic diglyceride fraction, containing approximately equivalent amounts of ester and aldehydogenic groups, is eluted first (Tubes 4 to 6) and is quite well separated from the conventional α,β-diglyceride (Tubes 11 to 14). Each fraction was combined, taken down to dryness, and rechromatographed in the same system. Recovery of both fractions was almost quantitative and each appeared to be substantially free of the other. The ratio of aldehyde to ester in the plasmalogenic diglyceride fraction was 0.90:1.0, but it is not certain if the color values of the reactive groups in the lipid are exactly the same as those given by the standards in the analytical methods used. The α,β-diglyceride fraction contained only small amounts of aldehydogenic material.

The solvents were removed from the plasmalogenic diglyceride and diglyceride fractions under vacuum, and each fraction was emulsified in 0.025 M Tris buffer at pH 8.0, containing 1% of "Tween 20" (polyoxyethylene sorbitan monolaurate from Atlas Powder Company) in a final concentration of 10 or 20 μmoles per ml.

Other Materials and Methods—Synthetic α,β-diolein was the generous gift of Drs. Baer and Buchnea. CDP-choline labeled with choline-1,2-Cl4 was synthesized by the method of Kennedy (14).

Stearic aldehyde (Aldrich Chemical Company) and myristic aldehyde (Tombarel Products Corporation) were redistilled under reduced pressure immediately before use.

Protein determinations were made by the method of Lowry et al. (15).

Experimental Procedure

Estimation of Enzymatically Synthesized Choline Plasmalogen—Attempts in our own and other laboratories to separate native choline plasmalogen from lecithin have been with little success. The plasmalogen, however, can be readily distinguished from lecithin and from sphingomyelin by the following properties: (a) Upon treatment with mild alkali the plasmalogen yields a lysoplasmalogen which is preferentially soluble in chloroform, whereas lecithin yields a water-soluble derivative, glycerophosphorylcholine. (b) Upon treatment with acetic acid, the plasmalogen yields a lysolecithin under conditions in which little or no hydrolysis of lecithin or sphingomyelin takes place.

Since it had been shown by Sribney and Kennedy (16) that there is very little formation of alkali-stable lipids from CDP-choline in enzyme systems from liver in the absence of suitable acceptors, the production of alkali-stable radioactive lipid from CDP-choline in the presence of plasmalogenic diglyceride was adopted as a routine assay for measuring the formation of choline plasmalogen.

At the end of the incubation period, the enzymatic reaction, usually in a volume of 0.50 ml, was stopped by the addition of 3 ml of ethanol. The tubes were kept at 37°C for at least 15 minutes to allow the denatured protein to flocculate, after which the precipitate was removed by centrifugation and extracted twice more with 2 ml portions of ethanol. The combined ethanolic extracts were taken up in chloroform, and the chloroform solution thoroughly washed with 2 M KCl and water as previously described (5). Portions of the washed chloroform phase were then plated in aluminum cups and counted under conditions of negligible self-absorption, giving a measure of the conversion of radioactive CDP-choline (or CDP-ethanolamine) to total lipid.

The conversion of CDP-choline to alkali-stable lipid was measured on another aliquot of the washed chloroform phase, which was taken to dryness under a jet of air, taken up in 0.2 ml of 0.2 N methanolic NaOH, and incubated at 37°C for 15 minutes with occasional shaking. These conditions were suggested by the useful method of Dawson (17). The samples were then chilled in an ice bath and 0.3 ml of 0.5 M Tris buffer at pH 6.7.
was added. Chloroform (5 ml) was then added after which the samples were vigorously shaken. The extract was then washed with 2 M KCl and water essentially as described (5). A portion of the chloroform phase was counted as described above.

Evidence that the alkali-stable lipid which is formed from CDP-choline is in fact plasmalogen will be presented in a later section of this paper.

Effect of Plasmalogenic Diglyceride on Conversion of CDP-Choline to Total and Alkali-Stable Phospholipid—When labeled CDP-choline is incubated with \( \alpha, \beta \)-diglycerides of conventional structure in the presence of a particulate enzyme fraction from liver, a rapid conversion to lecithin takes place, as has been previously reported (5). However, no significant synthesis of alkali-stable phospholipid is noted under these conditions (Fig. 3). In contrast, when CDP-choline is incubated with plasmalogenic diglyceride, a rapid labeling of the total lipid and of the alkali-stable lipid fraction occurs, as shown in Fig. 4.

It will be noted that only about half of the radioactivity present in the total lipid is recovered in the alkali-stable fraction in the experiment shown in Fig. 4. Control experiments indicate that the recovery of lyso-phospholipid after treatment of native choline plasmalogen (II) with alkali is not quantitative. Therefore, the values shown for conversion of CDP-choline to the alkali-stable fraction represent minimal estimates of the amount of plasmalogen formed. On the other hand, it is possible that the addition of plasmalogenic diglyceride somehow also stimulates the synthesis of lecithin of conventional structure (I), since only half of the total radioactive lipid is labile to mild treatment with acetic acid, as described below. The exact explanation for this finding is not understood. However, in any case it is clear that a considerable synthesis of alkali-stable lipid does occur when plasmalogenic diglyceride is added.

Identification of Product as Choline Plasmalogen—As mentioned above, choline plasmalogen can be distinguished from lecithin not only by its stability to mild alkali, but also by its ready conversion to lysolecithin (which can be separated from lecithin by chromatography) upon treatment with acetic acid (6, 18, 19) under conditions to which lecithin is stable. Accordingly, the total lipid fraction, obtained from an experiment in which labeled CDP-choline had been incubated with plasmalogenic diglyceride essentially as described in Fig. 3, was chromatographed on silicic acid with increasing concentrations of methanol in chloroform by gradient elution. All of the radioactivity was recovered in a single peak in the region expected for lecithin. This is consistent with the fact that native choline plasmalogen cannot easily be separated from lecithin (20). The material in this peak was concentrated to dryness under vacuum and treated with 5 ml of 90% acetic acid at 38°C for 17 hours, to cleave off the aldehydogenic moiety of any plasmalogen present (6). The product was then taken up in chloroform, washed with 2 M KCl and water, and rechromatographed (upper portion of Fig. 5). Approximately 52% of the radioactivity was recovered in the region of lysolecithin, and must therefore have been derived from choline plasmalogen. In a control experiment, the radioactive lipid obtained when CDP-choline was incubated with \( \alpha, \beta \)-diolein was carried through the same procedure. Only negligible amounts of lysolecithin were obtained, showing both the stability of lecithin under these conditions and the specificity of the enzyme system for plasmalogenic diglyceride.

In the experiment to be described in the next section, chemically measurable increase in the content of aldehydogenic groups of the lecithin fraction (isolated on silicic acid) was also observed. These results, together with the requirement for plasmalonomic diglyceride as acceptor, leave little doubt that choline plasmalogen is being formed.

Net Synthesis of Choline Plasmalogen—The total labeled phospholipid derived from an experiment run in a final volume of 10 ml was chromatographed on a silica gel without treatment of alkali. The lecithin fraction (which also contains the choline plasmalogen) was analyzed for total P, aldehydogenic groups and

![Fig. 3](http://www.jbc.org/)

![Fig. 4](http://www.jbc.org/)
FIG. 5. Isolation of radioactive lysolceithin after treatment of enzymatically synthesized choline plasmalogen with acetic acid. The conditions of the experiment are described in the text. In the upper portion of the figure, it is seen that about 52\% of the total radioactivity is recovered as lysolceithin (Tubes 12 to 16). In a control experiment, shown in the lower portion of the figure, radioactive lecithin (obtained from CDP-choline + nospholipid diglyceride) was subjected to exactly the same treatment. Negligible amounts of radioactivity were recovered in the region in which lysolceithin is expected.

**Table I**

Net synthesis of choline plasmalogen by rat liver particles

<table>
<thead>
<tr>
<th>Addition</th>
<th>Phospholipid recovered in lecithin fraction</th>
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<tbody>
<tr>
<td></td>
<td>Total phos-</td>
</tr>
<tr>
<td></td>
<td>phorus</td>
</tr>
<tr>
<td>None</td>
<td>1.25</td>
</tr>
<tr>
<td>Plasmalogenic diglyceride, 20 (\mu)moles</td>
<td>1.88</td>
</tr>
<tr>
<td>No increase</td>
<td>0.63</td>
</tr>
</tbody>
</table>

It was found (Table I) that the addition of plasmalogenic diglyceride led to a net increase in total phosphorus and of aldehydogenic groups in this fraction, as compared to a control without acceptor, as well as an increase in choline derived from CDP-choline. The amount of endogenous plasmalogen in the enzyme particles is small compared to the lecithin content, in keeping with the observation that the amount of plasmalogen in liver is quite low (21).

The increase in total P is in good agreement with the increase in choline derived from CDP-choline, as expected if phosphorylcholine is transferred as a unit from CDP-choline to the acceptor.

**Specificity for Plasmalogenic Diglyceride**—If the plasmalogenic diglyceride is treated with alkali before incubation, to destroy the ester linkage and to generate a compound which is presumably a plasmalogenic monoglyceride, no activity in stimulating the synthesis of total or alkali-stable lipid from CDP-choline is noted. The presence of an ester linkage is therefore necessary. As noted above, conventional \(\alpha,\beta\)-diglycerides show no activity, indicating the necessity for at least one unsaturated ether linkage as in IV. No synthesis of alkali-stable lipid from CDP-choline could be detected when the plasmalogenic diglyceride was replaced by long chain fatty aldehydes.

**Possible Identity of Enzymes Catalyzing Synthesis of Lecithin and Choline Plasmalogen**—If varying amounts of \(n,\alpha\),\(\beta\)-diglyceride are added to an enzyme system containing plasmalogenic diglyceride and CDP-choline, there is little effect on the amount of alkali-stable labeled lipid formed, consistent with the idea that only one \(\alpha,\beta\)-unsaturated bond is essential in this reaction.

FIG. 6. Inhibition of the synthesis of lecithin and of choline plasmalogen by calcium ion. The conditions of the experiment were closely similar to those described in Fig. 3, except that \(n,\alpha\),\(\beta\)-diglyceride (1 \(\mu\) mole) was the acceptor in one set of tubes (solid line) and plasmalogen diglyceride (1 \(\mu\) mole) was the acceptor in another set (broken line). The amounts of calcium ion added to both sets were varied as shown. The radioactivity of the total phospholipid fraction was then measured and the results are expressed as percentage of inhibition, with the level of synthesis in each set in the absence of calcium taken as 100\% activity (0 inhibition).

**Table II**

Synthesis of ethanolamine plasmalogen

<table>
<thead>
<tr>
<th>Additions (2 (\mu)moles each)</th>
<th>C\textsuperscript{4}-phospholipid synthesized</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
</tr>
<tr>
<td></td>
<td>(\mu)moles</td>
</tr>
<tr>
<td>None</td>
<td>2.0</td>
</tr>
<tr>
<td>(n,\alpha,\beta)-Diglyceride</td>
<td>21.8</td>
</tr>
<tr>
<td>Plasmalogenic diglyceride</td>
<td>18.4</td>
</tr>
</tbody>
</table>

The inhibitory effect of calcium ion on the synthesis of lecithin and of choline plasmalogen by rat liver particles is illustrated in Fig. 6 (Table II). The calcium inhibition is described in detail in the text.
that separate enzymes are involved. However, it is virtually impossible to saturate the system either with plasmalogenic diglyceride (Fig. 4) or \( \alpha, \beta \)-diglyceride (5), so that this observation carries little weight.

In a further effort to obtain information as to whether separate enzymes catalyze the reactions between CDP-choline and \( \alpha, \beta \)-diglyceride or plasmalogenic diglyceride respectively, the effect of calcium ions on these reactions was studied (Fig. 6). It is known that the lecithin-synthesizing enzyme is inhibited by low concentrations of calcium ion (5). It was found that the percentage of inhibition by calcium ion was the same, if \( \alpha, \beta \)-diglyceride or plasmalogenic diglyceride was added as acceptor, suggesting that the same enzyme catalyzes both reactions, or if different enzymes are involved, these must have strikingly similar properties.

Reaction of CDP-Ethanolamine with Plasmalogenic Diglyceride—When plasmalogenic diglyceride was incubated with labeled CDP-ethanolamine rather than CDP-choline, the formation of a radioactive, alkali-stable phospholipid, presumably the ethanolamine plasmalogens, was observed (Table II). The experiments described in this paper offer evidence that choline- and ethanolamine containing plasmalogens may be synthesized by reactions between plasmalogenic diglyceride (IV) and CDP-choline or CDP-ethanolamine, respectively. Such results are consistent with (but do not prove) the occurrence of a series of reactions essentially similar to those leading to the biosynthesis of lecithin (22), but involving aldehydogenic intermediates. Such a series might include the formation of a plasmalogenic phosphatidic acid and its subsequent phosphorylation by phosphatidic acid phosphatase, with the production of a plasmalogenic diglyceride. Experiments in general consistent with such a scheme have also been described briefly by Gambal and Monty (23).

However, an alternative interpretation is by no means ruled out. It may be that the enzymes catalyzing the synthesis of lecithin and phosphatidylethanolamine are not completely specific for diglycerides of conventional structure, and will react with plasmalogenic diglycerides even though such compounds may not be encountered in vivo. It is not possible at present to resolve this difficulty. Further progress is hindered by lack of organic chemical procedures for the synthesis of the suspected intermediates.

**SUMMARY**

1. Prolonged enzymatic hydrolysis of the choline-containing plasmalogen of beef heart, catalyzed by the lecithinase \( D \) of *Clostridium perfringens*, leads to the complete removal of the phosphorylethanolamine portion of the molecule. The other product of the reaction is an aldehydogenic lipid closely resembling a \( \alpha, \beta \)-diglyceride, except that one fatty acid ester bond is replaced by an \( \alpha, \beta \)-unsaturated ether linkage. The name "plasmalogenic diglyceride" is suggested for compounds of this type.

2. Methods for the purification of plasmalogenic diglycerides and the separation of these substances from conventional diglycerides by chromatography on silicic acid are described.

3. Evidence is presented for the occurrence of the following reactions, catalyzed by particulate enzyme preparations from liver:

\[
\text{CDP-choline} + \text{plasmalogenic diglyceride} \rightarrow \text{plasmalogenic phosphatidylcholine} + \text{cytidine 5' diphosphate}
\]

4. A chemically measurable net synthesis of plasmalogenic phosphatidylethanolamine has been observed.

5. The significance of the above reactions in a possible pathway leading to the biosynthesis of plasmalogens in vivo is discussed.

**REFERENCES**

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