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The Structure of Beef Heart Plasmalogens[1]

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The total phosphatides and the lecithin and phosphatidylethanolamine fractions of beef heart were isolated and reduced with hydrogen. These lipids were degraded to give a 68–72% yield of long chain α-glycerol ethers. Hence the major part of the plasmalogens of beef heart have the aldehyde attached to the α-position and the fatty acid attached to the β-position of glycerol. The unreduced beef heart lecithin fraction was hydrolyzed by snake venom lecithinase A to yield a mixture of the typical monoester type lysolecithin and the monoether type lysoplasmalogen. These lysophosphatides were reduced with hydrogen and hydrolyzed with acid. A 68% yield of long chain α-glycerol ether was obtained from the lysoplasmalogen. These data demonstrate that snake venom lecithinase A can hydrolyze the β-linked fatty acid on the plasmalogen. Data on the reactivity of the plasmalogens and lysoplasmalogens toward the Schiff reagent and methanolic iodine are given.

Previous studies[2,3] on the structure of pig heart plasmalogens have shown that the fatty acid is linked predominantly to the β-position of glycerol and the aldehyde is linked to the α-position. On the other hand studies on beef heart plasmalogens are not in accord since Klenk and Debuch[4] report that the aldehyde in these lipids is linked to the α-position of glycerol whereas Rapport and co-workers[5,6] and Gray[7] report that the aldehyde is attached to the β-position. Debuch[8] has later been willing to accept the β-structure for plasmalogens based on the work of Rapport and co-workers[5,6] and Gray.[7]

In order to clarify this problem we subjected beef heart plasmalogens to the same sequence of degradation reactions as those employed for pig heart plasmalogens.[2] A consistent yield (68–72%) (7 independent experiments) of α-glycerol ethers has now been obtained from beef as well as from pig heart lipids. The α-glycerol ethers have been unequivocally identified by elementary analysis and by comparison of their infrared spectra, paper chromatographic mobility and reactivity toward periodate with an authentic sample of α-octadecyl glycerol ether.[2,9] The dimedon derivative of formaldehyde has also been isolated in 82% yield after periodate oxidation of the heart glycerol ethers.

Our first preliminary experiment[2] indicated that beef heart plasmalogens yielded a smaller amount of α-glycerol ethers than did pig heart plasmalogens. Furthermore, the yield of material in the chloroform fraction which was obtained by column chromatography of the acid hydrolyzed glycerol phosphate ether was greater with beef plasmalogens than with the pig plasmalogens. The failure of the lipid in this chloroform fraction to react with periodate and the Schiff reagent, its complete lack of N, P and ester groups, and its infrared spectrum, suggested that it contained β-glycerol ethers. However, subsequent analysis (see Experimental Section N) of this fraction have shown that at least two components are present. The minor component has the properties of a long chain carbonyl-containing lipid. The major component has the properties of an α,β-glycerol diether. The presence of a small amount of β-glycerol ether is still possible but conclusive evidence on this compound has not yet been obtained.

The important point therefore in the present paper is that we have now consistently (seven independent experiments) obtained a 68–72% theoretical yield of α-glycerol ether from both beef heart and pig heart plasmalogens. In the case of beef heart a similar yield of α-glycerol ether has also been obtained from the enzymatically produced lysoplasmalogen. Hence the plasmalogens of both pig and beef heart are predominantly α-ether, β-ester derivatives of glycerylphosphorylcholine or glycerylphosphorylethanolamine. For sake of convenience these phosphatides shall be designated as α- or β-plasmalogens depending on the position of attachment of the aldehyde group. The existence of β-plasmalogens by our procedure has not yet been conclusively demonstrated. It is believed that some α,β-plasmalogens (*i.e.*, plasmalogens containing two aldehyde groups) may exist in beef heart, in particular in the cephalin fraction. It is noteworthy that Carter and co-workers[10] and Karnovsky and Bruum[11] have also isolated α-glycerol ethers from natural products.

The question arises as to how the results of our experiments can be reconciled with those of Rapport and co-workers[5,6] and of Gray.[7] As to the former, which is based on the specificity of action of snake venom phospholipase A, we have been unable in five attempts to confirm the work of Hanahan.[12] In our hands[13] the exact procedure outlined by Hanahan yields a mixture of four water soluble compounds on hydrolysis of the permanganate oxidized lysolecithin. These phosphate compounds have chromatographic mobilities similar to glycerol phosphate, phosphoglyceric acid, phosphorylcholine and orthophosphate. The production of several phosphate esters is not in agreement with the phospholipase splitting off only the α-linked fatty acid but rather is evidence that the enzyme hydrolyzes either the α- or β-ester linkage and thus gives rise to both α- and β-lysolecithins. Conse-

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(3) G. V. Marinetti, J. Erbland and E. Stotz, *Biochem. et Biophys. Acta*, **26**, 429 (1957).

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(5) M. M. Rapport and R. E. Franzl, *J. Biol. Chem.*, **225**, 851 (1957).

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(10) H. E. Carter, D. B. Smith and D. M. Jones, *ibid.*, **232**, 681 (1958).

(11) M. L. Karnovsky and A. F. Bruum, *ibid.*, **216**, 689 (1955).

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(13) G. V. Marinetti, J. Erbland and E. Stotz, *Biochem. et Biophys. Acta*, in press.

quently this enzyme cannot be used to establish the structure of the plasmalogens. In support of the nonspecific action of snake venom phospholipase A are the results of our studies on the action of Crotalus adamanteus or Naia naia venom on beef and pig heart lecithin (mixture of the diester and plasmalogen forms). We have found that these venoms act more rapidly on the diester lecithin than on the plasmalogen form but ultimately both forms are completely hydrolyzed to the corresponding lyso derivatives. Since by our results the choline plasmalogens have the fatty acid ester group predominantly on the β-position of glycerol, the venoms must hydrolyze the fatty acid on the β-position of glycerol.

With regard to the work of Gray,[7] the publication of experimental details are awaited before an evaluation of the data is made. However, the yield of methylglyoxal must be considered. In our hands the permanganate oxidation of lysolecithins has not yielded a single water-soluble phosphate ester.

A criticism which may be directed at our work is the possible isomerism of the glycerol ethers during the prolonged acid hydrolysis. In order to answer such an inquiry we have subjected the pure synthetic α-octadecyl glycerol ether to the same prolonged acid hydrolysis and have found no change in the properties of this compound either by paper chromatography or reactivity toward periodate. Hence no migration of the ether group to the β-position occurs. Although this does not rule out β to α-migration, the known stability of saturated ethers makes the migration very unlikely. We feel confident therefore that the isolation in good yield of an α-glycerol ether from both pig and beef heart phosphatides constitutes reliable proof for their containing predominantly α-plasmalogens. The fact that the yield of α-glycerol ethers have been only 68–72% does leave open the possibility that β- or α,β-plasmalogens do exist.

The reactivity of the native choline and ethanolamine plasmalogens and of the enzymatically prepared lysocholineplasmalogen toward the Schiff reagent bears mentioning. The rates of reaction were found to vary quite markedly. Thus the time required to reach full color intensity for the native plasmalogens was 120–140 minutes whereas the lysoplasmalogen required only 17 minutes. Under identical conditions a standard tetradecaldehyde required 7 minutes. After reaction of all the plasmalogens with methanolic iodine,[14] the native plasmalogens exhibited a decrease in extent of reaction with the Schiff reagent whereas the lysoplasmalogen showed no change in extent of this reaction in spite of the fact that it took up exactly one mole of iodine. Thus it appears that the nature of the aldehydogenic linkage of the two plasmalogens is different. It is peculiar that one mole of lysoplasmalogen takes up 1 mole of iodine, yet the reactivity of the vinyl ether structure toward the Schiff reagent is unaffected. In addition to the reactivity toward the Schiff reagent the lysoplasmalogen was far more sensitive toward acetic acid than the native plasmalogen. Indeed a major portion of it was readily hydrolyzed when chromatographed in the diisobutyl ketone–acetic acid–water solvent on silicic acid impregnated paper. In contrast, the native plasmalogens were only very slightly hydrolyzed during chromatography under identical conditions. It appears that the exact structure of this aldehydogenic linkage needs further clarification, although strong evidence for a vinyl ether structure stems from the work of Rapport and Franzl[14] and of Debuch.[8]

(14) M. M. Rapport and R. E. Franzl, *J. Neurochem.*, **1**, 303 (1957).

Experimental

Preparation and Reduction of the Total Beef Heart Lipids (I).—The beef heart lipids I were extracted and reduced with hydrogen as described previously.[2] The reduced lipids II were washed three times with excess acetone and dried.

B. **Hydrolysis of the Reduced Total Phosphatides.**—The reduced acetone washed lipides II (9.14 g. containing 172 mg. of P)[15] were refluxed for 2 hr. in 180 ml. of 1 *N* H_2SO_4. The mixture was cooled and extracted with ethyl ether. The ethyl ether extracts were washed with water, dried and evaporated to dryness to yield 7.36 g. of product which consisted of fatty acids III, glycerol phosphate ether IV and a small amount of partial hydrolysis products of the phosphatides.

C. **Isolation of the Glycerol Phosphate Ether IV by Column Chromatography.**—The mixture of III and IV (7.2 g.) was fractionated on 100 g. of silicic acid which was prepared as a slurry in 500 ml. of 20% methanol in chloroform and displaced with chloroform. The material was put on the column in chloroform. Elution was carried out with the following solvents: (a) 300 ml. of chloroform; (b) 500 ml. of 20% methanol in chloroform; (c) 300 ml. of 85% methanol in chloroform; (d) 100 ml. of methanol–water–chloroform 82:4:14. The free fatty acids III (6.1 g., no P) occurred in the chloroform eluate (a). The crude glycerol phosphate ether IV[16] (650 mg.) (5.63% P) occurred mainly in the 20% methanol eluate (b). A lesser amount also occurred in fraction (c) (105 mg.) (6.2% P) and fraction (d) (39 mg.—5.13% P). The recovery of lipid P put on the column was 92%.

D. **Hydrolysis of the Crude Glycerol Phosphate Ether IV.**—The glycerol phosphate ether IV (511 mg. containing 28.8 mg. of P) which occurred in eluate b mentioned in section C above was refluxed for 45 hours in 6 *N* aqueous H_2SO_4. The mixture was cooled and extracted with ether. The ether extracts were washed with water, dried and evaporated to dryness to yield 424 mg. of product (2.03% P) which consisted of a mixture of glycerol ether V and unhydrolyzed glycerol phosphate ether IV. The aqueous phase was found to contain 20.05 mg. P (all as orthophosphate) and the ether phase contained 8.5 mg. P. The extent of hydrolysis of IV was 69.8%.

E. **Isolation of the Glycerol Ether V by Column Chromatography.**—The mixture of IV and V (411 mg.) mentioned in section D was fractionated on 10 g. of silicic acid which was prepared as mentioned in section C. Elution was carried out as follows: (a) 40 ml. of chloroform, (b) 100 ml. of ethyl ether, (c) 100 ml. of 20% methanol in chloroform, (d) 100 ml. of methanol. The chloroform used to this point was Mallinckrodt "analytical reagent" which contained 0.75% ethanol. This small amount of alcohol is sufficient to cause the elution of some glycerol ether. Hence in subsequent steps the alcohol was removed by passing the chloroform through a molecular sieve[17] and redistilling. The yield of material in fractions a–c was as follows: (a) 202.5 mg., (b) 145 mg., (c) 32 mg., (d) 6.3 mg. The recovery of the material put on the column was 94%. Each of the above fractions was analyzed for P and subjected to paper chromatographic analysis as described previously[2] (see section I, page 1626). The glycerol ether V was found in eluates a and b, whereas the glycerol phosphate ether IV occurred in fractions c and d.

(15) This material also contained saturated triglycerides, saturated cholesterol esters and free cholesterol.

(16) This compound was identified on the basis of its P content and by paper chromatography.[2]

(17) The molecular sieve, type 4A powder was obtained from the Linde Air Products Co., 250 Delaware Ave., Buffalo, N. Y. Two volumes of chloroform were passed through 1 volume of sieve.

A portion of glycerol ether-containing fractions a and b were rechromatographed on silicic acid (10 g.). Elution was carried out stepwise with alcohol-free redistilled chloroform, 20% ethyl ether in chloroform and chloroform–methanol 1:1. The 20% ethyl ether in chloroform was found to contain nearly all of the glycerol ether. A total of 131 mg. of pure α-glycerol ether V was isolated by this procedure. However, making corrections for the fact that the hydrolysis of the original glycerol phosphate ether IV (section D) was only 67.8% and that only a portion of the glycerol phosphate ether and other materials isolated at each step was used, the expected yield, had all the material been used, would have been 328 mg. This is based on the following: the total lipid P in the starting material II was 172 mg. (5.55 m*M* of P). Since lecithin and phosphatidyl ethanolamine contain essentially all the plasmalogens of beef heart and since these 2 lipids comprise 60% of the total lipid P and contain 40% of the plasmalogen form, then 24% of the total lipid P can give rise to glycerol phosphate ether. Hence 1.33 m*M* of P or 1.33 m*M* of glycerol phosphate ether (or glycerol ether) could theoretically be obtained. Assuming a molecular wt. of 444 for the glycerol phosphate ether and 344 for the glycerol ether, the theoretical yields of these compounds are 590 and 457 mg., respectively. The overall yield of glycerol ether was thus 71.8%.

F. Properties of the Glycerol Ether V.—The glycerol ether V had the same R_f value in 2 solvent systems as an authentic synthetic α-octadecyl glycerol ether and as the pure α-glycerol ether which was isolated from pig heart.[2] The glycerol ether V also had the identical infrared spectrum[2] and reacted mole per mole with periodate as did the synthetic and pig heart glycerol ether. Periodate oxidation was carried out as described previously.[2] We have since observed that the periodate oxidation of the glycerol ethers is complete within 0.5 hr. if carried out in 90% acetic acid. The same reaction carried out in ethanol–water required about 5 hr. to reach completion.

G. Column Chromatography of the Total Unreduced Beef Heart Lipids I.—The total lipids I (14.0 g.) of beef heart were fractionated on silicic acid as described previously[2] (section K.). The lecithin (VI) (3.2 g.) and cephalin (phosphatidyl ethanolamine) (VII) (1.84 g.) fractions were isolated. The following analytical data were obtained.[18]

	N, %	P, %	N/P	Ester/P	Aldehyde/P
Lecithin (VI)	1.87	4.05	1.02	0.92	0.89
Cephalin (VII)	2.18	4.08	1.18	0.88	0.89

Both fractions were subjected to paper chromatographic analyses. In addition to the major spot for the expected lecithin and phosphatidyl ethanolamine, minor spots of the corresponding lyso-phosphatides were also observed. Hence either during the extraction of these lipids or during the column fractionation a small amount of hydrolysis had occurred.

H. Hydrolysis Studies on the Isolated Lecithin and Cephalin Fractions.—The lecithin (VI) and cephalin (VII) fractions were subjected to the same sequence of reduction and NaOH hydrolysis as given in section O of our previous paper.[2] Under these conditions 40–45% of the reduced lecithin phosphorous and 38–40% of the reduced cephalin phosphorus were rendered alkali stable and ether extractable. Hence the amount of plasmalogen in each of these phosphatides by this procedure is 40–45% and 38–40%, respectively.

I. Production of Lyso-phosphatides from Beef Lecithin and Cephalin.—The beef lecithin (VI) and cephalin (VII) were hydrolyzed in acetic acid and subjected to paper chromatography as described previously[2] (section P). The amount of lysolecithin and lysocephalin which were produced as well as the amount of each fraction which was stable toward acetic acid was determined by quantitative paper chromatography. In the case of lecithin, 40% was converted to lysolecithin and in the case of cephalin 37% was converted to lysocephalin. The reduced lecithin and cephalin were stable toward hydrolysis with acetic acid. Moreover, before reduction the lecithin and cephalin were Schiff positive, but after reduction they were Schiff negative. These experiments support the findings in the aforementioned section H with respect to the relative amount of plasmalogen in each phosphatide fraction. Indeed the acetic acid hydrolysis followed by quantitative paper chromatography of the formed lyso-phosphatides offers a simple and direct method for the analysis of the plasmalogen content of tissue phosphatides.[19]

J. Hydrolysis of Beef Heart Lecithin with Snake Venom Phospholipase A.—Unreduced beef lecithin (VI) (268 mg.) was dissolved in 150 ml. of ether (Merck, anhydrous, peroxide free) and 2.0 ml. of a solution of snake venom (Crotalus adamanteus from Ross Allen) (3 mg. of venom in 2.0 ml. of 0.1 *M* phosphate buffer *p*H 6.9) were added. The mixture was shaken and let stand 5 hr. at room temperature. After 4 hr. 3 mg. more of venom was added. The solution was allowed to stand overnight. The next day 3 mg. more of enzyme was added. The mixture was allowed to stand for 6 days at room temperature. At each time interval the mixture was shaken and aliquots were taken for paper chromatographic analysis. This clearly showed that after 24 hr. the diester lecithin was nearly completely hydrolyzed whereas the plasmalogen form was only very slightly hydrolyzed. After the reaction was complete, a white gelatinous precipitate formed. The precipitate was separated from the supernatant fluid and washed 3 times with ether. The ether extracts were washed with water, dried and evaporated to dryness to yield 49.3 mg. of fatty acids (yellow-brown oil), VIII which was essentially free of P and Schiff reacting material. The gelatinous precipitate IX was dried and yielded 180 mg. of white solid residue. This material contained essentially all the original lipid P and Schiff reacting material. Both VIII and IX were subject to paper chromatographic analysis.[2] It was found that VIII consisted of free fatty acids, whereas IX contained a mixture of lysolecithin (X) and lysophosphatidal choline (XI). A small amount of free aldehyde also occurred in IX. Chemical analysis of the mixture IX gave

	P, %	Ester P	Aldehyde P
Lysophosphatide fraction IX	5.6	0.49	0.40

A portion of fraction IX (90 mg.) was dissolved in methanol and reduced with hydrogen over PtO_2. The reduced product (86 mg.) was entirely negative toward the Schiff reagent. The material was then refluxed with 1 *N* H_2SO_4 (aqueous) for 1 hr., cooled and extracted with ether. The water-washed ether extracts yielded 49.3 mg. of material XII which contained 1.84 mg. of P. The H_2O phase contained 2.75 mg. of P of which only 0.21 mg. was present as orthophosphate and 2.54 mg. was present as glycerolphosphate. Hence 40% of the lipide P occurred in the ether phase and represents the glycerol phosphate ether derived from the lysophosphatidal choline (XI). Thus 60% of the lysophosphatide fraction IX is the typical ester type lysolecithin (X) and 40% is the lysoplasmalogen (XI).

The ether residue XII (49 mg.) was fractionated on silicic acid in order to separate the fatty acid and glycerol phosphate ether. There was obtained 23 mg. of free fatty acid (mixture of stearic and palmitic) and 26 mg. of glycerol phosphate ether. The latter compound contained 6.8% P and gave 1 spot on paper chromatography.[2]

The glycerol phosphate ether (20 mg.) was hydrolyzed with 6 *N* H_2SO_4 for 49 hr. The extent of hydrolysis was 70.5%. The glycerol ether was obtained as outlined above (section E). The actual yield of pure α-glycerol ether was 8.1 mg. (32% yield). However, the expected yield, had all the material in each step been used and had the hydrolysis been 100%, would have been 17 mg. The theoretical yield based on the P content of the original lysophosphatide (IX) and on the fact that 40% was lysoplasmalogen (XI) can be shown to be 25 mg. Hence the yield of α-glycerol ether was 68%. The glycerol ether had the same infrared spectrum and paper chromatographic mobility[2] as that isolated from the total beef heart phosphatides and also reacted with periodate mole/mole. Hence starting with either the total phosphatides II or with the enzymatically produced lysolecithins (IX) and α-glycerol ether can be obtained in essentially the same over-all yield.

K. Reactivity of the Native Plasmalogens and the Enzymatically Produced Lyso-plasmalogen toward Schiff Re-

(18) The ester and aldehyde analyses were done as mentioned previously (G. V. Marinetti, J. Erbland and J. Kochen, *Federation Proc.*, **16**, 837 (1957)). These analyses are not precise.

(19) G. V. Marinetti, J. Erbland and E. Stotz, *Biochim. et Biophys. Acta*, in press.

agent.—Small portions (100–200 μg.) of the unreduced lecithin (VI), cephalin (VII) and lysoplasmalogens (IX) were dissolved in 0.5 ml. of 90% HAc and 6.0 ml. of the Schiff reagent added immediately. The time required to reach full color intensity was then noted. The following was observed (color intensity at 575 mμ).

Sample	Time, in min. to reach full color intensity
Beef heart lecithin (VI)	140
Beef heart cephalin (VII)	120–130
Beef heart lysoplasmalogen (IX)	17
Std. tetradecaldehyde	7

As can be seen the lysoplasmalogen is far more reactive than the lecithin from which it is derived. The reason for this is not understood. The aldehydogenic material in the lysoplasmalogen is not free aldehyde since on reduction with hydrogen the glycerol phosphate ether can be obtained in good yield.

L. Reaction of the Plasmalogens with Methanolic-I_2.—The unreduced beef heart lysophosphatides (IX) (79.5 mg.) were dissolved in 25 ml. of redistilled absolute MeOH. To the solution was added dropwise from a buret a standard 0.01 *N* solution of I_2–KI solution. The iodine uptake was immediate. The solution was titrated until a faint yellow color of excess I_2 persisted. This yellow color was compared to a similar blank solution which contained an equal volume of methanol, water and 0.1 ml. of the I_2–KI solution. The lysoplasmalogens required 12.17 ml. of the 0.01 *N* I_2 solution, which is equivalent of 0.122 meq. The theoretical expected amount of I_2 was calculated to be 0.122 meq. This was calculated as follows. The 79.5 mg. of lysophosphatides has 40% of the acetal derivative, or 31.8 mg. of lysophosphatidal choline. Assuming a chain length of 18 C, the molecular weight of the lysoplasmalogen is 524. Hence the no. of m*M* of lysoplasmalogen is 0.061. Since 1 m*M* of lysocompound requires 1 mole of I_2, this will require 2 meq. of I_2, or $2 \times 0.061 = 0.122$ meq.

We have observed that the lysoplasmalogen after iodination in methanol is still reactive toward the Schiff reagent. This was unexpected since the total beef heart plasmalogens after reaction with methanolic I_2 show a much reduced reactivity toward the Schiff reagent. Hence the lysophosphatidal choline not only reacts at a much faster rate with the Schiff reagent than the native phosphatidal choline, but also retains this reactivity after iodination whereas the native plasmalogen does not. The reason for this difference is not yet understood.

M. Characterization of Formaldehyde after Periodate Oxidation of the Glycerol Ether V.—After periodate oxidation[2] of 58.2 mg. of glycerol ether in 90% acetic acid in water, the excess periodate was removed by addition of arsenious acid. The resulting solution was neutralized with NaOH and then 125 mg. of dimedon reagent in 1 ml. of 95% ethanol was added. The solution was let stand overnight. The formaldimedone derivative which precipitated out was removed by centrifugation and washed with small portions of water and chloroform. The yield of formaldimedone was 40 mg. (theoretical yield 49 mg.). Some formaldimedone was in the chloroform washes but this was not recovered. Hence the minimum yield of formaldimedone was 82% The melting point of the derivative was 188–189°. The melting point of an authentic sample of formaldimedone which was prepared from formaldehyde was 189–189.5°. Mixed melting point was 188–189°.

N. Studies on the Chloroform Eluted Fraction.—The chloroform eluates which were obtained by column chromatography of a portion of the glycerol ether fractions (a) and (b) mentioned in section E above were subjected to chemical, chromatographic and spectral analysis. These materials from both pig and beef heart were nearly identical. The yield from pig heart was 24 mg. and the yield from beef heart was 37 mg. These materials contained no N, P, ester or aldehyde but did give a weak positive test with 2,4-dinitrophenylhydrazine. The infrared spectrum was in agreement with a diether derivative of glycerol but did contain a relatively weak carbonyl band near 5.8 μ. Hence some contaminating ketone is present. The mobility of the material both on column and on paper showed that it was a less polar compound than a monoether derivative of glycerol.

Acetylation with acetic anhydride in pyridine yielded an ester derivative. Ester analyses were done by the method of Rapport and Alonzo.[20] Based on the molecular extinction coefficient of several esters (tripalmitin, monopalmitin, lecithin and cholesterol acetate) the ester compound was in agreement with a monoester derivative. The molecular weight of the compound as approximated by the ester analysis and assuming it to be a monoester was between 560 and 600. The molecular weights for a C-18 and C-20 diether derivative of glycerol are 597 and 653, respectively.

Elementary analysis of the material from the pig and beef heart chloroform fractions are[21]

	C, %	H, %
Found:		
Pig heart fraction	80.35	13.46
Beef heart fraction	80.25	13.54
Calculated:		
C-18 diether $C_{39}H_{80}O_3$ (597.03)	78.50	13.50
C-20 diether $C_{43}H_{88}O_3$ (651.13)	79.10	13.57

The combined data given above are in agreement (*although not conclusive*) for an α,β-long chain diether of glycerol. The authors wish to sincerely apologize for their previous misjudgment[2] in assuming that the chloroform fraction was mainly β-glycerol ether. At the time this interpretation was made, it seemed reasonable on the basis of the existing evidence. However, when the C and H analyses were later obtained, these were not in agreement with a monoether derivative of glycerol but rather with a diether derivative of glycerol. Further work is in progress to confirm the structure of this chloroform eluted material.

Addendum.—After this manuscript was submitted for publication a further paper by Gray on the structure of beef heart plasmalogens appeared in print.[22] These points should be considered between our work and that of Gray since there is an apparent lack of agreement on the structure of the beef plasmalogens: (a) Our work demonstrates that at least 70% of the plasmalogens are of the α-type. The remaining 30% may represent plasmalogens of either the β-type or the α,β-type. (b) Borgstrom[23] and Hirsch and Ahrens[24] have shown that during passage of 2-monoglycerides on silicic acid there occurs isomerization of the fatty acid to the C-1 position of glycerol. Because of this knowledge we did not subject our lysophosphatides to silicic acid treatment as did Gray. Isomerization of the lysolecithins prior to the oxidation of these compounds would lead to erroneous conclusions regarding their structure, (c) Gray obtained only a 45% yield of the methylglyoxal. One must consider this relatively low yield in the final analysis of this problem, (d) The prolonged treatment of the lysolecithins to strong acetic acid may bring about ester interchange and isomerization. It is apparent that isomerization of functional groups is important to this problem. We consider isomerization of the ester function far more facile than that of the saturated ether function.

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