[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF ROCHESTER SCHOOL OF MEDICINE AND DENTISTRY]

The Structure of Pig Heart Plasmalogens

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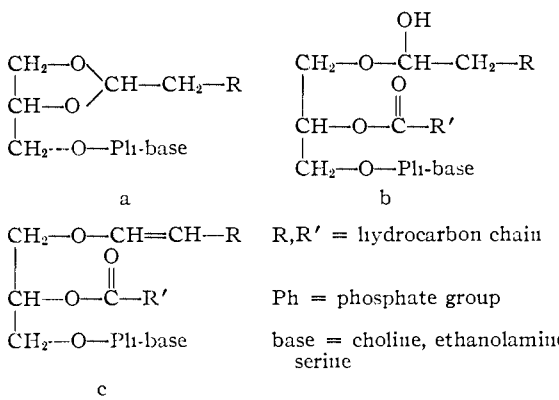
A long chain glycerol ether has been isolated by prolonged acid hydrolysis of reduced total pig heart phosphatides, pig heart lecithin and pig heart cephalin. In all cases the glycerol ethers react with one mole of periodic acid per mole of compound and therefore must be α-ethers. The infrared spectra and paper chromatographic mobility of the isolated glycerol ethers are identical to those of an authentic synthetic sample of *d*-α-octadecyl glycerol ether. Elementary analysis is also in agreement with this latter structure. The pig heart lecithin was purified by column chromatography and shown to be a mixture of the diester lecithin (60%) and the monoester–acetal type lecithin (40%). This was demonstrated by chemical analysis and by mild acid hydrolysis. The latter treatment converted 40% of the total lecithin to lysolecithin with the concomitant liberation of a long chain aldehyde. These findings demonstrate that some of the plasmalogens of pig heart muscle contain the long chain aldehyde on the α-carbon atom of the glycerol moiety and the long chain fatty acid on the β-carbon atom of this alcohol.

Plasmalogens were first isolated in 1939 by Feulgen and Bersin.[1] Later, Thannhauser and collaborators[2] obtained a similar compound from brain. The cyclic acetal structure (a) first proposed for these plasmalogens has now been shown to be different from the naturally occurring plasmalogen. Schmidt and co-workers[3,4] observed that the plasmalogens which are isolated by alkaline treatment did not correspond to the native plasmalogen which is extracted directly from tissues without the use of alkali, since the former but not the latter yields all of its phosphorus in a water-soluble form upon mild acid hydrolysis. Moreover, Klenk and Debuch[5] showed that the phosphorus content of the native plasmalogen was too low for a cyclic acetal structure shown by (a). These workers suggested several possible structures which were consistent with the known data. Two of these structures are indicated by (b) and (c).



Although the plasmalogens which were first isolated contained ethanolamine, evidence for similar phosphatides containing choline has been obtained.[6,7] Rapport and Alonzo[8] have recently reported that a Pangborn preparation of beef heart lecithin exists to the extent of 60% as the plasmalogen which they have named "phosphatidal choline." Klenk, *et al.*,[9] have analyzed a beef heart lecithin fraction and report that 40% exists as the plasmalogen. Studies from our laboratory[10] also show that 40% of the pig heart lecithin occurs as the plasmalogen.

The position of attachment of the aldehyde group to the glycerol moiety of the plasmalogens has been investigated by Klenk and Debuch,[9] Rapport and co-workers[11,12] and Marinetti and collaborators.[10,13] The results appear to be at variance since the work of Klenk and Debuch[9] and of Marinetti and Erbland[10] demonstrates that the aldehyde is attached to the α-carbon atom of glycerol whereas the work of Rapport and co-workers places the aldehyde function on the β-position. It is noteworthy that Rapport previously reported[14] that the glycerol ether obtained from beef heart lecithin did react in part with periodic acid. One can assume then that the product exists partially as the α-glycerol ether. Although the existence of plasmalogens having the aldehyde group on the β-position has not been ruled out exclusively, in pig heart at least, such isomers can only be present in small amount. A species difference may explain the discrepancy between the work mentioned above.

The data given in this paper represent an extension of our previous work[10,13] and demonstrate by a chemical method that some of the plasmalogens of pig heart are of the α-type (*i.e.*, the aldehyde group is attached to the α-carbon atom of glycerol and the ester group is linked to the β-carbon atom). A long chain glycerol ether has been isolated from the reduced pig heart lecithin and cephalin fractions and from the total pig heart phosphatides. An outline of the procedure used is given in Fig. 1 and the sequence of reactions giving rise to the glycerol ether is given in Fig. 2. In all cases the glycerol ether reacts with one mole of periodate per

(1) R. Feulgen and T. Bersin, *Z. physiol. Chem.*, **260**, 217 (1939).

(2) S. J. Thannhauser, N. F. Boncoddo and G. Schmidt, *J. Biol. Chem.*, **188**, 417 (1951).

(3) G. Schmidt, B. Ottenstein, N. Zoellner and S. J. Thannhauser, Abs. XIIth Inter. Congress of Pure and Applied Chem., New York, 104, Sept. 1951.

(4) G. Schmidt, B. Ottenstein and M. J. Bessman, *Federation Proc.*, **12**, 265 (1953).

(5) E. Klenk and H. Debuch, *Z. physiol. Chem.*, **296**, 179 (1954).

(6) M. H. Hack, *Biochem. J.*, **54**, 602 (1953).

(7) E. Klenk and G. Gehrmann, *Z. physiol. Chem.*, **292**, 110 (1953).

(8) M. M. Rapport and N. Alonzo, *J. Biol. Chem.*, **217**, 199 (1955).

(9) E. Klenk and H. Debuch, *Z. physiol. Chem.*, **299**, 66 (1955).

(10) G. V. Marinetti, J. Erbland and J. Kochen, *Federation Proc.*, **16**, 837 (1957).

(11) M. M. Rapport and R. E. Franzl, *J. Biol. Chem.*, **225**, 851 (1957); *J. Neurochem.*, **1**, 303 (1957).

(12) M. M. Rapport, B. Lerner, N. Alonzo and R. E. Franzl, *ibid.*, **225**, 859 (1957).

(13) G. V. Marinetti and J. Erbland, *Biochim. et Biophys. Acta*, **26**, 429 (1957).

(14) M. M. Rapport, in G. Popjak and E. LeBreton, "Proc. of the 2nd International Conference on the Biochemical Problems of Lipids," New York, N. Y., 1956, p. 102.

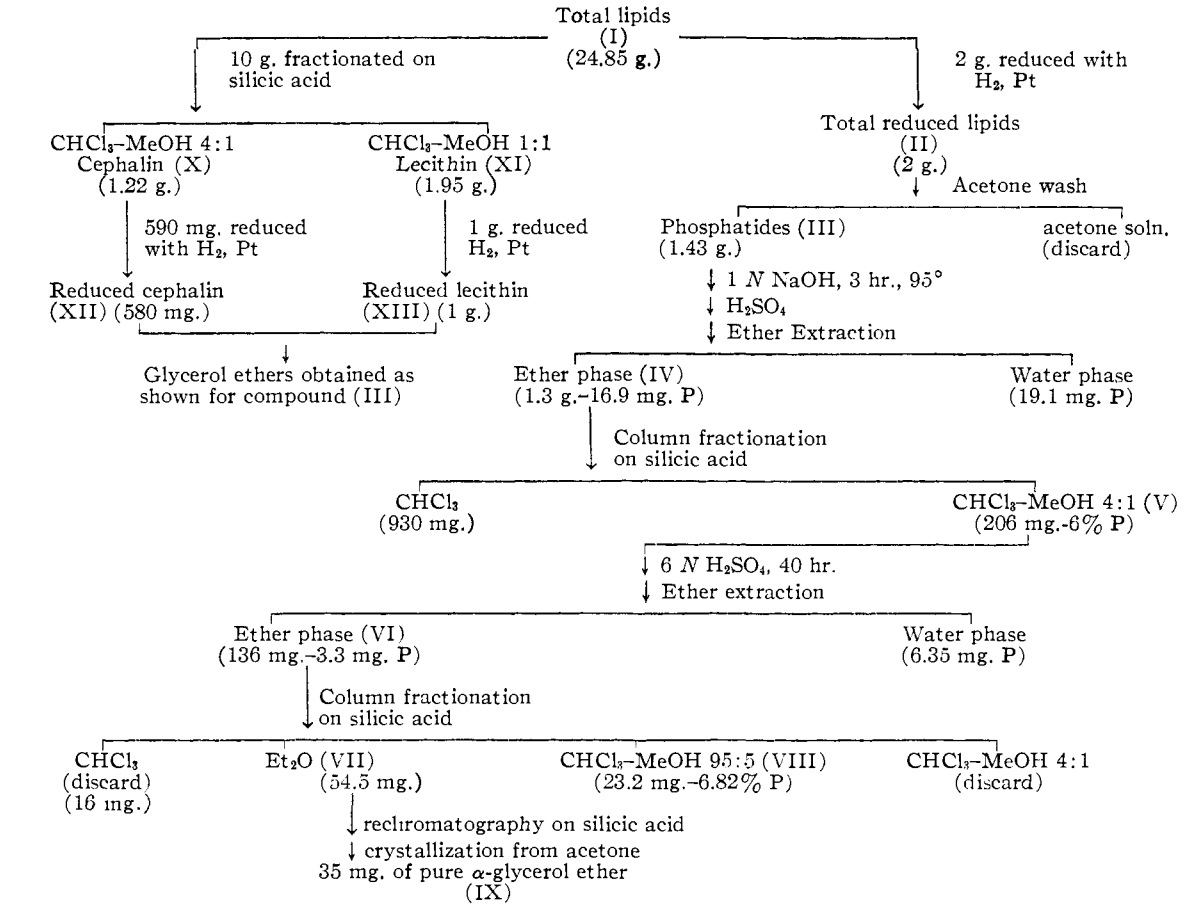


Fig. 1.—Flow diagram for demonstrating the preparation of the glycerol ethers from the pig heart plasmalogens.

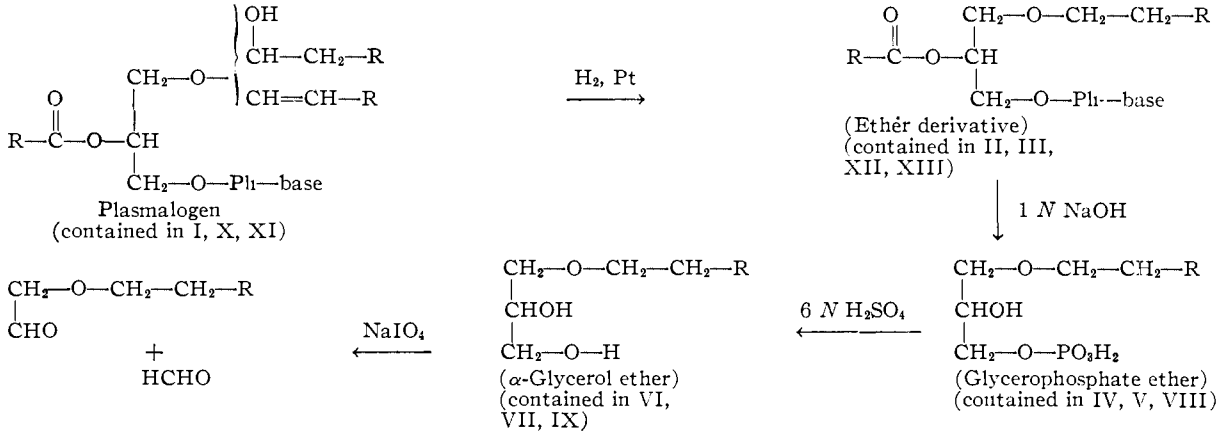


Fig. 2.—Reaction scheme demonstrating the production of the α-glycerol ether from the reduced plasmalogens: Ph–phosphate group; base–choline, ethanolamine, serine; R– hydrocarbon chain. Roman numerals refer to fractions in the Experimental section.

mole of compound. Furthermore, all the glycerol ethers have the same chromatographic mobility and infrared spectrum as a pure synthetic *d*-α-octadecyl glycerol ether[15] and react with periodate at the same rate and to the same extent as the synthetic ether. In addition, hydrolysis of the native plasmalogens with acetic acid produces lysophosphatides and free aldehyde. Hence the structure of the pig heart plasmalogens are those shown by structures (b) and (c). The ease of reduction of these compounds and their reactivity toward methanolic iodine[11] is evidence which supports the unsaturated ether structure (c).

(15) Kindly donated by Dr. E. Baer of the University of Toronto.

Experimental

A. Preparation of Total Pig Heart Lipids I.—Wet pig heart (728 g.) was homogenized in a Waring blendor in 100 ml. of methanol. To the homogenate were added 100 ml.

of methanol and 360 ml. of chloroform. Another 400 ml. of methanol–chloroform 3:1 was added and the homogenate was centrifuged at 3000 r.p.m. The supernatant fluid was decanted and saved and the residue extracted twice more with methanol–chloroform 1:1. The combined supernatant fluids were washed twice with a 3-fold volume of water. The washed chloroform phase was filtered through a sintered glass funnel and the filtrate evaporated to dryness under nitrogen *in vacuo* at 40°. The yield of total lipid (I) was 24.85 g.

B. **Reduction of I with Hydrogen.**—Two grams of I was dissolved in 100 ml. of methanol–hexane 1:1, 400 mg. of platinum oxide added and the lipid reduced for 3 hours in a Parr apparatus under 60 lb. pressure of hydrogen at room temperature. The catalyst was removed by filtration and the filtrate evaporated to dryness as described in section A. Approximately 2 g. of reduced lipid II was obtained. The total lipids I gave a strong test for aldehydes with both the Schiff and 2,4-dinitrophenylhydrazine reagents. On the other hand the reduced lipids II were completely negative.

C. **Preparation of the Reduced Total Phosphatides III.**—The reduced lipids II were washed repeatedly at room temperature with acetone in order to remove the non-phosphatides. The acetone-insoluble material was dried to yield 1.43 g. of reduced phosphatides III (2.5% P).

D. **Hydrolysis of the Reduced Total Phosphatides III.**—The reduced phosphatides III (1.43. g.) were hydrolyzed for 3 hours at 95° in 60 ml. of 1 *N* aqueous NaOH. The mixture was cooled and acidified with 20 ml. of 10 *N* H_2SO_4 and allowed to stand for ½ hr. The lipid material was extracted three times with peroxide-free ethyl ether. The combined ether extracts were washed in a separatory funnel 4 times with water and evaporated to dryness to yield 1.3 g. of product IV which consisted of a mixture of free fatty acids, a long chain ether of glycerol phosphate V, and some partial hydrolysis products of the phosphatides. The ether phase contained 16.9 mg. of P and the water phase contained 19.1 mg. of P.

E. **Isolation of the Glycerol Phosphate Ether V by Column Chromatography.**—The mixture IV (1.3 g.) was fractionated on silicic acid (Mallinckrodt, 100 mesh, for chromatography) as follows: 10 g. of silicic acid was suspended in 40 ml. of chloroform–methanol 1:1 and the slurry poured into a column measuring 1.5 cm. diameter. The adsorbent was allowed to settle under an air pressure head and then washed with 25 ml. of chloroform. The mixture IV was dissolved in 5 ml. of chloroform and placed on the column. Elution with 75 ml. of chloroform removed all the fatty acids (930 mg.) and subsequent elution with 100 ml. of 20% methanol in chloroform yielded the glycerol phosphate ether V (206 mg.) which contained 6.0% P and 0.59% N. This latter fraction V contained small amounts of partial hydrolysis products of the phosphatides. The calculated yield of V based on the P content of III is 171 mg. The amount of glycerol phosphate ether in V based on the 6% P is 168 mg.

F. **Hydrolysis of the Glycerol Phosphate Ether (V).**—The product V (206 mg.) was suspended in 70 ml. of 6 *N* aqueous H_2SO_4 and hydrolyzed under reflux for 40 hours. The mixture was cooled and extracted three times with ethyl ether. The combined ether extracts were washed with water and evaporated to dryness to yield 136 mg. of waxy residue VI which contained 3.3 mg. of P. The residue VI was found to be a mixture of a long chain glycerol ether VII and the unhydrolyzed glycerol phosphate ether V. The water phase remaining after ether extraction contained 6.35 mg. of P. Hence the per cent. hydrolysis of the phosphorylated glycerol ether V was 64%. The estimated yield of glycerol ether in VI is about 90 mg. (theoretical, 135 mg.). However, 114 mg. would have been obtained if the hydrolysis were complete.

G. **Isolation of the Glycerol Ether VII by Column Chromatography.**—The mixture VI (136 mg.) was dissolved in 5 ml. of chloroform and adsorbed on a 10-gram column of silicic acid which was prepared as described in section E. The column was eluted with these various solvents in the order given: (a) 75 ml. of chloroform, (b) 100 ml. of ethyl ether, (c) 50 ml. of 5% methanol in chloroform, (d) 100 ml. of 20% methanol in chloroform. The glycerol ether VII was found exclusively in the ether eluate (eluate b). The yield was 54.5 mg. (theoretical, 135 mg.). This material contained only a very small amount of P (0.3%). The unhydrolyzed glycerol phosphate ether V occurred in the 5% methanol eluate c. The yield of this material VIII was 23.2 mg. Compound VIII contained 6.82% P (theoretical value for a C-18 ether is 7.3%).

H. **Further Purification of the Glycerol Ether VII.**—The glycerol ether VII was dissolved in chloroform and rechromatographed on silicic acid. The first eluate of 15 ml. of chloroform was discarded. Subsequent elution with 50 ml. of 20% ethyl ether in chloroform yielded 47 mg. of a white waxy material IX which was free of P, N, ester and aldehyde.

Forty mg. of IX was dissolved in 4 ml. of warm redistilled acetone and cooled at 0° for 24 hours. The white crystalline precipitate was removed by centrifugation and washed with cold acetone. The yield of recrystallized glycerol ether was 35 mg. The melting point of this compound depended on the rate of heating. With relatively rapid heating this compound melted at 65–68°. The authentic sample of *d*-α-octadecyl glycerol ether[15] melted at 68–70° under the same conditions (Baer and Fischer report a melting point of 71–72° for this material[16]). Mixed melting point of our product with the synthetic glycerol ether was 67–70°.

The elementary analysis of the recrystallized glycerol ether IX was[17]: C, 73.70; H, 12.66 (theoretical values for an octadecyl glycerol ether $C_{21}H_{44}O_3$ are C,73.05; H, 12.88). Although the analysis of the glycerol ether is in agreement with an octadecyl derivative, the melting point range of the compound indicates the possible presence of some C-16 and C-20 derivatives. Indeed, it would be unusual to isolate a single molecular species from a natural product of the type which was used.

The infrared spectra of the recrystallized glycerol ether IX and the synthetic octadecyl glycerol ether were run in KBr at a concentration of 0.6 mg. per gram of KBr.[18] The spectra of these two compounds were identical. The major spectral bands (in microns) were: 2.94, 3.43, 3.51, 6.82, 7.25, 7.53, 8.05, 8.94, 9.44, 10.70, 10.94, 11.6, 13.92.

I. **Paper Chromatographic Analysis of the Glycerol Ether IX and the Purified Phosphate Derivative VIII.**—Compounds VIII, IX and the synthetic octadecyl glycerol ether were analyzed by paper chromatography in two solvent systems. The first consisted of *n*-heptane–diisobutyl ketone 70:60 and employed silicic acid impregnated paper which was prepared as described previously.[10] Compound IX and the synthetic glycerol ether had the same R_f value (R_f 0.22). The glycerol ether phosphate VIII had an R_f value of 0.00 in this system. The second system consisted of acetic acid–water 90:10 (saturated with liquid petrolatum) and employed filter paper impregnated with a 10% solution of liquid petrolatum.[19] Both the natural glycerol ether IX and the synthetic octadecyl glycerol ether had the same R_f value (R_f 0.81). The R_f value of the glycerol phosphate ether VIII in this latter system was 0.52. The compounds were detected on chromatograms with Rhodamine 6G (0.001% aqueous solution) as described previously.[10] The natural glycerol ether IX and the synthetic glycerol ether appeared as yellow spots under ultraviolet light (on wet chromatograms) whereas the phosphate compound VIII appeared blue.

J. **Periodate Oxidation of the Glycerol Ether IX.**—The glycerol ether IX was dissolved in chloroform at a concentration of 1.87 mg. per 2.0 ml. Two-ml. aliquots containing 5.45 μ*M* of compound (based on a molecular wt. of 344) were pipetted into test-tubes and evaporated to dryness. To each tube was added 4.0 ml. of 95% ethanol. After the compound had dissolved, 0.1 ml. of 0.1 *M* phosphate buffer *p*H 7.4 was added. To one tube which served as the control was added 1.0 ml. of water and to the other tube was added 1.0 ml. of 0.01 *M* $NaIO_4$. The optical density of the solutions was determined at 300 mμ at 15-min. intervals for a period of 5 hours. The reaction was essentially complete by this time. The periodate consumption *vs.* time

(16) E. Baer and O. L. Fischer, *J. Biol. Chem.*, **140**, 397 (1941).

(17) Analysis was done by the Schwarzkopf Microanalytical Lab., Woodside, N. Y.

(18) The infrared spectra were run by Dr. W. B. Mason and Mr. A. Behringer of the Univ. of Rochester Atomic Energy Project and were made possible in part by funds from the U. S. Atomic Energy Commission. A Perkin-Elmer model 21 Double Beam Spectrophotometer (rock salt prism) was used.

(19) H. P. Kaufmann and W. H. Nitsch, *Fette und Seifen*, **56**, 154 (1954).

is given in Fig. 3. The data show that 5.45 μM. of glycerol ether IX consumed 5.8 μM. of periodate.

The periodate oxidation was also carried out on the synthetic material (see Fig. 1). The data show that 5.0 μM. of the synthetic glycerol ether consumed 5.2 μM. of periodic acid and did so at the same rate as the natural ether IX.

The natural glycerol ether IX and the synthetic glycerol ether were completely negative toward the Schiff reagent before periodate oxidation but both gave the expected purple color test after periodate oxidation. In order to determine the amount of long chain aldehyde which was produced after oxidation, 0.2-ml. aliquots of each reaction mixture were pipetted into test-tubes and evaporated just to dryness with a stream of nitrogen at room temperature. This was done to remove the formaldehyde which also was formed as a result of periodate oxidation. To each tube were added the following: 0.5 ml. of glacial acetic acid, 4.0 ml. of Schiff reagent[10] and 4.0 ml. of water. The color intensities were determined at 570 mμ after 15 minutes (a Bausch and Lomb "Spectronic" colorimeter was used). The amount of aldehyde produced also was checked against a calibration curve of *n*-tetradecaldehyde. The natural product IX produced 6.1 μM. of aldehyde and the synthetic glycerol ether produced 5.1 μM. of aldehyde. Hence on a molar basis the natural glycerol ether IX and the synthetic glycerol ether consumed essentially one mole of periodate with the concomitant formation of one mole of long chain aldehyde.

K. Column Chromatography of the Total Pig Heart Lipids I.—In order to obtain the lecithin and cephalin fractions of the pig heart phosphatides, 10 g. of I was chromatographed on 160 g. of silicic acid. The column (3.5 cm. diameter) was prepared in chloroform–methanol 4:1 and the excess methanol displaced with chloroform. The lipids I were applied in chloroform and eluted with the following solvents in the order given: (a) 430 ml. of chloroform, (b) 500 ml. of chloroform–methanol 4:1, (c) 750 ml. of chloroform–methanol 1:1, (d) 700 ml. of absolute methanol. Fractions of 25-ml. volume were taken. The cephalin fraction X was eluted with chloroform–methanol 4:1. The yield was 1.22 g. The lecithin fraction XI was eluted with chloroform–methanol 1:1. The yield was 1.95 g. These fractions represent the center cuts of each of the corresponding peaks and hence do not represent the total amount of each of these phosphatides, although they do account for the major part of each respective fraction. Fractions X and XI were subjected to paper chromatographic analysis and chemical analysis. With regard to the latter, the P, N, ester, aldehyde and fatty acid contents were determined, with the results shown

	% N	% P	N/P	Ester/P	Aldehyde/P	% fatty acid
Cephalin X	2.50	2.60	2.1	1.0	0.96	41
Lecithin XI	1.66	3.89	1.05	1.3–1.5	0.89	48

The cephalin fraction X was shown by paper chromatography and hydrolysis studies to be mainly a mixture of the diester and plasmalogen forms of phosphatidyl ethanolamine and a smaller amount of phosphatidyl serine. After hydrolysis with 1 *N* HCl the bases serine and ethanolamine were identified by paper chromatography using the solvent systems described previously.[20]

The lecithin fraction XI also was shown to be a mixture of the diester and plasmalogen forms. After hydrolysis with 1 *N* HCl, choline was the only base which was detected on paper chromatograms.[20] The principal spots which were observed on chromatograms of unhydrolyzed X and XI had the same R_f values as authentic synthetic samples[15] of lecithin and phosphatidyl ethanolamine and gave the expected color tests with either the ninhydrin or choline reagents.[21]

L. Reduction of the Cephalin X and Lecithin XI Fractions.—Fraction X (590 mg.) was dissolved in a solution of 25 ml. of methanol and 20 ml. of benzene, 200 mg. of platinum oxide added, and the material was reduced under 67 lb. pressure of H_2 for 3 hr. as described in section B. The solution was flushed with N_2, heated and filtered. The filtrate was evaporated to dryness *in vacuo* under N_2 to yield about 580 mg. of reduced cephalin XII.

(20) G. V. Marinetti, D. J. Scaramuzzino and E. Stotz, *J. Biol. Chem.*, **224**, 819 (1957). The high N/P ratio of X is suggestive of a phosphatide-dipeptide.[10]

(21) C. Levine and R. Chargaff, *J. Biol. Chem.*, **192**, 465 (1951).

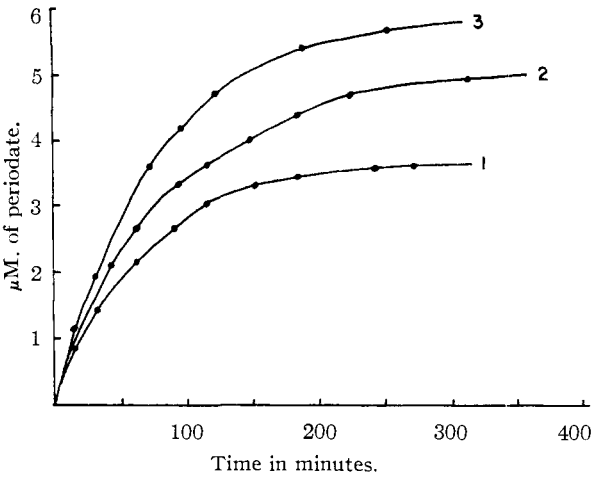


Fig. 3.—Periodate consumption of the natural and synthetic glycerol ethers. Curve 1 represents the rate of periodate consumption by 4.0 μM. of glycerol ether obtained from either the reduced cephalin XII or the reduced lecithin XIII. Curve 2 represents similar data on 5.0 μM. of synthetic *d*-α-octadecyl glycerol ether.[15] Curve 3 represents similar data on 5.45 μM. of glycerol ether IX. Experimental details are given in the text.

Fraction XI (1 g.) was dissolved in 50 ml. of methanol, 200 mg. of platinum oxide added, and the lipid was reduced as described above. The yield of reduced lecithin XIII was about 1 g. Compounds X and XI gave a strong test for aldehyde with the Schiff and 2,4-dinitrophenylhydrazine reagents but after reduction compounds XII and XIII were completely negative, The cephalin fraction X did contain a small amount of phosphatidyl serine. Since Klenk and Bohm[22] and Ansell and Norman[23] have shown that serine-containing plasmalogens are present in tissues, some glycerol ether may arise from this phosphatide.

M. Isolation of the Glycerol Ether from Reduced Cephalin XII and Reduced Lecithin XIII.—The procedure outlined in sections D–G was used to isolate the long chain glycerol ether from compounds XII and XIII. Approximately 20 mg. of product was obtained from XII and 30 mg. from XIII. Paper chromatographic analysis of the glycerol ethers was carried out as described in section I. The R_f values of the glycerol ethers from XII and XIII were identical to that of both compound IX and the synthetic octadecyl glycerol ether. Furthermore, the infrared spectra of all the glycerol ethers were essentially identical.

N. Periodate Oxidation of the Glycerol Ethers Obtained from XII and XIII.—The periodate oxidation and the Schiff analysis of the liberated aldehyde were carried out on the glycerol ethers obtained from XII and XIII. The method used was the same as that described in section J. On a molar basis (4.0 μM. consumed 3.8 μM. of periodate) both of the glycerol ethers obtained from XII and XIII consumed essentially one mole of periodic acid (Fig. 3) with the concomitant release of 1 mole of long chain aldehyde. Hence all the glycerol ethers are predominantly if not exclusively α-derivatives.

O. Further Studies on the Pig Heart Lecithin XI and XIII.—Fifty-mg. samples of compounds XI and XIII were hydrolyzed in 1 *N* NaOH and 1 *N* HCl for 2 hr. under reflux. The alkaline solution was acidified with H_2SO_4 and the solutions from both the hydrolyses were extracted three times with ethyl ether. The ethyl ether extracts were washed with water and made to a known volume. Phosphorus analyses[10] were performed on both the ethyl ether extracts and the respective water phases remaining after ether extraction. After NaOH hydrolysis 7–11% of the total P of XI and 39.8% of the total P of XIII occurred in the ether phase. After HCl hydrolysis, 6% of the total P of XI and 41% of the total P of XIII occurred in the ether extract. Hence after reduction of the lecithin fraction a

(22) E. Klenk and P. Bohm, *Z. physiol. Chem.*, **98**, 288 (1951).

(23) G. B. Ansell and J. M. Norman, *Biochem. J.*, **59**, ix (1955).

much greater amount of P is rendered ether soluble. Indeed, this P was shown to be due to the long chain ether of glycerol phosphate V.

P. Production of Lysolecithin and Long Chain Aldehyde by Mild Acid Hydrolysis of Lecithin XI.—The unreduced lecithin fraction XI (7.4 mg.) was dissolved in 1 ml. of glacial acetic acid, 2 drops of saturated $HgCl_2$ solution added, and the mixture heated in a boiling water-bath for 20 minutes and then let stand at room temperature for 20 hours. After the hydrolysis free aldehyde was liberated and lysolecithin was produced. However, only 40% of XI was hydrolyzed by this procedure. The products were easily detected by chromatography on silicic acid impregnated paper using diisobutyl ketone–acetic acid–water 40:20:3 as solvent.[10] Chromatography was carried out at 0–5°. The R_f values of lecithin, lysolecithin and the long chain aldehyde were 0.38, 0.22 and 0.90, respectively. The lecithin and lysolecithin were analyzed quantitatively by paper chromatography as described previously.[10] For this analysis the acetic acid solution was evaporated to dryness under nitrogen and the residue dissolved in exactly 0.74 ml. of isoamyl alcohol–benzene 1:1. Six 20-μl. aliquots were placed on silicic acid impregnated paper and the chromatograms developed in the solvent mentioned above. The lipids were detected on the chromatograms with Rhodamine 6G. The lecithin and lysolecithin spots were cut off and analyzed for total lipid P. The results showed that after hydrolysis 60% of the P occurred in the lecithin fraction and 40% in the lysolecithin fraction. Control chromatograms of the lecithin which was not hydrolyzed with acetic acid showed that only trace amounts of lysolecithin were present. Furthermore, when the reduced lecithin XIII was hydrolyzed with acetic acid as described above, no lysolecithin or free aldehyde was produced. These results are in agreement with the data given in section O. The data also show that 40% of the pig heart lecithin exists as the plasmalogen and 60% as the classical diester structure.

In order to clarify the apparent discrepancy between our findings and those of Rapport and co-workers,[11,12] we have prepared the phosphatides of beef heart and subjected these to the same reactions given in this paper. The beef heart plasmalogens, as well as those of pig heart, occur mainly in the lecithin and cephalin fractions. By our method beef heart lecithin and cephalin contain 40 and 37%, respectively, of the plasmalogen form, the remaining being the diester type. When the hydrolysis products of the glycerophosphate ethers were chromatographed on silicic acid as outlined in section G (Experimental section), we have subsequently found that the beta-glycerol ethers occur predominantly in the chloroform eluate whereas the alpha-glycerol ethers occur predominantly in the ethyl ether eluate. This procedure, coupled with chemical, infrared spectral, and paper chromatographic analysis and with the reactivity of these ethers toward periodate has now shown that the pig heart glycerol ethers are mainly alpha-derivatives (75% alpha and 25% beta) but the beef heart glycerol ethers are predominantly beta-derivatives (87% beta and 13% alpha). Hence a species difference explains the apparent discrepancy between our work and that of Rapport and collaborators.

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ROCHESTER, N. Y.

[CONTRIBUTION NO. 2257 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

Observations on the Chromatographic Heterogeneity of Normal Adult and Fetal Human Hemoglobin: A Study of the Effects of Crystallization and Chromatography on the Heterogeneity and Isoleucine Content

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By chromatographic methods, it has been shown that both crystallized and uncrystallized normal adult human hemoglobin contain a main component that comprises about 90% of the heme proteins. Three minor heme proteins and a non-heme protein also have been detected. The several components are not artifacts of preparation or chromatography, and they have been shown to differ in their content of isoleucine. Cord blood hemoglobin also contains minor components. These results are discussed in relation to other reports of the heterogeneity of human hemoglobin. The methods described may be used for the isolation of preparative amounts of the main components.

Introduction

Although there have been numerous reports of the heterogeneity of normal adult human hemoglobin,[1] it has generally been assumed that hemoglobin carefully crystallized, for example by Drabkin's method,[2] is homogeneous. Thus, crystallized hemoglobin has been used for amino acid analysis,[3–5] for the determination of N-terminal amino acids[6] and for other measurements in this and other laboratories. Indeed, Rhinesmith, Schroeder and Pauling[6] reported that crystallized hemoglobin did not show the heterogeneity on starch block electrophoresis that Kunkel and Wallenius[7] described for uncrystallized hemoglobin. In the course of experiments that required pure ferrihemoglobin cyanide, a study of the homogeneity of hemoglobin preparations was prompted by the observation that a minor component could be detected chromatographically in ferrihemoglobin cyanide that had been prepared from crystallized oxyhemoglobin. Later experiments showed that oxyhemoglobin and carbonmonoxyhemoglobin likewise were heterogeneous and that the minor component was not an artifact of preparation or chromatography nor could it be removed by crystallization under the most exacting conditions.

Although the chromatographic techniques employed in this study differ from those of previous workers, their published descriptions of the use of the ion exchange resin IRC-50 were of great assistance in the selection of proper conditions. The

(1) H. Itano, *Ann. Rev. Biochem.*, **25**, 331 (1956).

(2) D. L. Drabkin, *Arch. Biochem.*, **21**, 224 (1949).

(3) W. A. Schroeder, L. M. Kay and I. C. Wells, *J. Biol. Chem.*, **187**, 221 (1950).

(4) A. Rossi-Fanelli, D. Cavallini and C. de Marco, *Biochem. et Biophys. Acta*, **17**, 377 (1955).

(5) D. W. Allen and W. A. Schroeder, *J. Clin. Invest.* **36**, 1343 (1957).

(6) H. S. Rhinesmith, W. A. Schroeder and L. Pauling, THIS JOURNAL, **79**, 609 (1957).

(7) H. G. Kunkel and G. Wallenius, *Science*, **122**, 288 (1955).