[CONTRIBUTION NO. 1485 FROM THE STERLING CHEMISTRY LABORATORY, AND THE BINGHAM OCEANOGRAPHIC LABORATORY, YALE UNIVERSITY]

Contributions to the Study of Marine Products. XLVI. Phospholipids of a Sea Anemone[1]

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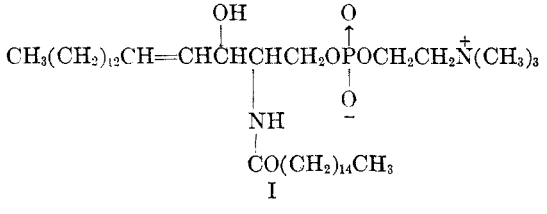
The phospholipids of the sea anemone, *Anthopleura elegantissima*, have been isolated and analyzed. The principal, if not the only, representatives were *sphingomyelin* and *plasmalogen* in a ratio of twenty to one. The sphingomyelin consisted mainly of the *N-palmityl* type. The plasmalogen fraction was obtained in a high state of uniformity. Its rather small degree of unsaturation, its infrared spectrum, and the presence of only one chain of methylene groups showed the acetal attachment of the aldehyde moiety in agreement with Feulgen's original formulation.

In connection with our comparative studies on the composition and evolution of the lipids of marine invertebrates, we have investigated the phospholipids of the common sea anemone of the West Coast, *Anthopleura elegantissima*.[2] The composition of the phospholipid mixture was of an exceptional simplicity which made possible a relatively facile isolation in a pure state of its only representatives, sphingomyelin and plasmalogen. They occurred in a ratio of about twenty to one, and plasmalogen, the minor component, constituted about 0.1 per cent of the dry weight of the anemone.

The sphingomyelin, a colorless powder, m.p. 181.5–183° (dec.) appeared to be free of other phospholipids when subjected to paper strip chromatography. Its elementary analysis indicated that it was a sphingomyelin with an *N*-palmityl group (I). In accordance with this structure the



I

sphingomyelin upon hydrolysis afforded choline, sphingosine, and palmitic acid. The choline was isolated as its reineckate, and the sphingosine was characterized as its triacetyl derivative, m.p. 97°; $[\alpha]_D$ −18.2°. The palmitic acid was isolated as its methyl ester and determined by means of gas chromatography.[3] Besides methyl palmitate the ester mixture contained small amounts of esters of acids of the order C_{12} to C_{20}. The low iodine number of the ester mixture indicated unsaturation not exceeding 0.1 double bond per mole. Similar results were obtained by means of gas chromatography of the brominated esters, and catalytic hydrogenation of spingomyelin. In the latter only 0.1 mole of hydrogen was consumed in addition to the one mole required to saturate the double bond in the sphingosine moiety. The infrared spectrum of the dihydrosphingomyelin differed from the unsaturated material only in the diminution of the absorption band at 10.3 microns, which is attributable to the disappearance of the *trans*-substituted double bond.

The simplicity of the phospholipid mixture made possible the isolation of the plasmalogen fraction without taking recourse to hydrolyses. Only selective treatments with organic solvents at room temperature were required, ensuring the isolation of an unaltered plasmalogen fraction. The material thus obtained appeared to be of a uniformity rarely if ever equaled before. It was a white wax, which upon heating decomposed above 200°. Its optical activity, $[\alpha]_D$ −7.85°, agreed in direction and magnitude with that of Rapport's plasmalogen fraction,[4] but not with that of Thannhauser's sample; $[\alpha]_D$ +6.25°.[5] The plasmalogen rapidly gave a positive Schiff's aldehyde test. Its elementary analysis, and the quantitative determinations of choline and glycerol showed that the aldehyde group constituted the only long methylene chain in the molecule. Hydrolysis afforded a mixture of aldehydes in a yield of nearly ninety per cent. The aldehydes showed the well-known tendency to polymerize, and some crystalline polymeric product was obtained. The unpolymerized material was converted to the 2,4-dinitrophenylhydrazones, whose elementary

(1) This investigation was supported by a research grant, Nonr 253(00) from the Office of Naval Research.

(2) The authors express their gratitude to Dr. E. C. Dougherty of the University of California School of Medicine for a generous supply of this anemone.

(3) The authors are greatly indebted to Dr. S. R. Lipsky of the Yale University School of Medicine for his help in performing the gas chromatographic analyses.

(4) M. M. Rapport, B. Lerner, N. Alonzo, and R. E. Franzl, *J. Biol. Chem.*, **225**, 859 (1957).

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

TABLE I

COMPARISON OF CEREBROSIDE AND PHOSPHOLIPID CONTENTS

Anemone	Cerebroside, %	Lecithin, %	Cephalin, %	Sphingomyelin, %	Plasmalogen, %
Anthopleura	<0.1	<0.1	<0.1	2.00	0.11
Gyrostoma	0.83	3.08	0.97	0.25	..

analysis corresponded best with the derivative of a C_{18}-aldehyde. It must be assumed, however, that this derivative represented mainly the lower homologs of the aldehyde mixture, and that the polymer was composed of the higher homologs which are particularly prone to polymerize. The iodine number of the hydrazone was equivalent to 0.23 double bond per molecule. Almost the same degree of unsaturation was observed for the plasmalogen itself. All unsaturation of the latter therefore appears to reside in the aldehyde moiety.

The infrared spectrum of the plasmalogen (Fig. 1] is notably void of a carbonyl band. Its absence

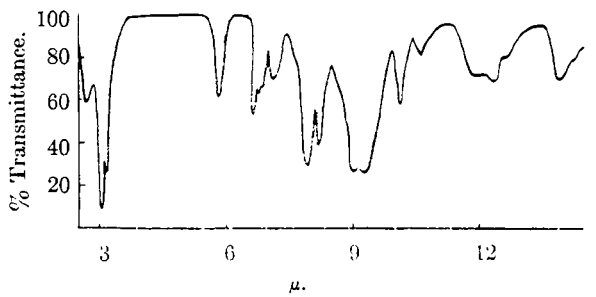


Fig. 1. Infrared absorption spectrum of sea anemone plasmalogen

not only excludes significant quantities of any of the common phosphatides with ester or amide groups, but also the presence of an ester group in the plasmalogen itself. This supports the chemical observation that the aldehydogenic unit is the only aliphatic chain in the molecule. The infrared spectrum also shows a broad hydroxyl band at 3.04 microns which is also shown by a typical lecithin such as has been prepared by Baer *et al.*[6] Since lecithin possesses no free C—OH group, this broad band is due either to the P-OH or the N-OH group, the same groups must be the cause for the analogous band in the plasmalogen spectrum. Absent in the latter is a sharp peak at lower wavelengths such as would be indicative of the presence of either an α or β-hydroxyl group in the glycerol fragment of the plasmalogen.

DISCUSSION

Last year, Rajagopal and Sohonie[7] reported the results of their titrimetric analyses of certain complex lipids of the sea anemone, *Gyrostoma sp.*, which is common along the shores of Bombay. By making standard assumptions and without isolating any of the lipid components they estimated by standard procedures that the dry animal contained various phospholipids in amounts listed in Table I, where they are compared with the present findings. The differences between the two findings are surprisingly large, the more so because both species are fairly closely related members of the *Actinaria.* While it is possible that seasonal and environmental differences may account for some variations in lipid content, they can hardly be held responsible for such extraordinary discrepancies. These point, however, to the dangers which are inherent in a casual transfer of methods of estimation from higher to lower animals. An *a priori* assumption of considerable chemical similarity among quite unrelated organisms may easily obscure the true purpose of a comparative study. One cannot always regard as a true measure of sphingomyelin the difference between total and acid-soluble phosphorus, nor estimate correctly the amount of phospholipids on the assumption that they contain four per cent of phosphorus. Equally unreliable is a procedure of separating phospholipids which is based on suspected differences in solubilities. Thus Baer *et al.*[6] have shown recently that contrary to expectations hydrolecithins are quite difficultly soluble in ether. Additional examples of the well-known influence of impurities on solubilities will be found in the experimental section of this report.

In our investigation we have attempted to work as quantitatively as possible, and to isolate and characterize all phospholipid fractions present in more than trace amounts. The success met by such measures is best illustrated by the fact that it was possible to isolate from two kilograms of anemones a 450-mg. sample of plasmalogen of remarkable uniformity. Had they been present in equal or even significantly smaller amounts, lecithins and cephalins, and also cerebrosides, would not have been overlooked. An explanation of the remarkable absence of such compounds must await further study. Whatever its outcome may be, it is doubtful that it will detract from the dominant position of the sphingosides among the phospholipids of sea anemones.

(6) E. Baer, D. Buchnea, and A. G. Newcombe, *J. Am. Chem. Soc.*, **78**, 232 (1956).

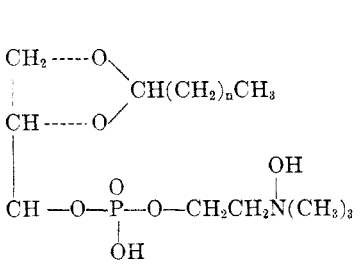
(7) M. V. Rajagopal and K. Sohonie, *Biochem. J.*, **65**, 34 (1957).

The plasmalogen which was isolated as the minor component belongs to the group of widely distributed phosphatides whose most characteristic property is the release of higher fatty aldehydes when treated with weak mineral acids. The adequacy of an acetal structure (II) for plasmalogens, first proposed by their discoverers, Feulgen and Bersin[8] and more recently supported by Thannhauser *et al.*[5] has been questioned repeatedly.[4,9–12] The alternate structures which have been proposed, III,[10,11] IV,[4,11] and V[12] are based on a disparity between the rates of aldehydogenesis of native plasmalogen and a synthetic glyceryl acetal,[4] the possible presence of a second aliphatic chain, unsaturation,[4,11] and hydrogenation to a compound believed to be the butyl alcohol phosphoric acid.[10,12] While these structures adequately express the behavior of previously reported plasmalogens, some admittedly impure, they do not agree with the physical and chemical properties of sea anemone plasmalogen which we hold to be pure.[13] Elementary aualyses, determinations of glycerol and choline, and the results of hydrolysis, coupled with the absence in the infrared spectrum of an ester carbonyl band (Fig. 1), exclude structures IIIb, IVb, Vb. The absence of a C—OH band in the infrared spectrum of the present plasmalogen, and the presence of less than one fourth of a double bond equivalent of unsaturation, exclude structures IIIa, IVa, and Va (lysoplasmalogens).[4] The only structural formula therefore which is in best agreement with our observations is Feulgen's original acetal structure II, in which choline is the base and the aldehyde chain represents a mixture of saturated and unsaturated units of an average length of twenty or twenty-two carbon atoms.[14]

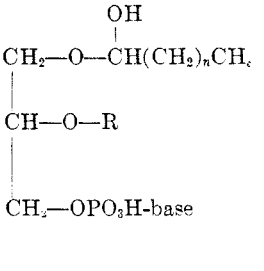
EXPERIMENTAL

Isolation of the phospholipids. The alcohol-preserved sea anemones, *Anthopleura elegantissima* (2 kg.), were ground in 200-g. lots in a Waring blender for 2–3 min. with 150 ml. of a mixture of two volumes of chloroform and one of methanol. The homogenate was filtered by suction, the fleshy parts treated as before and finally washed with 200 ml. of chloroform. After air-drying the residual material weighed 351 g.

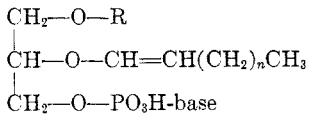
All extracts were combined and washed according to the procedure recommended by Folch *et al.*[15] The extract was placed in a beaker at the bottom of a cylindrical tank which was carefully filled with water of a volume ten times that of the extract. After standing overnight, the water was siphoned off except for a thin layer containing a small amount of fluffy material which adhered to the surface of the chloro-



II

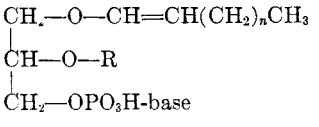


III



IV

a) R = H; b) R = $CO(CH_2)_mCH_3$;



V

base = $-CH_2CH_2\overset{+}{N}(CH_3)_3\ \bar{O}H$ or $-CH_2CH_2NH_2$

(8) R. Feulgen and Th. Bersin, *Z. physiol. Chem.*, **260**, 217 (1939).

(9) M. Anchel and H. Waelsch, *J. Biol. Chem.*, **152**, 50 (1944).

(10) E. Klenk and H. Debuch, *Z. physiol. Chem.*, **296**, 179 (1954); **299**, 66 (1955).

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

(12) G. V. Marinetti and J. Erbland, *Biochim. Biophys. Acta* **26**, 429 (1957); G. V. Marinetti, J. Erbland and E. Stotz, *J. Am. Chem. Soc.*, **80**, 1624 (1958).

(13) We have recently isolated from the sponge, *Spheciospongia vesparia*, an aldehydogenic lecithin whose properties are best expressed by the plasmalogen structure first proposed by Rapport *et al.*,[14,11] and supported by Marinetti.[12] This compound contains an aldehyde in an enol ether linkage and one fatty acid attached to the glycerylphosphorylcholine moiety. It appears therefore that there are at least two types of plasmalogens occurring in nature. If the term plasmalogen is to be retained it should be used to designate the aldehydogenic phospholids in general rather than a special type.

(14) A related compound, derived from palmital and ethanolamine has been synthesized by M. J. Egerton and T. Malkin, *J. Chem. Soc.*, 2800 (1953).

(15) J. Folch, I. Ascoli, M. Lees, J. A. Meath, and F. N. LeBaron, *J. Biol. Chem.*, **191**, 833 (1951).

form solution remaining in the beaker. The final separation was carried out in a separatory funnel, and the water and fluffy material were extracted twice with 25 ml. of chloroform which was then combined with the main extract.

The combined extracts were added to 3.5 times their volume of acetone which brought about precipitation of the bulk of the phospholipids. The mixture was kept at 5° overnight and the precipitate was collected by centrifugation; *Fraction A;* 8.3 g. The solvent of the supernatant liquid was removed at 25° in a rotary evaporator. There remained 49.5 g. (12.5% of the dry weight) of a yellow-brown oil. It was digested with 200 ml. of alcohol-free acetone, and the precipitate which separated was collected by centrifugation; *Fraction B;* 1.3 g. The dried remains of the anemones were extracted further in Soxhlet apparatus, first with ether for 40 hr., and then with ethanol for 90 hr. Evaporation of the ether extract gave only a small amount of oil, which upon treatment with acetone afforded less than 100 mg. of an untractable gum, which was discarded. Evaporation of the ethanol-extract left a more significant amount of residue which was dissolved in chloroform. The chloroform solution was washed with water, concentrated to 50 ml. and then mixed with 175 ml. of acetone. A dark, waxy precipitate formed, which was collected by centrifugation; *Fraction C;* 0.35 g.

Sphingomyelin. Fraction A, 8.3 g., was a light tan colored material, rather stable in the presence of air and moisture. Its infrared spectrum showed an amide carbonyl band. The fraction contained nitrogen, phosphorus, and choline in a ratio of 2:1:1. It gave a negative carbohydrate test. The material was dissolved in 35 ml. of chloroform and precipitated by addition of 3.5 volumes of acetone, and this purification was repeated once more. It did not entail a noticeable loss of phosphorus-containing material. The final product was a nearly white powder, 8.16 g., m.p. 181.5–183° (dec.). The properties of *Fraction C* were quite similar to those of Fraction A. Its purification afforded an additional amount of sphingomyelin.

The sphingomyelin was subjected to paper strip chromatography[16] on a Whatman No. 1 paper and with a solvent mixture of chloroform and ethanol, 4:1, saturated with water. Upon development with phosphomolybdic acid and acidic stannous chloride only one spot was obtained. In a separate test no spots were developed with ninhydrin. All but less than 1 mg. of a 100-mg. sample of sphingomyelin was soluble in 10 ml. of cold glacial acetic acid, indicating that the material was essentially free of cerebrosides.

Anal. Calcd. for $C_{39}H_{81}N_2O_7P$(*N*-palmityl derivative): P, 4.30; N, 3.89; choline, 16.81. Found:[17] P, 4.51; N, 3.95; choline, 17.1. N/P, 1.94; choline/P, 0.97.

Hydrolysis of sphingomyelin[18] *and identification of choline.* One g. of sphingomyelin was refluxed for 4 hr. with 90 ml. of 2*N* sulfuric acid in methanol. The methyl esters were then extracted with four 50-ml. portions of petroleum ether. The methanolic layer was made alkaline (*p*H 8) with 30% KOH in methanol, filtered, acidified to *p*H 6 with glacial acetic acid, and then concentrated *in vacuo* to a volume of 25 ml. About 15 ml. of water was added to dissolve the salts, the solution was made alkaline and was then extracted with three portions of 50 ml. of ether to remove the sphingosine. The remaining methanolic solution was acidified to *p*H 4 with glacial acetic acid, and was mixed with an excess of a 2% aqueous solution of ammonium reineckate. The mixture was cooled for 1 hr. to 4°, and the precipitate was then collected on a glass filter. The choline reineckate thus obtained was washed with cold water, cold ethanol, dried, and then recrystallized from acetone; λ_{max}: 327 (ϵ = 5.88 × 10^3); 527. Reported[19] λ_{max}: 327 (ϵ 5.81 × 10^3); 526. The ether extract containing the sphingosine was washed with two 25-ml. portions of water and dried over anhydrous sodium sulfate. The solvent was then removed *in vacuo,* and the pale yellow, waxy residue, 0.30 g. (71%) was triturated twice with petroleum ether to give the crude sphingosine in the form of a white powder, m.p. 60–61°. It was at once converted into the triacetate by heating with pyridine and acetic anhydride.[18] The triacetate was recrystallized three times from hexane when a further recrystallization did not raise the melting point, m.p. 96–97°, soft needles; $[\alpha]_D^{23°} = -18.2°$.

Anal. Calcd. for $C_{24}H_{43}NO_5$: C, 67.73; H, 10.18; N, 3.29. Found: C, 67.67; H, 10.0, N, 3.46.

Hydrogenation of sphingomyelin. The sphingomyelin was reduced with an Adam's catalyst in acetic acid in Ogg and Cooper's micro-hydrogenation apparatus.[20] On the basis of the molecular weight of sphingomyelin (721), the hydrogen uptake was 1.09 moles, which included 0.08 mole for the fatty acid moiety. The recovery of dihydrosphingomyelin was nearly quantitative; m.p. 186–186.5°.

Anal. Calcd. for $C_{39}H_{83}N_2O_7P$: N, 3.88: P. 4.28. Found: N, 4.02; P, 4.20.

Isolation of the acidic fragment. The petroleum ether solution containing the methyl esters formed during the acid hydrolysis of the sphingomyelin was washed quickly with 50 ml. of sodium bicarbonate solution and 50 ml. of water, and was then dried over anhydrous sodium sulfate. Removal of the solvent *in vacuo* afforded 0.35 g. (95+%) of nearly colorless esters. Analysis of the esters by gas chromatography[3,21] showed methyl palmitate to be the only major component and the methyl esters of other acids from C_{12} to C_{20} to be rather minor constituents; m.p. 30–34°, $n_D^{45°}$ 1.4315. (Methyl palmitate, m.p. 30°, $n_D^{45°}$ 1.4318.)

Anal. Calcd. for $C_{17}H_{34}O_2$: C, 75.56; H, 12.59. Found: C, 75.77; H, 12.29.

The iodine number of the ester mixture, as determined by Yasuda's method,[22] was only seven, corresponding to not more than 0.08 double bond per mole of methyl palmitate. A sample of the ester mixture, 113 mg., was dissolved in 25 ml. of ether and brominated with Yasuda's reagent. After standing for 15 min., the mixture was washed with water, twice with 5% sodium bicarbonate solution, and twice more with water. After drying the solution over anhydrous sodium sulfate, the ether was removed and the residue of saturated and brominated esters, 116 mg. (95+%), was submitted to chromatographic analysis under the same conditions as before. The only observable changes in the chromatogram were the disappearance of the small peaks at C_{12} and C_{20}.

Plasmalogen. The infrared spectrum of Fraction B, 1.3 g., and a positive reaction with digitonin indicated the presence of significant amounts of sterols and esters. The fraction was therefore washed twice with 100 ml. each of acetone, and when this did not bring about complete removal of the sterols twice more with 100 ml. each of anhydrous ether. The product thus obtained was free of sterols. It was dissolved in 10 ml. of chloroform and precipitated by addition of 35 ml. of acetone. After an additional reprecipitation, the plasmalogen was obtained as a white, semi-crystalline, waxy material; 0.45 g. (0.11% of total dry weight of anemone). Depending on the rate of heating the plasmalogen softened around 100° and decomposed above 200°. With Schiff's reagent, fuchsin and sulfurous acid, plasmalogen

(16) T. H. Bevan, G. I. Gregory, T. Malkin, and A. G. Poole, *J. Chem. Soc.,* 841 (1951).

(17) P and N analyses by Schwarzkopf Microanalytical Laboratory, Woodside 77, N. Y. Choline was determined gravimetrically as the reineckate.

(18) H. E. Carter, W. P. Norris, F. J. Glick, G. E. Phillips, and R. Harris, *J. Biol. Chem.,* **170,** 269 (1947).

(19) R. W. Engel, W. D. Salmon, and C. J. Ackerman, *Methods of Biochemical Analysis,* **1,** 274 (1954).

(20) C. L. Ogg and F. J. Cooper, *Anal. Chem.,* **21,** 1400 (1949).

(21) S. R. Lipsky and R. A. Landowne, *Biochim. et Biophys. Acta,* **27,** 666 (1958).

(22) M. Yasuda, *J. Biol. Chem.,* **94,** 401 (1931).

gives a fairly rapid positive test; $[\alpha]_D^{27°} = -7.85°$ (1.905% in absolute ethanol, $\alpha = -0.15°$).

Anal. Calcd. for $C_{30}H_{64}NO_7P$: P, 5.32; N, 2.41; glycerol, 15.8; choline, 20.62. Found:[17] P, 5.30; N, 2.42; glycerol, 15.5, choline, 20.6. N/P, 1.01; choline/P, 0.99.

Hydrolysis of plasmalogen. A 0.1-g. sample was heated to boiling on a steam bath with 10 ml. of ethanol and 5 ml. of concentrated hydrochloric acid. Fifteen ml. of water and an additional 2 ml. of hydrochloric acid were added and the heating continued for a few minutes. The mixture was left standing at room temperature overnight when a flocculent precipitate was formed. This was collected by centrifugation and dissolved in low-boiling petroleum ether. The acid solution remaining from the plasmalogen hydrolysis was extracted three times with 25 ml. each of low-boiling petroleum ether. All extracts were combined, dried over anhydrous sodium sulfate, and the solvent was evaporated *in vacuo*. The residue consisted of a mixture of aldehydes in form of a soft wax, 50 mg. (89%). Its infrared spectrum showed a strong carbonyl band at 5.84 microns.

A significant part of the aldehyde mixture rather rapidly polymerized to form a product quite difficultly soluble in ethanol. Recrystallization of the polymer from ethanol gave needle-shaped crystals, m.p. 74–77°. The polymers of hexadecanal and octadecanal melt near 73°.

The soluble, unpolymerized part of the aldehyde fraction was treated with Brady's reagent in the standard manner. The 2,4-dinitrophenylhydrazones were purified by chromatography over alumina in a benzene solution and by two recrystallizations from 80% ethanol; m.p. 90–105°. The dinitrophenylhydrazone of hexadecanal has been reported to melt at 105–107°, and its higher homologs somewhat higher.

Anal. Calcd. for $C_{24}H_{40}N_4O_4$: C, 64.29; H, 8.93. Found: C, 64.81; H, 8.82.

Unsaturation. The unsaturation of the unhydrolyzed plasmalogen and the 2,4-dinitrophenylhydrazones of the aldehyde mixture was determined according to Yasuda's method[22] with 0.02*N* pyridine sulfate dibromide as the brominating agent. The average of two closely agreeing determinations were as follows: plasmalogen: iodine number, 10; double bonds per mole, 0.24; aldehyde-2,4-dinitrophenylhydrazones: iodine number, 13; double bonds per mole, 0.23.

NEW HAVEN, CONN.

[CONTRIBUTION NO. 1490 FROM THE STERLING CHEMISTRY LABORATORY, YALE UNIVERSITY]

Contributions to the Study of Marine Products. XLVII 22-Dehydrocholesterol[1]

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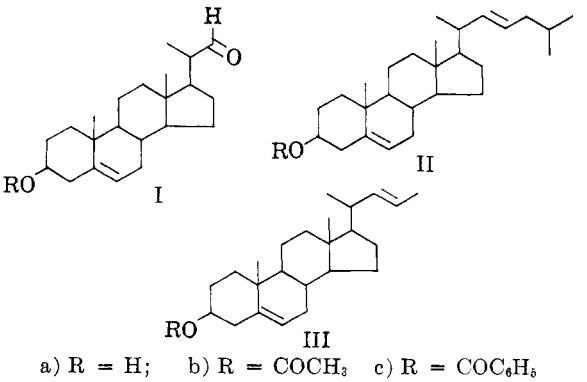
22-Dehydrocholesterol and 3β-hydroxy-$\Delta^{5,22}$-choladiene have been prepared by means of the Wittig reaction.

Among naturally occurring sterols with a methyl or ethyl group at the 24 position of their side chain those with a Δ^{22}-*trans*-oriented double bond are relatively common. The best known examples are ergosterol and stigmasterol. One might expect on biogenetic grounds that the corresponding derivative of cholesterol is also present in natural sterol mixtures. As yet, however, the natural occurrence of 22-dehydrocholesterol (II), while often suspected, has not been convincingly established. In connection with the synthesis of other sterols now in progress in this laboratory this unknown sterol has now been prepared, not only in order to obtain a reference sample to guide isolation studies but also to obtain starting material for the preparation of the ergosterol analog of cholesterol and its irradiation products.

The sterol was prepared from 3β-acetoxy-5-cholen-22-al (1) by means of the Wittig reaction which had previously been used with conspicuous success in the synthesis of 24-methylenecholesterol,[2,3] 24-[4,5] and 25-dehydrocholesterol.[3,5] Although the Wittig reaction is known to be nonstereospecific,[6] in the present synthesis the interaction between the aldehyde and the ylide generated



a) R = H; b) R = $COCH_3$ c) R = COC_6H_5

(1) This investigation was supported by a research grant, Nonr 253(00) from the Office of Naval Research.

(2) W. Bergmann and J. P. Dusza, *Ann.*, **603**, 36 (1957).

(3) D. R. Idler and U. H. M. Fagerlund, *J. Am. Chem. Soc.*, **79**, 1988 (1957).

(4) U. H. M. Fagerlund and D. R. Idler, *J. Am. Chem. Soc.*, **79**, 6473 (1957).

(5) W. Bergmann and J. P. Dusza, *J. Org. Chem.*, **23**, 459 (1958).

(6) G. Wittig and U. Schöllkopf, *Ber.*, **87**, 1318 (1954).