207–208°. The more soluble product from the cleavage, 2-hydroxy-3-phenyl-5-methoxypyrazine, could not be freed of its isomer by recrystallization.

P. 2,3-Dihydroxypyrazine.—A mixture of 2.0 g. (0.014 mole) of 2,3-dimethoxypyrazine and 60 ml. of 42% hydrobromic acid was refluxed for 15 minutes, and then it was evaporated to dryness under vacuum. The solid residue was recrystallized from 250 ml. of water to give 1.3 g. (81%) of the dihydroxypyrazine as light gray flat prisms which did not melt below 320°. This compound also has been prepared in 50% yield by acid hydrolysis of 1,2-di-(N^4-acetylsulfanilyl)-pyrazine.[13]

Anal. Calcd. for $C_4H_4N_2O_2$: N, 25.00. Found: N, 25.16.

Q. 2,5-Dihydroxy-3,6-dimethylpyrazine.—A mixture of 1.8 g. (0.0107 mole) of 2,5-dimethoxy-3,6-dimethylpyrazine and 25 ml. (0.093 mole) of 20% methanolic sodium methoxide was heated in a sealed tube at 175° for 40 hr. and then poured into 180 ml. of warm (60°) water. After the yellow solid had dissolved, the solution was cooled to 25°, filtered and acidified with 8.0 ml. of acetic acid to precipitate the dihydroxy compound, which was filtered off immediately. This was dried in air and recrystallized by extraction from a Soxhlet thimble with methanol. The yield was 1.0 g. (67%) of small yellow granules which did not melt below 320°.

Anal. Calcd. for $C_6H_8N_2O_2$: N, 20.00; C, 51.46; H, 5.76. Found: N, 19.81; C, 51.54; H, 5.75.

If the alkaline solution of cleavage products was neutralized by bubbling carbon dioxide through it for several hours, the dihydroxy compound was precipitated and then slowly redissolved to form a golden yellow *neutral* solution. When heated with phosphorus oxychloride at 170° for 12 hr., the dihydroxypyrazine gave 2,5-dichloro-3,6-dimethylpyrazine, identical with an authentic specimen.[3]

R. 2,5-Dihydroxy-3,6-diphenylpyrazine. 1.—A mixture of 1.0 g. (0.0034 mole) of 2,5-dimethoxy-3,6-diphenylpyrazine and 20 ml. (0.074 mole) of 20% methanolic sodium methoxide was heated in a sealed tube at 182° for 60 hr. and then poured into 180 ml. of water. After warming to 80° to dissolve the yellow solid, the solution was cooled to 40°, filtered, and the filtrate was neutralized with carbon dioxide to precipitate the dihydroxy compound. This was recrystallized by dissolving it in 750 ml. of hot acetone and rapidly boiling the solution down to a volume of 50 ml. The yield was 0.85 g. (94%) of bronze-colored flakes, m.p. 295–300° dec.

Anal. Calcd. for $C_{16}H_{12}N_2O_2$: N, 10.69. Found: N, 10.39.

When heated with phosphorus oxychloride at 180° for 40 hr., this gave 2,5-dichloro-3,6-diphenylpyrazine, identical with an authentic specimen.[10]

2.—A mixture of 1.0 g. of 2,5-dimethoxy-3,6-diphenylpyrazine, 50 ml. of acetic acid and 50 ml. of 42% hydrobromic acid was refluxed for 15 minutes and then concentrated under vacuum. The residue was dissolved in warm 1% aqueous sodium hydroxide, and the yellow solution was filtered and neutralized with carbon dioxide. After recrystallization from acetone as described above, the yield of bronze flakes was 0.1 g. (11%), m.p. 295–300° dec.

S. 2-Hydroxy-5-carboxy-3,6-diphenylpyrazine.—A mixture of 4.0 g. (0.01 mole) of 2,5-dibromo-3,6-diphenylpyrazine and 16 g. of cuprous cyanide in 60 ml. of dry γ-picoline was refluxed for 7 hr. and then poured into 1000 ml. of 4 *N* hydrochloric acid. To this was added 500 ml. of chloroform, and the mixture was warmed to 40° and stirred mechanically for 10 minutes and then filtered. The chloroform phase was concentrated and the tarry residue was distilled to give 2.5 g. of pasty distillate which boiled at 170–220° (0.01 mm.). No pure product could be obtained from this mixture by recrystallization and so it was hydrolyzed by refluxing for 9 days in 100 ml. of ethanol containing 16 g. of potassium hydroxide. Water (500 ml.) was added to the clear solution, and then it was neutralized with carbon dioxide, filtered, and the filtrate was acidified with hydrochloric acid to precipitate a yellow solid. This was recrystallized from acetic acid to give 1.0 g. (33%) of yellow prisms, m.p. 264–265° with evolution of carbon dioxide and resolidification followed by a m.p. of 292–294°. This is the behavior of 2-hydroxy-5-carboxy-3,6-diphenylpyrazine, described by Gastaldi,[6] which decarboxylates to form 2-hydroxy-3,6-diphenylpyrazine.

T. 2-Hydroxy-3-cyano-5,6-diphenylpyrazine.—Two grams (0.0059 mole) of 2-bromo-3-methoxy-5,6-diphenylpyrazine was refluxed with 1.5 g. (0.017 mole) of cuprous cyanide in 40 ml. of dry γ-picoline for 3 hr., and then the hot solution was poured with stirring into a mixture of 500 ml. of cold 3 *N* hydrochloric acid and 100 ml. of chloroform. After 15 minutes of mechanical stirring the solid was filtered off, washing well with 100 ml. more chloroform. The chloroform portion of the filtrate was evaporated to dryness, and the residue was recrystallized from 25 ml. of ethanol to give 1.3 g. (72%) of long yellow prisms, m.p. 230–232°.

Anal. Calcd. for $C_{17}H_{11}N_3O$: N, 15.39. Found: N, 15.09.

U. 2-Hydroxy-3-carboxy-5,6-diphenylpyrazine.—One gram (0.0037 mole) of 2-hydroxy-3-cyano-5,6-diphenylpyrazine was refluxed for 7 hr. in 50 ml. of 15% aqueous potassium hydroxide, and then the yellow solution was diluted with 200 ml. of water, acidified with hydrochloric acid and extracted with two 120-ml. portions of chloroform. After evaporation of the chloroform extracts, the residue was recrystallized from ethanol to give 0.7 g. (65%) of yellow granules. This hydroxy-acid melted at 225–227°, with evolution of carbon dioxide, to form 2-hydroxy-5,6-diphenylpyrazine,[8] m.p. 239–240°.

Anal. Calcd. for $C_{17}H_{12}N_2O_3$: N, 9.58. Found: N, 9.56.

Acknowledgments.—We are grateful to the Ortho Research Foundation, which has permitted us to perform the experimental work in its laboratories, and to Mr. Joseph Grodsky for all of the microanalyses.

RARITAN, NEW JERSEY

(13) F. G. McDonald and R. C. Ellingson, THIS JOURNAL, **69**, 1036 (1947).

[CONTRIBUTION FROM THE CONVERSE MEMORIAL LABORATORY OF HARVARD UNIVERSITY]

On the Structure of an Intermediate in the Biological Demethylation of Lanosterol

BY F. GAUTSCHI AND K. BLOCH

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A new intermediate in the biological demethylation of lanosterol to cholesterol is described. It is isolated by chromatography of the non-saponifiable fraction of liver and intestinal tissue of rats which had been injected with C^{14}-acetate. Evidence is presented that the intermediate contains a *gem*-dimethyl substituent at C_4, a double bond between C_{24} and C_{25} and an inert nuclear double bond. On enzymatic conversion to cholesterol it yields two moles of CO_2. These properties suggest that the intermediate is a 4,4-dimethylcholestadienol.

Introduction

In a recent report,[1] we have described briefly the isolation of a new intermediate in the biological synthesis of cholesterol. Its source was the non-saponifiable fraction of liver and intestinal tissue from rats which had received C^{14}-acetate by injec-

(1) P. B. Schneider, R. B. Clayton and K. Bloch, *J. Biol. Chem.*, in press.

tion and were sacrificed soon thereafter. The intermediate is an alcohol, slightly more polar than lanosterol as shown by alumina chromatography, and readily converted to cholesterol in liver homogenates. These properties fit those of a partially demethylated lanosterol. When isolated from moderate amounts of tissue (about 100 g.) the radioactive column fraction is too small in weight (less than 1 mg.) to allow characterization by the conventional methods of organic chemistry and we are, therefore, unable to report any physical properties. It has nevertheless been possible to demonstrate a number of structural features which characterize the metabolite, designated here as X_1, as a C_{29} sterol or 14-norlanostadienol.

Isolation of the Intermediate.—When C^{14}-acetate is injected into rats intraperitoneally and the animals are killed a few minutes later, the non-saponifiable fraction of the combined liver and intestinal tissue contains several chromatographically separable components. With deactivated alumina, the mixture of radioactive substances can be readily resolved into squalene, a "lanosterol" fraction, cholesterol and more polar metabolites. By chromatography on a large excess of alumina the "lanosterol" peak has previously been shown to be inhomogeneous and to consist of a mixture of lanosterol and at least one slightly more polar material.[1] Since related sterols of the lanosterol type are more effectively separated as the acetates[2] this "lanosterol" fraction was acetylated and subjected to chromatography on alumina Woelm, Grade II. This effected a complete separation of lanosterol from the more polar unknown material which could not be further resolved by additional chromatography. It showed the elution diagram expected of a single substance (Fig. 1). Intermediate X_1 is, therefore, assumed to be homogeneous, although it is realized that in the absence of conventional physical criteria this claim must be regarded as provisional.

Structural Studies.—In approaching the structural elucidation of the intermediate, a close relation to lanosterol was suspected from its chromatographic behavior. It was reasoned that after mixing of radioactive X_1 and non-radioactive lanosterol, radioactivity should remain associated with some of the known transformation or degradation products which are typical of lanosterol. This is the principle used for the characterization of the unknown metabolite in the present experiments. For example when such a mixture was oxidized with osmium tetroxide,[3] the column fraction containing lanostenetriol was radioactive. On further oxidation with lead tetraacetate, acetone was obtained and this had the expected C^{14}-content. The unknown, therefore, yields acetone under the same conditions as lanosterol or other Δ^{24}-stenols and must, therefore, contain a double bond in the same position. That a Δ^{24}-stenol is the major constituent of the radioactive X_1 fraction becomes clear from the following calculation: 100 mg. of non-isotopic lanosterol was added to radioactive X_1 to give a specific activity (c.p.m. of $BaCO_3$ at infinite thickness) of 52 in the mixture. It is quite safe to assume that in lanosterol formed from 1-C^{14}-acetate, 12 of the 30 carbon atoms including C_{25} are labeled[4] and that the S.A. (specific activity) of a single labeled carbon atom will be 30/12 = 2.5 times that of the S.A. of lanosterol itself. The S.A. of the derived acetone which, in this case, will have two unlabeled (C_{26} and C_{27}) and 1 labeled carbon atom (C_{25}) will, therefore, be $(2 \times 0 + 2.5)/3 \times 100 = 83\%$ of that of lanosterol itself. A result, in accord with these assumptions, already has been obtained for lanosterol in this Laboratory.[5] If the new metabolite contained 29 carbon atoms instead of 30, the S.A. of the acetone is similarly calculated to be 80% of that of the whole molecule or 41.6 c.p.m. in the present case. The value actually observed was 40 c.p.m. or 96% of the theoretical for a C_{29} compound.[6]

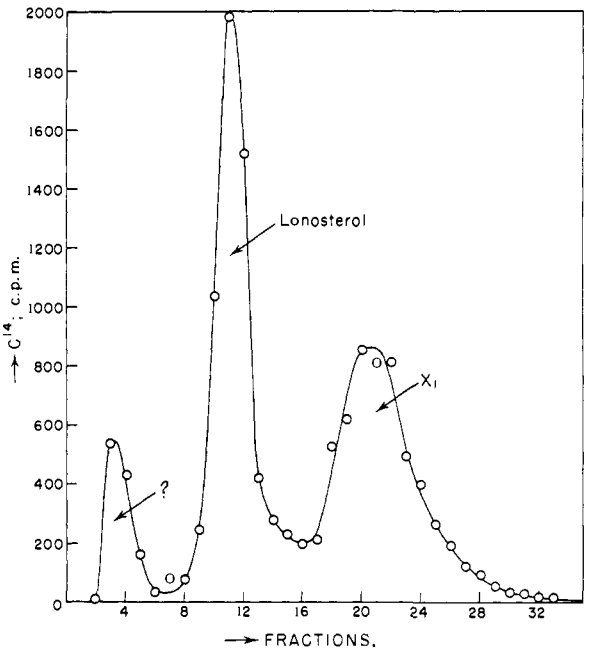

Fig. 1.—Chromatographic separation of biosynthetic C^{14}-lanosterol and C^{14}-X_1 in the form of their acetates.

The presence in the intermediate of a 4,4-*gem*-dimethyl substituent has been demonstrated in an analogous fashion. A mixture of radioactive X_1 and lanosterol was catalytically reduced in a neutral medium to saturate the Δ-24 double bond and then subjected to "Wagner–Meerwein" rearrangement with PCl_5 to yield the isopropylidene derivative of Ring A, typical of 3-β-hydroxy-4,4-dimethyl sterols.[7] The specific activity of the hydrocarbon fraction and of the acetone obtained

(2) D. Johnston, F. Gautschi and K. Bloch, *J. Biol. Chem.*, in press.
(3) H. Wieland and W. Benend, *Z. physiol. Chem.*, **274**, 215 (1942).
(4) J. Wyersch, R. L. Huang and K. Bloch, *J. Biol. Chem.*, **195**, 439 (1952).
(5) R. B. Clayton and K. Bloch, *ibid.*, **218**, 305 (1956).
(6) In relating the S.A. of the acetone to that of the whole molecule, a slight error is introduced into the calculation by the fact that the acetone derived from the C_{29} compound comprises $^3/_{29}$ of the total number of carbon atoms in the molecule, whereas the acetone derived from lanosterol represents $^3/_{30}$ of the total. Calculations using various arbitrary weight ratios of X_1 and lanosterol show this error to be negligible.
(7) L. Ruzicka, M. Montavon and O. Jeger, *Helv. Chim. Acta*, **31**, 818 (1948); C. Dorée, J. F. McGhie and F. Kurzer, *J. Chem. Soc.*, S167 (1949).

from it by oxidation establishes the presence of this structural feature in the intermediate.

Because it is slightly more polar than lanosterol and contains the 4,4-dimethyl grouping, X_1 was thought to differ from lanosterol either by the absence of the 14-α-methyl substituent or by the presence of additional polar groupings in a C_{30} structure. For the purpose of testing the first of these possibilities, we have exploited the fact that in the biological demethylation of lanosterol to cholesterol the three methyl substituents are oxidized to CO_2[8] probably by way of intermediate steroid carboxylic acids. These substituents can be labeled biosynthetically by the use of methyl-labeled acetate as a precursor. The resulting "methyl-labeled" lanosterol yields on incubation with liver homogenates approximately 3 moles of labeled CO_2 for every mole of cholesterol formed.[9] The stochiometric relationship between the radioactivities in the products is calculated as follows. In "methyl-labeled" lanosterol 18 carbon atoms, including the 4,4-*gem*-dimethyl carbon atoms and that of the 14-methyl substituent can be assumed to contain isotopic carbon.[10,5] On conversion to cholesterol, 15 of these remain in the steroid structure and three branched methyls are released as CO_2, which may be expressed as follows, the asterisks denoting the labeled carbon atoms

$$C^*_{18}C_{12}H_{50}O \longrightarrow C^*_{15}C_{12}H_{46}O + 3CO^*_2$$

$$\frac{\text{c.p.m.C}^{14}\text{ in cholesterol}}{\text{c.p.m.C}^{14}\text{ in CO}_2} = \frac{15}{3} = 5$$

Analytical data in accord with this ratio have been obtained,[9] but occasionally, as in the present series of experiments, these ratios are somewhat smaller. We ascribe this deviation from the calculated value in part to the accumulation of intermediates in the demethylation process whose formation is attended by CO_2 production without cholesterol formation, a situation which would lower the cholesterol–CO_2 ratio. The possibility that the CO_2 produced originates in part from carbon atoms other than those under discussion has been rendered unlikely by the finding that "methyl"-labeled cholesterol or "carboxyl"-labeled lanosterol fails to yield radioactive CO_2 under the same experimental conditions.[9]

Despite this slight deviation from the required ratio the relative production of cholesterol and CO_2 nevertheless can serve as a valid measure of the number of methyl groups removed from a precursor when it is oxidized to cholesterol. Table I lists the results obtained on comparing CO_2 production from lanosterol and from intermediate X_1. The two cholesterol precursors were incubated with liver homogenates in a closed system, the CO_2 was absorbed in alkali and cholesterol isolated chromatographically. The yields of radioactive cholesterol from the two substrates were nearly identical but significantly less CO_2 was produced from X_1. The average cholesterol:CO_2 ratio in five experiments with lanosterol was 4.2; for intermediate X_1 this ratio was 6.9. Thus, the quantity of CO_2 produced per mole of cholesterol formed from the unknown was only about $^2/_3$ as great as was formed from lanosterol as the substrate or, stated differently, intermediate X_1 contains two oxidizable methyl substituents compared to the three of lanosterol. In assessing the quantitative significance of these results, it should be pointed out again that the theory requires a ratio of 5 for the radioactivities in cholesterol and CO_2, the two products derived from lanosterol. Since the experimentally realizable value for lanosterol in the present series was 4.2 or 84% of theory, it is not unreasonable to anticipate also slightly lower values for the ratio of radioactivities resulting from the metabolic demethylation of intermediate X_1. On the assumption that we are dealing with a dimethylcholestane derivative the transformation to cholesterol requires

$$C^*_{17}C_{12}H_{48}O \longrightarrow C^*_{15}C_{12}H_{46}O + 2CO^*_2$$

$$\frac{\text{c.p.m.C}^{14}\text{ in cholesterol}}{\text{c.p.m.C}^{14}\text{ in CO}_2} = 7.5$$

The value observed was 6.9 (or 92% of 7.5), in satisfactory agreement with that required by theory. The comparative data on CO_2 production thus lead to the conclusion that in the conversion to cholesterol, intermediate X_1 loses two carbon atoms and hence is a C_{29} sterol. Moreover, since the compound contains the two extra methyl carbons in the form of the 4,4-dimethyl substituent, it also follows that X_1 lacks the methyl group at C_{14} and, hence, has the carbon skeleton of 4,4-dimethylcholestane.

Location of Double Bonds.—As shown by the production of radioactive acetone on oxidation, intermediate X_1 contains an isoöctenyl side chain with a double bond in the Δ-24 position. There are also indications for the presence of a double bond in the nuclear portion of the molecule, though only circumstantial evidence is available thus far. On biogenetic grounds, we consider it improbable that in the course of the demethylation reaction, the 8,9-double bond of lanosterol is first eliminated to form a stanol and subsequently reintroduced either at the same or another location. In view of the report by Schwenk, *et al.*, that zymosterol ($\Delta^{8,24}$-cholestadienol) is converted to cholesterol,[11] a finding substantiated in this Laboratory,[12] it has become a distinct possibility that both double bonds of lanosterol are retained while the three methyl substituents are being removed. Assuming this to be the case, all partial demethylation products of lanosterol, including intermediate X_1, would be expected to have a nuclear double bond as well as one in the side chain. Some light has been thrown on this point by the response of catalytically reduced intermediate X_1 to oxidative treatment. Allylic oxidation to 7-monoketones and 7,11-enediketones by chromic acid under relatively mild conditions is characteristic for $\Delta^{8,9}$-sterols of the lanosterol type[13] and was, therefore, considered to be suitable for testing the presence of a nuclear double bond in X_1. For this purpose use was made

(8) J. A. Olson and K. Bloch, *Federation Proc.*, **15**, 323 (1956).
(9) J. A. Olson, M. Lindberg and K. Bloch, *J. Biol. Chem.*, in press.
(10) J. W. Cornforth and G. Popjak, *Biochem. J.*, **58**, 403 (1954).
(11) E. Schwenk, G. F. Alexander, T. H. Stoudt and C. A. Fish, *Arch. Biochem. Biophys.*, **55**, 274 (1955).
(12) D. Johnston and K. Bloch, unpublished.
(13) L. Ruzicka, E. Rey and A. C. Muhr, *Helv. Chim. Acta*, **27**, 472 (1944).

of the fact that $\Delta^{8,24}$-dienes such as lanosterol are converted on catalytic reduction in a neutral medium to the 24,25 dihydro derivatives, but retain the $\Delta^{8,9}$-bond. It could, therefore, be argued that X_1—if it contained a nuclear double bond either at 8,9 or at other inert positions—should be converted to the 24,25-dihydro derivative on exposure to Pt–H_2 in a neutral medium, but should remain susceptible to oxidation after such treatment. When a mixture of catalytically reduced and acetylated intermediate X_1 and non-radioactive dihydrolanosteryl acetate was oxidized in chromic–acetic acid at 45–50° and the products chromatographed, the results shown in Fig. 2 were obtained. Small amounts of radioactivity derived from X_1 accompanied both the monoketone (peak A) and the ene-diketone (peak B), fractions which were checked for identity by measurement of their typical absorption at 255 and 275 mμ, respectively.[13] The nuclear double bond in the intermediate X_1 therefore appears to impart to the molecule a comparable susceptibility to oxidation as the $\Delta^{8,9}$-double bond in known stenols. However, this evidence is not sufficient for specifying the location of the double bond in intermediate X_1. For example, the chromatographic coincidence of some 10% of the radioactivity with the monoketone and ene-diketone derivatives of lanosterol[14] may be fortuitous and the strong possibility exists that products of similar polarity can arise also from Δ^{7}- and $\Delta^{8,14}$-stenols.[15] A further uncertainty arises from the probable influence of a 14-methyl substituent on the nature and yield of oxidative products from Δ^{8}-stenols. Under the conditions employed in the present experiments the two principal neutral oxidation products of *lanosterol* were the Δ^{8},7-ketone (peak A, 40%) and the Δ^{8},7,11-diketone (peak B, 14%) whereas the bulk of the *radioactivity* derived from X_1 (28%) was found in a column fraction of still greater polarity (peak C, Fig. 2). Evidently, with X_1 oxidation proceeds further than with lanosterol and this is not unexpected if, as the present evidence suggests, X_1 differs from lanosterol, *inter alia*, by the absence of the methyl substituent at C_{14}. The C_{14}-position in X_1 will be susceptible to oxidation, allowing for the formation of 14-hydroxy derivatives. The response of X_1 to oxidizing conditions is thus not inconsistent with the structure under consideration. While the site of the nuclear double bond in X_1 cannot be definitely localized at this time, some further arguments may be presented to support the view that such a double bond is in fact present and moreover, that it is situated in the area of the B/C ring junction. Catalytically reduced and acetylated X_1 forms various oxidation products on treatment with chromic acid under mild conditions and for this reason the reduction product cannot be a stanol. Since catalytic reduction fails to saturate the molecule, the resistant double bond can be located only at $\Delta^{8,9}$, $\Delta^{7,8}$, $\Delta^{8,14}$ or $\Delta^{9,11}$. All other double bonds of the steroid ring system are known to be reduced by Pt–H_2 in neutral medium.[15,16] There is one other site of unsaturation which is not ruled out by this argument. We have observed that 4,4-dimethylcholesterol[17] fails to take up hydrogen on catalytic hydrogenation, presumably because of hindrance of the 5,6-double bond by the two methyl substituents at C_4. However, for other reasons, we do not consider the 5,6-position to be a likely site for the double bond in X_1. Radioactive 4,4-dimethylcholesterol, prepared from C^{14}-cholesterol according to Woodward, *et al.*,[17] is not a precursor of cholesterol.[18] Moreover, on cocrystallization of reduced X_1 (24,25-dihydro-X_1) with non-isotopic 4,4-dimethylcholesterol, radioactivity separates from the carrier. Of the four remaining sites of unsaturation in X_1 none is ruled out by the experimental evidence available, but it appears to us that $\Delta^{9,11}$ is the least likely and $\Delta^{8,9}$ the most attractive on biogenetic grounds. On the other hand a mechanistic argument can be made in favor of a $\Delta^{5,14}$-double bond on the basis of the following considerations. The demethylation of lanosterol is an oxidative process,[9] and appears to proceed by way of steroid carboxylic acids. If, as the present experiments suggest, the 14-methyl group is the first one to be oxidized, the decarboxylation of an intermediate carboxylic acid (4,4-dimethyl-14-carboxy-$\Delta^{8,24}$-cholestadienol) would be assisted by a double bond in the β,γ-position. The 8,9-double bond would, however, be expected to shift to the adjoining 8,14-position as a consequence of decarboxylation.

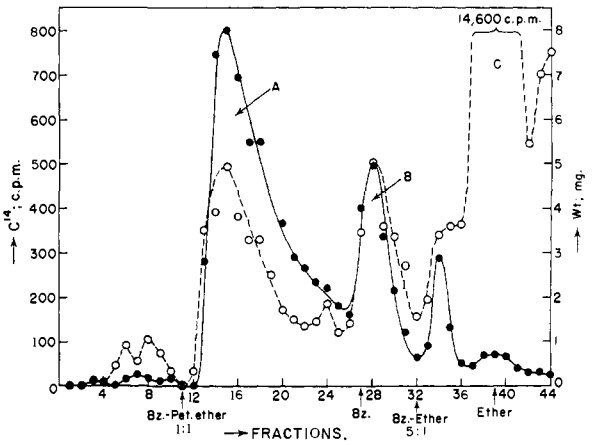

Fig. 2.—Chromatogram of products obtained on chromic acid oxidation of a mixture of dihydrolanosteryl acetate and catalytically reduced and acetylated X_1; solid line, weight curve of products derived from dihydrolanosteryl acetate; broken line, c.p.m. of C^{14} in products derived from X_1.

Although the position of the nuclear double bond remains to be elucidated, the structure of X_1 is al-

(14) Since intermediate X_1 is a C_{29} compound, the oxidation products, even if otherwise identical with those of lanosterol, will be slightly more polar. However, it would seem likely that the difference in chromatographic behavior due to one methyl group is masked by the presence of the additional much more polar ketone groups.

(15) L. F. Fieser and M. Fieser, "Natural Products Related to Phenanthrene," Reinhold Publ. Corp., New York, N. Y., 3rd Ed., 1949, pp. 227–246.

(16) D. H. R. Barton and J. D. Cox, *J. Chem. Soc.*, 214 (1949).

(17) R. B. Woodward, A. A. Patchett, D. H. R. Barton, D. A. J. Ives and R. B. Kelly, THIS JOURNAL, **76**, 2852 (1954); A. A. Patchett, Dissertation, Harvard University, 1955.

(18) By itself this argument is not valid because X_1 contains a double bond also at 24,25. However, we have found (Rilling, Schneider and Bloch, unpublished) that the 24,25-dihydro derivative of lanosterol is also converted to cholesterol, and hence it may be reasoned that the reduction of the Δ^{24}-double bond of cholesterol precursors should not abolish their biological activity.

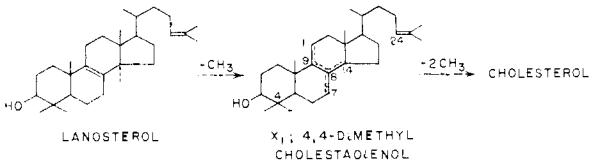

Fig. 3.—Proposed structure of intermediate X_1; the dotted lines indicate possible sites of the nuclear double bond.

ready sufficiently defined to provide an important clue to the mechanism of the biological demethylation of lanosterol to cholesterol. An intermediate of the complex over-all process $C_{30}H_{50}O \rightarrow C_{27}H_{46}O$ has been shown to have the carbon skeleton of 4,4-dimethylcholestane and, for this reason, the branched methyl substituents of lanosterol must be oxidized in sequence, the 14-methyl group being the first of the three to be eliminated.

Experimental

Isolation of Radioactive Intermediate X_1.—Male white rats, weighing approximately 100 g., received each 0.1 millicurie of either 1-C^{14}-acetate or 2-C^{14}-acetate by intraperitoneal injection. The animals were sacrificed 7 min. later, the combined liver and intestinal tissue suspended in methanol and homogenized in a Waring blendor. For saponification, the tissue was heated under reflux in methanolic KOH for 1 hr. The non-saponifiable fraction, isolated in the usual manner, contained on the average 1–2% of the injected C^{14}. The non-saponifiable materials, when subjected to chromatography on a 50-fold excess of deactivated alumina, yielded the following preliminary fractions: (a) squalene, eluted by Skellysolve, (b) 3 fractions of a polarity intermediate between squalene and cholesterol, eluted by benzene–Skellysolve 1:4, the middle fraction containing a mixture of lanosterol and X_1; (c) cholesterol, eluted by benzene and (d) a highly polar fraction eluted by methanol–ether 1:1. The column fractions containing lanosterol and X_1 were pooled and, after removal of the solvent, the mixture was acetylated under the mild conditions described earlier.[2] For further resolution, the mixed sterol acetates were placed on a column (7 mm. diam.) of 3 g. of neutral alumina, Woelm, Grade II, with Skellysolve (distilled over sodium) as the solvent and only eluent. 8.5-ml. fractions were collected with an automatic fraction collector, evaporated to a small volume and aliquots plated on aluminum planchets for C^{14}-assay. The elution diagram obtained by chromatography of 700,000 c.p.m. of an acetylated "lanosterol" fraction is shown in Fig. 1. The least polar material (fractions 2–6) which is unidentified, contained 50,000 c.p.m., the lanosterol peak (fr. 8–16) 275,000 c.p.m. and the X_1 peak (fr. 17–50) a total of 234,000 c.p.m. The identity of lanosterol was established by cocrystallization with authentic lanosterol; the specific activity of the crystals and mother liquor material remained constant on successive crystallizations. Additional chromatography under the same conditions failed to give a further resolution of fraction X_1, indicating homogeneity. The material so obtained was used for the degradation studies and after saponification, for the incubation experiments described below. The weight of all three fractions was negligible.[19]

Characterization of X_1. (a) **Side Chain Degradation.**—A quantity of X_1, biosynthesized from carboxyl-labeled acetate and containing 9,500 c.p.m. when counted as infinitely thin sample, was mixed with 110 mg. of non-isotopic carrier lanosterol and oxidized with osmium tetroxide.[3] The crude fraction containing Δ^8-lanostene-3,24,25-triol was treated with lead tetraacetate and the acetone derived from C_{25}, C_{26} and C_{27} of the sterol side chain was isolated as the Hg complex as described,[5] yield of acetone, calcd. from the weight of the Hg complex, 5.5 mg. or 40%. For C^{14}-analysis the acetone–Hg complex was subjected to wet combustion[20] and the CO_2 precipitated as $BaCO_3$. As infinitely thick samples of $BaCO_3$, duplicate samples of acetone gave 39 and 40 c.p.m., and the starting material (X_1 plus non-isotopic lanosterol) 51 and 53 c.p.m. The calculations based on these data, showing that X_1 yielded C^{14}-acetone with the specific activity expected from a C_{29}-Δ^{24}-stenol, are given in the text.

(b) **"Wagner–Meerwein" Rearrangement of Ring A.**—Acetylated "carboxyl-labeled" X_1 (125,000 c.p.m. as infinitely thin sample) and 300 mg. of carrier lanosteryl acetate were mixed and catalytically reduced with Pt–H_2 in ethyl acetate until 1 mole of H_2 had been consumed. After reduction, the mixture of acetates was saponified and a sample of the free sterols analyzed for C^{14}: 71 and 74 c.p.m. as infinitely thick samples of $BaCO_3$. For the conversion to the known isopropylidene five-membered ring A rearrangement product, the mixture of reduced sterols was treated with PCl_5 (300 mg.) in 12 ml. of Skellysolve in the usual fashion.[7] Chromatography of the crude reaction product on a column of 7 g. of alumina (Woelm, Grade III) yielded 217 mg. of a hydrocarbon fraction having the same specific activity (73 c.p.m.) as the starting sterol mixture. Conversion of the isopropylidene derivative to the ring A diol by use of OsO_4 was carried out as under (a) and yielded, after chromatography of the reaction mixture, 58 mg. of diol fraction, specific activity 80 c.p.m. All radioactivity was, therefore, retained on conversion to the rearrangement and oxidation products typical for 3β-hydroxy-4,4-dimethylsterols. The diol fraction was oxidized with lead tetraacetate to yield acetone which was isolated in the form of the Hg complex and subjected to wet combustion, as described above. A sample gave $BaCO_3$ having 42 c.p.m. as infinitely thick sample. The carbonyl carbon of the acetone so obtained is derived from C_4 of the sterol and the 2 methyl carbons of acetone from the opposite *gem*-dimethyl group. Starting from 2-C^{14}-acetate as the precursor only C_4 will be labeled.[21] The calculated specific activity of the acetone should, therefore, be equal to that of the acetone derived from the isopropyl portion of the sterol side chain (see text), namely, 80% that of the whole molecule, assuming this to be C_{29}-sterol. Since the starting material, before rearrangement, had 73 c.p.m., the calculated value for acetone is 58 c.p.m. The experimental value of 42 c.p.m. is 72% of that predicted by theory for the acetone derived from a 4,4-dimethylsterol.

(c) **Biological Demethylation of X_1 to Cholesterol.**—Lanosterol and X_1 isolated from animals after injection of methyl-labeled acetate as described above, were used for these experiments. The radioactive materials were dissolved in small amounts of acetone and added to a solution of 0.5 mg. of Tween 80 in 0.2 ml. of water. The acetone was removed by a stream of nitrogen and the clear sterol suspensions added to rat liver homogenates prepared according to Bucher.[22] Each flask contained 3 ml. of homogenate and 1500–2000 c.p.m. of radioactive substrate. The flasks were stoppered tightly with vaccine ports and a small glass tube containing 0.2 ml. of 30% KOH for absorption of CO_2 suspended inside.[9] Incubations were carried out for 3 hr. at 38° in a Dubnoff shaker. Thereafter, the reaction mixture was acidified by injecting N H_2SO_4 through the rubber vaccine ports in order to liberate CO_2. Following a suitable equilibration period, the flasks were opened and the CO_2 absorbed in the KOH traps precipitated as $BaCO_3$. Per flask, an average of 10–11 mg. of $BaCO_3$, derived for the most part from the respiration of the liver tissue, were obtained. The samples were plated and assayed for C^{14}. To the homogenates 2 vol. of methanol was added and KOH to make a final concentration of approximately 20%. After heating on the steam-bath for 1 hr., the non-saponifiable fraction was isolated in the usual manner and separated chromatographically on a column containing 1.6 g. of deactivated alumina into (a) remaining starting material, (b) cholesterol and (c) more polar products. The conversions of lanosterol and X_1 to cholesterol and CO_2 were determined in parallel experiments with aliquots of the same liver homogenate with the results shown in Table I.

Oxidation of Reduced X_1 with Chromic Acid.—A quantity of acetylated X_1 containing 52,000 c.p.m. was shaken with Pt–H_2 in ethyl acetate for 2 hr., the catalyst removed by filtration and 104 mg. of dihydroanosteryl acetate added as

(19) In experiments using the isotope dilution method, K. S. McCully has found in this Laboratory that the lanosterol content of hog liver is approximately 0.05% of the non-saponifiable fraction or 0.04% of the cholesterol content of this tissue.

(20) D. D. Van Slyke and J. Folch, *J. Biol. Chem.*, **136**, 509 (1940).

(21) J. W. Cornforth, G. D. Hunter and G. Popjak, *Biochem. J.*, **54**, 590 (1953).

(22) N. L. R. Bucher, THIS JOURNAL, **75**, 498 (1953).

TABLE I

CONVERSION OF "METHYL"-LABELED LANOSTEROL AND "METHYL"-LABELED X_1 TO CHOLESTEROL AND CO_2 IN LIVER HOMOGENATES

Substrate	$BaCO_3$, mg.	CO_2, total c.p.m.	Cholesterol, total c.p.m.	Cholesterol CO_2
Lanosterol (1500 c.p.m.)	10.85	203	929	4.6
	10.82	193	928	4.8
	10.89	184	590	3.2
	10.76	205	825	4.1
	11.83	220	848	3.9
			Av.	4.2
X_1 (1500 c.p.m.)	10.58	147	918	6.2
	10.44	133	932	7.0
	10.55	145	960	6.6
	10.17	116	921	7.9
	11.95	130	866	6.7
			Av.	6.9

In another series of 4 experiments with X_1 the total c.p.m. in cholesterol averaged 340, and in CO_2 50 c.p.m., giving a ratio of 6.8 for the two radioactivities.

carrier. The solvent was removed and the mixture oxidized with chromic acid in acetic acid at 45–50° for 1.5 hr., conditions which afford the 7-keto and the 7,11-diketo derivative of dihydrolanosteryl acetate as the principal products.[13] The neutral reaction products were chromatographed on 5 g. of alumina (Merck) and the various fractions weighed and assayed for C^{14} (Fig. 2). The combined fractions 13–24 weighing 46 mg. and having a total of 3500 c.p.m. or 6.7% of the starting radioactivity may be assumed to contain 3β-acetoxy-7-keto-Δ^8-lanostene as well as radioactive α,β-unsaturated monoketones. The peak fraction 15 had λ_{max} 255 mμ, log ϵ_{255} 3.84; reported[13] λ_{max} 255 mμ, log ϵ_{255} 4.08. The second weight and radioactivity peak (B) comprising fractions 27 to 31, contained 16.3 mg. of solid material and 1810 c.p.m. or 3.3% of the original C^{14}. On the basis of ultraviolet absorption (λ_{max} 268 mμ, log ϵ_{268} 3.87, reported for ene-diketones,[13] λ_{max} 275 mμ, log ϵ_{275} 3.94) peak B is assumed to contain 3β-acetoxy-7,11-diketo-Δ^8-lanostene and ene-diketones derived from X_1. The more polar eluates (peak C, fr. 36–42) accounted for 28% of the initial radioactivity, but contained only 3.4 mg. of unidentified solids. The introduction of a hydroxyl group at C_{14}, in addition to the 2 keto groups elsewhere in the molecule would explain the high polarity of fraction C.

4,4-Dimethylcholesterol (Expt. carried out by R. B. Clayton).—This was synthesized according to Patchett[17] starting from biosynthetic C^{14}-cholesterol. The product, m.p. 149–151°, had a specific activity of 1250 c.p.m./mg. To test the conversion to cholesterol 2 mg. was suspended in a solution containing 0.5% bovine serum albumin and incubated with 40 ml. of rat liver homogenate[22] for 4.5 hr. at 37°. The non-saponifiable fraction was isolated in the customary manner and chromatographed on alumina. The cholesterol fraction was devoid of radioactivity.

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CAMBRIDGE, MASSACHUSETTS

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, CARNEGIE INSTITUTE OF TECHNOLOGY]

A New Synthesis of Flavone Involving Cyclization *via* Displacement of Aromatic Chlorine

BY PHILIP L. SOUTHWICK AND JACK R. KIRCHNER

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The compound β-morpholino-2-chlorochalcone, which has been made by addition of morpholine to *o*-chlorophenylbenzoylacetylene, cyclizes when heated to yield 4-morpholinoflavylium chloride. The latter substance is readily hydrolyzed to flavone.

We wish to describe the results of the initial investigation of a new cyclization reaction which can be used for the preparation of flavone (VI) and probably will prove applicable to the synthesis of some types of substituted flavones. The cyclization in question occurs when β-morpholino-2-chlorochalcone (II) is heated to the proper temperature in a solvent. The initial reaction product is not flavone itself but a related salt, 4-morpholinoflavylium chloride (V), which yields flavone upon hydrolysis. The salt is representative of a class of substances which are most conveniently referred to as 4-aminoflavylium salts, as represented in this case by the contributing form V, although other contributing forms such as Va are undoubtedly very important. Apparently such compounds have hitherto been little known[1] and never previously obtained directly from a cyclization procedure.[2]

We have found that β-morpholino-2-chlorochalcone (II) cyclizes slowly when heated in di-*n*-butyl ether to the temperatures slightly above 140° reached in refluxing solutions. In a heating period of 3.5 hr. under these conditions the salt V was obtained in 43% yield and 48% of the starting material was recovered unchanged. Compound V crystallizes from the refluxing solution in rather pure form as the reaction proceeds.

The assignment of the indicated structure to this substance is based upon its composition, its salt-like properties and its ready hydrolysis in dilute acid to flavone (VI). That the substance is a salt is indicated by its insolubility in non-polar solvents, its high melting point and the apparently ionic nature of its chlorine, which is immediately

(1) The only compounds of this type which we have found to be described in the literature are the flavone benzylimine methiodide of W. Baker, G. G. Clarke and J. B. Harborne, *J. Chem. Soc.*, 998 (1954), and the less closely similar 4-amino-5,6,7,4'-tetramethoxyflavylium picrate and chloride of R. Robinson and G. Schwarzenbach, *ibid.*, 822 (1930).

(2) Methods of preparation and reactions of flavones and flavylium salts have been reviewed recently by Wawzonek; see R. C. Elderfield, "Heterocyclic Compounds," Vol. II, John Wiley and Sons, Inc., New York, N. Y., 1951, pp. 229–342.