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The Transfer of Hydrogen from C-24 to C-25 in Ergosterol Biosynthesis

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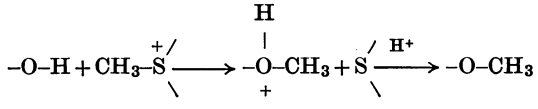
1. A convenient synthesis of 3-hydroxytrisnorlanost-8-en-24-al and its conversion into [24-^{3}H]lanosterol and [26,27-$^{14}C_2$]lanosterol is described. 2. A method for the efficient incorporation of lanosterol into ergosterol by the whole cells of *Saccharomyces cerevisiae* is also described. 3. It is shown that in the biosynthesis of ergosterol from doubly labelled lanosterol the C-24 hydrogen atom of lanosterol is retained in ergosterol. 4. On the basis of unambiguous degradations it is shown that the *C*-alkylation step in ergosterol biosynthesis is accompanied by the migration of a hydrogen atom from C-24 to C-25. 5. The mechanism for the biosynthesis of the ergosterol side chain is presented. 6. Mechanisms of other *C*-alkylation reactions are also discussed.

A survey of the literature on *S*-adenosylmethionine-linked biological alkylations (for reviews see Greenberg, 1963; du Vigneaud & Rachele, 1965; Lederer, 1965; Alexander, Gold & Schwenk, 1957; Badar, Guglielmetti & Arigoni, 1964; Castle, Blondin & Nes, 1963; Parks, 1958) suggests that the driving force for the transfer of a C_1 unit in these reactions is the nucleophilic attack of a suitable electron-rich group on the methyl carbon atom attached to the positively charged sulphur atom. When the nucleophilic attack is initiated by hydroxyl or amino groups, electrical neutrality is maintained by the loss of a proton, e.g. with a hydroxyl group:



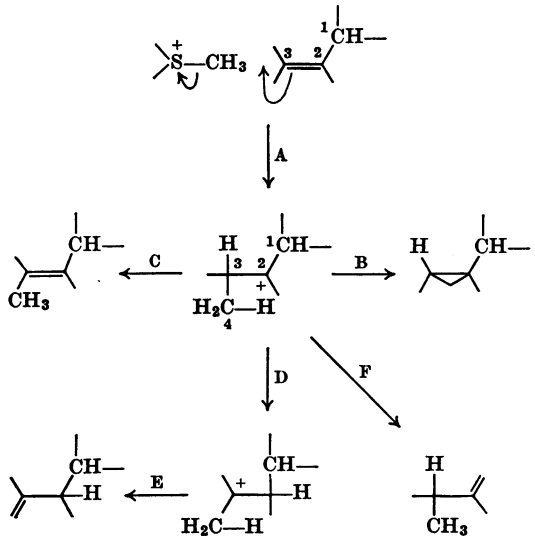
However, when the nucleophilic group is C=C, then the initial attack should in principle result in the formation of a carbonium ion (reaction A in Scheme 1). The subsequent fate of the carbonium ion intermediate may involve one of the many possibilities (see also Clayton, 1965) illustrated in Scheme 1.

The present investigation was made to examine experimentally certain aspects of this hypothetical scheme, and the biosynthesis of the ergosterol side chain was chosen as an illustration.

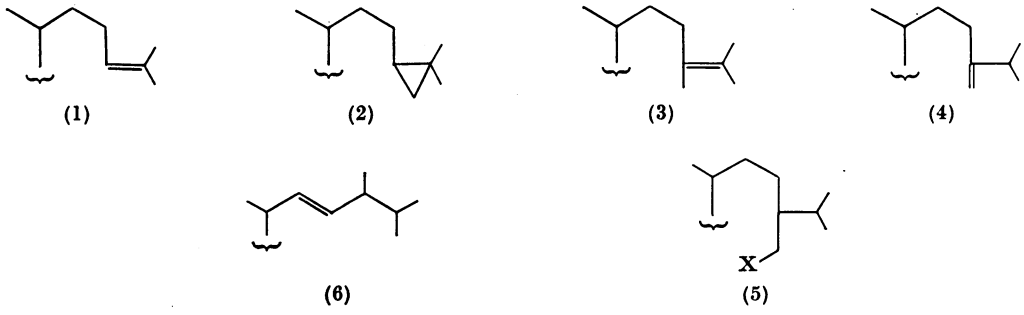
The direct participation of *S*-adenosylmethionine in the elaboration of the normal steroid side chain of type (1) (Scheme 2) into the ergosterol side chain of type (6) was first demonstrated by Parks (1958). In an elegant work using mass spectrometry, Lederer (1964) showed that when ergosterol was biosynthesized in the presence of [*Me*-$^2H_3$]methionine only two of the deuterium atoms were incorporated into ergosterol. This observation gave rise to considerable speculation, and a number of intermediates of types (2), (3), (4) and (5) (Scheme 2) were postulated as precursors of ergosterol (for review see Lederer, 1964). The controversy, in our view, has now been resolved by work (Akhtar, Parvez & Hunt, 1966*b*; Akhtar, Hunt & Parvez, 1966*a*; Barton, Harrison & Moss, 1966) in which it is shown that 24-methylenelanosterol [side chain as (4) in Scheme 2] is efficiently incorporated into ergosterol by whole cells of *Saccharomyces cerevisiae* (LK_2G_{12}). The present paper describes the chemical synthesis of [24-^{3}H]lanosterol and [26,27-$^{14}C_2$]-lanosterol and shows that the *C*-alkylation step in ergosterol biosynthesis is accompanied by the migration of a hydrogen atom from C-24 to C-25. A part of this work has been described in a preliminary communication (Akhtar *et al.* 1966*a*).

RESULTS AND DISCUSSION

Synthesis of [24-^{3}H]*lanosterol* (XI) *and* [26,27-$^{14}C_2$]*lanosterol* (XII). Lanosteryl acetate monoepoxide (V) (Bellamy & Dorée, 1941), on treatment with aqueous perchloric acid–methanol, gave the methoxy alcohol (VI). Cleavage of the C-24–C-25 bond in compound (VI) was achieved by the application of a new procedure involving photolysis in the presence of lead tetra-acetate, when the aldehyde (VII) was obtained in 40–50% yield.



Scheme 1. A, Formation of carbonium ion; B, $-H^+$ from C-4; C, $-H^+$ from C-3; D, hydride transfer from C-3 to C-2; E, $-H^+$ from C-4; F, $-H^+$ from C-1.



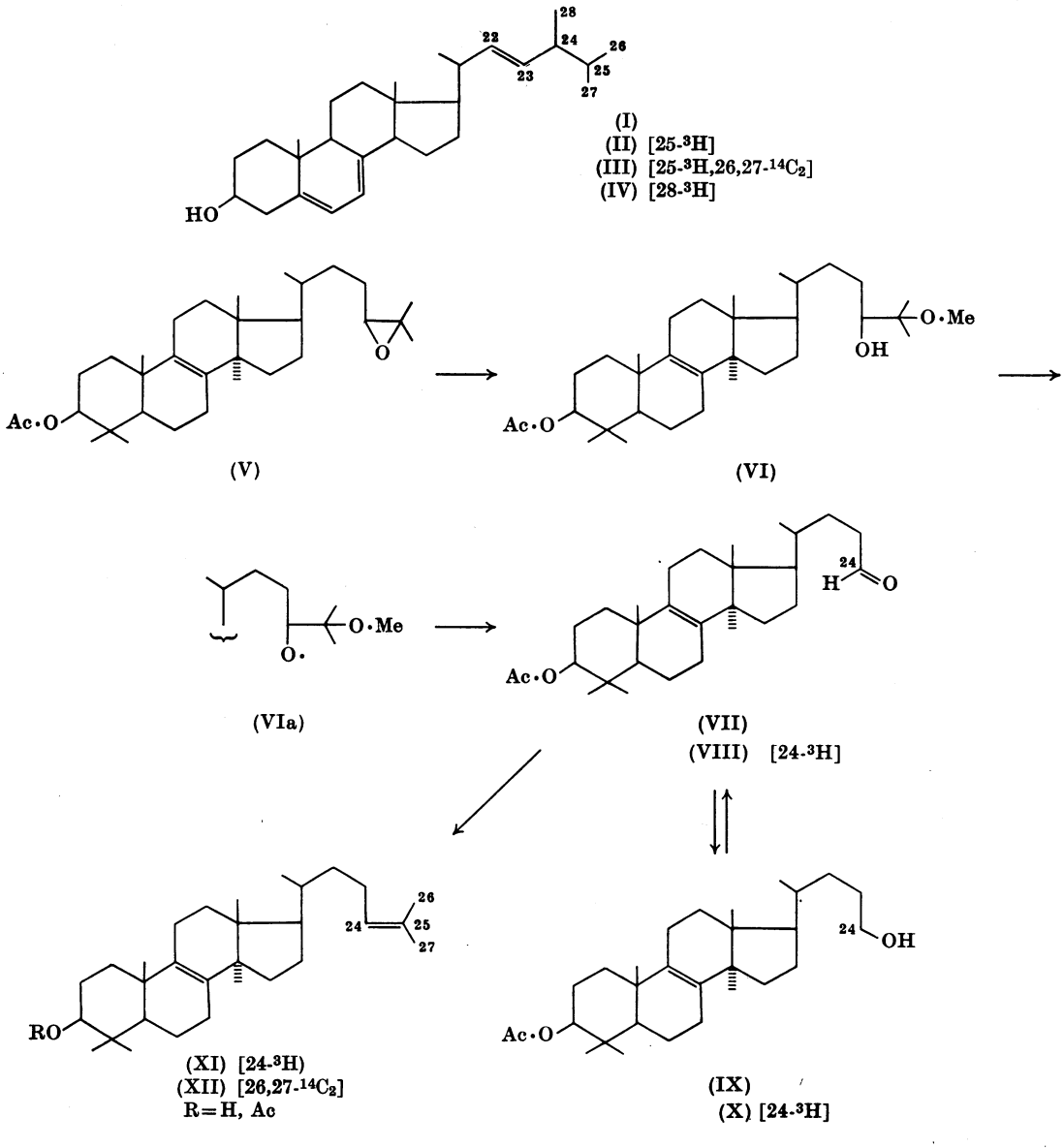
Scheme 2.

The synthesis of [26,27-$^{14}C_2$]lanosterol (XII) was completed by reaction of the aldehyde (VII) with [1,3-$^{14}C_2$]isopropylidenephosphorane, followed by lithium aluminium hydride reduction.

For the preparation of the tritiated analogue the aldehyde (VII) was reduced with tritiated sodium borohydride and the resulting alcohol (X) oxidized without purification by the method of Pfitzner & Moffat (1965) to furnish the tritiated aldehyde (VIII). This was converted into [24-^{3}H]lanosterol (XI) by the method described above. Radiochemical purity of the compounds (XI) and (XII, R = H) was established by conventional methods.

It is suggested that the useful modification of the conventional method for the cleavage of 1,2-diols occurs via a free-radical mechanism as shown in the sequence (VI)–(VII). The initial step in the conversion is the formation of a lead ester, which gives the alkoxy radical (VIa). Although the alkoxy radical (VIa) could theoretically decompose in a number of ways (Akhtar, 1964; Heusler & Kalvoda, 1964), formation of the aldehyde (VII) is favoured, since this involves the elimination of a stable radical fragment $(CH_3)_2\dot{C}\cdot O\cdot CH_3$.

Conversion of lanosterol into ergosterol. We have recently described a method for the incorporation of sterols into whole yeast cells (Akhtar *et al.* 1966*b*; see also Kodicek, 1959; Andreasen & Stier, 1953). By this procedure doubly labelled lanosterol (XI + XII; $^3H/^{14}C$ ratio 14·0) was converted into ergosterol (III; $^3H/^{14}C$ ratio 13·7) in 14·7% yield. The derived ergosterol retained all the tritium of



Scheme 3. Ac, $CH_3 \cdot CO$.

its precursor lanosterol, showing that in the biological conversion of lanosterol into ergosterol the C-24 hydrogen atom of the former is retained. Stone & Hemming (1965) have made similar observations with [4*R*-^{3}H]mevalonate.

Analysis of C-23 *and* C-24 *hydrogen atoms.* The next main objective was to determine the location of tritium in the biosynthetic ergosterol. Ergosterol (III; $^3H/^{14}C$ ratio 13·7) was ozonized (Hanahan & Wakil, 1953) and 2,3-dimethylbutyraldehyde isolated as the dimedone derivative. The derivative had the same $^3H/^{14}C$ ratio, namely 14·4, as the ergosterol, thus showing, not unexpectedly, that the tritium was located in the fragment constituting C-23–C-28 of ergosterol. When 2,3-dimethylbutyraldehyde was equilibrated under basic conditions before the formation of the dimedone derivative no tritium was lost ($^3H/^{14}C$

ratio 14·7). This provided the first direct experimental evidence that the *C*-alkylation step in ergosterol biosynthesis is accompanied by the migration of the C-24 hydrogen atom. The validity of our argument, however, depended entirely on the ability of the α-hydrogen atom of 2,3-dimethylbutyraldehyde to exchange freely with the hydrogen atoms of the medium. This was demonstrated by equilibrating non-radioactive aldehyde under the same conditions but in the presence of tritiated water. The specific radioactivity/m-mole of the aldehyde was then equal to the specific radioactivity/mg.atom of hydrogen in the medium, indicating complete equilibration of one atom of hydrogen in the aldehyde molecule.

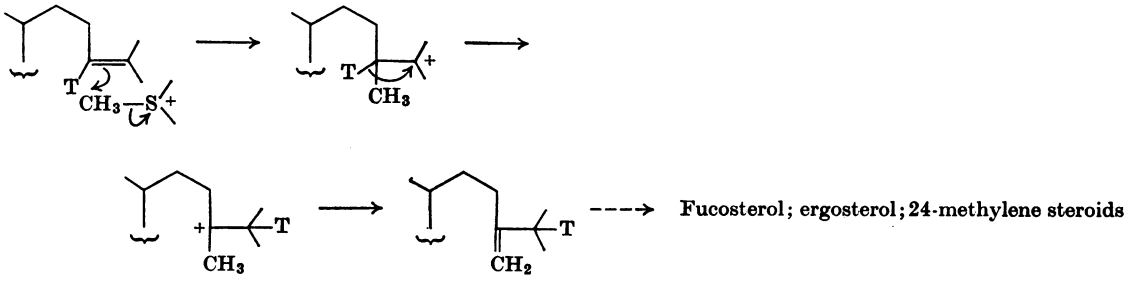
The unlikely possibility that during the biosynthesis of ergosterol the C-24 hydrogen atom of lanosterol might have migrated to position 23 in ergosterol was eliminated when 2,3-dimethylbutyraldehyde from the biosynthetic ergosterol was oxidized to furnish the corresponding acid ($^3H/^{14}C$ ratio 14·8). This conversion took place with complete retention of tritium radioactivity.

Kuhn–Roth oxidation and the location of tritium at C-25. Location of tritium at C-25 required a degradative procedure that would distinguish positions 25 and 28, and isolation of the 26-, 27- and 28-methyl groups by Kuhn–Roth (Kuhn & Roth, 1933) oxidation seemed a likely possibility. It was necessary, however, to carry out control experiments to determine the extent of equilibration of methyl hydrogen atoms under the drastic conditions used. Two model compounds, [19-^{3}H]-cholesteryl acetate (Akhtar & Gibbons, 1965) and [28-^{3}H]ergosterol (IV), were synthesized and subjected to Kuhn–Roth oxidation. The derived acetic acid was converted into sodium acetate and in each case 50–60% of the original radioactivity was recovered. Sodium acetate derived under identical conditions from biosynthetic ergosterol (II) retained only 4% of the ^{3}H activity. The following experiment shows that the radioactivity remaining was due to some unspecified impurities and not to acetic acid. A sample of ergosterol (III; $^3H/^{14}C$ ratio 3·58) was oxidized and the resulting sodium acetate was crystallized after the addition of carrier. The sodium acetate ($^3H/^{14}C$ ratio 0) was completely devoid of ^{3}H activity (see the Experimental section).

The cumulative experimental evidence presented below shows that in ergosterol biosynthesized from [24-^{3}H]lanosterol the labelled hydrogen atom is not at C-22, C-23, C-24, C-26, C-27 or C-28; it is therefore located at C-25.

Mechanism of the C-*alkylation step in ergosterol biosynthesis.* These results and the fact that 24-methylenelanosterol is efficiently incorporated into ergosterol (Akhtar *et al.* 1966*a*,*b*) establish the sequence of events shown in Scheme 4 for the *C*-alkylation step in ergosterol biosynthesis. A mechanism of this type was first considered by Castle *et al.* (1963) (see also Goad, Hammam, Dennis & Goodwin, 1966; Akhtar *et al.* 1966*a*) for the biosynthesis of ethyl sterols. Since the substrate specificities of enzymes involved in the biosynthesis of ergosterol from 24-methylenelanosterol are not known, the stage at which alkylation occurs still remains to be established. In an earlier communication (Akhtar *et al.* 1966*a*) we expressed the view that this sequence (Scheme 4) may also be involved in the biosynthesis of tuberculostearic acid (Jauréguiberry, Law, McClosky & Lederer, 1965), 24-methylene steroids, polyporenic acids, fucosterol (Goad & Goodwin, 1966) and related compounds. We have now learnt from Professor Lederer that he has independently established the hydride-transfer mechanism for the biological conversion of stearic acid into tuberculostearic acid (Lenfant, Audier & Lederer, 1966).

The intermediacy of cyclopropane derivatives in the biosynthesis of methyl and methylene groups has been considered (Law, Zalkin & Kaneshiro, 1963; Stone & Hemming, 1965; Barton *et al.* 1966); however, our results make such proposals unlikely,



Scheme 4.

at least for ergosterol. We consider that the cyclopropane ring in Nature represents only an alternative mode of reaction of the carbonium ion (step B in Scheme 1) rather than being a precursor of a methyl or methylene group.

EXPERIMENTAL

Microanalyses were by Weiler and Strauss, Microanalytical Laboratory, Oxford. Infrared (i.r.) spectra were determined on a Unicam SP.200 spectrometer. All compounds described below gave the expected i.r. spectra. Nuclear-magnetic-resonance (n.m.r.) spectra were determined in deuterochloroform on a Varian model A60 spectrometer. Optical rotations were measured with a Hilger–Nutting visual spectrophotometer, in chloroform solutions. For preparative thin-layer chromatography silica gel HF254 (E. Meek A.-G., Darmstadt, Germany) was used. All radioactivity measurements except one were taken on an Isotope Developments Ltd. (Beenham, Berks.) scintillation counter model 6012A in conjunction with an Ekco (E. K. Cole and Co. Ltd., Southend-on-Sea, Essex) model N529D scaler, with Nuclear Enterprises (G.B.) Ltd. (Edinburgh) scintillator no. NE213. Both isotopes, ^{14}C and ^{3}H, were counted at an efficiency of 15%; corrections for quenching were made either by 'cross-addition' or by an internal standard.

Conversion of [24-^{3}H,26,27-$^{14}C_2$]*lanosterol into ergosterol.* Doubly labelled lanosterol (XI+XII, R=H; 1mg. containing $6{\cdot}16\times10^5$ counts/100sec. of ^{3}H and $4{\cdot}4\times10^4$ counts/100sec. of ^{14}C; $^{3}H/^{14}C$ ratio 14·0) was emulsified in Tween 80 and incubated (Akhtar *et al.* 1966*b*) with *Saccharomyces cerevisiae* LK_2G_{12} in a liquid culture medium (Klein, Eaton & Murphy, 1954). The cells were harvested and sterols isolated as described by Akhtar *et al.* (1966*b*). The non-saponifiable fraction, which contained 61% ($2{\cdot}72\times10^4$ counts/100sec. at the ^{14}C setting) of the original radioactivity, was spotted on a preparative thin-layer silica-gel plate (20cm.×10cm., 2mm.-thick layer) and developed in chloroform four times and viewed under ultraviolet light to give two main radioactive bands A (R_F 0·65) and B (R_F 0·5). The less-polar band A contained 29% ($1{\cdot}28\times10^4$ counts/100sec. at the ^{14}C setting) of the original radioactivity (perhaps lanosterol) and was not characterized. The more-polar band B contained 32% ($1{\cdot}44\times10^4$ counts/100sec. at the ^{14}C setting) of the original radioactivity and was extracted with ether, diluted with ergosterol (100mg.) and crystallized to constant radioactivity (63 counts/100sec./mg., total activity $63\times100=6{\cdot}3\times10^3$ counts/100sec. at the ^{14}C setting; and 863 counts/100sec./mg. total activity $863\times100=8{\cdot}63\times10^4$ counts/100sec. at the ^{3}H setting). Thus 43% of the activity of the band B was present as ergosterol, which represents 14·7% conversion of lanosterol into ergosterol (21μg. of lanosterol converted/g. dry wt. of yeast cells). The $^{3}H/^{14}C$ ratio of the precursor lanosterol was 14·0 and that of biosynthetic ergosterol 13·7.

Conversion of [24-^{3}H]*lanosterol into ergosterol.* [24-^{3}H]-Lanosterol (1·3mg.; $2{\cdot}7\times10^6$ counts/100sec.) was converted by the above method into ergosterol. The more-polar band (ergosterol) contained 41·8% ($1{\cdot}13\times10^6$ counts/100sec.) of the original radioactivity; dilution with ergosterol (1g.) and crystallization to constant radioactivity gave 650 counts/100sec./mg. (total activity $650\times1000=6{\cdot}5\times10^5$ counts/100sec.). This represents 24% incorporation of labelled lanosterol into ergosterol (44μg. of lanosterol converted/g. dry wt. of yeast cells).

Preparation of [28-^{3}H]*ergosterol.* Lanosterol (10mg.) in the presence of [*Me*-^{3}H]methionine (15mg.; 100μc) was converted into [28-^{3}H]ergosterol (630 counts/100sec./mg.) by the method described above.

Preparation of 3-*acetoxy*-24,25-*epoxylanost*-8-*ene* (V). *m*-Chloroperbenzoic acid (18·4g.; 85% of per-acid) in chloroform (250ml.) was added dropwise over 40min. to a stirred solution of lanosteryl acetate (Lewis & McGhie, 1956) (34g.) in chloroform (250ml.) at −5°. The solution was stirred for a further 2½hr., followed by successive washing with aqueous solutions of Na_2SO_3, $NaHCO_3$, $Na_2S_2O_3$, NaCl and finally with water. After removal of the solvent the residue was crystallized from ether–methanol to give the crude monoepoxide (21g.) (containing a little diepoxide). Recrystallization gave the monoepoxide (V), m.p. 181–182°, $[\alpha]_D^{20}$ +55° (Found: C, 78·6; H, 10·7. $C_{32}H_{52}O_3$ requires C, 79·3; H, 10·8%).

Preparation of 3-*acetoxy*-25-*methoxylanost*-8-*en*-24-*ol* (VI). Perchloric acid (60%, w/v; 10·5ml.) was added to a cooled suspension of the monoepoxide (V) (21g.) in methanol (1700ml.). The mixture was stirred at room temperature until most of the solid had dissolved (1hr.). Addition of saturated aq. $NaHCO_3$ and filtration gave a solid. Recrystallization from ether–light petroleum (b.p. 40–60°) gave the methoxy compound (VI) (13g.), m.p. 132°, $[\alpha]_D^{20}$ +47°; the n.m.r. spectrum showed a singlet at 3·21p.p.m. (CH_3–O) (Found: C, 76·1; H, 10·6. $C_{33}H_{56}O_4$ requires C, 76·7; H, 10·9%).

Preparation of 3-*acetoxytrisnorlanost*-8-*en*-24-*al* (VII). A stirred solution of the methoxy compound (VI) (1g.) in dry benzene (50ml.) was photolysed (high-pressure mercury lamp, 125w; A.E.I. Ltd., Leicester) for 20min. in the presence of lead tetra-acetate (1·2g.). The benzene solution was filtered on silica gel and the combined filtrates were washed with water and dried (anhydrous Na_2SO_4). The solvent from eight such batches gave, on crystallization from ether–methanol, a solid (4g.). Chromatography on silica gel gave the aldehyde (VII) (2·5g.), m.p. 144–146°, $[\alpha]_D^{20}$ +58°; the n.m.r. spectrum showed a triplet at 9·80p.p.m. (aldehydic hydrogen) (Found: C, 78·7; H, 10·4. $C_{29}H_{46}O_3$ requires C, 78·7; H, 10·5%).

Preparation of [1,3-$^{14}C_2$]*isopropyltriphenylphosphonium iodide.* Isopropyl iodide (0·2ml.) containing [1,3-$^{14}C_2$]-isopropyl iodide (0·2mc) and triphenylphosphine (1g.) in benzene (1·7ml.) were sealed in a Pyrex tube that was then maintained at 90° for 48hr. The resulting crystalline solid (403mg.) was filtered off and washed with ether; it had m.p. 186–190°.

Preparation of [26,27-$^{14}C_2$]*lanosterol* (XII, R=H). *n*-Butyl-lithium solution (20%, w/v; 0·48ml.) was added to a stirred suspension of [1,3-$^{14}C_2$]isopropyltriphenylphosphonium iodide (435mg.) (dried under vacuum over P_2O_5) in anhydrous tetrahydrofuran (25ml.) under an atmosphere of N_2. The red phosphorane solution was stirred for 30min. and the 24-aldehyde (VII) (500mg., dried as iodide) was added. The reaction mixture was refluxed for 2hr., evaporated to dryness and the residue extracted with ether. The ether solution was washed with methanol–water (1:4, v/v) containing HCl and dried (anhydrous Na_2SO_4). After removal of the solvent the residue was acetylated. Chromatography of the product

mixture on silica gel gave, after crystallization from ether–methanol, [26,27-$^{14}C_2$]lanosteryl acetate (XII, R=$CH_3 \cdot CO$) (175mg.), m.p. 124–126°. The ^{14}C-labelled acetate (XII) (50mg.) in dry ether was refluxed for 2hr. with $LiAlH_4$ (150mg.). Work-up as usual gave [26,27-$^{14}C_2$]-lanosterol (XII, R=H) (42mg.; activity 300000 counts/100sec./mg.).

Determination of radiochemical purity. (*a*) Labelled lanosterol (XII, R=H) was spotted on a thin-layer silica-gel plate that was then run in chloroform and developed lightly with iodine. The plate was divided into horizontal strips 1cm. wide that were separately eluted and assayed for radioactivity. All radioactivity was confined to the lanosterol zone.

(*b*) Labelled lanosterol (XII, R=H) (1mg.) was diluted with highly purified carrier (200mg.) and the mixture was precipitated from solution. During three subsequent crystallizations the specific activity remained the same as that of the precipitated solid.

Preparation of 3-acetoxytrisnorlanost-8-en-24-ol (IX). The 24-aldehyde (VII) (200mg.) in methanol (20ml.) was treated with $NaBH_4$ (100mg.) and left at room temperature for 1hr. After addition of excess of water and extraction into ether, 3-acetoxytrisnorlanost-8-en-24-ol (140mg.), m.p. 175–176°, was crystallized from ether–methanol, $[\alpha]_D^{20}$ +52°; the n.m.r. spectrum showed hydroxyl hydrogen at 1·70p.p.m. (1·59p.p.m. on dilution) (Found: C, 78·1; H, 10·9. $C_{29}H_{48}O_3$ requires C, 78·3; H, 10·9%). The 24-alcohol (IX) was identical (m.p.; i.r. spectrum; thin-layer chromatography) with a sample prepared from trisnorlanostanoic acid (Curtis & Silberman, 1952) by $LiAlH_4$ reduction to the 3,24-diol followed by acetylation and partial hydrolysis to the 24-alcohol 3-acetate.

Preparation of [24-^{3}H]*lanosterol* (XI, R=H). The 24-aldehyde (VII) (200mg.) in methanol (20ml.) was reduced as above with tritiated $NaBH_4$ (12mg., 10mc). Thin-layer chromatography showed complete conversion into the 24-alcohol (IX), which was not isolated but reoxidized by the method of Pfitzner & Moffat (1965). The crude 24-aldehyde was subjected to a Wittig reaction as above to give [24-^{3}H]lanosteryl acetate (XI, R=$CH_3 \cdot CO$) (dilution with 50mg. of unlabelled lanosteryl acetate was necessary). The acetate was reduced with $LiAlH_4$ to give [24-^{3}H]lanosterol (52mg.; activity $3{\cdot}7 \times 10^6$ counts/100sec./mg.). Radiochemical purity was demonstrated as above.

Degradation of the side chain of labelled ergosterol. A suspension of ergosterol (1g.) from the incubation of doubly labelled lanosterol (XI+XII, R=H) in acetic acid (10ml.) was ozonized (Hanahan & Wakil, 1953). The ozonide was reduced with zinc dust (2·5g.), and 2,3-dimethylbutyraldehyde was isolated by steam-distillation. The distillate was neutralized with aq. NaOH and a portion (12·5ml.) was treated with dimedone (200mg.) in ethanol (6ml.). The dimedone derivative of 2,3-dimethylbutyraldehyde (m.p. 152°) was filtered off after 1hr. The remainder of the distillate was equilibrated under N_2 for 15hr. with aq. 10% (w/v) NaOH (3ml.). After neutralization with acetic acid, the dimedone derivative was prepared as above. The dimedone derivative of the non-equilibrated aldehyde had 539 counts/100sec./mg. at the ^{3}H setting and 37 counts/100sec./mg. at the ^{14}C setting ($^3H/^{14}C$ ratio 14·4). The derivative of the equilibrated aldehyde had 570 counts/100sec./mg. at the ^{3}H setting and 39 counts/100sec./mg. at the ^{14}C setting ($^3H/^{14}C$ ratio 14·7), and the original lanosterol (XI+XII, R=H) had $6{\cdot}16 \times 10^5$ counts/100sec./mg. at the ^{3}H setting and $4{\cdot}4 \times 10^4$ counts/100 sec./mg. at the ^{14}C setting ($^3H/^{14}C$ ratio 14·0).

A control experiment was carried out by equilibrating non-radioactive aldehyde in the presence of tritiated water so that the medium had $3{\cdot}98 \times 10^4$ counts/min./mg.atom of hydrogen. The resulting dimedone derivative had $3{\cdot}87 \times 10^4$ counts/min./m-mole, which represents 0·98mg.-atom of tritium incorporated/m-mole of aldehyde. When tritiated water was added to the neutralized solution immediately before the addition of dimedone the resulting derivative was non-radioactive, i.e. there was no random incorporation by the reagent.

Preparation of 2,3-dimethylbutyric acid. The aldehyde distillate from the ozonolysis of labelled ergosterol (III) (1g.) was neutralized and again steam-distilled. The distillate was made alkaline and treated with saturated aq. $KMnO_4$ (Hanahan & Wakil, 1953). After work-up the resulting 2,3-dimethylbutyric acid (73mg.) in methanol (20ml.) was titrated against 0·05N-NaOH. The sodium salt was evaporated to dryness repeatedly with benzene followed by methanol and dissolved in water. The aqueous solution was extracted twice with light petroleum spirit (b.p. 40–60°) and the water was removed under vacuum; the residue was redissolved in methanol for counting. The sodium salt of 2,3-dimethylbutyric acid had 1416 counts/100sec./mg. at the ^{3}H setting and 96 counts/100sec./mg. at the ^{14}C setting ($^3H/^{14}C$ ratio 14·8). Lanosterol had 3900 counts/100sec./mg. at the ^{3}H setting and 257 counts/100sec./mg. at the ^{14}C setting ($^3H/^{14}C$ ratio 15·2).

Table 1. *Kuhn–Roth oxidation of tritiated sterols*

Details are described in full in the Experimental section. Each compound used theoretically liberates 5moles of acetic acid on Kuhn–Roth degradation. Oxidation for 3hr. gives a comparable value, and hence equilibration appears to take place only in the initial stages of degradation. With a tritium atom located on one of the *gem*-methyl groups (at C-26 and C-27) one would expect 25–30% recovery of activity.

	10^{-4} × Sp. activity (counts/100sec./m-mole)	10^{-4} × Sp. activity of acetic acid (counts/100sec./5m-moles)	Activity recovered in acetic acid (%)
Ergosterol (II)	28·6	1·2	4·25
[28-^{3}H]Ergosterol (IV)	23·7	14·0	59
[19-^{3}H]Cholesteryl acetate	24·0	12·4	52

Kuhn–Roth oxidation procedure. Labelled steroid (50mg.) was refluxed for 15hr. with CrO_3 (3g.) in water (20ml.) and conc. H_2SO_4 (5ml.). Water (100ml.) was added and 100ml. of distillate was collected, which was neutralized with aq. 10% (w/v) NaOH. After re-acidification with H_3PO_4 (sp.gr. 1·75) (0·5ml.), a further quantity of water (50ml.) was added and the solution redistilled. The distillate (100ml.) was titrated against 0·05N-NaOH and the aqueous solution evaporated to dryness (10mg. of acetic acid was obtained). After repeated evaporation with benzene and methanol, a methanolic solution of the sodium acetate was counted. The results are summarized in Table 1.

Ergosterol (III) (150mg.) was oxidized by the above procedure and the resulting sodium acetate (47mg.) was diluted with carrier (70mg.) and recrystallized twice from methanol–ether (yield 30mg.). A sample of the recrystallized material (10mg.) had no activity at the ^3H setting and 100 counts/100sec. at the ^{14}C setting ($^3H/^{14}C$ ratio 0), compared with the ergosterol (III) (10mg.), which had 2700 counts/100sec. at the ^3H setting and 750 counts/100sec. at the ^{14}C setting ($^3H/^{14}C$ ratio 3·58). In this experiment radioactivity measurements were made on a Packard Tri-Carb model 3003 scintillation spectrometer, through the courtesy of the Zoology Department.

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REFERENCES

Akhtar, M. (1964). *Advanc. Photochem.* **2**, 263.
Akhtar, M. & Gibbons, C. J. (1965). *J. chem. Soc.* p. 5964.
Akhtar, M., Hunt, P. F. & Parvez, M. A. (1966*a*). *Chem. Commun.* p. 565.
Akhtar, M., Parvez, M. A. & Hunt, P. F. (1966*b*). *Biochem. J.* **100**, 38c.
Alexander, G. J., Gold, A. M. & Schwenk, E. (1957). *J. Amer. chem. Soc.* **79**, 2967.
Andreasen, A. A. & Stier, T. J. B. (1953). *J. cell. comp. Physiol.* **41**, 23.
Badar, S., Guglielmetti, L. & Arigoni, D. (1964). *Proc. chem. Soc., Lond.*, p. 16.
Barton, D. H. R., Harrison, D. M. & Moss, G. P. (1966). *Chem. Commun.* p. 595.
Bellamy, L. J. & Dorée, C. (1941). *J. chem. Soc.* p. 176.
Castle, M., Blondin, G. & Nes, W. R. (1963). *J. Amer. chem. Soc.* **85**, 3306.
Clayton, R. B. (1965). *Quart. Rev. chem. Soc., Lond.*, **19**, 168.
Curtis, R. G. & Silberman, H. (1952). *J. chem. Soc.* p. 1187.
du Vigneaud, V. & Rachele, J. R. (1965). In *Transmethylation and Methionine Biosynthesis*, p. 1. Ed. by Shapiro, S. K. & Schlenk, F. Chicago: University of Chicago Press.
Goad, L. J. & Goodwin, T. W. (1966). *Biochem. J.* **99**, 735.
Goad, L. J., Hammam, A. S. A., Dennis, A. & Goodwin, T. W. (1966). *Nature, Lond.*, **210**, 1322.
Greenberg, D. M. (1963). *Advanc. Enzymol.* **25**, 395.
Hanahan, D. J. & Wakil, S. J. (1953). *J. Amer. chem. Soc.* **75**, 273.
Heusler, K. & Kalvoda, J. (1964). *Angew. Chem. (int. Engl. Ed.)*, **3**, 525.
Jauréguiberry, G., Law, J. H., McClosky, J. A. & Lederer, E. (1965). *Biochemistry*, **4**, 347.
Klein, H. P., Eaton, N. R. & Murphy, J. C. (1954). *Biochim. biophys. Acta*, **13**, 591.
Kodicek, E. (1959). *Ciba Found. Symp.: On the Biosynthesis of Terpenes and Sterols*, p. 173. London: J. and A. Churchill Ltd.
Kuhn, R. & Roth, H. (1933). *Ber. dtsch. chem. Ges.* **66**, 1274.
Law, J. H., Zalkin, H. & Kaneshiro, T. (1963). *Biochim. biophys. Acta*, **70**, 143.
Lederer, E. (1964). *Biochem. J.* **93**, 449.
Lederer, E. (1965). *Israel J. med. Sci.* **1**, 1129.
Lenfant, M., Audier, H. & Lederer, E. (1966). *Bull. Soc. chim. Fr.* p. 2775.
Lewis, D. A. & McGhie, J. F. (1956). *Chem. & Ind.* p. 550.
Parks, L. W. (1958). *J. Amer. chem. Soc.* **80**, 2023.
Pfitzner, K. E. & Moffat, J. G. (1965). *J. Amer. chem. Soc.* **87**, 5670.
Stone, K. J. & Hemming, F. W. (1965). *Biochem. J.* **96**, 14c.