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Assay of the Possible Organization of Particle-Bound Enzymes with Squalene Synthetase and Squalene Oxidocyclase Systems

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Lanosterol was biosynthesized in pig liver homogenate from [4,8,12-$^{14}C_3$]farnesyl pyrophosphate and [4*S*-4-^{3}H]NADPH through the intermediary formation of squalene labelled asymmetrically with ^{3}H. The biosynthetic lanosterol, freed from labelled 24,25-dihydrolanosterol, which was also synthesized, was converted into 24,25-dihydrolanosteryl acetate and subjected to chemical degradations to locate the position(s) of the ^{3}H label in the molecule. The ratio of ^{3}H at C-11 to that at C-12 was found to be 1·28. Although a certain inequality of labelling was thus indicated, experimental uncertainties did not permit the conclusion that the asymmetrically labelled squalene might have been cyclized preferentially from one end.

Many enzymes participating in cell metabolism are not in solution but associated with structural elements of the cell. The microsomes obtainable by differential centrifugation of disrupted cells are mostly fragments of the endoplasmic reticulum. The two enzyme systems, squalene synthetase, which synthesizes squalene from two molecules of farnesyl pyrophosphate, and squalene oxidase, which oxidizes squalene to squalene epoxide, are associated with microsomal particles. If these two enzyme systems were associated in a structural unit, the newly synthesized squalene might be oxidized, and thereafter cyclized, by an attack from one end only; in other words the spatial arrangement of the enzymes could introduce a discrimination between the two ends of the symmetrical molecule of squalene. This might be the case if, and only if, the newly synthesized squalene does not dissociate from the cell particles on which it is synthesized but is immediately attacked by the neighbouring oxidase. We decided to test this possibility despite the report by Samuelson & Goodman (1964) that the squalene molecule is attacked at both ends. These authors, using a microsomal preparation of rat liver, produced cholesterol in two stages; first, they permitted squalene to be synthesized anaerobically from [^{14}C]farnesyl pyrophosphate and [^{3}H]NADPH; then, in a second aerobic phase, the [^{3}H,^{14}C]squalene synthesized was converted into cholesterol after addition of a soluble supernatant of liver homogenate and unlabelled NADPH. If the [^{3}H,^{14}C]squalene had been oxidized and cyclized from one end only, the cholesterol should have contained ^{3}H only at the 11α- or the 12β-position; they demonstrated that it was in fact labelled with ^{3}H in both of these positions and therefore concluded that squalene is cyclized by a random attack at either end.

A doubt persisted, nevertheless, as to whether the experiment of Samuelson & Goodman (1964) represented the natural events, since in their experiment the squalene accumulated during the first anaerobic phase of incubation must have become dissociated from its site of synthesis, unless each active centre of the synthetase synthesized only one molecule of squalene; hence attachment to the oxidizing and cyclizing enzyme system randomly from either end was almost an inevitable consequence.

There existed the possibility that squalene might be cyclized preferentially from one end if it were produced in an enzyme preparation capable of oxidizing and cyclizing it immediately. We report here the results of such an experiment.

DESIGN OF EXPERIMENT

Previous work showed that, when squalene is synthesized from two molecules of farnesyl pyrophosphate (I) (Scheme 1), the *S* hydrogen atom from C-1 of one of the two farnesyl pyrophosphate molecules is eliminated (Popják, Goodman, Cornforth, Cornforth & Ryhage, 1961; Popják, Cornforth, Cornforth, Ryhage & Goodman, 1962; Cornforth, Cornforth, Donninger & Popják, 1966*b*; Donninger & Popják, 1966)

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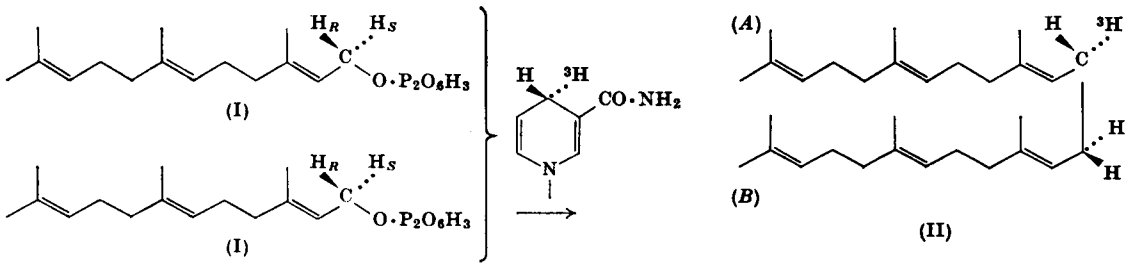
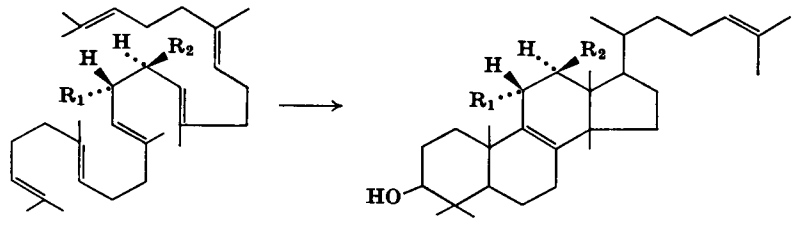


Scheme 1.



Scheme 2.

and is replaced by the *S* hydrogen atom at C-4 of NADPH (Popják, Schroepfer & Cornforth, 1962; Cornforth *et al.* 1966*a*), and that the configuration around C-1 of the second farnesyl residue, not involved in the hydrogen exchange, is inverted (Cornforth *et al.* 1966*b*). It follows that, if the synthesis takes place in the presence of [4*S*-4-^{3}H]NADPH, an asymmetrically labelled squalene (II) (Scheme 1) is formed in which the two ends, (*A*) and (*B*), are different. A consequence of this artifically induced asymmetry, which would not in itself affect in any way the course of enzymic epoxidation or cyclization, is that, if the squalene labelled as in (II) is attacked at the end (*A*), the squalene oxide (III*a*) (Scheme 2) is formed and the lanosterol, cholesterol and other sterols will have the ^{3}H in the 11α-position. If the oxidation takes place from the end (*B*), the squalene oxide formed will be labelled as in (III*b*) (Scheme 2), and 12β-^{3}H-labelled sterols will be formed. If, however, there is no discrimination between the two ends, the 11α- and the 12β-position of the sterols will have an equal distribution of radioactivity. To test these possibilities we decided to study lanosterol (IV) (Scheme 2), the direct product of the cyclization.

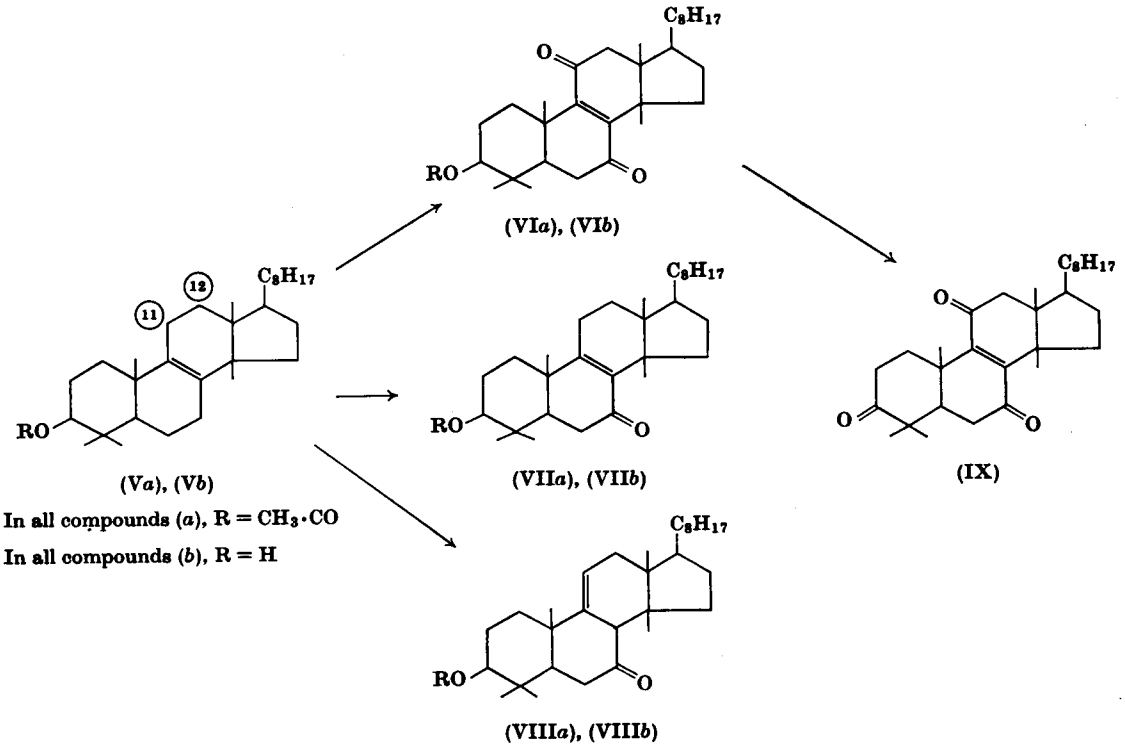
It is known that the chromic acid oxidation of 24,25-dihydrolanosteryl acetate (V*a*) (Scheme 3) leads to a mixture of compounds, in which compounds (VI) and (VII) (Scheme 3) have been recognized (Ruzicka, Rey & Muhr, 1944). If, during this oxidation of dihydrolanosteryl acetate, there is no exchange of protons at C-12 with the acetic acid medium, this molecule could be used for our purposes, for it is then sufficient to oxidize the labelled compound with chromic acid and to isolate compound (VI).

Determination of the ^{3}H content of compound (VI), before and after equilibration with base, should then provide the answers to the questions posed.

Since Tchen & Bloch (1957) showed that the product of squalene cyclization in pig liver homogenates made in a Waring Blendor was lanosterol, we decided to use these preparations for the synthesis of [^{3}H,^{14}C]lanosterol from [4,8,12-^{14}C$_3$]farnesyl pyrophosphate and [4*S*-4-^{3}H]NADPH. Because it was known from previous work (Popják *et al.* 1961) that ^{3}H in NADH and NADPH exchanges rapidly in full homogenates with the hydrogen of the water in the medium, we used a system that generated [4*S*-4-^{3}H]NADPH continuously, instead of adding prepared [^{3}H]NADPH to the homogenates. It is known that glucose 6-phosphate dehydrogenase is a B-side specific enzyme, transferring hydrogen from C-1 of glucose 6-phosphate to the 4*S* position of NADP. We therefore converted [1-^{3}H]glucose with hexokinase and ATP into [1-^{3}H]glucose 6-phosphate, and added it to the liver homogenate containing [^{14}C]farnesyl pyrophosphate and NADP.

MATERIALS AND METHODS

Chemical and biochemical reagents. All solvents used were A.R.-grade reagents, supplied by Hopkin and Williams Ltd., Chadwell Heath, Essex. Lanosterol was obtained from Croda Ltd., Cowick Hall, Snaith, Goole, Yorks., and from Mann Research Laboratories, New York, N.Y., U.S.A. [2-^{14}C]Mevalonolactone was purchased from The Radiochemical Centre, Amersham, Bucks., and D-[1-^{3}H]glucose



Scheme 3.

(5 mc/2 mg.) from New England Nuclear Corp., Boston, Mass., U.S.A. ATP, NAD^+, $NADP^+$ and NADPH were obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A., and hexokinase and glucose 6-phosphate dehydrogenase from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany.

[^{14}C]*Farnesyl pyrophosphate.* This was a specimen biosynthesized from [2-^{14}C]mevalonate and purified by a new procedure (G. Popják, J. Edmond, K. Clifford & V. Williams, unpublished work); its specific radioactivity was 0·06 μc/μmole.

Spectrometry. Infrared and ultraviolet spectra were taken with Perkin–Elmer Infracord, the Perkin–Elmer UV–Visible and Cary (Applied Physics Corp., Monrovia, Calif., U.S.A.) spectrophotometers.

Mass spectra were obtained in an MS-9 high-resolution instrument (A.E.I. Ltd., Manchester) at 70 ev ionization potential. The samples were introduced on a quartz probe directly into the ion source.

Proton-resonance spectra were taken in a Varian A 60 nuclear - magnetic - resonance spectrometer (Varian Associates, Palo Alto, Calif., U.S.A.) with tetramethylsilane as reference.

Chromatographic techniques. Gas–liquid chromatography of lanosterol, 24,25-dihydrolanosterol, cholesterol and their trimethylsilyl ethers was carried out at 200° with a Varian Aerograph model 1520 instrument fitted with a 5 ft. long × 4 mm. diam. column packed with 3% QF_1 on Chromosorb. For thin-layer chromatography, glass plates, 5 cm. × 20 cm., 20 cm. × 20 cm. and 100 cm. × 20 cm., coated with silica gel G or HF ('nach Stahl'; E. Merck A.-G., Darmstadt, Germany) or alumina (M. Woelm, Eschwege, Germany) were used. The analytical plates were coated with 0·2–0·4 mm.- and the preparative plates with 1 mm.-thick layers. The spots on the plates were located either by spraying the plates with 0·1% Rhodamine G solution in ethanol or by exposing them to iodine vapour.

Assay of radioactivity. ^{3}H and ^{14}C were measured in a Packard Tri-Carb scintillation spectrometer (model 3003) by the 'channels-ratio' technique in a standard 2,5-diphenyloxazole scintillation solution. Calibrations were made with [^{3}H]- and [^{14}C]-toluene solutions of known radioactivity. Sufficient counts were taken so that the standard error of sample counts (total counts minus background) did not exceed ±1·2%.

Radioactivity on thin-layer plates was located with a Packard chromatogram scanner (Packard Instrument Co., La Grange, Ill., U.S.A.).

EXPERIMENTAL

Preparation of pig liver homogenate. A 250 g. sample of fresh pig liver from the slaughterhouse, kept in ice before use, was homogenized, as described by Tchen & Bloch (1957), for 20 sec. in a Waring Blendor with 2 vol. of cold 0·1 M-potassium phosphate buffer, pH 7·4. The preparation was

first centrifuged at 12000*g* for 5min. at 0° and then the supernatant was centrifuged once more under the same conditions for 1hr. The supernatant from the second centrifugation was filtered through cotton wool moistened with buffer and then stored at −20° in 20ml. batches. The preparation remained active for at least 2 months, giving reproducible results.

Incorporation of [2-^{14}C]*mevalonate into squalene and sterols.* To find out the optimum conditions for the synthesis of lanosterol in pig liver homogenates preliminary experiments were carried out with [2-^{14}C]mevalonate and various amounts of cofactors. We found that the pig liver homogenates, containing 1mM-[2-^{14}C]mevalonate and fortified with nicotinamide (30mM), ATP (3mM), NAD^+ (3mM) and $MgCl_2$ (5mM), converted 44% of the substrate into unsaponifiable substances after aerobic incubation for 2hr. at 37°. Surprisingly enough, the addition of NADPH (2mM final concn.) decreased the utilization of mevalonate to about one-half of that observed in its absence. This inhibition, confirmed several times, was never seen by us in rat liver homogenates made by the procedure of Bucher & McGarrahan (1956). The concentration of [^{3}H]NADPH in the final experiments made for the preparation of [^{3}H,^{14}C]-lanosterol did not exceed 0·5mM.

Synthesis of squalene and sterols from [4,8,12-$^{14}C_3$]*farnesyl pyrophosphate and* [^{3}H]*NADPH.* Before the setting up of the incubations, [1-^{3}H]glucose 6-phosphate was generated in an incubation (1·2ml.) containing potassium phosphate buffer, pH7 (100μmoles), D-[1-^{3}H]glucose (12μmoles), excess of ATP (75μmoles), $MgCl_2$ (5μmoles) and hexokinase (70 units). Preliminary experiments carried out with unlabelled glucose showed complete phosphorylation of sugar (assayed with glucose 6-phosphate dehydrogenase) after a 3hr. incubation at 30°.

The entire solution of [1-^{3}H]glucose 6-phosphate was added to 10ml. of pig liver homogenate together with 3·2ml. of a solution containing $NADP^+$ (7·5μmoles), $MgCl_2$ (75μmoles), KF (100μmoles) and nicotinamide (450μmoles). The mixture was first incubated for 3min. at 37°, then 4μmoles of [4,8,12-$^{14}C_3$]farnesyl pyrophosphate were added to it and the incubation was continued aerobically for 3hr. The homogenates contained enough glucose 6-phosphate dehydrogenase to reduce all the $NADP^+$.

The mixture was saponified with 2N-KOH at 80° for 1hr.; the unsaponifiable material was then extracted with light petroleum (b.p. 40–60°); evaporation of the solvent left 3mg. of residue.

Identification of components of the unsaponifiable material. Chromatography of the unsaponifiable material on thin-layer plates (silica gel HF) with benzene–ethyl acetate (1:1, v/v) gave mainly two visible spots: one migrated like squalene (R_F 0·71) and the other like cholesterol (R_F 0·45). Scanning of the plates for radioactivity showed three radioactive zones: two of these coincided with squalene and cholesterol, and the third had the same R_F, 0·55, as lanosterol (Fig. 1).

The fastest-moving radioactive material was identified as squalene only by its chromatographic behaviour: on $AgNO_3$-impregnated plates developed with chloroform it remained at the origin.

The substance with R_F 0·45 was identified as cholesterol by mass spectrometry: it gave molecular ions at *m/e* 386 and the other fragment ions characteristic of this substance.

For the identification of what was suspected to be radioactive lanosterol, a few hundred micrograms of commercial lanosterol were added to the radioactive material eluted from the thin-layer plates. We found, by gas–liquid chromatography, that the commercial specimens of lanosterol contained 30–50% of 24,25-dihydrolanosterol as well as lanosterol. These two substances were separated by thin-layer chromatography on silica-gel G plates impregnated with $AgNO_3$ (15%) and developed with chloroform. Lanosterol had R_F 0·49, dihydrolanosterol R_F 0·55; both were identified by their mass spectra. When the radioactive material obtained from the biosynthetic experiment was similarly chromatographed with the carrier, both lanosterol and dihydrolanosterol proved to be radioactive.

Hydrogenation of lanosterol. Lanosterol was hydrogenated in acetic acid in the presence of PtO_2 as catalyst. On a large scale 1g. of the compound was dissolved in 10ml. of acetic acid and shaken, in an atmosphere of H_2, with 60mg. of PtO_2 for 3·5hr.; on a small scale 20–30mg., dissolved in 1ml. of acetic acid, was hydrogenated in the presence of 10mg. of PtO_2. The catalyst was removed by filtration through a column (5cm. × 1cm.) of silicic acid (Mallinckrodt analytical grade, 100 mesh), the column being eluted with chloroform.

Dihydrolanosteryl acetate. Dihydrolanosterol (300mg.) was acetylated in pyridine (1ml.) with acetic anhydride (0·5ml.). The preparation, after standing overnight, was worked up in the usual way and gave 306mg. of crude dihydrolanosteryl acetate.

The dihydrolanosteryl acetate (166mg.) was purified by chromatography on a column of silica gel ('Kieselgel, 0·05–0·2mm.'; E. Merck A.-G.) made up of 3·2g. of powder packed over a 3mm. pad of Celite 545 in a 1cm.-diameter tube. The column was eluted by the following schedule: (1) light petroleum (b.p. 40–60°), 30ml.; (2) light petroleum–benzene (9:1, v/v), 10ml.; (3) light petroleum–benzene (8:2, v/v), 10ml.; (4) three fractions of 10ml. each of light petroleum–benzene (7:3, v/v). The fractions (4) contained the pure dihydrolanosteryl acetate (94·9mg.). The remainder of the specimen (unknown impurities) was eluted later with benzene and ether.

Chromic acid oxidation of dihydrolanosteryl acetate. At first the conditions described by Ruzicka *et al.* (1944) were followed: 20mg. of dihydrolanosteryl acetate dissolved in 0·9ml. of warm acetic acid was oxidized with 13mg. of CrO_3 in 0·12ml. of 90% acetic acid. After the mixture had been shaken for 4hr. at 40°, the excess of CrO_3 was decomposed with methanol; the oxidation products were extracted with diethyl ether, and the ether layer was washed with dilute alkali and then with water. The ethereal solution was dried over $MgSO_4$, and the products of the oxidation were chromatographed on two 20cm. × 20cm. silica gel HF plates (0·2mm.) with light petroleum (b.p. 40–60°)–diethyl ether (3:2, v/v). Three main bands separated and were identified as (*a*) compound (VI*a*) (13·6mg., R_F 0·46); (*b*) compound (VII*a*) (0·55mg., R_F 0·40); (*c*) a mixture in which the compound (VIII*a*) was present (0·91mg., R_F 0·57) (Scheme 3).

In a second oxidation milder conditions were employed: 75·5mg. of dihydrolanosteryl acetate dissolved in 3·4ml. of acetic acid was oxidized with 20mg. of CrO_3 dissolved in 0·3ml. of 90% acetic acid. After 45min. at 40° the reaction mixture was treated as before. The products purified by plate chromatography were: (*a*) compound (VI*a*) (7·9mg.); (*b*) compound (VII*a*) (13·2mg.); (*c*) the mixture containing compound (VIII*a*) (23·5mg.).

Identification of the products of chromic acid oxidation. (*a*) The yellow crystals of compound (VI*a*), after three crystallizations from methanol and after drying *in vacuo* over P_2O_5 for 4hr. at 80°, had m.p. 158–159° (uncorrected); the ultraviolet spectrum of compound (VI*a*), taken in ethanol, showed $\lambda_{max.}$ 273mμ and $\log\epsilon$ 3·89 (reported: m.p. 156·5–158·5°, $\lambda_{max.}$ 'about' 275mμ, $\log\epsilon$ 3·94; Ruzicka *et al.* 1944). The infrared spectrum taken in CCl_4 in NaCl cells of 0·5mm. light-path gave a band at 1735cm.$^{-1}$ (attributable to the C=O group of the acetate) and a sharp band at 1676cm.$^{-1}$ due to the two conjugated keto groups. The mass spectrum confirmed the structure by giving molecular ions at *m/e* 498 and further characteristic fragment ions. The proton-resonance spectrum of compound (VI*a*), taken in $CDCl_3$, was identical with that reported by Hemmert, Lacoume, Levisalles & Pettit (1966) for an authentic sample. The absorption bands attributable to the protons of methyl groups attached to the nucleus were found at: 57cyc./sec. and 54·5cyc./sec. (4α- and 4β-methyl protons respectively); 79·5cyc./sec. (C-19 protons); 48cyc./sec. (C-18 protons); 70·2cyc./sec. (C-28 protons).

(*b*) Compound (VII*a*), recrystallized as for compound (VI*a*), gave colourless needles, m.p. 149–151°, $\lambda_{max.}$ 254mμ, $\log\epsilon$ 3·98 (reported: m.p. 151·5–152·5°, $\lambda_{max.}$ 255mμ, $\log\epsilon$ 4·08; Ruzicka *et al.* 1944). The infrared spectrum, taken as for compound (VI*a*), showed absorption bands at 1740cm.$^{-1}$ (C=O stretching of the acetate group) and at 1666cm.$^{-1}$ (conjugated C=O). The mass spectrum gave molecular ions at *m/e* 484 and further characteristic fragment ions. The proton-resonance spectrum, taken as for compound (VI*a*), gave bands at: 57cyc./sec. (4α- and 4β-methyl protons); 72cyc./sec. (C-19 protons); 40·5cyc./sec. (C-18 protons); 58cyc./sec. (C-28 protons). These values are in agreement with those reported by Hemmert *et al.* (1966).

(*c*) The presence of compound (VIII*a*) in the band migrating with R_F 0·57 on thin-layer plates was inferred from the following observations. (i) The infrared spectrum of the specimen showed absorptions at 1735cm.$^{-1}$ (C=O of acetate), 1660cm.$^{-1}$ (conjugated ketone) and 1715cm.$^{-1}$ (unconjugated ketone). (ii) In the ultraviolet, maximum absorption was found at $\lambda_{max.}$ 258mμ ($\log\epsilon$ 3·57; about 45% of that for the conjugated ketone). Another sample of the mixture migrating with R_F 0·57 contained only 13% of the conjugated ketone as judged from its ultraviolet spectrum. The presence of compound (VIII*a*) in this mixture was proved by its partial isomerization to compound (VII*a*) with BF_3 as described by Mijović, Voser, Heuser & Jeger (1952): 6·9mg. of the specimen dissolved in 0·5ml. of dry benzene was treated with 2 drops of 48% (w/w) BF_3 solution in diethyl ether and left at room temperature for 3 days. After the usual working up, thin-layer chromatography of the products showed that part of the mixture had been converted into compound (VII*a*), which was identified by its R_F and infrared and ultraviolet spectra.

Chromic acid oxidation of dihydrolanosteryl acetate in the presence of $CH_3\cdot CO_2D$. To test the possibility of a hydrogen exchange at C-12 of dihydrolanosteryl acetate during its conversion into the ketones, the chromic acid oxidation was repeated in the presence of $CH_3\cdot CO_2D$.

The $CH_3\cdot CO_2D$ was prepared by refluxing for 15min. a mixture of 10ml. of freshly distilled acetic anhydride with 2·3ml. of 99·7% D_2O.

The oxidation was carried out as follows: 20mg. of dihydrolanosteryl acetate (V*a*) was dissolved in 1ml. of $CH_3\cdot CO_2D$ containing 13mg. of CrO_3. The mixture was kept at 40° for 90min. and then 0·2ml. of D_2O was added to it. After a further period of 2hr. at 40° the products were isolated and chromatographed as described before. The yields were: compound (VI*a*), 12·1mg.; compound (VII*a*), 1·3mg.; the mixture containing compound (VIII*a*), 1·3mg. Each fraction was examined in the mass spectrometer.

Alkali equilibration of compound (VI*a*). Compound (VI*a*) (7·2mg.) was dissolved in methanol (0·7ml.) and then sodium (15mg.) was added in small portions while the mixture was cooled. When all the metal had dissolved, water (0·12ml.) was added and the tube containing the mixture was sealed under N_2 and then kept at 80° for 4 days (cf. Williams, Wilson, Budzikiewicz & Djerassi, 1963). After the usual treatments, 7·5mg. of residue was recovered, from which, by chromatography on a silica gel HF plate with light petroleum (b.p. 40–60°)–diethyl ether (3:2, v/v), 5·1mg. of the alcohol (VI*b*) (Scheme 3) was obtained. This alcohol was identified as compound (VI*b*) by its acetylation to compound (VI*a*) and by the comparison of its R_F, 0·175, with that of a sample of the alcohol obtained by saponification of compound (VI*a*) under mild conditions. The specimen for this comparison was made by refluxing 11·2mg. of the acetate (VI*a*) with 2ml. of 5% (w/v) KOH in methanol for 90min. After the usual working up, 11mg. of a substance with R_F 0·175 was obtained. The identity of compound (VI*b*) as obtained from the alkali equilibration was established further by its oxidation to the 3,7,11-triketone (IX) (see below).

Oxidation of the alcohol (VI*b*) *to the triketone* (IX) (*Scheme* 3). A 3mg. portion of CrO_3, dissolved in 0·5ml. of 10% (v/v) acetic acid, was added to 11mg. of compound (VI*b*) dissolved in 1·3ml. of acetic acid. The precipitated substance was brought back into solution by the addition of 0·2ml. of acetic acid and by gentle warming; the solution remained clear throughout the reaction, which took 18hr. at room temperature. After the usual working up and purification by thin-layer chromatography [silica gel HF with, as developing solvent, light petroleum (b.p. 40–60°)–diethyl ether (3:2, v/v)] 5·1mg. of the triketone (IX) (R_F 0·42) and 0·5mg. of unchanged alcohol (VI*b*) were obtained.

The triketone (IX) was identified by its mass and infrared spectra. The mass spectrum gave a molecular weight of 454. The infrared spectrum showed no OH absorption, but absorption bands at 1720cm.$^{-1}$, attributable to the C=O at C-3, and at 1695cm.$^{-1}$ with a shoulder at about 1680cm.$^{-1}$ due to the conjugated C=O groups at C-7 and C-11.

RESULTS AND DISCUSSION

Two samples of radioactive lanosterol, uncontaminated by the dihydro compound, were prepared: one was biosynthesized from [2-^{14}C]-mevalonate and the other from [4,8,12-$^{14}C_3$]-farnesyl pyrophosphate and [4*S*-4-^{3}H]NADPH.

The first one of these was used to increase the ^{14}C content of the specimen derived from the farnesyl pyrophosphate. This had a low specific radioactivity with respect to ^{14}C but a high content of ^{3}H.

Although not strictly relevant to the present

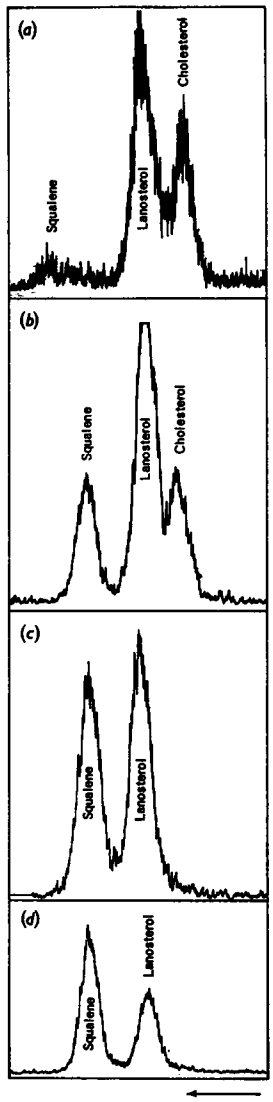


Fig. 1. Records of the scanning of thin-layer plates, showing varying distribution of ^{14}C in components of unsaponifiable material synthesized from [2-^{14}C]mevalonate in pig liver homogenates. (*a*) Fresh homogenate fortified with ATP and $NADP^+$; (*b*) 1-week-old homogenate fortified with ATP alone; (*c*) 2-week-old homogenate fortified with ATP and NADPH; (*d*) 1-month-old homogenate fortified with ATP, NAD^+ and NADPH. The seeming correlation between the age of homogenate and the increased accumulation of squalene and disappearance of cholesterol was not borne out by all experiments.

investigation, some of our observations from several incubations of pig liver homogenates with mevalonate are worth recording in general terms. The percentage conversion of [^{14}C]mevalonate into total unsaponifiable matter was most reproducible (see the Experimental section), but there was variable distribution of radioactivity among squalene, lanosterol and cholesterol. Figs. 1(*a*), 1(*b*), 1(*c*) and 1(*d*) illustrate this variation, which could not be correlated with the addition of a particular coenzyme; it is more likely that this was due to the enzymic properties of the homogenates. Broadly, we can confirm the observation by Tchen & Bloch (1957) that pig liver homogenates made in a Waring Blendor in a medium free of nicotinamide and Mg^{2+} give good yields of lanosterol.

As mentioned in the Experimental section, the spot corresponding to lanosterol on silica gel G or HF plates could be resolved into lanosterol and 24,25-dihydrolanosterol on silver nitrate-impregnated plates. In one experiment, we found that 30% of the radioactivity in the crude lanosterol fraction was associated with the dihydro compound and the remainder with lanosterol itself.

In the 15ml. incubation of [4,8,12-$^{14}C_3$]farnesyl pyrophosphate with [1-^3H]glucose 6-phosphate and $NADP^+$ (see the Experimental section) 9·5% of the ^{14}C from the farnesyl pyrophosphate and 0·032% of the ^3H (1·44μc) were incorporated into total unsaponifiable material. The ^{14}C radioactivity was distributed among three fractions as follows: squalene, 17%; lanosterol + dihydrolanosterol, 36%; cholesterol, 47%.

The biosynthetic dihydrolanosterol, of course, had to be separated from the lanosterol, because it must be presumed to contain ^3H, not only in the nucleus, but also in the side chain from NADPH-dependent reduction of the C-24–C-25 double bond (Avigan, Goodman & Steinberg, 1963). Absence of biosynthetic dihydrolanosterol from the lanosterol used for chemical degradations was ensured by mixing it with an equal amount of non-radioactive dihydrolanosterol and repeating the separation on silver nitrate-treated silica gel.

Catalytic hydrogenation of lanosterol in acetic acid was not accompanied by any exchange of the labelling ^3H; this was demonstrated by hydrogenating a sample of lanosterol having a $^3H/^{14}C$ ratio 12·1. After hydrogenation this ratio was essentially the same (12·15), and a ratio 12·05 was found in dihydrolanosteryl acetate prepared from this specimen and once more hydrogenated.

Further, no exchange of hydrogen attached to C-12 (or other positions) with the medium occurred during chromic acid oxidation of dihydrolanosteryl acetate. To demonstrate this, oxidation in $CH_3 \cdot CO_2D$ was executed as described in the Experimental section, and the mass spectrum of the purified product, compound (VI*a*), was examined. The ratio $(M+1)/M$ for the molecular ion was found to be the same (0·362) as had been observed for compound (VI*a*) prepared in acetic

acid containing no excess of deuterium. Notably, the other oxidation products, compounds (VII*a*) and (VIII*a*), both contained excess of deuterium: respectively a 4·8% and a 6·75% increase in the ratio $(M+1)/M$ was observed.

It could then be concluded (i) that the dihydrolanosteryl acetate used for oxidation was labelled with ^3H to the same extent and in the same positions as was the parent lanosterol, and (ii) that the enedione (VI*a*) contained all of the ^3H which had been at C-12 of lanosterol and none of that which had been at C-11. Accordingly, a sample of dihydrolanosteryl acetate (19·3mg.) (^3H content: 1420 disintegrations/min./mg.), having a $^3H/^{14}C$ ratio 7·76 (values obtained: 7·83, 7·6, 7·76, 7·86), was oxidized and the enedione (VI*a*) was isolated (13·6mg.). The $^3H/^{14}C$ ratio in this compound was 4·04 (values obtained: 4·3, 3·8, 4·04). After equilibration of 9mg. of the enedione (VI*a*) with methanolic sodium methoxide, as described in the Experimental section, the $^3H/^{14}C$ ratio of the total product was 1·14 (values obtained: 1·21, 1·07), and that of the alcohol (VI*b*) isolated from this product was 1·07. Thus it appeared that the molecule contained a small proportion of ^3H not attached to C-12. Further equilibration with methanolic sodium methoxide did not remove this ^3H; nor had ^3H been introduced at C-3 by an enzymic oxidation–reduction, since oxidation of the alcohol (VI*b*) to the triketone (IX) did not diminish the $^3H/^{14}C$ ratio.

It is difficult to ascribe the presence of this 'non-exchangeable' ^3H to synthesis of lanosterol from, for instance, endogenous acetate, for Bucher & McGarrahan (1956) were unable to show synthesis of sterols from acetate in liver homogenates made in a Waring Blendor.

The location and origin of this 'exchange-resistant' ^3H hence remain unexplained. Thus the exact proportions of ^3H present at C-11 and C-12 in the original lanosterol are rendered somewhat uncertain, but their probable limits can be defined on the basis of certain assumptions.

If it is assumed that the 'residual ^3H' was originally present in lanosterol at positions unaffected by the chemical degradation, then the proportion of tritium at C-11 relative to that at C-12 is given by $(7·76-4·04)/(4·04-1·14)=1·28$. A ratio much closer to 1·00 than this value could only be derived if (*a*) a substantial part of the ^3H not originally at C-11 or C-12 was located (e.g. at C-7) where it would not have appeared in the oxidation product, or (*b*) during the chemical degradation (including equilibration) some ^3H was transferred from C-12 to a part of the molecule where it was not exchangeable. Both these suppositions are regarded as unlikely.

Our experiment has therefore indicated a certain inequality of labelling at positions C-11 and C-12; but in view of the experimental uncertainties we hesitate to ascribe this to asymmetry in the enzymic utilization of squalene. What seems certain is that this asymmetry, if real, is not marked in the conditions of our experiment.

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