Investigations on the Biosynthesis of Steroids and Terpenoids. Part XII.[1] Biosynthesis of 3β-Hydroxy-triterpenoids and -steroids from (3*S*)-2,3-Epoxy-2,3-dihydrosqualene

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(3*S*)-2,3-Epoxy-2,3-dihydrosqualene has been shown to be the precursor of lanosterol in pig liver, of lanosterol and ergosterol in yeast, and of cycloartenol, β-amyrin, and lupeol in germinating pea seedlings.

2,3-EPOXY-2,3-DIHYDROSQUALENE is firmly established as the precursor of most polycyclic triterpenoids in nature.[2] The concerted nature of the cyclisation processes [3] is supported by all available evidence.[2] On this basis the (3*S*)-isomer of 2,3-epoxy-2,3-dihydrosqualene should be the precursor of the 3β-hydroxy-triterpenoids. The antipodes of 2,3-epoxy-2,3-dihydrosqualene have recently become available.[4] We now report the results from feedings of chiral 2,3-epoxy-2,3-dihydro-[4-$^3H_2$]squalenes to a range of organisms.

Trisnorsqualene aldehyde, prepared from thiourea-clathrate purified squalene by established methods,[5] was tritiated by base-catalysed exchange with tritiated water. The [2-$^3H_2$]trisnorsqualene aldehyde obtained

[1] (*a*) Part XI, T. R. Jarman, A. A. L. Gunatilaka, and D. A. Widdowson, *Bio-org. Chem.*, in the press; (*b*) preliminary communication, D. H. R. Barton, T. R. Jarman, K. C. Watson, D. A. Widdowson, R. B. Boar, and K. Damps, *J.C.S. Chem. Comm.*, 1974, 861.

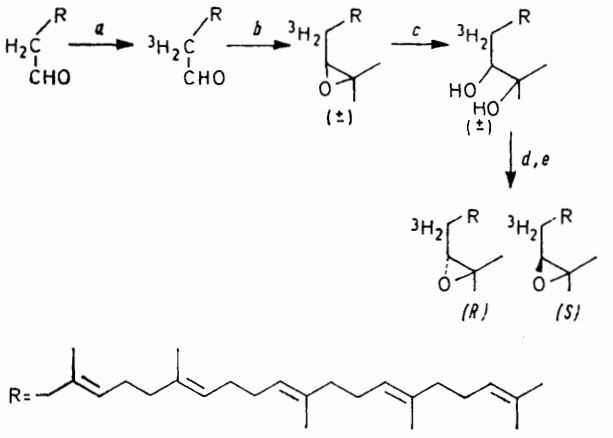
[2] For reviews see H. H. Rees and T. W. Goodwin, Chem. Soc. Specialist Periodical Reports, 'Biosynthesis,' vol. 1, p. 68; vol. 2, p. 25; R. B. Clayton, *Quart. Rev.*, 1965, **19**, 168, 201.

[3] A. Eschenmoser, L. Ruzicka, O. Jeger, and D. Arigoni, *Helv. Chim. Acta*, 1955, **38**, 1890.

[4] R. B. Boar and K. Damps, *Tetrahedron Letters*, 1974, 3731.

[5] R. G. Nadeau and R. P. Hanzlik, *Methods Enzym.*, 1969, **15**, 346.

was treated with diphenylsulphonium isopropylide [6] to yield (RS)-2,3-epoxy-2,3-dihydro[4-$^3H_2$]squalene (Scheme). This was hydrolysed with aqueous perchloric acid to (RS)2,3-dihydro[4-$^3H_2$]squalene-2,3-diol which



SCHEME *a*, pyridine–^3H_2O; *b*, $Ph_2S{=}CMe_2$; *c*, $HClO_4$–H_2O; *d*, resolution; *e*, TsCl–pyridine/KOH–EtOH

was resolved into the (+)-(3*R*)- and (−)-(3*S*)-[4-$^3H_2$]-enantiomers by chromatography of its 3β-acetoxyandrost-5-ene-17β-carboxylate ester. Reduction with lithium aluminium hydride [4] regenerated the diols([α]$_D$ ±10·7°). Treatment of the resolved diols with toluene-*p*-sulphonyl chloride in pyridine followed by addition of ethanolic potassium hydroxide solution gave (+)-(3*R*)- and (−)-(3*S*)-2,3-epoxy-2,3-dihydro[4-$^3H_2$]squalene ([α]$_D$ +2 and −1·8°, respectively).

The (*R*)-, (*S*)-, and (*RS*)-2,3-epoxy-2,3-dihydro[4-$^3H_2$]-squalenes were incubated with the microsomal fraction of pig liver [7] under conditions found to be optimal for oxidosqualene cyclase activity.[8] The lanosterol (I) produced was purified as its benzoate by preparative layer chromatography (p.l.c.) on silica–silver nitrate after addition of inactive carrier, and recrystallised to constant specific activity. The incorporation of (3*S*)-2,3-epoxy-2,3-dihydro[4-$^3H_2$]squalene into lanosterol was 76·8%, to be compared with only 1·9% for the (3*R*)-isomer (Table 1).

The conventionally assumed [9] (3*S*)-enantiomer is indeed the *in vivo* precursor. The majority of the (3*R*)-epoxide was recovered unchanged from incubation mixtures. The incorporation of (*RS*)-2,3-epoxy-2,3-dihydro[4-$^3H_2$]-squalene (39·2% for an unresolved sample, 37·3% for a sample obtained by recombination of the resolved enantiomers) was *ca.* 50% of the value for the resolved (3*S*)-epoxide.

Similar incubations were carried out with a cell-free 500 *g* supernatant of a homogenate of germinating peas (*Pisum sativum*).[10] Incorporations into the immediate cyclisation products β-amyrin (II), lupeol (III), and cycloartenol (IV) were measured. After additions of inactive carrier the triterpenoids were separated by p.l.c. on silica–silver nitrate and recrystallised to constant specific activity. β-Amyrin (26·9% incorporation) was formed efficiently from (3*S*)-2,3-epoxy-2,3-dihydro[4-$^3H_2$]-squalene and comparatively low incorporations into lupeol (0·59%) and cycloartenol (0·59%) were obtained (Table 1). Incorporation of the (3*R*)-epoxide was less than 4% of that for the (3*S*)-epoxide for all three triterpenoids, whereas incorporation of (*RS*)-epoxide (both unresolved and that formed by recombination of resolved enantiomers) was, as before, *ca.* 50% of that for the (3*S*)-epoxide in all cases. The rate of β-amyrin formation from (*RS*)-2,3-epoxy-2,3-dihydrosqualene by the crude homogenate used was 0·018 nmol h^{-1} (mg protein)$^{-1}$ which is higher than the rate reported for a purified preparation of β-amyrin-2,3-oxidosqualene cyclase from peas.[7]

The incorporation of the stereochemical forms of 2,3-epoxy-2,3-dihydro[4-$^3H_2$]squalene into lanosterol by a 20,000 *g* supernatant of a cell-free preparation of yeast (*Saccharomyces cerevisiae*, strain NCYC 1236) was also investigated. The incubation conditions used were those reported to be optimal for yeast oxidosqualene cyclase activity.[8] The results (Table 2) show a high transformation of (3*S*)-2,3-epoxy-2,3-dihydro[4-$^3H_2$]squalene into lanosterol and a very low transformation of the (3*R*)-epoxide. Incorporation of (*RS*)-2,3-epoxy-2,3-dihydro-[4-$^3H_2$]squalene was 67% of that obtained with the

TABLE 1

Incorporation of resolved and racemic 2,3-epoxy-2,3-dihydro[4-$^3H_2$]squalene into 3β-hydroxy-triterpenoids and -steroids by liver, pea, and yeast systems

	% Incorporations					
	Pig liver microsomal fraction	Yeast whole-cell culture		Pea homogenate		
Squalene oxide incubated	Lanosterol	Lanosterol	Ergosterol	β-Amyrin	Lupeol	Cycloartenol
(3*S*)-[4-$^3H_2$]	76·8 (100) [a]	0·61 (100)	6·2 (100)	26·9 (100)	0·59 (100)	0·57 (100)
(3*R*)-[4-$^3H_2$]	1·9 (2·5)	0·0075 (1·2)	0·22 (3·5)	0·76 (2·8)	0·02 (3·4)	0·02 (3·5)
(*RS*)-[4-$^3H_2$] (unresolved)	39·2 (51)	0·053 (8·7)	1·12 (18)	14·1 (52)	0·33 (56)	0·31 (54)
(*RS*)-[4-$^3H_2$] [formed by recombination of (3*R*)- and (3*S*)-]	37·3 (49)			13·1 (49)	0·32 (54)	0·27 (47)

[a] The figures in parentheses indicate the incorporation obtained as a percentage of that obtained with (3*S*)-[4-$^3H_2$]squalene oxide.

[6] E. J. Corey, M. Jautelat, and W. Oppolzer, *Tetrahedron Letters*, 1967, 2325.

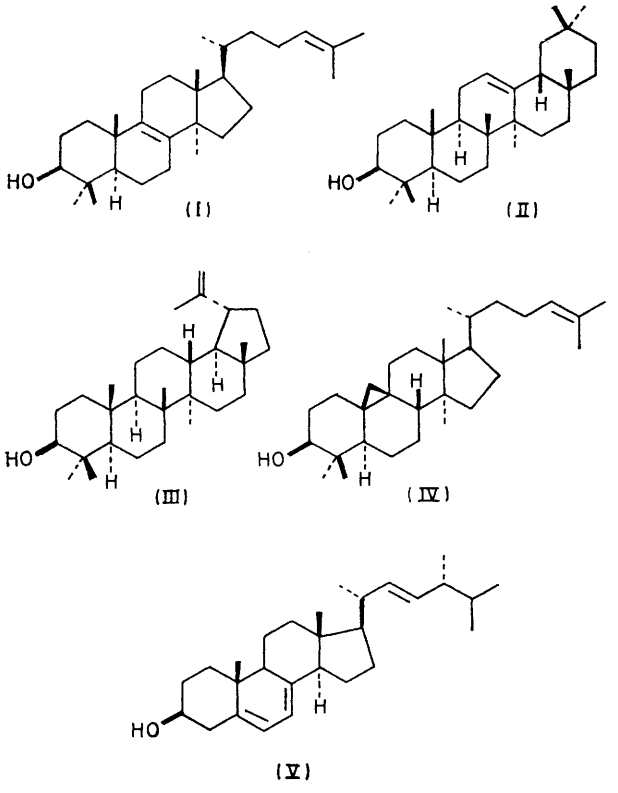
[7] P. D. G. Dean, *Methods Enzym.*, Academic Press, New York, 1969, vol. 15, p. 495.

[8] I. Schechter, F. W. Sweat, and K. Bloch, *Biochim. Biophys. Acta*, 1970, **220**, 463.

[9] G. P. Moss and S. A. Nicolaides, *Chem. Comm.*, 1969, 1022; G. P. Cotterrell, T. G. Halsall, and M. J. Wriglesworth, *J. Chem. Soc.* (*C*), 1970, 739.

[10] E. Capstack, N. Rosin, G. A. Blondin, and W. R. Nes, *J. Biol. Chem.*, 1965, **240**, 3258.

(3*S*)-epoxide and was apparently independent of the concentration of labelled precursor in the range studied. The



enzyme preparation has a relatively short lifetime and only a limited quantity of squalene oxide is cyclised in each run. Consequently both the (*S*)- and (*RS*)-incubations involve an excess of (*S*)-isomer with respect to the

TABLE 2

Lanosterol formation from resolved and racemic squaleneoxide by cell-free preparations of yeast

Squalene oxide precursor	Amount of precursor added (nmol)	Lanosterol formed [a,b] (nmol)
(3*S*)-[4-$^3H_2$]	20	5·52
(3*R*)-[4-$^3H_2$]	20	0·13
(*RS*)-[4-$^3H_2$] [formed by recombination of (3*S*)- and (3*R*)-]	20	3·70
(*RS*)-[4-$^3H_2$]	75	3·73

[a] Average of >2 runs. [b] Corrected for boiled enzyme blank.

turnover capability of the system and in the limit would give equal incorporation. The lower incorporation obtained with (*RS*)- as compared with (3*S*)-epoxide would then be due to an inhibition by the (3*R*)-enantiomer rather than to a concentration effect. The rate of lanosterol formation from the (*RS*)-epoxide, which was 2·4 nmol h^{-1} (mg protein)$^{-1}$, was similar to that previously obtained.[8]

When the 2,3-epoxy-2,3-dihydro[4-$^3H_2$]squalenes were incubated with aerobically growing cultures of yeast (*S. cerevisiae*, strain A184D [11]) under previously defined conditions,[1a] the pattern of the incorporation of the (3*S*)- and (3*R*)-epoxides into lanosterol (I) and ergosterol (V) corresponded to the results obtained with other systems (Table 1). After addition of inactive carriers, lanosterol and ergosterol were separated by p.l.c. Lanosterol was further purified as its benzoate by p.l.c. on silica–silver nitrate and ergosterol as its trimethylsilyl ether by p.l.c. on alumina–silver nitrate. Recrystallisation of lanosterol benzoate and ergosterol (after hydrolysis) gave samples of constant specific activity. The incorporation of the (*RS*)-epoxide deviated from the pattern obtained previously, being only 18% of the incorporation of the (3*S*)-epoxide into ergosterol and 8·4% of that into lanosterol. This is explicable either by inhibition by (*R*)-isomer as above, or if the turnover of substrate is relatively independent of concentration in the medium; then the quantity of (*S*)-epoxide metabolised in a given time will then be proportionately higher for the (*RS*)- than for the pure (*S*)-isomer for equal initial concentrations. The difference between the lanosterol and ergosterol would be a consequence of the continuing metabolisms of these sterols in the whole cell system. These results are in agreement with previous indirect evidence obtained using a liver preparation.[12] The comparatively low incorporation obtained with samples of the resolved (3*R*)-epoxide still permit the possibility that this enantiomer is also cyclised, however inefficiently, to 3β-hydroxy-triterpenoids. More probably these low incorporations represent the limits of the resolution method.[4] In no case was incorporation of the (3*R*)-epoxide greater than 2% of the amount added or greater than 3·5% of the incorporation obtained from the (3*S*)-epoxide.

EXPERIMENTAL

Scintillation counting was performed on a Nuclear Enterprises Ltd. NE 8310 Counter with toluene (15 ml) containing 2,5-diphenyloxazole (5 g l^{-1}) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (0·2 g l^{-1}) as scintillation fluid. When necessary quench corrections were made by the external standard method. T.l.c. was carried out on 0·1 mm thick plates of silica gel GF_{254} (Merck) or alumina GF_{254} (Merck). Silica–silver nitrate and alumina–silver nitrate plates refer to plates impregnated with silver nitrate (10% w/w). P.l.c. was performed on plates 1 mm thick. Light petroleum refers to the fraction of b.p. 40—60°.

Protein estimations were made by a modification of the Lowry method [13] with dried bovine serum albumin as standard.

[2-$^3H_2$]*Trisnorsqualene Aldehyde*.—To trisnorsqualene aldehyde (1 g; prepared from thiourea-clathrate purified squalene [5]) in tetrahydrofuran (30 ml) and triethylamine (2 ml) was added tritiated water (1 ml; activity 1 Ci ml^{-1}). The solution was heated at 50—55° for 15 h, then evaporated. A solution of the residual oil in light petroleum was dried (K_2CO_3), filtered, and evaporated to give [2-$^3H_2$]trisnorsqualene aldehyde (0·65 g).

(RS)-2,3-*Epoxy*-2,3-*dihydro*[4-$^3H_2$]*squalene*.— [2-$^3H_2$]Trisnorsqualene aldehyde (0·23 g) was added under anhydrous conditions at −70° to diphenylsulphonium isopropylide

[11] M. Bard, R. A. Woods, D. H. R. Barton, J. E. T. Corrie, and D. A. Widdowson, *J.C.S. Perkin I*, 1974, 1326.

[12] T. Shishibori, T. Fukui, and T. Suga, *Chem. Letters*, 1973, 1137.

[13] E. F. Hartree, *Analyt. Biochem.*, 1972, **48**, 422.

(generated [6] *in situ*) in 1,2-dimethoxyethane (10 ml). After stirring for 1 h at −70° the solution was allowed to warm to room temperature, quenched with water (30 ml), and extracted with light petroleum (3 × 30 ml). The combined extracts were washed (H_2O), dried ($MgSO_4$), and evaporated. The residue was taken up in methanol (30 ml) and sodium borohydride (30 mg) was added with stirring.[5] After 30 min, the solution was filtered and evaporated and the epoxide purified by p.l.c. on silica (eluant 5% EtOAc–light petroleum). Further purification *via* the thiourea clathrate [5] gave the final sample (42 mg) of specific activity $0{\cdot}53 \times 10^5$ disint. s^{-1} mg^{-1}.

2,3-*Dihydro*[4-$^3H_2$]*squalene*-2,3-*diol*.—Crude 2,3-epoxy-2,3-dihydro[4-$^3H_2$]squalene, prepared as above but without purification, was treated with aqueous 3% perchloric acid (0·6 ml) in 1,2-dimethoxyethane (7 ml) at room temperature for 3 h. The mixture was quenched with aqueous 5% sodium hydrogen carbonate (20 ml) and extracted with light petroleum (3 × 30 ml), and the combined extracts were washed (H_2O), dried ($MgSO_4$), and evaporated to yield an oil. The residue from two such preparations was chromatographed on a column of silica (deactivated with 6% H_2O) (75 g). This was eluted with a gradient of benzene to 10% ethyl acetate–benzene to give the diol (0·12 g).

(3R)- *and* (3S)-2,3-*Dihydro*[4-$^3H_2$]*squalene*-2,3-*diol*.—(*RS*)-2,3-Dihydro[4-$^3H_2$]squalene-2,3-diol was resolved *via* chromatography of its 3β-acetoxyandrost-5-ene-17β-carboxylate ester and regenerated by reduction with lithium aluminium hydride reduction.[4] This gave the optically pure (+)-(3*R*)- and (−)-(3*S*)-stereoisomers.[4]

(3S)- and (3R)-2,3-*Epoxy*-2,3-*dihydro*[4-$^3H_2$]*squalene*.—Separate solutions of (+)-(3*R*)- (26 mg) and (−)-(3*S*)-2,3-dihydro[4-$^3H_2$]squalene-2,3-diol (19 mg) in pyridine (0·2 ml) at −20 °C were treated with toluene-*p*-sulphonyl chloride (120 mg). After 16 h at 3—5 °C the reactions were quenched with ice (0·3 ml). Ethanol (0·5 ml) and potassium hydroxide (0·1 g) were added and the mixtures were shaken vigorously at 5 °C for 3 h. Water (10 ml) was added and the solutions were extracted with light petroleum (3 × 10 ml). The combined extracts were washed (H_2O), dried ($MgSO_4$), and evaporated. The residues were fractionated by t.l.c. on silica (eluant 30% EtOAc–light petroleum) to yield (−)-(3*S*)-2,3-epoxy-2,3-dihydro[4-$^3H_2$]squalene (12·3 mg) (specific activity $5{\cdot}23 \times 10^6$ disint. s^{-1} mg^{-1}) and the (+)-(3*R*)-isomer (9·2 mg) (specific activity $6{\cdot}48 \times 10^6$ disint. s^{-1} mg^{-1}). These oxides were used without further purification.

Incubation with Liver Microsomal Fraction.—Pig liver microsomal fraction [7] was resuspended in 10mM-phosphate buffer, pH 7·5, to a protein concentration of 16·5 mg ml^{-1}. Incubation mixtures [8] which contained in 15 ml: resuspended microsomal fraction (11·5 ml), sodium deoxycholate (1·5 mg), potassium chloride (6 mmol), and 2,3-epoxy-2,3-dihydro-[4-$^3H_2$]squalene (0·2 mg), were added as a dispersion in 0·5% (w/w) Tween 80 (1 ml). The components were mixed at 0—1 °C, the flasks were flushed with nitrogen, and the reaction was started by warming the flasks rapidly to 37 °C. The flasks were shaken for 2 h at 37 °C before addition of 15% potassium hydroxide in methanol (15 ml). After addition of carrier lanosterol (100 mg) the mixtures were saponified by heating at 70 °C for 2 h, added to water (50 ml), and extracted with ether (3 × 50 ml). The combined extracts were washed (H_2O) to neutrality, dried ($MgSO_4$), and evaporated. The residue was benzoylated with benzoyl chloride–pyridine and lanosterol benzoate was purified by t.l.c. on silica–silver nitrate (eluant 30% benzene–light petroleum). After elution with benzene the lanosterol benzoate was recrystallised to constant specific activity from chloroform–methanol (incorporation given in Table 1).

Incubation with Germinating Pea Homogenate.—A homogenate of germinating peas (*Pisum sativum*, var. Onward), as described in ref. 10 except that sucrose, glutathione, and magnesium sulphate were omitted from the suspending medium, was centrifuged at 500 *g* for 20 min at 5 °C to remove whole cells and debris. Incubation mixtures contained the 500 *g* supernatant of the pea homogenate (25 ml; protein concentration 37·5 mg ml^{-1}) plus 2,3-epoxy-2,3-dihydro[4-$^3H_2$]squalene (0·2 mg), added as a dispersion in 0·5% (w/w) Tween 80 (1 ml). The incubation mixtures were shaken under nitrogen at 250 rev. min. on a gyratory shaker for 4 h at 30 °C before addition of 15% (w/w) potassium hydroxide in methanol Inactive β-amyrin (100 mg), lupeol (60 mg), and cycloartenol (60 mg) were added and the mixtures were heated at 70 °C for 3 h before extraction with ether (3 × 50 ml). The combined extracts were washed (H_2O) to neutrality, dried ($MgSO_4$), and evaporated. The triterpenoids were benzoylated (benzoyl chloride–pyridine) and separated by p.l.c. on silica–silver nitrate (eluant 30% benzene–light petroleum). The β-amyrin benzoate, cycloartenol benzoate, and lupeol benzoate thus obtained were recrystallised to constant specific activity (incorporation given in Table 1).

Incubation with a Cell-free Preparation of Yeast.—*Saccharomyces cerevisiae* (strain NCYC 1236) was grown aerobically on yeast complete medium [11] in shake culture at 30 °C as previously described.[1a] After harvesting, the wet cell paste was suspended in 0·2M-phosphate buffer, pH 7·5 (equal vol.), and the cells were broken by passage through a Hughes pressure cell.[14] The broken-cell suspension was diluted with water (1 vol) and centrifuged at 2000 *g* for 20 min at 2 °C, and the supernatant was further centrifuged at 20,000 *g* for 20 min at 2 °C. The resultant supernatant was taken and stored, frozen, at −20 °C.

Incubation mixtures [8] contained in a volume of 1 ml: the 20,000 *g* supernatant (0·4 ml) of cell-free preparation (protein concentration 8 mg ml^{-1}), Triton X-100 to a final concentration of 0·2% (v/v), and 2,3-epoxy-2,3-dihydro[4-$^3H_2$]-squalene (usually 20 nmol) added as a dispersion in 0·1 ml of 0·1% (w/v) Tween 80 in 10 mM-phosphate buffer (pH 7·5). The reactions were started by addition of the squalene oxide, conducted at 37 °C for 30 min, and stopped by addition of acetone (1 ml). The quenched mixtures were extracted with 25% acetone–benzene (3 × 4 ml), which was dried ($MgSO_4$), filtered, and evaporated. Carrier lanosterol (1 mg) was added to the residue, which was fractionated by t.l.c. on silica (eluant 7·5% ethyl acetate–benzene). Regions of the plate corresponding to lanosterol were scraped into scintillation vials and counted. The counts obtained were corrected by subtraction of the counts obtained from boiled-enzyme controls. The results (Table 2) are the average of at least two separate incubations.

Incubation with Whole-cell Cultures of Yeast.—2,3-Epoxy-2,3-dihydro[4-$^3H_2$]squalene was incubated aerobically for 24 h with a previously anaerobically grown 200 ml culture of yeast (*S. cerevisiae*, strain A184 [11]) as described previously.[1a] After harvesting, the wet cell paste was suspended in 15% (w/v) potassium hydroxide in methanol (70 ml) and saponified by refluxing under nitrogen for 2·5 h. After cooling, carrier ergosterol (300 mg) and lanosterol (150 mg) were

[14] D. E. Hughes, J. W. T. Wimpenny, and D. Lloyd, *Methods Microbiol.*, 1971, **5B**, 1.

added, and the mixture was added to water (100 ml) and extracted with ether (3 × 150 ml). The combined extracts were washed (H_2O) to neutrality, dried ($MgSO_4$), and evaporated. Lanosterol and ergosterol were separated by p.l.c. on silica (eluant 7·5% ethyl acetate–benzene). Lanosterol was benzoylated and purified by p.l.c. on silica–silver nitrate (eluant 25% benzene–light petroleum). The lanosterol benzoate was eluted with benzene and recrystallised to constant specific activity from chloroform–methanol. Ergosterol was converted into its trimethylsilyl ether [15] and purified by t.l.c. on alumina–silver nitrate (eluant 20% benzene–light petroleum). After elution with benzene the trimethylsilyl ether was hydrolysed to the free sterol by shaking in aqueous methanol. The sterol was extracted into ether after addition of water and the ergosterol obtained recrystallised to constant specific activity from chloroform–methanol (Table 1).

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[15] M. Makita and W. W. Wells, *Analyt. Biochem.*, **1963**, **5**, 523.