Studies on Phytosterol Biosynthesis: Metabolism of 24,25-Dihydrolanosterol and Cholesterol in *Ochromonas malhamensis*

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(Received 8 March 1972)

The conversion of several potential phytosterol precursors, including lanosterol, into poriferasterol (III) by *Ochromonas malhamensis* has been demonstrated (Hall *et al.*, 1969; Lenton *et al.*, 1971). Since a cell-free extract of peas can alkylate lanosterol, cycloartenol or desmosterol but not 24,25-dihydrolanosterol (I) or cholesterol (II), it has been suggested that sterols without a Δ^{24} -bond cannot be alkylated (Russell *et al.*, 1967). However, there is an isolated report of the conversion of cholesterol into 22,23-dihydrostigmasterol (presumably sitosterol) by the flowers of *Wistaria sinensis* (Badiello *et al.*, 1967). Therefore the biosynthetic role of 24,25-dihydrolanosterol, which has been isolated from *O. malhamensis* (G. H. Beastall, unpublished work), is uncertain. This sterol may undergo C-24 alkylation and further metabolism into poriferasterol, the major sterol in the organism (Gershengorn *et al.*, 1968), or it may be a specific precursor of a C_{27} sterol such as cholesterol. Cholesterol cannot be detected in the free and esterified sterol fractions of *O. malhamensis*, but low concentrations of the compound have been characterized by g.l.c.–mass spectrometry in the sterol glycoside ester and water-soluble sterol fractions of the organism (G. H. Beastall, unpublished work).

The present communication describes the conversion of 24,25-dihydro[2- 3H_2]lanosterol (I) into both cholesterol (II) and poriferasterol (III) by *O. malhamensis*. Since plant systems can alkylate exogenous Δ^{24} -sterol at both the C_{30} and the C_{27} level (for review see Lederer, 1969), the specificity of *O. malhamensis* to alkylate saturated-side-chain sterols was tested. In this respect the conversion of [4- ^{14}C]-cholesterol into poriferasterol by the organism is also reported.

Experimental and results

Incorporation of 24,25-dihydro[2- 3H_2]lanosterol into *O. malhamensis*. 24,25-Dihydro[2- 3H_2]lanosterol was prepared by Dr. A. G. Smith by base-catalysed equilibration of lanost-8-en-3-one with tritiated water followed by reduction with $LiAlH_4$. The purity of the radioactive substrate, in particular the absence of Δ^{24} -compounds, such as lanosterol, was established by t.l.c. on $AgNO_3$ -impregnated silica gel and by radio-g.l.c. Dihydro[2- 3H_2]lanosterol (140 μCi ;

15.3 $\mu Ci/mg$) in 0.1 ml of ethanol was introduced into two 3-day cultures of *O. malhamensis* growing on a defined medium (Aaronson & Baker, 1959). After 4 days the cells were harvested, washed and freeze-dried, and the total lipid (220 mg) was extracted under reflux with acetone, followed by chloroform–methanol (2:1, v/v). The residue was refluxed three times with water for a total of 9 h and the combined aqueous extract was freeze-dried. There was a 14% incorporation of radioactivity from the dihydrolanosterol into the total lipid, but no detectable radioactivity was associated with sterol after hydrolysis (Adams & Parks, 1967) of the water-soluble extract.

(I) 24,25-Dihydrolanosterol

(II) Cholesterol

(III) Poriferasterol

Table 1. *Distribution of radioactivity between the sterol-containing fractions of O. malhamensis after the incorporation of 24,25-dihydro[2-³H₂]lanosterol by the organism*

24,25-Dihydro[2-³H]lanosterol (140 μ Ci) in ethanol was added to 3-day-old cultures of *O. malhamensis*. Cells were harvested after a further 4 days' growth.

Sterol fraction	Radioactivity (μ Ci)	Incorporation (%)
Free sterols	2.3	1.7
Sterols from sterol esters	3.9	2.8
Sterols from sterol glycoside esters	0.03	0.02
Total 4,4-dimethyl sterols	8.1	5.8
Total 4 α -methyl sterols	0.2	0.2

The sterol esters, free sterols and sterol glycoside esters were isolated from the total lipid by alumina column chromatography and t.l.c. on silica gel. The sterol esters were saponified and the sterols were separated from the 4,4-dimethyl sterol + 4 α -methyl sterol fraction. The 4,4-dimethyl sterol + 4 α -methyl sterol fractions from free and esterified sources were combined and further purified by reversed-phase t.l.c. The radioactivity associated with each fraction was determined (Table 1). Negligible radioactivity chromatographed with squalene, indicating that no breakdown of the substrate into small molecules and reincorporation had occurred.

A portion (19mg) of the free sterol fraction was mixed with non-radioactive cholesterol and subjected to g.l.c. on 1% HiEff-8BP. Samples were collected at 1 min intervals in glass capillary tubes at ambient temperature and radioassayed. Approx. 40% of the recovered radioactivity was associated with cholesterol, and the remainder was associated with poriferasterol. To ensure that the radioactivity co-chromatographing with poriferasterol could not be attributed to a C₂₇ Δ^7 -monoene or a C₂₇ $\Delta^{5,7}$ -diene, a further portion of the free sterol fraction was subjected to t.l.c. on AgNO₃-impregnated silica gel. More than 95% of the eluted radioactivity was located in the cholesterol/poriferasterol band, whereas negligible label was associated with the 5 α -cholest-7-en-3 β -ol and cholesta-5,7-dien-3 β -ol regions. The cholesterol/poriferasterol band was subjected to preparative g.l.c., and the two eluted peaks were diluted with the respective non-radioactive compounds and recrystallized both as the free sterols and the sterol acetates until constant specific radioactivity was attained (poriferasterol, 2627 d.p.m./mg; cholesterol, 1309 d.p.m./mg). Radio-g.l.c. of the sterols of the sterol esters revealed that more than 90% of the radioactivity was associated with cholesterol, the remainder co-chromatographing with poriferasterol. Similar treatment of the sterol released by acid hydrolysis (Thornton *et al.*, 1940) of the sterol glycoside esters again revealed radioactivity co-chromatographing with both cholesterol and poriferasterol.

The total 4,4-dimethyl sterol fraction was acetylated. T.l.c. on AgNO₃-impregnated silica gel followed by radio-g.l.c. showed that unchanged dihydrolanosterol was the sole component of this fraction. Radio-g.l.c. of the total 4 α -methyl sterol fraction revealed a single radioactive peak co-chromatographing with authentic 24-methylenelophenol. This was not characterized further.

Incorporation of [4-¹⁴C]cholesterol into O. malhamensis. [4-¹⁴C]Cholesterol (15 μ Ci; 59.2mCi/mmol) in ethanol (0.1ml) was added to a 3-day culture of *O. malhamensis*. After a further 4 days the cells were harvested and extracted by direct saponification. The total sterol (8.1 mg; 3.6 μ Ci) was isolated from the non-saponifiable lipid (30mg; 4.0 μ Ci). Radio-g.l.c. of a portion of this sterol fraction on 1% HiEff-8BP showed that 16% of the eluted radioactivity was associated with poriferasterol, whereas the remainder chromatographed with cholesterol and was unchanged substrate. The poriferasterol was isolated by preparative g.l.c., diluted with carrier material and recrystallized to constant specific radioactivity both as the free sterol and the sterol acetate (712 d.p.m./mg). No evidence could be obtained for possible biosynthetic intermediates between cholesterol and poriferasterol, either by radio-g.l.c. or radioautography of an AgNO₃-impregnated-silica-gel chromatogram of the total sterol.

Discussion

Administration of labelled dihydrolanosterol to *O. malhamensis* produced radioactive cholesterol and poriferasterol in the free sterols, sterol ester and probably sterol glycoside ester fractions, but no incorporation of radioactivity into the water-soluble sterols was observed. The approximate specific radioactivities of the poriferasterol in the free sterol and sterol ester fractions after dihydrolanosterol incorporation were of the same order, suggesting that the free and esterified sterols may share a common pool of poriferasterol. However, cholesterol, which

could not be detected by mass in either the free or the esterified sterol fractions, was the predominantly labelled component of the sterol ester fraction but was labelled to a much smaller extent in the free sterol fraction. This suggests that, in contrast with the poriferasterol of the same fractions, specific esterification of the cholesterol derived from dihydrolanosterol was taking place. Although 60% of the radioactivity of the free sterol fraction was in poriferasterol, its estimated specific radioactivity was, as in the sterol ester fraction, considerably less than that of cholesterol. It therefore seems that the major role of dihydrolanosterol in *O. malhamensis* is as a precursor of cholesterol.

The conversion of cholesterol into poriferasterol in good yield (4.3%) by *O. malhamensis* demonstrates that the organism is capable of two successive alkylation reactions at the C₂₇ level. No labelled intermediates between cholesterol and poriferasterol could be detected. It is conceivable that the conversion of dihydrolanosterol into poriferasterol could proceed via cholesterol as well as through a C-24-alkylated 4 α -methyl sterol, although the conversion of the 4 α -methyl sterols 24-methylenelophenol and 24-ethylidenelophenol into poriferasterol by *O. malhamensis* has been demonstrated (Lenton *et al.*, 1971). Alkylation in *O. malhamensis* will apparently proceed independently of the degree of nuclear methyl substitution and saturation, owing to the presence of non-specific enzymes, including, presumably, a dehydrogenase that introduces the unsaturation at C-24 necessary for reaction with *S*-adenosylmethionine (Parks, 1958).

The results of these experiments indicate that *O. malhamensis* will transform exogenous 24,25-dihydrolanosterol and cholesterol into poriferasterol. However, it is difficult to assess the significance of these conversions in the normal metabolism of the organism, since the concentrations of the two precursors are probably strictly regulated. The conversion of cholesterol into campesterol and stigmasterol by *Nicotiana tabacum* has also been reported (Tso & Cheng, 1971).

We thank the Science Research Council for financial assistance and Dr. A. G. Smith for the gift of 24,25-dihydro[2-³H₂]lanosterol.

- Aaronson, S. & Baker, H. (1959) *J. Protozool.* **6**, 282
Adams, B. G. & Parks, L. W. (1967) *Biochem. Biophys. Res. Commun.* **28**, 490
Badiello, R., Tamba, M. & Breccia, A. (1967) *Ric. Sci.* **37**, 1156
Gershengorn, M. C., Smith, A. R. H., Goulston, G., Goad, L. J., Goodwin, T. W. & Haines, T. H. (1968) *Biochemistry* **7**, 1698
Hall, J., Smith, A. R. H., Goad, L. J. & Goodwin, T. W. (1969) *Biochem. J.* **112**, 129
Lederer, E. (1969) *Quart. Rev. Chem. Soc.* **23**, 453
Lenton, J. L., Hall, J., Smith, A. R. H., Ghisalberti, E. L., Rees, H. H., Goad, L. J. & Goodwin, T. W. (1971) *Arch. Biochem. Biophys.* **143**, 664
Parks, L. W. (1958) *J. Amer. Chem. Soc.* **80**, 2023
Russell, P. T., van Aller, R. T. & Nes, W. R. (1967) *J. Biol. Chem.* **242**, 5802
Thornton, M. H., Kraybill, H. R. & Mitchell, J. H. (1940) *J. Amer. Chem. Soc.* **62**, 2006
Tso, T. C. & Cheng, A. L. S. (1971) *Phytochemistry* **10**, 2133