Phytosterol Biosynthesis: Evidence for a 24-Ethylidene Intermediate during Sterol Formation in *Ochromonas malhamensis*

By A. R. H. SMITH, L. J. GOAD and T. W. GOODWIN

Department of Biochemistry, University of Liverpool

and E. LEDERER

Institut de Chimie des Substances Naturelles, Gif-sur-Yvette, Seine-et-Oise, France

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The 24-ethyl and 24-ethylidene groups present in various phytosterols arise by two successive trans-methylations from methionine (Castle, Blondin & Nes, 1963; Bader, Guglielmetti & Arigoni, 1964; Villanueva, Barbier & Lederer, 1964). A knowledge of the fate of the hydrogen atoms of the methionine methyl group is important to the elucidation of the transmethylation mechanisms in operation. During ergosterol biosynthesis it has been shown that one of the hydrogen atoms of the methionine methyl group is lost (Jauréguiberry, Law, McCloskey & Lederer, 1965). Possible mechanisms have been discussed (Lederer, 1964) and include the inter-

mediate production of a 24-methylene derivative; this idea has now gained support by the isolation of 24-methylenelanolsterol from fungi (Goulston, Goad & Goodwin, 1967) and by the demonstration that this compound is converted into ergosterol by yeast cultures (Akhtar, Parvez & Hunt, 1966; Barton, Harrison & Moss, 1966).

Proposed biosynthetic schemes (Castle *et al.* 1963; Lederer, 1964; Clayton, 1965; Goad, Hammam, Dennis & Goodwin, 1966) have suggested that 24-ethyl- and 24-ethylidene-sterols arise from a 24-methylene intermediate (I) to give a carbonium ion (II). This carbonium ion can conceivably be

stabilized in a number of ways, as indicated in Scheme 1 (cf. Jauréguiberry, Lenfant, Das & Lederer, 1966). In a previous study we used [$Me-^{14}C,^3H$]methionine and obtained evidence supporting the intermediate formation of a 24-ethylidene steroid (III) during β -sitosterol biosynthesis in maize and larch leaves (Goad *et al.* 1966). However, Lenfant, Zissmann & Lederer (1967) have incorporated [$Me-^2H_3$]methionine into stigmast-22-en-3 β -ol in the slime mould *Dictyostelium discoideum* and have shown that all five hydrogen atoms of the 24-ethyl group arise from methionine. This clearly eliminates a 24-ethylidene steroid from the biosynthetic sequence in this organism. Since experiments with 3H -labelled compounds may be questioned on the basis of possible hydrogen isotope effects we decided to reinvestigate this problem with [$Me-^2H_3$]methionine, as employed by Lenfant *et al.* (1967). The phytoflagellate *Ochromonas malhamensis* was chosen as the biological system, since previous work had shown it to contain relatively large amounts of one sterol, poriferasterol (the 24-epimer of stigmasterol) (Williams & Goodwin, 1966). This organism also has the advantage that it is fast-growing and can be cultured on a well-defined medium.

Experimental and results. *Ochromonas malhamensis* (933/1A), obtained from the Cambridge Culture Collection, was cultured in 100ml. batches in 250ml. flasks on the medium of Aaronson & Baker (1959). Each flask was inoculated with 5ml. of a fully grown culture and was then maintained at 25–28° with

continuous shaking for 6 days. Continuous illumination was provided by a bank of fluorescent lights. The cells were harvested by centrifugation and washed with a little fresh culture medium. They were then suspended in 10ml. of ethanol, and 1ml. of 60% (w/v) KOH solution was added. The mixture was refluxed for 90min., the non-saponifiable material was extracted in the usual way and the poriferasterol was obtained by preparative thin-layer chromatography on silica gel (Goad & Goodwin, 1966). The poriferasterol was checked for purity by gas-liquid chromatography on a Varian Aerograph 1522 instrument fitted with flame ionization detectors. With columns (6ft. $\times$ $\frac{1}{8}$ in.) of 1% QF-1 or 1% SE-30 it was shown that the *Ochromonas* sterol contained only one major component (98%) with retention data identical with those of poriferasterol. A minor peak (1%) corresponding to brassicasterol was also observed.

Although methionine appeared to be an essential component of the culture medium it was advisable to show that the methyl group of the added methionine was appreciably incorporated into poriferasterol by *O. malhamensis*. Four 100ml. cultures were grown containing 25, 50, 100 and 200mg. of L-methionine respectively, and including 1 μ C of L-[$Me-^{14}C$]methionine in each case. The specific activities of the isolated poriferasterol samples (7750, 9480, 8000 and 4590 disintegrations/min./mg.) corresponded to incorporations of the methionine methyl group into the 24-ethyl group of poriferasterol of approx. 12, 30, 50 and 60% respectively.

Fig. 1. Mass spectrum of poriferasterol biosynthesized by *Ochromonas malhamensis* in the presence of [$Me-^2H_3$]-methionine.

On the basis of these results duplicate 100ml. cultures of medium were prepared, each incorporating 100mg. of [$Me-^2H_3$]methionine instead of unlabelled methionine. The cultures were inoculated and grown as described above. The isolated poriferasterol was checked by gas-liquid chromatography (98% pure) and then analysed by mass spectrometry (Fig. 1). A large peak at m/e 412 was noted and represents the molecular ion peak for the non-deuterated poriferasterol present. Large peaks were also observed at m/e 413, 415 and 416, and were due to poriferasterol containing 1, 3 and 4 deuterium atoms/mol. respectively. The molecular ions at m/e 414 and 417 were of the order expected for the ^{13}C isotope content of the peaks at m/e 413 and 416 respectively. The ions at m/e 397, 379, 369 and 351, corresponding to the loss of methyl, methyl plus water, terminal isopropyl and terminal isopropyl plus water respectively were all accompanied by ions at m/e values +1, +3 and +4. The peak at m/e 300 for loss of part of the side chain (Lenfant *et al.* 1967) was unaccompanied by higher m/e peaks, thus providing evidence that all four deuterium atoms were present in the sterol side chain. The peaks at m/e 271 and 255 also supported this conclusion.

This result establishes that in *O. malhamensis* alkylation of the sterol side chain proceeds via a mechanism leading to the intermediate formation of a 24-ethylidene steroid (III), which is subsequently reduced to give the 24-ethyl derivative (VII). This is in agreement with our previous results, which also indicated that during β -sitosterol synthesis in higher plants a 24-ethylidene steroid is an intermediate (Goad & Goodwin, 1966, 1967; Goad *et al.* 1966). However, Lenfant *et al.* (1967) have established that in the slime mould *D. discoideum* an alternative mechanism for the stabilization of the carbonium ion (II) is in operation (e.g. see Scheme 1). The recent isolation from plant sources

of clerosterol (Sucrow, 1966; Manzoor-i-Khuda, 1966), which has side chain (IV), also supports the conclusion that an alternative mechanism can occur as indicated in Scheme 1. In this Scheme the problem of the formation of the 22,23-double bond frequently encountered in phytosterols is not considered.

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Reduction of a Disulphide Bond of Ribonucleotide Reductase by the Dithiol Substrate

By E. VITOLS, H. P. C. HOGENKAMP, CAROL BROWNSON, R. L. BLAKLEY and J. CONNELLAN

*Department of Biochemistry, John Curtin School of Medical Research,
 Australian National University, Canberra, A.C.T., Australia*

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A ribonucleotide reductase from *Lactobacillus leichmannii* catalyses the reduction of ribonucleoside triphosphates to the corresponding deoxyribonucleotides by dithiols, and has an absolute

requirement for deoxyadenosylcobalamin (Blakley, 1966). Dihydrolipoic acid and other 1,3- or 1,4-dithiols are suitable reductants at high concentrations (30mM), whereas monothiols such as 2-