Short Communications

Observations on the Sterol Side-Chain Alkylation Mechanism in a *Trebouxia* species

By L. J. GOAD, F. F. KNAPP, J. R. LENTON and T. W. GOODWIN

Department of Biochemistry, University of Liverpool, P.O. Box 147, Liverpool L69 3BX, U.K.

(Received 12 May 1972)

The sterols of most higher plants and algae are C_{28} and C_{29} compounds in which the additional alkyl group at C-24 is derived by transmethylation from methionine (Lederer, 1969). Ergosterol* biosynthesized by *Neurospora crassa* in the presence of $[Me-^2H_3]$ methionine contained two deuterium atoms in the 24-methyl group (Jaureguiberry *et al.*, 1965), demonstrating the intermediacy of a 24-methylene sterol (Scheme 1, I→II→III→IV). In the chrysophyte *Ochromonas malhamensis* four deuterium atoms derived from $[Me-^2H_3]$ methionine were found in poriferasterol (Smith *et al.*, 1967), showing that a 24-ethylidene sterol is formed and subsequently reduced (Scheme 1, I→II→III→V→VI→VII→VIII) (Knapp *et al.*, 1971). In contrast the chlorophytes *Chlorella vulgaris* and *Chlorella ellipsoidea* incorporated three and five deuterium atoms into the C_{28} and C_{29} sterols respectively when grown in media containing $[Me-^2H_3]$ methionine (Tomita *et al.*, 1970, 1971). Similarly a slime mould, *Dictyostelium discoideum*, incorporated five deuterium atoms into 5 α -stigmast-22-en-3 β -ol (Lenfant *et al.*, 1969). In these organisms a 24-methylene sterol is not reduced to give a 24-methyl sterol, nor is a 24-ethylidene compound (VI) an intermediate in 24-ethyl sterol formation. Several alternative mechanisms have been proposed for the stabilization of cations (II) and (V) as indicated in Scheme 1 (Castle *et al.*, 1963; Lederer, 1969; Smith *et al.*, 1967; Tomita *et al.*, 1971; Ellouz & Lenfant, 1971; Goad & Goodwin, 1972).

We now report relevant investigations on the sterol C-24 alkylation reactions which occur in a species of *Trebouxia*, the algal symbiont of a lichen.

Methods

Trebouxia species 213/3 was obtained from the Cambridge Culture Collection. It was cultured either in the organic medium I of Ahmadjian (1967) or in Bold's mineral medium (Ahmadjian, 1967). Flasks containing 200 ml of media were inoculated with 5 ml

of *Trebouxia* culture and then shaken for 20–26 days at 20°C with illumination under an atmosphere of 5% CO_2 in air.

Sterol analysis was generally performed as described previously (Lenton *et al.*, 1971). Cells were harvested by centrifugation, the non-saponifiable lipids extracted and the sterols obtained by t.l.c. on silica gel developed with chloroform. Sterol acetates were purified by t.l.c. on 10% (w/w) $AgNO_3$ -silica gel developed with ethanol-free chloroform. G.l.c. was done on glass columns [1.52 m $\times$ 0.635 cm (5 ft $\times$ $\frac{1}{8}$ in)] packed with either 3% SE-30 or 3% OV-17 on 100–120 mesh Gas-Chrom Q. For sample collection an effluent splitter was used and samples were trapped at 1 min intervals in capillary tubes at ambient temperature. Mass spectra were determined with an AEI MS12 instrument coupled to a gas-liquid chromatograph and radioactivity was assayed by liquid-scintillation counting.

L- $[Me-^2H_3]$ Methionine was synthesized by the method of Dolphin & Endo (1970). The synthesis of $[23,25-^3H_3]$ cycloeucalenol (11.33 mCi/mmol) was described previously (Lenton *et al.*, 1971). 31- $[2,4-^3H_3]$ -Norcyclolaudenol (1.62 mCi/mmol) was prepared from unlabelled material by methods used previously to label other 4 α -methyl sterols (Lenton *et al.*, 1971). The labelled compounds were administered in ethanol (0.2 ml) to cultures of *Trebouxia* growing in the organic medium I.

Results and discussion

G.l.c. and mass-spectral analysis showed that the sterols of the *Trebouxia* species were ergost-5-en-3 β -ol (M^+ at m/e 400), poriferasterol (M^+ at m/e 412) and clionasterol (M^+ at m/e 414). When the alga was grown in the presence of $[Me-^2H_3]$ methionine three deuterium atoms were incorporated into the side chain of ergost-5-en-3 β -ol (M^+ at m/e 403) and two, three or five deuterium atoms were present in the poriferasterol (M^+ at m/e 414, 415 and 417) and clionasterol (M^+ at m/e 416, 417 and 419). Thus in *Trebouxia* the C-24 alkylation reactions are similar to those operating in *Chlorella* and do not involve $\Delta^{24(28)}$ intermediates (III or VI) in C_{28} (X) and C_{29} (XII or XIII) sterol formation. However, consideration of the postulated alkylation mechanisms

* Systematic names of sterols used in this paper: 31-norcyclolaudenol, 4 α ,14 α -dimethyl-9 β ,19-cyclo-5 α -ergost-25-en-3 β -ol; cycloeucalenol, 4 α ,14 α -dimethyl-9 β ,19-cyclo-5 α -ergost-24(28)-en-3 β -ol; poriferasterol, (24*R*)-24-ethylcholesta-5,22-dien-3 β -ol; clionasterol, (24*S*)-24-ethylcholest-5-en-3 β -ol; ergosterol, ergosta-5,7,22-trien-3 β -ol.

Table 1. *Percentage composition of the sterol mixtures isolated from Trebouxia grown in the presence of either methionine or [Me-²H₃]methionine*

In Expt. 1 the cells were cultured in 200ml of Bold's mineral medium containing either 100mg of methionine or 100mg of [Me-²H₃]methionine. For Expt. 2 the same media were used except that 2% (w/v) glucose was added. All cultures were grown for 26 days under identical conditions. The sterols were analysed by g.l.c. (SE-30).

	Sterol composition (%)			
	Expt. 1		Expt. 2	
	Methionine	[Me- ² H ₃]- Methionine	Methionine	[Me- ² H ₃]- Methionine
Ergost-5-en-3 β -ol (C ₂₈ , Δ^5)	23.0	39.5	23.0	41.5
Poriferasterol (C ₂₉ , $\Delta^{5,22}$)	64.5	55.5	61.0	53.0
Clonasterol (C ₂₉ , Δ^5)	12.5	5.0	16.0	5.5

indicates that the alga should produce a 24-methylene sterol (III) as a mandatory intermediate in the second transmethylation step (III $\rightarrow$ V) that is required to produce the C₂₉ sterols.

Comparison of the composition of the sterol mixtures obtained from the cultures grown on normal and deuterium-labelled methionine showed that the proportion of C₂₈ sterol was markedly increased in the cultures grown in the presence of [Me-²H₃]methionine (Table 1). This reveals the operation of a pronounced deuterium isotope effect during the stabilization of cation (II) and indicates that proton loss from C-28 is the rate-limiting step in $\Delta^{24(28)}$ sterol formation. Consequently with C-28 deuterium-labelled species of cation (II) the kinetic isotope effect may favour stabilization by loss of a proton from another position to give, for example, a Δ^{25} (IXa) or Δ^{24} (IXb) compound, which could then be reduced to give the saturated side chain (X). Diversion of the sterol precursor (I) in this manner into C₂₈ sterol (X) biosynthesis would have the consequential effect of decreasing the production of $\Delta^{24(28)}$ sterol (III), which is required for C₂₉ sterol (XII and XIII) formation. The operation of a deuterium isotope effect should thus increase the proportion of C₂₈ sterol and lower the content of C₂₉ sterols in the cell, as was observed experimentally (Table 1). These results therefore establish that the operation of isotope effects can in some circumstances significantly alter the course and equilibrium of metabolic sequences, and further emphasize the caution required in the interpretation of results obtained when deuterium- or tritium-labelled compounds are used for biochemical investigations.

The observed deuterium-labelling results are compatible with several of the routes shown in Scheme 1. One mechanism involving Δ^{25} compounds (IXa and XIa) is attractive, since such compounds

occur naturally (Goad & Goodwin, 1972) and their mode of biosynthesis apparently conforms to the route indicated in Scheme 1 (Ghisalberti *et al.*, 1969; Bolger *et al.*, 1970). The possibility that a Δ^{25} intermediate (IXa) is involved in ergost-5-en-3 β -ol synthesis was tested by administration of 31-[2,4-³H₃]-nortetrolaudenol (8.6 μ Ci) to a culture of *Trebouxia*. The isolated sterols (2.61 $\times 10^5$ d.p.m.) were examined by g.l.c. with sample-trapping, which showed that radioactivity was only present in ergost-5-en-3 β -ol; no radioactivity was associated with the poriferasterol or clonasterol peaks. The exclusive labelling of ergost-5-en-3 β -ol was confirmed by t.l.c. of the sterol acetates on AgNO₃-silica gel; radioactivity was only present in the ergost-5-en-3 β -yl acetate band, and the poriferasteryl acetate was unlabelled. This demonstration of Δ^{25} -bond reduction adds support to the suggestion that one product of the first alkylation reaction may be a Δ^{25} compound (IXa), which then acts as a precursor of the C₂₈ sterol of *Trebouxia*. The absence of label in the C₂₉ sterols shows that conversion of compound (IXa) into compound (III) does not occur readily, if at all.

The intermediacy of a $\Delta^{24(28)}$ sterol (III) in C₂₉ sterol synthesis was demonstrated by growth of *Trebouxia* in the presence of [23,25-³H₃]cyclo-eucalenol (10.0 μ Ci). G.l.c. of the isolated sterols (3.60 $\times 10^5$ d.p.m.) showed that poriferasterol and clonasterol were both labelled but that ergost-5-en-3 β -ol was devoid of radioactivity. The labelling of poriferasterol was confirmed by acetylation and purification of poriferasteryl acetate (7.85 $\times 10^4$ d.p.m.) on AgNO₃-silica gel followed by addition of carrier (31.0mg) and crystallization to constant specific radioactivity (2530, 2600, 2680, 2720, 2630, 2690, 2600 d.p.m./mg for successive crystallizations). Lack of radioactivity in ergost-5-en-3 β -ol shows that in *Trebouxia* a $\Delta^{24(28)}$ sterol (III) is not reduced to

give a 24-methyl sterol (IV), a conclusion further substantiated by the absence of ergost-5-en-3 β -ol containing two deuterium atoms in the [$Me-^2H_3$]-methionine experiments. Thus all the $\Delta^{24(28)}$ sterol (III) produced in the first transmethylation must be utilized to produce C_{29} sterols (XII and XIII) by the second transmethylation step. A possible role for a Δ^{25} (XIa) or Δ^{24} (XIb) intermediate (Tomita & Uomori, 1970) in C_{29} sterol formation by *Trebouxia* remains to be evaluated.

We thank the Science Research Council for financial assistance. F. F. K. was the recipient of a National Institutes of Health Postdoctoral Fellowship.

- Ahmadjian, V. (1967) *The Lichen Symbiosis*, p. 119, Blaisdell Publishing Co., Waltham, Mass.
- Bolger, L. M., Rees, H. H., Ghisalberti, E. L., Goad, L. J. & Goodwin, T. W. (1970) *Biochem. J.* **118**, 197
- Castle, M., Blondin, G. & Nes, W. R. (1963) *J. Amer. Chem. Soc.* **85**, 3306
- Dolphin, D. & Endo, K. (1970) *Anal. Biochem.* **36**, 338
- Ellouz, R. & Lenfant, M. (1971) *Eur. J. Biochem.* **23**, 544
- Ghisalberti, E. L., De Souza, N. J., Rees, H. H., Goad, L. J. & Goodwin, T. W. (1969) *Chem. Commun.* 1401
- Goad, L. J. & Goodwin, T. W. (1972) *Progr. Phytochem.* **3**, 113
- Jaureguiberry, G., Law, J. H., McCloskey, V. A. & Lederer, E. (1965) *Biochemistry* **4**, 347
- Knapp, F. F., Greig, J. B., Goad, L. J. & Goodwin, T. W. (1971) *Chem. Commun.* 707
- Lederer, E. (1969) *Quart. Rev. Chem. Soc.* **23**, 453
- Lenfant, M., Ellouz, R., Das, B. C., Zissmann, E. & Lederer, E. (1969) *Eur. J. Biochem.* **7**, 159
- Lenton, J. R., Hall, J., Smith, A. R. H., Ghisalberti, E. L., Rees, H. H., Goad, L. J. & Goodwin, T. W. (1971) *Arch. Biochem. Biophys.* **143**, 664
- Smith, A. R. H., Goad, L. J., Goodwin, T. W. & Lederer, E. (1967) *Biochem. J.* **104**, 56c
- Tomita, Y. & Uomori, A. (1970) *Chem. Commun.* 1416
- Tomita, Y., Uomori, A. & Minato, H. (1970) *Phytochemistry* **9**, 555
- Tomita, Y., Uomori, A. & Sakurai, E. (1971) *Phytochemistry* **10**, 573