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S-Adenosyl-L-Methionine–Cycloartenol Methyltransferase Activity in Cell-Free Systems from *Trebouxia* sp. and *Scenedesmus obliquus*

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1. Homogenates prepared from *Trebouxia* sp. 213/3 and *Scenedesmus obliquus* exhibited *S*-adenosyl-L-methionine–cycloartenol methyltransferase activity. 2. The products of the reaction, with cycloartenol as the substrate, were 24-methylenecycloartanol and cyclolaudenol. 3. Optimal enzyme activity was found in homogenates prepared at pH 7.6 and the transmethylase was distributed between the supernatant and microsomal fractions of the *Trebouxia* homogenate. 4. The relevance of these results is discussed in relation to C_{28} and C_{29} sterol production in the algae.

The C-24 methyl or ethyl groups of C_{28} and C_{29} sterols present in most higher palnts, algae and fungi are derived by transmethylation from L-methionine (Lederer, 1969 and references therein). Several different mechanisms for the introduction of a C-24 methyl group have been proposed as shown in Scheme 1 (Castle *et al.*, 1963; Smith *et al.*, 1967; Lederer, 1969; Tomita *et al.*, 1971; Ellouz & Lenfant, 1971; Goad *et al.*, 1972; Goad & Goodwin, 1972). A soluble partially purified enzyme preparation from yeast has been described (Katsuki & Bloch, 1967; Moore & Gaylor, 1969, 1970) that is capable of synthesis of 24-methylene sterols (III) from Δ^{24} compounds (I) and *S*-adenosylmethionine as indicated in Scheme 1 by the sequence I → II → III. Similarly, production of 24-methylene derivatives was observed when a crude cell-free system from germinating peas was incubated with cycloartenol (XI) or lanosterol (XII) and methionine (Russell *et al.*, 1967; Malhotra & Nes, 1971) and the formation of 24-methylene cycloartanol (XIIIb) from cycloartenol by a microsomal enzyme of *Rubus fruticosus* has been reported (Heintz & Benveniste, 1972). The utilization of 24-methylene (III) and 24-ethylidene (VIII) compounds in sterol biosynthesis by the Chrysophyte *Ochromonas malhamensis* has been demonstrated (Lenton *et al.*, 1971; Knapp *et al.*, 1971). In particular *O. malhamensis* and the related organism *Ochromonas danica* grown in the presence of [*Me*-$^2H_3$]methionine produced brassicasterol (C-24 methyl) and poriferasterol (C-24 ethyl) that contained two and four deuterium atoms respectively (Smith *et al.*, 1957; Lederer, 1969; W. Sach & L. J. Goad, unpublished results). This agrees with the formation of 24-methylene and 24-ethylidene sterols as intermediates (route I → II → III → VI and route I → II → III → VII → VIII → X respectively). By contrast, in similar experiments with the chlorophytes *Chlorella vulgaris* and *Chlorella pyrenoidosa* (Tomita *et al.*, 1970, 1971), *Scenedesmus obliquus* (W. Sach & L. J. Goad, unpublished work) and a *Trebouxia* sp. (Goad *et al.*, 1972) three and five deuterium atoms were incorporated into the C_{28} and C_{29} sterols respectively and this eliminates 24-methylene and 24-ethylidene intermediates in these species. An alternative alkylation mechanism was proposed (Goad *et al.*, 1972, Doyle *et al.*, 1972) involving the production of 25-methylene intermediates (IV and IX) and gained substantial support by the demonstration that the 25-methylene compound 31-norcyclolaudenol (side chain IV) was converted into C_{28} sterol (VI) by *Trebouxia* sp. (Goad *et al.*, 1972). However, this work also showed that a 24-methylene compound, cycloeucalenol (side chain III), could act as a substrate for the second transmethylation step leading to C_{29} sterols (X). We now report evidence showing that both 24-methylene and 25-methylene derivatives are formed from cycloartenol (XI) and *S*-adenosylmethionine by cell-free preparations from the algae *S. obliquus* and *Trebouxia* sp. 213/3.

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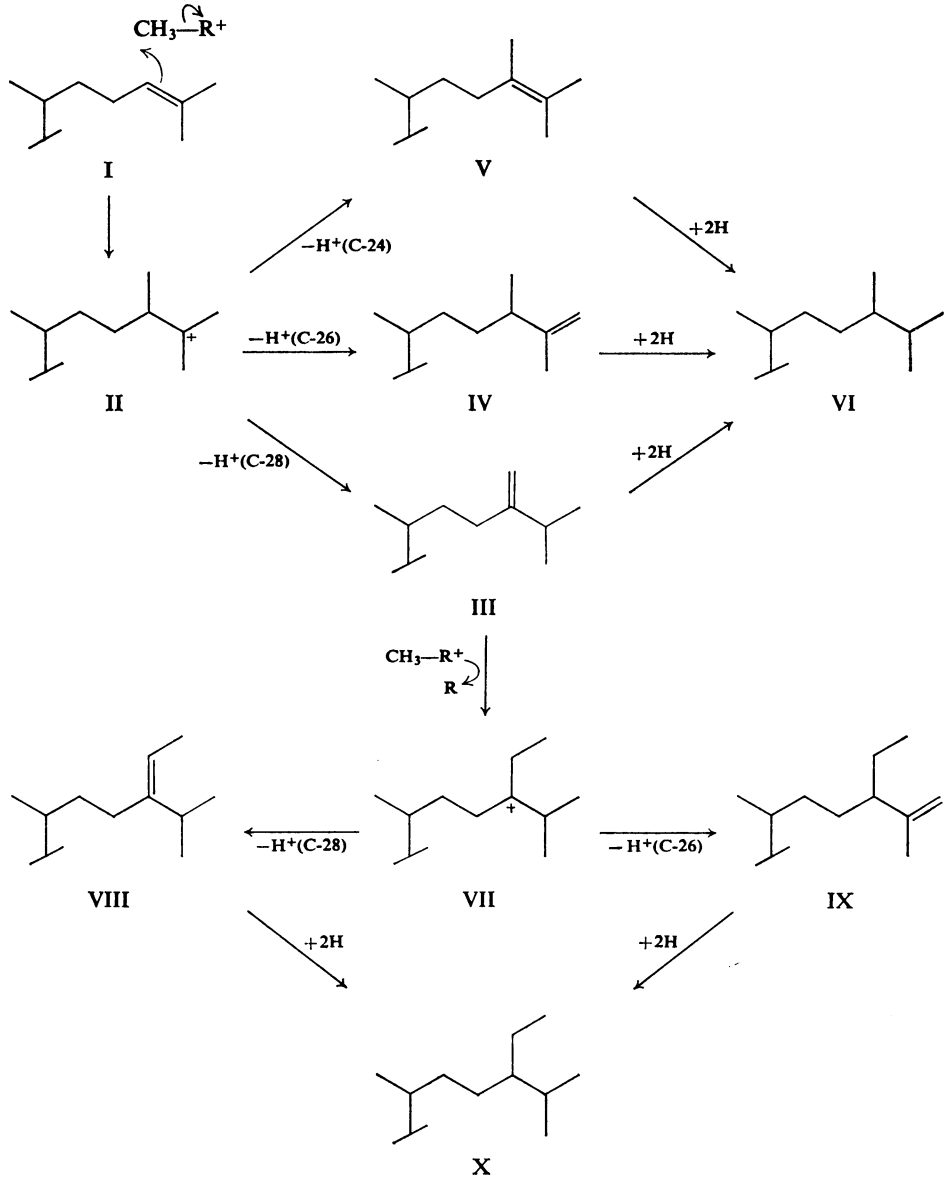
Experimental

Nomenclature

Lanosterol, lanosta-8,24-dien-3β-ol; 24,25-dihydrolanosterol, lanost-8-en-3β-ol; cycloartenol, 9β,19cyclolanosta-8,24-dien-3β-ol; 24-methylene cycloartanol, 4,4,14α-trimethyl-9β,19-cyclo-5α-ergost-24(28)-en-3β-ol; cyclolaudenol, 4,4,14α-trimethyl-9β,19-cyclo-5α-ergost-25-en-3β-ol; 31-nor-cyclolaudenol, 4α,14α-dimethyl-9β,19-cyclo-5α-ergost-24(28)-en-3β-ol; cycloeucalenol, 4α,14α-dimethyl-9β,19-cyclo-5α-ergost-25-en-3β-ol; 24-methylene-



Scheme 1. *Alternative mechanisms for sterol C-24 methylation*

lophenol, 4α-methyl-5α-ergosta-7,24(28)-dien-3β-ol; brassicasterol, ergosta-5,22-dien-3β-ol; clionasterol, (24*S*)-24-ethylcholest-5-en-3β-ol; poriferasterol, (24*R*)-24-ethylcholesta-5,22-dien-3β-ol.

Materials

Trebouxia species, 213/3 was obtained from the Cambridge Culture collection. *S. obliquus*, strain D_3, was obtained from Dr. R. Powls, Department of Biochemistry, Liverpool University. *S*-Adenosyl-L-[*Me*-^{14}C]methionine (53 mCi/mmol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Sterols used as substrates and carriers were obtained as follows: cycloartenol acetate, obtained from *Strychnos nux vomica* by Mr. D. Abramson; cycloartenol and cyclolaudenol, from a 4,4-dimethyl sterol fraction of *Papaver somniferum* provided by Dr. F. F. Knapp; 24-methylenecycloartanol and 24-methylenelophenol isolated from barley seeds;

lanosterol and 24,25-dihydrolanosterol, obtained by t.l.c. on $AgNO_3$-impregnated silica gel of a commercial source of lanosterol (Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K.); cycloeucalenol, isolated from tallow wood by Dr. F. F. Knapp; 5α-ergosta-7,24(28)-dien-3β-ol, isolated from *Asterias rubens* by Mr. I. Rubinstein. The identity and purity of all the above sterols was checked by t.l.c., g.l.c. and mass spectrometry.

General procedures

Chromatography. T.l.c. of sterols and their derivatives was performed on Kieselgel G developed with chloroform (system I); chloroform–methanol (97:3, v/v) (system II); chloroform–methanol (92:8, v/v) (system III); or on 15% (w/w) $AgNO_3$-impregnated Kieselgel G developed with ethanol-free redistilled chloroform (system IV).

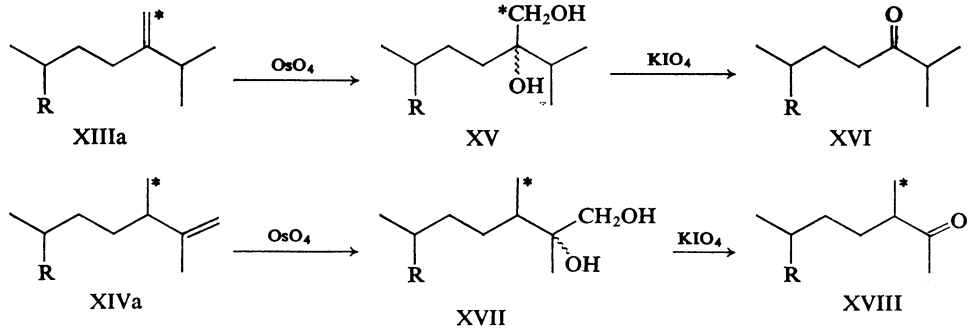
Radioassay. Radioactivity was assayed by liquid-scintillation counting with an Intertechnique SL40 instrument. Samples were dissolved in 10ml of scintillation solution containing 0.5% (w/v) 2,5-diphenyloxazole in toluene.

Melting points. These were determined on a Reichert hot-stage apparatus and are corrected.

Culture of algae. Trebouxia sp. 213/3 was cultured on Bold's mineral medium containing 1% (w/v) peptone and 2% (w/v) glucose (Ahmadjian, 1967). Flasks containing 200ml of medium were inoculated with 5.0ml of *Trebouxia* culture and incubated for 8–24 days at 20°C with continuous illumination and aeration. The *Trebouxia* culture used in this work was originally grown in a medium that did not contain glucose and was slow growing. After several re-inoculations into the glucose-supplemented medium a distinct increase in growth rate was observed and was possibly due to an adaptation of the cells to the glucose medium. *S. obliquus* was grown on a medium containing yeast extract, glucose and mineral salts (Kessler *et al.*, 1957). Flasks containing 1 litre of medium were inoculated with 200ml of *S. obliquus* culture and grown for 8 days at 28°C in the dark with continuous aeration.

Preparation of cell-free homogenates. The cells were harvested by centrifugation, washed three times with ice-cold 0.1M-sodium phosphate buffer, pH7.6, and resuspended in the same buffer (70ml/50g of wet cells). Portions (35ml) of the suspension were placed into 50ml flasks containing 10g of glass beads (0.25–0.30mm diam.) and shaken in a Braun Cell Homogenizer (4000 oscillations/min) at about 0°C for 5min. The homogenate was then centrifuged at 4000*g* for 20min to provide the cell-free supernatant used for most experiments. For the preparation of a microsomal fraction the supernatant was centrifuged at 20000*g* for 20min and the resulting supernatant recentrifuged at 105000*g* for 1h. The microsomal pellet was then resuspended in 0.1M-sodium phosphate buffer, pH7.6, to restore the initial volume.

Incubation with S-adenosyl-L-[Me-^{14}C]methionine. The incubation mixture contained 5.0ml of freshly prepared homogenate, 0.1ml of $MgCl_2$ (40μmol), 0.2ml of reduced glutathione (20μmol), 0.5ml of sterol emulsion (0.25mg) and 0.5ml of *S*-adenosyl-L-[*Me*-^{14}C]methionine (0.5μCi). For large-scale incubations to provide radioactive products for characterization these volumes were scaled up 10-fold (*S. obliquus*) or 14-fold (*Trebouxia*). The sterol substrate emulsions were prepared by sonication of the sterol suspension in aqueous 1% (v/v) Tween 80 immediately before use. The incubation mixture was maintained at 33°C for 18h and the reaction was terminated by the addition of an equal volume of 10% (w/v) KOH in aq. 75% (v/v) ethanol. Samples were then refluxed for 30min, diluted with 5vol. of water



Scheme 2. *Degradation of* 24-*methylenecycloartanyl acetate* (*XIIIa*) *and cyclolaudenyl acetate* (*XIVa*)

The radioactive carbon derived from *S*-adenosyl[*Me*-^{14}C]methionine is indicated (*).

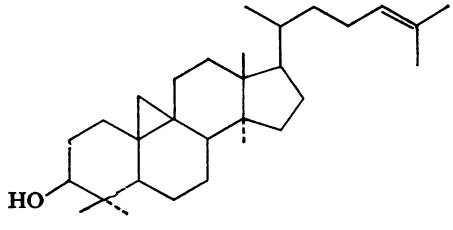
and the non-saponifiable lipids extracted with light petroleum (b.p. 40–60°C)–diethyl ether (1:1, v/v) and assayed for radioactivity (Table 1).

Characterization of the radioactive products (*Scheme* 2). The non-saponifiable lipid (735000d.p.m.) obtained from a large-scale incubation of a *Trebouixa* homogenate was mixed with cyclolaudenol (2mg) and 24-methylenecycloartanol (2mg) and purified by t.l.c. on system I and subsequently by t.l.c. on system IV. To the recovered material (654000d.p.m.) cyclolaudenol (58.4mg) and 24-methylenecycloartanol (90.5mg) were added and the mixture was acetylated with pyridine–acetic anhydride (1:2, v/v). The steryl acetates (155.7mg) were dissolved in pyridine (5.5ml) and OsO_4 (120mg) was added. The mixture was stored at room temperature overnight, then water (3.0ml) and sodium bisulphite (500mg) were added and the mixture stirred for 2h. After dilution with water (50ml) the reaction mixture was extracted with diethyl ether (3 × 50ml) which after drying and evaporation gave the crude diol mixture (143mg). This material was separated by preparative t.l.c. on system III (Table 2) to give the two isomeric diols (XV) derived from 24-methylene cycloartanol (A, 50.1mg; B, 28.6mg) and the two diols (XVII) derived from cyclolaudenol (C, 25.3mg; D, 16.5mg). These diols were crystallized several times (Table 2) and then the isomeric pairs combined. Diols A plus B (46.2mg) were dissolved in dioxan (5.0ml) and potassium metaperiodate (50mg) in water (2.5ml) was added. The mixture was stirred at room temperature overnight and then diluted with water (10vol.) and extracted with diethyl ether. The resulting 24-oxocycloartanyl acetate (XVI, 35.2mg) was purified by t.l.c. on system II. Treatment of diols C plus D (28.1mg) in a similar manner gave 25-oxo-26-norcyclolaudenyl acetate (XVIII, 16.9mg).

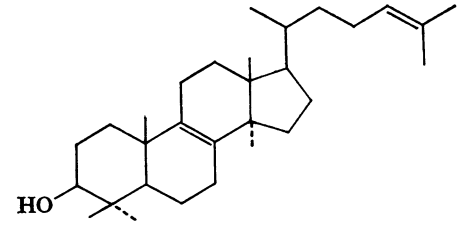
The radioactive products from a large-scale incubation of *S. obliquus* homogenate were characterized in a similar manner (Table 3).

Results

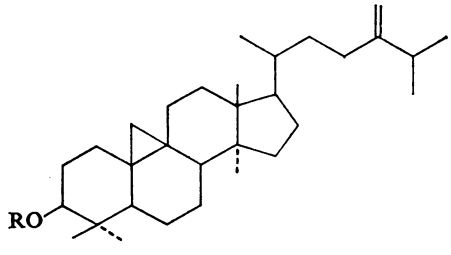
The formation of a radioactive product in the non-saponifiable lipid was observed when cell-free preparations from *Trebouxia* sp. or *S. obliquus* were incubated with *S*-adenosyl[*Me*-^{14}C]methionine and emulsified cycloartenol (XI). Under the conditions employed (see the Experimental section) the incorporation of ^{14}C into the non-saponifiable lipid was about 0.4% for *S. obliquus* and up to 50% with *Trebouxia* sp. Only a very low incorporation was obtained when cycloartenol was omitted (Table 1) showing that the cell-free preparations of *Trebouxia* did not contain significant amounts of endogenous acceptors for transmethylation. A stimulation of $^{14}CH_3$ group incorporation was, however, observed with lanosterol (XII) as substrate, but it was considerably lower than that obtained with cycloartenol (XI) (Table 1). This result agrees with observations that both higher plants (Russell *et al.*, 1967; Hewlins *et al.*, 1969; Gibbons *et al.*, 1971) and algae (Hall *et al.*, 1969; Lenton *et al.*, 1971) can utilize cycloartenol and lanosterol for phytosterol biosynthesis



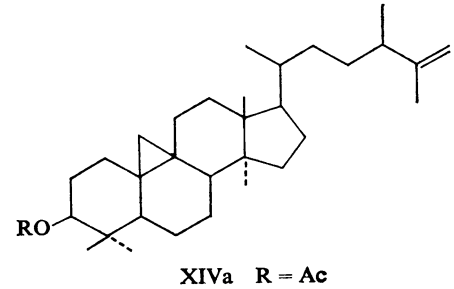
XI



XII



XIIIa R = Ac
XIIIb R = H



XIVa R = Ac
XIVb R = H

Table 1. *Activity of S-adenosyl–sterol methyltransferase in the 4000g supernatant from a Trebouxia homogenate*

Incubation conditions were as described in the Experimental section.

Sterol substrate	Radioactivity (d.p.m.) incorporated into the non-saponifiable lipid
None (control)	4877
Cycloartenol	157017
Lanosterol	18842
24,25-Dihydrolanosterol	5123
24-Methylenecycloartanol	5789
Cycloeucalenol	4667
24-Methylenelophenol	4667
5α-Ergosta-7,24(28)-dien-3β-ol	4842
Cycloartenol, glutathione omitted	76105

in vivo. This is probably due to a relatively low substrate specificity of the transmethylating enzyme. Complete lack of stimulation of $^{14}CH_3$ group incorporation by 24,25-dihydrolanosterol (Table 1) supports the necessity for a Δ^{24} bond (Russell *et al.*, 1967). A preliminary investigation of the effect of pH showed that the homogenates prepared and incubated at pH7.6 had maximal activity.

Radioscanning of the non-saponifiable lipids after t.l.c. separation (system I) showed that with both *S. obliquus* and *Trebouxia* sp. practically all the radioactivity was associated with the 4,4-dimethyl sterol fraction; the 4α-methyl and 4-demethyl sterols were not labelled. Therefore under the conditions employed the enzyme preparations did not catalyse further transformations of the methylated product (for example demethylation at C-4) even in the presence of air. On $AgNO_3$-impregnated silica gel (system IV) the acetylated radioactive product chromatographed with the same mobility as 24-methylenecycloartanyl acetate (XIIIa) and cyclolaudenyl acetate (XIVa), which ran together. Unequivocal differentiation between 24-methylenecycloartanol (XIIIb) and cyclolaudenol (XIVb) by t.l.c. is impossible and therefore the procedure outlined in Scheme 2 was used to demonstrate that both compounds were labelled. The non-saponifiable lipid obtained from an incubation of *Trebouxia* homogenate with cycloartenol and *S*-adenosyl[*Me*-^{14}C]methionine was added to carrier cyclolaudenol and 24-methylenecycloartanol. The mixture was purified by t.l.c. (system I) and repurified by t.l.c. on $AgNO_3$-impregnated silica gel. The purified material was then acetylated and converted into the corresponding diols (XV and XVII) by treatment with OsO_4 (Ghisalberti *et al.*, 1969). The diols were separated by t.l.c., which gave four bands, two representing the isomeric diols derived from 24-methylenecycloartanyl acetate, and the other two derived from cyclolaudenyl acetate (Table 2). Radioscanning and radioautography showed that all the radioactivity was associated with these bands. All these compounds were crystallized several times to constant specific radioactivity (Table 2). The isomeric pairs of diols were then combined and cleaved with periodate to give the corresponding ketones (XVI and XVIII), which were purified by t.l.c. and crystallized several times (Table 2). With the ketone (XVI) derived from 24-methylenecycloartanyl acetate (XIIIa) essentially all the radioactivity was lost as would be expected if the 24-methylene group were to arise from the labelled methyl group transferred from *S*-adenosyl[*Me*-^{14}C]methionine (Scheme 1). By contrast the ketone (XVIII) produced from cyclolaudenyl acetate (XIVa) retained all its radioactivity as predicted by the alkylation mechanism (Scheme 1). The results presented in Table 2 therefore demonstrate that the radioactive products formed by the *Trebouxia* homogenate are 24-methylenecycloartanol (76%) and cyclolaudenol (24%). Analysis by this procedure revealed that the radioactive products produced by *S. obliquus* homogenate (Table 3) were also 24-methylenecycloartanol (74%) and cyclolaudenol (26%).

The *S*-adenosylmethionine–cycloartenol methyltransferase activity was found to be distributed approximately equally between the microsomal (105000*g* pellet) and soluble (105000*g* supernatant) fractions of the homogenate, but further purification of the enzyme activity has not yet been achieved. Consequently, an examination of metal ion or other cofactor requirements has not been made but a stimulation of activity was noted by glutathione (Table 1). Some 24-methylene sterols representing possible potential acceptors in the second transmethylation step leading to C_{29} sterols were tested with the *Trebouxia* homogenate but in no case was incorporation of radioactivity obtained from *S*-adenosyl[*Me*-^{14}C]-methionine (Table 1).

Table 2. *Characterization of the radioactive products formed from cycloartenol and S-adenosyl[Me-^{14}C]methionine after incubation with a Trebouxia homogenate*

Details are described in the Experimental section.

			T.l.c.		M.p. (°C) Crystallization	...	Specific radioactivity (d.p.m./mg)				
			R_F	System			Initial	1st	2nd	3rd	4th	5th
Derivatives of 24-methylene-cycloartanyl acetate (XIIIa)	Diols (XV)	Isomer A	0.72	III	183–184		5243	5401	5521	5600	5585	5607
		Isomer B	0.68	III	180–182		5056	5464	5613	5467	5336	5500
		24-Ketone (XVI)	0.65	II	119–121		124	117	112	—	—	—
Derivatives of cyclolaudenyl acetate (XIVa)	Diols (XVII)	Isomer C	0.61	III	197–199		2266	2498	2630	2494	2525	2637
		Isomer D	0.55	III	200–203		2399	2516	2765	2791	2793	—
		25-Ketone (XVIII)	0.58	II	138–140		2804	2774	2790	2869	2786	2812

Discussion

Previous investigations employing [*Me*-$^2H_3$]-methionine (Goad *et al.*, 1972) demonstrated that in *Trebouxia* the C_{28} sterol, ergost-5-en-3β-ol incorporated three deuterium atoms and therefore was not produced by reduction of a 24-methylene compound. It was therefore suggested that a 25-methylene sterol, such as cyclolaudenol (XIVb), is produced from cation II (Scheme 1). Likewise the C_{29} sterols, poriferasterol and clionasterol, contained five deuterium atoms, thereby eliminating a 24-ethylidene precursor, and again a 25-methylene intermediate (IX) has been implicated (Goad *et al.*, 1972; Doyle *et al.*, 1972). However, although a 24-methylene sterol is not a C_{28} sterol precursor its production by *Trebouxia* is still required (Scheme 1), since a 24-methylene compound is presumed to be the substrate leading to C_{29} sterols (Goad & Goodwin, 1972). The observation that 31-norcyclolaudenol (side chain IV) and cycloeucalenol (side chain III) were converted exclusively into C_{28} and C_{29} sterols respectively by *Trebouxia* (Goad *et al.*, 1972) strongly supported the above views. Nevertheless, these conversions do not provide conclusive evidence, because of the possibility that the alga contains enzymes that can utilize non-physiological substrates. The results obtained in the present work with a cell-free homogenate of *Trebouxia* now provide compelling evidence for the production of both 24-methylene and 25-methylene compounds. Differentiation of the routes leading to C_{28} and C_{29} sterols thus occurs at the level of cation II stabilization (Scheme 1) to give either cyclolaudenol (XIVb) used for C_{28} sterol production or 24-methylenecycloartanol (XIIIb) required for the biosynthesis of C_{29} sterols. It is noteworthy that the ratio of cyclolaudenol to 24-methylenecycloartanol (24:76) synthesized by the *Trebouxia* homogenate from cycloartenol was approximately equal to the ratio of C_{28} to C_{29} sterol (23:77) found previously in this species (Goad *et al.*, 1972).

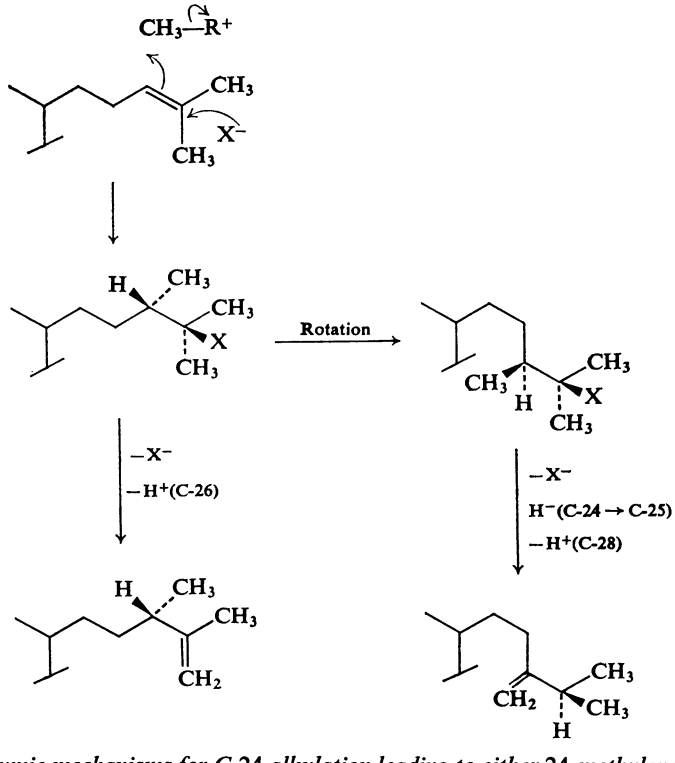
The present results, which demonstrate the production of cyclolaudenol and 24-methylenecycloartanol by the *S. obliquus* homogenate, permit similar conclusions to be drawn concerning the mechanisms of C_{28} and C_{29} sterol synthesis in this organism. The observed incorporation of three and five deuterium atoms from [*Me*-$^2H_3$]methionine into the C_{28} and C_{29} sterols of *S. obliquus* (W. Sach & L. J. Goad, unpublished work) is also in accord with this view.

Some details of the mechanism of cyclolaudenol biosynthesis in a higher plant have been investigated (Ghisalberti *et al.*, 1969). However, it remains an open question whether the formation of both cyclolaudenol and 24-methylenecycloartanol as the products of cycloartenol methylation is mediated by one methyltransferase or by two different enzymes. The observation (Goad *et al.*, 1972) that a strong isotope effect is operative in the stabilization of cation II when

Table 3. *Characterization of the radioactive products obtained from an incubation of cycloartenol and S-adenosyl-[Me-^{14}C]methionine with a S. obliquus homogenate*

Methods were as described in the Experimental section.

			Specific radioactivity (d.p.m./mg)			
		Crystallization ...	Initial	1st	2nd	3rd
Derivatives of 24-methylene-cycloartanyl acetate (XIIIa)	Diols (XV)	Isomer A	478	532	494	470
		Isomer B	453	492	512	572
	24-Ketone (XVI)		0	—	—	—
Derivatives of cyclolaudenyl acetate (XIVa)	Diols (XVII)	Isomer C	215	230	256	249
		Isomer D	204	259	241	239
	25-Ketone (XVIII)		242	276	296	292



Scheme 3. *Possible enzymic mechanisms for C-24 alkylation leading to either 24-methylene or 25-methylene derivatives*

[*Me*-^{2}H$_3$]methionine has been utilized may indicate that only one enzyme is involved, and the mechanisms indicated in Scheme 3 have been suggested as a possible explanation for the production of both 24-methylene and 25-methylene compounds (Goad & Goodwin, 1972). Clearly a final decision must await the purification of the enzyme or enzymes involved.

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