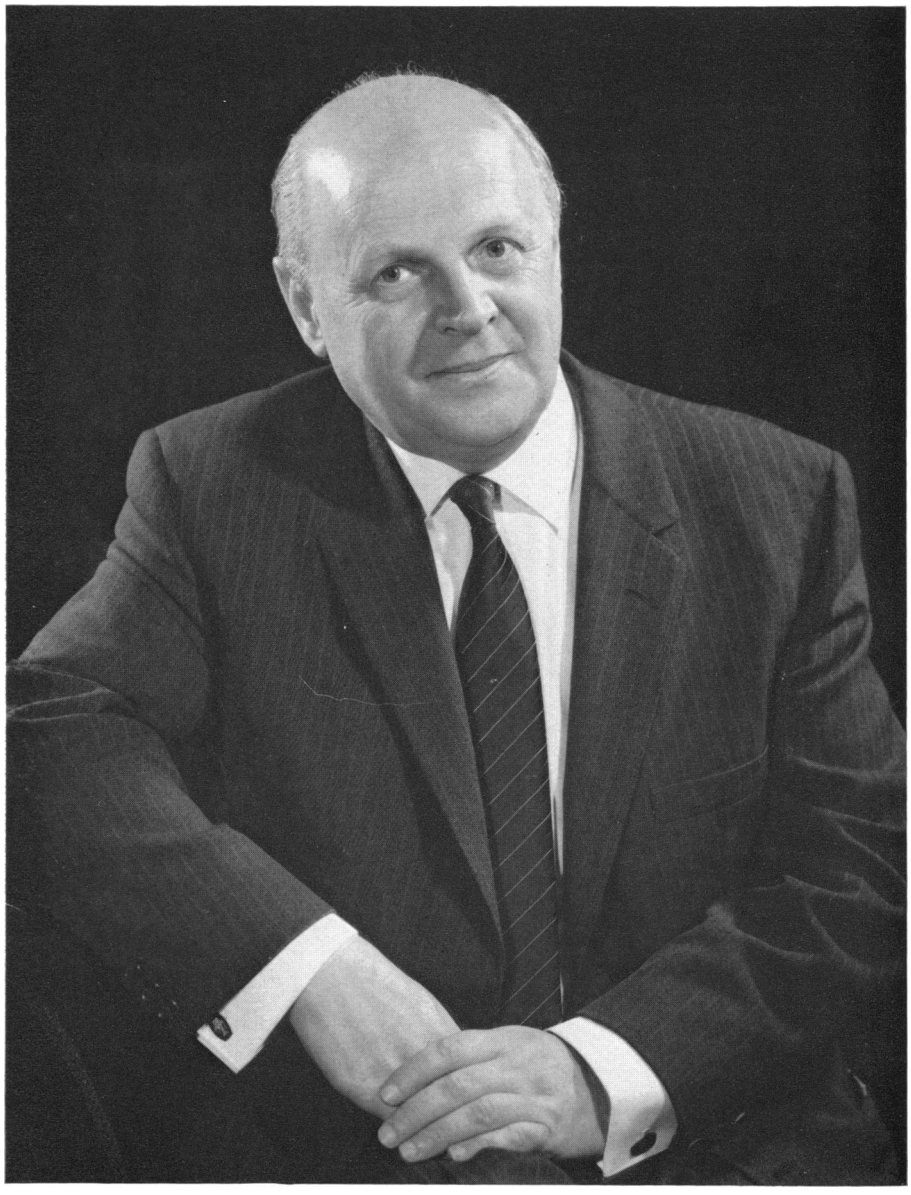

PROFESSOR T. W. GOODWIN, F.R.S.

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Biosynthesis of Carotenoids and Plant Triterpenes

THE FIFTH CIBA MEDAL LECTURE

By T. W. GOODWIN*

Delivered at a Meeting of the Biochemical Society on 16 October 1970 at the Royal College of Surgeons of England, Lincoln's Inn Fields, London WC2A 3PN, U.K.

Introduction

In presenting this fifth CIBA Medal Lecture, I feel that in some ways it can be considered Chapter Two of a saga, Chapter One of which was the first CIBA Medal Lecture. In that lecture Popják & Cornforth (1966) described their elegant studies on the stereochemistry of squalene biosynthesis, investigations made possible only by their equally elegant syntheses of various species of stereospecifically labelled mevalonates (see e.g. Cornforth & Cornforth, 1970). Many of the experiments I describe below would also not have been possible without the availability of stereospecifically labelled substrates, and I am most grateful to Professor Cornforth and Professor Popják for generous gifts of these compounds, particularly in the early days when they were in such short supply.

It must be clear that I am indebted to numerous graduate students and postdoctoral fellows for so effectively carrying out our research programme, but I am sure they would all agree that I am especially indebted during the last few years to my colleagues Dr L. J. Goad, Dr G. Britton and Dr H. H. Rees. They have played a major role in the planning and execution of our investigations at Liverpool and they continue to play an increasingly important part. My debt to them is great. Much of the work described below was begun while I was at Aberystwyth, and I am grateful to all my former colleagues there for help, but I would particularly like to thank Dr E. I. Mercer and Dr B. H. Davies for their pioneering efforts.

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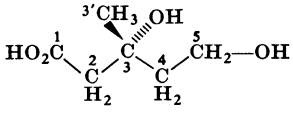
Biosynthesis of phytoene

It is now well known that mevalonate is the first specific precursor of terpenes and that the biologically active isomer has the 3*R* configuration (I) (Eberle & Arigoni, 1960). It is unique in that it has three prochiral centres (C-2, C-4 and C-5), all of which are involved in some aspect of sterol and carotenoid biosynthesis in the sense that one of the two hydrogen atoms attached to each of these carbon atoms is removed at a specific stage in the biosynthetic sequences. In chemical reactions these hydrogen atoms would be indistinguishable, but in enzyme-catalysed reactions it was highly likely that the process of removal would be stereospecific. This view, as we shall see, has been fully borne out in experiments with stereospecifically labelled mevalonates.

Mevalonate is first converted into the basic C_5 terpene precursor isopentenyl pyrophosphate (II) (Scheme 1), which is then isomerized to dimethylallyl pyrophosphate. The action of the isomerase can be formally represented by Scheme 2. Thus a hydrogen atom originally attached to C-2 of mevalonate is removed. Dimethylallyl pyrophosphate (III) acts as a starter for chain elongation to produce the C_{15} and C_{20} precursors of sterols and carotenoids respectively; the enzyme prenyltransferase first transfers an isopentenyl residue from isopentenyl pyrophosphate to dimethylallyl pyrophosphate to form geranyl pyrophosphate (C_{10}) in a reaction formally indicated in Scheme 3. Again it is a hydrogen atom originating from C-4 of mevalonate that is lost. Similarly another hydrogen atom is lost from C-4 of mevalonate in producing farnesyl



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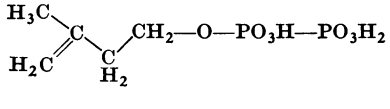

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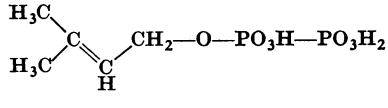

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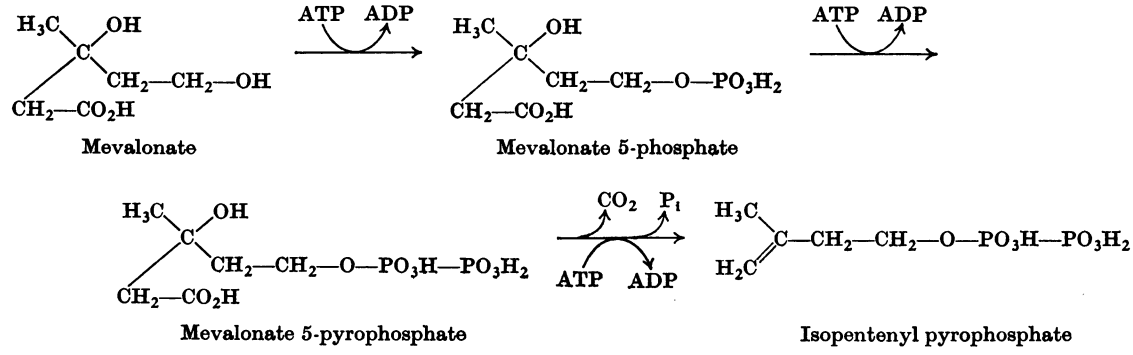
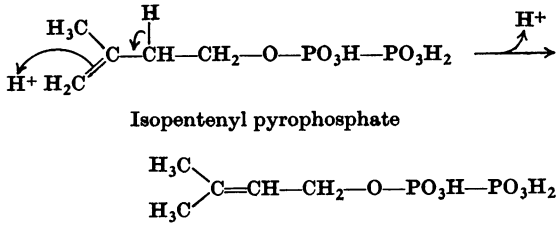


Scheme 1. Formation of isopentenyl pyrophosphate from mevalonate.



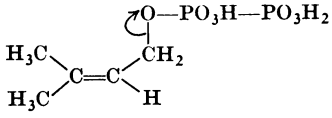
Scheme 2. Formation of dimethylallyl pyrophosphate from isopentenyl pyrophosphate.

pyrophosphate [the C_{15} precursor of squalene (IV), the first C_{30} precursor of all triterpenes] and yet another in forming geranylgeranyl pyrophosphate [the C_{20} precursor of phytoene (V), the first C_{40} precursor of carotenoids].

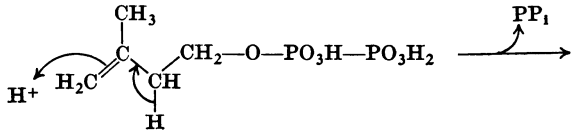
Phytoene (V) is not the C_{40} homologue of squalene (IV); it contains an additional double bond at the centre of the molecule; the C_{40} homologue of squalene is lycopersene (VI), and it was early work at Aberystwyth by Dr Mercer and Dr Davies that clarified a confused situation in showing by rigorous experimentation that it was not possible to demonstrate lycopersene either as a natural product or as a biosynthetic intermediate in carotenogenesis (Mercer, Davies & Goodwin, 1963; Davies, Jones & Goodwin, 1963). In work begun at Aberystwyth Dr Simpson was later able to show that 'lycopersene' in a red yeast was an unknown contaminant present in the culture medium (Scharf & Simpson, 1968). The key position of phytoene, rather than lycopersene, in carotenoid biosynthesis has now been emphasized on numerous occasions (see e.g. Porter & Anderson, 1967; Goodwin, 1971*b*).

Cornforth and Popják, using two stereospecifically labelled species of mevalonate, namely [2-^{14}C,(4*R*)-4-$^{3}H_1$]mevalonate (VII) and [2-^{14}C,-(4*S*)-4-$^{3}H_1$]mevalonate (VIII), showed that in squalene synthesized in liver preparations there is stereospecific removal of a hydrogen atom from C-4 of mevalonate at each step in the formation of squalene (Fig. 1) and that it is always in the same sense: the pro-*S*-hydrogen atoms are removed and the pro-*R*-hydrogen atoms are retained; i.e. with [2-^{14}C,(4*R*)-4-$^{3}H_1$]mevalonate the $^{14}C/^{3}H$ ratio is the same in squalene as in the starting material whereas with [2-^{14}C,(4*S*)-4-$^{3}H_1$]mevalonate no tritium is present in squalene. This result incidentally eliminates the simple mechanistic concept of Scheme 3; in order to remove the pro-*S*-hydrogen atom by a *trans* elimination the intermediate involvement of a nucleophile [X^-] (presumably an enzyme) must be invoked (Popják & Cornforth, 1966) (Scheme 4). By using the same two mevalonate substrates we were able to show that the same stereospecificity was observed in squalene biosynthesis in plants and also in the formation of the carotenoid precursor phytoene (Fig. 1) (Goodwin & Williams, 1966). All the double bonds involving C-4 of mevalonate in squalene and phytoene are *trans*; in rubber they are *cis*, and in this case it is the pro-*R*-hydrogen atoms from C-4 of mevalonate that are eliminated (Archer *et al.* 1966). Certain polyprenols are now known that are of mixed biogenetic origin, part of the molecule being *trans*-derived and part *cis*-derived, so that pro-*S*- and pro-*R*-hydrogen atoms from C-4 of mevalonate are lost in the same molecule (Hemming, 1970).

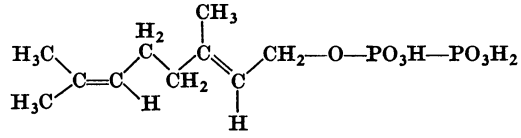
The tail-to-tail reaction of two farnesyl pyrophosphate molecules to form squalene involves on paper no loss of hydrogen at the centre from each C-1 of the participating farnesyl pyrophosphate molecules, whereas that of phytoene involves loss of hydrogen from C-1 of each geranylgeranyl pyrophosphate molecule involved. The situation at the centre of the molecule of squalene as it is formed is not as simple as it appears; the pro-*S*-hydrogen atom from C-1 of one molecule of farnesyl pyro-



Dimethylallyl pyrophosphate

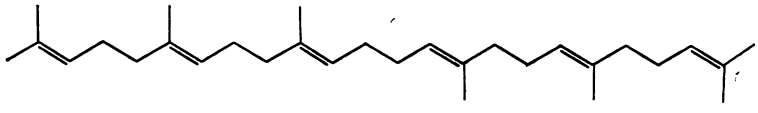


Isopentenyl pyrophosphate



Geranyl pyrophosphate

Scheme 3. Formation of geranyl pyrophosphate from dimethylallyl pyrophosphate and isopentenyl pyrophosphate.



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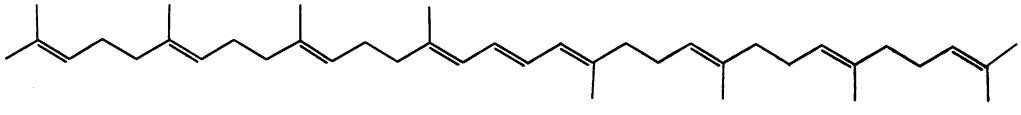

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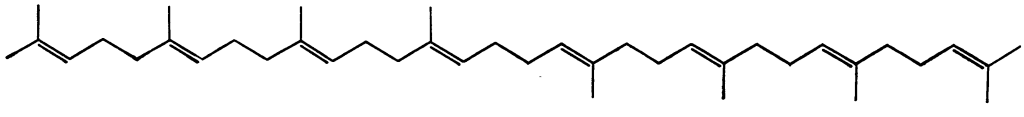
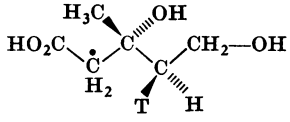
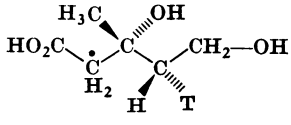


(VI)

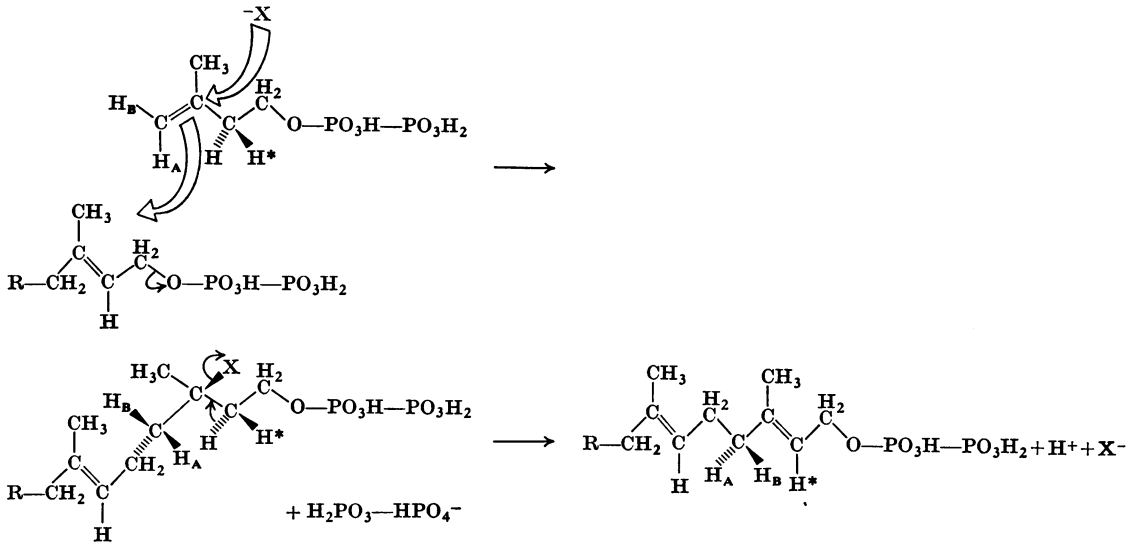


(VII)



(VIII)

phosphate (i.e. a pro-*S*-hydrogen atom arising from C-5 of mevalonate) is removed and a hydrogen atom from the B side of NADPH inserted (Fig. 2) (Popják & Cornforth, 1966). By using [2-^{14}C,(5*R*)-5-^{3}H$_1$]mevalonate (IX) and [2-^{14}C,5-^{3}H$_2$]mevalonate (X), synthesized by Dr Britton [[2-^{14}C,(5*S*)-5-^{3}H$_1$]mevalonate has been synthesized only very recently], we showed that two pro-*S*-hydrogen atoms are lost, one from C-1 of each geranylgeranyl pyrophosphate, in the formation of phytoene in tomato fruit and bean leaves (Williams, Britton, Charlton & Goodwin, 1967*b*). In these experiments the internal standard used was squalene, and it was reasonably assumed that with [2-^{14}C,(5*R*)-5-^{3}H$_1$]mevalonate the atomic ratio would be the same as that observed in squalene formed in liver, i.e. that the ^{14}C/^{3}H ratio would be 6:6. This uncertainty was removed by experiments with chloroplasts isolated by a non-aqueous technique. Such chloroplasts, but not those prepared by more conventional methods, will synthesize phytoene from mevalonate (Charlton, Treharne & Goodwin, 1967) and in addition



Scheme 4. Two-step mechanisms for the formation of polyisoprenoids (Popják & Cornforth, 1966).

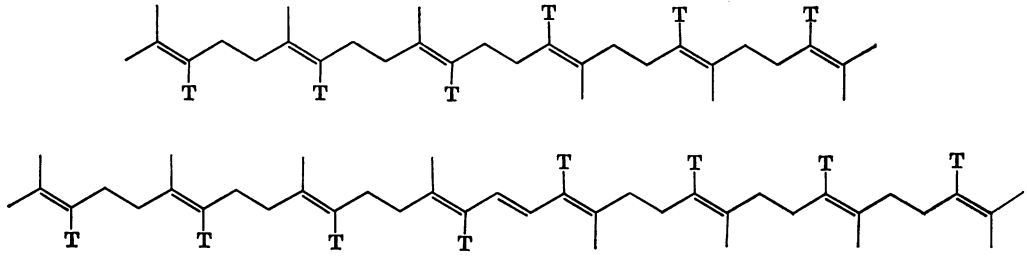


Fig. 1. Labelling of squalene and phytoene from [(4*R*)-4-$^3H_1$]mevalonate. T indicates ^3H.

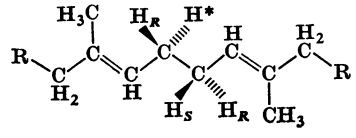
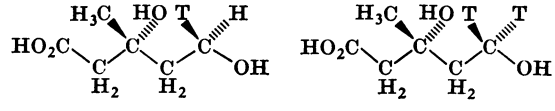
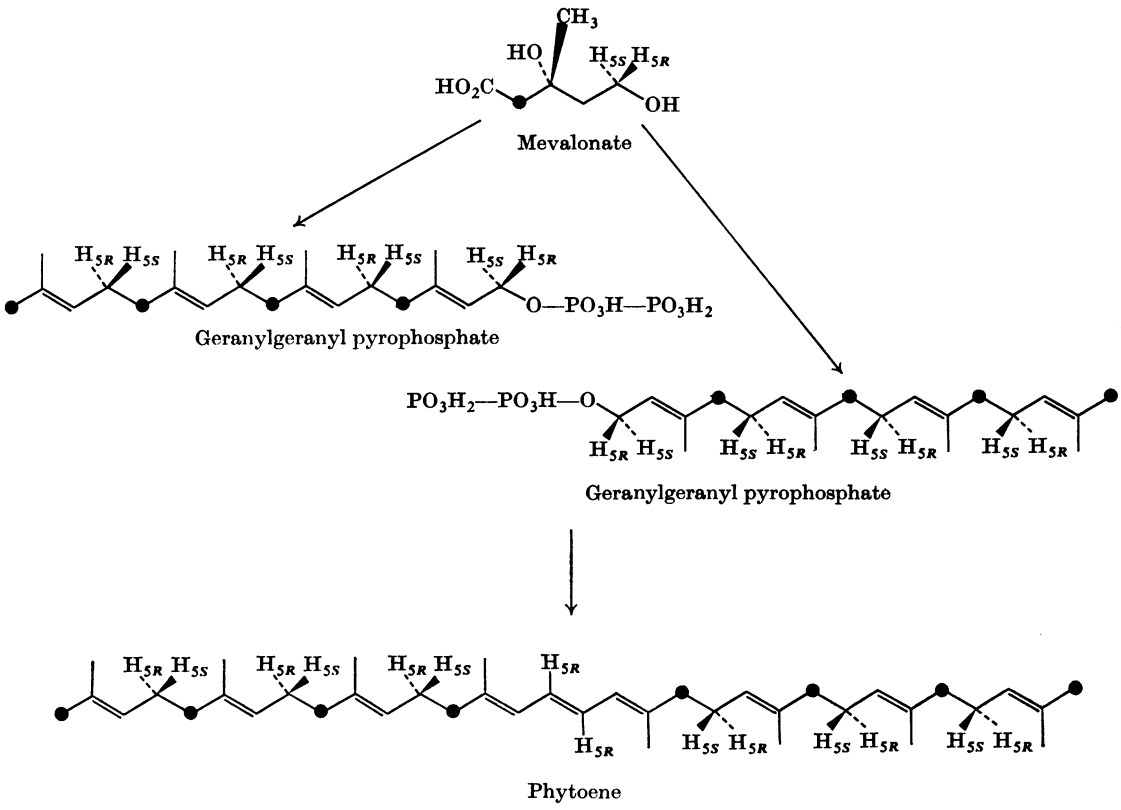


Fig. 2. Source of the hydrogen atoms at the centre of the squalene molecule. H_R indicates the pro-*R*-hydrogen atom from C-5 of mevalonate; H_S indicates the pro-*S*-hydrogen atom from C-5 of mevalonate; H* indicates the hydrogen atom from the B side of NADPH.



(IX) (X)

accumulate considerable amounts of geranylgeraniol. As indicated in Scheme 5 the formation of geranylgeraniol does not result in loss of hydrogen atoms from C-5 of mevalonate and it is thus an excellent internal standard. Our experiments showed clearly that phytoene contains two fewer tritium atoms than does geranylgeraniol and that it is the pro-*S*-hydrogen atoms that are lost (Buggy, Britton & Goodwin, 1969). The naturally occurring phytoene in higher plants is said to have a *cis* configuration at the centre (Davis, Jackman, Siddons & Weedon, 1961; Jungalwala & Porter, 1965), and a mechanism for its formation with this configuration can be postulated that depends on the loss of two pro-*S*-hydrogen atoms (Scheme 6); loss of two pro-*R*-hydrogen atoms would give the same compound, but the loss of one pro-*R*- and one pro-*S*-hydrogen atom would lead to the *trans* configuration (Williams *et al.* 1967*b*). Recently the naturally occurring



Scheme 5. Conversion of mevalonate into geranylgeranyl pyrophosphate and phytoene: the fate of the pro-5*R*- and pro-5*S*-hydrogen atoms. ● indicates ^{14}C; H_{5R} and H_{5S} indicate the pro-5*R*- and pro-5*S*-hydrogen atoms of mevalonate respectively.

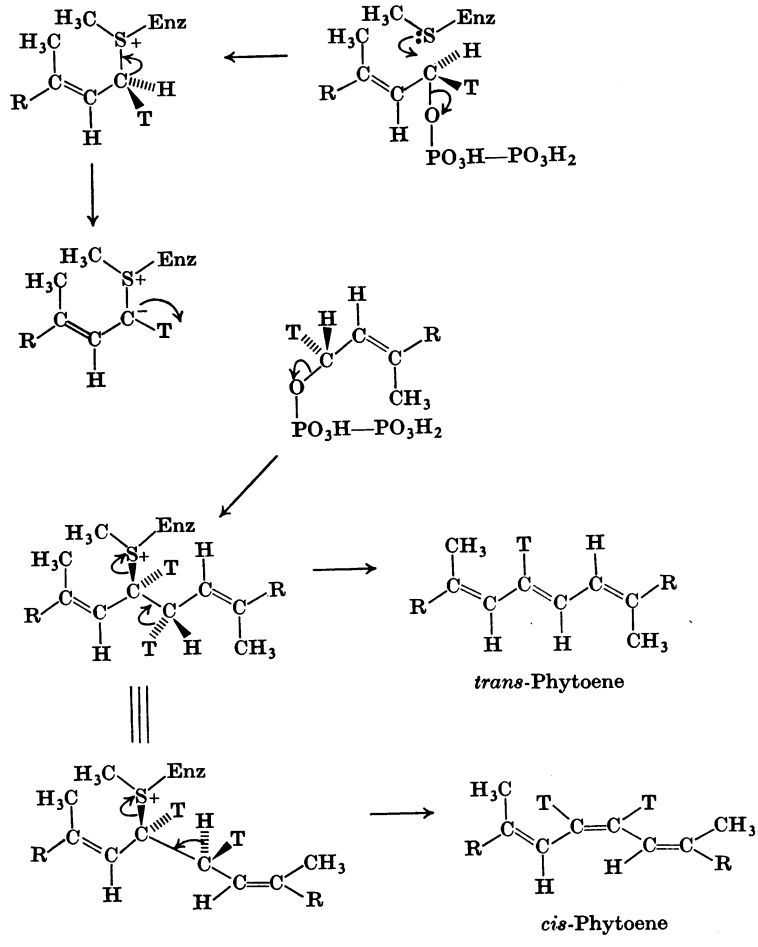
phytoene in some bacteria has been said to have the *trans* configuration at the centre of the molecule (Weeks, 1971).

Desaturation of phytoene

Considerable evidence has accumulated to define the pathway of conversion of phytoene into lycopene in higher plants, algae and fungi as the stepwise desaturation indicated in Scheme 7 (see Porter & Anderson, 1967; Goodwin, 1971*b*), and the enzymic conversion of [^{14}C]phytoene into phytofluene and lycopene by an enzyme system from spinach chloroplasts has been reported (Subbarayan, Kushwaha, Suzue & Porter, 1970). On the evidence that phytoene and phytofluene (Jungalwala & Porter, 1967) have the *cis* configuration at their centres, this enzyme system must not only desaturate but also, at the phytofluene → ζ-carotene stage, carry out an isomerization, for there is yet no evidence for the existence in nature of a *cis*-ζ-carotene.

In spite of many attempts we have not been able to demonstrate desaturation of phytoene in our non-aqueous chloroplast preparations, but by using intact systems we have been able to study some aspects of the stereochemistry of double-bond formation. Each of the four additional double bonds formed in converting phytoene into lycopene involves loss of a hydrogen atom from each of the carbon atoms arising from C-2 and C-5 of mevalonate (Scheme 8).

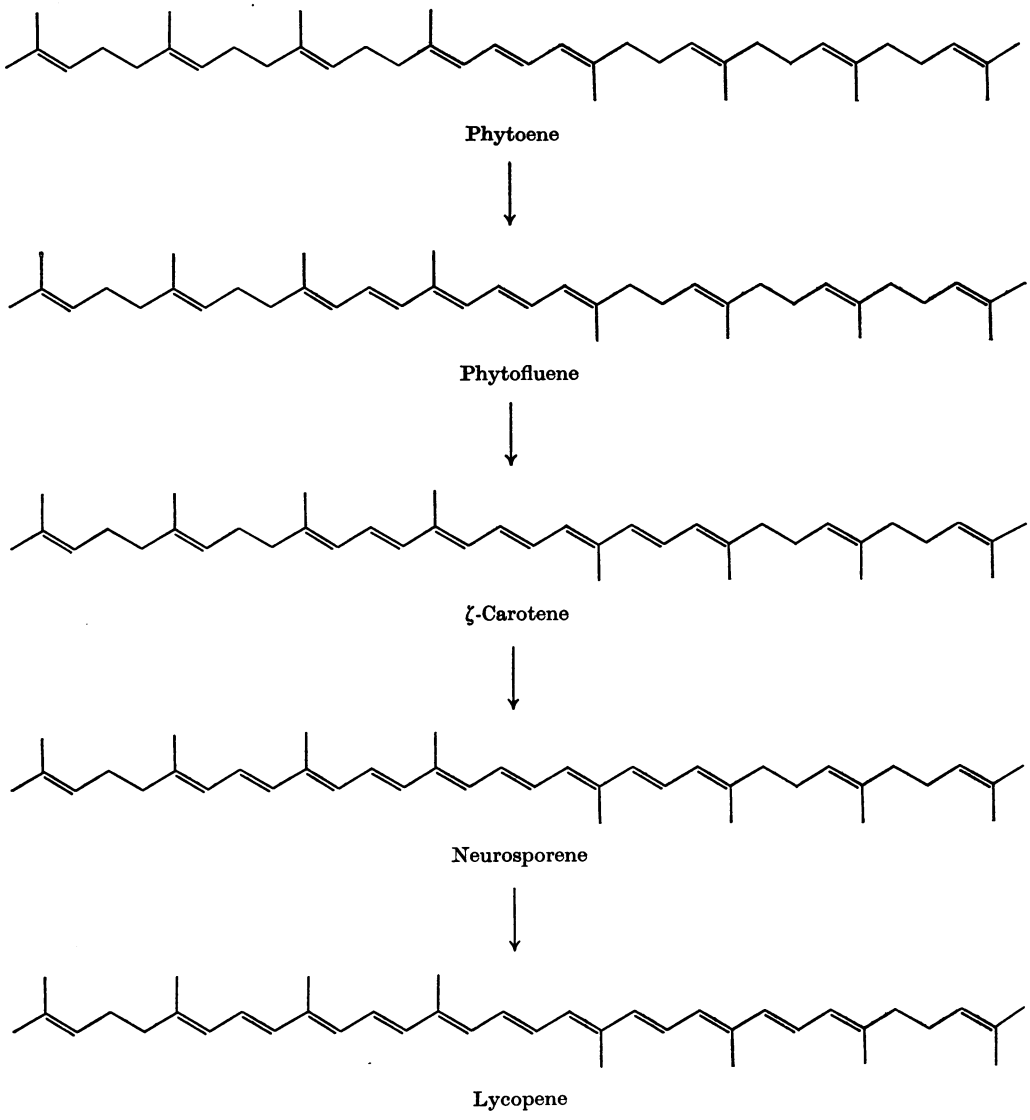
Experiments with [2-^{14}C,(5*R*)-5-$^{3}H_1$]- and [2-^{14}C,5-$^{3}H_2$]-mevalonate in tomato slices and maize seedlings showed that there was stereospecific loss of the 5-pro-*R*-hydrogen atom during the formation of each double bond (Williams *et al.* 1967*b*; Walton, Britton & Goodwin, 1969). Similar experiments with [2-^{14}C,(2*R*)-2-$^{3}H_1$]mevalonate (XI) and [2-^{14}C,(2*S*)-2-$^{3}H_1$]mevalonate (XII) indicated that there was considerable loss of stereospecificity of the tritium atoms, and this was also observed with studies on plant sterol biosynthesis (see below and Goad, 1970), although no such phenomenon occurred with sterol synthesis in animals (Popják & Cornforth, 1966; Goad, 1970). The



Scheme 6. Possible mechanism for phytoene formation. T indicates ^3H.

probable reason for this is that the prenyltransferase in plants is a much more sluggish enzyme than that in animals, thus allowing the enzyme catalysing the only freely reversible step in the terpenoid biosynthetic pathway, the isopentenyl pyrophosphate–dimethylallyl pyrophosphate isomerase, time to scramble the label from C-2 of mevalonate. Scheme 9 shows how isopentenyl pyrophosphate arising from [2*R*-$^3H_1$]mevalonate can give rise to isopentenyl pyrophosphate with the configuration that it would have if it arose from [2*S*-$^3H_1$]mevalonate and vice versa; the production of unlabelled isopentenyl pyrophosphate is also a possibility. However, in an attempt to overcome this randomization geranylgeranyl pyrophosphate was prepared from [2-^{14}C,(2*R*)-2-$^3H_1$]- or [2-^{14}C,(2*S*)-2-$^3H_1$]-mevalonate by a very active enzyme from *Echinocystis macrocarpa* (Oster & West, 1968) so that randomization was not appreciable. Preliminary results with these substrates indicate that the hydrogen atoms lost in desaturation are the pro-2*S*-hydrogen atoms of mevalonate (J. R. Vose, G. Britton & T. W. Goodwin, unpublished work), so that the stereochemistry of desaturation is probably as indicated in Scheme 8.

In our efforts to study the desaturation process further we began to look for possible epoxide intermediates between phytoene and lycopene. None was found, but Dr Britton did discover phytoene 1,2-oxide, the analogue of squalene 2,3-oxide, in tomato fruit (Britton & Goodwin, 1969). Since then he has isolated the epoxides of the other components of the phytoene → lycopene sequence (G. Britton, unpublished work). Mass-spectrum measurements were particularly effective in characterizing this first member of a new group of

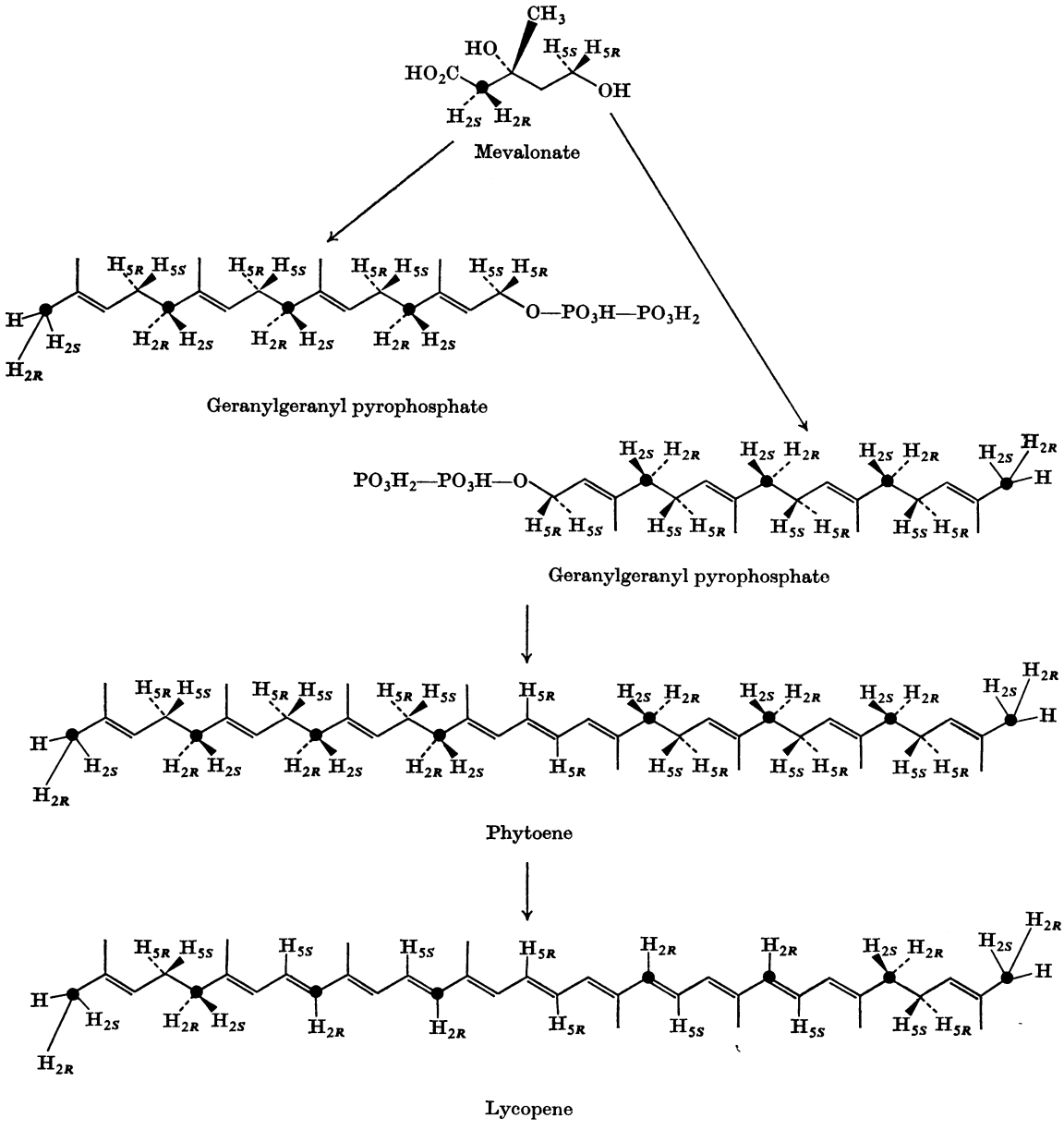


Scheme 7. Pathway of conversion of phytoene into lycopene in higher plants, algae and fungi.

carotenoids. For example, the spectrum showed a parent ion, M^+, at m/e 560.4979 ($C_{40}H_{64}O$) and fragment ions at m/e 491 ($M-69$), 475 ($M-85$), 423 ($M-137$), 407 ($M-153$), 355 (strong, $M-205$) and 339 (strong, $M-221$); no peak was observed at m/e 542 ($M-H_2O$) (Fig. 3). At the moment no biological significance can be attached to these carotenoid 1,2-oxides. Maturing fruit represent a senescing system, and it may be that the phytoene series leak out of the disintegrating chromoplasts and are attacked in the cytoplasm by squalene oxidase, which is not a particularly specific enzyme. A rather similar phenomenon occurs in senescing leaves; the xanthophylls that are liberated by disintegrating chloroplasts are esterified in the cytoplasm (Goodwin, 1958).

Cyclic carotenoids

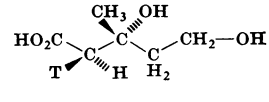
So far only the acyclic carotenoids have been considered, but the major carotenoids in chloroplasts, for example, contain terminal cyclohexene rings, either α-rings (XIII) or β-rings (XIV). Typical examples of cyclic carotenes are α-carotene



Scheme 8. Stereochemistry of the desaturation of phytoene to lycopene. ● indicates C-2 of mevalonate; H_{2R}, H_{2S}, H_{5R} and H_{5S} indicate the pro-2*R*-, pro-2*S*-, pro-5*R*- and pro-5*S*-hydrogen atoms of mevalonate respectively.

(XV) with one α- and one β-ring, and β-carotene (XVI) with two β-rings.

Evidence is now accumulating that lycopene is the precursor of α- and β-carotene. Dr Rogers at Aberystwyth obtained incorporation of [^{14}C]-lycopene into β-carotene in isolated bean chloroplasts (Hill & Rogers, 1969), and Kushwaha,



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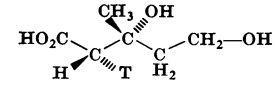

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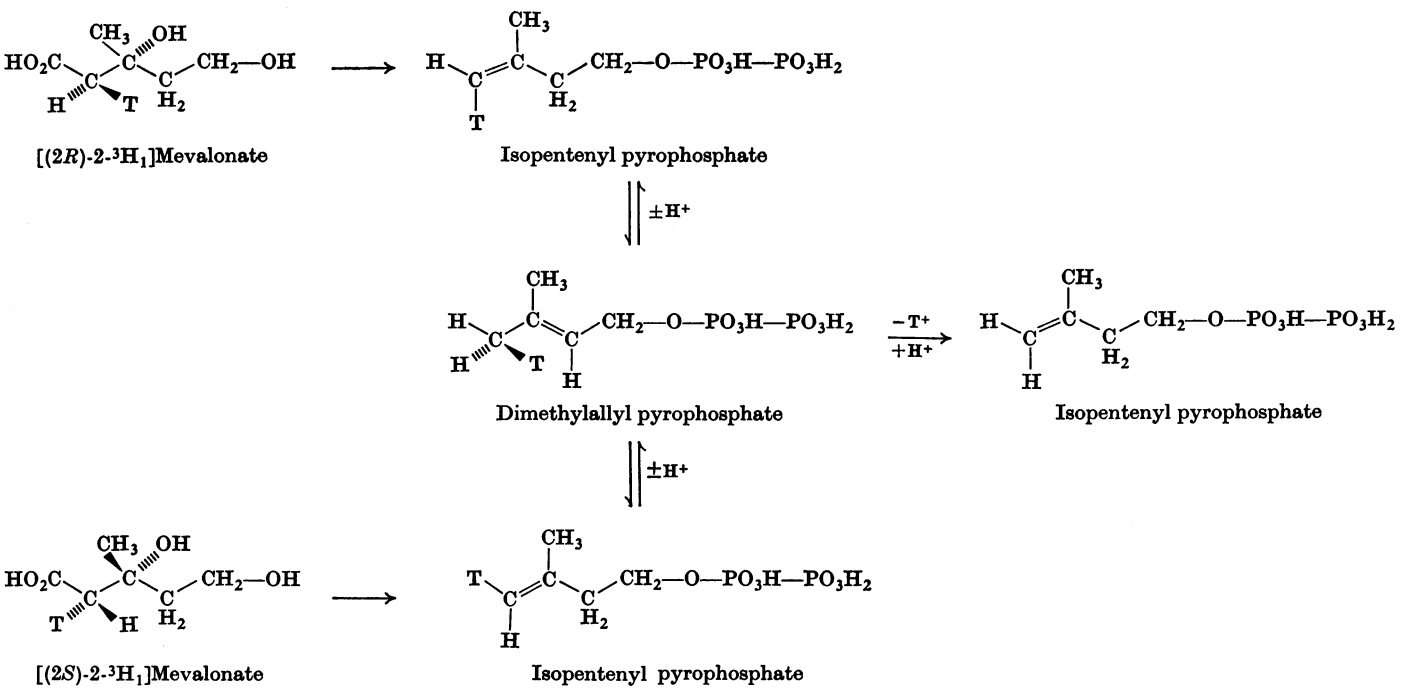


Scheme 9. Randomization of label from [(2*R*)-2-$^3H_1$]mevalonate and [(2*S*)-2-$^3H_1$]mevalonate caused by reversibility of the isopentenyl pyrophosphate–dimethylallyl pyrophosphate isomerase. T indicates ^3H.

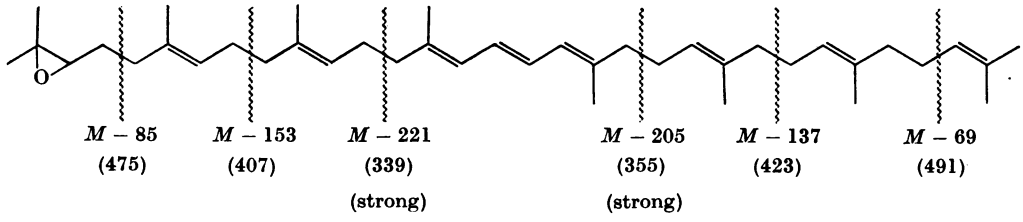
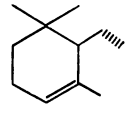


Fig. 3. Mass-spectrum cracking pattern of phytoene 1,2-epoxide.



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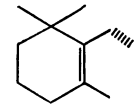
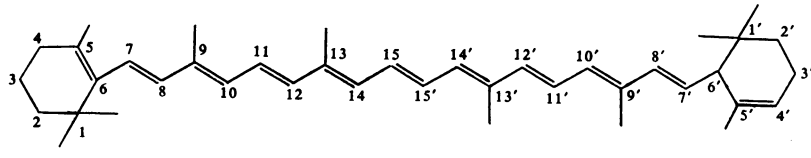
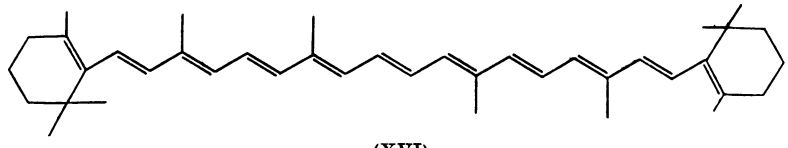


(XIV)



(XV)



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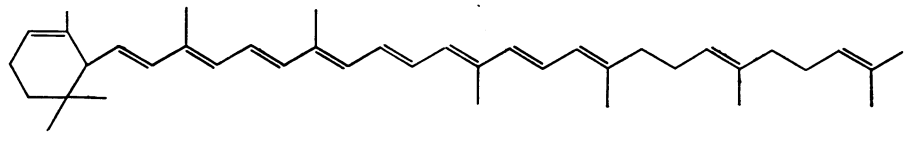
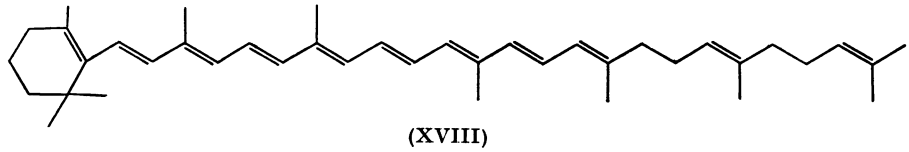
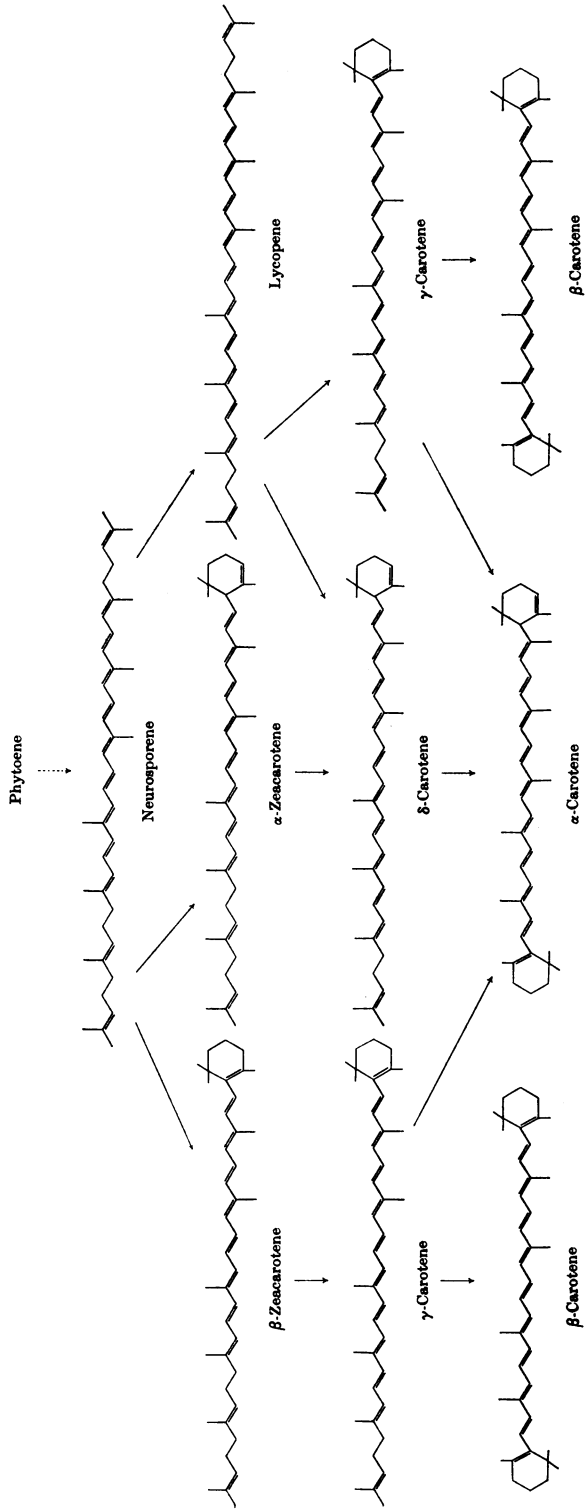


(XVII)

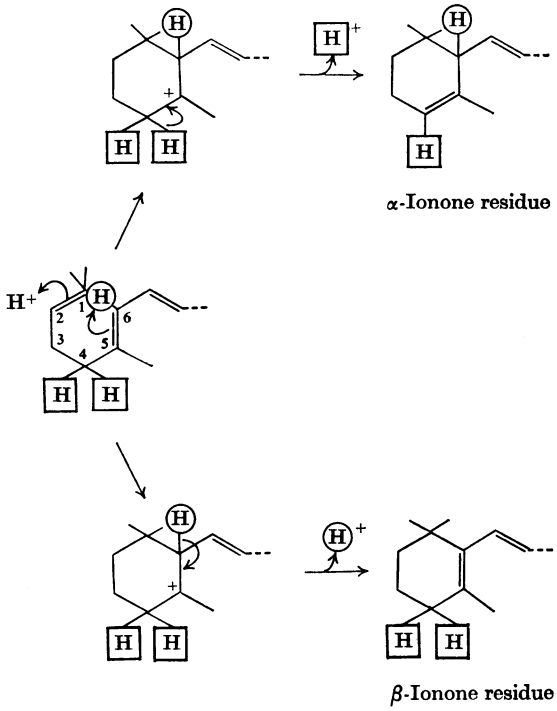


(XVIII)

Subbarayan, Beeler & Porter (1969) prepared extracts of spinach and tomato chloroplasts that cyclized [15,15′-$^3H_2$]lycopene to various cyclic carotenes. Howes & Batra (1970) have demonstrated that nicotine inhibits cyclization in a *Mycobacterium* sp. with the accumulation of lycopene. When the inhibitor was washed out lycopene disappeared and the cyclic compounds appeared. We have confirmed Batra's observations in *Phycomyces blakesleeanus* and in a *Flavobacterium* sp. With the latter organism, which normally synthesizes zeaxanthin, we have shown that the cyclization of lyco-



Scheme 10. Possible pathways of synthesis of cyclic carotenes from neurosporene and lycopene.



(H) indicates ^3H from [(4*R*)-4-$^3H_1$]mevalonate;

[H] indicates ^3H from [2-$^3H_2$]mevalonate.

Scheme 11. Basic mechanism for formation of α- and β-rings in carotenoids.

pene to β-carotene is an anaerobic process (J. C. B. McDermott, G. Britton & T. W. Goodwin, unpublished work).

Cyclization of neurosporene, i.e. at the biosynthetic step preceding lycopene, must also take place, because of the existence in Nature of α- and β-zeacarotene (XVII and XVIII), first isolated from maize oil (Petzold, Quackenbush & McQuistan, 1959). α- and β-Zeacarotene could theoretically be converted into δ-carotene and γ-carotene respectively, which could then cyclize to give α- and β-carotene (Scheme 10). No direct evidence for this pathway is yet forthcoming, but β-zeacarotene accumulates under conditions that lead to inhibition of the synthesis of β-carotene, such as in *Rhodotorula rubra* cultured at low temperatures or in the presence of β-ionone or methylheptenone (Simpson, Nakayama & Chichester, 1964) or in *Phycomyces blakesleeanus* grown in culture media containing diphenylamine (Williams, Davies & Goodwin, 1965). The β-zeacarotene of maize was shown to be identical with the synthetic all-*trans* compound (Simpson & Goodwin, 1965) and corresponded to β_1-zeacarotene described in the original investigations. No reports of the existence of cyclic compounds less saturated than neurosporene have yet appeared. The search for a good source of neurosporene that we could label and use to carry out cyclization experiments led us to examine *Rhodopseudomonas viridis*, the chief pigments of which were said to be neurosporene and lycopene (K. E. Eimhjellen, unpublished work cited

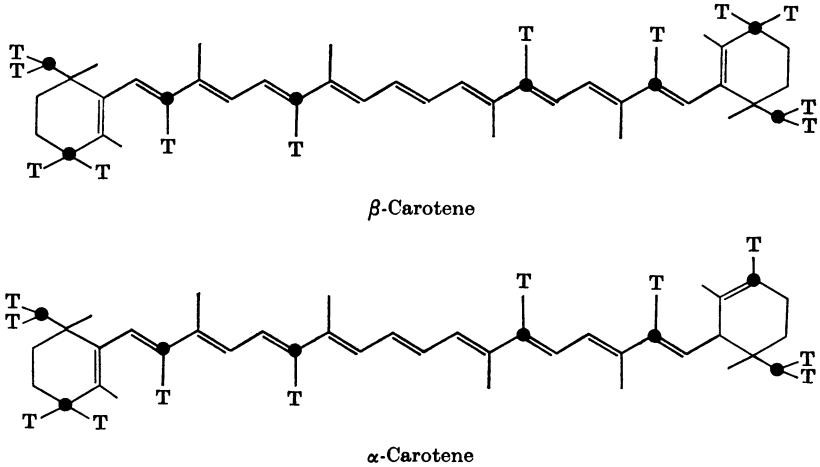
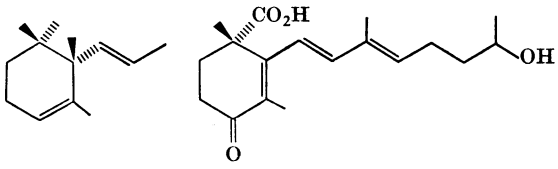


Fig. 4. Expected labelling of α- and β-carotene from [2-^{14}C,2-$^3H_2$]mevalonate on the assumption that the rings are formed separately (see Scheme 11). ● indicates ^{14}C from [2-^{14}C]mevalonate; T indicates ^3H from [2-$^3H_2$]mevalonate.



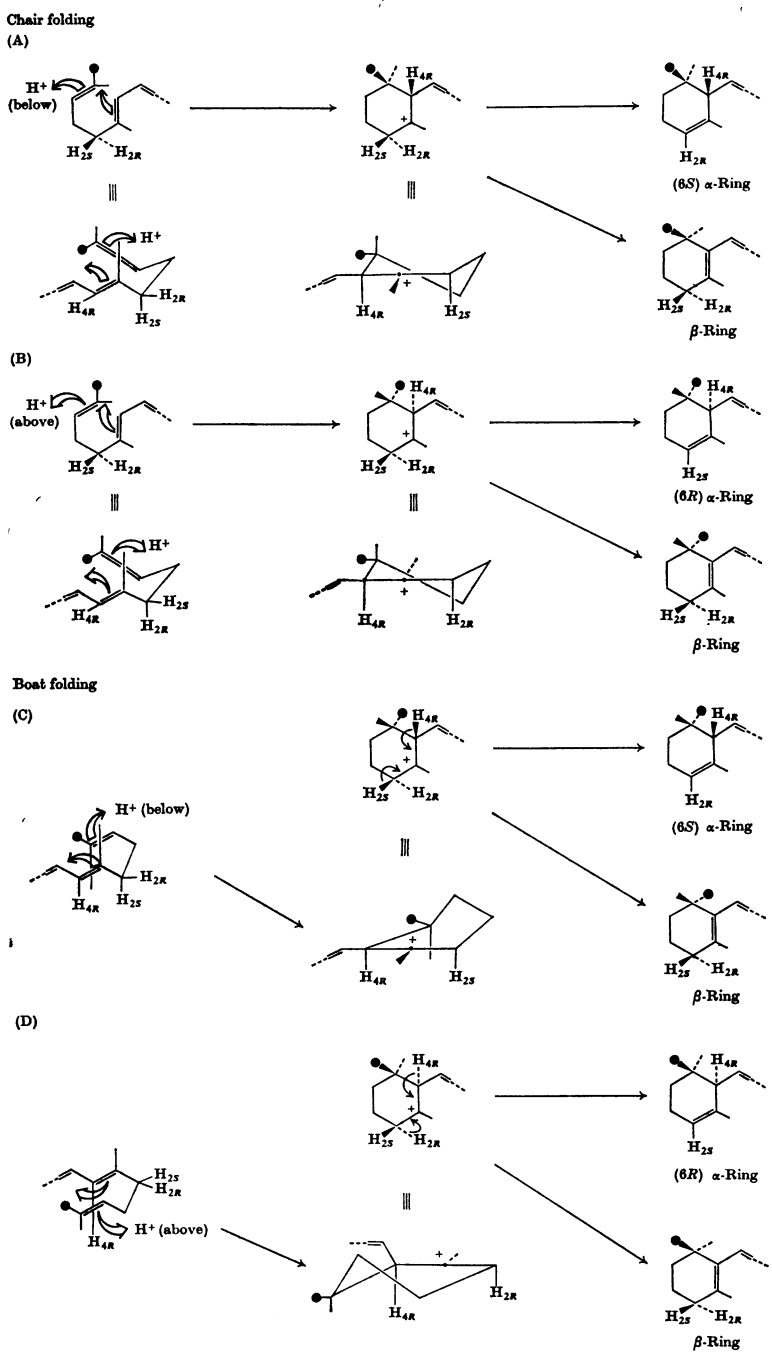
(XIX) (XX)

by Thornber, Olson, Williams & Clayton, 1969). We found that, although these two pigments were present in small amounts, the major pigments were 1,2-dihydroneurosporene and 1,2-dihydrolycopene (Malhotra, Britton & Goodwin, 1970*a*). Further investigations showed that 1,2-dihydro derivatives of phytoene, phytofluene and ζ-carotene were also present, as were traces of 1,2,1′,2′-tetrahydrolycopene and 1,2,1′,2′-tetrahydrophytofluene (Malhotra, Britton & Goodwin, 1970*b*). Mass spectrometry was again particularly useful in this investigation. Although we are still without a good source of neurosporene, we had discovered yet another new class of naturally occurring carotenoids.

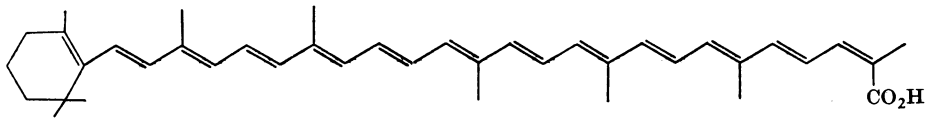
Irrespective of the nature of the immediate substrate for cyclization, it has been possible to study the mechanism of cyclization with the use of various species of labelled mevalonate. An attractive mechanism is illustrated in its simplest form in Scheme 11. Proton attack on the terminal double bond would yield an intermediate, which is indicated formally in Scheme 11 as a carbonium ion. This could be stabilized in the presence of a β-cyclase by ejection of a proton from C-6 to yield a β-ring; similarly loss of a proton from C-4 in the presence of an α-cyclase would yield an α-ring. With [2-^{14}C,(4*R*)-4-^{3}H$_1$]mevalonate as substrate the acyclic precursor would have a tritium atom at C-6; thus if the mechanism indicated in Scheme 11 were operative the ^{14}C/^{3}H atomic ratio of β-carotene would be 8:6 compared with that of the acyclic precursors phytoene → lycopene normalized to 8:8. This has now been demonstrated on numerous occasions in various systems (carrot slices, maize seedlings, tomato fruit and fungi) (Goodwin & Williams, 1965*a*; Williams *et al.* 1967*b*; Williams, Britton & Goodwin, 1967*c*; Goodwin, Britton & Walton, 1968). In similar experiments α-carotene was found to have the ^{14}C/^{3}H atomic ratio 8:7 (Goodwin & Williams, 1965*b*; Williams *et al.* 1967*c*), thus indicating that the α-ring was not formed from the β-ring, as had been earlier suggested (see e.g. Porter & Anderson, 1962). With [2-^{14}C,2-^{3}H$_2$]mevalonate as substrate the possible ^{14}C/^{3}H atomic ratios, compared with phytoene normalized to 8:16, are 8:11 for α-carotene and either 8:12 or 8:10 for β-carotene, according to whether the β-rings arose directly or by isomerization of performed α-rings (Fig. 4). Experiments by Williams *et al.* (1967*c*) showed that in carrots the ratio was 8:11.39 for α-carotene, and that β-carotene contained one more tritium atom and not one fewer (ratio 8:12.25). Thus there was no detectable conversion of an α-ring into a β-ring, and the conclusion must be that both rings are formed separately via the same or similar enzyme–substrate complexes (carbonium ions).

When the details of the stereochemistry of the cyclization are considered note must be taken of: (*a*) the absolute configuration (6′-*R*) of the α-ring in α-carotene (XIX), which has been demonstrated by Eugster, Buchecker, Tscharner, Uhde & Ohloff (1969); (*b*) which methyl group of the *gem*-dimethyl groups of the β-ring arises from C-2 of mevalonate and which from C-3′. The pro-*R*-methyl group of trisporic acid (XX) arises from C-2, and on the assumption that trisporic acid is a metabolite of β-carotene in *Blakeslea trispora* the same configuration must exist in β-carotene itself (Bu'Lock, Austin, Snatzke & Hruban, 1970). A number of stereochemical possibilities exist for ring formation depending on (i) whether the acyclic precursor is folded in a boat or a chair conformation and (ii) which side the entering proton attacks (Scheme 12). The possibilities (A) and (C) (Scheme 12) are ruled out for α-carotene formation because they would give the unnatural enantiomer; possibilities (B) and (D) (Scheme 12) would give the natural enantiomer of α-carotene, and possibility (D), but not possibility (B), would also give β-carotene with the correct methyl group labelled from C-2 of mevalonate, if the stereochemical analogy with trisporic acid is correct. Thus possibility (D) would allow both α- and β-rings to be formed from a common carbonium ion intermediate (Scheme 11). Preliminary biochemical investigations with [2-^{14}C,(2*R*)-2-^{3}H$_1$]- and [2-^{14}C,(2*S*)-2-^{3}H$_1$]-mevalonate, however, suggest that it is not the axial (pro-2*R*) hydrogen atom that is lost in forming α-carotene (J. R. Vose, G. Britton & T. W. Goodwin, unpublished work), but much further work is required in this area.

The assumption that the *gem*-dimethyl groups in the α- and β-rings of cyclic carotenes retain their individuality is highly likely, but still requires direct experimental proof. Information is, however, available on the acyclic end group of torularhodin (XXI), produced by *Rhodotorula rubra*, in which one of the methyl groups at C-1 is oxidized to a carboxyl group. Dr Simpson has now completed an investigation begun with us in which he showed that the carboxyl group at C-1′ arises exclusively from C-2 of mevalonate and that the methyl group at C-1′ must therefore arise from C-3′ of mevalonate (Tefft, Goodwin & Simpson, 1970). No indication of lack of individuality of the two groups was observed.



Scheme 12. Effect of different foldings of an acyclic precursor to yield α-carotene and β-carotene of the correct configurations. ● indicates C-2 of mevalonate; H_{2R}, H_{2S}, H_{4R} and H_{4S} indicate the pro-2*R*-, pro-2*S*-, pro-4*R*- and pro-4*S*-hydrogen atoms cf mevalonate respectively.



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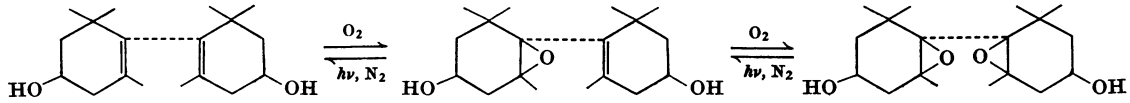
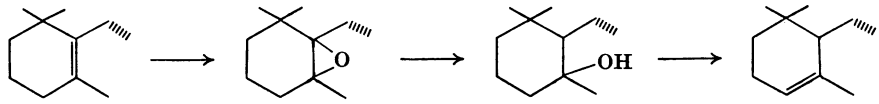
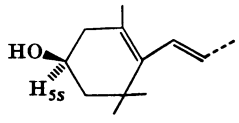


Scheme 13. Carotenoid interconversions that occur in chloroplasts.



Scheme 14. Pathway for the possible conversion of zeaxanthin into lutein via violaxanthin.



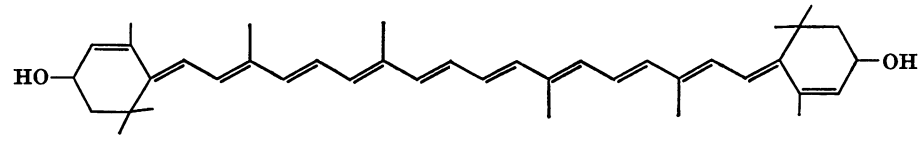
(XXII)

Xanthophyll synthesis

Xanthophylls are oxygen-containing carotenoids and the major xanthophylls in green tissues have hydroxyl groups at C-3 and C-3′ (e.g. lutein, 3,3′-dihydroxy-α-carotene); all the available evidence indicates that in general they are formed at the end of the biosynthetic sequence (Goodwin, 1971*b*). For example β-carotene is probably the precursor of zeaxanthin (3,3′-dihydroxy-β-carotene) in a *Flavobacterium* sp. (J. C. B. McDermott, G. Britton & T. W. Goodwin, unpublished work). Our experiments with stereospecifically labelled mevalonates indicated that in maize seedlings there was no detectable interconversion of α- and β-rings of chloroplast xanthophylls, i.e., specifically, lutein is not converted into zeaxanthin (Goodwin *et al.* 1968; Walton *et al.* 1969); this was important to check, because such interconversion had been suggested by indirect experiments. It is now clear that the reversible reaction indicated in Scheme 13 can take place in chloroplasts (see Goodwin, 1971*b*), but it had been claimed that the de-epoxidation of violaxanthin could yield an α-ionone ring (Scheme 14).

Experiments with [2-^{14}C,(5*R*)-5-$^{3}H_1$]- and [2-^{14}C,5-$^{3}H_2$]-mevalonate have shed some light on the hydroxylation mechanism because C-3 and C-3′ of zeaxanthin and lutein arise from C-5 of mevalonate. With [2-^{14}C,5-$^{3}H_2$]mevalonate these pigments lost only two more tritium atoms than did the corresponding hydrocarbons, thus ruling out a 3- or 3′-oxo intermediate; with [2-^{14}C,(5*R*)-5-$^{3}H_1$]-mevalonate the xanthophylls also had two fewer tritium atoms than did the parent hydrocarbons. Thus there is stereospecific removal of the 3-pro-*R*- and 3′-pro-*R*-hydrogen atoms in forming the 3,3′-dihydroxycarotenes (Walton *et al.* 1969). If one assumes that a mixed-function oxidase is involved and that the hydroxyl group replaces the pro-*R*-hydrogen atom arising from C-5 of mevalonate without inversion, then the absolute configuration at C-3 that emerges (structure XXII) is that shown by Weedon (1965) from chemical studies. Incidentally these studies, which show that the xanthophylls synthesized from [5-$^{3}H_2$]mevalonate contain fewer tritium atoms than do the corresponding carotenes, rule out the possibility suggested previously that carotenes are formed from xanthophylls (Costes, 1965).

The unique retro-carotenoid eschscholtzxanthin (XXIII) has the same $^{14}C/^{3}H$ ratio as β-carotene when formed in the petals of *Eschscholtzia californica* in the presence of [2-^{14}C,(4*R*)-4-$^{3}H_1$]mevalonate (Williams, Britton & Goodwin, 1966). These results suggested the mechanism outlined in Scheme 15 for the conversion of antheraxanthin



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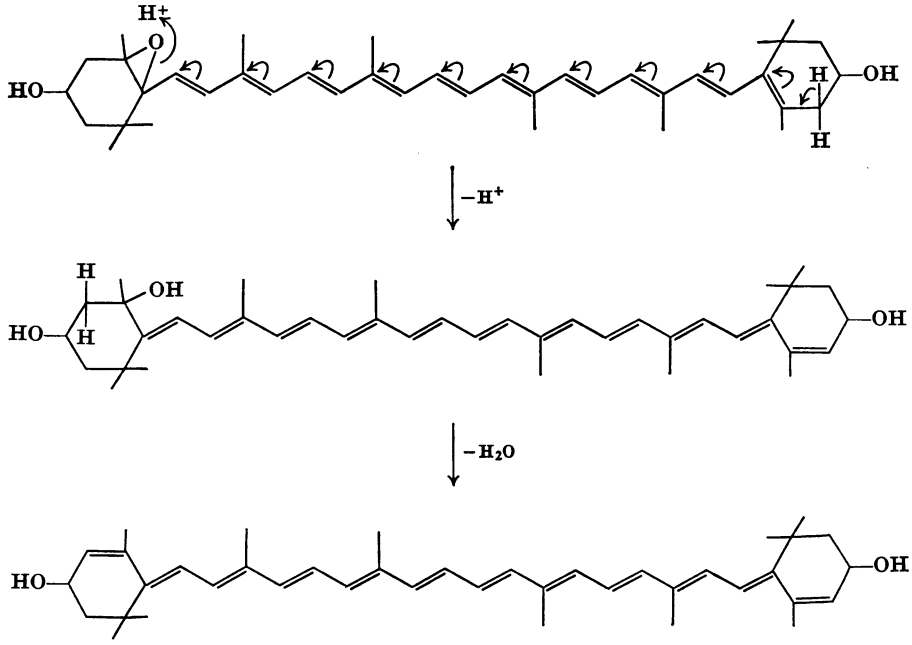
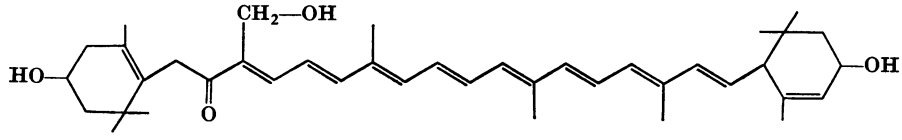


Scheme 15. Conversion of antheraxanthin into eschscholtzxanthin.

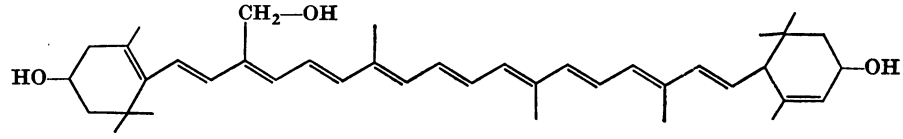


(XXIV)

into eschscholtzxanthin with the retention of all the hydrogen atoms arising from C-4 of mevalonate.

Algal carotenoids

The chloroplast carotenoids of higher plants are qualitatively very similar, but those in algal chloroplasts vary considerably and the carotenoid distribution among algal classes has been used as a biochemical marker in studies on evolution of algae (Goodwin, 1971*a*). One of the very characteristic xanthophylls found in algae is siphonaxanthin, which, together with its ester siphonein, represents the major part of the xanthophyll fraction of the siphonaceous green algae (Strain, 1958). The structure (XXIV), which we have recently worked out (Walton, Britton, Goodwin, Diner & Moshier, 1970), contains two novel features: (i) an in-chain methyl group has been oxidized to a hydroxymethyl group and (ii) the presence of an in-chain oxo group. Such groups have been observed previously only separately as in loroxanthin (XXV) (Aitzetmüller, Strain, Svec, Grandolfo & Katz, 1969) and fucoxanthin (XXVI).



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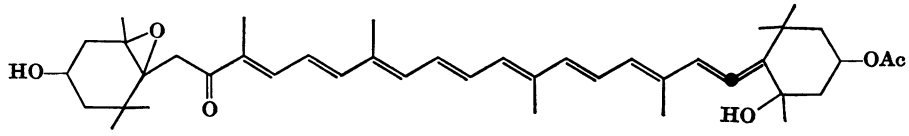

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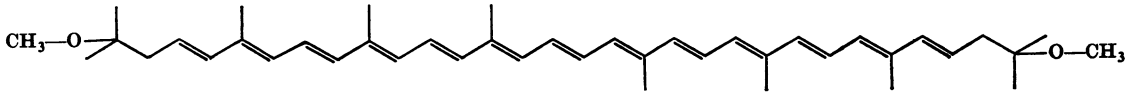


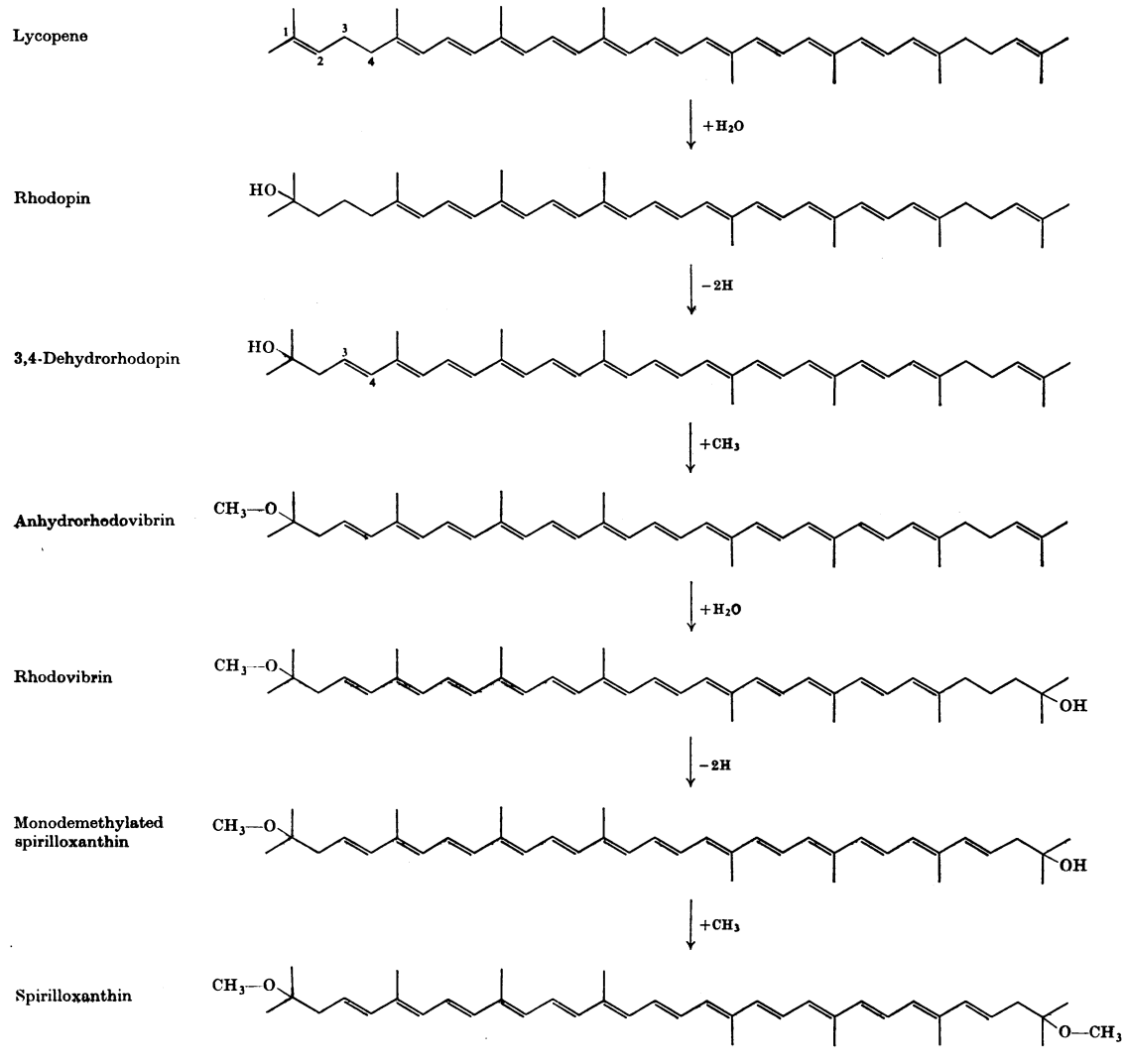
(XXVII)

Photosynthetic bacteria

As long ago as 1953–1954 we showed that synthesis of spirilloxanthin (XXVII) in *Rhodospirillum rubrum* was inhibited by diphenylamine with the simultaneous accumulation of more-saturated compounds (Goodwin & Osman, 1953, 1954). Later investigations in greater detail indicated that spirilloxanthin was synthesized from lycopene as outlined in Scheme 16, and that lycopene was formed from phytoene by the pathway already described (Scheme 7) (Liaaen-Jensen, Cohen-Bazire & Stanier, 1961). A recent important observation by Dr B. H. Davies at Aberystwyth showed that the desaturation of phytoene in this organism did not proceed as indicated in Scheme 7, but that desaturation of phytofluene led to 7,8,11,-12-tetrahydrolycopene and then to neurosporene and lycopene (Scheme 17); ζ-carotene (7,8,7′,8′-tetrahydrolycopene) was not involved (Davies, 1970*a*,*b*). The patterns of synthesis in Schemes 16 and 17 were based on the accumulation of intermediates in the presence of diphenylamine and on some kinetic studies. However, our recent studies on diphenylamine-inhibited cultures have revealed that hydration across the C-1–C-2 bond followed by methylation at C-1 can occur at any stage of desaturation and not, as previously assumed, only at a late stage in the biosynthetic sequence leading to spirilloxanthin. We have isolated monomethoxy carotenoids with three, five, seven, nine, eleven and twelve conjugated double bonds, and dimethoxy derivatives with seven, nine, eleven and twelve conjugated double bonds (corresponding to the compounds listed in Schemes 7 and 17) (Malhotra, Britton & Goodwin, 1970*c*,*d*). It remains to be seen whether these newly discovered compounds are products of side reactions forced on somewhat unspecific enzymes by the pressure of the inhibitor, or whether *in vivo* a matrix of reactions occurs that eventually results in the accumulation of spirilloxanthin.

The more recent studies on the diphenylamine effect on *Rhodospirillum rubrum* show that the organism is also forced into synthesizing pigments with eight and ten conjugated double bonds [e.g. spheroidene (XXVIII) and 11′,12′-dihydrospheroidene (XXIX)] (Davies, Holmes, Loeber, Toube & Weedon, 1969; Malhotra, Britton & Goodwin, 1969, 1970*c*,*d*), compounds that were originally considered to be confined to *Rhodopseudomonas spheroides* and related species. For example, *Rhodopseudomonas gelatinosa* synthesizes both spheroidene and spirilloxanthin, and a pathway of synthesis of spirilloxanthin via spheroidene has been suggested (Scheme 18) (Eimhjellen & Liaaen-Jensen, 1964); it now seems that under some circumstances this pathway also operates in *Rhodospirillum rubrum*. In *Rhodopseudomonas spheroides* as soon as the cells are exposed to oxygen spheroidene is rapidly converted into spheroidenone (XXX) (van Niel 1947; Goodwin, Land & Sissins, 1956); this does not happen in *Rhodospirillum rubrum*.

The formation of 1,2-dihydro derivatives in *Rhodopseudomonas viridis* has already been mentioned, but in the present context the coexistence



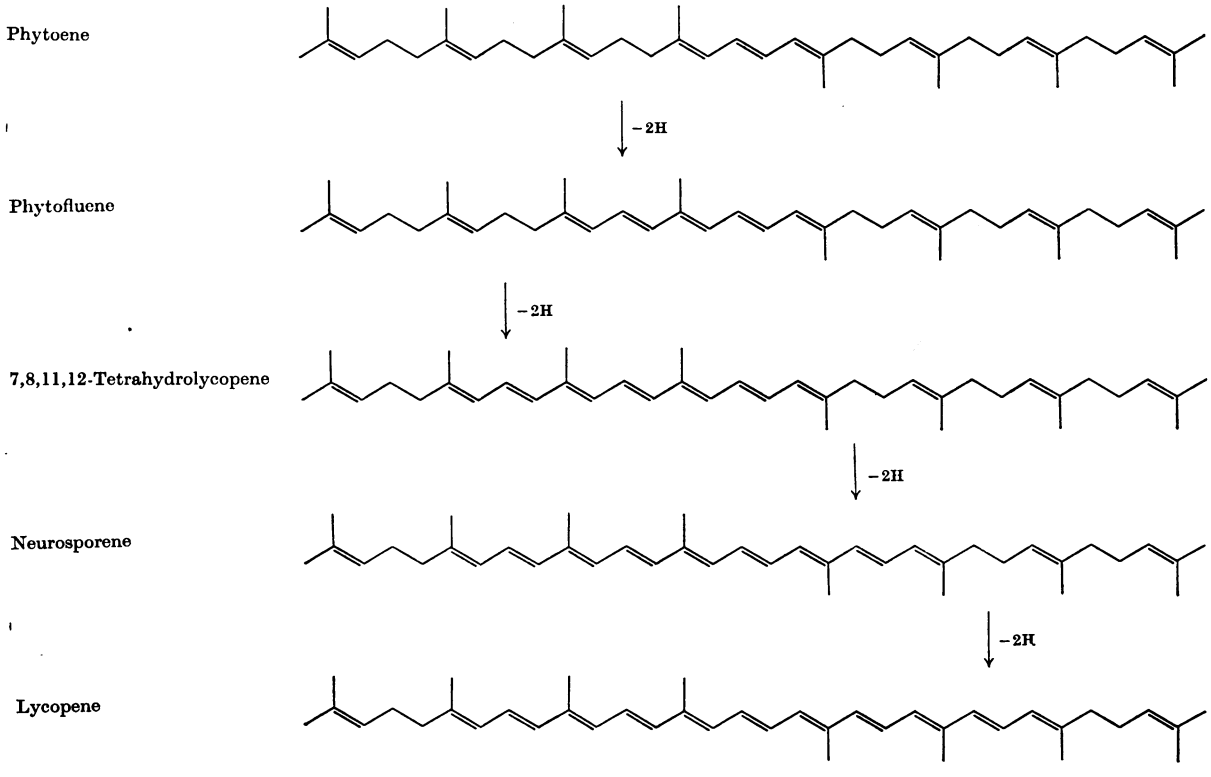
Scheme 16. Proposed pathway of spirilloxanthin synthesis in *Rhodospirillum rubrum*.

of 1,2-dihydro-ζ-carotene and 1,2,7,8,11,12-hexahydrolycopene in the same organism is rather unexpected (Malhotra *et al.* 1970*b*). Previously it was thought that ζ-carotene occurred only in higher plants and 7,8,11,12-tetrahydrolycopene only in photosynthetic bacteria.

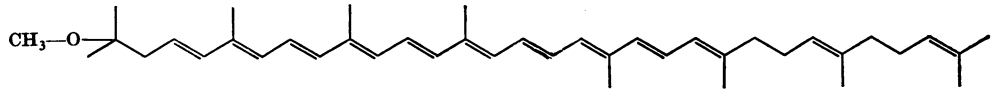
Rhodomicrobium vannielii is unique among the Athiorhodaceae in that it synthesizes, as well as spirilloxanthin, the cyclic β-carotene (Volk & Pennington, 1950; Ryvarden & Liaaen-Jensen, 1964). Our recent examination of this organism has revealed the presence of a pigment 'half-spirilloxanthin–half-β-carotene' [3′,4′-didehydro-1′,2′-dihydro-1′-methoxy-γ-carotene (XXXI)] (A. Ben-Aziz, G. Britton & T. W. Goodwin, unpublished work). We have also unexpectedly isolated β-cryptoxanthin (3-hydroxy-β-carotene); in an earlier section it was suggested that hydroxylation of carotenes in higher plants occurs via a mixed-function oxidase, but this cannot be the mechanism in an obligate anaerobe.

Biosynthesis of plant sterols and related compounds: general comments

Our interest in carotenoid biosynthesis in particular and plant biochemistry in general led us naturally a few years ago to begin to study the



Scheme 17. Pathway from phytoene to lycopene in *Rhodospirillum rubrum*.



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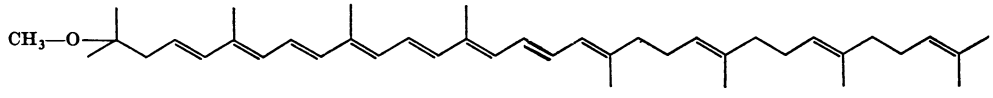

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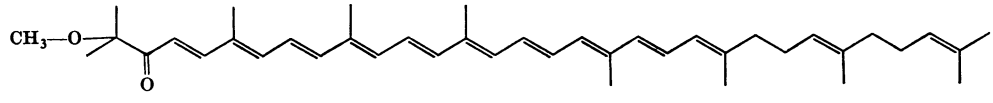

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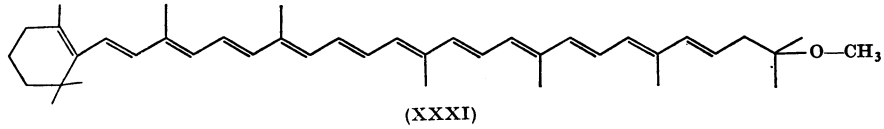

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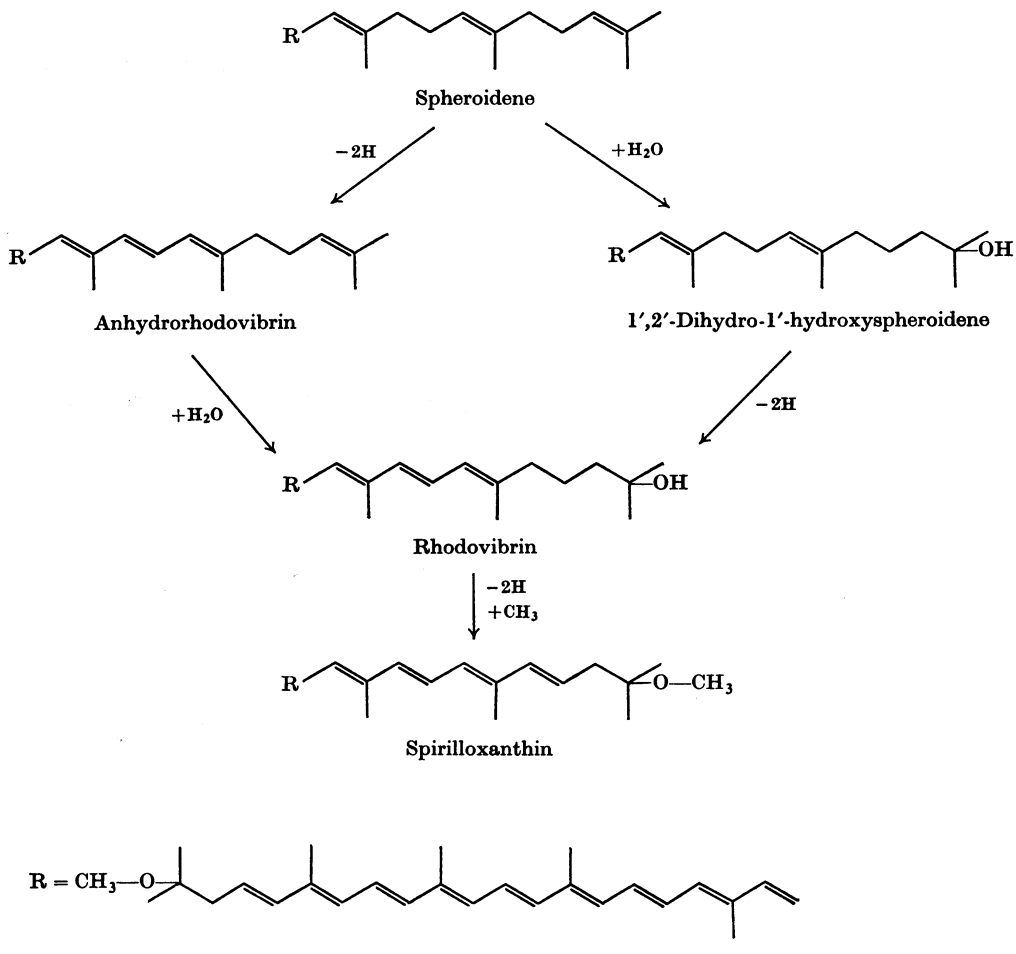
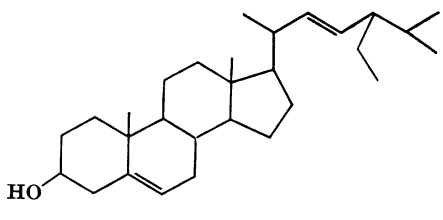


Scheme 18. Formation of spirilloxanthin from spheroidene.



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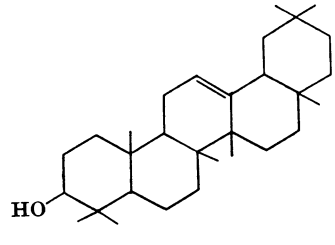
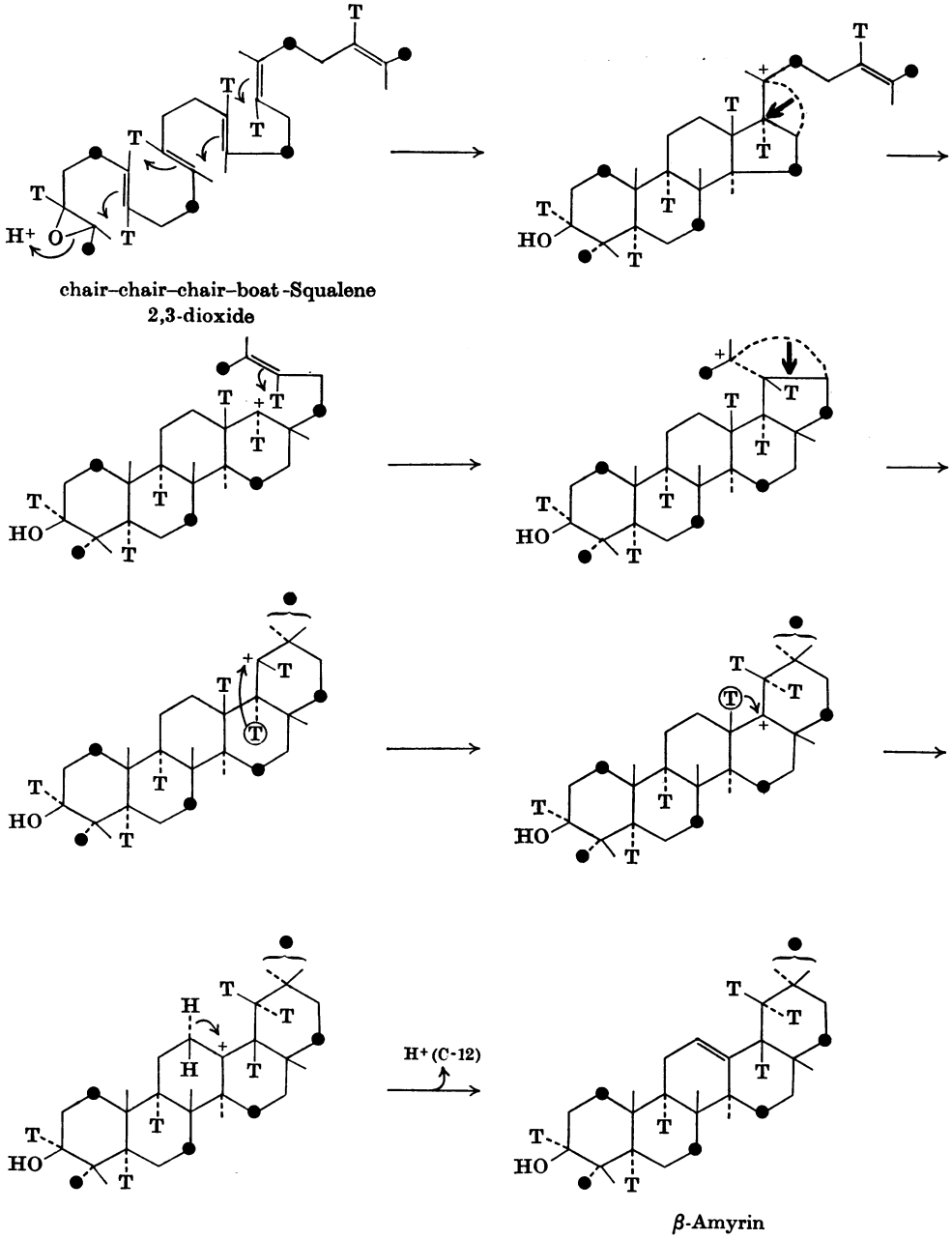


(XXXIII)

formation of plant sterols. The time was ripe because analytical methods were becoming available for unequivocal separations, identifications and structural determinations; furthermore stereospecific species of mevalonate could be used to study many details of the biosynthetic process. Many plant sterols differ from cholesterol, which used to be considered a typical animal sterol but which is now known to be present in traces in most plants examined (see e.g. Goodwin & Goad,



Scheme 19. Mechanism for the formation of β-amyrin from squalene 2,3-oxide.

1971), in having additional C_1 or C_2 residues at C-24 and/or a double bond at C-22, as typified by stigmasterol (XXXII). We have investigated these two problems, but we first looked into the mechanism of cyclization of squalene 2,3-oxide to give β-amyrin (XXXIII) and the nature of the first cyclization product of squalene 2,3-oxide in plant sterol biosynthesis.

β-Amyrin biosynthesis

A possible mechanism for cyclization of squalene 2,3-oxide to yield β-amyrin (XXXIII) is indicated

in Scheme 19. The mechanism demands no loss of either ^{14}C or ^{3}H from squalene 2,3-oxide in forming β-amyrin. This was demonstrated by Rees, Mercer & Goodwin (1966). Later Dr Rees degraded the molecule and located five of the six tritium atoms in positions C-3, C-9, C-18 and C-19 (two), as expected from the proposed mechanism (Rees, Britton & Goodwin, 1968*a*). There is no serious doubt that the final tritium atom is located at C-5, as indicated in Scheme 19.

First cyclic precursor of plant sterols

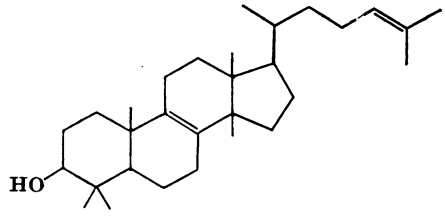
In animals the first cyclic precursor of cholesterol is lanosterol (XXXIV), but the existence of lanosterol in higher plants is extremely restricted and probably it is present only in the latex of the Euphorbiaceae (see e.g. Goad, 1970); its place appears to be taken by cycloartenol (XXXV) (von Ardenne, Osske, Schreiber, Steinfelder & Tümmler, 1965; Benveniste, Hirth & Ourisson, 1966*a*,*b*; Goad & Goodwin, 1966, 1967; Williams, Goad & Goodwin, 1967*a*; Kemp, Hammam, Goad & Goodwin, 1968). Labelled acetate (Benveniste *et al.* 1966*a*,*b*; Ehrhardt, Hirth & Ourisson, 1967) and mevalonate (Goad & Goodwin, 1966, 1967; Goad, Gibbons, Bolger, Rees & Goodwin, 1969; Kemp *et al.* 1968; Rees, Goad & Goodwin, 1968*b*) quickly label cycloartenol, but no labelled lanosterol could be detected. Cycloartenol is also present in red algae (Alcaide, Devys & Barbier, 1968), green algae (Gibbons, Goad & Goodwin, 1968*a*), the phytoflagellates *Ochromonas malhamensis* and *Ochromonas danica* (Gershengorn *et al.* 1968) and brown algae (*Fucus spiralis*) (Goad & Goodwin, 1969).

With [2-^{14}C,(4*R*)-4-$^{3}H_1$]mevalonate we showed that cycloartenol is a product of direct cyclization of squalene 2,3-oxide and is not produced by isomerization of lanosterol (Rees *et al.* 1968*b*; Goad & Goodwin, 1969). Scheme 20 shows that the $^{14}C/^{3}H$ atomic ratio falls from 6:6 in squalene to 6:5 in lanosterol; in cycloartenol derived from lanosterol the ratio would also be 6:5, but if it were formed directly the ratio would be 6:6, owing to the retention of the sixth tritium atom at C-8. The ratio was found to be 6:6, and the extra tritium atom was located at C-8 in experiments with potato leaves.

Under anaerobic conditions in liver preparations squalene oxide is converted into lanosterol, which accumulates because the next step (demethylation) in sterol biosynthesis is oxidative. Under similar conditions cell-free preparations from *Ochromonas malhamensis* and beans will convert squalene 2,3-oxide into cycloartenol but not into lanosterol (Rees, Goad & Goodwin, 1968*c*, 1969). Furthermore labelled lanosterol cannot be isolated in trapping experiments in plant tissue cultures (Eppenberger, Hirth & Ourisson, 1969). Finally, labelled cycloartenol is effectively converted into phytosterols in *Ochromonas malhamensis* (7%) (Hall, Smith, Goad & Goodwin, 1969; Lenton *et al.* 1971) and in tissue cultures of *Nicotiana tabacum* (Hewlins, Ehrhardt, Hirth & Ourisson, 1969) without any degradation into smaller units, which could have been resynthesized into sterols. However, lanosterol is also an effective precursor of phytosterols in *Euphorbia peplus* (Baisted, Gardner & McReynolds, 1968), *Ochromonas malhamensis* (Hall *et al.* 1969; Lenton *et al.* 1971) and tissue cultures of *Nicotiana tabacum* (Hewlins *et al.* 1969); at the moment this is taken to mean that the enzymes concerned with the further metabolism of 4,4-dimethyl sterols are not completely specific, and that lanosterol is not a naturally occurring intermediate in higher plants and algae.

Possible pathway of synthesis of phytosterols

Before we consider the details of the transformations involved in sterol synthesis in higher plants let us consider a possible pathway (Scheme 21). The evidence that such a pathway may exist is based mainly on the observation that all the intermediates indicated have now been found as natural products (Goad, 1970). Additional evidence that we have obtained includes: (i) labelled 24-methylenecycloartanol (Scheme 21) is converted into phytosterols in *Ochromonas malhamensis* (Hall *et al.* 1969; Lenton *et al.* 1971); (ii) both cycloeucalenol and obtusifoliol (Scheme 21) are present in many plant sources that we have examined (Goad, Williams & Goodwin, 1967; Kemp *et al.* 1968) and become rapidly labelled when larch (*Larix decidua*)



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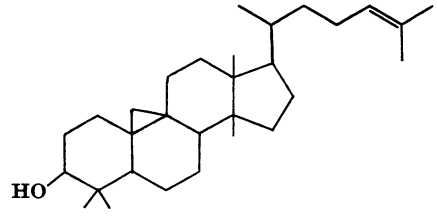

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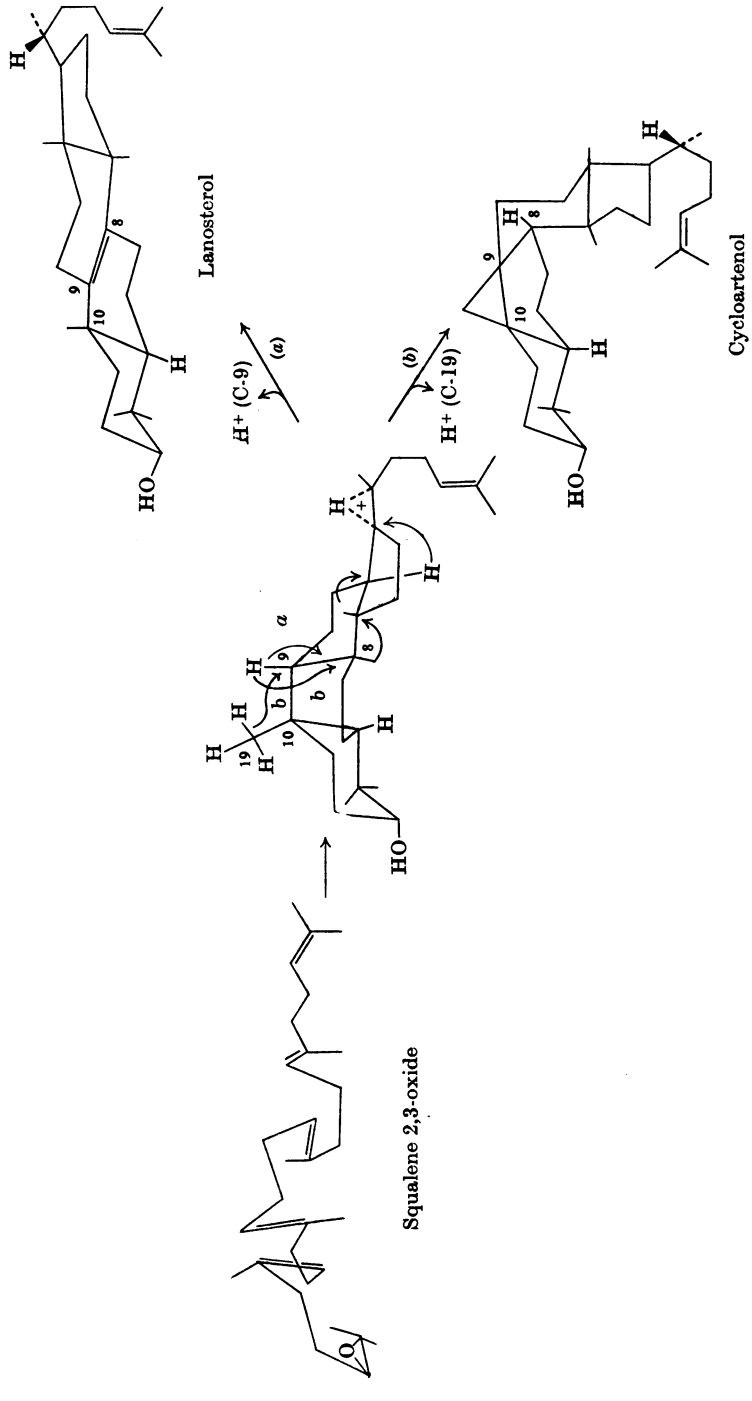
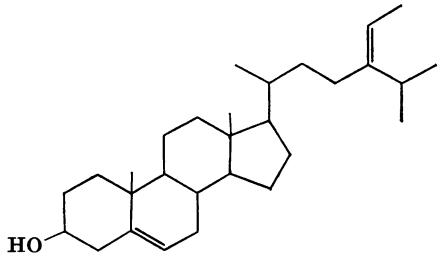


Scheme 20. Mechanism for lanosterol and cycloartenol formation from squalene 2,3-oxide.

Scheme 21. Possible pathway for the formation of phytosterols from cycloartenol.



(XXXVI)

leaves (Goad & Goodwin, 1967) and tobacco cultures (Benveniste, 1968) are incubated with [2-^{14}C]-mevalonate; (iii) 4α-methyl sterols with 24-methylene and 24-ethylidene substituents are often present in plants at least in trace amounts (Osske & Schreiber, 1965; Williams *et al.* 1967*a*; Goad & Goodwin, 1967); (iv) 28-isofucosterol (XXXVI) has been found in various higher plants (see Goad, 1970), and becomes labelled when pine (*Pinus pinea*), larch (*Larix decidua*) and pea (*Pisum sativum*) seedlings are incubated with [2-^{14}C]-mevalonate (van Aller, Chikamatsu, de Souza, John & Nes, 1968; Goad *et al.* 1969) and is converted into sitosterol by *Pinus pinea* seedlings (van Aller *et al.* 1968).

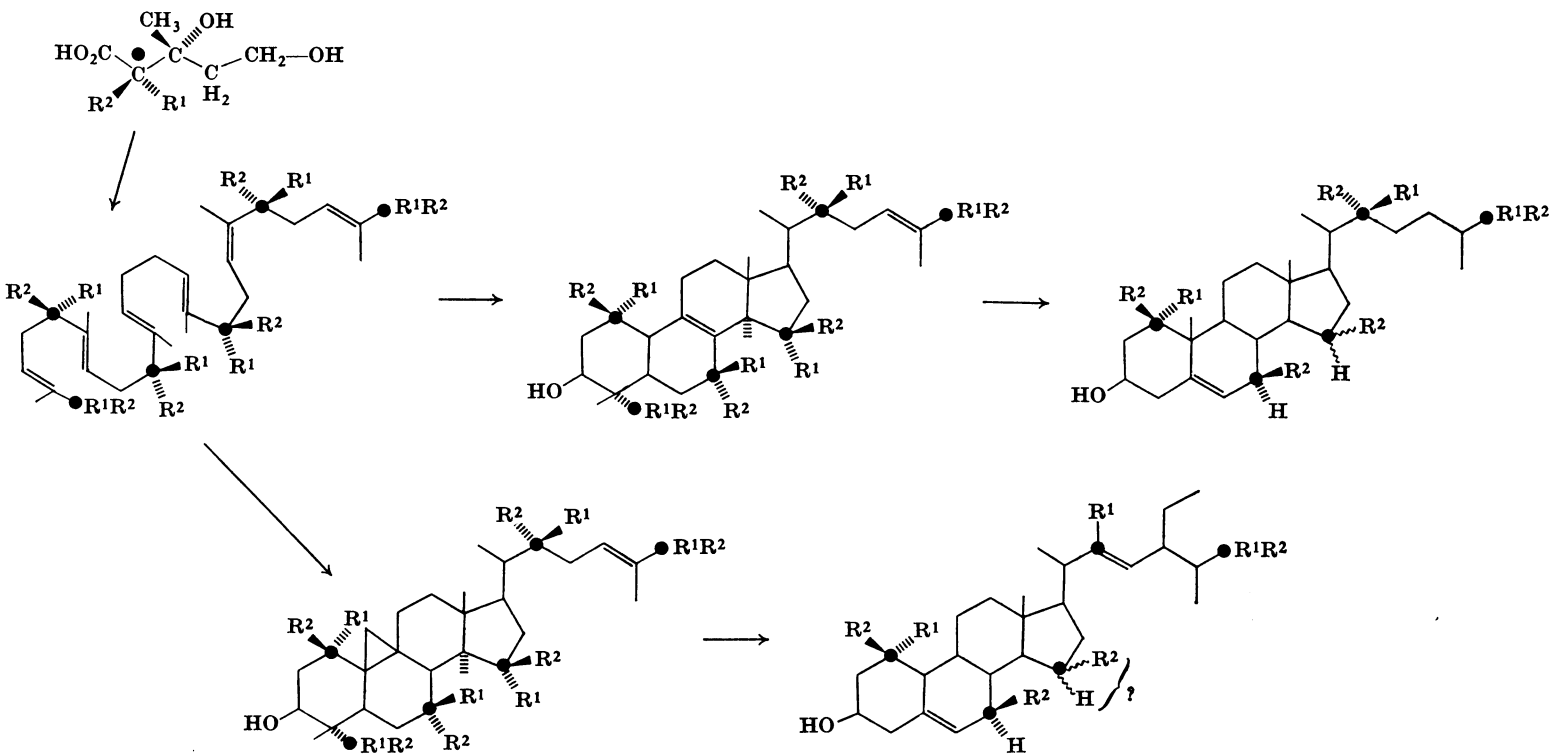
The presence of lanosterol in the Euphorbiaceae raises the question whether it is an immediate product of squalene 2,3-oxide, as is cycloartenol, or whether it is produced from the primary cyclization product cycloartenol. The latter possibility is emphasized by the fact that latex of *Euphorbia lathyris* will convert [25-^{14}C]cycloartenol into lanosterol (Ponsinet & Ourisson, 1968). It will be important to check this point in *Ochromonas malhamensis*, where we have recently found, on g.l.c.–mass-spectrometry analysis, traces of a material that may be lanosterol (G. H. Beastall & H. H. Rees, unpublished work).

Demethylation of C-14 *and* C-4

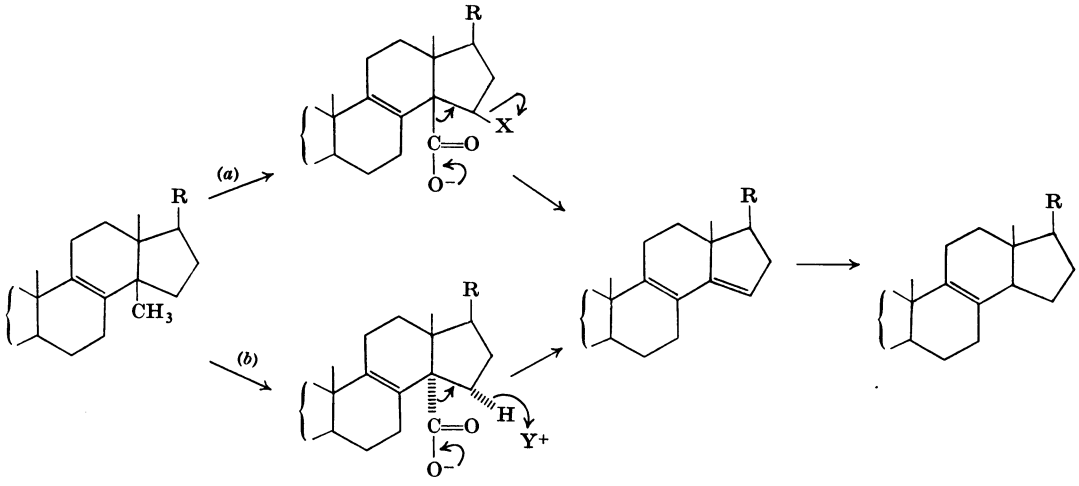
Use of stereospecifically labelled mevalonates has helped in elucidating the mechanism of demethylation of lanosterol and cycloartenol in their conversion into sterols. Squalene formed from [(2*R*)-2-$^{3}H_1$]mevalonate has the configuration indicated in Scheme 22, and leads to lanosterol labelled with tritium at the 1β-, 7α-, 15β-, 22-*R*-, 26- or 27-, and 30- or 31-positions. With [(2*S*)-2-$^{3}H_1$]-mevalonate the labelling is at the 1α-, 7β-, 15α-, 22-*S*-, 26- or 27-, and 30- or 31-positions. In the conversion of lanosterol into cholesterol a hydrogen atom is removed from C-7, and in experiments with mevalonates stereospecifically tritiated at C-2 it was shown that the 7β-hydrogen atom was lost (a pro-2*S*-hydrogen atom of mevalonate) (Canonica *et al.* 1968*a*,*b*; Caspi, Grieg, Ramm & Varma, 1968; Gibbons *et al.* 1968*a*). However, one more tritium atom was lost in cholesterol formed from [(2*S*)-2-$^{3}H_1$]mevalonate than was at first expected; it did not take long to realize that the only position that could be involved was C-15, and this conclusion was soon proved by Gibbons, Goad & Goodwin (1968*b*), Canonica *et al.* (1968*c*) and Akhtar, Watkinson, Rahimtula, Wilton & Munday (1969). We also showed that the pro-*R*-hydrogen atom of C-2 of mevalonate present at C-15 in lanosterol is retained at C-15 in cholesterol (Gibbons *et al.* 1968*b*). These experiments indicated that a $\Delta^{8,14}$-diene may be involved in demethylation at C-14, and a number of laboratories quickly reported that 5α-cholesta-8,14-dien-3β-ol and 4,4-dimethyl-5α-cholesta-8,14-dien-3β-ol are effectively converted into cholesterol by rat liver homogenates (see Goad, 1970). The saturation of the Δ^{14}-bond involves NADPH; the hydride ion from C-4 becomes the 14α-hydrogen atom, and a proton from water is added at C-15 (Akhtar *et al.* 1969). The configuration taken up by the entering proton is β (Ramm & Caspi, 1969). A possible overall mechanism of demethylation at C-14 that accommodates all the known facts is indicated in Scheme 23.

With regard to the removal of the two methyl groups at C-4, we showed that the 4α-methyl group of cycloartenol arises from C-2 of mevalonate (Ghisalberti, de Souza, Rees, Goad & Goodwin, 1969*b*). Cycloartenyl acetate synthesized from [2-^{14}C,(4*R*)-4-$^{3}H_2$]mevalonate by *Polypodium vulgare* was converted chemically into the dihydroxy compound and finally into methyl 4-oxo-3,4-*seco*-30-norlanost-8-en-3-oate (Scheme 24). The observed increase in the $^{3}H/^{14}C$ ratio in the oxo derivative compared with that in the *seco* methyl ester was consistent with the carbon atom of the methylene group originating from C-2 of mevalonate. Thus, as the methylene group of the *seco* compound arises from the 4α-methyl group of the parent triterpene (Moss & Nicolaidis, 1969), the 4α-methyl group of cycloartenol must arise biosynthetically from C-2 of mevalonate. *Polypodium vulgare* rhizomes also produce 31-norcycloartanol (XXXVII), in which the methyl group remaining at C-4 has the α-configuration. We found that the methyl group removed biologically arose from C-2 of mevalonate, so the 4α-methyl group must be the first group removed from cycloartenol and the 4β-methyl group must take up the 4α-configuration in 31-norcycloartanol. The pathway involved is probably that indicated in Scheme 25. This sequence of events agrees with the finding in animal tissues (Sharpless *et al.* 1968, 1969).

We had previously shown that the 3α-hydrogen atom of cycloartenol is lost during its conversion



Scheme 22. Incorporation of [2-^{14}C,(2*R*)-2-$^{3}H_1$]- and [2-^{14}C,(2*S*)-2-$^{3}H_1$]mevalonate into cholestero l and poriferasterol. ● indicates carbon atoms arising from C-2 of mevalonate. R^1 indicates ^{3}H arising from [(2*S*)-2-$^{3}H_1$]mevalonate; R^2 indicates ^{3}H arising from [(2*R*)-2-$^{3}H_1$]mevalonate.



Scheme 23. Possible mechanisms for demethylation of lanosterol at C-14.

into phytosterols (Rees *et al.* 1966; Goad & Goodwin, 1969). In the investigation on *Polypodium vulgare* the 3-oxo derivative of 31-norcycloartanol was formed chemically from the parent compound biosynthesized from [2-^{14}C,(4*R*)-4-$^{3}H_1$]mevalonate and it had the same $^{14}C/^{3}H$ ratio as 31-norcycloartanol, thus demonstrating for the first time the involvement of C-3 in the loss of the first methyl group from C-4 (Ghisalberti *et al.* 1969*b*).

$\Delta^8 \rightarrow \Delta^5$ *transformation*

The transformations involved in the intramolecular movement of the double bond from Δ^8 to Δ^5 in cholesterol formation are $\Delta^8 \rightarrow \Delta^7 \rightarrow \Delta^{5,7} \rightarrow \Delta^5$, and these have been well established by numerous definitive investigations (see Goad, 1970). We have obtained evidence for some stereochemical aspects of the sequence. As already indicated (Scheme 22) experiments with [(2*S*)-$^{3}H_1$]- and [(2*R*)-$^{3}H_1$]-mevalonate demonstrated that in the isomerization $\Delta^8 \rightarrow \Delta^7$ in animals the 7β-hydrogen atom is eliminated (Gibbons *et al.* 1968*a*), and the same situation obtains in the alga *Ochromonas malhamensis* (Smith, Goad & Goodwin, 1968*a*) and higher plants (Bimpson, 1970; Sharma, 1970). In yeast, however, it is the 7α-hydrogen atom that is removed (Caspi & Ramm, 1969).

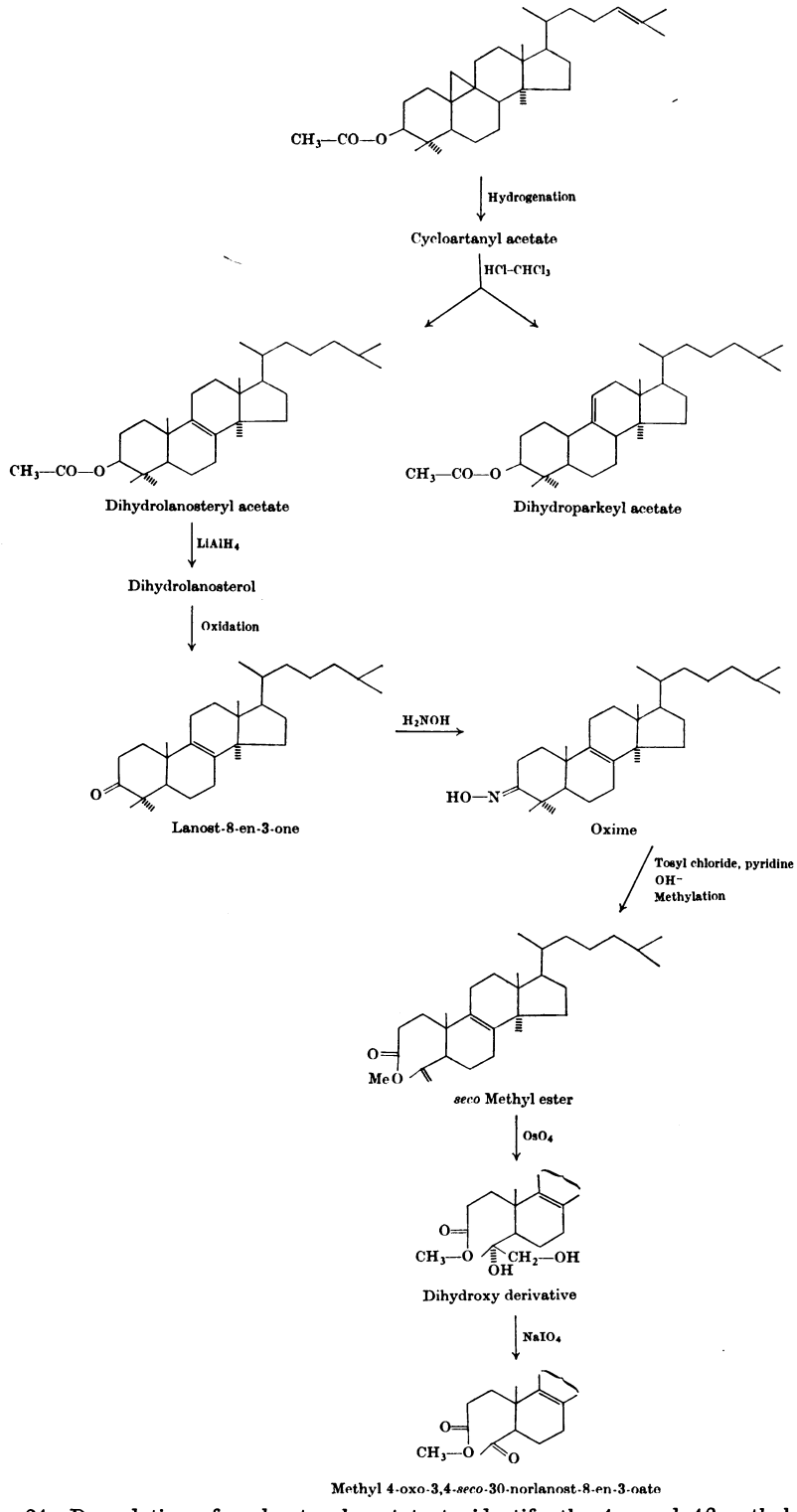
[2-^{14}C,(5*R*)-5-$^{3}H_1$]Mevalonate yielded lanosterol labelled as indicated in Scheme 26, and ultimately cholesterol with a tritium atom retained at C-6, indicating elimination of the α-hydrogen atom at C-6 during the formation of the Δ^5-bond (Goad *et al.* 1969). This confirmed the earlier conclusion, drawn from different types of experiments, of Akhtar & Marsh (1967) and Paliokas & Schroepfer (1967,1968). The loss of the 6α-hydrogen atom in ergosterol formation in *Aspergillus fumigatus* was demonstrated by Bimpson, Goad & Goodwin, (1969*a*). Akhtar and Schroepfer also showed that the 5α-hydrogen atom was removed in cholesterol biosynthesis, and thus the formation of the double bond must involve a *cis* elimination.

Finally the saturation of the Δ^7-bond in cholesterol formation involves *trans* addition of the hydride ion from the B side of NADPH to position 7α and of a proton to position 8β. The hydrogen originally at position 7α in lanosterol takes up the 7β-position (Wilton, Munday, Skinner & Akhtar 1968). Studies with [2-^{14}C,(2*R*)-2-$^{3}H_1$]mevalonate confirmed these conclusions, because it was shown that the tritium atom retained at C-7 of cholesterol from this substrate was in the β-position (Caspi *et al.* 1968; Gibbons *et al.* 1968*a*). The details of this step in plants are not yet known.

The protozoan *Tetrahymena pyriformis* will desaturate exogenous cholesterol to cholest-5,7,22-trien-3β-ol (XXXVIII) (Mallory, Conner, Landray & Iyengar, 1968). We have shown by using specimens of cholesterol biosynthesized from [2-^{14}C,(2*R*)-2-$^{3}H_1$]- and [2-^{14}C,(2*S*)-2-$^{3}H_1$]-mevalonate as substrates that it is the 7β-hydrogen atom that is removed in Δ^7-bond formation (Bimpson, Goad & Goodwin, 1969*b*).

Formation of the Δ^{22}-trans-*bond in higher plants, algae and fungi*

This particular biochemical elaboration is characteristic of higher plants and fungi and is not encountered in higher animals. As far as we can see it has no connexion with alkylation at C-24, another characteristic plant reaction, unlike the formation



Scheme 24. Degradation of cycloartenyl acetate to identify the 4α- and 4β-methyl groups.

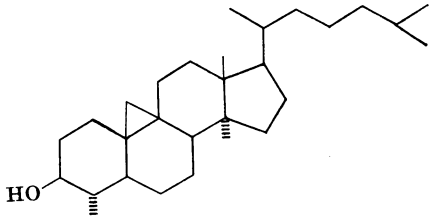
of the Δ^{25}-bond, which is directly connected with the alkylation reaction (see below). Our interest has been in defining the sterochemistry of the desaturation at the C-22–C-23 bond. C-22 and C-23 arise from C-2 and C-5 of mevalonate respectively, so that experiments with [2-^{14}C,(2*R*)-2-$^{3}H_1$]-, [2-^{14}C,(2*S*)-2-$^{3}H_1$]- and [2-^{14}C,(5*R*)-5-$^{3}H_1$]-mevalonate should indicate the stereochemistry of the hydrogen removal at these two carbon atoms. Appropriate degradations of labelled poriferasterol from *Ochromonas malhamensis* showed that C-23 contained no tritium when [5*R*-5-$^{3}H_1$]mevalonate was the substrate (Smith, Goad & Goodwin, 1968*b*). With [2*S*-2-$^{3}H_1$]mevalonate considerable tritium was retained at C-22, whereas with [2*R*-2-$^{3}H_1$]-mevalonate only a small amount of label was retained (Smith *et al.* 1968*a*). The reason for the randomization when mevalonate stereospecifically labelled with tritium at C-2 is used in experiments with plants has already been discussed. In these experiments only degradations gave meaningful results; $^{14}C/^{3}H$ ratio measurements were of little value. In ergosterol formation in *Ochromonas danica* the 23-pro-*R*-hydrogen atom is also lost (Smith, 1969). In complete contrast, the 22-pro-*S*- and 23-pro-*S*-hydrogen atoms are eliminated in forming the Δ^{22}-bond in ergosterol in *Aspergillus fumigatus* (Bimpson *et al.* 1969*a*). The same stereochemistry with regard to C-22 has been observed in another fungus, *Blakeslea trispora* (Bimpson, 1970).

The stereochemistry of desaturation of cholesterol at C-22 and C-23 by *Tetrahymena pyriformis* is the same as that observed in poriferasterol in *Ochromonas malhamensis*, but opposite to that found in ergosterol in *Aspergillus fumigatus* (Bimpson *et al.* 1969*b*).

Alkylation reactions

It is now well established that the methyl group at C-24 in plant sterols arises by transmethylation and that the ethyl group arises by a sequential double transmethylation (see Lederer, 1969). Possible mechanisms and pathways of alkylation are indicated in Scheme 27. There is little doubt that in ergosterol formation in yeast route *A* is followed (Lederer, 1969), and that in the synthesis of 24-ethyl sterols in the slime mould *Dictyostelium discoideum* (Lederer, 1969) and the alga *Chlorella vulgaris* (Tomita, Uomori & Minato, 1970) the routes are different from that in *Ochromonas* spp. In early experiments with [*Me*-^{14}C,*Me*-$^{3}H_3$]methionine we suggested that routes *B*, *C*, *E* and *F* were functioning in higher plants and algae (Goad, Hammam, Dennis & Goodwin, 1966). There is always a degree of uncertainty about experiments of this type because of possible isotope effects; this was resolved when in collaboration with Professor Lederer we found that in *Ochromonas malhamensis* [*Me*-$^{2}H_3$]methionine yielded poriferasterol with only four deuterium atoms present in the ethyl side chain (Smith, Goad, Goodwin & Lederer, 1967); this contrasts with *Dictyostelium discoideum* and *Chlorella vulgaris*, in which five deuterium atoms are retained. The pathway proposed (Scheme 27) involves the shift of a hydrogen atom from C-24 to C-25. We have demonstrated this in the case of fucosterol (XXXIX) in *Fucus spiralis* (Goad & Goodwin, 1965, 1969) and poriferasterol in *Ochromonas malhamensis* (Smith, 1969), and Raab, de Souza & Nes (1968) found the same situation in 28-isofucosterol (XXXVI) in *Pinus pinea*.

To clinch the situation the conversion of a 24-methylene sterol or a 24-ethylidene sterol into the



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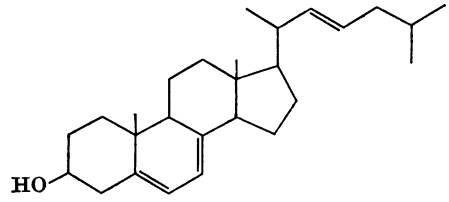

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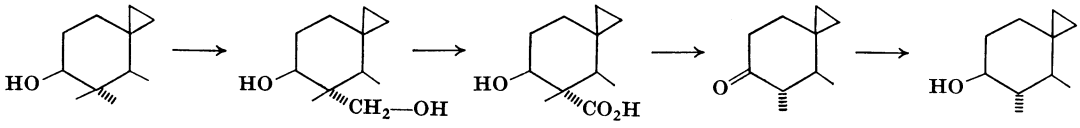
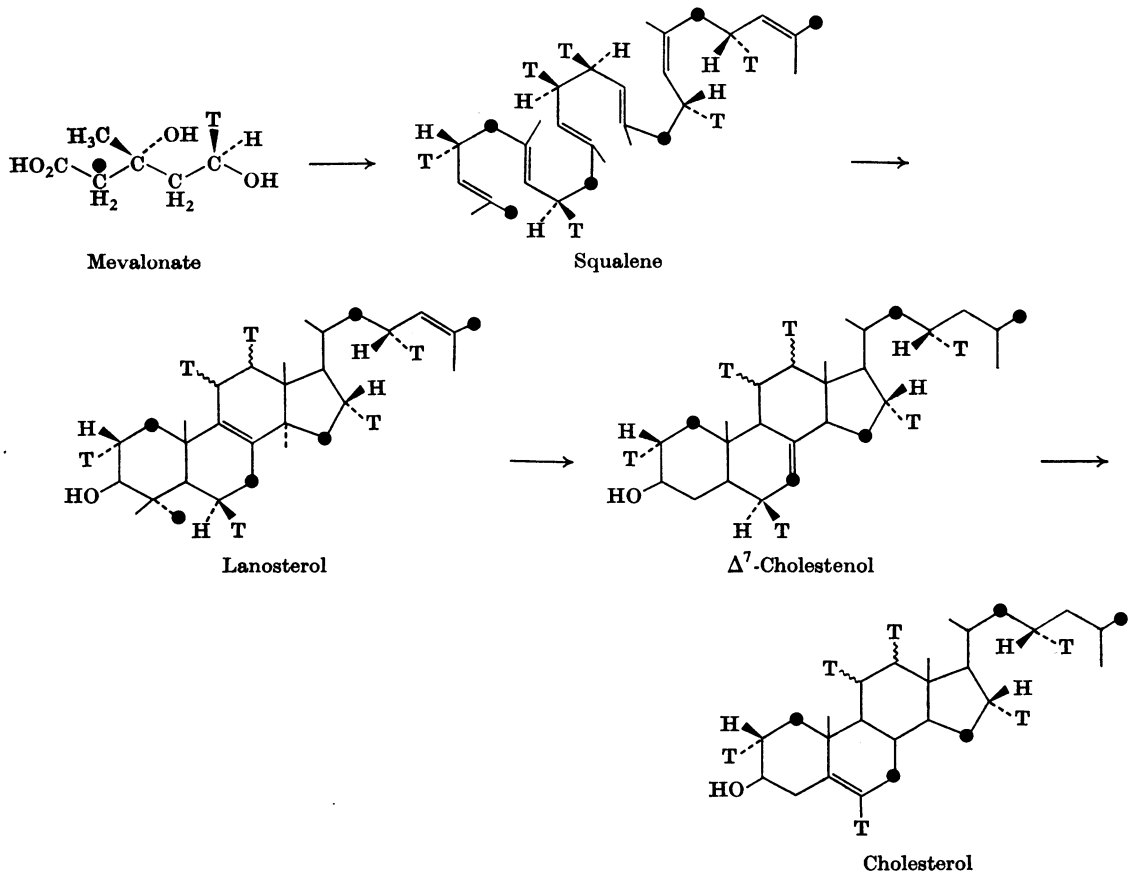


Scheme 25. Probable pathway for the removal of the first methyl group at C-4 of cycloartenol.

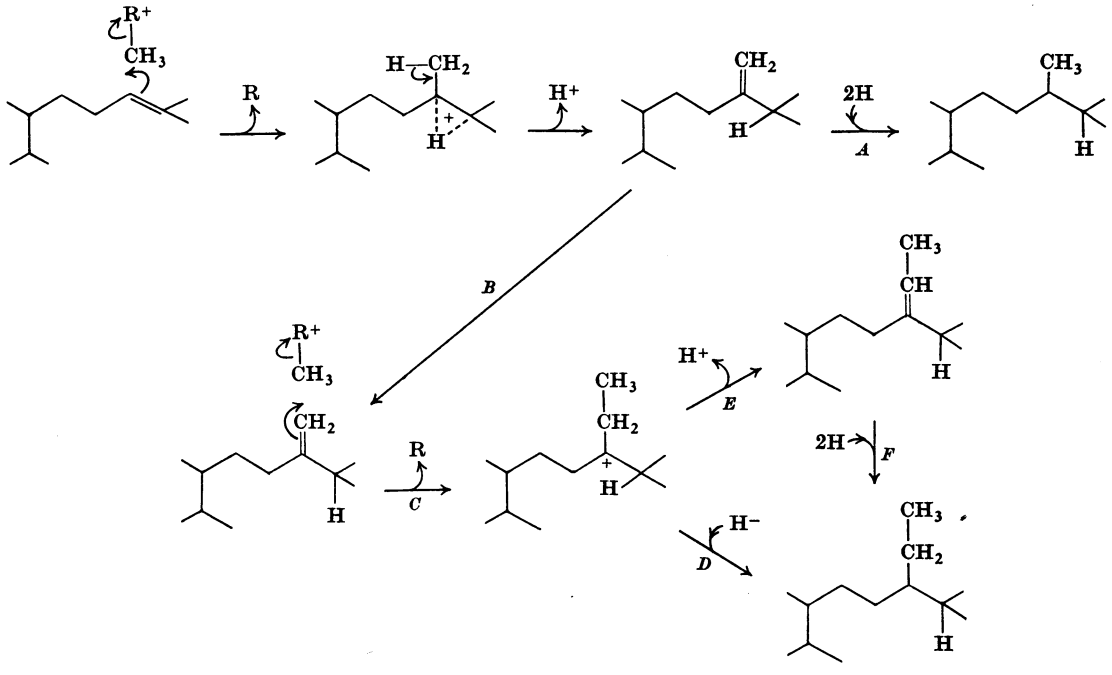


Scheme 26. Labelling of lanosterol and cholesterol from [(5*R*)-5-$^3H_1$]mevalonate. ● indicates ^{14}C from [2-^{14}C]mevalonate; T indicates ^3H from [(5*R*)-5-$^3H_1$]mevalonate.

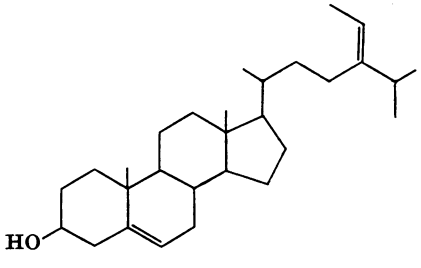
corresponding saturated product must be demonstrated, and this has been achieved in yeast, where 24-methylenelanost-8-en-3β-ol (XL) is converted into ergosterol (Akhtar, Parvez & Hunt, 1966; Barton, Harrison & Moss, 1966) and in *Ochromonas malhamensis* where 24-methyl- and 24-ethylidenelophenol are converted into poriferasterol (Lenton *et al.* 1971). There is as yet only one short report on the conversion of an ethylidene sterol (28-isofucosterol) into an ethyl sterol, sitosterol (van Aller *et al.* 1968). At the moment we are concentrating on the synthesis of labelled isofucosterol of high specific radioactivity, to test it as a precursor. Isofucosterol is more likely to be the precursor than fucosterol. Fucosterol appears to be present only in Phaeophyceae and some Phycomycetes, whereas isofucosterol is widely distributed in trace amounts in higher plants (see Goad *et al.* 1969; Patterson, 1971), and has also been identified in some green algae (Gibbons, Goad & Goodwin, 1968*c*).

Formation of Δ^{25}*-bonds*

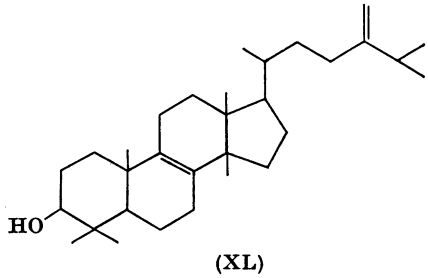
A number of triterpenoids have a 26-methylene instead of a methyl group; those we have been particularly interested in are cyclolaudenol (XLI) and 31-norcyclolaudenol (XLII), and a new sterol, 24-ethylcholesta-5,22,25-trien-3β-ol (XLIII) from *Clerodendrum campbellii* (Bolger, Rees, Ghisalberti, Goad & Goodwin, 1970). As already indicated insertion of the Δ^{22}-bond has no apparent relationship with alkylation at C-24, but insertion of a double bond at C-25–C-26 must have some effect because the shift of the hydrogen atom at C-24 to C-25, e.g. in fucosterol biosynthesis, cannot take place. In the methylated triterpene cyclolaudenol a mechanism indicated in Scheme 28 is probably involved. Experiments with [2-^{14}C,(4*R*)-4-$^3H_1$]-mevalonate showed that cyclolaudenol and 31-norcyclolaudenol, synthesized by rhizomes of *Polypodium vulgare* Linn., retained the hydrogen atom



Scheme 27. Possible pathways of alkylation in biosynthesis of phytosterols.



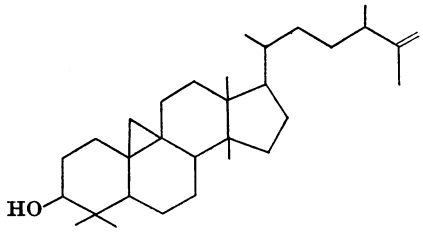
(XXXIX)



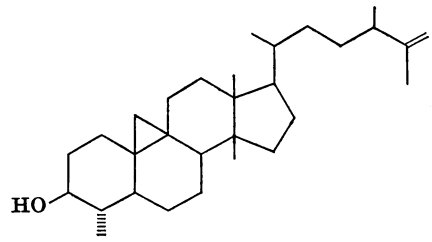
(XL)

originally present at C-24. Experiments with [*Me*-$^3H_3$]methionine indicated that all three hydrogen atoms of the methyl group of methionine were retained in cyclolaudenol, but a definitive experiment remains to be carried out with [*Me*-$^2H_3$]-methionine. Thus the probable mechanism of forming the double bond at C-25–C-26 involves methylation at C-24, with the formation of an intermediate carbonium ion, which is stabilized by loss of a proton at C-26 with double-bond formation (Scheme 28) (Ghisalberti, de Souza, Rees, Goad & Goodwin, 1969*a*).

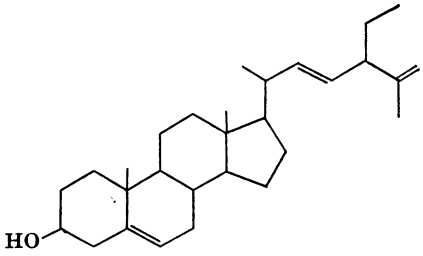
In the case of the Δ^{25}-sterol (XLIII) from *Clerodendrum campbellii* similar results were obtained in experiments with [2-^{14}C,(4*R*)-4-$^3H_1$]mevalonate, and again direct demonstration of the presence of a tritium atom at C-24 was achieved, by converting the sterol into 24-ethyl-25-oxo-26-norcholesta-5,22-dien-3β-yl acetate (XLIV) and isomerizing it to 24-ethyl-25-oxo-26-norcholesta-5,23-dien-3β-yl acetate (XLV). This was accompanied by loss of one tritium atom, which must have been located at C-24. Scheme 29 therefore indicates the probable mechanism involved (Bolger *et al.* 1970). The first alkylation involves the formation of a methylene derivative in which the hydrogen atom at C-24 migrates to C-25. Methylenecycloartenol is a likely intermediate; it is produced by *Clerodendrum*



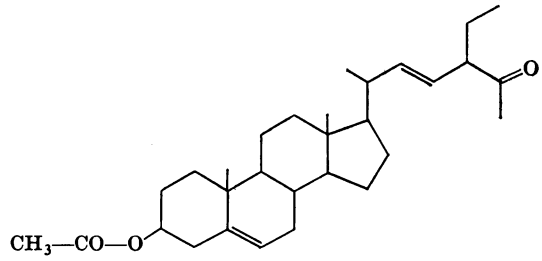
(XLI)



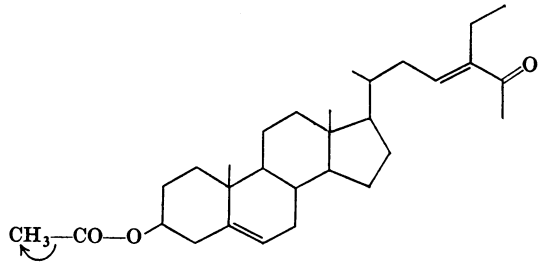
(XLII)



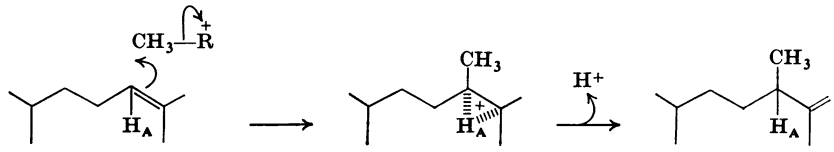
(XLIII)



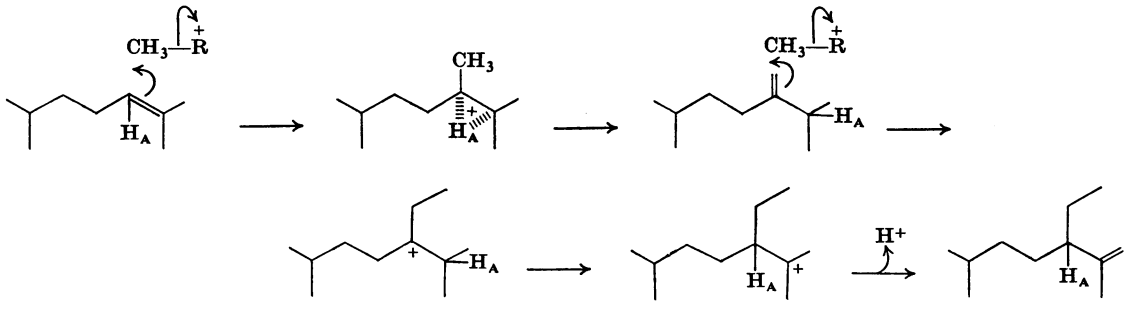
(XLIV)



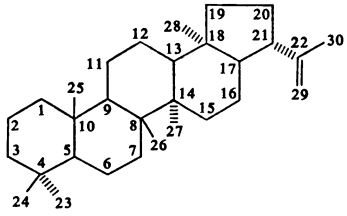
(XLV)



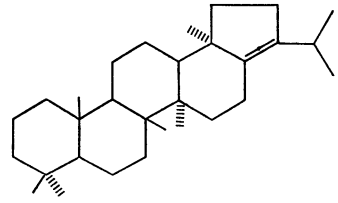
Scheme 28. Probable mechanism of methylation of Δ^{24}-sterols with the formation of Δ^{25}-sterols.



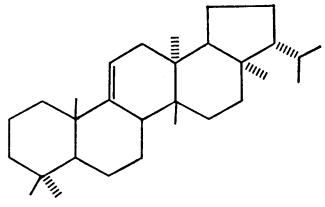
Scheme 29. Probable mechanism of ethylation of Δ^{24}-sterols with the formation of Δ^{25}-sterols.



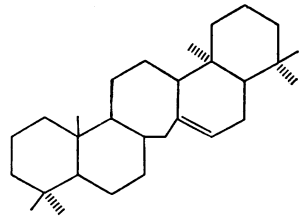
(XLVI)



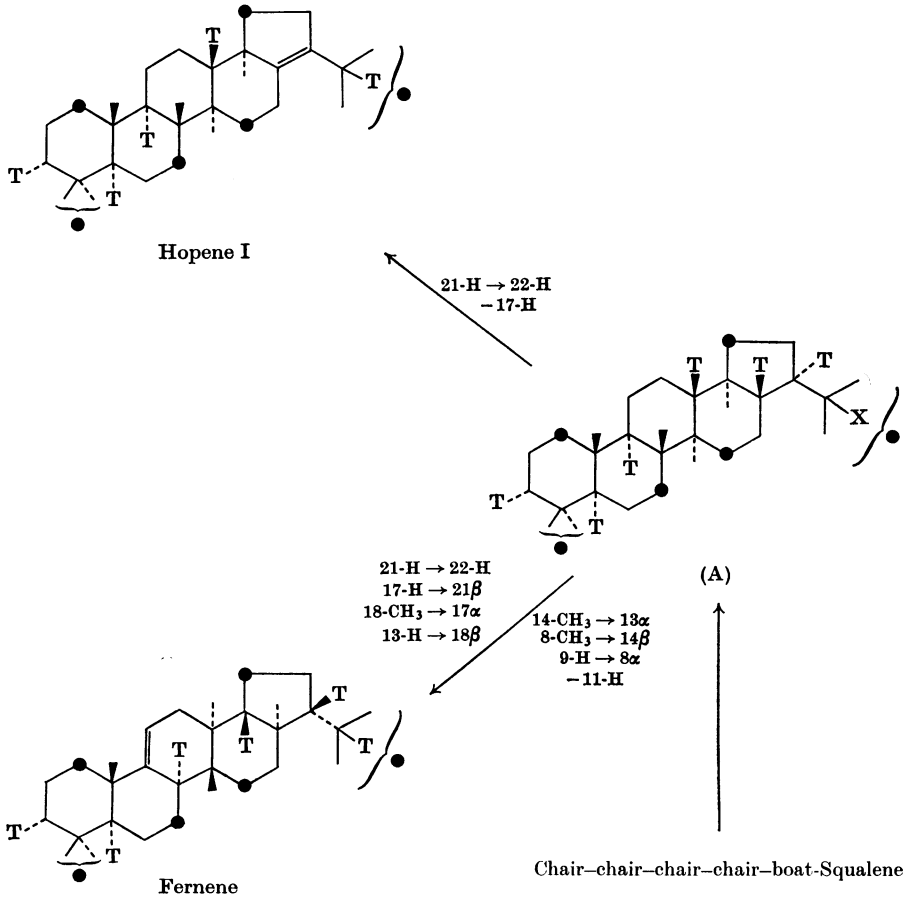
(XLVII)



(XLVIII)



(XLIX)



Scheme 30. Mechanism of formation of fernene and hopene I in *Polypodium vulgare*. ● indicates carbon atoms from C-2 of mevalonate; T indicates ^3H from [(4*R*)-4-$^3H_1$]mevalonate.

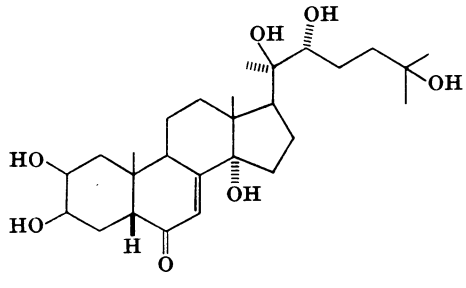
campbellii and retains all the tritium atoms when formed from [4*R*-4-$^3H_1$]mevalonate, as does cycloartenol. The second alkylation step involves the formation of a carbonium ion at C-24, followed by stabilization of the molecule by migration of the hydrogen atom at C-25 back to C-24 and elimination of a proton from C-26 with the simultaneous formation of a Δ^{25}-bond. A bonus from these two investigations was obtained during the degradation of the labelled terpenoids: in neither case was the methylene carbon (C-26) labelled with ^{14}C, indicating that the two terminal methyl groups never became equivalent during the biosynthetic sequence from squalene and that the methylene carbon in these compounds arose exclusively from C-3′ of mevalonate.

Pentacyclic triterpene hydrocarbons

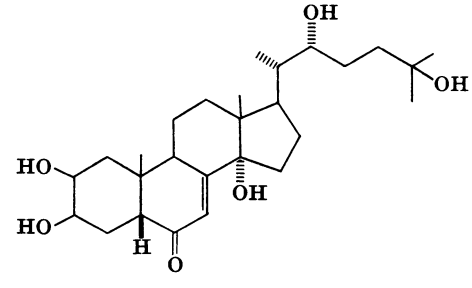
Pentacyclic triterpene hydrocarbons are rather rare in Nature, but *Polypodium vulgare* synthesizes a family of at least four, namely diploptene (XLVI), hopene I [hop-17 (21)-ene] (XLVII), fernene (XLVIII) and serratene (XLIX). It had recently been shown that these hydrocarbons were formed by proton-catalysed cyclization of squalene and that squalene 2,3-oxide was not involved (Barton, Gosden, Mellows & Widdowson, 1969). By using [2-^{14}C,(4*R*)-4-$^3H_1$]mevalonate we found that all six tritium atoms were retained in fernene and only five in hopene I. This indicates that no double bonds are involved in the final rearrangement leading to fernene (Ghisalberti, de Souza, Rees & Goodwin, 1970). A possible mechanism in which a product, (A), formed by cyclization of chair–chair–chair–chair–boat-squalene undergoes seven 1,2-shifts to form fernene with retention of all tritium atoms arising from C-4 of mevalonate, is illustrated in Scheme 30. The pathway to hopene-I is also indicated.

Biosynthesis of insect moulting hormones in plants

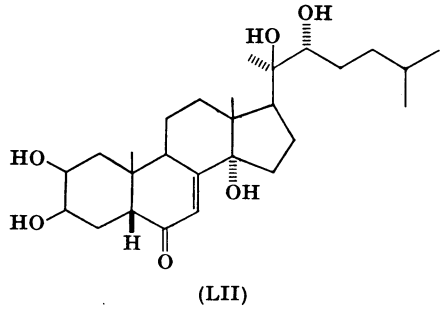
Ecdysterone (L) is widely distributed in many plants (see Berkoff, 1969) and occurs in the rhizomes of the fern *Polypodium vulgare* in concentrations up to 1.0% of the total dry matter (Jizba & Herout, 1967). The first insect moulting hormone isolated, ecdysone (LI), is much less widely distributed, but it does occur in *Polypodium vulgare*. We have been able to show that [^{14}C]mevalonate is incorporated into ecdysterone and ponasterone A (LII) in the leaves of *Taxus baccata*, but the incorporation is much slower than into the sterols and the mevalonate had to be painted on the leaves every alternate day for 30 days before significant label appeared in the products (de Souza, Ghisalberti, Rees & Goodwin, 1969). Similarly with *Polypodium vulgare* incorporation of [2-^{14}C]mevalonate into ecdysone, ecdysterone and 5β-hydroxyecdysterone (LIII) was slight in both leaves and rhizomes in spite of the high concentrations of the



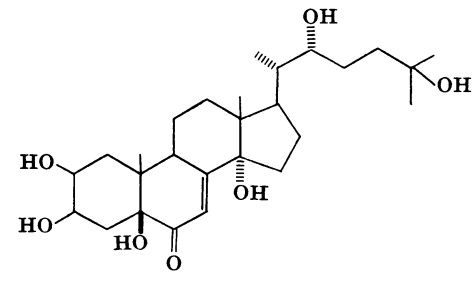
(L)



(LI)



(LII)



(LIII)

products in the rhizomes (de Souza, Ghisalberti, Rees & Goodwin, 1970). The significance of this low incorporation is not clear, but it suggests that the ecdysterones may be located in some organelle that exogenous mevalonate finds it difficult to enter. This tends to occur in carotenoid synthesis, which is localized in the chloroplast (see above).

In *Polypodium vulgare* [^{14}C]cholesterol was significantly incorporated into all three ecdysones, and traces of what appears to be 7-dehydrocholesterol were detected. Although this suggests the involvement of a $\Delta^{5,7}$-sterol in the biosynthetic pathway much work is needed before the pathway is fully clarified (de Souza *et al.* 1970).

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REFERENCES

Aitzetmüller, K., Strain, H. H., Svec, W. A., Grandolfo, M. & Katz, J. J. (1969). *Phytochemistry*, **8**, 1761.

Akhtar, M. & Marsh, S. (1967). *Biochem. J.* **102**, 462.

Akhtar, M., Parvez, M. A. & Hunt, P. F. (1966). *Biochem. J.* **100**, 38 c.

Akhtar, M., Watkinson, T. A., Rahimtula, A. D., Wilton, D. C. & Munday, K. A. (1969). *Biochem. J.* **111**, 757.

Alcaide, A., Devys, M. & Barbier, M. (1968). *Phytochemistry*, **7**, 329.

Archer, B. L., Barnard, D., Cockbain, E. G., Cornforth, J. W., Cornforth, R. H. & Popják, G (1966). *Proc. R. Soc.* B, **163**, 519.

Baisted, D. J., Gardner, R. L. & McReynolds, L. A. (1968). *Phytochemistry*, **7**, 945.

Barton, D. H. R., Gosden, A. F., Mellows, G. & Widdowson, D. A. (1969). *Chem. Commun.* p. 184.

Barton, D. H. R., Harrison, D. M. & Moss, G. P. (1966). *Chem. Commun.* p. 595.

Benveniste, P. (1968). *Phytochemistry*, **7**, 951.

Benveniste, P., Hirth, L. & Ourisson, G. (1966*a*). *Phytochemistry*, **5**, 31.

Benveniste, P., Hirth, L. & Ourisson, G. (1966*b*). *Phytochemistry*, **5**, 45.

Berkoff, C. E. (1969). *Q. Rev. chem. Soc.* **23**, 372.

Bimpson, T. (1970). Ph.D. Thesis: University of Liverpool.

Bimpson, T., Goad, L. J. & Goodwin, T. W. (1969*a*). *Chem. Commun.* p. 297.

Bimpson, T., Goad, L. J. & Goodwin, T. W. (1969*b*). *Biochem. J.* **115**, 857.

Bolger, L. M., Rees, H. H., Ghisalberti, E. L., Goad, L. J. & Goodwin, T. W. (1970). *Biochem. J.* **118**, 197.

Britton, G. & Goodwin, T. W. (1969). *Phytochemistry*, **8**, 2257.

Buggy, M. J., Britton, G. & Goodwin, T. W. (1969). *Biochem. J.* **114**, 641.

Bu'Lock, J. D., Austin, D. J., Snatzke, G. & Hruban, L. (1970). *J. chem. Soc.* D, p. 255.

Canonica, L., Fiecchi, A., Kienle, M. G., Scala, A., Galli, G., Paoletti, E. G. & Paoletti, R. (1968*a*). *Steroids*, **11**, 749.

Canonica, L., Fiecchi, A., Kienle, M. G., Scala, A., Galli, G., Paoletti, E. G. & Paoletti, R. (1968*b*). *Steroids*, **12**, 445.

Canonica, L., Fiecchi, A., Kienle, M. G., Scala, A., Galli, G., Paoletti, E. G. & Paoletti, R. (1968*c*). *J. Am. chem. Soc.* **90**, 3597.

Caspi, E., Grieg, J. B., Ramm, P. J. & Varma, K. R. (1968). *Tetrahedron Lett.* no. 35, p. 3829.

Caspi, E. & Ramm, P. J. (1969). *Tetrahedron Lett.* no. 3, p. 181.

Charlton, J. M., Treharne, K. J. & Goodwin, T. W. (1967). *Biochem. J.* **105**, 205.

Cornforth, J. W. & Cornforth, R. H. (1970). *Biochem. Soc. Symp.* no. 29, p. 5.

Costes, C. (1965). *Annls Physiol. vég., Paris*, **7**, 105.

Davies, B. H. (1970*a*). *Biochem. J.* **116**, 93.

Davies, B. H. (1970*b*). *Biochem. J.* **116**, 101.

Davies, B. H., Holmes, E. A., Loeber, D. E., Toube, T. P. & Weedon, B. C. L. (1969). *J. chem. Soc.* C, p. 1266.

Davies, B. H., Jones, D. & Goodwin, T. W. (1963). *Biochem. J.* **87**, 326.

Davis, J. B., Jackman, L. M., Siddons, P. T. & Weedon, B. C. L. (1961). *Proc. chem. Soc.* p. 261.

de Souza, N. J., Ghisalberti, E. L., Rees, H. H. & Goodwin, T. W. (1969). *Biochem. J.* **114**, 895.

de Souza, N. J., Ghisalberti, E. L., Rees, H. H. & Goodwin, T. W. (1970). *Phytochemistry*, **9**, 1247.

Eberle, M. & Arigoni, D. (1960). *Helv. chim. Acta*, **43**, 1508.

Ehrhardt, J. D., Hirth, L. & Ourisson, G. (1967). *Biochemistry, Easton*, **6**, 815.

Eimhjellen, K. E. & Liaaen-Jensen, S. (1964). *Biochim. biophys. Acta*, **82**, 21.

Eppenberger, U., Hirth, L. & Ourisson, G. (1969). *Eur. J. Biochem.* **8**, 180.

Eugster, C. H., Buchecker, R., Tscharner, C., Uhde, G. & Ohloff, G. (1969). *Helv. chim. Acta*, **52**, 1729.

Gershengorn, M. C., Smith, A. R. H., Goulston, G., Goad, L. J., Goodwin, T. W. & Haines, T. H. (1968). *Biochemistry, Easton*, **7**, 1698.

Ghisalberti, E. L., de Souza, N. J., Rees, H. H., Goad, L. J. & Goodwin, T. W. (1969*a*). *Chem. Commun.* p. 1401.

Ghisalberti, E. L., de Souza, N. J., Rees, H, H., Goad, L. J. & Goodwin, T. W. (1969*b*). *Chem. Commun.* p. 1403.

Ghisalberti, E. L., de Souza, N. J., Rees, H. H. & Goodwin, T. W. (1970). *Phytochemistry*, **9**, 1817.

Gibbons, G. F., Goad, L. J. & Goodwin, T. W. (1968*a*). *Chem. Commun.* p. 1212.

Gibbons, G. F., Goad, L. J. & Goodwin, T. W. (1968*b*). *Chem. Commun.* p. 1458.

Gibbons, G. F., Goad, L. J. & Goodwin, T. W. (1968*c*). *Phytochemistry*, **7**, 983.

Goad, L. J. (1970). *Biochem. Soc. Symp.* no. 45.

Goad, L. J., Gibbons, G. F., Bolger, L., Rees, H. H. & Goodwin, T. W. (1969). *Biochem. J.* **114**, 885.

Goad, L. J. & Goodwin, T. W. (1965). *Biochem. J.* **96**, 79 p.

Goad, L. J. & Goodwin, T. W. (1966). *Biochem. J.* **99**, 735.

Goad, L. J. & Goodwin, T. W. (1967). *Eur. J. Biochem.* **1**, 357.
Goad, L. J. & Goodwin, T. W. (1969). *Eur. J. Biochem.* **7**, 502.
Goad, L. J., Hammam, A. S. A., Dennis, A. & Goodwin, T. W. (1966). *Nature, Lond.*, **210**, 1322.
Goad, L. J., Williams, B. L. & Goodwin, T. W. (1967). *Eur. J. Biochem.* **3**, 232.
Goodwin, T. W. (1958). *Biochem. J.* **68**, 503.
Goodwin, T. W. (1971*a*). In *Aspects of Terpene Biochemistry*, p. 315. Ed. by Goodwin, T. W. London: Academic Press (Inc.) Ltd.
Goodwin, T. W. (1971*b*). In *Carotenoids*. Ed. by Isler, O. Basle: Birkhauser (in the Press).
Goodwin, T. W., Britton, G. & Walton, T. J. (1968). *Pl. Physiol., Lancaster*, **43** (Suppl.), 46.
Goodwin, T. W. & Goad, L. J. (1971). *Progress in Phytochemistry* (in the Press).
Goodwin, T. W., Land, D. G. & Sissins, M. E. (1956). *Biochem. J.* **64**, 486.
Goodwin, T. W. & Osman, H. G. (1953). *Biochem. J.* **53**, 541.
Goodwin, T. W. & Osman, H. G. (1954). *Biochem. J.* **56**, 222.
Goodwin, T. W. & Williams, R. J. H. (1965*a*). *Biochem. J.* **94**, 5 C.
Goodwin, T. W. & Williams, R. J. H. (1965*b*). *Biochem. J.* **97**, 28 C.
Goodwin, T. W. & Williams, R. J. H. (1966). *Proc. R. Soc.* B, **163**, 515.
Hall, J., Smith, A. R. H., Goad, L. J. & Goodwin, T. W. (1969). *Biochem. J.* **112**, 129.
Hemming, F. W. (1970). *Biochem. Soc. Symp.* no. 29, p. 105.
Hewlins, M. J. E., Ehrhardt, J. D., Hirth, L. & Ourisson, G. (1969). *Eur. J. Biochem.* **8**, 184.
Hill, H. M. & Rogers, L. J. (1969). *Biochem. J.* **113**, 31 P.
Howes, C. D. & Batra, P. P. (1970). *Biochim. biophys. Acta*, **222**, 174.
Jizba, J. & Herout, V. (1967). *Coll. Czech. chem. Commun.* **32**, 2867.
Jungalwala, F. B. & Porter, J. W. (1965). *Archs Biochem. Biophys.* **110**, 291.
Jungalwala, F. B. & Porter, J. W. (1967). *Archs Biochem. Biophys.* **119**, 209.
Kemp, R. H., Hammam, A. S. A., Goad, L. J. & Goodwin, T. W. (1968). *Phytochemistry*, **7**, 447.
Kushwaha, S. C., Subbarayan, C., Beeler, D. A. & Porter, J. W. (1969). *J. biol. Chem.* **244**, 3635.
Lederer, E. (1969). *Q. Rev. chem. Soc.* **23**, 453.
Lenton, J. R., Hall, J., Smith, A. R. H., Ghisalberti, E. L., Rees, H. H., Goad, L. J. & Goodwin, T. W. (1971). *Archs Biochem. Biophys.* (in the Press).
Liaaen-Jensen, S., Cohen-Bazire, G. & Stanier, R. Y. (1961). *Nature, Lond.*, **192**, 1168.
Malhotra, H. C., Britton, G. & Goodwin, T. W. (1969). *Phytochemistry*, **8**, 1047.
Malhotra, H. C., Britton, G. & Goodwin, T. W. (1970*a*). *J. chem. Soc.* D, p. 127.
Malhotra, H. C., Britton, G. & Goodwin, T. W. (1970*b*). *Int. J. Vitam. Res*, **40**, 315.
Malhotra, H. C., Britton, G. & Goodwin, T. W. (1970*c*). *FEBS Lett.* **6**, 334.
Malhotra, H. C., Britton, G. & Goodwin, T. W. (1970*d*). *Phytochemistry*, **9**, 2369.
Mallory, F. B., Conner, R. C., Landray, J. R. & Iyengar, C. L. (1968). *Tetrahedron Lett.* no. 58, p. 6103.
Mercer, E. I., Davies, B. H. & Goodwin, T. W. (1963). *Biochem. J.* **87**, 317.
Moss, G. P. & Nicolaidis, S. A. (1969). *Chem. Commun.* p. 1077.
Osske, G. & Schreiber, K. (1965). *Tetrahedron*, **21**, 1559.
Oster, M. O. & West, C. A. (1968). *Archs Biochem. Biophys.* **127**, 112.
Paliokas, A. M. & Schroepfer, G. J. (1967). *Biochem. biophys. Res. Commun.* **26**, 736.
Paliokas, A. M. & Schroepfer, G. J. (1968). *J. biol. Chem.* **243**, 453.
Patterson, G. W. (1971). *Lipids* (in the Press).
Petzold, E. N., Quackenbush, F. W. & McQuistan, M. (1959). *Archs Biochem. Biophys.* **82**, 117.
Ponsinet, G. & Ourisson, G. (1968). *Phytochemistry*, **7**, 757.
Popják, G. & Cornforth, J. W. (1966). *Biochem. J.* **101**, 553.
Porter, J. W. & Anderson, D. G. (1962). *Archs Biochem. Biophys.* **97**, 520.
Porter, J. W. & Anderson, D. G. (1967). *A. Rev. Pl. Physiol.* **18**, 197.
Raab, K. H., de Souza, N. J. & Nes, W. R. (1968). *Biochim. biophys. Acta*, **152**, 742.
Ramm, P. J. & Caspi, E. (1969). *J. biol. Chem.* **244**, 6064.
Rees, H. H., Britton, G. & Goodwin, T. W. (1968*a*). *Biochem. J.* **106**, 659.
Rees, H. H., Goad, L. J. & Goodwin, T. W. (1968*b*). *Biochem. J.* **107**, 417.
Rees, H. H., Goad, L. J. & Goodwin, T. W. (1968*c*). *Tetrahedron Lett.* no. 6, p. 723.
Rees, H. H., Goad, L. J. & Goodwin, T. W. (1969). *Biochim. biophys. Acta*, **176**, 892.
Rees, H. H., Mercer, E. I. & Goodwin, T. W. (1966). *Biochem. J.* **99**, 726.
Ryvarden, L. & Liaaen-Jensen, S. (1964). *Acta chem. scand.* **18**, 643.
Scharf, S. S. & Simpson, K. L. (1968). *Biochem. J.* **106**, 311.
Sharpless, K. B., Snyder, T. E., Spencer, T. A., Maheshwari, K. K., Guhn, G. & Clayton, R. B. (1968). *J. Am. chem. Soc.* **90**, 6874.
Sharpless, K. B., Snyder, T. E., Spencer, T. A., Maheshwari, K. K., Nelson, J. A. & Clayton, R. B. (1969). *J. Am. chem. Soc.* **91**, 3394.
Simpson, K. L. & Goodwin, T. W. (1965). *Phytochemistry*, **4**, 193.
Simpson, K. L., Nakayama, T. O. M. & Chichester, C. O. (1964). *J. Bact.* **88**, 1688.
Smith, A. R. H. (1969). Ph.D. Thesis: University of Liverpool.
Smith, A. R. H., Goad, L. J. & Goodwin, T. W. (1968*a*). *Chem. Commun.* p. 926.
Smith, A. R. H., Goad, L. J. & Goodwin, T. W. (1968*b*). *Chem. Commun.* p. 1259.
Smith, A. R. H., Goad, L. J., Goodwin, T. W. & Lederer, E. (1967). *Biochem. J.* **104**, 56 C.
Strain, H. H. (1958). *A. Priestley Lect. Penn. State Univ.* no. 32.
Subbarayan, C., Kushwaha, S. C., Suzue, G. & Porter, J. W. (1970). *Archs Biochem. Biophys.* **137**, 547.

Tefft, R. E., Goodwin, T. W. & Simpson, K. L. (1970). *Biochem. J.* **117**, 921.

Thornber, J. P., Olson, J. M., Williams, D. M. & Clayton, M. L. (1969). *Biochim. biophys. Acta*, **172**, 351.

Tomita, Y., Uomori, A. & Minato, H. (1970). *Phytochemistry*, **9**, 555.

van Aller, R. T., Chikamatsu, H., de Souza, N. J., John, J. P. & Nes, W. R. (1968). *Biochem. biophys. Res. Commun.* **31**, 842.

van Niel, C. B. (1947). *Antonie van Leeuwenhoek*, **12**, 156.

Volk, W. A. & Pennington, D. (1950). *J. Bact.* **59**, 169.

von Ardenne, M., Osske, G., Schreiber, K., Steinfelder, K. & Tümmler, R. (1965). *Kulturpflänze*, **13**, 101.

Walton, T. J., Britton, G. & Goodwin, T. W. (1969). *Biochem. J.* **112**, 383.

Walton, T. J., Britton, G., Goodwin, T. W., Diner, B. & Moshier, S. (1970). *Phytochemistry*, **9**, 2545.

Weedon, B. C. L. (1965). In *Chemistry and Biochemistry of Plant Pigments*, p. 75. Ed. by Goodwin, T. W. London: Academic Press (Inc.) Ltd.

Weeks, O. B. (1971). In *Aspects of Terpene Biochemistry*, p. 291. Ed. by Goodwin, T. W. London: Academic Press (Inc.) Ltd.

Williams, B. L., Goad, L. J. & Goodwin, T. W. (1967*a*). *Phytochemistry*, **6**, 1137.

Williams, R. J. H., Britton, G., Charlton, J. M. & Goodwin, T. W. (1967*b*). *Biochem. J.* **104**, 767.

Williams, R. J. H., Britton, G. & Goodwin, T. W. (1966). *Biochim. biophys. Acta*, **124**, 200.

Williams, R. J. H., Britton, G. & Goodwin, T. W. (1967*c*). *Biochem. J.* **105**, 99.

Williams, R. J. H., Davies, B. H. & Goodwin, T. W. (1965). *Phytochemistry*, **4**, 759.

Wilton, D. C., Munday, K. A., Skinner, S. J. M. & Akhtar, M. (1968). *Biochem. J.* **106**, 803.