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# TERPENOID BIOSYNTHESIS BY CHLOROPLASTS ISOLATED IN ORGANIC SOLVENTS

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**Key Word Index**—*Phaseolus vulgaris*, Leguminosae, dwarf bean, non-aqueous chloroplasts, terpenoids, biosynthesis, geranylgeraniol, phytoene

**Abstract**—Chloroplasts isolated in organic solvents ("non-aqueous chloroplasts") incorporate [2-$^{14}$C]-mevalonate into geranylgeraniol and phytoene No incorporation into phytol or into other carotenoids was achieved, even in mixtures of non-aqueous chloroplasts with chloroplasts prepared in aqueous media The incorporation of geranylgeranyl pyrophosphate into phytoene by the non-aqueous chloroplast preparation has been demonstrated, and the stereochemistry of hydrogen loss in phytoene synthesis by non-aqueous chloroplasts has been determined

## INTRODUCTION

CONSIDERABLE evidence is available to suggest that the carotenoid pigments normally present in the chloroplasts of higher plants are synthesized, *in situ*, in the plastids [1] Early attempts to demonstrate carotenoid synthesis from mevalonic acid in chloroplasts isolated by a variety of aqueous techniques met with little success, the failures being attributed to losses of water-soluble components from the plastids during isolation [2] Chloroplasts isolated in organic solvents ("non-aqueous chloroplasts") however, were found to be capable of incorporating [2-$^{14}$C]-mevalonate into the carotenoid precursor phytoene [3] Preliminary work[3] suggested that labelled phytol was also formed from [2-$^{14}$C]-mevalonate by this system, and radioactivity was also detected in a chromatographic fraction containing xanthophylls (oxygenated carotenoids)

The possible synthesis of phytol by this system has now been re-examined, with the use of more rigorous purification techniques Also, in order to evaluate the non-aqueous chloroplast preparation as a cell-free system suitable for studying the biosynthesis of chloroplast carotenoids, the ability of the system to incorporate mevalonate into carotenoids other than phytoene has been investigated, and the potential usefulness of the system for detailed studies of phytoene biosynthesis has been explored

## RESULTS AND DISCUSSION

Incubation of [2-$^{14}$C]-mevalonate (12 $\mu$Ci) with non-aqueous chloroplasts gave good incorporation (1 068 900 dpm, 8% of the active isomer) into lipid material, in agreement with the work of Charlton *et al* [3] Of the total radioactivity incorporated, 37% was located in the chromatographic fraction containing carotene hydrocarbons, 15% in the fraction

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[1] GOODWIN, T W (1971) in *Carotenoids* (ISLER, O, ed), p 577, Birkhauser Basel

[2] TREHARNE, K J (1964) Ph D Thesis, University College of Wales Aberystwyth

[3] CHARLTON, J M TREHARNE K J and GOODWIN T W (1967) *Biochem J* **105**, 205

containing xanthophylls, and 48% in the fraction expected to contain sterols, triterpenes and terpenoid alcohols

Examination of TLCs of the first (hydrocarbon) fraction by autoradiography and by scanning for radioactivity showed the presence of one main radioactive zone corresponding to phytoene Carrier amounts of phytoene, phytofluene, α- β- and ζ-carotenes and lycopene were added to this fraction (279 300 dpm), and each of these carotenoids was purified and assayed for radioactivity The phytoene was highly labelled (176 540 dpm recovered) but the other compounds were all inactive

Examination of silica gel TLCs of the third (xanthophyll) fraction by autoradiography revealed the presence of a strong radioactive zone corresponding to lutein This lutein band was isolated and shown to contain almost all the radioactivity present in this third fraction After rechromatography of the lutein fraction on MgO however autoradiography revealed that the radioactivity did not correspond to lutein but to approximately four colourless zones near the solvent front These have not been identified After addition of carriers, purification and radioassay of lutein, violaxanthin and neoxanthin showed that no radioactivity was associated with these xanthophylls but most (23 100 dpm) of the initial radioactivity (33 100 dpm) was recovered from the solvent front zone of the MgO chromatograms

The present work, in which more rigorous techniques have been used for purification of the carotenoids confirms the earlier finding[3] that the non-aqueous chloroplast system is capable of synthesizing phytoene from mevalonate (*ca* 1% conversion of the active isomer) and shows that no synthesis of phytofluene, ζ-carotene, lycopene or any of the normal bean leaf carotenoids occurs

Examination of silica gel TLCs of the second chromatographic fraction by autoradiography and scanning for radioactivity showed that the activity corresponded to the terpenoid alcohols (phytol), and not to the triterpene or sterol positions By reversed phase TLC, however, it was shown that the radioactivity was in fact associated with geranylgeraniol and not with phytol After addition of carrier amounts of geranylgeraniol phytol farnesol geranyllinalool and sterol, geranylgeraniol samples purified by TLC reversed phase TLC and GLC were found to be heavily labelled (total activity recovered 206 000 dpm, from initial 356 800 dpm) whereas similarly purified samples of the other compounds contained no radioactivity When a sample of geranylgeraniol (20 000 dpm) was hydrogenated, the radioactivity remained associated with the perhydrogeranylgeraniol after purification by TLC reversed phase TLC and GLC (total activity recovered, 11 200 dpm)

This work therefore shows that the non-aqueous chloroplast system will incorporate mevalonate into geranylgeraniol, and not into phytol as suggested by the earlier preliminary work [3] The geranylgeraniol is presumably formed from geranylgeranyl pyrophosphate by the action of phosphatases the presence of which has been demonstrated by gel electrophoresis *

The non-aqueous chloroplast system is therefore capable of performing the early reactions of carotenoid biosynthesis, i e the reactions leading to geranylgeranyl pyrophosphate and phytoene It is likely that these reactions take place in an aqueous environment, requiring soluble enzymes, and preparation of chloroplasts in organic solvents should minimize losses of water-soluble enzymes and cofactors which may be leached from chloroplasts prepared

* Willis S R unpublished results

in aqueous media The enzymes acting later in the pathway of carotenoid biosynthesis, i e the desaturating, cyclizing and hydroxylating enzymes, utilize water-insoluble hydrocarbon substrates, and it seems likely that organic solvents would adversely affect the non-polar environment in which these reactions take place, so that no reactions after phytoene production can occur Isolation of chloroplasts in aqueous media, however, should not adversely affect these enzymes in this way

In the previous work,[3] attempts were made to use mixtures of non-aqueous chloroplasts with chloroplasts prepared by aqueous techniques, since it was considered that such mixtures might supply all the components necessary for synthesis of the normal chloroplast carotenoids In no case was any incorporation of radioactivity from [2-$^{14}$C]-mevalonate into any carotenoid except phytoene detected In the present work, in similar incubations of mixtures of non-aqueous chloroplasts prepared by some more recently developed aqueous procedures,[4–6] mevalonate was incorporated only into geranylgeraniol and phytoene, and not into any other carotenoid It thus appears that the non-aqueous chloroplast preparation is not of potential use, even in mixtures with chloroplasts isolated in aqueous media, in studies of the biosynthesis of carotenoids occurring later than phytoene in the biosynthetic sequence

The system should, however, be useful for studying details of the biosynthesis of phytoene Some preliminary investigations of some aspects of phytoene biosynthesis were therefore undertaken to evaluate the potential usefulness of the system

The formation of phytoene is generally considered[1] to involve a "tail-to-tail" condensation of two molecules of geranylgeranyl pyrophosphate in a reaction somewhat similar to the formation of squalene from farnesyl pyrophosphate, and is now believed to involve an intermediate, prephytoene pyrophosphate[7] (prelycopersene pyrophosphate[8]), a compound analogous to presqualene pyrophosphate The case of lycopersene, the $C_{40}$ analogue of squalene, as a possible intermediate in phytoene biosynthesis has recently been revived [8] Two aspects of the synthesis of phytoene have been examined with the non-aqueous chloroplast preparation

A small amount of geranylgeranyl pyrophosphate-[$^{14}$C] (10 000 dpm) was available, and this was used as substrate in the non-aqueous chloroplast system The main labelled product isolated was free geranylgeraniol (total activity recovered 5220 dpm), again demonstrating the presence in the chloroplast preparation of active phosphatase enzymes, but *ca* 8% incorporation into phytoene (770 dpm recovered) was achieved No radioactivity was detected in lycopersene This therefore confirms the formation of phytoene from geranylgeranyl pyrophosphate in the non-aqueous chloroplast preparation, but insufficient labelled geranylgeranyl pyrophosphate was available to permit further study of this conversion

The demonstration of the direct incorporation of geranylgeranyl pyrophosphate into phytoene, and the fact that phytoene and geranylgeraniol are the main terpenoids produced from mevalonate in this system, prompted an investigation of the stereochemistry of hydrogen loss in the formation of phytoene Details of the experiment have been published elsewhere [9] One hydrogen atom, originally from C-5 of mevalonate, is lost from C-1 of each

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of the two molecules of geranylgeranyl pyrophosphate from which phytoene is formed Incubation of the non-aqueous chloroplast system with [2-$^{14}$C, (5*R*)-5-$^{3}H_1$]-mevalonate and [2-$^{14}$C, 5-$^{3}H_2$]-mevalonate allowed a direct comparison to be made between the $^{14}$C $^{3}$H atomic ratios of phytoene and those of geranylgeraniol which, as the pyrophosphate has been established as a direct precursor The results of these experiments showed that in each case it was the 1-*pro-S* hydrogen atom of geranylgeranyl pyrophosphate (originally the 5-*pro-S* hydrogen atom of mevalonate) that was lost and the 1-*pro-R* hydrogen atom (originally the 5-*pro-R* hydrogen atom of mevalonate) that was retained

This use of the non-aqueous chloroplast system therefore established the stereochemistry of hydrogen loss in the biosynthesis of phytoene, and this must be taken into account in any postulated mechanism of phytoene biosynthesis, via prephytoene pyrophosphate, and via lycopersene if this is confirmed as an intermediate No incorporation of mevalonate into lycopersene has been detected, however in any experiment with the non-aqueous chloroplast system Even in the presence of NADH and NADPH, when small amounts of squalene are produced either by chloroplast enzymes or by enzymes from small amounts of contaminating cytoplasm, phytoene remains the only $C_{40}$ hydrocarbon produced [3]

These findings therefore indicate the potential usefulness of the non-aqueous chloroplast system in detailed studies of the biosynthesis of phytoene Since the early work on the development of the non-aqueous chloroplast system was reported several other cell-free systems have been developed for studying carotenoid biosynthesis in green tissues of higher plants A cell-free system from pea fruits has been described[10,11] which will incorporate mevalonate into phytoene and also into squalene and kaurene This pea system probably contains extra- and intra-chloroplastidic material, and is obviously potentially useful for investigating the controlling factors responsible for channelling biosynthetic intermediates into terpenoids of different classes The conversion of lycopene into cyclic carotenes by bean leaf[12] and spinach[13] chloroplast systems has been demonstrated, and the incorporation of isopentenyl pyrophosphate and phytoene into lycopene and cyclic carotenes by spinach chloroplast preparations has been described [14]

The non-aqueous chloroplast preparation provides the opportunity to study the details of the biosynthesis of phytoene in a simple and efficient system, not complicated by the ability to form other carotenoids and other terpenoids

## EXPERIMENTAL

*Preparation of chloroplasts* Seedlings of dwarf bean (*Phaseolus vulgaris* var Lightning) were grown for 12 14 days at 20–24 in the dark, followed by 24 hr illumination The leaf tissues were then freeze-dried and a preparation of developing chloroplasts was obtained by the non-aqueous technique described by Charlton *et al* [3]

*Incubations* The chloroplast pellet suspended in 0 1 M potassium phosphate buffer pH 7 4, was subjected to ultrasonic disintegration for 1 min as previously described before addition of co-factors ATP (10 $\mu$mol), $MgCl_2$ (20 $\mu$mol) and GSH (20 $\mu$mol) and appropriate substrate The mixture, final vol 1 5 ml, was incubated at 25° for 6 hr Substrates used were [2-$^{14}$C]-mevalonic acid (as $K^+$ salt, 2 $\mu$Ci per 1 5 ml incubation) and [4 8 12 16-$^{14}$C]-geranylgeranyl pyrophosphate (10 000 dpm)

*Preparation of chloroplasts in aqueous media* Seedlings of dwarf bean were grown as described above, and chloroplast preparations were obtained by the methods of Walker [4] Jensen and Bassham[5] and Cockburn *et al* [6] These aqueous chloroplasts were mixed with non-aqueous chloroplasts before disruption and incubation with [2-$^{14}$C]-mevalonate as described above

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*Extraction and purification of reaction products* After incubation, the reaction mixtures were saponified and the unsaponifiable lipid material was extracted by standard procedures[15] and chromatographed in light petrol (b p 40–60°) on a column of neutral alumina (activity grade III) Three fractions, eluted respectively with 0 5% $Et_2O$ in light petrol, 30% $Et_2O$ in light petrol and 2% EtOH in $Et_2O$, were collected From the first and second fractions respectively phytoene and geranylgeraniol were isolated and purified in several successive TLC systems as described elsewhere[9] In experiments to examine other carotenoids for radioactivity, appropriate carriers were added to the column chromatographic fractions, and these carotenoids were separated and each was purified by TLC according to standard procedures[15] Chromatograms at different stages in the TLC purification procedure were examined for radioactivity by scanning and by autoradiography Geranylgeraniol (diluted with carrier, 2 mg) was hydrogenated, in cyclohexane, with $PtO_2$ as catalyst, and the product, perhydrogeranylgeraniol was purified on the same TLC and reversed-phase TLC systems as were used for geranylgeraniol

*GLC* Samples of geranylgeraniol and perhydrogeranylgeraniol were purified by preparative GLC at a column temp of 190° and gas flow-rate ($N_2$) of 5 ml/min, on a 1 8 m column of 10% cyclohexanedimethanol succinate on Chromosorb W in an Aerograph A-700 Autoprep instrument equipped with a variable effluent splitter

*Detection and assay of radioactivity* Autoradiography was performed as previously described,[3] and samples were assayed for radioactivity by liquid scintillation counting as previously described[9] TLCs were examined for radioactivity with a Panax RTLS-1A scanner

[4,8,12,16-$^{14}$C]-*Geranylgeranyl pyrophosphate* Labelled geranylgeranyl pyrophosphate was prepared (by Dr J R Vose) biochemically by incubating [2-$^{14}$C]-mevalonate with *Echinocystis macrocarpa* endosperm,[16] the kind gift of Dr C A West, University of California, Los Angeles

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