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THE STEREOCHEMISTRY OF HYDROGEN TRANSFER DURING THE REDUCTION OF C-20 ISOPRENOIDS IN HIGHER PLANTS

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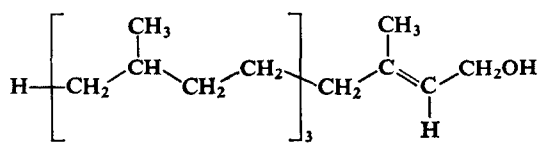
Abstract—Etiolated seedlings of *Phaseolus vulgaris* and *Avena sativa* were allowed to green in the light and to take up tritium-labelled NADPH (either 4R or 4S-diastereoisomers) by transpiration. A sterospecific transfer of hydrogen from the nicotinamide ring of NADPH (label from the 4R-diastereoisomer) to the isoprene residues of phytol and α-tocopherol during biogenesis was detected.

INTRODUCTION

SEVERAL aceclic terpenoid compounds having one or more saturated isoprenoid residue exist in nature. The saturated diterpenoid, phytol (I) has been shown to have three saturated



(I)

isoprene residues at the ω-end of the molecule.[1] The configuration of the ethylenic hydrogen atom with respect to the methyl group is *trans*[2] and the biogenesis of the saturated residues has been shown using [2-^{14}C-(4R)-4-^{3}H$_1$]- and [2-^{14}C-(4S)-4-^{3}H$_1$]-mevalonate, to be *trans* as well.[3]

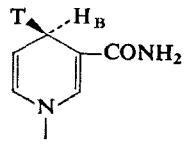
The phytyl side-chain also occurs naturally in higher plants in phylloquinone[4,5] where the residue nearest the quinone ring has the *trans* configuration.[6] There are reports of naphthoquinones with partially hydrogenated side-chains. In *Corynebacterium diphthiae* the presence of a menaquinone designated MK-8 (2H) has been reported,[7] whilst MK-9 (2H) has been reported in *Mycobacterium phlei*.[8] A recent report has shown both *cis* and

[1] F. G. FISCHER, *Liebigs Ann.* **469**, 69 (1928).
[2] J. W. K. BURRELL, L. M. JACKMAN and B. C. L. WEEDON, *Proc. Chem. Soc.* 263 (1959).
[3] A. R. WELLBURN, K. J. STONE and F. W. HEMMING, *Biochem. J.* **100**, 23c (1966).
[4] H. DAM, A. GEIGER, J. GLAVIND, P. KARRER, E. ROTHSCHILD and H. SALOMON, *Helv. Chim. Acta.* **22**, 310 (1939).
[5] P. KARRER and A. GEIGER, *Helv. Chim. Acta* **22**, 945 (1939).
[6] L. M. JACKMAN, R. RUËGG, G. RYSER, C. VON PLANTA, U. GLOOR, H. MAYER, P. SCHUDEL, M. KOFLER and O. ISLER, *Helv. Chim. Acta* **48**, 1332 (1965).
[7] P. B. SCHOLES and H. K. KING, *Biochem. J.* **97**, 766 (1965).
[8] P. H. GALE, P. H. ARISON, N. R. TRENNER, A. C. PAGE, K. FOLKERS and A. F. BRODIE, *Biochem.* **2**, 200 (1963).

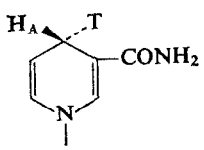
trans isomers of MK-9 (2H) occur in this organism.[9] A terminally saturated ubiquinone, Q-10 (2H-10) has been found in *Gibberella fujckuroi*.[10]

The side-chains of the tocopherolquinones and of the tocopherols bear a structural relationship to the phytyl side-chain save that all isoprene residues are saturated. However, a common biosynthetic origin would appear to be most likely.

The mechanism for the saturation of isoprene units is not understood. Cornforth *et al.* have shown that during the formation of squalene from farnesyl pyrophophate, several stereochemical features are present.[11] One of these, the stereospecificity of transfer of hydrogen from position 4 of the nicotinamide ring of NADPH, has been demonstrated using "A"-tritio NADPH (4R-diastereoisomer, II) and "B"-tritio NADPH (4S-diastereoisomer, III) respectively. The hydrogen atom H_B, or in their experiments the label from the



(II)



(III)

4S-diastereoisomer, exchanges with one of the hydrogens at C-1 of farnesyl pyrophosphate during the biosynthesis of squalene.

This paper describes a preliminary investigation into the reduction of certain isoprenoid compounds to be found in higher plants, with NADPH involved as a co-factor. In addition, a study of the stereochemistry of the hydrogen transfer from the nicotinamide ring is described.

RESULTS AND DISCUSSION

The introduction of labelled precursors or co-factors into higher plants is difficult. Impermeability of cell walls and selective permeability of cell membranes limit the number of techniques available and prevent the use of intact plants. Experiments involving injections of solution of labelled NADPH into the stems of *Phaseolus vulgaris* plants were unsuccessful. Insufficient label appeared in the isoprenoid fractions, presumably owing to the high efficiency of callus formation upon wounding. The conventional form of uptake by transpiration was adopted following this experience.

A dramatic synthesis of chlorophylls, plastoquinone and α-tocopherol occurs on greening etiolated seedlings[12] and the experiments were designed to take advantage of these changes during uptake of labelled NADPH. To accentuate the rapid formation of the chlorophylls, δ-aminolaevulinic acid, which is known to reduce the lag phase of greening,[13] was added to the incubation mixture. Mevalonate was also added in the hope that isoprenoid formation be enhanced and those moieties arising from the reduction of 3-hydroxy-3-methyl-glutaryl CoA to mevalonate (see later) be minimized. Both these considerations have yet to be substantiated by experiment.

The results of incubation of both *Phaseolus* and *Avena* seedlings with solutions containing either of the two tritium-labelled diastereoisomers of NADPH are shown in Table 1. Great

[9] P. J. DUNPHY, D. L. GUTNICK, P. G. PHILLIPS and A. F. BRODIE, *J. Biol. Chem.* **243**, 398 (1968).
[10] P. H. GALE, P. H. ARISON, N. R. TRENNER, A. C. PAGE and K. FOLKERS, *Biochem.* **2**, 196 (1963).
[11] J. W. CORNFORTH, R. H. CORNFORTH, C. DONNINGER, G. POPJÁK and G. J. SCHROEPFER, *Proc. R. Soc. B.* **163**, 492 (1966).
[12] W. T. GRIFFITHS, D. R. THRELFALL and T. W. GOODWIN, *Biochem. J.* **90**, 40P (1964).
[13] E. C. SISLER and W. H. KLEIN, *Physiol. Plantarum* **16**, 315 (1963).

TABLE 1. SPECIFIC ACTIVITIES OF ISOPRENOID COMPOUNDS ISOLATED FROM SEEDLINGS OF *Phaseolus vulgaris* AND *Avena sativa* AFTER TREATMENT WITH STEREOSPECIFICALLY LABELLED NADPH

Seedlings	Labelling of NADPH	Compound	Total disintegrations min^{-1}	Disintegrations min^{-1} $\mu mole^{-1}$	"Corrected" disintegrations min^{-1} $\mu mole^{-1}$
Phaseolus vulgaris	4R-diastereo isomer	Plastoquinone	5·5	14·1	0·0
		Phytyl acetate	1978·1	443·5	437·2
		α-Tocopheryl acetate	2759·8	574·9	568·6
		Squalene	43·8	13·8	4·4
		Sterol	35·3	16·0*	8·9*
	4S-diastereo isomer	Plastoquinone	21·2	23·0	0·0
		Phytyl acetate	339·8	95·2	85·0
		α-Tocopheryl acetate	42·4	16·1	5·9
		Squalene	49·6	40·6	23·3
		Sterol	50·0	35·7*	24·0*
Avena sativa	4R-diastereo isomer	Plastoquinone	9·5	21·7	0·0
		Phytyl acetate	1472·8	412·5	402·9
		α-Tocopheryl acetate	1239·8	444·2	434·6
		Squalene	106·0	18·9	4·4
		Sterol	44·3	14·3*	3·3*
	4S-diastereo isomer	Plastoquinone	9·0	15·2	0·0
		Phytyl acetate	46·2	15·6	8·9
		α-Tocopheryl acetate	80·1	33·1	26·4
		Squalene	379·2	74·2	64·1
		Sterol	211·8	66·2*	58·5*

* Disintegrations min^{-1} mg^{-1}.

care was taken to ensure the purity of each isoprenoid. During each chromatography on thin-layer plates, only the centre of the band of chromatographed material was eluted from the plate and therefore only the specific activity of each compound is significant.

To eliminate errors arising from randomization of tritium label during biogenesis or of incorporation of tritium from labelled NADPH during the formation of mevalonate, plastoquinone was used as an internal standard. It has been assumed that plastoquinone (PQ-9) has zero incorporation of label. The small amount of label incorporated into this compound was therefore assumed to be due to non-stereospecific reactions. The specific activities of the other compounds were therefore corrected by subtracting, in proportion to the isoprene units they contain, an amount related to the observed incorporation into plastoquinone. This correction makes little difference to the overall result, as few counts were found in any plastoquinone sample.

Owing to difficulties in purification, the specific activities of the sterols cannot be readily calculated. The "corrected" specific activities for sterol assume 1 μmole of plastoquinone-45 to be equivalent to 0·748 mg.

The results show that both phytol, and the closely related side-chain of α-tocopherol derive the hydrogen atoms for saturation stereospecifically from NADPH; in this case hydrogen atom H_A because the label is incorporated from the 4R-diastereoisomer.

The specific activities of those compounds of sesquiterpenoid origin are much lower. A slight preference for the stereospecific transfer of hydrogen atom H_B (the label coming

from the 4S-diastereoisomer) during the exchange of hydrogen occurring during the "tail-to-tail" condensation of C-15 units agrees with the findings of Cornforth *et al.*[11] who used rat-liver microsome preparations. The low specific activities found for squalene and the sterol fraction are most likely explained by the inhibition of sterol biosynthesis during greening.[14]

The full details of the transformation of the geranyl–geranyl unit, as the free alcohol, pyrophosphate or in other combination, to the phytyl chain, either in the free state or esterified in some way, have yet to be evaluated. Esterification with chlorophyllide takes place after photoreduction of protochlorophyllide[15] but the esterifying moiety has not been determined. The nature of the hydrophobic alcohol of protochlorophyll, which has been suggested not to be phytol,[16] may be significant. Consequently, it is not possible to decide in what form, or at what stage in chlorophyll biosynthesis, the geranyl–geranyl chain occurs immediately before stereospecific transfer to the ω-end of the molecule.

The case of α-tocopherol is slightly different. The existence of a complete series of tocotrienols comparable with that of the tocopherols[17] would indicate that the reduction of isoprenoid residues is a late stage in the biosynthesis of these compounds. Current opinion favours the side-chain of the tocotrienols to be derived from geranyl–geranyl-pyrophosphate and the tocotrienols to be the precursors of the tocopherols. It is not known if hydrogenation, involving the stereospecific transfer of hydrogen from NADPH, takes place before or after methylation of the benzenoid ring or chromanol formation but it seems certain to occur after attachment of the geranyl–geranyl side-chain to the quinone ring.

EXPERIMENTAL

A. Preparation of [4-^{3}H]-NADP$^+$ and the Formation of "A"-Tritio and of "B"-Tritio NADPH

Essentially the modified method of San Pietro[18] used by Cornforth *et al.*[11] was followed to prepare 65·4 μmoles of [4-^{3}H]-NADP$^+$ starting from 72·0 μmoles of NADP$^+$ (Sigma). The ^{3}H-content of the preparation was determined in a Nuclear Enterprises Scintillation Spectrometer (Model NE 8303) and reflector-coated vials and 5 ml of NE 240 scintillation fluid. The specific activity of the preparation was slightly higher than that reported by Cornforth *et al.*,[11] being $1{\cdot}34 \times 10^5$ disintegrations min^{-1} μmole^{-1}.

The reduction of [4-^{3}H]-NADP$^+$ to the "A"-tritio and "B"-tritio NADPH was accomplished with "B"-specific glucose-6-phosphate dehydrogenase and "A"-specific isocitrate dehydrogenase, together with appropriate substrates, as described by Cornforth *et al.*[11] Each incubation consisted of 0·1 M potassium phosphate buffer (pH 7·5), 5 mM $MgCl_2$ and 1 mM [4-^{3}H]-NADP$^+$ (10 ml) together with either 100 μg of glucose-6-phosphate dehydrogenase (Boehringer) dissolved in 0·4 ml of 2 per cent (w/v) bovine serum albumin (Sigma) and 20 μmoles of glucose-6-phosphate (B.D.H.), or 2 mg of isocitrate dehydrogenase (Boehringer) and 40 μmoles of DL-isocitrate (B.D.H.) in 2 per cent (w/v) bovine serum albumin (0·2 ml). Both solutions were incubated in stoppered test-tubes for 15 min at 37°.

Before uptake of label by seedlings 10 ml of 0·1 M potassium phosphate buffer, containing 30 μmoles of DL-mevalonic acid lactone (B.D.H.) and 10 μmoles each of ATP, di-sodium salt and δ-amino-laevulinic acid (Sigma), corrected to pH 7·5 with 6 N KOH was added to each of the solutions containing "A"-tritio or "B"-tritio NADPH.

B. Sources of Plant Material and Conditions of Incubation

Surface sterilized seeds of *Phaseolus vulgaris* var. Brown Dutch Haricot and *Avena sativa* var. Victory were germinated on vermiculite and allowed to grow for 6 days in the case of kidney beans, and for 3 days in the case of oats, in total darkness at 28°. Prior to incubation, the stems of the etiolated oat seedlings (about 6 cm high) were severed under water just above the vermiculite surface with a new razor blade. The beans (about

[14] A. S. A. HAMMAM, unpublished observations quoted by T. W. GOODWIN, *Biosynthetic Pathways in Higher Plants* (edited by J. B. PRIDHAM and T. SWAIN), p. 60, Academic Press, New York and London (1965).
[15] J. H. C. SMITH, *Comparative Biochemistry of Photoreactive Systems*, p. 257, Academic Press, New York and London (1960).
[16] F. G. FISCHER and W. RÜDIGER, *Ann. Chem.* **627**, 35 (1959).
[17] J. F. PENNOCK, F. W. HEMMING and J. D. KERR, *Biochem. Biophys. Res. Commun.* **17**, 542 (1964).
[18] A. SAN PIETRO, *J. Biol. Chem.* **217**, 579 (1955).

10 cm high) were cut in similar manner 5–6 cm below the cotyledons. Both the oat and bean seedlings were divided into equal portions by weight when the cut ends of the stems were transferred to small weighed reservoirs containing the prepared solutions of either "A"-tritio or "B"-tritio NADPH.

The cut seedlings were allowed to take up the labelled solutions by transpiration when placed in the growth chamber of an orbital incubator (Gallenkamp), with all 7 × 77 cm warm white fluorescent lights and circulation fans switched on and the temperature regulated to 28°. The light flux at plumule height, 25 cm below the lights, estimated with a light-meter (Type D7, Megatron Ltd.), was 6 klux or $1{\cdot}8 \times 10^3$ erg $sec^{-1}cm^{-2}$ over the range 400–700 nm calculated from the conversion figures given by Gaastra.[19] Uptake of most of the label under these conditions took about 4 hr. The labelled solution was followed in by 0·075 M potassium phosphate buffer for 24 hr and finally by sterilized tap-water for 44 hr. During this time, the oat leaves greened and lengthened by 0·3–0·5 cm, whilst the plumular hook of the etiolated beans uncurled and the first pairs of leaves were exposed and greened.

C. Lipid Extraction and Purification Procedures

Incubated cut seedlings of *P. vulgaris* were severed just above the level of the cotyledons whilst the stems of *A. sativa* were cut again 2 cm above the previous cut end. The lowermost portions were discarded. 75 g each of the incubated beans and 50 g each of the incubated oats, representing approximately 95 per cent of the total batch in each case, were extracted three times by maceration in 80 per cent (v/v) acetone (approx. 2 ml/g tissue) using an Ultraturrax gun homogenizer in bursts of 20 sec duration and filtered each time through a sintered-glass funnel and under reduced pressure. The combined acetone extracts, diluted with water (1:3), were extracted three times with freshly distilled di-ethyl ether. The ethereal extracts were washed with water, dried (Na_2SO_4), evaporated and the residues were blown to dryness under N_2.

The whole lipids were taken up in light petroleum (b.p. 40–60°) and chromatographed on 50-g columns of alumina (Woelm, weakened to Brockmann Grade III) using 1 l. of 2·5 per cent and 20 per cent (v/v) di-ethyl ether in petrol (E/P) and 5 per cent (v/v) methanol in ether in turn as eluents. The bulk of each fraction was reduced by distillation and the residues blown to dryness under N_2.

Each fraction was dissolved in 5 per cent (w/v) pyrogallol in 95 per cent (v/v) ethanol (5 ml) and saponified, to reduce problems arising from high levels of labelling in esterified fatty acids, with 60 per cent (w/v) KOH (2·5 ml) whilst gently refluxing for 30 min. The fractions were allowed to cool and diluted (1:3) before extraction three times with ether. The combined ethereal extracts were washed, dried and blown to dryness as described previously.

The saponified polar fractions containing free phytol were dissolved in redistilled toluene (1 ml), excess redistilled acetic anhydride together with two drops of redistilled pyridine added, and the mixture left overnight at room temperature. Afterwards, the mixture was poured slowly into ice-cold 5 N HCl and the whole extracted three times with 50 per cent (v/v) ether in petrol. The bulked extracts were washed with 5 N HCl to remove traces of pyridine and with water to remove acidic components. The extracts were dried, evaporated and blown to dryness as above.

The acetylated fractions were dissolved in cyclohexane and chromatographed as lines (7 mg lipid per plate) on layers of silica gel G (275 μ thick) with 1 per cent (v/v) methanol in benzene as developing solvent. All 20 × 20 cm silica gel plates used for TLC were freed from lipid by a preliminary development with freshly distilled di-ethyl ether. The position of the phytyl acetate bands, which had similar chromatographic properties to those of authentic phytyl acetate (R_f=0·54), were indicated by spraying with 0·01 per cent (w/v) fluorescein in ethanol,[20] eluted with ether and blown to dryness under N_2. The phytyl acetate fractions were purified a further three times with a different developing solvent each time (first solvent, 10 per cent (v/v) iso-propyl ether in petrol, R_f=0·57; second solvent, 0·5 per cent (v/v) methanol in benzene, R_f=0·41; third solvent, chloroform, R_f=0·67).

The purity of the phytyl acetate sample was checked by gas–liquid chromatography in a Pye 104 chromatogram (Model 4) and a 3 ft column packed with 10 per cent PEGA on 100/120 mesh Celite operated isothermally at 185° with a carrier gas (argon) flow rate of 45 ml/min^{-1}. Only a single peak with a retention time of 16·7 min corresponding to that of authentic phytyl acetate was detected.

Those saponified fractions, previously eluted from alumina columns with 20 per cent E/P, were chromatographed in the two-dimensional TLC systems of Pennock *et al.*[17] The materials having identical chromatographic properties to those of authentic α-tocopherol and stigmasterol on a marker plate, were eluted with ether, and blown to dryness as described above. The α-tocopherol fractions were acetylated to ensure there was no possibility of uptake of label by free hydroxyl groups, and the acetate purified by TLC a further three times (first solvent, 1 per cent (v/v) methanol in benzene, R_f=0·57; second solvent, 10 per cent (v/v) iso-propyl ether in petrol, R_f=0·71; third solvent, chloroform, R_f=0·62). The sterol fractions were also purified in like manner using authentic stigmasterol as a marker compound (first solvent, 3 per cent (v/v) methanol in benzene, R_f=0·43; second solvent, 20 per cent (v/v) ethyl acetate in benzene, R_f=0·29; third solvent, chloroform, R_f=0·34).

[19] P. GAASTRA, *Mededel. Landbouwhogeschool Wageningen* **59** (13), 1 (1959).
[20] P. J. DUNPHY, K. J. WHITTLE and J. F. PENNOCK, *Chem. Ind.* 1217 (1965).

The saponified non-polar fractions were also chromatographed as lines on TLC using 50 per cent (v/v) benzene in petrol as developing solvent. Bands with chromatographic properties similar to those of authentic squalene (R_f=0·70) and plastoquinone (PQ-9, R_f=0·27) were eluted with ether and blown to dryness under N_2. They were purified a further three times using different developing solvents each time (squalene: first solvent, 1 per cent (v/v) ether in petrol, R_f=0·47; second solvent, 0·5 per cent (v/v) methanol in benzene, R_f=0·64; third solvent, chloroform, R_f=0·74) (PQ-9: first solvent, 0·5 per cent (v/v) methanol in benzene, R_f=0·59; second solvent, benzene, R_f=0·46; third solvent, chloroform, R_f=0·74).

D. Radioactivity Counting Procedure

Reflector coated vials were dried under vacuum and weighed on an Oertling 147 microbalance to constant weight (±0·01 mg). The lipid samples were dissolved in cyclohexane and equal portions transferred to duplicate vials. The solvent was removed slowly under a stream of N_2. The vials were dried and reweighed. Scintillation fluid (10 ml, Type NE240, Nuclear Enterprises) was added to each vial and counting was carried out with a Nuclear Enterprises Automatic Liquid Scintillation Spectrometer (Model NE8303). Counts were corrected for background, quenching and the efficiency of counting.