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ISOLATION AND PROPERTIES OF A MONOTERPENE REDUCTASE FROM ROSE PETALS

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Abstract—Geraniol and nerol were reduced to citronellol by a solubilized enzyme preparation from rose petals. The terpene reductase was specific for primary terpene alcohols with a *cis* or *trans* allylic double bond. The cofactor requirement was fulfilled only by NADPH.

INTRODUCTION

AMONG the major terpenoids found in rose petals are the diunsaturated *trans* and *cis* monoterpene alcohols geraniol and nerol and the corresponding 2,3-dihydro derivative citronellol. The natural occurrence of partly saturated terpenoids is quite widespread and includes phytol in plants, some sterols in animals, the partly saturated menaquinones in the bacteria and ubiquinones in some fungi. The biosynthesis of these compounds probably occurs by enzymic reduction of the appropriate double bond. Phytol biosynthesized in leaves of *Ficus elastica* was shown to be biogenetically *trans*[1] possibly arising from geranylgeraniol (or pyrophosphate) presumably by hydrogen addition. Wilton *et al.*[2] have demonstrated the NADPH dependent reduction of 7-dehydrocholesterol to cholesterol using a rat liver microsomal preparation while Potty and Bruemmer have shown the enzymic reduction of the terminal isopropylidene group of limonene using an enzyme system from orange juice vesicles.[3] Similarly, cell free extracts from *Mentha piperita* convert ^{14}C-pulegone, in an anaerobic NADPH dependent reaction, into menthone and isomenthone.[4]

This paper reports on the enzymatic reduction of monoterpene alcohols by a cell free extract from rose petals and outlines some properties of the reductase.

RESULTS AND DISCUSSION

In the enzyme extract prepared from the acetone powder 89% of the geraniol reductase activity remained in the supernatant after centrifugation at 100 000 *g* for 3 hr.

Geraniol, nerol and a number of other compounds were reduced by the enzyme preparation to the corresponding dihydro derivatives. Table 1 shows the specificity of the enzyme(s) for various terpenoid and non-terpenoid substrates. Clearly the enzyme appears to be specific for acyclic terpene alcohols with a primary allylic hydroxyl group, while the other primary allylic compounds, tertiary allylic alcohols and cyclic terpenols were not reduced to any extent. The data reveal that the most favoured substrates for the enzyme(s) are geraniol and nerol, the only product being citronellol. The identity of the enzymatic product from geraniol was confirmed to be the 2,3-dihydroderivative by combined GLC–MS. The position of saturation in the product was demonstrated by GLC comparison with standards

[1] A. R. WELLBURN, K. J. STONE and F. W. HEMMING, *Biochem. J.* **100**, 23C (1966).
[2] D. C. WILTON, K. A. MUNDAY, S. J. M. SKINNER and M. AKHTAR, *Biochem. J.* **106**, 803 (1968).
[3] V. H. POTTY and J. H. BRUEMMER, *Phytochem.* **9**, 2319 (1970).
[4] J. BATTAILE, A. J. BURBOTT and W. D. LOOMIS, *Phytochem.* **7**, 1159 (1968).

TABLE 1. SPECIFICITY OF THE TERPENOID REDUCTASE

Substrate	Product	Relative reductase activity
Nerol	Citronellol	100
Geraniol	Citronellol	100*
Geranial	Citronellol, geraniol†	90
Geranyl acetate	‡	0
Geranyl pyrophosphate	—	0
Geranic acid	—	0
Linalool	—	0
cis/*trans*-Farnesol	2,3-Dihydrofarnesol	34
Limonene	—	0
Carveol	Dihydrocarveol	12
(±)-Carvone	—	0
Pulegone	—	0
trans-2-Hexenol	*n*-Hexanol	8

* Activity corresponds to 57% conversion of geraniol to citronellol after 60 min at 30° using substrates and cofactors at levels given in the Experimental.
† Both geraniol and citronellol produced by enzymatic reduction.
‡ 30% hydrolysis to geraniol occurred.

of citronellol, 6,7-dihydronerol, and 6,7-dihydrogeraniol (Table 2) synthesized by hydrazine hydrate reduction. There was no evidence for the enzymatic formation of the 6,7-dihydroterpenols. Further analysis of the enzymatic reduction product by argentation TLC (solvent 30% EtOAc in toluene) again confirmed that the only product formed was β-citronellol (R_f 0·57) while there was no evidence for the formation of the α-isomer (R_f 0·43) with the double bond in the terminal position (i.e. Δ^7). Incubation of citronellol with NADP failed to produce either geraniol or nerol indicating that the reductase did not catalyse the desaturation of citronellol to the allylic alcohols. Trace amounts of citronellal however were produced due to the presence of a terpenoid dehydrogenase in the preparation.

TABLE 2. GLC ANALYSIS OF ENZYMATIC REDUCTION PRODUCTS

Compound	Relative retention time*
Nerol	1·85
6,7-Dihydronerol	1·27
Geraniol	2·17
6,7-Dihydrogeraniol	1·47
Tetrahydrogeraniol	1·00
2,3-Dihydrogeraniol (citronellol)	1·54
Enzymatic product from nerol	1·54
Enzymatic product from geraniol	1·54

* Retention time for tetrahydrogeraniol = 7 min. For GLC conditions see Experimental.

The rate of formation of citronellol was directly proportional to the protein concentration over the range measured (i.e. up to 4 mg protein). The relative rates of reduction of geraniol and nerol were similar, indicating that the enzyme(s) was capable of reducing either a *cis*

TABLE 3. COFACTOR REQUIREMENT OF THE TERPENOID REDUCTASE

Cofactor	Substrate	% conversion to citronellol
NADPH	Geraniol	46
NADH	Geraniol	1
NADPH	Nerol	38
NADH	Nerol	2
Ascorbic acid	Geraniol	0
Hydroquinone	Geraniol	0

Substrate and nucleotide concentration and incubation conditions as in the Experimental.

or *trans* double bond, citronellol being formed from nerol at the rate of 2·12 and from geraniol at 1·97 mμmol/min/mg protein (1 mg soluble protein derived from 40 mg acetone powder). Reduction was completely dependent on the addition of NADPH as a cofactor and substitution by NADH, ascorbic acid or hydroquinone resulted in little or no formation of citronellol (Table 3). The enzymic formation of citronellol from geraniol showed a pH optimum at 8 falling off to 18% of maximum at pH 6·0 and 25% at pH 10·0.

TABLE 4. EFFECT OF INHIBITORS ON THE TERPENOID REDUCTASE

Incubation mixture*	citronellol formed mμmol
Control	60
Control + 0·5 mM PCMS	0
Control + 0·5 mM PCMS + 5 mM glutathione†	58
Control + 4 mM iodoacetic acid	59
Control + 4 mM *n*-ethylmaleimide	60

* Components and conditions as in the Experimental.

† *p*-Chloromercuriphenyl sulphonic acid (monosodium salt) preincubated with the enzyme preparation for 10 min prior to the addition of reduced gluthathione.

The effect of various sulphydryl reagents on the reductase is shown in Table 4. The enzymatic reduction of geraniol was completely inhibited by the addition of *p*-chloromercurcuriphenyl sulphonate and the inhibition reversed to the extent of 96% by reduced glutathione. Iodoacetate and *n*-ethylmaleimide were without effect on the enzyme. The ability of PCMS to inhibit the enzyme indicates at best the presence of one or more sulphydryl groups sufficiently near the active centre of the enzyme to interfere with its catalytic properties probably by combining directly with a —SH group rather than by denaturation since inhibition was completely reversed by reduced glutathione. The inability of iodoacetate or *n*-ethylmaleimide to effect inhibition may be a reflection of the relative inactivity of these —SH reagents when compared with the mercurials. A number of enzymes including urease[5,6] and lactic dehydrogenase[7] show inhibition of activity through —SH binding with

[5] L. HELLERMAN, *Cold Spring Harbor Symp. Quant. Biol.* **7**, 165 (1939).
[6] L. HELLERMAN, F. P. CHINARD and V. R. DEITZ, *J. Biol. Chem.* **147**, 443 (1943).
[7] J. B. NEILANDS, *J. Biol. Chem.* **208**, 225 (1954).

mercurials while compounds such as iodoacetate and iodosobenzoate though reacting with —SH groups in these enzymes do not interfere with their catalytic properties. Therefore the data on the reductase enzyme may be interpreted on the basis of the inaccessibility (differential reactivity) of specific enzyme —SH groups and or the different reactivities of the various —SH reagents with a sulphydryl group(s) at or near the active centre of the enzyme.

Clearly the presence of this enzyme in petal extracts accounts for the mode of synthesis of citronellol. Similar enzyme preparations from *Rosa damascena* Kazanlik showed the presence of the reductase, though this enzyme was not demonstrated in cell free extracts from petals of the hybrid tea rose 'Lady Seton'. Unlike *Rosa damascena* which contained citronellol as the major monoterpene alcohol, 'Lady Seton' produced only trace amounts of this compound. Geraniol or nerol can act equally well as precursors resulting in the formation of the same product. The apparent ability of the enzyme to bring about the reduction of both the *cis* and *trans* isomers can be accounted for in a number of ways. It is possible that one enzyme was involved in the reduction of both isomers, or that one enzyme reduced the *cis* and another the *trans*, or that only one isomer was reduced, the reduction of the compound of opposite geometry occurring after isomerization. The latter seems unlikely since both geraniol and nerol were reduced at about the same rate nor was there any evidence for the enzymatic formation of geraniol when nerol was the substrate or vice versa. It seems more likely that one enzyme is involved in the reduction of either the *cis* or *trans* isomer and this might occur through binding by the enzyme to and in the plane of the double bond of the substrate followed by attack by hydride ion (from NADPH) from the opposite unhindered side of the molecule.

Specificity studies with geranial as substrate were difficult to interpret since the enzyme preparation contained a very active NADP/NADPH terpene dehydrogenase which rapidly reduced geranial to geraniol, the latter compound acting as a substrate for the reductase. Therefore it was not possible at this stage of enzyme purification to exclude the enzymic hydrogenation of geranial to citronellal and subsequent reduction of the aldehyde to the alcohol.

In common with other known reductases, the reaction was NADPH dependent and appeared to have a requirement for —SH group(s).[8,9] The irreversibility of the reductase is in agreement with the findings of Steinberg and Avigan[8] on the Δ^{24}-sterol reductase from rat liver microsomes. Soluble enzyme extracts from a strain of *Myobacterium globiforme* was shown to effect both the reduction of Δ^{1}-sterols and the dehydrogenation of sterols to the Δ^{1}-products. On the basis of differences in enzyme inhibition, cofactor specificity and protein fractionation, it was demonstrated that the introduction of a double bond and double bond saturation were carried out by two different enzymes.[10,11]

The above results with the soluble enzyme preparation demonstrates the dual origin of citronellol from either the *trans* or *cis* monoterpene alcohols in rose petals. The properties of the dehydrogenase and the possible interelationship of geraniol and nerol will be dealt with in a separate communication.

[8] D. STEINBERG and J. AVIGAN, *Methods in Enzymology* (edited by R. B. CLAYTON), Vol. 15, p. 514, Academic Press, New York (1969).
[9] M. E. DEMPSEY, *Methods in Enzymology* (edited by R. B. CLAYTON), Vol. 15, p. 501, Academic Press, New York (1969).
[10] N. N. LESTROVAYA, M. I. BUKHAR and G. K. SKRYABIN, *Biokhimiya* **32**, 612 (1967).
[11] N. N. LESTROVAYA and M. I. BUKHAR, *Biokhimiya* **35**, 737 (1970).

EXPERIMENTAL

Chemicals. Pure geraniol, nerol and citronellol were obtained from A. Boake, Roberts & Co., Ltd., London E.17.

Plant material. The hybrid tea rose 'Fragrant Cloud' was used in all experiments. The plant was grown under greenhouse conditions at 22° and 16 hr day length. Flowers showed a fully opened phase lasting from 5 to 7 days and petals were harvested on the first or second day of this period.

Enzyme preparation. The petals from freshly picked flowers were frozen in liquid N_2 and ground to a fine powder which was washed four times with cold acetone. This dehydration step in addition to producing an acetone powder also removed terpenoids. The residual acetone was removed *in vacuo* and the powder stored at −8° under dry conditions until required. A soluble enzyme extract was prepared by stirring 1 g of acetone powder with 25 ml phosphate buffer (0·1 M, pH 8·0) containing 25 mM 2-mercaptoethanol for 30 min at 2°. The suspension was filtered through two layers of cheese cloth and the residue discarded. To the clear supernatant was added $(NH_4)_2SO_4$ to 70% saturation and the mixture stirred for 30 min then centrifuged. The precipitated protein was resuspended in phosphate buffer (0·1 M, pH 8·0) and respun at 1700 g/15 min to remove insoluble polysaccharides. This clear, coloured solution was used for incubation studies.

Soluble enzyme incubations. These were carried out at 30° using 2 ml of the above enzyme preparation (*ca.* 2 mg protein/ml) with added nucleotide or other electron donors (1·2 μmol/ml) and terpene (124 mμmol/ml) the latter added as a 5% solution in acetone. Reaction products were extracted with Et_2O and analysed by GLC on a Pye 104 gas chromatograph using a 2·75 m glass helical column (4 mm i.d.) packed with 10% FFAP on celite (100–120 mesh) and run at 145° isothermally with N_2 as carrier gas (flow rate 60 ml/min). Products were identified by comparison with known standards and by combined GLC–MS.

Preparation of dihydroterpenol. Geraniol or nerol (4 mg) in methyl cyanide (3 ml) was shaken at 60° in an atmosphere of O_2 with HOAc (40 μl) and 60% hydrazine hydrate (180 μl) for 4 hr.[12] The reaction was terminated by the addition of 2 N HCl and the products extracted with Et_2O. Both geraniol and nerol gave a mixture of three products, the 6,7-dihydro, the 2,3-dihydro and the tetrahydroalcohols. These were separated by GLC on FFAP.

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[12] E. D. Bitner and H. J. Dutton, *J. Am. Oil Chem. Soc.* **45**, 603 (1968).

Key Word Index—Rosa cultivars; Rosaceae; monoterpene reductase; geraniol; nerol; citronellol.