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MONOTERPENE INTERCONVERSIONS: METABOLISM OF PULEGONE BY A CELL-FREE SYSTEM FROM *MENTHA PIPERITA*

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Abstract—Cell-free extracts prepared from young vegetative shoot tips of *Mentha piperita* L. (peppermint) utilize ^{14}C-labelled pulegone. Menthone, isomenthone, and menthol have been tentatively identified as products. $NADPH_2$ is an essential cofactor. Anaerobic conditions enhance the yields of menthone and isomenthone, and menthol production has been observed only under anaerobic conditions. Preparation of active extracts requires anaerobic conditions, as well as the use of insoluble polyvinylpyrrolidone (Polyclar AT) to remove phenolic materials, and gel filtration to remove endogenous substrate and any remaining phenolic materials. Extracts prepared by a similar procedure from *M. arvensis* L. and from *M. sylvestris* L. have been shown to reduce pulegone to menthone and isomenthone in the presence of $NADPH_2$.

INTRODUCTION

IT IS commonly assumed by biochemists that monoterpenes, like other isoprenoid compounds, are produced by metabolic pathways which proceed from mevalonic acid. However, the experimental evidence supporting this assumption is slight, affording neither clear affirmation nor contradiction. Experiments performed with intact plants, or with plant tissues, have shown incorporation of label from mevalonate-2-^{14}C into monoterpenes, but yields have been consistently low,[1,2] and in some cases it appeared that the label had been incorporated via $^{14}CO_2$.[2,3] $^{14}CO_2$, in the light, has generally been the best precursor for monoterpenes in green plant tissues.[1,4] These findings have led to the suggestion[1] that mevalonic acid cannot be translocated readily to the site of monoterpene biosynthesis but must be produced *in situ* from other materials, such as sugars, which can be readily translocated—a situation very much like that observed in terpenoid biosynthesis in chloroplasts.[5–7] It is evident that the further elucidation of monoterpene biosynthesis calls for cell-free systems in which translocation and compartmentation problems can be eliminated.

A scheme of monterpene interconversions in peppermint (*Mentha piperita* L.), which was originally proposed by Reitsema[8] and subsequently modified,[1,9] assigns a central role

[1] W. D. LOOMIS, in *Terpenoids in Plants* (edited by J. B. PRIDHAM), p. 59. Academic Press, London and New York (1967).
[2] R. G. BATTU and H. W. YOUNGKEN, JR., *Lloydia* **29**, 360 (1966).
[3] D. ARIGONI. Personal communication: cited in Ref. 1.
[4] F. W. HEFENDEHL, E. W. UNDERHILL and E. VON RUDLOFF, *Phytochem.* **6**, 823 (1967).
[5] T. W. GOODWIN, in *Biosynthetic Pathways in Higher Plants* (edited by J. B. PRIDHAM and T. SWAIN), p. 57. Academic Press, London and New York (1965).
[6] L. J. ROGERS, S. P. J. SHAH and T. W. GOODWIN, *Biochem. J.* **100**, 14C (1966).
[7] J. M. CHARLTON, K. J. TREHARNE and T. W. GOODWIN, *Biochem. J.* **105**, 205 (1967).
[8] R. H. REITSEMA, *J. Am. Pharm. Assoc., Sci. Ed.* **47**, 267 (1958).
[9] A. J. BURBOTT and W. D. LOOMIS, *Plant Physiol.* **42**, 20 (1967).

to (+)-pulegone as the precursor of (+)-menthofuran, (−)-menthone, (+) isomenthone, and, through the menthones, of (−)-menthol, (+)-neomenthol, (+)-isomenthol, and (+)-neoisomenthol. Experimental support for the scheme has thus far consisted of analyses of seasonal changes in the composition of the peppermint oil, variations in composition of oil from tissues of different ages in a single plant,[10,11] sequential appearance in various monoterpenes of the label from $^{14}CO_2$ in time course studies,[2,4,11,12] and conversion in leaf tissue slices or intact leaves of ^{14}C-labelled pulegone to menthofuran and menthone[11] and of ^{14}C-labelled menthone to menthol.[12] In most of these studies isomenthone could not be distinguished from menthone, and no distinction could be made among the menthol isomers. Although the results of these investigations have supported the general features of the proposed scheme of terpene interconversions, it is clear that they could not yield detailed information about the reactions. Again, cell-free systems are clearly needed.

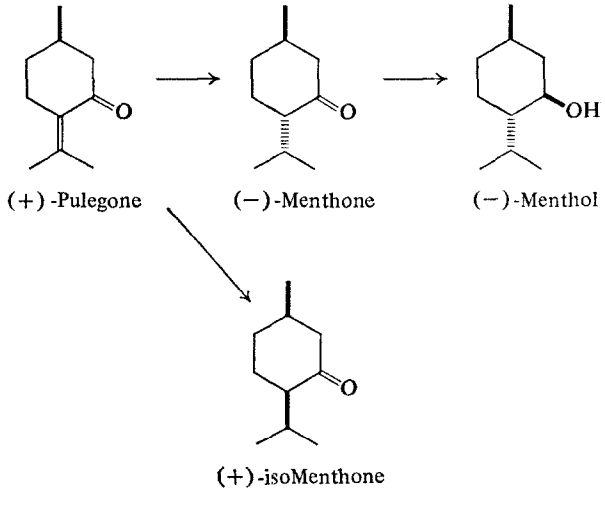

FIG. 1. ENZYMATIC REDUCTION OF PULEGONE.

Until recently attempts to isolate cell-free enzymes from monoterpene-producing plants met with little success. In previous publications,[13,14] we described methods for removing phenolic materials and thereby obtaining several active cell-free enzymes from peppermint and other plants which contain phenolic compounds. Using the same basic method, but with all operations carried out anaerobically, we have now been able to obtain cell-free extracts from *M. piperita* which are capable of metabolizing pulegone. Figure 1 shows the reactions catalyzed by these extracts. In the present communication we wish to describe the preparation and some of the properties of this system.

RESULTS AND DISCUSSION

Pulegone was chosen as substrate for the attempt to develop a cell-free system for monoterpene interconversions for two principal reasons: first, because of its central role

[10] R. H. REITSEMA, F. J. CRAMER and W. E. FASS, *J. Agr. Food Chem.* **5**, 779 (1957).
[11] J. BATTAILE and W. D. LOOMIS, *Biochim. Biophys. Acta* **51**, 545 (1961).
[12] R. H. REITSEMA, F. J. CRAMER, N. J. SCULLY and W. CHORNEY, *J. Pharm. Sci.* **50**, 18 (1961).
[13] W. D. LOOMIS and J. BATTAILE, *Phytochem.* **5**, 423 (1966).
[14] W. D. LOOMIS, in *Methods in Enzymology* (edited by J. M. LOWENSTEIN). In press. Academic Press, New York.

in monoterpene metabolism in peppermint and, second, because of the ease with which ^{14}C-labelled pulegone can be produced by biosynthesis from $^{14}CO_2$ in *Mentha pulegium* L. (pennyroyal).

The preparation of cell-free extracts from peppermint which are capable of utilizing pulegone depends on three principal precautions: (1) removal of phenolic materials by means of insoluble polyvinylpyrrolidone (Polyclar AT); (2) removal of endogenous substrate and remaining phenolic materials by gel filtration; (3) exclusion of air during preparation of the extracts and, for maximum activity, during the incubation with labelled pulegone as well. The method of enzyme extraction is basically the same as that which we described previously,[13] except that all operations are carried out in a nitrogen atmosphere. In the previous work we used large amounts of sodium ascorbate to reduce quinones which were formed by oxidation of phenols during the extraction, but suggested that this must be regarded as only an imperfect substitute for actual prevention of phenol oxidation. This contention is borne out by the present results.

In the cell-free systems prepared by the present method three different products of pulegone metabolism have been clearly observed, and other reactions are indicated less clearly. The three observed products are tentatively identified as menthone, isomenthone, and menthol. All three of these products are formed only in the presence of $NADPH_2$ or an $NADPH_2$ generating system; neither $NADH_2$ nor an $NADH_2$ generating system can substitute.

TABLE 1. EFFECTS OF COFACTORS AND OXYGEN ON THE METABOLISM OF ^{14}C-PULEGONE BY A CELL-FREE SYSTEM FROM PEPPERMINT

	Atmosphere	Cofactors added (3 mg) each				^{14}C Recovered in monoterpenes (cpm)*		
		NAD	NADP	$NADH_2$	$NADPH_2$	Pulegone	Menthone	Isomenthone
Boiled control	N_2	+	+	+	+	10,500	0	0
	Air	+	+	+	+	5,300	0	0
	N_2	+	+	+	+	6,300	1,600	6,600
	Air	+	+	+	+	1,800	1,200	1,900
	N_2	+	−	−	−	11,900	0	0
	Air	+	−	−	−	8,100	0	0
	N_2	−	+	−	−	7,800	0	0
	Air	−	+	−	−	3,500	0	0
	N_2	−	−	+	−	9,200	0	0
	Air	−	−	+	−	6,800	0	0
	N_2	−	−	−	+	700	2,200	5,200
	Air	−	−	−	+	0	650	1,100

* In all cases, 0·07 μmole ^{14}C-pulegone, 26,000 dpm, was used as substrate.

Table 1 summarizes the results of an experiment in which ^{14}C-pulegone was incubated with a peppermint extract for 3 hr at 37° in either an air or nitrogen atmosphere, and with various cofactors as indicated. The requirement for $NADPH_2$ is apparent from the table. It is also apparent that recovery of ^{14}C-label was lower from the air flasks than from the N_2 flasks. This loss was no doubt due in part to evaporation, since the air flasks were open, while the N_2 flasks were tightly closed. However, the very low recovery of label from the flask with $NADPH_2$ and air is striking and suggests that the extract may contain an enzyme

system dependent on $NADPH_2$ and O_2 which converts pulegone to compounds not recovered by the combination of hexane extraction and gas chromatography.

In the experiment described in Table 1, no detectable menthol was formed in any of the samples. However, ^{14}C-labelled menthol has been detected in several similar experiments. Production of menthol requires $NADPH_2$ and anaerobic conditions and is strongly favored by the use of an $NADPH_2$ generating system (glucose-6-phosphate plus glucose-6-phosphate dehydrogenase with NADP or $NADPH_2$).

Another enzyme extract, very similar to that used in the experiment shown in Table 1, was tested to determine its stability in storage. The pulegone-reducing ability was found to be very labile. Storage for 24 hr in a freezer at $-20°$ resulted in loss of over half the activity. In dry ice, more than one-third of the activity was lost in 24 hr, and only in liquid nitrogen was full activity preserved. We do not yet know whether this instability is intrinsic, or whether it is due to other substances present in the extract.

To date most of our investigations of the enzymatic reduction of pulegone have been carried out with cell-free extracts from *M. piperita*. However, we have also been able to demonstrate production of menthone and isomenthone from pulegone by cell-free extracts from *M. arvensis* L. and from *M. sylvestris* L. These extracts were prepared and assayed by essentially the same procedures as described here for peppermint. Reactions were carried out in a nitrogen atmosphere with $NADPH_2$ as the reducing agent.

EXPERIMENTAL

Mentha pulegium L. was propagated vegetatively in the greenhouse on approximately 15-hr days from a single original plant found growing wild near Corvallis. ^{14}C-Pulegone for use as a substrate was produced by exposing small cuttings of *M. pulegium*, consisting of the growing tip and three or four leaf pairs, to $^{14}CO_2$ in continuous light for 3 days. Exposures were carried out in a sealed glass chamber placed in a plant growth chamber with a light intensity of 60,000 lux (measured by a General Electric No. 213 light meter with selenium photocell) from Sylvania VHO Gro-Lux fluorescent lamps. For most preparations, six cuttings were used with approximately 1 mc of $^{14}CO_2$ of high specific activity. At the end of the exposure period the shoots were ground in liquid N_2 and extracted with Skellysolve-B (chiefly *n*-hexane). The hexane extract was dried with anhydrous Na_2SO_4 and decolorized with the minimum amount of charcoal (Norit A) sufficient to remove the yellow color. The extract was chromatographed by silica gel G TLC,[11,15] the pulegone area was scraped off, and the labelled pulegone was recovered by extraction with ethyl acetate. The labelled pulegone was transferred to the aqueous enzyme assay systems by layering an aliquot of the ethyl acetate solution on the buffer–water mixture to be used for an individual assay and evaporating the ethyl acetate under a stream of N_2. There are undoubtedly variable losses of the substrate by evaporation during this procedure, but this variability in the amount of substrate does not affect the validity of the results reported here.

Plant material used as the source of enzyme extracts was the Black Mitcham variety of *M. piperita* L., grown from a single clone[9] in a growth chamber maintained at 24° day temperature and 10° night temperature during a regular 24-hr cycle with 16-hr day. A light intensity of 11,000 lux was provided by a mixture of Sylvania VHO Gro-Lux lamps and incandescent bulbs. The growth chamber and lighting have been described previously.[9] These growing conditions lead to the production of peppermint oil consisting largely of menthone.[9] *M. arvensis* L. and *M. sylvestris* L. were propagated vegetatively in the greenhouse on approximately 15-hr days from planting stock donated by Dr. M. J. Murray of the A. M. Todd Co., Kalamazoo, Michigan.

The preparation of the cell-free system used for the experiment reported in Table 1 was as follows: In a dry box with cold N_2 atmosphere (from liquid N_2), 4·0 g of fresh peppermint shoot tips (growing tips plus immature leaves less than half expanded) were ground in liquid N_2 in a mortar. The resulting powder was stirred into an ice-cold slurry of 2·5 g of purified Polyclar AT[13] in 16 ml water plus 1 ml of 1 M K-phosphate buffer of pH 7·0. The resulting paste was squeezed through bolting silk, yielding 11 ml of crude extract. The entire extract was filtered through a 2·5 cm by 7·5 cm column of Bio-Gel P-10 (Bio-Rad Laboratories). Collection of eluate was begun with the first appearance of green color (chloroplast fragments) and terminated shortly before the color turned to yellow, yielding 10 ml of eluate. 3 ml of water were added to bring the volume to 13 ml.

For assay of the cell-free system, 1 ml aliquots of the gel-filtered extracts were pipetted, still in the N_2

[15] J. BATTAILE, R. L. DUNNING and W. D. LOOMIS, *Biochim. Biophys. Acta* **51**, 538 (1961).

atmosphere, into assay vessels (25 ml Erlenmeyer flasks) prepared previously outside of the dry box. For controls, 1 ml aliquots of the extract were removed from the dry box, heated for 5 min in a boiling water bath, and returned to the dry box for further manipulations. Each assay flask contained: 1·0 ml of fresh or boiled extract; 0·25 ml of 1 M K-phosphate buffer, pH 7·0; 0·75 ml glass distilled water; 0·07 μmole (26,000 dpm) of pulegone; and 3 mg each of one or more pyridine nucleotides, as indicated (Table 1). The pyridine nucleotides were administered in solutions containing 3 mg per 0·1 ml. The total volume was thus 2·4 ml for samples containing all four nucleotides, and 2·1 ml for the rest. Flasks which were to be incubated in an N_2 atmosphere were sealed tightly with Parafilm. Flasks which were to be incubated in air were left open. The samples were shaken for 3 hr at 37°.

After incubation each sample was extracted four times with 5-ml portions of Skellysolve-B. The combined extracts were decolorized with the minimum amount of Norit A charcoal and the volume of extract was reduced to about 100 μl in a stream of N_2. The concentrated extracts were analyzed by gas chromatography coupled with effluent counting by a Nuclear Chicago Biospan 4998 counter. Counting efficiency was about 95 per cent. The column used for gas chromatography was 1 per cent phenyl diethanolamine succinate (PDEAS) and 1·5 per cent sucrose acetate isobutyrate (SAIB) on 100–120 mesh Chromosorb G. The chromatographic separation was temperature-programmed from 100° to 160°. The procedures are described in more detail elsewhere.[9,16]

Although the details of the preparation and assay methods described here apply specifically only to the experiment described in Table 1, they represent general methods which have been applied successfully with minor variations in a number of assays. We have found that the amount of Polyclar AT used in these experiments was suboptimal, and that increasing the amount of Polyclar to 1 g per g of fresh tissue removes the plant phenolic compounds more effectively.

Tentative identifications of the products of pulegone metabolism have been reached by comparison with authentic samples of menthone, isomenthone and menthol[17] in gas chromatography on the PDEAS-SAIB column mentioned above, and on a 1 per cent Carbowax 20 M column. In addition, TLC on silica gel G[15] confirmed the formation of a substance or substances having the same R_f as menthone and isomenthone. The amounts of menthol formed were very small in most experiments, and in the experiment which was analyzed by thin-layer chromatography there was not enough menthol for detection on the chromatoplates.

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[16] A. J. BURBOTT and W. D. LOOMIS. To be published.
[17] Sources of these standards have been listed previously[9].