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CHEMOGENETIC EVIDENCE SUPPORTING MULTIPLE ALLELE CONTROL OF THE BIOSYNTHESIS OF (−)-MENTHONE AND (+)-ISOMENTHONE STEREOISOMERS IN *MENTHA* SPECIES

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Key Word Index -*Mentha*; Lamiaceae; mint; essential oil; monoterpenes; menthone; isomenthone; menthol isomers; biosynthesis; genetics; multiple alleles.

Abstract—The essential oils of certain *Mentha* species and chemotypes have proportions of (−)-menthone and (+)-isomenthone which differ but show a high degree of heritability in clonal propagation. Oil from an F_2 individual (69-296), selected from numerous 4*n* *M. longifolia* (4*n* = 48) × *M. crispa* (2*n* = 48) hybrids for high isomenthone content, had 41.3% isomenthone; the associated but seldom observed alcohols, 1.6% isomenthol, 10.3% *neoiso*-menthol; and 13% of their esters; in contrast to 8% menthone with 0.1% menthol, 5.0% *neo*-menthol, and 1.7% esters. Self-pollination of strain 69–296 gave a 3:1 ratio of high isomenthone: high menthone. Crosses with a true breeding high menthone plant having 80% menthone and 3.2% isomenthone gave a 1:1 ratio of the parental phenotypes by GLC analyses and herbage odor. This and data from high isomenthone and high menthone crosses with tester strains lead us to postulate the involvement of a single locus having multiple alleles with true breeding menthone having the genotype P^sP^s, true breeding isomenthone P^rP^r, 69–296 P^rP^s, and high pulegone *pp*. The P^r allele is not completely dominant over the P^s allele in 69–296 as about 18% of the total ketone derived from pulegone is menthone. Both are dominant over the recessive allele *p* that largely prevents menthone development. The quantitative amounts of the two isomers are believed to be controlled by the six combinations of the three alleles in a diploid species with graded effects obtained in the more complex genotypes possible in double diploid and octoploid species. 69–296 has (−)-piperitone even though (+)-piperitone is believed to be the common isomer in *Mentha*.

INTRODUCTION

The related stereoisomers (−)-menthone and (+)-isomenthone, often referred to as menthone and isomenthone, occur together in the oils of certain Lamiaceae (Labiatae) mint species of the genera *Pycnanthemum* [1], *Hedeoma* [2], *Thymus* [3], *Satureja* [4] and *Mentha* [5,6] in proportions typical of the particular species. The proportion of each isomer has a high degree of heritability in clonally propagated strains. The Black Mitcham clonal strain of *Mentha piperita* L., which is cultivated agriculturally as a source of peppermint oil, has essentially the same proportion of menthone and its derived products to isomenthone and its derived products, when grown in different states of the U.S.A. or other countries of the world [5–7]. Adequate chemogenetic data support the conclusion that a single dominant gene *P* allows the conversion of (+)-pulegone to (−)-menthone [8,9] but no chemogenetic data have been published to show the genetic basis for isomenthone production and its biogenetic origin.

The conversions given in Fig. 1 have been postulated upon the basis of stereochemical structure, the co-occurrence of related compounds in different strains, radioactive tracer studies and feeding experiments with cell-free enzyme extracts. A brief review of those results follows. Radioactive tracer work [10] with $^{14}CO_2$ showed that piperitenone is probably the precursor of pulegone and piperitone. Katsuhara [11] first suggested in 1966 that (+)-pulegone is the precursor of both (+)-isomenthone and (−)-menthone, a conclusion based solely upon the stereochemical structure of the compounds. Cell-free enzyme preparations [12,13] obtained from Mitcham peppermint were able to convert (+)-pulegone to menthone and also to isomenthone. Aviv and Galun [14] found that pulegone was converted to isomenthone, not menthone, when pulegone was added to a *Mentha* cell suspension, but it was not stated whether (+)-pulegone or (−)-pulegone was added.

The reduction of (+)-4*S*-piperitone to (−)-(1*R*-4*S*)-menthone was proposed by Reitsema [15], Katsuhara [11], Hendriks and van Os [16], and Lawrence [17]. Loomis [18] designated piperitone as (−)-piperitone and concluded that it might be converted to (+)-isomenthone but not to (−)-menthone. Lawrence [17] found that (+)-piperitone is the stereoisomer commonly occurring in the genus *Mentha*. Conversely, if (−)-piperitone exists it presumably could be converted to isomenthone as suggested by Loomis [18] and indeed a logical place to find the isomer would be in a *Mentha* strain having isomenthone as the predominant constituent.

The observation [19] that menthone isomerizes easily to isomenthone spontaneously or with heating may be

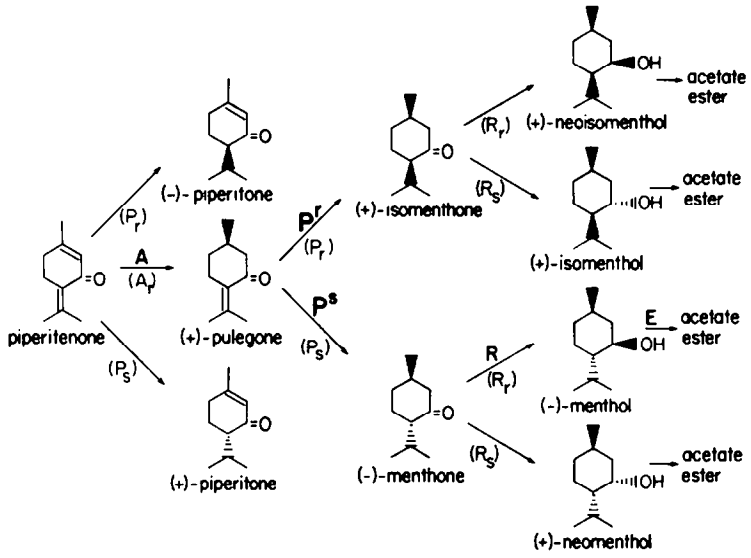

Fig. 1. Postulated biogenetic origin of stereoisomers (−)-menthone and (+)-isomenthone and their related compounds.

considered non-enzymatic in nature and at least is not a recognizable change in the oils of *M. piperita* and *M. arvensis* stored at room temperature for several years. Radioactive tracer studies by Hefendehl *et al.* [20] were less definite than desired and did not eliminate the possibility that isomenthone was reversibly converted to menthone. Lawrence [17] re-evaluated this possibility and concluded that while it is conceivable that an isomerase enzyme could convert isomenthone to menthone or vice versa, there was more likelihood that a stereospecific reduction of (+)-pulegone produced isomenthone as well as menthone, as originally suggested by Katsuhara [11] solely on the basis of stereochemical structure. Enzyme extract studies [13] also suggested that pure (+)-pulegone could be reduced to both menthone and isomenthone. Lawrence [17] in his own research looked for supportive evidence from the co-occurrence of the three compounds in certain species strains (especially members of the subgenus *Pulegium* and chemotypes of *Mentha arvensis*) and from deductive reasoning on the types of enzymes that might be responsible for these changes. The postulated enzymes [17], designated P_s, P_r, and A_r, are similar to those postulated by Hendriks *et al.* [16,21] with Hendrik's *P* equivalent to P_s and *X* equivalent to A_r.

The assumptions that piperitenone is converted to (+)-pulegone by the enzyme A_r or *X* and (+)-pulegone to (−)-menthone by the enzyme P_s or *P* are supported by genetic data [8, 9, 22], whereas the assumptions that piperitenone is converted to (+)-piperitone by the P_s or *P* enzyme and (+)-piperitone to (−)-menthone by A_r or *X* appear logical but are not supported by adequate genetic data. Lawrence [17] further postulates that the enzyme A_r converts (−)-carvone to (+)-*trans*-dihydrocarvone while a different enzyme, A_s, causes a stereospecific reduction of the 1,2-endocyclic double bond to convert (−)-carvone to (+)-*cis*-dihydrocarvone. The multiple effects of an enzyme are not unexpected in *Mentha* oil biogenesis since the enzyme controlled by the gene *R* causes conversion of menthone to menthol, carvone to carveol, dihydrocarvone to dihydrocarveol, and pulegone to pulegol [17, 23, 24].

To conclude, the original objective of the present chemogenetic research was to provide genetic information on how two stereoisomers can be derived from one compound in biogenesis and, if possible, determine how the quantitative amounts of each product were determined. Several fundamental questions have arisen since this research was completed. Does (−)-piperitone occur in *Mentha* species? Is menthone derived from either (+)-pulegone or (+)-piperitone, while isomenthone is only derived from (+)-pulegone? Does the P_s enzyme convert (+)-piperitenone oxide to (−)-*cis*-piperitone oxide, and piperitenone to (+)-piperitone as suggested [17]?

RESULTS

Selection of isomenthone parent

The first requirement of a genetic study of isomenthone is a fertile, easily hybridized parent having large amounts of isomenthone and its products, with low amounts of menthone and its products. All members of the subgenus *Pulegium* are unusable since emasculation and hybridization are difficult or impossible. Sizable amounts of menthone and isomenthone were found only in fertile *M. spicata L.* ($2n = 48$), *M. crispa L.* ($2n = 48$) and *M. arvensis L.* ($2n = 96$), in specific natural strains or in S_1 inbred individuals. No sterile hybrid species or strains with menthones could be hybridized unless one utilized colchicine-induced polyploid strains which often have duplicate genes.

The data in Table 1 show that some selected strains had large amounts of menthone, others isomenthone, and some about equal amounts. Certain strains, as 69–292, with nearly equal amounts of menthone and isomenthone may have over a 90% ketone content whereas others, as 69–240, less than 47%. The selection program provided

Table 1. Assay data for selected menthone strains to illustrate variability found in (+)-isomenthone and (−)-menthone content

Strain	Isomenthone (%)	Menthone (%)	Piperitone (%)	Other (%)
High isomenthone:				
69–296 F_1 or F_2 selection from 4*n* *M. longifolia* × *M. crispa*	68.2	7.7	0.5	23.6
69–241 F_1 or F_2 selection from 4*n* *M. longifolia* × *M. crispa*	47.3	11.8	4.4	36.5
69–299 F_1 or F_2 selection from 4*n* *M. longifolia* × *M. crispa*	47.1	10.4	1.3	41.2
69–240 F_1 or F_2 selection from 4*n* *M. longifolia* × *M. crispa*	36.9	6.9	2.7	53.5
Isomenthone and menthone about equal:				
69–292 F_1 or F_2 selection from 4*n* *M. longifolia* × *M. crispa*	51.9	41.8	2.1	4.2
69–245 F_1 or F_2 selection from 4*n* *M. longifolia* × *M. crispa*	44.1	41.1	3.6	11.2
69–285 F_1 or F_2 selection from 4*n* *M. longifolia* × *M. crispa*	41.6	30.1	2.8	25.5
69–48 Sterile *M. spicata*	33.3	35.0	0.6	31.1
Isomenthone lower than menthone:				
69–269 F_1 selection from 4*n* *M. longifolia* × *M. crispa*	34.4	47.2	0.3	18.1
69–196 *M. crispa* S_1	23.1	52.2	2.2	22.5
69–198 *M. crispa* S_1	16.9	56.3	1.6	25.2
M. spicata Missiones strain	13.1	51.9	0.5	34.5
69–188 *M. crispa* S_1	13.9	43.0	0.4	42.7
Mitcham peppermint*	3.9	35.6	0.9	59.6
Mitcham peppermint†	2.7	11.5	0.4	85.4

* Harvested 3 June near South Bend, Ind.

† Harvested early September in Yakima Valley, Wash.

basic information on the limits of selection as well as producing strains of plant breeding interest. Wholly fertile polyploid *M. Longifolia* (L.) Huds. ($4n = 48$) having the ketone piperitone and its oxide hybridized with fertile *M. crispa* having carvone produced some vigorous, strong stemmed, *Verticillium* and *Puccinia* rust resistant, agronomically useful F_1 and F_2 selections, having menthones that were useful as parents in crosses to improve the disease resistance of such cultivars as *M. arvensis*. (See U.S. Plant Patents 1926–28.) This practical selection program did not include the *Verticillium* and *Puccinia* rust-susceptible Line 1 strain of *M. spicata* which has been subsequently studied by Tucker [25] who found that isomenthone predominated over menthone in five self-pollinated S_1 progeny of this strain.

Chemical composition of a high isomenthone strain

The highest isomenthone strain 69–296, an F_2 selection from cross *M. crispa* S_0 × 4*n* *M. longifolia* from Table 1, was chosen for use in crosses and for detailed chemical study (Table 2). Table 3 is a rearranged summary of Table 2 data showing that isomenthone and its derived alcohols

Table 2. Oil composition of strain 69–296

	Compound	%		Compound	%
1	α-Pinene	0.5	16	*neoiso*-Menthyl acetate	10.7
2	Camphene	Trace	17	(+)-*neo*-Menthol*	5.0
3	β-Pinene	0.8	18	Caryophyllene	3.4
4	Sabinene	0.4	19	(+)-*neo*-Isomenthol*	10.3
5	Myrcene	0.9	20	Menthol	0.1
6	(−)-Limonene	0.7	21	Unknown	0.2
7	β-Phellandrene	0.1	22	(+)-Isomenthol*	1.6
7a	Cineol	5.0	23	Pulegone	0.3
8	*cis*-β-Ocimene	0.2	24	Unknown	0.1
9	*trans*-β-Ocimene	Trace	25	α-Terpineol	0.3
10	Terpinolene	Trace	26	Germacrene D	1.3
11	Unknown	0.2	27	(−)-Piperitone*	2.0
12	(−)-Menthone*	8.0	28	Carvone	0.6
13	(+)-Isomenthone*	41.3	29	Bicyclogermacrene	0.7
14	β-Bourbonene	0.6	30	Unknown	0.2
14a	*neo*-Menthyl acetate	1.6		Trace peaks (all under 0.1%)	
15	*iso*-Menthyl acetate	2.3		numbered and unnumbered	0.5
15a	Menthyl acetate	0.1			100.0

* It was not possible to determine exact values of rotation because of very low amounts of compounds available. It is therefore possible that the enantiomers are also present in certain quantities.

Table 3. Rearranged summary of Table 2

Principal ketones	%	Alcohols	%	Esters	%	Total (%)
Menthone	8.0	Menthol	0.1	Menthyl acetate	0.1	
		neo-Menthol	5.0	*neo*-Mentyyl acetate	1.6	
Total	8.0		5.1		1.7	14.8
Isomenthone	41.3	Isomenthol	1.6	*iso*-Menthyl acetate	2.3	
		neo-Isomenthol	10.3	*neo*-Isomenthyl acetate	10.7	
Total	41.3		11.9		13.0	66.2

and esters account for about two-third (66.2 %) of the oil in comparison to 14.8% for menthone, its alcohols and esters. *Neo*-Menthol, as well as menthol, is assumed to be derived from menthone as often postulated [6, 18]. Piperitone and pulegone, the postulated intermediate compounds in the synthesis of isomenthone, are not greater in 69–296 than in similar high menthone strains such as *M. arvensis* (Table 4). There are, in contrast, sizable amounts of the menthol isomers isomenthol and *neo*-isomenthol not often identified in *M. arvensis* commercial oil or other *Mentha* species. *Neo*-Menthol is greater than menthol, the opposite of *M. arvensis* and *M. piperita* L. oils.

Selfed progeny and intercrosses of isomenthone and menthone strains

A high menthone-selected S_1 *M. arvensis* var. *piperascens* ($2n = 96$) parent with 80% menthone, 3.2% isomenthone, 3.2% piperitone and 0.01% pulegone was true breeding for high menthone/low isomenthone in 402 S_2 progeny and has the known genotype *cc AA PP rr FF*. The cc AA genotype allows conversion of piperitone to pulegone [22], the dominant *P* gene allows the conversion of (+)-pulegone to (−)-menthone [9], and menthone is accumulated since the recessive *rr* genotype prevents its partial conversion to menthol [23] and the *FF* genotype partial conversion to menthofuran [26] (Fig. 1). Conversely, a dominant *C* gene would cause carvone, the recessive *aa* genotype with *cc* would result in piperitenone or piperitone formation, and the dominant *R* gene allows the conversion of menthone to menthol [23].

Self-pollination of strain 69–296 with 41% isomenthone, 8% menthone, 2% piperitone and 0.3% pulegone gave 39 isomenthone-odored to 10 menthone-odored S_1 individuals. This is a ratio of 3 high isomenthone to 1 high menthone ($P = 0.6$). No menthone-odored individuals were assyed but 21 isomenthone-odored field plants were and had high isomenthone and low menthone. The alcohols and esters of the 21 S_1 individuals were not identified but the means and standard deviations of the following compounds were determined, namely: α-pinene 0.17/0.11, β-pinene, sabinene, and myrcene 3.5/1.4, limonene 0.62/0.24, cineole trace only, menthone 6.6/2.8, isomenthone 65.0/9.1, pulegone 0.48/0.34, piperitone 2.7/1.4 and sesquiterpenes 1.2/0.24. The data show that the 69–296 strain is heterozygous for a single dominant gene that causes high isomenthone in the parental clone and three-fourths of its S_1 progeny, while all S_1 individuals having the recessive genotype are similar in herbage odor and genotype to the high menthone/low isomenthone tester strain of *M. arvensis*. These data also indicate that the *P* gene has three alleles which may be designated as P^r, P^s and *p* using the subscripts assigned the enzymes [17] as superscripts to avoid unnecessary duplication of symbols and still conform to standard genetic usage. The P^r and P^s alleles appear dominant over the third allele *p* in the series since the recessive *pp* genotype clearly inhibits the formation of sizable amounts of either menthone of isomenthone [9]. The high isomenthone allele, designated P^r, is not completely dominant over the high menthone allele, designated P^s, since the oil of 69–296 with the heterozygous genotype P^rP^s has 14.8% menthone and products. There is about five times as much isomenthone and products as menthone and products (18%) resulting from the reduction of pulegone. The S_1 assays also show that both alleles function in the heterozygous individuals.

The isomenthone strain 69–296 having the postulated heterozygous genotype P^rP^s hybridized with the high menthone strain of *M. arvensis* having the true-breeding genotype P^sP^s should, and did, give a test cross 1:1 ratio of the two parental types; namely, 52 high isomenthone-odored: 41 high menthone-odored ($P = 0.2$), GLC assays for the menthone isomers of 28 random individuals are plotted in Fig. 2. Twenty-six of the individuals clearly belong to two classes. One unusual plant had 45.1% menthone and 40.3% isomenthone, a second 27.1% menthone and 17% isomenthone. If the latter two individuals are placed with the high menthone class, a perfect 1:1 ratio of the classes occurs. The proportions of each isomer is partially influenced by conversion to the derived alcohols and esters (Fig. 1). Similar test cross results of 40 isomenthone-odored to 39 menthone or menthol-odored were obtained when the *M. arvensis* parent had the *Rr* genotype and menthol.

Interaction with other genes

The first cross of Table 4 was to test whether 69–296 F_1 hybrids with a strain having about equal amounts of pulegone and menthone would show increased amounts of either menthone or isomenthone, the postulated products of pulegone. (See bottom of Table 4 for assay of 62–213 and other parental strains.) In comparison to the 62–213 parent, the two F_1 hybrids showed a 14-fold decrease of pulegone, no increase in menthone and a 10-fold increase in isomenthone. There were 210 isomenthone-odored: 78 menthone-odored, a 3:1 ratio ($P = 0.4$). The 62–213 genotype seems to be P^rP^s with

Table 4. GLC data* for F_1 hybrids, namely tester strains × 69–296 having 41% isomenthone and 8% menthone and as controls certain tester strains × strains having 50–70% menthone and 2–10% isomenthone

	Pulegone (%)	Menthone alcohols and esters (%)	Menthofuran (%)	Piperitone (%)	Isomenthone (%)
ms† *M. crispa* S_1 strain 213 33% pulegone × 69 296	2.4	43.4	0.1	2.1	24.5
	0.2	47.3		1.4	25.5
ms *M. spicata* S_1 strain 199 57% menthone × 69–296	2.6	34.4		2.9	27.0
ms 4*n M. longifolia* 63% piperitone oxide × 69–296	22.1	13.9	0.5	—	26.8
	4.1	29.9		1.1	33.6
ms *M. citrata* strain 1 86% linalool and ester × 69–296‡	15.8	2.2	31.1	0.4	0.6
ms *M. citrata* strain 5 87% linalool and ester × 69 296	0.7	39.4	5.4	1.6	11.6
ms *M. aquatica* Dutch strain 80% menthofuran × 69–296	18.0	8.7	1.6	0.7	30.9
	11.1	8.8	1.2	0.8	41.5
ms *M. aquatica* Dutch strain × *M. spicata* S_1 strain 199 57% menthone§	1.1	58.1	4.1	1.6	5.0
ms *M. aquatica* Dutch strain × *M. spicata* Missiones strain 52% menthone‖	3.0	48.5	4.8	1.5	8.3
ms *M. aquatica* Dutch strain × *M. crispa* S_1 strain 213 33% pulegone¶	4.3	40.6	30.5	0.6	1.8
ms *M. aquatica* Pritchard strain 79% menthofuran × 69–296	0.6	13.5	12.1	0.4	24.5
	0.3	14.7	12.0	0.1	22.2
	0.8	7.2	3.0	0.2	9.2
ms 78% menthol *M. arvensis* × 69 296	0.8	47.1		1.2	23.0
	0.5	54.0		1.8	23.5
ms 78% menthol *M. arvensis* × *M. crispa* S_1 55% 55% menthone	0.2	85.4	-	4.5	3.9
M. arvensis 80% menthol cultivar control	0.1	85.6		3.2	1.8
M. arvensis S_1 80% menthone cultivar control	tr	81.4	tr	3.2	3.2
M. spicata S_1 strain 199 menthone tester strain	2.5	51.0	—	0.5	13.1
M. crispa S_1 strain 213 pulegone/menthone tester strain	33.2	43.8	—	2.4	2.3
4*n. M. longifolia* piperitone oxide tester strain	—	-		65.6	—
69–296 high isomenthone data from Table 1 from GLC column used in above assays	0.3	7.7	—	0.5	68.2

* α-Pinene, β-pinene, sabinene, myrcene, α-terpineol, limonene, cineole, isopulegone, β-caryophyllene and other minor constituents do not vary greatly in the assays and are not given.

† ms = genic male-sterile, 100% seed fertile.

‡ One lavender-odored hybrid had 13.1% linalol to 68.1% linalyl acetate with no menthones.

§ Average of 25 F_1 hybrids.

‖ Average of 5 F_1 hybrids.

¶ Average of 9 F_1 hybrids.

modifying genes as the F_1 hybrids with 69–296 ($P^r P^s$) should, and did, have a 3:1 ratio.

Strain 69–296 with the genotype $P^r P^s$ hybridized with a true breeding high menthone strain $P^s P^s$ produced certain F_1 hybrids, as the one cited, which have about equal amounts of menthone and isomenthone whereas others are similar to the high menthone parent. A 1:1 ratio of the two parental types would be expected but the data were 95 isomenthone-odored: 53 menthone-odored, a 2:1 ratio ($P = 0.5$). This is apparently due to close linkage between the menthone P^s allele and the previously known heterozygous recessive semilethal dwarf gene which is present in strain 199 and also in original 69–296. Other menthone tester strains as those from *M. arvensis* and *M. crispa* do not have this recessive lethal and were used to avoid obtaining unusual ratios difficult to interpret and data supporting the erroneous conclusion that a strain is true breeding when it is not.

The cross with 4*n M. longifolia* having 2.5% piperitone, 63.1% piperitone oxide, 1.7% piperitenone and 1.4% piperitenone oxide was to test whether piperitone is a precursor of isomenthone or of menthone. The cross gave 27 isomenthone: 32 menthone: 13 piperitone-odored individuals, or a ratio of 3:3:2($P = 0.3$). These data could result from crossing the genotype *aa aa* $P^s P^s$ × *Aa Aa* $P^r P^s$ but the important fact is that several piperitone-odored hybrids did occur in this cross and show that the recessive *aa* genotype inhibits isomenthone as well as menthone. This is definitely known from GLC assays of a series of segregant individual from the *M. crispa* × 4*n. M. longifolia* crosses and from assays of several strains of *M. longifolia*. In comparison to the 4*n M. longifolia* parent which has no pulegone, menthone or isomenthone, the two F_1 hybrids have intermediate amounts of these ketones: namely, a great increase of pulegone and a 2–4-fold increase of menthone over the 69–296 parent, but only 50% as much isomenthone.

The limonene tester strain with the known genotype Lm_1 lm_1 Lm_2 lm_2 [27, 28] crossed with 69–296 gave a duplicate gene test cross ratio of 3 limonene-odored to 1

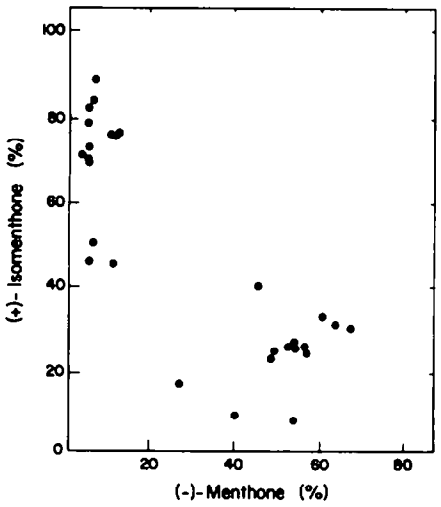

Fig. 2. Strain 69–296 having the P^rP^s genotype and the high isomenthone/low menthone phenotype hybridized with *M. arvensis* having the true breeding P^sP^s genotype and the high menthone/low isomenthone phentoype gave 28 F_1 hybrids with menthone–isomenthone GLC values plotted to show that 50% are like the 69–296 parent and 50% like the *M. arvensis* parent.

menthone-odored proving that the *Lm* gene is epistatic over isomenthone, as it was known to be for menthone. No assays are cited. Data were 63:21, a perfect 3:1 ratio.

M. citrata strains 1 and 5 with the known *Ii* genotype [29] crossed with 69–296 gave a ratio of 1 lavender-odored to 1 not, proving that the *I* gene is epistatic over the P^r allele of the *P* gene. Data were 18:17 and 10:16. The major compounds of the first non-lavender hybrid were pulegone and its oxidation product, menthofuran, with the reduction products less than 5%. In contrast, the second individual had almost 40% menthone and 11.6% isomenthone accounting for about 50% of the oil.

The 80% menthol *M. arvensis* cultivar, the 80% menthone *M. arvensis* S_1 and their hybrids with 50–70% menthone *M. crispa* S_1's had only 1–5% isomenthone. The 78% menthol *M. arvensis* F_1 hybrid with 69–296 had 23% isomenthone, while a similar F_1 hybrid with an *M. crispa* S_1 strain having 55% menthone had 3.9% isomenthone. Two Dutch strain *M. aquatica* hybrids with 69–296 had 30–41% isomenthone, whereas similar *M. aquatica* hybrids with high menthone *M. spicata* parents had less than one fifth as much isomenthone. The 69–296 hybrids with the Pritchard strain of *M. aquatica* are of two kinds. Two had 22–25% and six had 8.7–10.1% isomenthone as in two *M. citrata* hybrids having an *ii* genotype. These data show that the P^r allele in heterozygous 69–296 P^rP^s genotype has a dominant or epistatic effect in increasing the amount of isomenthone in a part (presumably one half) of the F_1 hybrids with high menthone strains of several kinds having the postulated P^sP^s or possibly P^sp genotypes. The range of 69–296 hybrids presumably having a P^r allele was 22–41.5% whereas those having a P^s allele had a range of 0.6–13.1%. Some of these strains, especially the *M. aquatica* hybrid having 41.5% isomenthone, may not differ greatly from the 69–296 parent if the totals of the assayed alcohols and esters are included.

DISCUSSION

A multiple allelic series of three alleles can produce the following six combinations: P^rP^r, P^rP^s, P^sP^s, P^rp, P^sp and *pp*. The genotypes P^rP^r, P^rp, and P^sp were not identified and assayed, but P^rP^s had 66.2% isomenthone to 14.8% menthone, P^sP^s 3.2% isomenthone to 80% menthone, and *pp* 5.5% to 6.4%. A graded series could be obtained since double diploids ($2n = 48$) like *M. spicata* and *M. crispa* would have alleles on two or more chromosome pairs and the 6*n* and 8*n* strains of *M. arvensis* with 72 and 96 somatic chromosomes could have alleles on three and four chromosome pairs. The total amount of the two related ketones could be influenced by the amount of pulegone produced and by the amount of pulegone converted to the alcohol pulegol or oxidized to menthofuran. An ideal comparison of the six allelic combinations would require a lengthy project to study each in a common genetic background, but it is quite clear that P^r, the isomenthone allele, is mostly dominant over P^s, the menthone allele, and that both are highly dominant over the recessive *p* or pulegone allele. An unanswered question is whether the 1–5% presence of menthone and isomenthone in a *pp* genotype [30] is due to non-enzymatic reduction catalysed by distillation heat or whether it is derived from the reduction of (+)-piperitone to menthone [17] and (−)-piperitone to isomenthone [18].

The generalization that all piperitone found naturally in *Mentha* species is (+)-piperitone [17] has at least one exception as (−)-piperitone is reported in 69–296 strain (Table 2). Further work would seem necessary to determine whether both isomers may occur in the same plant and how extensively the (−)-piperitone occurs.

The summarized data of this paper do not require assuming that the genes P^r and P^s have multiple effects on biogenesis as postulated [16, 17] and conversely neither confirm nor refute the logical conversions that could occur. Dominant gene substitutions by convergent backcrossing would afford more positive proof than the interaction data that are the supporting evidence at this time. The genetic control of monoterpene synthesis in *Mentha* has recently been reviewed [31–33].

Alcohols and esters

Lawrence [17] has postulated that two kinds of *R* or reductase enzymes control the development of the four menthol isomers found in 69–296 (Table 3). The R_r enzyme (Fig. 1) is believed to convert (−)-menthone to (−)-menthol and (+)-isomenthone to (+)-*neo*-isomenthol while the R_s one converts (−)-menthone to (+)-*neo*-menthol and (+)-isomenthone to (+)-isomenthol. If two enzymes are controlled by the multiple alleles, R^r, R^s and *r*, then the *rr* genotype should largely prevent the development of all alcohols and menthyl esters derived from them. In *M. arvensis* with a ketone genotype P^sP^s, the *RR* and *Rr* genotypes are indistinguishable from their assays and the *rr* genotype reduces total alcohols and esters from 83% to 1.4%. This reduction is largely in menthol since 1.8% *neo*-menthol was reduced to 0.3% [23]. The alcohols of (−)-menthone are *neo*-menthol with a woody, musty odor and menthol, widely utilized for its familiar cooling odor. The alcohols isomenthol and *neo*-isomenthol are believed to be derived

from isomenthone [17, 18]. Strain 69–296 with 41.3% isomenthone and 8% menthone (P^rP^s) should have and does have more isomenthone-derived alcohols (11.9%) and esters (13%) than menthone-derived alcohols (5.1%) and esters (1.7%) (Table 3). The fact that *neo*-menthol is greater than menthol in 69–296 (Table 3) could mean that the R^s allele is dominant over the R^r allele, but then isomenthol should be greater than *neo*-isomenthol and is not. The S_1 strains of *M. crispa* with 50–70% menthone would have menthol or neomenthol if these strains had an R^r or R^s allele. *M. spicata* strains with isomenthone would have isomenthol or *neo*-isomenthol if the strain had either R^r or R^s alleles. In contrast, polyploid *M. longifolia* S_1 does not have either pulegone, menthone, isomenthone, menthols or esters, but it could have either R^r or R^s and must have both if 69–296 has the genotype *cc AaAa* P^rP^s R^rR^s *EE*.

To conclude, the postulated multiple allele control of two reductase enzymes that convert ketones to alcohols is logical, but the available genetic evidence for an R^s allele is not critical. A strain having a large amount of *neo*-menthol should be studied, if one can be found. There is no evidence at this time that more than one *E* or ester gene occurs.

EXPERIMENTAL

A field nursery grown near Kalamazoo, Michigan or South Bend, Indiana had 7-plant single row plots 1.8 m long of 27 menthone *M. crispa* S_1 strains, 13 menthone-odored F_1 (4*n* *M. longifolia* × *M. crispa*) hybrids and 47 F_2 (4*n* *M. longifolia* × *M. crispa*) hybrids which were maintained by vegetative propagation for 20 years as vigorous, disease resistant strains to be used as possible pollen parents in plant breeding research needing a menthone parent. These 87 strains and all species and hybrid material were grown on organic soil in similar plots or as single plants spaced 1.8 m equidistant. The strains used in this research may be obtained from the Mentha Foundation Nursery maintained by the U.S. Dept. of Agriculture at Oregon State University, Corvallis, Oregon. All crosses were made in a screened greenhouse.

In the genus *Mentha*, the basic species of the subgenus *Menthastrum*, *M. suaveolens* Ehrh. (*M. rotundifolia* (L.) Huds.) and *M. longifolia* (L.) Huds. (*M. sylvestris* L.) have 12 bivalent pairs of chromosomes which disjoin regularly to give monogenic ratios and also perfect seed fertility in the absence of severe disease and when growing in suitable soil in well-adapted climatic conditions, especially long day length and cool nights. The pollen is wholly fertile too, unless the individual has a dominant male-sterile gene causing rudimentary anthers, a condition that may be confused with interspecific hybrid sterility of both seed and pollen due to the presence of univalent genomes or partially or completely unlike genomes in the hybrid individual.

A high amount of quadrivalent pairing occurs in spontaneous, or heat-induced or colchicine-induced polyploids of *Datura stramonium*, *Zea mays*, *Hordeum vulgare*, *Trifolium*, *Apium graveolens* and melons (Cucurbitaceae) and is associated with a 'high degree' of sterility, as well as tetrasomic segregation where observed. In contrast, the colchicine-induced polyploids of the basic *Mentha* species are completely seed and pollen fertile apparently due to a high degree of bivalent pairing as judged by direct observation, high fertility and duplicate gene ratios rather than tetrasomic ones. This difference in chromosome pairing in polyploid strains may be due to the short length of the *Mentha* chromosomes that presumably have fewer chiasmata than the longer chromosomes of maize and *Datura*.

The colchicine-induced polyploids of *M. suaveolens* and *M. longifolia* do not produce the 48-chromosomed species of section *Spicatae* whereas the latter are derived from both basic species to give a double diploid which has bivalent pairing and which is equivalent to 4*n* *Avena* or 4*n* *Triticum* in having bivalent chromosome pairing.

The natural 48-chromosomed species *M. spicata* L., *M. crispa* L. (*M. spicata* var. *crispata* Schrad.) and *M. cordifolia* of the *Spicatae* group of *Menthastrum* are double diploids (amphidiploids or allotetraploids) in origin and have 24 bivalent pairs of chromosomes which disjoin to produce complete seed and pollen fertility as well as monogenic or duplicate gene segregation. Cytogenetic or pollen fertility observations should never be made on greenhouse plants grown during the winter or in low light growth chambers. This is especially true of *M. crispa*, certain strains of *M. arvensis* and of *M. aquatica*. The same winter conditions also affect oil composition. The highly sterile cultivar native spearmint, often inappropriately included with fertile 48-chromosomed *M. spicata*, is an allotriploid with 36 chromosomes, perhaps an F_1 hydrid between *M. cordifolia* and *M. longifolia*. Colchicine-induced polyploid native spearmint is wholly fertile. All 48-chromosomed natural strains are wholly fertile except for genic male sterility, vary greatly in appearance, lose vigor rapidly on inbreeding and do not breed true for appearance characteristics or for the genes A and C controlling major differences in oil composition.

In the *Verticillatae* group of *Menthastrum*, the North American varieties of *M. arvensis* L. have 48 bivalent chromosome pairs and are completely fertile except for dominant or recessive genic male sterility. Some European strains of *M. arvensis* have 36 bivalent pairs and are fertile, whereas the cultivar *M. arvensis* var. *piperascens* has 48 bivalent pairs and is fertile.

In the *Capitatae* section of *Menthastrum*, the octoploid species ($2n = 96$) *M. aquatica* L. and *M. citrata* Ehrh. have 48 bivalent chromosome pairs and except for a monogenic dominant male sterile gene are wholly fertile and have monosomic or duplicate gene ratios. In certain *M. citrata*–*M. aquatica* F_1 hybrids, rare quadrivalent association and crossovers between unlike chromosomes have given unusual individuals having 70% limonene or large amounts of isopinocamphone. Strains of each hexaploid and octoploid species are morphologically very similar in appearance and their self-pollinated inbred individuals are identical or nearly so in appearance. Two or three generations of inbreeding results in very little loss of vigor.

The analytical data in Table 2 was done by F. W. Hefendehl using the following methods:

Fractionation of the oil: (a) Prefractionation by 'dry column chromatography' [34, 35] 100 g silicic acid, Woelm 60–150 mesh, activity III, in nylon tube; solvent: 100 ml hexane, 100 ml hexane-Et_2O (9:1); number of fractions 9. (b) Fractionation into single compounds by prep. GLC.

Gas chromatography: GC—Varian Aerograph 1860-4 and Hewlett-Packard 5750. Columns: preparative: 3 m × 6 mm 15% QF-1 or PEG 20 M 10% on Chromosorb W, DMCS-treated 60–80 mesh. Analytic: Thin film capillary columns 50 m × 0.25 mm, OV 17 or PEG 20 M.

Identification of isolated compounds: IR as film (NaCl or AgCl plates) Perkin Elmer 257. UV, Zeiss-Spectrophotometer PMQ II. Reaction-chromatography [24]. GLC assays for Fig. 2 and Table 4 by Lincoln were made using a 7.3 m × 3.2 mm column packed with 3% Carbowax 20 M on Gas Chrom Q at 135° and 30 ml/min N_2 carrier, FID, digital integrator. Using similar equipment all selections for high menthone and isomenthone,

including those in Table 1 made by Murray, were assayed in A. M. Todd Co. Chemistry Laboratory.

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REFERENCES

1. Lawrence, B. M., Morton, J. K. and Chambers, H. L. (1974) *Proc. VIth Int. Congr. on Essent. Oils.* San Francisco, Microfilm.
2. Handa, K. L., Smith, D. M., Nigam, I. C. and Levi, L. (1964) *J. Pharm. Sci.* **53**, 1407.
3. Vernet, P. (1976) Ph.D. thesis, Univ. Montpellier, France.
4. Lincoln, D. E. and Langenheim, J. H. (1978) *Biochem. Syst. Ecol.* **6**, 21.
5. Guenther, E. (1949) *The Essential Oils*, Vol. II, D. Van Nostrand, Princeton, N. J.
6. Lawrence, B. M., Hogg, J. W. and Terhune, S. J. (1972) *Flavour Ind.* **3**, 467.
7. Smith, D. M. and Levi, L. (1961) *J. Agric. Food Chem.* **9**, 230.
8. Murray, M. J., Marble, P. M. and Lincoln, D. E. (1971) *J. Hered.* **62**, 363.
9. Lincoln, D. E. and Murray, M. J. (1978) *Phytochemistry* **17**, 1727.
10. Battaile, J. and Loomis, W. D. (1961) *Biochim. Biophys. Acta* **51**, 545.
11. Katsuhara J. (1966) *Koryo* No. 83, 51.
12. Battaile, J., Burbott, A. J. and Loomis, W. D. (1968) *Phytochemistry* **7**, 1159.
13. Burbott, A. J., Croteau, R., Shine, W. E. and Loomis, W. D. (1974) *Proc. VIth Int. Congr. on Essent. Oils.* Paper No. 17, San Francisco, Microfilm.
14. Aviv, D. and Galun, E. (1978) *Planta Med.* **33**, 70.
15. Reitsema, R. H. (1958) *J. Am. Pharm. Assoc. Sci. Ed.* **47**, 265.
16. Hendriks, H. and van Os, F. H. L. (1976) *Phytochemistry* **15**, 1127.
17. Lawrence, B. M. (1978) Doctoral thesis, Rijksuniversiteit te Groningen, Netherlands.
18. Loomis, W. D. (1967) in *Biosynthesis and Metabolism of Monoterpenes in Terpenoids in Plants* (Pridham, J. B., ed.) Chap. 4. Academic Press, New York.
19. Fujita, S. and Fujita, Y. (1970) *Agric. Biol. Chem.* **34**, 1511.
20. Hefendehl, F. W., Underhill, E. W. and von Rudloff, E. (1967) *Phytochemistry* **6**, 823.
21. Hendriks, H., van Os, F. H. L. and Feenstra, W. J. (1976) *Planta Med.* **30**, 154.
22. Murray, M. J. (1960) *Genetics* **45**, 931.
23. Murray, M. J. (1960) *Genetics* **45**, 925.
24. Hefendehl, F. W. and Murray, M. J. (1972) *Phytochemistry* **11**, 189.
25. Tucker, A. (1975) Ph.D. thesis, Rutgers Univ., New Brunswick.
26. Murray, M. J. and Hefendehl, F. W. (1972) *Phytochemistry* **11**, 2469.
27. Murray, M. J. and Hefendehl, F. W. (1973) *Phytochemistry* **12**, 1875.
28. Lincoln, D. E., Marble, P. M., Cramer, F. J. and Murray, M. J. (1971) *Theor. Appl. Genet.* **41**, 365.
29. Murray, M. J. and Lincoln, D. E. (1970) *Genetics* **65**, 457.
30. Von Rudloff, E. and Hefendehl, F. W. (1966) *Can. J. Chem.* **44**, 2015.
31. Murray, M. J. (1972) *An. Acad. Bras. Cien.* **44**(Suppl), 24.
32. Hefendehl, F. W. and Murray, M. J. (1973) *Riv. Ital. Essenze, Profumi, Piante Off., Aromi, Saponi, Cosmet., Aerosol* **55**, 791.
33. Hefendehl, F. W. and Murray, M. J. (1976) *Lloydia* **39**, 39.
34. Loev, B. and Snader, K. M. (1965) *Chem. Ind. (London)* 15.
35. Loev, B. and Goodman, M. M. (1967) *Chem. Ind. (London)* 2026.