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APPARENT ENERGY DEFICIENCY IN MONO- AND SESQUI-TERPENE BIOSYNTHESIS IN PEPPERMINT

RODNEY CROTEAU, ALICE J. BURBOTT and W. DAVID LOOMIS
Department of Biochemistry and Biophysics, Oregon State University, Corvallis, OR 97331, U.S.A.

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Key Word Index—*Mentha piperita*; Labiatae; peppermint; monoterpenes; sesquiterpenes; biosynthesis; mevalonate-^{14}C incorporation; stimulation by sucrose and CO_2.

Abstract—Radioactivity from mevalonate-2-^{14}C is incorporated into the mono- and sesqui-terpenes of peppermint (*Mentha piperita* L.) cuttings when this precursor is fed through the cut stems. When unlabeled sucrose is fed along with labeled mevalonate, the incorporation of mevalonate into mono- and sesquiterpenes is markedly increased, the turnover period of the labeled terpenes is extended but with little change in turnover rate, and the proportion of label in the two major sesquiterpenes is shifted. These results are discussed in relation to the morphology of peppermint secretory structures and in relation to possible compartmentalization of mono- and sesqui-terpene biosynthesis within these structures, and it is suggested that sucrose may be satisfying an energy requirement at the biosynthetic sites. Exposure to 5% CO_2 in the light, coupled with mevalonate-2-^{14}C feeding, produces results similar to the co-administration of sucrose. The addition of unlabeled sodium acetate slightly increases incorporation of mevalonate-2-^{14}C into mono- and coupled with mevalonate-2-^{14}C feeding, produces results similar to the co-administration of sucrose. The addition of unlabeled sodium acetate slightly increases incorporation of mevalonate-2-^{14}C into mono- and sesqui-terpenes, probably by 'sparing' mevalonate rather than by satisfying an energy requirement.

INTRODUCTION

ONE OF the most striking findings to emerge from the numerous *in vivo* tracer studies of monoterpene biosynthesis is the almost universally poor incorporation of exogenous labeled substrates, especially MVA-^{14}C. Typically from 0·01 to 0·1% of MVA* or acetate label is incorporated into monoterpenes.[1,2] The only notable exception to date is the case of rose petals.[3] In some instances, slightly higher incorporations of MVA label into 'volatile oils' have been reported,[4,5] but without positive identification of the labeled components. Poor incorporations of MVA-^{14}C into certain sesquiterpenes were also reported[6,7] but these studies were less extensive, and few experimental details were published. In several cases[6–10] degradation of the mono- and sesqui-terpene products indicated direct incorporation of MVA-2-^{14}C *via* the isoprenoid pathway, but in other cases extensive randomization of label was observed.[1,2]

* Abbreviations used: MVA, mevalonic acid; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate.

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[10] R. CROTEAU and W. D. LOOMIS, *Phytochem.* **11,** 1055 (1972).

Such results might suggest an alternate, non-mevalonoid, pathway for the biosynthesis of mono- and sesqui-terpenes were it not for the degradative evidence cited, and the absence of other data supporting alternative pathways. Low incorporations might also be attributed to poor uptake of MVA by the plant or to the inhibition of essential enzymes by the concentrated doses of RS–MVA often administered. However, a number of investigators have carried out translocation and substrate-dilution studies, and even when optimum dose levels and methods of administration are utilized, high levels of MVA incorporation (i.e. greater than 1%) are not realized.[9–12] The 'physiological' source of carbon in green plants is CO_2, and $^{14}CO_2$, in the light, was found to be a relatively good monoterpene precursor, much better than MVA-^{14}C or acetate-^{14}C.[1,5,13] From these results we suggested[1] that the sites of monoterpene biosynthesis are isolated from the rest of the plant, and that the bulk of MVA utilized in monoterpene synthesis must arise at the site of synthesis from translocated photosynthate, probably sugars.

In search of other substrates that might be able to penetrate to the apparently isolated site of synthesis, we tested a number of labeled compounds as monoterpene precursors in peppermint.[14,15] Glucose and CO_2 were the most efficient substrates, and acetate and mevalonate were the least effective. The preferential incorporation of label from glucose, and especially from glucose-6-^{14}C, suggested a preferential transport of sugars. Thus, the findings supported our earlier suggestion that the sites of monoterpene biosynthesis in peppermint are compartmentalized, and isolated from the mainstream of plant metabolism.

We have tested various techniques for feeding MVA-2-^{14}C, and the effects of various forms and concentrations of this material on the efficiency of incorporation by peppermint cuttings, and, under what are presently considered to be optimum conditions, have obtained levels of about 0·03% incorporation of the *R*-isomer into monoterpenes and 0·3% into sesquiterpenes in the 6-hr maximal incorporation period.[10] This finding is particularly interesting because the sesquiterpenes constitute less than 2% of peppermint essential oil, while the monoterpenes constitute most of the remaining 98%. In contrast to these results, we have found label from ^{14}C-sugars and $^{14}CO_2$ to be incorporated into monoterpenes much more efficiently than into sesquiterpenes[16] (in a ratio roughly approximating the natural proportion of these materials in the essential oil) and have suggested that the two groups of terpenoids are synthesized at separate compartmentalized sites, probably within the oil glands.[10,15,16] It appeared that both types of sites are about equally accessible to translocated sugars, but that the sesquiterpene sites are more accessible to exogenous MVA.

Burmeister and von Guttenberg[17] studied the accumulation of essential oils under low-oxygen conditions, and under the influence of metabolic inhibitors, and on the basis of their findings they suggested that the biosynthesis of essential oil is a partially anaerobic process, which occurs as an adaptation to limited oxygen supply. The morphology of most types of oil glands is such as to suggest a degree of isolation both from the rest of the plant and from the atmosphere. For example, peppermint has two types of glandular epidermal

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[15] W. D. LOOMIS and R. CROTEAU, in *Recent Advances in Phytochemistry* (edited by V. C. RUNECKLES), Vol. 6, Academic Press, New York (1972).
[16] R. CROTEAU, A. J. BURBOTT and W. D. LOOMIS, *Phytochem.* **11,** 2459 (1972).
[17] J. BURMEISTER and H. VON GUTTENBERG, *Planta Med.* **8,** 1 (1960).

trichomes, both heavily cutinized, and attached to the leaf by a single stalk cell.[18,19] It seems quite possible that biosynthetic sites within such a gland are not readily accessible either to carbon substrates or to oxygen. Furthermore, electron microscope studies of peppermint glands,[18,19] and of oil glands from several other plant species (reviewed in Ref. 15) show characteristic membrane degeneration in the secretory cells at a very early stage of development. Thus it is likely that these cells are deficient in functional mitochondria. Should either or both of these considerations apply in the biosynthesis of mono- and sesquiterpenes (i.e. limited ability of O_2 to penetrate to the site of biosynthesis, or lack of functional mitochondria at the site), the metabolic consequences, in terms of energy production via mainly fermentative processes, would be similar. If, at the same time, the supply of photosynthate to the secretory cells is limited, these cells might be very energy deficient.

In vivo biosynthesis of acetyl-CoA from sugars yields, concomitantly, ATP and reduced pyridine nucleotides (i.e. energy), both of which are required for utilization of acetyl-CoA in terpene biosynthesis. However, when exogenous MVA-2-^{14}C is fed alone, necessary cofactors for terpene biosynthesis must still be generated endogenously. This requirement may pose a formidable problem for terpene biosynthesis within an isolated oil gland, as in peppermint, where photosynthate may not be readily available and where primarily fermentative mechanisms may be operative. Thus, the low incorporation of label from MVA-2-^{14}C into lower terpenes that is observed in many plant tissues may result from two related effects of compartmentalization: the relative inaccessibility of the glandular biosynthetic site to precursor MVA-2-^{14}C, and the inability to effectively utilize precursor MVA-2-^{14}C that does reach the site because of an inherent energy deficiency. Such a situation would make the cells of the oil glands extremely sensitive to the types and amounts of fermentable substrate (e.g. sucrose) available to them from adjacent cells.

To test these hypotheses, we have studied the effects of co-administration of unlabeled metabolic substrates on the incorporation of MVA-2-^{14}C into mono- and sesqui-terpenes of peppermint. The results, which are reported here, support the hypothesis that the biosynthesis of these terpenoid compounds is a largely fermentative process, requiring sugar as an energy source, and suggest that the biosynthetic sites are energy deficient.

RESULTS

Effect of Sucrose on the Incorporation of MVA-2-^{14}C

Aqueous solutions of MVA-2-^{14}C (5 μCi, 1 μmol, in 0·1 ml) were fed to individual peppermint cuttings through the cut stems, either alone or with 1 μmol of unlabeled sucrose or mannitol. Feedings were carried out in the light, under the same conditions as in previous studies.[10] After various time intervals the mono- and sesqui-terpenes were extracted, and analyzed by gas radiochromatography. The results are shown in Fig. 1. When MVA-2-^{14}C was fed alone, the time-course of terpene labeling was similar to that reported previously:[10] sesquiterpenes were labeled much more effectively than monoterpenes, while both classes of terpenes acquired maximum label in about 6 hr and then lost label. Co-administration of 1 μmol of unlabeled mannitol did not appreciably affect the results, whereas 1 μmol of unlabeled sucrose had a definite stimulatory effect on the incorporation of MVA into both mono- and sesqui-terpenes at all time periods studied. The fact that

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[19] F. Amelunxen, *Planta Med.* **13,** 457 (1965).

mannitol did not stimulate MVA utilization indicates that the stimulation by sucrose is not due to osmotic effects. Sucrose had relatively little effect on the absolute rate of loss of label after the 6-hr maximum but, the increased incorporation of label results in a slower relative turnover rate and an extended turnover period. Sucrose stimulated the incorporation of MVA into all of the individual terpenes that could be analyzed by gas radiochromatography (representing over 90% of the essential oil), and so the mono- and sesqui-terpenes are plotted as classes (i.e. the sums of gas radiochromatographic peaks) in Fig. 1 rather than as individual components.

The bulk of the essential oil label in this experiment was found in the two major sesquiterpenes, caryophyllene and γ-muurolene. Figure 2 shows the time-course of labeling in these two compounds. The qualitative trends shown in Figs. 1 and 2 were verified in several similar time-courses, though there was some quantitative variation from one experiment to another. For example, caryophyllene and γ-muurolene were always the principal labeled components, but their relative activities varied, and occasionally γ-muurolene was the more highly labeled of the two.

The effect of sucrose level (up to 10 μmol in 0·1 ml) on 6-hr MVA incorporations was then studied. The results for a set of matched cuttings, which showed moderate stimulation of MVA incorporation, are shown in Fig. 3, along with a set of mannitol controls. The degree of stimulation by sucrose was somewhat variable, due in part to unavoidable variability in the cuttings employed, but the presence of sucrose even at low levels always measurably increased the incorporation of MVA into both mono- and sesqui-terpenes. The presence of mannitol had little or no effect on MVA incorporation. The degree of MVA incorporation into monoterpenes increased linearly with increasing amounts of sucrose up to about 1 μmol per cutting, while the incorporation of MVA into sesquiterpenes reached its maximum between 0·25 and 0·5 μmol of sucrose per cutting. Thus, the sesquiterpene system appeared to reach saturation at lower levels of exogenous sucrose than the monoterpene system, consistent with the previous suggestion that the sesquiterpene biosynthetic sites are less isolated than the monoterpene biosynthetic sites.[10,15] At levels of sucrose above 3 μmol per cutting, incorporation of MVA into both mono- and sesqui-terpenes declined. MVA incorporation in the controls appeared to decrease slightly at the level of 10 μmol of mannitol per cutting, suggesting an osmotic effect at the higher levels of sucrose or mannitol. It seems likely that the reduced ^{14}C-incorporation at intermediate levels of sucrose was due to competition between exogenous MVA-2-^{14}C and MVA derived from the sucrose.

Several further experiments similar to this one were carried out. Although there was some variability, the results were similar to those shown in Fig. 3. In the range of 1–8 μmol of sucrose per cutting, MVA incorporation into monoterpenes was stimulated 3–10-fold, and into sesquiterpenes 3–7-fold over zero-sucrose or equimolar-mannitol controls. On several occasions cuttings that showed unusually high MVA incorporations were noted. In these cuttings it appeared that when the incorporation of MVA into sesquiterpenes was greatly stimulated (6- or 7-fold), the incorporation into monoterpenes was only 2–3 times that of controls. In other visually identical cuttings the reverse situation applied: when monoterpene biosynthesis was stimulated 8–10-fold, sesquiterpene biosynthesis was barely doubled.

Interactions of MVA and Sucrose

The interactions of sucrose and MVA in the biosynthesis of mono- and sesqui-terpenes

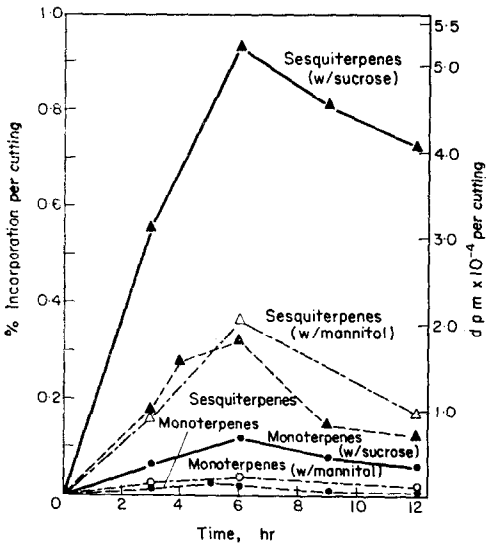

FIG. 1. TIME-COURSE OF LABELING OF PEPPERMINT MONO- AND SESQUI-TERPENES FROM MVA-2-^{14}C WITH AND WITHOUT 1 μmol ADDED SUCROSE OR MANNITOL.

Data presented are from a single set of visually matched cuttings fed 1 μmol RS-MVA-2-^{14}C per cutting (with or without 1 μmol added sucrose or mannitol) in 0·1 ml H_2O. Replication of the experiment showed a similar time-course. Data points for mono- and sesqui-terpenes represent the sums of activities in gas radiochromatographic peaks, and % incorporation is calculated by assuming that only R-MVA is physiologically active. Time is from start of MVA feeding.

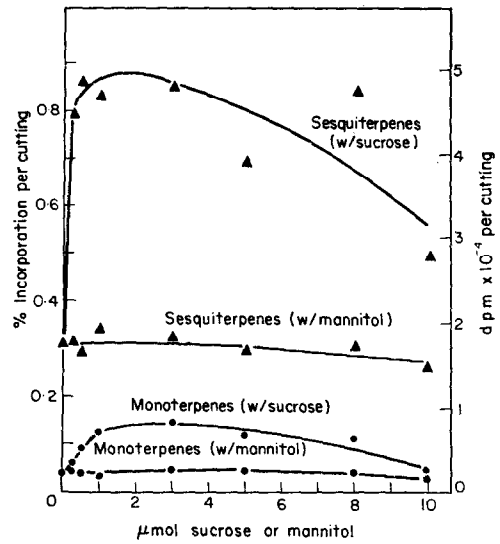

FIG. 3. EFFECT OF DIFFERENT LEVELS OF SUCROSE OR MANNITOL ON INCORPORATION OF MVA-2-^{14}C INTO PEPPERMINT MONO- AND SESQUI-TERPENES.

Data presented are from a single set of visually matched cuttings fed 1 μmol RS-MVA-2-^{14}C per cutting (with sucrose or mannitol added as shown) in 0·1 ml H_2O. Data points for mono- and sesqui-terpenes represent the sums of activities in gas radiochromatographic peaks, and % incorporation is calculated by assuming that only R-MVA is physiologically active. Incorporation time is 6 hr from start of MVA feeding.

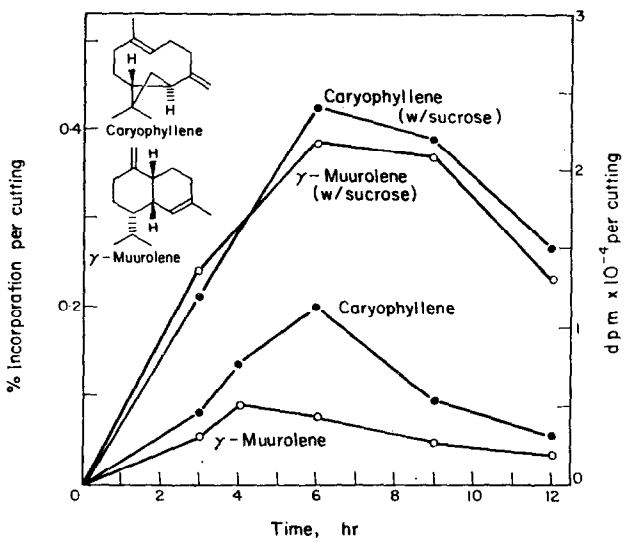

FIG. 2. TIME-COURSE OF LABELING OF CARYOPHYLLENE AND γ-MUUROLENE FROM MVA-2-^{14}C WITH AND WITHOUT 1 μmol ADDED SUCROSE. SAME EXPERIMENT AND EXPERIMENTAL DETAILS AS FIG. 1.

were further examined by feeding various combinations of labeled and unlabeled substrates to peppermint cuttings. The results are shown in Table 1. As before, unlabeled sucrose stimulated incorporation of label from MVA-2-^{14}C into both mono- and sesqui-terpenes (Expt. b vs. Expt. a). On the other hand, 1 μmol of unlabeled MVA suppressed the incorporation of sucrose-U-^{14}C into mono-terpenes by about 20% and into sesquiterpenes by 10% (Expt. c vs. Expt. e). That the suppression was not due to uptake or translocation differences between (c) and (e) is shown by the osmotic control (d), in which the addition of 1 μmol of adipate had no effect on sucrose-U-^{14}C incorporation. That exogenous sucrose and exogenous MVA are in fact utilized simultaneously for mono- and sesqui-terpene biosynthesis is shown by the fact that the results of Expt. (b) plus Expt. (e) are about equal to Expt. (f). Thus it appears that while sucrose stimulates the incorporation of exogenous MVA-^{14}C into mono- and sesqui-terpenes, it also serves as a source of acetyl-CoA, and thence of MVA, which competes with the exogenous MVA as a terpenoid precursor.

TABLE 1. INTERACTION OF SUCROSE AND MVA IN THE BIOSYNTHESIS OF PEPPERMINT MONO- AND SESQUI-TERPENES

Substrates*		Products			
		Monoterpenes		Sesquiterpenes	
Labeled	Unlabeled	dpm	% Incorp.†	dpm	% Incorp.†
(a) MVA-^{14}C	—	2 700	0·038	21 100	0·30
(b) MVA-^{14}C	Sucrose	15 000	0·21	131 000	1·9
(c) Sucrose-^{14}C	—	141 000	1·2	10 700	0·092
(d) Sucrose-^{14}C	Adipate	139 000	1·2	11 100	0·096
(e) Sucrose-^{14}C	MVA	111 000	0·96	9500	0·082
(f) Sucrose-^{14}C + MVA-^{14}C	—	125 600	—	144 000	—
Sum of (b) and (e)		126 000	—	140 500	—

* In 0·1 ml of H_2O. Each specified substrate was present at a level of 1 μmol (1 μmol RS-MVA = 0·5 μmol R-MVA). ^{14}C-substrates were RS-MVA-2-^{14}C ($10{\cdot}4 \times 10^6$ dpm) and sucrose-U-^{14}C ($11{\cdot}6 \times 10^6$ dpm). Adipic acid (neutralized with NaOH) was used as an osmotic control because it is similar to MVA, but not a common metabolite.

† Per cent incorporation represents the sum of activities in gas radiochromatographic peaks after a 6-hr incorporation period and is calculated for MVA by assuming that only the R-isomer is physiologically active. Results are averages of two cuttings, except Expt. (f) which represents a single cutting.

Effects of CO_2 *and Acetate on the Incorporation of MVA*-2-^{14}C

If the effect of sucrose on MVA-2-^{14}C incorporation is that of providing a suitable energy source at the site of biosynthesis, then the administration of CO_2 in the light during stem feeding of MVA might be expected to produce similar results. Two types of experiments were carried out to test this possibility. Three cuttings were first allowed to take up MVA-2-^{14}C solution (1–1·5 hr) and then placed in an atmosphere of 5% CO_2 in air in the light for 1 hr. Another set of three cuttings were fed MVA-2-^{14}C during a 1-hr exposure to 5% CO_2 in the light. At the end of the CO_2 exposure period both sets of cuttings were flushed with air and maintained in the light until 6 hr had elapsed from the start of MVA feeding. Controls were fed MVA-2-^{14}C while being maintained in normal air in the light for the entire 6-hr period. Individual cuttings were then extracted, and the essential oil was analyzed by gas radiochromatography. The results of these experiments are shown in Table 2. In both experiments 5% CO_2 approximately doubled the incorporation of label

into sesquiterpenes. Incorporation into monoterpenes was stimulated 2–3-fold by CO_2 administered after MVA-^{14}C and 3–5 fold by simultaneous CO_2. This latter observation may reflect a more nearly coincident arrival of MVA-^{14}C and translocated photosynthate at the site of synthesis when MVA and CO_2 are fed simultaneously.

TABLE 2. EFFECT OF CO_2 ON THE INCORPORATION OF MVA-2-^{14}C INTO MONO- AND SESQUI-TERPENES OF PEPPERMINT

Conditions	Ratio of % incorporation with CO_2 treatment* to % incorporation in controls† Monoterpenes‡	Sesquiterpenes‡
MVA-^{14}C fed before CO_2 exposure	1·9, 2·3, 2·9	1·8, 1·8, 2·2
MVA-^{14}C fed during CO_2 exposure	2·9, 4·1, 4·7	1·7, 1·9, 2·1

* Per cent incorporation represents the sum of activities in gas radiochromatographic peaks after a 6-hr incorporation period in the light with 1 hr exposure to 5% CO_2, and is calculated by assuming that only R-MVA-2-^{14}C is physiologically active. Each cutting received 1 μmol RS-MVA ($10{\cdot}4 \times 10^6$ dpm).

† Average of two controls run in air = 0·038 % incorporation of R-MVA-2-^{14}C into monoterpenes; 0·32% incorporation into sesqui-terpenes.

‡ Values given are for three individual cuttings.

In another set of experiments the effect of co-administration of sodium acetate on MVA-2-^{14}C incorporation was tested. Cuttings were fed 1 μmol of MVA-2-^{14}C with up to 12 μmol of added sodium acetate or NaCl in 0·1 ml H_2O. As shown in Table 3, 3–8 μmol of sodium acetate per cutting produced a slight stimulation of MVA incorporation into mono- and sesqui-terpenes, up to about 1·5 times the level of the NaCl or zero-acetate controls. NaCl had no apparent effect at these levels. Solutions containing acetate or NaCl at levels of 12 μmol per cutting were taken up quite slowly by the cuttings, and both produced a decrease in incorporation of MVA, probably due to osmotic effects. Exogenous acetate is not a good precursor of either mono- or sesqui-terpenes in peppermint,[14,15] and presumably is not a fermentable substrate. It seems likely from the present results that acetate does exert an MVA-sparing effect, thus allowing more the labeled MVA to reach the sites of essential oil synthesis.

At the 6-hr sampling time, CO_2 had much the same effect on the distribution of label between caryophyllene and γ-muurolene as did sucrose at 6 hr, as shown in Fig. 2 (i.e. γ-muurolene label was enhanced more than caryophyllene label). This effect was not observed in the acetate experiments. It should be kept in mind that these results are based on 6-hr incorporation periods, which may not be strictly comparable, because the time-course curves might shift with changes in sucrose or acetate concentration. As shown in Fig. 1, however, a 6-hr maximum incorporation was exhibited by cuttings with either 1 μmol of added sucrose or with no added sucrose.

Although some investigators routinely co-administer ATP with MVA-^{14}C, no stimulation of MVA utilization by exogenous ATP has actually been demonstrated, and it seems unlikely that exogenous ATP would be able to enter the cells. In a final set of experiments the effect of co-administration of ATP and related compounds on 6-hr MVA-^{14}C incorporation in peppermint was tested. ATP (2·5 μmol) had little or no effect on MVA-2-^{14}C (0·5–1·0 μmol) incorporation into either mono- or sesqui-terpenes. Inorganic pyrophosphate

(10 μmol) also had little or no effect on MVA-2-^{14}C incorporation, while AMP (2·5 μmol) plus inorganic pyrophosphate (10 μmol) substantially decreased MVA-^{14}C incorporation. Co-administration of fructose-1,6-diphosphate (2·5 μmol) had no appreciable effect on MVA-^{14}C incorporation.

TABLE 3. EFFECT OF SODIUM ACETATE CONCENTRATION ON THE INCORPORATION OF MVA-2-^{14}C INTO MONO- AND SESQUI-TERPENES OF PEPPERMINT

μmol Na acetate or NaCl* per cutting	% incorporation into monoterpenes†		% incorporation into sesquiterpenes†	
	With NaCl	With acetate	With NaCl	With acetate
0	0·037	0·037	0·40	0·40
3	0·034	0·041	0·37	0·44
5	0·041	0·066	0·44	0·53
8	0·034	0·054	0·38	0·53
12	0·026	0·024	0·29	0·31

*μMol Na acetate or NaCl plus 1 μmol RS-MVA-2-^{14}C (10·4 $\times$ 10^6 dpm) in 0·1 ml H_2O.

†Per cent incorporation represents the sum of activities in gas radiochromatographic peaks after a 6-hr incorporation period in the light and is calculated by assuming that only R-MVA-2-^{14}C is physiologically active. Data presented are for a single set of cuttings. Repetition of the experiment at selected acetate concentrations yielded similar results.

DISCUSSION

All the results reported here are consistent with the concept that the sites of biosynthesis of mono- and sesqui-terpenes in peppermint are highly compartmentalized, largely dependent on fermentative metabolism for energy and for acetyl-CoA production, and energy deficient due to a lack of sugars. The monoterpene biosynthetic sites would appear to be more rigidly compartmentalized and more energy deficient than the sesquiterpene sites, consistent with earlier findings.[10] Though other factors, such as permeability effects, or MVA-sparing effects, cannot be excluded, none of these seems to offer a rational cause-and-effect explanation of all of the observations. Although the slight stimulation of MVA-^{14}C incorporation by acetate is probably due to an MVA-sparing effect, it seems unlikely on theoretical grounds that the effects of supplemental sucrose are due to MVA-sparing. For example, complete conversion of 1 μmol of sucrose to MVA would yield 1·33 μmol of R-MVA, which if totally utilized to spare MVA-2-^{14}C would only effectively increase the R-MVA-^{14}C level administered from 0·5 to 1·83 μmol, while under these conditions a greater than 4-fold increase in MVA was incorporation routinely noted, and 7–10-fold increases were sometimes seen.

Our previous tracer studies with peppermint were routinely carried out in the light in a growth chamber, since it was assumed that the cuttings needed energy from photosynthesis, especially when $^{14}CO_2$ was used as the substrate. However, the present results suggest that the transport of photosynthate to the essential oil-producing cells may normally be very restricted. Feeding of exogenous sucrose or glucose through the xylem of the cut stem probably provides luxury levels of sugars to the whole cutting, overcoming normal control mechanisms. If peppermint is a 'high-compensation' plant, a 5% CO_2 atmosphere would be expected to enhance net photosynthesis, by increasing the rate of CO_2 fixation,

be inhibiting photorespiration, or by a combination of these effects.[20,21] Thus it is not surprising that supplemental sucrose and supplemental CO_2 have similar effects on MVA-2-^{14}C utilization. It seems reasonable to suggest that the effect in both cases is to supply metabolic energy in the form of sugars to the essential oil-producing cells, and that some of this energy can be utilized for the metabolism of exogenous MVA-^{14}C. This suggestion is strengthened by the observed competition between MVA and sucrose as carbon substrates for the biosynthesis of mono- and sesqui-terpenes, as seen in Table 1. 'Normal' biosynthesis of mono- and sesqui-terpenes in peppermint is probably a largely fermentative process, with glycolysis providing both energy and acetyl-CoA. Tracer evidence based on the incorporation of label from specifically ^{14}C-labeled glucoses into peppermint monoterpenes indicated extensive involvement of the pentose phosphate pathway as well.[16] It is not possible at this point to judge whether this pathway is directly involved in terpene synthesis, or only contributes incidentally to the scrambling of carbon atoms, but it could serve as a source of NADPH needed for the biosynthesis of MVA.

From the data of Table 1 it can be calculated that 1 μmol of sucrose increased the incorporation of MVA-2-^{14}C (1 μmol of RS-MVA administered) into mono- and sesquiterpenes by about 8·7 nmol. This enhancement would require 26 nmol of ATP, since three ATP's are required for each MVA utilized in terpenoid synthesis. Conversely, 1 μmol of RS-MVA decreased the incorporation of sucrose-U-^{14}C (1 μmol of sucrose administered) into mono- and sesqui-terpenes by about 2·6 nmol. If these data are representative, and if the effects of sucrose on MVA-2-^{14}C incorporation and of MVA on sucrose-U-^{14}C incorporation are truly additive, it would seem reasonable, as a first approximation, to suppose that 2·6 nmol of sucrose provided the ATP for utilization of 8·7 nmol of MVA, or 26 nmol of ATP. This would require a yield of 10 nmol of ATP per nmol of sucrose, about 2·5 times the 4 nmol of ATP to be expected from anaerobic fermentation, but much less than the 76 nmol of ATP to be expected from completely aerobic respiration. (These ATP yields assume that the energy of the glycosidic bond of sucrose is not preserved, since known mechanisms for accomplishing this in plants require inorganic pyrophosphate for pyrophosphorylation of UDP-glucose.) In view of the assumptions made, and the fact that the ratio 10:1 is calculated for total mono- and sesqui-terpenes and thus represents a composite of events that may be taking place at different biosynthetic sites in which the efficiency of sucrose utilization may vary greatly, no precise quantitative conclusions can be reached from these calculations. It does seem reasonable to suggest, though, that the orders of magnitude are such as to agree with a largely fermentative metabolism of sucrose.

It thus becomes pertinent to enquire whether purely fermentative processes could account for terpene production. We have previously suggested[1] that in the biosynthesis of the *p*-menthanoid monoterpenes, the first cyclic product is either α-terpineol or a *p*-menthadiene (limonen or terpinolene). The theoretical derivation of a monoterpene molecule at this oxidation-reduction level by fermentation of sucrose can be formulated as follows (assuming that the energy of the glycosidic linkage is lost, and that NADH can transfer electrons to NADP):

$$1\tfrac{1}{2}\ \text{Sucrose} \rightarrow 6\ \text{acetyl-CoA} + 6\ Co_2 + 12\ \text{NADH} + 6\ \text{ATP} \qquad (1)$$

$$6\ \text{Acetyl-CoA} + 4\ \text{NADPH} + 6\ \text{ATP} \rightarrow \text{terpinolene} + 2\ CO_2 \qquad (2)$$

$$1\tfrac{1}{2}\ \text{Sucrose} \rightarrow \text{terpinolene} + 8\ CO_2 + 8\ \text{NADH}. \qquad (1+2)$$

[20] I. ZELITCH, *Ann. Rev. Plant Physiol.* **20,** 329 (1969).
[21] W. A. JACKSON and R. J. VOLK, *Ann. Rev. Plant Physiol.* **21,** 385 (1970).

In peppermint, a substantial part of the monoterpene produced is further metabolized to menthones and menthols. The reduction of pulegone to menthone and isomenthone, and the reduction of menthone to menthol are known to utilize NADPH.[22] The other steps are not yet known, but it seems likely that terpinolene is oxygenated to piperitenone, which is then reduced to pulegone by NADPH. The oxygenation may be postulated to utilize O_2 plus either 1 or 2 NADPH.[23] Thus the conversion of the first cyclic monoterpene to menthol probably utilizes a maximum of 5 NADPH:

$$\text{Terpinolene} + 5\ \text{NADPH} + O_2 \rightarrow \text{menthol} + H_2O. \qquad (3)$$

The overall scheme, from sucrose to menthol, would then be:

$$1\tfrac{1}{2}\ \text{Sucrose} + O_2 \rightarrow \text{menthol} + 8\ CO_2 + 3\ \text{NADH} + H_2O. \qquad (1+2+3)$$

Similar considerations would apply in sesquiterpene biosynthesis. Thus, a simple fermentative conversion of sucrose to lower terpenes would produce an unbalanced system, providing barely adequate ATP, and an excess of NADH. Several possible mechanisms for reoxidation of the excess NADH may be suggested. Although it appears that the essential oil-producing cells do not carry out 'normal' respiration, they may be able to respire limited amounts of NADH, or they may oxidize NADH by uncoupled electron transport, without ATP production. Other possible explanations involve mixed fermentations. For example, if the terpene-producing cells simultaneously produce cuticle lipids as a major product, the reductions and oxygenations involved, plus the lower amounts of ATP required, might produce a balanced system overall. A further possibility is that lower terpenes are the product of a mixed fermentation in which some other fermentation product (e.g. ethanol or α-glyceryl phosphate) serves as a 'shuttle' to be reoxidized in adjacent cells that are capable of normal respiration. Some of the excess NADH might also be utilized, incidentally, in processes such as NO_3^- reduction.[24]

The effects of sucrose and CO_2 on the incorporation of MVA into lower terpenes suggest that the *in vivo* biosynthesis of these compounds is directly influenced by the presence of sucrose, or equivalent products of photosynthesis, which might in turn be controlled by the balance between photosythesis and the utilization of photosynthate. This balance does appear to influence mono-terpene biosynthesis and metabolism in long-term experiments with intact peppermint plants.[15,25] In a recent preliminary experiment we attempted to examine the short-term effects of this balance on the biosynthesis of lower terpenoids from MVA-2-^{14}C in peppermint cuttings. In all previous experiments, cuttings were taken from mint plants in the morning. 2–2·5 hr after the beginning of the light period, and allowed to take up and metabolize MVA-2-^{14}C in the light. When cuttings were taken at the end of the normal 16-hr daylight period (when they presumably contain more accumulated photosynthate) and allowed to take up and metabolize MVA in the light, they incorporated only slightly more MVA lebel at the 6-hr peak than did cuttings taken from plants in the morning; however, the turnover period was greatly extended. Although these preliminary results are from a single experiment using only two sets of cuttings, they do appear to be consistent with the results of co-administration of sucrose or CO_2 on MVA incorporation (i.e. abundance of carbohydrate appears, generally, to promote terpene synthesis and/or reduce terpene turnover).

[22] J. BATTAILE, A. J. BURBOTT and W. D. LOOMIS, *Phytochem.* **7,** 1159 (1968).
[23] B. S. COHEN and R. W. ESTABROOK, *Arch. Biochem. Biophys.* **143,** 46, 54 (1971).
[24] L. KLEPPER, D. FLESHER and R. H. HAGEMAN, *Plant Physiol.* **48,** 580 (1971).
[25] A. J. BURBOTT and W. D. LOOMIS, *Plant Physiol.* **43,** 20 (1967).

It should be kept in mind that the results described are for cuttings and, although useful conclusions may be drawn from this work concerning the biosynthesis of lower terpenes, the results for cuttings may not exactly reflect the metabolism of the intact plant. For example, recent experiments have shown that on exposure to $^{14}CO_2$, cuttings incorporate somewhat more label into mono- and sesqui-terpenes and shown a faster rate of turnover than rooted plants.[14]

Accumulation or secretion of any quantity of mono- or sesqui-terpenes is generally, perhaps always, associated with the presence of recognizable glandular structures: notably oil cells, glandular hairs, oil or resin ducts, or glandular epidermis.[15] It is commonly supposed that the essential oil components are produced within the 'secretory cells' of the oil glands, although there is evidence to suggest that secretory activity may spread to other cells as well.[15] The universally poor incorporation of exogenous MVA into mono- and sesqui-terpenes in vegetative tissues suggests that the secretory cells in these tissues are isolated. The present observations suggest that, at least in peppermint, they are also energy deficient.

Tracer studies of monoterpene biosynthesis in flower tissues provide a striking contrast to all of the reported studies of mono- and sesqui-terpene biosynthesis in vegetative tissues. Rose petals were found to incorporate up to 22% of applied R-MVA into monoterpenes within 1 hr,[3] while chrysanthemum ovules incorporated over 2% of applied R-MVA-2-^{14}C into monoterpenoid chrysanthemum acids in 24 hr.[26] This unique behaviour of flower tissues is probably related to the presence of unique scent glands or 'osmophors' in flowers.[27] Osmophors consist of a glandular epidermis, which appears to be in intimate contact with the phloem and with storage cells in the mesophyll. In some species it has been observed that starch from the storage cells is depleted rapidly during formation of the volatile secretion.[27] It also appears that osmophore cells are more permeable to vital stains than are other flower cells, indicating increased membrane permeability.[27] In contrast to other types of oil glands the glandular epidermis seems to be designed for maximum exposure of the secretory cells both to the atmosphere and to the energy-rich mesopyll. It seems unlikely that these cells suffer from deficiency of either oxygen or carbohydrate. The secretory structures of rose petals have the appearance of typical osmophors.[28–30] The relationship of the glandular epidermis of rose petals to the phloem, or to reserves in the mesophyll, apparently remains to be examined, but the tracer studies indicate that the monoterpene-synthesizing cells of rose petals take up MVA readily and do not lack energy to utilize it. Thus it appears that there may be two fundamental physiological types of essential oil secretory sites in plants: isolated energy-deficient sites (as in peppermint), and non-isolated energy-rich sites (as in rose petals).

EXPERIMENTAL

Plant material. Peppermint plants were the Black Mitcham cultivar of *Mentha piperita* L., propagated vegetatively from the clone used previously,[25] and grown in a growth chamber maintained at 24° day temp. and 10° night temp. during a regular 24-hr cycle with 16-hr day under 10 500–11 000 lx light intensity as

[26] M. P. Crowley, P. J. Godin, H. S. Inglis, M. Snarey and E. M. Thain, *Biochim. Biophys. Acta* **60**, 312 (1962).

[27] S. Vogel, *Akad. Wiss. Lit., Mainz, Abh. Math.-Naturwiss. Kl.*, No. 10, pp. 599–763 (pages also numbered separately: 1–165) (1962).

[28] W. Mazurkiewicz, *Z. Allg. österr. Apoth.-Verein.* **51**, 241 (1913).

[29] G. Weichsel, in *Die Ätherischen Öle* (edited by E. Gildemeister, F. Hoffman and W. Treibs), Vol. I, p. 233, Akademie, Berlin (1956).

[30] J. M. Stubbs and M. J. O. Francis, *Planta Med.* **20**, 211 (1971).

determined with a Se photocell.[22,25] Illumination was from Sylvania VHO Gro-Lux and Wide-Spectrum Gro-Lux lights in equal numbers. Cuttings, consisting of the tuft of youngest leaves at the growing tip plus the next 2 leaf pairs, were taken in the morning 2–2·5 hr after the beginning of the light period unless otherwise specified in the text. Stems were cut under H_2O, and the cuttings were carefully tested before feeding began to insure that they were able to take up H_2O actively. Fr. wts of cuttings were between 250 and 300 mg, and cuttings were matched visually as closely as possible.

Administration of mevalonate-2-^{14}*C, sucrose, acetate* and CO_2. For all experiments RS-mevalonate-2-^{14}C (N,N-dibenzylethylenediamine salt) obtained from New England Nuclear Corp., Boston, Massachusetts was employed. This substance was reported by the manufacturer to possess a specific activity of 4·7 μCi/μmol and a radiochemical purity greater than 97% as determined by PC. Cuttings were placed in vials in a small growth chamber under day conditions as described above and given aq. solutions of RS-MVA-2-^{14}C (5 μCi in 0·1 ml per cutting) through the cut stems. After the uptake of labeled material (1–1·5 hr) the vials were kept filled with distilled H_2O. Cuttings were removed at appropriate time intervals and the essential oil was immediately extracted. To co-administer sucrose, sodium acetate, mannitol, sodium chloride and other unlabeled compounds to the cuttings, appropriate amounts of aqueous solutions of these materials were first placed in the vials. The H_2O was then removed under vacuum before the addition of MVA-2-^{14}C solution. Sucrose-U-^{14}C (5·22 μCi/μmol) was obtained from New England Nuclear Corp. and was reported by the manufacturer to possess a radiochemical purity greater than 99%. Unlabeled RS-mevalonate (*N,N*-dibenzylethylenediamine salt) was obtained from Calbiochem, La Jolla, California. Co-administration of these substrates was essentially as described above. To co-administer CO_2 6 cuttings at a time were placed in a 250-ml glass exposure chamber, and a 5% CO_2 in air atmosphere was generated by the addition of perchloric acid to aqueous K_2CO_3. After 1 hr the chamber was flushed with air and opened, and the cuttings were maintained under day conditions for the remainder of the incorporation period.

Isolation and gas radiochromatographic analysis of essential oil. Cuttings were extracted with hexane in the presence of anhydrous Na_2SO_4, and the extracts were decolorized with Norit A activated charcoal as previously described.[25] Radioactive essential oil components were analyzed using a Beckman Thermotrac temperature programmer fitted with a Carle Micro-Detector (thermal conductivity) attached directly to a Nuclear-Chicago Biospan 4998 continuous gas flow counter. The column employed was 6·1 m × 3 mm stainless steel with 1% phenyl diethanolamine succinate (PDEAS) and 1·5% sucrose acetate isobutyrate (SAIB) coated on 100–120 mesh Chromosorb G and was programmed from 125 to 165° at 1°/min with a helium flow rate of 25 ml/min. The instrument was calibrated with toluene-^{14}C, and peak areas were determined with a Disc integrator. The procedures, and the composition of peppermint oil, are described in detail elsewhere.[10,13,16]

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