

CHROMATOGRAPHIC ANALYSIS AND BIOSYNTHESIS
OF PEPPERMINT OIL TERPENES

by

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TABLE OF CONTENTS

	Page
INTRODUCTION	1
CHROMATOGRAPHY OF PEPPERMINT OIL	8
Introduction	8
Experimental	10
Preparation and Use of Chromatograms	11
Detection of Colorless Compounds	13
Experimental Results	14
Dependence of R_f on the Composition of the Solvent	16
Dependence of R_f on Temperature	17
Discussion	19
Theoretical Treatment	20
METABOLISM OF 1-C ¹⁴ -ACETATE BY EXCISED PEPPERMINT LEAVES	29
Tracer Study I	29
Time Study	30
Chromatography	30
Tracer Study II	32
Time Study	33
Chromatography	33
Discussion	37
SUMMARY	38
BIBLIOGRAPHY	39

LIST OF TABLES

	Page
Table 1 - Variation of R_f Values with the Composition of the Solvent	16
Table 2 - Range of R_f Values for Some Representative Chromatograms	17
Table 3 - Outline of Tracer Study II	33

LIST OF FIGURES

Figure 1 - Structure of Menthone and Menthol	2
Figure 2 - A Representative Chromatoplate	15
Figure 3 - Plot of R_f against Temperature	18
Figure 4 - Plot of R_f against the Ethyl Acetate in Solvent	25
Figure 5 - Plot of $\ln R_f$ against Reciprocal of Temperature	27
Figure 6 - Total Counts per Minute of C^{14} against Time - Time Study I	31
Figure 7 - Total Counts per Minute of C^{14} against Time - Time Study II	34

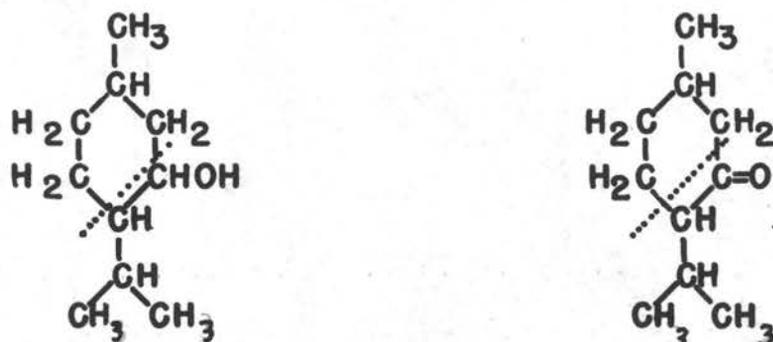
CHROMATOGRAPHIC ANALYSIS AND BIOSYNTHESIS OF PEPPERMINT OIL TERPENES

INTRODUCTION

Chemically, peppermint oil is a complex mixture containing several terpenes as well as simpler substances such as acetic acid, isovaleric acid and isovaleraldehyde. The major component of peppermint oil is menthol, which comprises 45-60% of the oil depending on the time of the year (17, p.595). Some of the menthol is esterified with acetic acid and isovaleric acid. Menthone, the ketone corresponding to menthol, makes up from 2-13% of the oil, also varying with the time of the year. As the plant matures, the content of menthol increases, with a simultaneous decrease in menthone. Various other terpenes, such as cineole, 1-limonene, terpinene, cadinene and menthofuran, are present in small amounts (17, pp.616-617).

Terpenes have much in common with rubber, cholesterol and carotenoids in that the structure of each may be broken down into five-carbon units related to isoprene. The structures of menthone and menthol are shown in Figure 1, with dotted lines indicating the division into isoprene-like units.

Figure 1



It is generally felt that all of these substances arise biologically via common five-carbon branched-chain intermediates. The presence in peppermint oil of isovaleraldehyde and isovaleric acid, both of which have the isoprene carbon skeleton, is consistent with this hypothesis.

Various schemes have been suggested for the formation of the isoprenoid precursors. On the basis of the composition of mint oil, Kremers (26, pp.31-34) in 1922 postulated a scheme for the biosynthesis of the terpenes involving a condensation of acetone with acetaldehyde to form 3-methylbutenal. This would then undergo dimerization and cyclization to form the terpenes. Guenther (16, pp.50-58) has suggested that acetone condenses with pyruvate followed by a decarboxylation to form 3-methylbutenal. Francesconi (16, p.55 and 12, pp.33-36) suggested that isoamyl alcohol

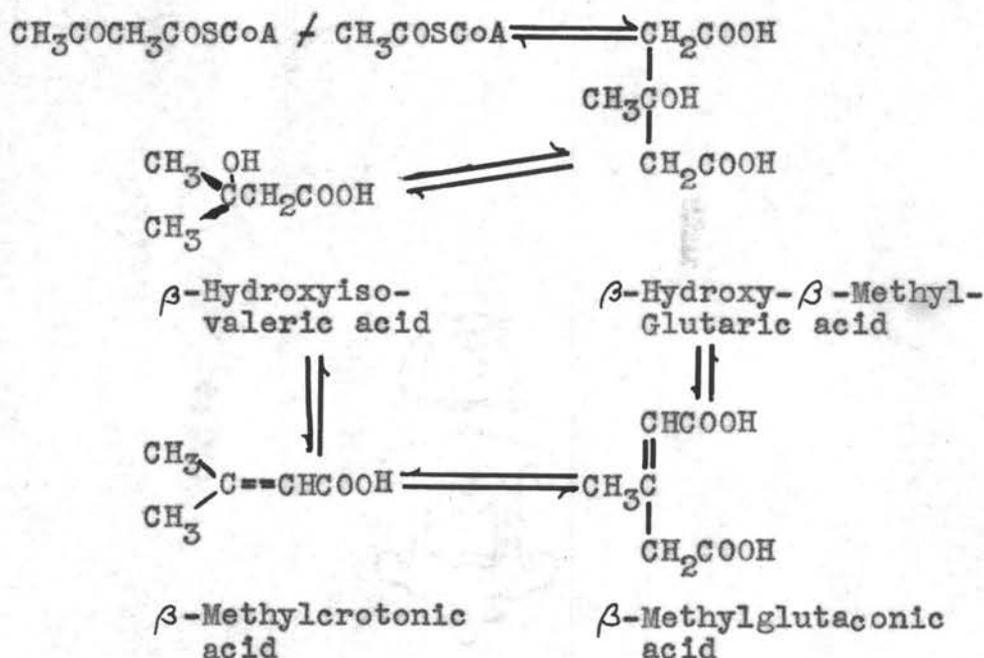
is important in the formation of these precursors while Simpson (41, pp.33-36) visualizes a coupling of acetoacetic acid with acetone to obtain monocyclic terpenes. Emde (10, pp.881-911) suggests a synthesis from sugars through levulinic acid-like molecules followed by a loss of carbon dioxide. Hall (18, pp.305-343) attributes the formation of terpenes to the condensation and breakdown of sugar derivatives. The above mentioned schemes are based on structural relationships of compounds known to occur in the plants. Until recently there was no direct evidence based on metabolic studies.

It was shown by Bonner (4, pp.628-631 and 5, pp.109-124) that carbon labeled acetate can be used in the formation of rubber in guayule and that unlabeled acetate stimulates the biosynthesis of rubber. Beta-methylcrotonate also stimulates rubber biosynthesis and was in fact more effective than acetate. Bonner et al. (1, pp.234-247) have shown further that beta-methylcrotonic acid becomes radioactive in plant tissue supplied with C¹⁴-labeled acetate, suggesting that beta-methylcrotonic acid arises from acetate. Millerd and Bonner (30, pp.343-355) and Bonner et al. (6, p.549) have demonstrated the reactions:
acetate → acetoacetate → beta-hydroxy-beta-methylglutarate
with plant enzyme systems. The formation of beta-methylcrotonic acid and beta-hydroxy-beta-methylglutaric acid

to all of these methyls arise from the carboxyl carbon atoms of acetate (14, pp.250-251 and 15, pp.1908-1912). This is what one would expect if carotene is synthesized via the pathway shown above.

Investigations of the biosynthesis of cholesterol in animals suggest a similar pathway. Rat liver extracts which possess the ability to incorporate C¹⁴-labeled acetate into cholesterol (32, pp.345-346) are also able to synthesize beta-hydroxy-beta-methylglutaric acid and beta-methylcrotonic acid (33, pp.307-313; 34, p.3037; and 39, pp.1698-1699). Trans-beta-methylglutaconic acid also has been reported in liver (35, p.5168). Bloch (2, pp.103-109) concluded that the position of the label in cholesterol and squalene from C¹⁴-acetate indicates a five-carbon intermediate formed by the condensation of three molecules of acetic acid. This work also suggests that the triterpene, squalene, must be a precursor of cholesterol. Dauben and Takemura (7, pp.6302-6304) found that the isoprene units of squalene arise from acetate, with the 2, 4, and 4' carbons coming from the methyl group of acetate and the 1 and 3 carbons from the carboxyl group. It has also been shown that beta-hydroxy-beta-methylglutaric, beta-hydroxy-isovaleric, and beta-methylcrotonic acids are used in rats to form cholesterol without prior breakdown to acetate (36, p.1295). Rudney (38, pp.2595-2596) has demonstrated

the formation of beta-methylcrotonic acid from beta-hydroxyisovaleric acid in liver, although this has not been demonstrated in plants (13, pp.497-522). Bloch (3, pp.687-699) proposes the following mechanisms for the formation of beta-methylcrotonic acid, supported by the over-all conversion of various branched chain acids to cholesterol and the observed isotope distribution pattern in his work.



There is as yet no experimental evidence in the literature that the pathways such as those proposed by Bonner and by Bloch are operative in terpene synthesis. The structural relationship, however, between the terpene and cholesterol, rubber, and carotene suggests a common precursor for all of them.

One of the main problems, and probably a cause of the lack of information on terpene metabolism, is the analytical difficulty inherent in work with essential oils.

CHROMATOGRAPHY OF PEPPERMINT OIL

Introduction

As peppermint oil is a complex mixture, and makes up only about 0.1-0.3% of the fresh weight of the leaf (17, pp.595-617), chromatography appeared to offer the best method of separating it into its components. Paper chromatography is unsuitable for analysis of the terpenes because the adsorbing strength is not great enough (22, pp.420-425), although Leandro Montes et al. chromatographed the 2,4-dinitro-phenylhydrazone of camphor on paper (28, pp.17-20). Paper impregnated with adsorbent has been used, but was not found to be useful in the case of terpenes (22, pp. 420-425). Kirchner and Miller (23, pp.318-320) successfully separated several terpenes on a silicic acid column but did not work with mint terpenes. Wang and Bang (43, pp.113-115) removed objectionable odor-causing substances from peppermint oil by column chromatography. Varma, Burt and Schwarting (42, pp.318-320) used Norit A with alcohol, and silicic acid with chloroform for the analysis of various terpenes. For our purposes, because of the small quantities of mint oil to be used, a column separation seemed undesirable.

Kirchner et al. (23, pp.420-425 and 24, pp.1107-1109) have developed a method that combines the advantages of

paper chromatography and column chromatography. In this method, silicic acid, with a suitable binder, is mixed with water and the resulting slurry is spread on glass strips for one-dimensional chromatography or on plates for two-dimensional chromatography. The names "chromato-strips" and "chromatoplates" were suggested. Reitsema (37, pp.960-963) adapted the chromatostrip technique to mint oil but found the R_f values to be extremely variable. He overcame this difficulty by using wider strips and chromatographing various known reference compounds beside the unknown. Ito et al. (19, pp.413-416 and 20, p.699) found that the variation in R_f values could be minimized by carefully controlling the temperature at which the plates were developed.

Various methods of identifying the colorless terpenes may be found in the literature. Unsaturated compounds can be detected by spraying the chromatogram with a 0.5% solution of fluorescein dye, exposing to bromine vapors and observing under ultra-violet light (22, pp.420-425). Aldehydes have been detected by spraying with a solution of o-dianisidine in glacial acetic acid (22, pp.420-425). Kirchner et al. (22, pp.420-425 and 24, pp.1107-1109) incorporated a zinc-cadmium sulfide-zinc silicate phosphor into the chromatostrips, as suggested by Sease (40, p.13630),

for the identification of compounds that absorb ultra-violet light. On such a chromatogram ultra-violet-absorbing materials appear as dark spots against a fluorescent background. Reitsema (37, pp.960-963) and Labat and Leandro Montes (27, pp.166-176) incorporated a fluorescent dye, rhodamine 6G, into the adsorbent before spreading it on the strips or plates. These authors (27, pp.166-176 and 29, pp.273-281) ran their chromatograms with the 2,4-dinitrophenylhydrazones of terpenes that will form such derivatives. The 2,4-dinitrophenylhydrazones will absorb ultra-violet light.

Experimental

Materials

For the preparation of the chromatoplates and chromatostrips, Mallinckrodt silicic acid and Jean Vivaudou plaster of Paris were used. Mitcham peppermint oil was kindly supplied by Dr. C. E. Horner. Menthol, menthone and isovaleraldehyde were Eastman Organic Chemicals, practical grade, and cineole and pulegone were Eastman Organic Chemicals, white label. Geraniol was Matheson, Coleman and Bell technical grade. Beta-methylcrotonic acid was prepared in this laboratory (9, pp.27-29).

Preparation and Use of Chromatograms

The plates and strips were prepared as follows: 20 ml. of distilled water were added to 10 grams of silicic acid and 2 grams of plaster of Paris and thoroughly mixed. Although Kirchner and Miller (22, pp.420-425) and Reitsema (37, pp.960-963) used both starch and plaster of Paris as a binder, we found plaster of Paris superior. The resulting slurry, which is quite liquid, is quickly spread on a 7" x 11" glass plate or on 3 or 4 glass strips (1" x 11") with a large spatula. The plate is then gently shaken to smooth the surface, and placed in the oven for four hours at 65°C. The chromatograms are used as soon as they cool. No attempt was made to closely control the thickness of the adsorbent on the plates as there was no indication that the R_f values depend on the thickness. The surface of the plates and strips, though, was smooth and the coating appeared to be of uniform thickness. It is important that the slurry be spread on quickly, as it solidifies very rapidly.

After preparation, the chromatograms are spotted with the terpenes to be resolved. The developing solvents were various concentrations of ethyl acetate in petroleum ether (B.P. 30-60°C.). The R_f values are very sensitive to changes in the per cent of ethyl acetate in the solvent or to the presence of polar compounds in the petroleum ether. Because of this, the petroleum ether was purified

by passing it through alumina. The chromatograms were then developed in a large battery jar which was placed in a constant-temperature cabinet. The tops of the battery jars were covered with a glass plate sealed with a rubber-base gasket seal. Stopcock grease was found unsatisfactory for sealing the covers, as the solvent vapors dissolve the grease and contaminate the solvent. The stopcock grease fluoresces, and this may be seen as a faint blue fluorescence on the plates or strips.

Development takes two to five hours, depending on the temperature. The higher the temperature the slower the rate of ascent of the solvent. After the chromatogram has been developed, the front is marked with a pencil, and the chromatogram is dried in a hood. This takes only a few minutes.

In investigating the chromatographic methods the substances most frequently employed were menthone, menthol, pulegone, geraniol, cineole and peppermint oil. Some work was done with isovaleraldehyde and its 2,4-dinitrophenylhydrazone, and with beta-methylcrotonic acid and carotene. The substances were dissolved in petroleum ether or alcohol for application to the chromatogram. Five microliters of a 1% solution of the individual compounds or 10 microliters of a 2% solution of peppermint oil were used for each spot.

Detection of Colorless Compounds

Many of the terpenes do not have detectable functional groups, and a general method of detection was desired. One of the first methods tried was that of Kirchner and Miller (22, pp.420-425) of spraying the chromatograms with a 5% solution of nitric acid in concentrated sulfuric acid. Although this will bring out the spots, it leaves much to be desired as it destroys the compounds. Some success was found with the use of an aqueous vanillin spray followed by a concentrated sulfuric acid spray followed by heating (19, pp.413-416 and 20, p.699).

A more satisfactory method of detecting the spots was developed using a water soluble fluorescent dye, rhodamine B. The dye, instead of being incorporated into the chromatogram, is sprayed on after development, as a 0.05% aqueous solution, and the chromatogram is observed under ultra-violet light. As the spraying continues, the spots appear dark against a bright orange fluorescent background. As a source of ultra-violet light a Mineralite short-wave ultra-violet lamp was used. A long wave length ultra-violet lamp does not show up the spots as readily.

Any compound that absorbs ultra-violet light will show up immediately on spraying the chromatogram lightly with the dye. Other spots become visible only after the

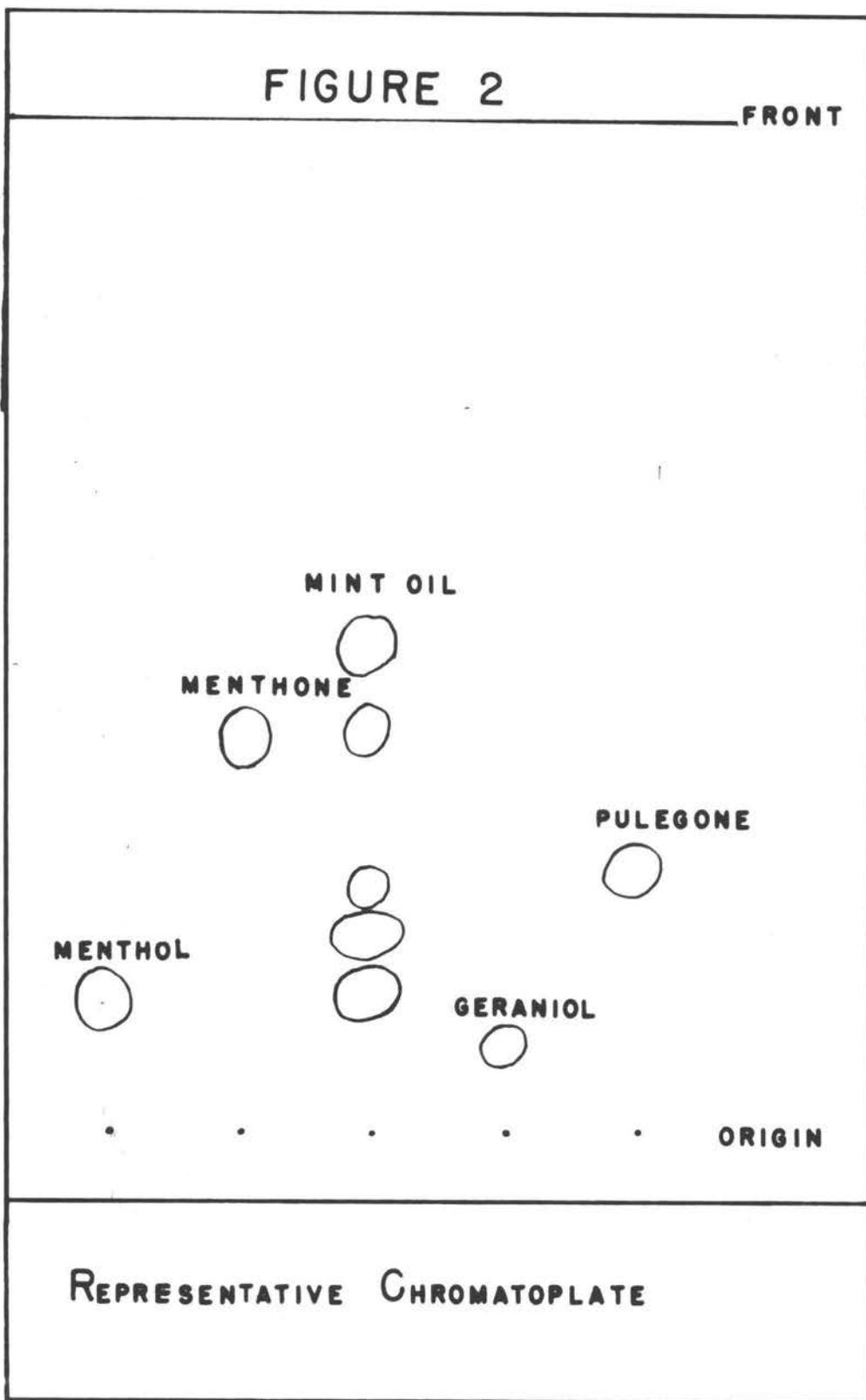
plates have been saturated with the dye. Any of the substances that are slightly soluble in water, such as menthol and geraniol, diffuse as the spraying continues. Their spots thus appear as spreading rings. The spots may also be seen on the back of the chromatograms in daylight as white spots against a pink background, because of the water repellent action of the oils.

Experimental Results

In the chromatograms of peppermint oil, five and sometimes more spots can be seen. Using 5% ethyl acetate as the solvent and keeping the temperature at 24.5°C., the conditions found most satisfactory for general use, the chromatograms appear as shown in Figure 2.

If less than 0.2 mgm. of oil is used, it is impossible to discern all of the spots although three of them still appear with as little as 0.1 mgm. of oil. Two of these spots are menthol and menthone.

Because of the dependence of R_f on both the temperature and the per cent of ethyl acetate in the solvent, a study was made of the variation of the R_f with temperature, holding the composition of the solvent constant and the variation of R_f with the concentration of ethyl acetate in the solvent with constant temperature. Menthol, menthone and pulegone were used as test substances in these studies.



Dependence of R_f on the Composition of the Solvent

There is a definite dependence of the value of the R_f on the composition of the solvent. If the solvent used is pure petroleum ether, the R_f values for the three test substances are zero. As ethyl acetate is added in increasing amounts, the R_f values increase, as shown in Table 1.

Table 1

Variation of R_f Values with
the Composition of the Solvent

Composition of Solvent in per cent Ethyl Acetate	$R_f \times 100$		
	Menthol	Menthone	Pulegone
0	0	0	0
1	3.7	13	5.9
2	7.0	20	14
3	10	29	21
4	13	35	22
5	15	47	30
6	20	52	34
7	26	58	40
8	28	68	49
10	49	90	75

The R_f values are given as R_f times 100. The temperature was 24.5°C.

The deviation from the mean by the R_f values at constant solvent composition and isothermal conditions ranged from 0.05 to as low as 0.005, taking the solvent front as unity. Table 2 shows the range of R_f values in representative concentrations of ethyl acetate.

Table 2

Range of R_f Values for
some Representative Chromatograms

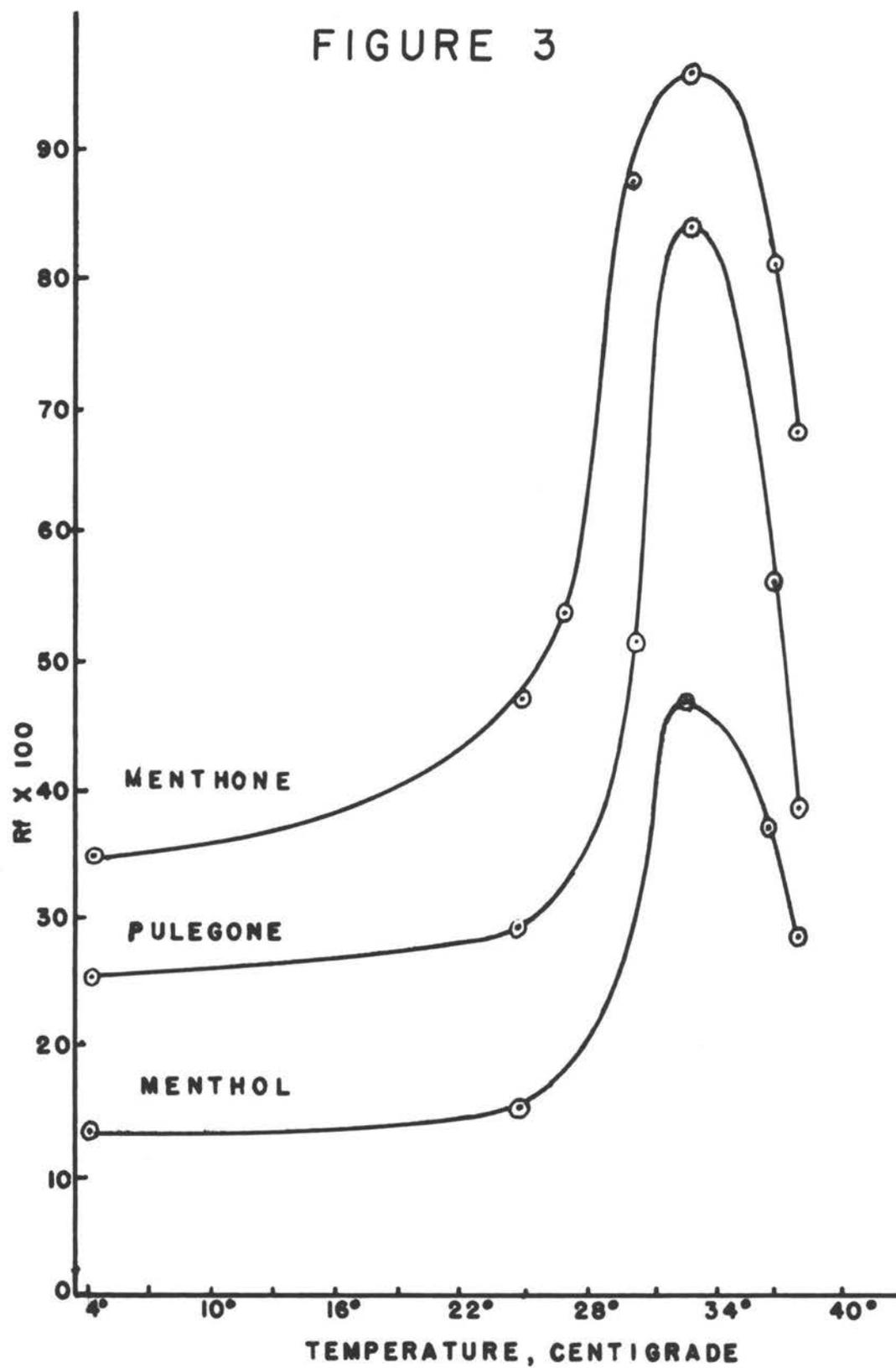
Compound	Concentration of Ethyl Acetate in Solvent			
	3%	5%	6%	8%
Menthol	0.098-0.103	0.11-0.19	0.167-0.24	0.22-0.36
Menthone	0.30-0.38	0.45-0.53	0.497-0.54	0.64-0.74
Pulegone	0.205-0.216	0.26-0.39	0.28-0.39	0.45-0.53

In all cases both the composition of the solvent and the temperature are held constant.

In the cases where an especially wide range is indicated this is usually due to wide deviation of a single chromatogram. Such is the case with the values for pulegone in 5% ethyl acetate. The R_f values obtained were 0.39, 0.25, 0.26, 0.29 and 0.30; the mean value of all five is 0.30.

Dependence of R_f on Temperature

In the temperature studies, the solvent was kept at a constant composition of 5% (V/V) ethyl acetate. The variation of the R_f values with temperature is very marked in the region of 24°-38° and it is quite interesting to note that there is a peak at 32.5°-33.0°C. A plot of temperature against R_f value is shown in Figure 3. Despite the large temperature coefficient of the R_f values in the region of 30°C., at lower temperatures; i.e., between 4° and 24°, the R_f values appear to be fairly constant.

PLOT OF R_f AGAINST TEMPERATURE

Discussion

It can be seen that if the temperature and composition of the solvent are kept constant, the variation of the R_f values may be kept within the extreme limits of 0.10-0.15, with most variations less than this.

By controlling the temperature and the per cent of ethyl acetate in the solvent it is possible to broaden a portion of the chromatogram as wished. Our principal interest is in the terpenes of which the substance with the lowest R_f value (0.15 at 24.5°C. and 5% ethyl acetate) was menthol. There also is interest in some of the spots between the menthol spot and the origin. One such spot, which is of considerable interest, is that of isovaleraldehyde. As it is close to the origin, it is desirable to broaden this portion in order to move it higher on the chromatogram. This may be accomplished by varying either the temperature or the per cent of ethyl acetate in the solvent. If an 8% solvent is used, it is possible to raise the R_f of menthol to 0.30. In this case isovaleraldehyde has an R_f of 0.14. With a 3% solvent, the R_f values of menthol and isovaleraldehyde are so close as to overlap, but the portion of the chromatogram above menthone is broadened.

Although most of the work was done with terpenes and peppermint oil, this method is quite versatile. Carotene

goes to the front on these as on other chromatograms but other more polar compounds will chromatograph with usable R_f values. Beta-methylcrotonic acid will chromatograph on these plates with an R_f value between menthol and the origin, about the same as isovaleraldehyde, the exact R_f depending again on the temperature and the per cent of ethyl acetate in the solvent.

About 45 micrograms were used for each spot of the individual compounds. When peppermint oil was chromatographed, about 200 micrograms were used. The commercial samples of menthone, pulegone and isovaleraldehyde, when developed, showed more than one spot. In menthone, a spot that corresponds to menthol appears. In the case of pulegone four spots, and sometimes additional faint spots, other than pulegone, could be discerned. Although no attempt was made to determine the limits of sensitivity, this would seem to indicate a sensitivity as low as at least 10 micrograms. It must be said that the impurities appear only after extensive spraying of the dye and water and that these spots are much smaller than the main component.

Theoretical Treatment

Theoretically, these chromatograms pose an interesting problem. If this were partition chromatography, one would expect ethyl acetate to form the stationary phase. The

R_f values of the polar substances, such as the ketones, aldehydes and alcohols, should, therefore, decrease as the solvent becomes richer in ethyl acetate. Since this is contrary to the experimental observation, it appears that we are not dealing with partition chromatography.

Assuming this to be an adsorption phenomenon, and restricting our consideration to the adsorption of the first monolayer, it is possible to start with the adsorption equation given by Emmett (11, pp.1-36) in the derivation of the Brunauer, Emmett, and Teller equation and derive a relationship between the value of the R_f , the composition of the solvent and the temperature. We will start with two relationships, one for the adsorption of the ethyl acetate and one for the adsorption of the terpene being chromatographed.

$$(1) \quad K_1 C_1 \frac{S_0}{S_1} = e^{-\frac{E_1}{RT}}$$

$$(2) \quad K_2 C_2 \frac{S_0}{S_2} = e^{-\frac{E_2}{RT}}$$

K_1 and K_2 are constants.

S_0 is the surface area on which nothing is adsorbed.

S_1 is the surface area with adsorbed ethyl acetate.

S_2 is the surface area with adsorbed terpene.

C_1 is the concentration of ethyl acetate.

C_2 is the concentration of the terpene being chromatographed at the spot that it occupies.

The terpene and the ethyl acetate will compete for the available surface area at the spot where the terpene is located. The total surface area is the sum of the three surface area terms.

$$S_T = S_0 + S_1 + S_2$$

If the reciprocal of the R_f is set proportional to the surface area occupied by the terpene divided by the sum of the surface area terms in which the terpene is not involved, we have:

$$(3) \quad \frac{1}{R_f} = \frac{1}{K_r} \cdot \frac{S_2}{S_0 + S_1}$$

or:

$$(4) \quad \frac{R_f}{K_r} = \frac{S_0}{S_2} + \frac{S_1}{S_2}$$

rearranging and solving for $\frac{S_0}{S_1}$

$$(5) \frac{S_2}{S_1} \frac{R_f}{K_r} - 1 = \frac{S_0}{S_1}$$

substituting from equation (1)

$$(6) \frac{S_2}{S_1} \frac{R_f}{K_r} - 1 = \frac{e^{-\frac{E_1}{RT}}}{C_1 K_1}$$

then multiplying through by $\frac{S_0}{S_2}$ gives

$$(7) \frac{S_0}{S_1} \frac{R_f}{K_r} - \frac{S_0}{S_2} = \frac{S_0}{S_2} \frac{e^{-\frac{E_1}{RT}}}{C_1 K_1}$$

Substituting from equation (1) and (2) we have

$$(8) \frac{e^{-\frac{E_1}{RT}}}{C_1 K_1} \frac{R_f}{K_r} - \frac{e^{-\frac{E_2}{RT}}}{C_2 K_2} = \frac{e^{-\frac{E_1 - E_2}{RT}}}{C_1 C_2 K_1 K_2}$$

then multiplying through by $C_1 K_1 e^{\frac{E_1}{RT}}$ and rearranging we get

$$(9) \frac{R_f}{K_r} = \frac{C_1 K_1}{C_2 K_2} e^{\frac{E_1 - E_2}{RT}} + \frac{e^{-\frac{E_2}{RT}}}{C_2 K_2}$$

When the concentration of ethyl acetate is zero, the R_f values are also zero, so

$$(10) \frac{e^{-\frac{E_2}{RT}}}{C_2 K_2} = 0$$

therefore:

$$(11) \frac{R_f}{K_r} = \frac{K_1 C_1}{C_2 K_2} e^{\frac{E_1 - E_2}{RT}}$$

The amount of the substance being chromatographed does not seem to have any effect on the R_f values. It appears that if an increased amount is placed on the chromatogram, a larger spot is formed instead of increasing the concentration within the spot. Therefore it should be permissible to assume that C_2 is constant at any given temperature, and define a constant

$$(12) \quad D = \frac{K_1 K_r}{K_2 C_2}$$

We thus arrive at the relationship

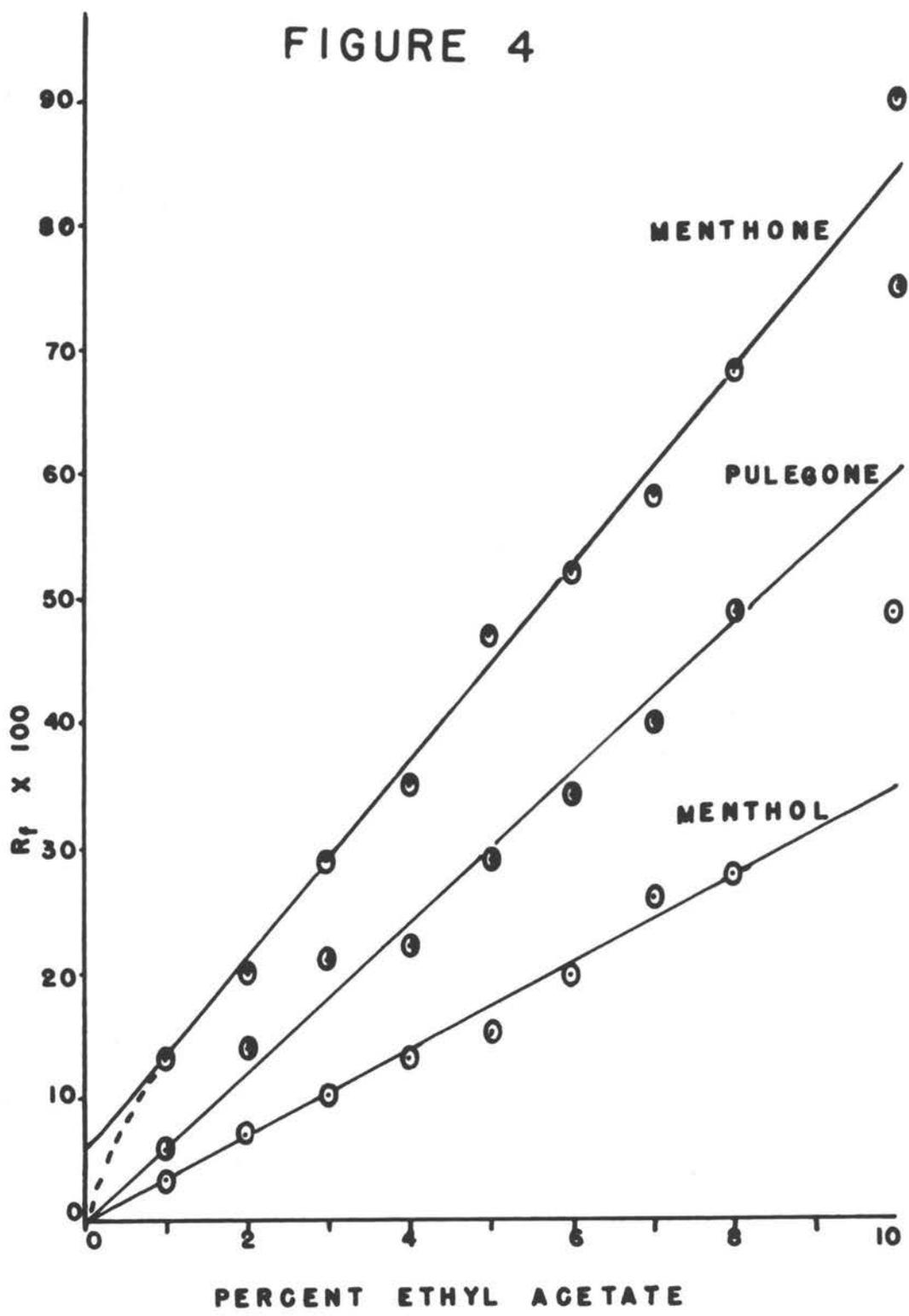
$$(13) \quad R_f = D C_1 e^{\frac{E_1 - E_2}{RT}}$$

From equation (13) it can be seen that under isothermal conditions the R_f should vary linearly with the concentration of ethyl acetate in the solvent. Figure 4 shows the dependence of the R_f on the concentration of ethyl acetate in the solvent and is in good agreement with equation (13) up to 8% ethyl acetate. It may be seen from equation (9) that if the curve does not go through the origin the intercept should be equal to

$$(14) \quad \frac{e^{-\frac{E_2}{RT}}}{C_2 K_2}$$

In the case of menthone, the theoretical curve extrapolates not to zero but somewhat above, although at zero concentration the observed R_f of menthone departs from the linear

FIGURE 4



PLOT OF R_f AGAINST THE ETHYL ACETATE IN SOLVENT

relationship and goes to zero. In the case of menthol and pulegone, the intercept is equal to zero. The slope of the curve is, from equation (13):

$$(15) \quad D e^{\frac{E_1 - E_2}{RT}}$$

If equation (13) is converted to the logarithmic form

$$(16) \quad \ln R_f = \ln (D C_1) + \frac{E_1 - E_2}{R} \cdot \frac{1}{T}$$

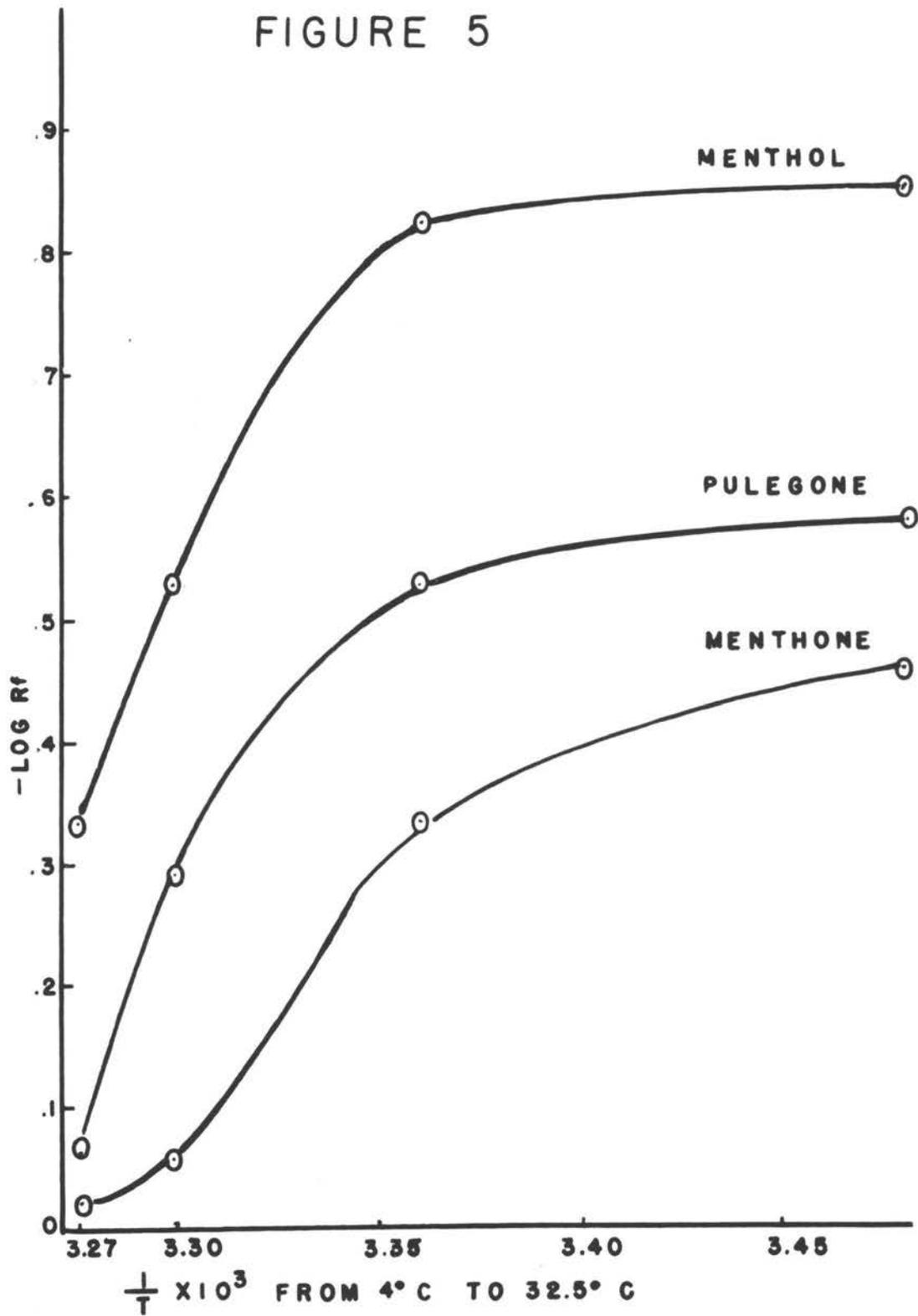
it can be seen that the plot of $\ln R_f$ against $1/T$ should give a straight line with the intercept equal to $\ln (D C_1)$

and the slope equal to $\frac{E_1 - E_2}{R}$ if the C_2 term of D and

the $E_1 - E_2$ term are independent of temperature. A plot of this may be seen in Figure 5. Heats of adsorption are known to vary with temperature, so it is not surprising that the observed values do not give a straight line.

Although it is not possible to say definitely whether the C_2 term varies with temperature, the fact that no change in size of the spot was noticed with increased temperature suggests that C_2 is independent of temperature. If this is the case, the slopes of the curves in Figure 5 at any given temperature should be equal to the difference in the heat of adsorption between the terpene and ethyl acetate.

FIGURE 5



PLOT OF $\ln R_f$ AGAINST RECIPROCAL OF THE TEMPERATURE

It is interesting to note that the lack of a large variation of the R_f value between 4° and 24° would be explained if $E_1 - E_2$ is approximately a linear function of T up to 24° . This would then cancel out the $1/T$ term causing the R_f to approach constancy. Above 24° this no longer holds.

METABOLISM OF 1-C¹⁴ ACETATE BY
EXCISED PEPPERMINT LEAVES

As mentioned before, it is thought that terpenes originate from isoprenoid precursors such as beta-methylcrotonic acid, and that acetate can serve as a precursor of these isoprenoid compounds. We therefore attempted to show incorporation of radioactivity from acetate into the terpenes by intact leaves. The 1-C¹⁴ sodium acetate was prepared by the method described by Murry (30, pp.10-14).

Tracer Study I

A time study was made involving twelve leaves divided into three groups of four leaves each. Each leaf was placed in an individual cup with the petiole immersed in 0.5 ml. of a solution containing 2 microcuries of 1-C¹⁴ sodium acetate. A stream of air was drawn through a series of gas-washing bottles to remove moisture and carbon dioxide. The bottles were arranged in the order: soda-lime, sodium hydroxide solution and concentrated sulfuric acid. The sulfuric acid bottle was used to dry the air and thereby increase the transpiration rate of the leaves. It was replaced by a bottle of water as soon as all of the liquid had been taken up by the leaves (which required several hours).

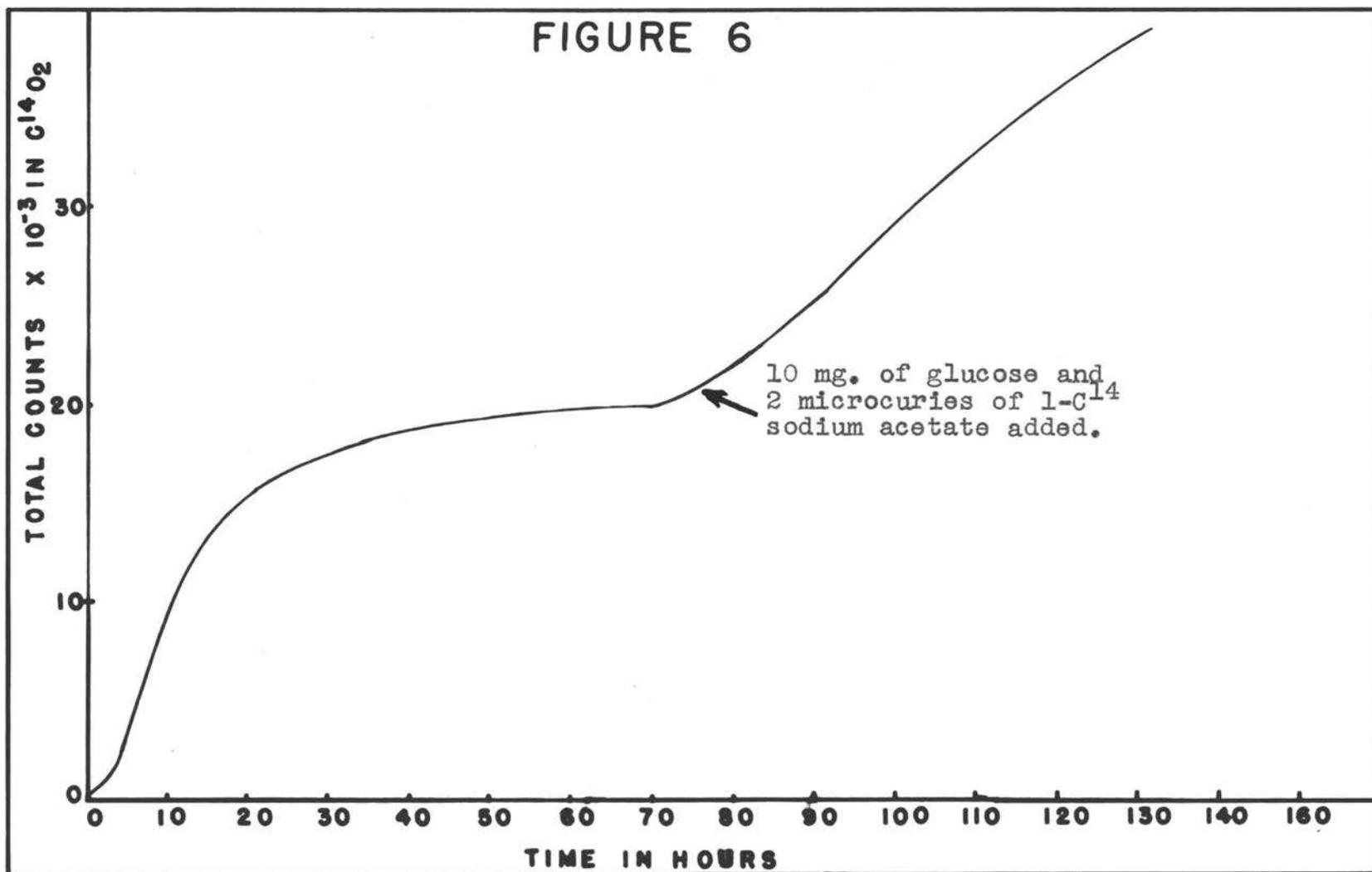
Time Study

The respired CO_2 was collected in 0.5N sodium hydroxide, which was removed and replaced every four to eight hours. The resulting carbonate was precipitated as barium carbonate, plated on planchets, and counted with a Geiger counter. The counts were corrected for self-absorption and coincidence and plotted as total counts per minute respired against time, as shown in Figure 6. The maximum rate of evolution of C^{14}O_2 is reached after five hours. After 20 hours the rate of evolution of C^{14}O_2 had decreased sharply. After 73 hours, four of the leaves were removed and to each of the remaining eight were added 2 microcuries of 1-C^{14} acetate and 10 mg. of glucose. It was hoped that glucose, by serving as a respiratory substrate, would divert the acetate into anabolic pathways. The output of radioactive CO_2 again increased, reaching a maximum at the end of 90 hours. The leaves were still respiring C^{14}O_2 at the end of 168 hours.

Chromatography

The leaves in the 96 hour group were placed into a mortar and ground in sodium bicarbonate. After grinding, the leaves were extracted with two portions of skelly B and the extract was washed twice with a 5% solution of sodium bicarbonate. Then 0.5 ml. of the resulting 3.5 ml. of extract was placed on a silicic acid chromatostrip and

FIGURE 6



developed with 15% ethyl acetate in petroleum ether. The developed chromatograms were counted by means of a Geiger counter with automatic strip-counter attachment. Four radioactive spots were shown, one at the origin, one at the front, and two between the origin and the menthol spot. The most radioactive spot was at the front and coincided with a band of carotene.

Tracer Study II

A second run was made using a higher level of radioactivity and a shorter time period. Since, in the previous experiment, most of the C^{14} was respired as CO_2 , it was decided to supply glucose to half of the leaves in order to get quantitative information on the effect of glucose on the metabolism, and in the hope of getting a larger amount of acetate into anabolic pathways.

Six brown glass jars were set up with four leaves in each jar. The plants from which the leaves were taken had been starved by placing them in the dark for 48 hours and spraying with approximately 0.1 M calcium nitrate. The leaves were put two to a cup with the petioles immersed in an aqueous solution of $l-C^{14}$ sodium acetate. 18.5 microcuries (0.13 mg.) of the sodium acetate were placed into each cup. To three of the six sets of leaves were added 20 mg. of glucose per cup. The total liquid volume was 0.4 ml. in each cup. The experiment is outlined in Table 3.

Table 3
Outline of Tracer Study II

Jar	Mg. glucose/leaf	Wt. of leaves/jar	Hours run
1	10	0.381 gm.	18
2	0	0.388	18
3	10	0.430	12
4	0	0.414	12
5	10	0.397	6
6	0	0.404	6

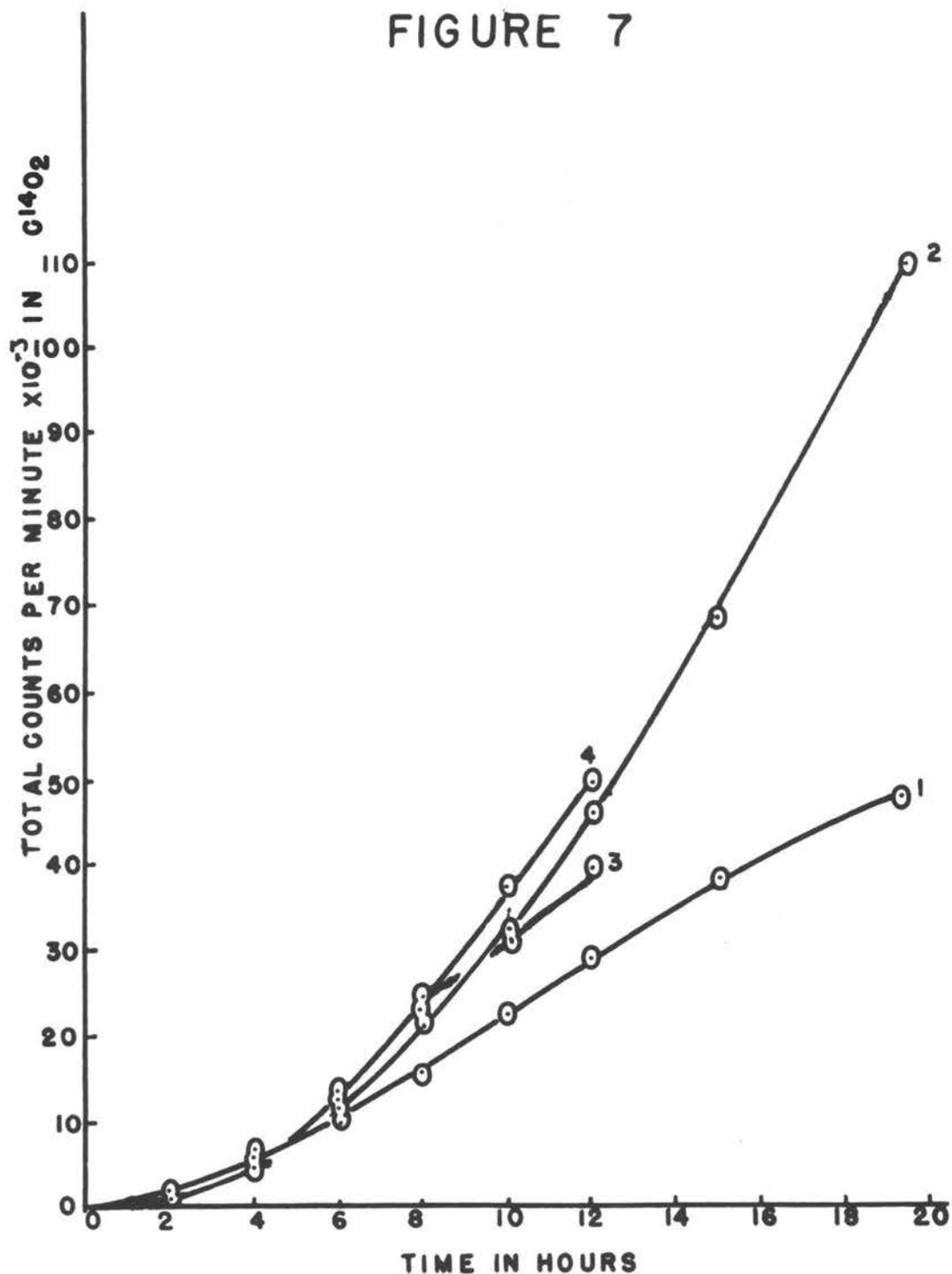
Time Study

The time course of $C^{14}O_2$ evolution is shown in Figure 7. It can be seen that up to six hours there was no significant difference between the flasks with glucose and those without. The curves of the evolution of $C^{14}O_2$ from jars 1 and 3 have the same slope after twelve hours, as do the curves from jars 2 and 4. As jars 1 and 3 had glucose, while 2 and 4 did not, it is evident that the presence of glucose inhibits the catabolism of the radioactive acetate in incubation period of more than six hours. After 18 hours it can be seen that there is a wide difference between the amounts of radioactive CO_2 respired by jars 1 and 2.

Chromatography

The leaves of jar 2 were thawed and ground in a mortar with approximately their own bulk of sodium bicarbonate. Water was then added and the mixture was transferred to a centrifuge tube resulting in a final volume

FIGURE 7



TOTAL COUNTS PER MINUTE OF C^{14} AGAINST TIME - TIME STUDY II. 1 and 3 have 10 mg. of glucose per leaf. 2 and 4 have no glucose.

of 6.0 ml. To this were added about 5 ml. of petroleum ether that had been purified by passing it through alumina; the whole mixture was well mixed and then centrifuged. The pale yellow extract was washed with 2.3 ml. of a dilute sodium bicarbonate solution. One milliliter of the 4.7 ml. extract was placed on a chromatostrip and chromatographed. The remaining 3.7 ml. of extract were hydrolyzed for four hours with 50% NaOH at room temperature. 1.8 ml. of the hydrolyzed extract was put on a chromatostrip and chromatographed. All these chromatograms were developed in 5% ethyl acetate in alumina-purified petroleum ether. No difference was detected between the hydrolyzed and unhydrolyzed extracts. The chromatograms were run through the counter and activity appeared at the front, at the origin, and at a spot that coincides with isovaleraldehyde. A band of carotene also appeared at the front.

As it was desirable to remove the fat soluble compounds such as carotene and the fats from the oil, it was decided to release the oil from the leaves by refluxing them with water, and extracting the aqueous extract with petroleum ether. This proved to be a satisfactory method of eliminating carotene from the oil extract and so was adopted in place of the method used earlier.

This method was used in the analysis of the leaves from jars 1, 3, and 4. The leaves were placed in a 50 ml.

round bottom flask outfitted with a reflux condensor. About 20 ml. of distilled water were placed in the flask. The solution was made alkaline by the addition of sodium bicarbonate and refluxed for an hour. The water was removed and extracted with two portions of petroleum ether. In the case of jar 4, the extract was then dried over anhydrous sodium sulfate. The petroleum ether extract was then chromatographed on chromatostrips. The chromatogram of the oil from the leaves of jar 1 (18 hours, plus glucose) showed very high radioactivity at the origin and also activity at a position between menthol and the origin coinciding with isovaleraldehyde with an R_f of 0.087. Six spots, corresponding to those achieved with other samples of peppermint oil, were discerned, indicating that the extraction technique with hot water will remove the oil from the leaves. In the leaves from jar 3 (12 hours plus glucose) there was activity at the front, at a spot above where menthone was, and a spot that exactly coincides with the R_f of isovaleraldehyde. Known isovaleraldehyde chromatographed at the same time in the same solvent, gave an R_f value of 0.146 and the R_f of the spot on the strip was 0.143. No activity was apparent in the oil from the leaves of jar 4.

In all of the chromatograms run with the petroleum ether extract, the usual distribution of spots appeared

as is found when known oil is used. With the exception of the spot corresponding in R_f value to isovaleraldehyde, there was no detectable radioactivity in the spots known to originate from the peppermint oil.

Discussion

Although no activity could be seen in the terpenes in the oil from the peppermint leaves after the leaves had been fed C^{14} labeled acetate, there was some activity in the spot that corresponds to isovaleraldehyde. If it is isovaleraldehyde at this spot, this indicates that acetate is being synthesized into a compound with the isoprene structure.

The time studies of the amount of radioactive CO_2 respired by the plant indicate that the presence of glucose somewhat inhibits the catabolism and increases the anabolism of the acetate in the plants. This is born out by the fact that the leaves with glucose respired less radioactive CO_2 than those without and by the fact that in the twelve-hour leaves (jars 3 and 4) radioactivity could be detected in the oil fraction only when glucose was supplied (jar 3).

SUMMARY

1. A method of chromatography on silicic acid chromatoplates and chromatostrips was applied to peppermint oil and a new method of identifying colorless spots was developed.
2. The variation of the R_f values with temperature and with the concentration of ethyl acetate in the solvent was studied.
3. A theory was developed to explain the mechanism of this type of chromatography. Good agreement of the experimental results with the theory was obtained in the case of the variation of R_f values with composition of the solvent under isothermal conditions.
4. Time studies were run on the $C^{14}O_2$ respired in excised peppermint leaves to determine the effect of glucose on the metabolism of 1- C^{14} acetate. The silicic acid chromatostrip technique was applied to the peppermint oil extracts from these time studies.

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