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Metabolism of Labeled Ethylene in the Avocado

APPEARANCE OF TRITIUM IN THE METHYL GROUP OF TOLUENE

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The metabolic fate of ethylene in fruit under conditions wherein ethylene hastens the post-harvest maturation, *i.e.* the acceleration of the onset of the climacteric, has received only limited attention (1). Therefore, a study of this type of hydrocarbon metabolism in green avocados was undertaken, the fruit being treated with labeled ethylene. To follow the fate of both elements of the ethylene molecule, ethylene-C^{14} and ethylene-H^3 were used. The present report is concerned with the demonstration that approximately one-eighth of the total incorporated radioactivity of metabolized ethylene-H^3 resides in the methyl group of toluene, and that approximately one-fortieth of the tritium in the toluene is in the benzenoid portion.

MATERIALS AND METHODS

Mature green avocados, picked 1 day before, were used throughout these studies. The fruit was exposed to the radioactive ethylene for 4 hours in a closed container. Three large fruits, Haas variety, were used in each experiment. A pump consisting of a mercury leveling bulb was attached to the container so that periodic movement of the air within the container could be achieved by lowering and raising the bulb. The container was of such size that the amount of oxygen consumed (measured by CO_2 formation) during the incubation did not significantly alter the oxygen content of the atmosphere. After the incubation, CO_2-free air was passed through the container; the ethylene was collected in cold mercuric perchlorate solution (2), and the CO_2 in $Ba(OH)_2$ solution. The flesh and skin of the fruit, 660 g in each case, were then rapidly ground in a large blender with 1850 ml of 95% alcohol. After filtration, the pulp was extracted twice more with 2700 ml of 70% ethanol. The three extracts were combined.

Uniformly labeled C_2H_4-H^3 was purchased from New England Nuclear Corporation, Boston, Massachusetts.[1] To avoid polymerization of the highly radioactive ethylene (40 mc per mmole), the manufacturer prepared the ethylene just before use and shipped it packed in Dry-Ice.

The radioactivity of the tritium was counted by the liquid scintillation system in a naphthalene-dioxane scintillation solution (3) with the Tri-Carb spectrometer manufactured by Packard Instrument Company, Inc., La Grange, Illinois. The quenching effect (decrease in counting efficiency) of the several toluene derivatives was determined by adding various amounts of the unlabeled derivative to standard tritiated water in the scintillation solution.

EXPERIMENTAL PROCEDURE AND RESULTS

Amount of Ethylene Metabolized—The amount of ethylene-H^3 incorporated into the avocados as measured by the radioactivity in the ethanol extract is shown in Table I. At the lower level fed (250 p.p.m.), 0.015% of the total in the surrounding atmosphere was taken up; at the higher level (2000 p.p.m.), 0.042% was metabolized. Hence, neither the fraction nor the total incorporated was directly proportional to the amount of ethylene in the surrounding atmosphere.

Separation of Volatile Metabolites—Evaporation of portions of the 70% alcoholic extracts in vacuum at a maximal temperature of 55° revealed that approximately three-fourths of the incorporated ethylene was volatile in each case (Table II). Two liters of the more highly active extract (from fruit treated at 2000 p.p.m.) were then evaporated as described, and the volatile material was collected in a Dry-Ice trap followed by a liquid nitrogen trap. The material in the liquid nitrogen trap was diluted with 380 ml of 70% alcohol and combined with that in the Dry-Ice trap. The total activity in both traps was 1.15×10^6 c.p.m. This solution was distilled, after a $2\frac{1}{2}$-hour reflux, in a 10-plate Fenske packed column with a ratio of reflux to takeoff of 125:5.

The results obtained on the first five fractions are given in Table III. The first fraction, corresponding to 0.4% of the total solution, contained 43.8% of the radioactivity. The radioactivity contained in the first four fractions was 83.6%. The remainder of the alcohol contained in the solution was collected in eight additional fractions, all of which possessed the same radioactivity, 33 c.p.m. per ml. The first three aqueous fractions had a radioactivity of 240 to 400 c.p.m. per ml. However, the subsequent eight aqueous fractions also possessed a constant radioactivity, 150 c.p.m. per ml. If the constant radioactivity contained in the alcoholic and aqueous fractions was due to exchangeable tritium, the maximal amount of such tritium contained in the alcohol solution was 13% of the volatile total.

Characterization of Low Boiling Fractions—To 1.5 ml of water were added 50 μl of the first distillation fraction, and the solution was then extracted with 1.5 ml of cyclohexane. Determination of the radioactivity in both phases demonstrated that the distribution ratio of radioactive components between cyclohexane and water was 125:1. This result suggested that the radioactive compounds in this fraction were hydrocarbons or possessed solubilities similar to hydrocarbons. The ultraviolet absorption spectrum of the first fraction showed the presence of

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[1] Reference to a company or product name does not imply approval or recommendation of the product by the Department of Agriculture to the exclusion of others that may also be suitable.

both benzene and toluene. The second and third fractions were found to be transparent to ultraviolet light at wave lengths of 220 mμ and above. A comparison with known solutions of benzene and of toluene in ethanol indicated that the first fraction contained approximately 3 mg each of toluene and benzene, or approximately 3 mg of each per 200 g of fruit. The ethanol used for extracting the fruit was ruled out as a source of these hydrocarbons. When 2 liters of the 70% ethanol used for extraction were subjected to the same distillation procedure as the extract of the avocados, spectroscopic observations of the first distillation fraction revealed the absence of benzene and the presence of toluene, but only in the amount of 240 μg, *i.e.* 8% of that found in the avocado extract.

To ascertain whether or not toluene was labeled and, if so, where, three series of reactions were carried out on the material in the first fraction, which had been transferred to nonpolar solvents. They were as follows: Reaction I, toluene → β-(*p*-methylbenzoyl)propionic acid → *p*-toluic acid → terephthalic acid → terephthalic acid diethyl ester; Reaction II, toluene → 4-nitrotoluene + 2,4-dinitrotoluene → 4-aminotoluene + 2,4-diaminotoluene → 4-acetaminotoluene + 2,4-diacetaminotoluene; Reaction III, toluene → benzoic acid.

I. β-(p-Methylbenzoyl)propionic Acid and p-Toluic Acid—The radioactivity contained in 3.0 ml (1.56×10^5 c.p.m.) of the first fraction of distillate (Table III) was transferred to cyclohexane by diluting the alcohol with 15 ml of H_2O and extracting this solution with 5 ml of cyclohexane. After separation of the aqueous phase, the cyclohexane solution was diluted with 5 ml of 1,1,2,2-tetrachloroethane and dried over anhydrous Na_2SO_4. The solution was further diluted with 10 ml of tetrachloroethane and 1.0 ml of toluene and immediately subjected to the Friedel-Craft reaction with succinic anhydride by the procedure of Reinheimer and Taylor (4), except that the aluminum chloride was added in 15 ml of tetrachloroethane. After steam distillation, the hot aqueous solution of β-(*p*-methylbenzoyl)propionic acid was cooled and the crystalline acid was harvested.[2] A yield of 1290 mg (69% of theoretical) of β-(*p*-methylbenzoyl)propionic acid (m.p. observed, 127°; reported, 127–128.5° (4)) was obtained. The product was first recrystallized from water and then from *n*-heptane. The specific activity of the compound (Table IV) was lowered by the recrystallization from water, but was unaffected by the subsequent recrystallization in *n*-heptane. When adjusted on the assumption of a 100% yield of the added toluene in the Friedel-Craft reaction, the activity found in the β-(*p*-methylbenzoyl)propionic acid was 38% of the total in the sample used to prepare it, and represents 12.5% of the total C_2H_4-H^3 incorporated into the avocados. Hence toluene represents a major metabolite of C_2H_4-H^3.

TABLE I

Amount of ethylene-H^3 incorporated into three avocados in 4 hours

Amount fed		Amount incorporated	
Weight	Radioactivity	Radioactivity*	Per cent of total fed
mg	*mc*	*μc*	
3†	4.0	0.60	0.015
24‡	36	15.0	0.042

* Corrected for counting efficiency.

† The surrounding atmosphere contained 250 p.p.m.

‡ The surrounding atmosphere contained 2000 p.p.m.

TABLE II

Volatile and nonvolatile metabolites of ethylene-H^3

Concentration to which fruit was exposed	Volatile	Nonvolatile
p.p.m.	%	%
250	74	26
2000	73	27

TABLE III

First five distillation fractions of 70% alcohol solution of volatile metabolites

Fraction	Reflux temperature	Volume	Radioactivity	Per cent of total
		ml	*c.p.m./ml*	
1	78.0°	9.7	52,000	43.8
2	78.1	10.4	8,430	7.5
3	78.1	9.5	6,270	5.2
4	78.1	250	1,250	27.1
5	78.2	260	49	1.1

TABLE IV

Specific radioactivities of β-(p-methylbenzoyl)propionic acid and p-toluic acid

Compound	Treatment	Radioactivity
		c.p.m./mmole
β-(*p*-Methylbenzoyl)-propionic acid		6700*
	Recrystallized from H_2O	6300*
	Subsequently recrystallized from *n*-heptane	6300*
p-Toluic acid	Recrystallized from H_2O	6200†
	Twice recrystallized from H_2O	6200†

* Corrected for quenching of β-(*p*-methylbenzoyl)propionic acid.

† *p*-Toluic acid, at the concentrations used in scintillation counting, did not quench.

A portion (600 mg) of the *n*-heptane-recrystallized product was oxidized to *p*-toluic acid by alkaline sodium hypochlorite (4). The yield of *p*-toluic acid was 380 mg (89%), m.p. 182° (reported, 181° (5)). After recrystallization from water, the specific activity of the *p*-toluic acid was essentially the same as that of the β-(*p*-methylbenzoyl)propionic acid from which it was prepared (Table IV). These results give additional support to the possibility that toluene is a metabolite of tritiated ethylene.

To determine whether or not the benzene contained in the first fraction possessed radioactivity, benzene was used as a diluent before the Friedel-Craft reaction. For this purpose the radioactive compounds in the first distillation fraction from another experiment were used. The radioactive compounds were transferred to cyclohexane as described above; 5 ml of the cyclo-

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[2] The tetrachloroethane phase of the steam distillate contained 35% of the radioactivity originally present in the 3.0 ml of the first distillation fraction.

TABLE V

Specific radioactivities of acetamino derivatives of toluene

Compound	No. of recrystallizations*	Radioactivity†
		c.p.m./mmole
4-Acetaminotoluene		5900
	1	6000
	2	6200
	3	6400
	4	6400
2,4-Diacetaminotoluene		5400
	1	6300
	2	6200

* From water.

† Corrected for quenching of the individual compound.

hexane contained 61,000 c.p.m. The cyclohexane solution was diluted with 1.0 ml of benzene and subjected to the Friedel-Craft reaction as above. A yield of 69% of the β-benzoylpropionic acid (m.p. 113°; reported m.p., 117° (4)) was obtained. The apparent specific activity of the compound was 3500 c.p.m. per mmole. However, recrystallization of a portion of the compound from water, followed by two recrystallizations from *n*-heptane, decreased the apparent specific activities to 3300, 3000, and 2600 c.p.m. per mmole, respectively. These results indicated that if the β-benzoylpropionic acid were radioactive, a better method of separating it from the accompanying β-(*p*-methylbenzoyl)propionic acid should be sought.

It was found that a mixture of β-benzoylpropionic, β-(*p*-methylbenzoyl)propionic, and β-(*p*-ethylbenzoyl)propionic acids was readily separated by adsorption on a Dowex 1-formate column and elution with 4 N formic acid. (Concentrations of formic acid as low as 0.75 N were likewise effective eluents.) The order of successive elution was β-benzoylpropionic acid, β-(*p*-methylbenzoyl)propionic acid, and β-(*p*-ethylbenzoyl)propionic acid.

Accordingly, 670 mg (3.77 mmoles) of the radioactive β-benzoylpropionic acid were adsorbed on a Dowex 1-formate column and eluted with 4 N formic acid. The eluent was collected in 10-ml fractions; the absorption at 254 mμ was continuously recorded. The fractions constituting the β-benzoylpropionic acid and those of the β-(*p*-methylbenzoyl)propionic acid were separately evaporated to dryness, and aliquots thereof were assayed for radioactivity. It was found that the β-benzoylpropionic acid possessed 3%, and the β-(*p*-methylbenzoyl)propionic acid 97%, of the radioactivity. The specific activity of the former was 130 c.p.m. per mmole. The latter was purified by solution in water, followed by evaporation and recrystallization from 1 ml of *n*-heptane. The melting point of the 0.8 mg of β-(*p*-methylbenzoyl)propionic acid so obtained was 125°. A mixed melting with an authentic sample was 127°. Its specific activity was 755,000 c.p.m. per mmole. This result affords additional evidence that toluene is a metabolite of C_2H_4-H^3. The possibility exists that benzene may also be a metabolite.

II. 4-Acetaminotoluene and 2,4-Diacetaminotoluene—For further confirmation that toluene is a metabolite of C_2H_4-H^3, the acetamino derivatives of toluene were prepared. For this purpose another 3.0-ml portion (1.56×10^5 c.p.m.) of the first fraction of distillate (Table III) was transferred to CCl_4 by first diluting the alcoholic solution with 15 ml of H_2O and then extracting with 5 ml of CCl_4. To the CCl_4 solution was added 1.0 ml of toluene, and the acetamino derivatives thereof were prepared according to the procedure of Iaptieff and Schmerling (6). As opposed to the direct nitration of toluene (6), its nitration in CCl_4 resulted in the formation of both 4-nitrotoluene and 2,4-dinitrotoluene, thus giving, on reduction and acetylation, both 4-acetaminotoluene and 2,4-diacetaminotoluene. The mixture was separated by solution in 25 ml of hot H_2O, from which, on cooling, 160 mg of 4-acetaminotoluene were recovered. The 4-acetaminotoluene, on recrystallization from water, possessed a melting point of 146° (recorded m.p., 145° (6)). On evaporation of the mother liquor to 10 ml, 95 mg of 2,4-diacetaminotoluene (m.p. 223°; recorded m.p., 221° (6)) were obtained. The specific radioactivity of the monoderivative was constant after three recrystallizations, and that of the diderivative after one (Table V). The fact that the two acetamino derivatives, as well as the β-(*p*-methylbenzoyl)propionic acid, and *p*-toluic acid all had essentially the same specific activity indicates that toluene is indeed a metabolite of C_2H_4-H^3.

III. Location of Tritium in Toluene—To locate the tritium in the toluene, the methyl group was oxidized to a carboxyl group. *p*-Toluic acid, prepared as described above, was oxidized to terephthalic acid, and toluene was oxidized to benzoic acid.

A 100-mg portion of the twice recrystallized *p*-toluic acid was oxidized to terephthalic acid by heating it with 3 ml of H_2O, 1.0 g of $K_2Cr_2O_7$, and 1.1 ml of H_2SO_4 on a steam bath under reflux for 48 hours, followed by 6 hours of boiling under reflux. The reaction mixture was diluted with water and the product was recovered. A portion of the diluted reaction mixture was distilled. The radioactivity found in the distillate was 75% of that of the original *p*-toluic acid taken. Because of the insolubility of terephthalic acid, the acid was converted to its diethyl ester (7). The yield of the ester (m.p. 42°; recorded m.p., 42–44° (7)) obtained was 94 mg (58% of theory). The specific activity of ester was 230 c.p.m. per mmole or 4% of that of the *p*-toluic acid. Therefore, by far the major portion of the tritium is located in the methyl group of the toluene.

Confirmation of the labeling pattern was obtained by oxidation of toluene to benzoic acid. For this purpose the radioactivity contained in 3.0 ml of the first distillation fraction (the same fraction as that used for the Friedel-Craft and nitration reactions) was transferred to 7.0 ml of CCl_4 by the same procedure as that described above. To the CCl_4 solution were added 1.0 ml of toluene and 50 ml of 5% $KMnO_4$ in 2% Na_2CO_3 solution. The reaction mixture was heated under reflux for 20 hours, after which the excess $KMnO_4$ was decomposed by the addition of solid $NaHSO_3$. The MnO_2 and CCl_4 were separated by centrifugation, and the aqueous solution was acidified with 6 N HCl. A yield of 418 mg (36% of theoretical) of benzoic acid (m.p. 120.5°) was obtained. The specific activity of the benzoic acid was 390 c.p.m. per mmole. Recrystallization of the benzoic acid from water raised the melting point to 121.5° but failed to change the specific activity. This radioactivity was 6% of that of *p*-toluic acid (Table IV) or that of the acetamino derivatives (Table V). These results confirm the finding that toluene formed by the metabolism of C_2H_4-H^3 is labeled primarily in the methyl group.

DISCUSSION

The first distillation fraction of the alcoholic solution of volatile metabolites (used here for the identification of toluene) also contains nonaromatic hydrocarbon-like compounds, since the amount of radioactivity recovered as β-(p-methylbenzoyl)propionic acid was only 38% of what was to be expected if all had been present as toluene. Moreover, the second and third fractions, although relatively high in radioactivity, were transparent to ultraviolet light at 220 mμ and above. Yet the solubility of all the radioactivity in nonpolar solvents indicated hydrocarbon-like properties for these unknown substances. Their nature is currently under investigation. The possibility exists that they may be terpenes.

Clearly, a small part of the metabolized C_2H_4-H^3 resides in the benzenoid rings since the specific activities of the β-benzoylpropionic acid, terephthalic acid ester, and benzoic acid were all of the same order: 130, 230, and 390 c.p.m. per mmole, respectively. More complete evidence for this will be the subject of a subsequent publication.

A comparison of the toluene arising from C_2H_4-H^3 and that from C_2H_4-C^{14} metabolism is rather striking. Approximately 25% of the C^{14} metabolites are volatile.[3] On distillation of the aqueous alcohol solution of these volatile metabolites, the first distillation fraction contained benzene and toluene in approximately the same amount as with C_2H_4-H^3. However, on transferring the radioactive components (1.6×10^5 c.p.m.) to CCl_4, diluting with 1.0 ml of toluene, and oxidizing the toluene to benzoic acid, only 1% of the radioactivity was found in the benzoic acid. The specific activity of the acid was 160 c.p.m. per mmole. The C^{14} radioactivity in the toluene was therefore comparable with the H^3 activity of the benzene ring of toluene. The comparison suggests that in the metabolism of ethylene by the avocado, a considerable part of the hydrogen is removed from the ethylene, perhaps by dehydrogenation, and proceeds along a pathway different from that followed by the carbon. Nevertheless, part of the hydrogen and the carbon of ethylene are metabolized along the same pathway. Proof of this hypothesis must await the isolation and characterization of other metabolites of both C_2H_4-H^3 and C_2H_4-C^{14}.

SUMMARY

1. Ethylene-H^3 (40 mc per mmole) was incorporated into avocado fruit from the surrounding atmosphere to a small extent in 4 hours, as measured by the radioactivity of 70% alcohol extracts of exposed fruit. At the levels of 250 p.p.m. and 2000 p.p.m. of ethylene in the atmosphere, 0.015% and 0.042% of the ethylene, respectively, were incorporated by the fruit.

2. Approximately three-fourths of the radioactive metabolites found in the 70% alcohol extracts were volatile. Fractional distillation of the aqueous alcohol solution resulted in the concentration of the radioactive components in the first fractions. Not more than 13% of the volatile components resided in exchangeable tritium.

3. The radioactive components in the first distillation fraction, which corresponded to 0.4% of the total solution and contained 43.8% of the radioactivity, possessed the solvent distribution properties of hydrocarbons. Ultraviolet absorption spectra of this fraction showed the presence of benzene and toluene.

4. Toluene produced by the metabolism of C_2H_4-H^3 was shown to be labeled by dilution of the first distillation fraction with toluene and the preparation therefrom of β-(p-methylbenzoyl)-propionic acid, p-toluic acid, 4-acetaminotoluene, and 2,4-diacetaminotoluene. All of these derivatives on recrystallization possessed essentially the same specific radioactivity. The radioactivity found in the toluene derivatives corresponded to 12.5% of the total radioactivity incorporated by the ethylene metabolism.

5. By dilution of the first distillation fraction with benzene, preparation therefrom of β-benzoylpropionic acid, and chromatographic separation of the β-benzoylpropionic acid from the accompanying small amount of β-(p-methylbenzoyl)propionic acid, it was found that 97% of the radioactivity of the mixture resided in the β-(p-methylbenzoyl)propionic acid. This toluene derivative was characterized by recrystallization, melting point, and mixed melting point with an authentic sample. It possessed the high specific radioactivity to be expected.

6. Approximately 95% of the radioactivity of toluene was located in its methyl group. This was shown by oxidation of the toluene to benzoic acid and p-toluic acid (from β-(p-methylbenzoyl)propionic acid) to terephthalic acid.

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[3] A more detailed account of these results will be published later.