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L-LEUCINE AS A PRECURSOR OF *ISO*AMYL ALCOHOL AND *ISO*AMYL ACETATE, VOLATILE AROMA CONSTITUENTS OF BANANA FRUIT DISCS

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Abstract—Incubation of ripe banana tissue discs resulted in biological conversion of L-leucine-U-^{14}C to volatile constituents. A large proportion (up to 81 per cent) of the volatile radioactivity was found in *iso*amyl alcohol. Relatively little (up to 10 per cent) was found in *iso*amyl acetate. Hydrolysis of *iso*amyl acetate showed its radioactivity to be derived from acetate as well as from the alcohol. Compounds which are major banana aroma constituents were, therefore, produced biologically from leucine in quantities sufficient to allow their isolation and further analysis.

INTRODUCTION

INVESTIGATIONS of banana fruit aroma have dealt primarily with the isolation and identification of volatiles.[1-4] They are primarily esters (acetates and butyrates), alcohols (C_1 through C_7) and carbonyls. In general, as ripening proceeds the amount and number of volatiles increases.[5]

The biogenesis of volatile constituents, other than carbon dioxide and ethylene, during ripening of fruit has received little attention. The earliest work was that of Hultin and Proctor[6] who demonstrated that addition of crude enzyme extracts from ripe banana pulp to heat-processed banana puree, regenerated fresh banana aroma. Aroma production was increased by addition of pyruvic acid, valine, or oleic acid. Drawert *et al.*[7] reported enzymatic formation of 2-hexenal and *n*-hexanal from linoleic and linolenic acids in apple, banana, pear, plum and grape homogenates. Conversion of alanine, leucine and valine to carbonyls and alcohols by tomato enzyme extracts[8] has also been described.

The present investigation employed banana discs (3 × 20 mm) immersed in aqueous solution.[9,10] Conditions were generally similar to those used by Sacher[11] and Bauer and

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[1] H. O. HULTIN and B. E. PROCTOR, *Food Tech.* **15**, 440 (1961).
[2] E. L. WICK, A. I. MCCARTHY, M. MYERS, E. MURRAY, H. NURSTEN and P. ISSENBERG, in *Flavor Chemistry*, Adv. in Chem. Series No. 56, p. 241, American Chemical Society (1966).
[3] K. E. MURRAY, J. K. PALMER, F. B. WHITEFIELD, B. H. KENNET and G. STANLEY, *J. Food Sci.* **33**, 632 (1968).
[4] E. L. WICK, T. YAMANISHI, A. KOBAYASHI, S. VALENZUELA and P. ISSENBERG, *J. Agr. Food Chem.* **17**, 751 (1969).
[5] A. I. MCCARTHY, J. K. PALMER, C. P. SHAW and E. E. ANDERSON, *J. Food Sci.* **28**, 379 (1963).
[6] H. O. HULTIN and B. E. PROCTOR, *Food Tech.* **16**, 108 (1961).
[7] F. DRAWERT, W. HEIMANN, R. EMBERGER and R. TRESSL, *Ann. Chem.* **694**, 200 (1966).
[8] M. YU, L. E. OLSON and D. K. SALUNKHE, *Phytochem.* **7**, 561 (1968).
[9] E. H. BUCKLEY, *Plant Physiol.* **37**, xlvi (1962).
[10] J. K. PALMER and W. B. MCGLASSON, *Australian J. Biol. Sci.* **22**, 87 (1969).
[11] J. A. SACHER, *Plant Physiol.* **41**, 701 (1966).

Workman[12] in studies of cell permeability, respiration and amino acid incorporation in banana tissue.

Since little is known about the biosynthesis of volatiles within ripening fruit, selection of a potential precursor compound was arbitrary. L-Leucine was finally chosen because, like valine and other amino acids, it accumulates in ripening bananas of both the Gros Michel and Valery variety[2,13] and correlation between these increases and flavor development has been observed.[2] Since *Saccharomyces cerevisiae* converts branched-chain amino acids to branched-chain alcohols,[14] it was thought that the branched-chain alcohols and esters, which are major banana aroma components, might be derived similarly. Therefore, an investigation of the relationship between L-leucine-U-^{14}C and the production of *iso*amyl alcohol (3-methylbutanol) and its acetate was carried out using banana tissue slices at various stages of ripeness.

Previous research, which employed direct vapor analyses over banana slices, had demonstrated the production of *iso*amyl acetate only by unripe slices but that no *iso*amyl alcohol was formed at any ripeness stage.[15] The present investigation was expected to aide interpretation of these results.

RESULTS

Hands of green bananas (*Musa cavendishii* cv. Valery) were ripened at 20° and high relative humidity. Less than ripe fruit was defined as green-yellow to yellow with green tips; ripe fruit was full yellow and overripe fruit was yellow with brown flecks.

Slices from ripe tissue was shaken at 26° in the presence of L-leucine-U-^{14}C in phosphate buffer, pH 6·0, for periods from 10 min to 5 hr. Condensible volatile constituents were isolated by vacuum distillation and investigated as outlined in Fig. 1. Uptake of leucine into ethanol-extractable compounds by the ripe discs is shown in Fig. 2 together with the radioactivity in the aqueous condensates after vacuum distillation (Fig. 1). After 10 min about 17 per cent of the applied label was found in the ethanol-extractable compounds. This increased to about 30 per cent after 2 hr and then remained essentially constant. Incorporation of radioactivity from leucine to the volatiles, on the other hand, was essentially maximum (0·98 per cent vs. 1·26 per cent of applied label) in 10 min.

Radioactivity in Iso*amyl Alcohol and* Iso*amyl Acetate in Aqueous Condensates*

Odor concentrates, isolated in the manner outlined in Fig. 1, were separated by gas chromatography and twenty-three fractions were collected separately as shown in Fig. 3. Radioactivity in peaks eluted before *iso*amyl acetate and after *iso*amyl alcohol was determined by summation of counts found in each individually trapped peak (Table 1). *Iso*amyl acetate (fraction 6), *iso*amyl alcohol (fraction 8) and fraction 7 (the baseline between the acetate and alcohol) were also counted separately. No significant activity was found in fraction 7. The total radioactivity contained in *iso*amyl alcohol and *iso*amyl acetate in the aqueous condensates from ripe discs was calculated by using values obtained for the recovery of *n*-heptanol (fraction 16), and internal standard.

[12] J. R. BAUR and M. WORKMAN, *Plant Physiol.* **34**, 540 (1964).
[13] F. C. STEWARD, A. C. HULME, S. R. FREIBERG, M. P. HEGARTY, J. K. POLLARD, R. RABSON and R. A. BARR, *A. Botany London*, **24**, 117 (1960).
[14] J. F. GUYMON, *Develop. Ind. Microbiol.* **7**, 88 (1966).
[15] M. J. MYERS, P. ISSENBERG and E. L. WICK, *J. Food Sci.* **34**, 504 (1969).

Changes in the specific activity of *iso*amyl alcohol and *iso*amyl acetate isolated from slices at the three ripeness stages are summarized in Table 2. Both compounds were produced at all three ripeness stages and in all cases studied, the activity of *iso*amyl alcohol was greater than that of *iso*amyl acetate. Incubation in the presence of 0·01 M cyanide inhibited production of both compounds.

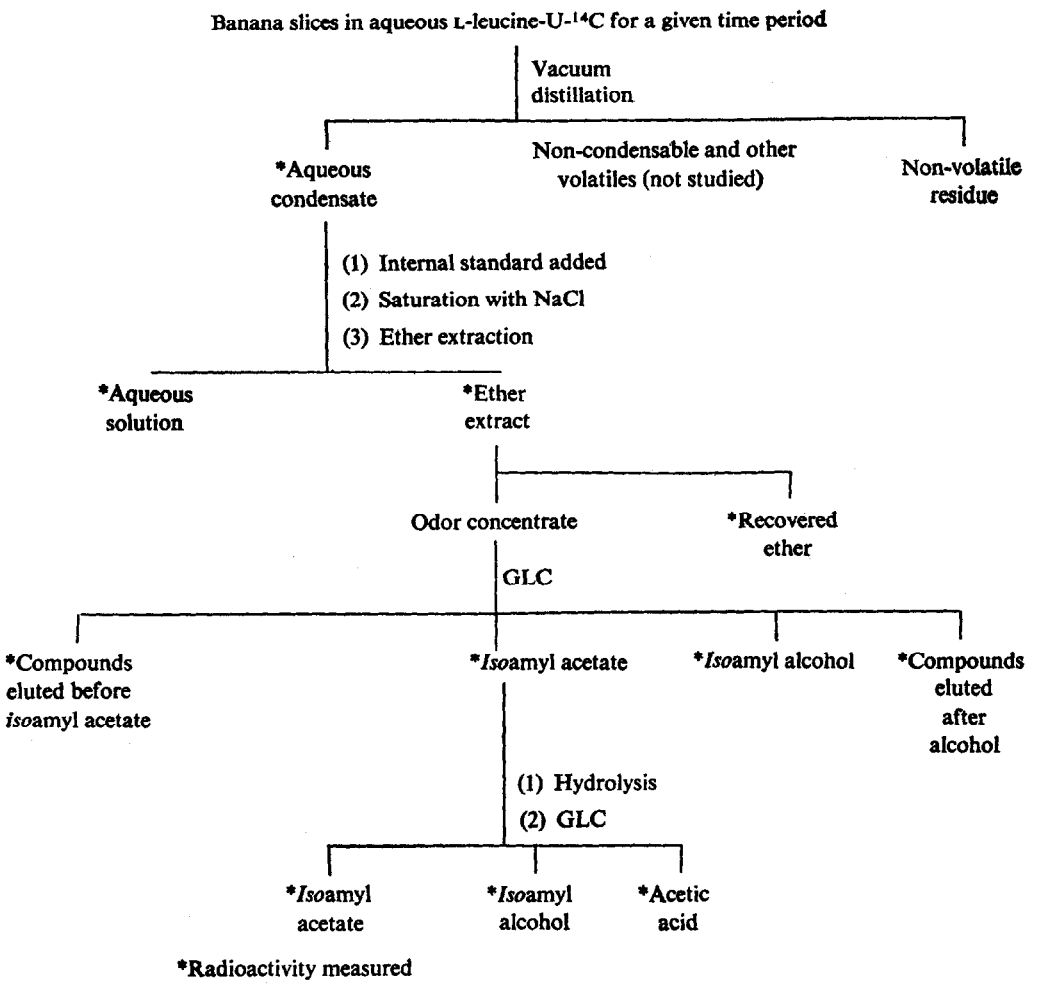

FIG. 1. ISOLATION AND INVESTIGATION OF VOLATILE CONSTITUENTS FROM RIPE BANANA FRUIT.

Radioactivity in the hydrolysis products of *iso*amyl acetate isolated from ripe slices after 1-, 2-, and 5-hr incubations is shown in Table 3. Leucine was the source of carbon atoms in both the *iso*amyl alcohol and acetic acid portions of *iso*amyl acetate. As incubation periods lengthened, radioactivity in acetic acid decreased while activity in the alcohol increased.

Distribution of Radioactivity Among Volatile Constituents

It can be seen from Table 1 that by far the largest proportion of radioactivity (52–81 per cent) was found in *iso*amyl alcohol. Relatively little (1–10 per cent) was incorporated

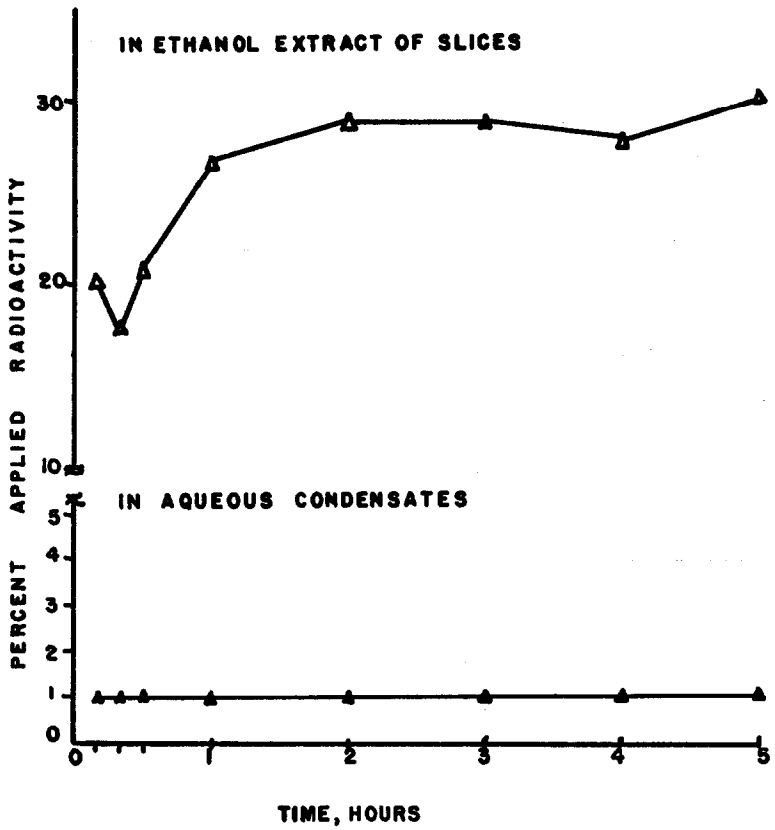

FIG. 2. "UPTAKE" OF L-LEUCINE-U-^{14}C INTO RIPE BANANA DISCS AND "INCORPORATION" OF RADIOACTIVITY INTO VOLATILE COMPONENTS OF AQUEOUS CONDENSATES.

Conditions: twelve slices incubated in 14·4 ml of 0·05 M phosphate buffer, pH 6·0, and 0·5 ml of L-leucine-U-^{14}C (50 μc, 0·2 μmole).

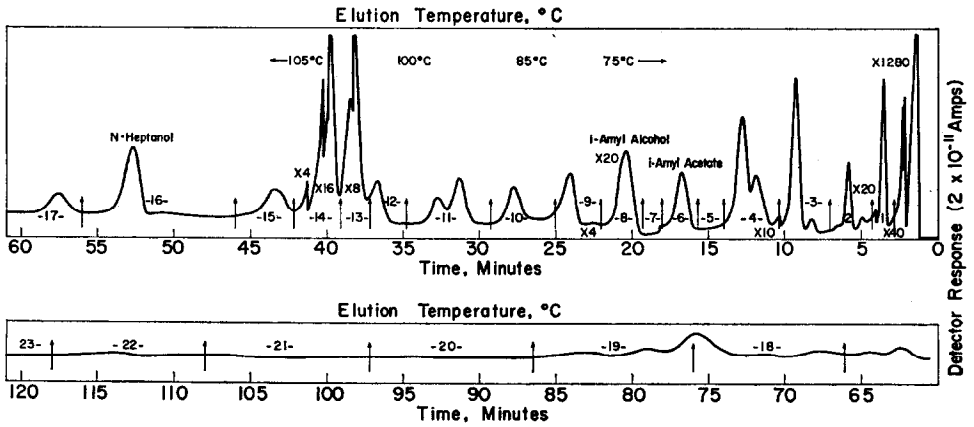

FIG. 3. TYPICAL GAS CHROMATOGRAPHIC SEPARATION OF A VOLATILE CONCENTRATE FROM RIPE BANANA FRUIT.

TABLE 1. TIME COURSE OF THE DISTRIBUTION OF RADIOACTIVITY AMONG VOLATILE CONSTITUENTS FROM RIPE BANANA DISCS

	Per cent total radioactivity in volatiles*							
	10 min	20 min	30 min	1 hr	2 hr	3 hr	4 hr	5 hr
Peaks eluted before *iso*amyl acetate	14·5	22·6	17·0	6·9	4·0	2·6	1·4	1·9
*Iso*amyl acetate	1·5	2·2	2·3	3·6	5·7	7·6	7·0	10·0
*Iso*amyl alcohol	57·6	60·0	64·7	77·7	81·0	78·3	76·4	78·3
Peaks eluted after *iso*amyl alcohol	26·4	15·2	16·0	11·8	9·3	11·5	15·2	9·8
Total DPM in traps	59,555	76,655	97,872	166,286	233,543	286,250	311,976	565,000

Conditions: twelve slices incubated in 14·5 ml of 0·05 M phosphate buffer, +0·5 ml L-leucine-U-^{14}C (50 μc, 0·2 μmoles).

* Per cent total radioactivity $= \frac{\text{DPM in a given trap}}{\text{Total DPM in traps}} \times 100.$

TABLE 2. COMPARISON OF SPECIFIC ACTIVITIES OF *iso*AMYL ACETATE AND *iso*AMYL ALCOHOL IN BANANA DISCS

	Specific activities (dpm/nmole)		
Incubation time	Less than ripe	Ripe	Overripe
*Iso*amyl acetate			
10 min	—	4	—
20 min	—	9	—
30 min	—	14	—
1 hr	359 (*)[a]	46	17 (*)[a]
2 hr	—	103 (26)[a]	—
3 hr	234 (93)[a]	159	39 (10)[a]
4 hr	—	200	—
5 hr	312 (171)[a]	232	123 (57)[a]
*Iso*amyl alcohol			
10 min	—	50	—
20 min	—	79	—
30 min	—	111	—
1 hr	912 (34)[a]	188	60 (18)[a]
2 hr	—	274 (65)[a]	—
3 hr	527 (128)[a]	366	83 (76)[a]
4 hr	—	382	—
5 hr	504 (353)[a]	367	175 (143)[a]

* Not statistically significant at the 99 per cent level.
[a] Specific activity when incubation was carried out in the presence of 0·01 M KCN.

into *iso*amyl acetate. The rest was found in the range of compounds eluted before *iso*amyl acetate and after *iso*amyl alcohol. As incubation periods lengthened, the proportion of radioactivity in both *iso*amyl acetate and *iso*amyl alcohol increased until, at the end of 5 hr, 88 per cent was in these two compounds (78 per cent in the alcohol and 10 per cent in the acetate).

TABLE 3. RADIOACTIVITY IN HYDROLYSIS PRODUCTS OF *iso*AMYL ACETATE-^{14}C ISOLATED FROM RIPE BANANA DISCS

	Apparent dpm	Ratio = $\frac{\text{dpm } iso\text{amyl alcohol}}{\text{dpm acetic acid}}$
1-hr Incubation		
*Iso*amyl alcohol	366	3·0
Acetic acid	120	
3-hr Incubation		
*Iso*amyl alcohol	688	9·7
Acetic acid	71	
5-hr Incubation		
*Iso*amyl alcohol	3300	23·1
Acetic acid	143	

DISCUSSION

Incubation of ripe banana tissue discs in the presence of L-leucine-U-^{14}C resulted in its uptake by the discs and its conversion to volatile aroma constituents, especially *iso*amyl alcohol. Although this was not unexpected in view of the experiments with yeasts,[14] in earlier work on bananas no increase in the vapor concentration of *iso*amyl alcohol at any ripeness stage or of *iso*amyl acetate at ripe and overripe stages had been observed using direct vapor analysis.[15] The striking difference between the increasing specific activities of the alcohol and acetate and their essentially unchanging concentration in the vapor overripe slices is shown in Fig. 4. The implications of these results are important in that they demonstrate the need to interpret direct vapor analyses over ripening fruit or vegetables with caution. Insufficient information is available to explain the observed steady vapor concentrations.

Hydrolysis of *iso*amyl acetate showed that part of its radioactivity was derived from acetate as well as *iso*amyl alcohol. A small proportion of radioactivity was detected among the other constituents that were either more volatile or less volatile than *iso*amyl alcohol and *iso*amyl acetate. It is probable that these substances were labelled non-specifically, in part via the acetate pathway.

This investigation employed slices that were continually immersed in aqueous solution. Such treatment may have caused "unnatural" behavior[16,17] so that the observed biogenesis of *iso*amyl alcohol and *iso*amyl acetate in the banana discs and in intact fruit may differ.[18,19] Future work which incorporates the model slice system of Palmer and McGlasson,[10] in combination with the analytical procedures developed in this investigation, should allow realistic assessment of this question. Incubation conditions must also be found that permit the accurate quantitative determination of both the time-course of incorporation of radioactivity into the general range of volatile components, and into *iso*amyl alcohol and *iso*amyl acetate, the two aroma components of interest, so that the relative importance of volatiles and their relationships to general metabolism can be more clearly understood.

[16] R. L. BEILESKI and G. G. LATIES, *Plant Physiol.* **38**, 586 (1963).
[17] G. G. LATIES, *Plant Physiol.* **39**, 391 (1964).
[18] S. P. BURG and K. V. THIMANN, *Plant Physiol.* **35**, 24 (1960).
[19] W. B. MCGLASSON and H. K. PRATT, *Plant Physiol.* **39**, 128 (1964).

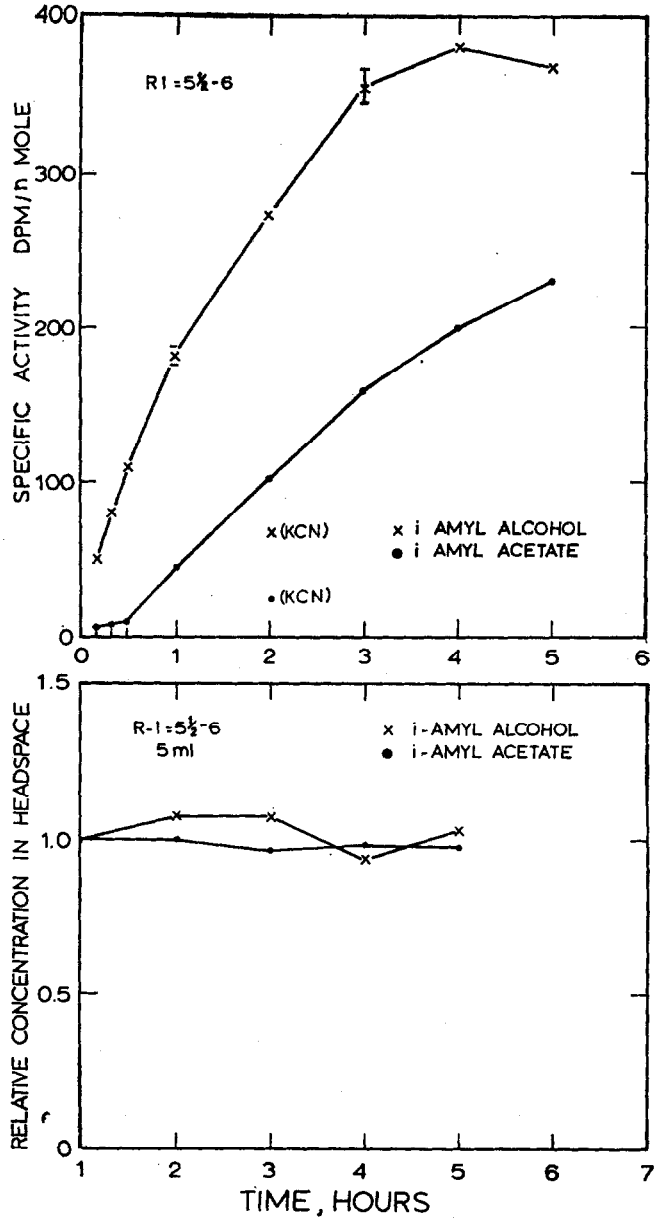

FIG. 4. COMPARISON OF CHANGES IN THE SPECIFIC ACTIVITY OF *iso*AMYL ACETATE AND *iso*AMYL ALCOHOL FROM RIPE SLICES (R.I. 5½–6) WITH CHANGES IN THEIR VAPOR CONCENTRATION.

EXPERIMENTAL

General

All glassware, solutions and utensils were sterilized before use and the banana fingers were swabbed with 70% EtOH.

Incubation of Slices

Slices (3 × 20 mm) prepared from banana pulp tissue with a hand microtome and a No. 14 cork-borer were randomized and soaked in tap water for up to 1 hr. Sets of twelve slices (about 12 g) were placed in

flasks which contained 13·0–14·4 ml, 0·05 M phosphate buffer, pH 6·0, and 0·5 m L-leucine-U-^{14}C (50 μc, 0·2 μmoles, New England Nuclear Corp.). The flasks were covered with Al foil and shaken at room temperature (26°). At appropriate times liquid N_2 was added to selected samples to stop the reaction. The flasks were stoppered and stored at −40° until distillation was carried out.

Isolation of Volatiles

Vacuum distillation was carried out at $<0{\cdot}2 \times 10^{-3}$ torr according to the method of Merritt *et al.*[20] but only the so-called "water fraction" was isolated. The condensate was thawed at room temperature, treated with an internal standard (8 μg of *n*-heptanol in EtOH, 0·1% (w/v)) and made up to 50 ml with H_2O. After removal of aliquots for measurement of radioactivity, the solution was saturated with NaCl, extracted with Et_2O and the extract dried (Na_2SO_4). The extract was concentrated at 35°.[2] The final concentrate (about 10 μl) exhibited a "typical" banana aroma and contained some Et_2O and EtOH from the internal standard.

Separation and Isolation of Volatiles and Radioactivity Determinations

Separation of the odor concentrate was achieved (Fig. 3) on a 2·5 m × 3 mm o.d. stainless-steel column containing 10% Ucon 50 HB 2000 on Anachrom ABS 100/110 (Analabs, Hamden, Conn.). The column was maintained at about 75° until *iso*amyl alcohol was eluted and then temperature-programmed at 2°/min. H_2 flow was 15 ml/min. Injector temperature was 205° and temperature at the effluent splitter was 140°. Sample sizes ranged from 4 to 20 μl.

The chromatograph was fitted with a flame ionization detector. Selected fractions were trapped in capillary tubes. For radioactivity determinations traps were broken and dropped into scintillation fluid[21] and vigorously shaken. The efficiency of counting for every sample was determined by use of toluene ^{14}C as internal standard ($5{\cdot}26 \times 10^5$ dpm/ml). Trapping efficiency from the chromatographic column was determined by chromatographing, trapping, and counting 3 μl of a standard solution of *iso*amyl acetate-^{14}C in EtOH (7000 dpm/μl). The acetate had been prepared by acetylation of *iso*amyl alcohol with acetic-1-^{14}C anhydride. The radioactivity in all trapped fractions was corrected for inefficiencies in trapping.

Peaks 6, 8 and 16 in Fig. 3, which contained *iso*amyl alcohol, *iso*amyl acetate and *n*-heptanol, respectively, were identified by comparison of their retention times with those of authentic samples. The amounts of these components were determined by comparison of their peak areas (calculated by triangulation) to standard curves which related mass to peak areas. The recovery in the trapped fractions of volatile compounds originally isolated in the aqueous condensates was found by determining the recovery of *n*-heptanol. Rechromatography of fraction 6 (*iso*amyl acetate) after hydrolysis and of fraction 8 (*iso*amyl alcohol) under conditions known to separate the 2-methyl- and 3-methyl butanols, gave no evidence for the presence of the 2-methyl isomer.[22]

Leucine Uptake

Sets of twelve slices were incubated for the selected times in L-leucine-U-^{14}C (1 ml, 1 μc, 0·2 μmoles) and 14·0 ml of 0·05 M phosphate buffer (pH 6·0). The slices were removed, rinsed for 1 min in H_2O and then extracted by vigorous shaking with 50 ml of 95% EtOH for 16 h. The extracted slices were washed 4× with 95% EtOH and the washings combined. Radioactivity in aliquots of the EtOH extract was determined.

Hydrolysis of Isoamyl Acetate

*Iso*amyl acetate trapped in fraction 6 was treated with 1 μl of 1 N HCl. The trap was centrifuged, sealed and placed in an oven at 160° for 16 hr. On opening, 3 μl of EtOH was added and the mixture centrifuged. A solution (2 μl) which contained equal portions of EtOH, *iso*amyl acetate, *iso*amyl alcohol, and acetic acid was added to serve as a carrier and to provide detectable quantities of the hydrolysis products. The resulting solution was centrifuged and then separated on a 3 m × 3 mm stainless-steel gas chromatographic column containing 20% NPGS (neopentyl glycol succinate) + 2% H_3PO_4 on firebrick 60/80 maintained at 95° for about 23 min and programmed at 10°/min to 130°. Carrier gas (N_2) flow was 15 ml/min. Injector temperature was 205°. Sample sizes were 4·5 to 4·7 μl.

Hydrolysis of *iso*amyl acetate under these conditions was more than 95 per cent complete. The requisite fractions were trapped and their radioactivity determined. Trapping efficiency was determined by chromatography of a standard solution of acetic-1-^{14}C acid in EtOH (1700 dpm/μl).

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[20] C. MERRITT, S. R. BRESNICK, M. L. BAZINET, J. T. WALSH and P. ANGELINI, *J. Agri. Food Chem.* 7, 784 (1959).
[21] G. A. BRUNO and J. E. CHRISTIAN, *Anal. Chem.* 33, 1216 (1961).
[22] M. J. MYERS, Ph. D.Thesis, Massachusetts Institute of Technology (1968).