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THE BIOSYNTHESIS OF PINOSYLVIN IN THE SAPWOOD OF *PINUS RESINOSA* AIT*

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Abstract—Acetate-1-^{14}C, glucose-G-^{14}C, phenylalanine-G-^{14}C, phenylalanine-[COOH]-^{14}C, and cinnamic acid-β-^{14}C were administered to wounds on paraffin-treated sections of live red pine branches. The wounds were treated with solid carbon dioxide, and the branch sections were kept at 22° for 10 days after which the wood was extracted and radioactive pinosylvin and its monomethyl ether isolated. The incorporation of activity was 0·2–1·7 per cent, except in the experiment with cinnamic acid where only 0·03 per cent incorporation was obtained. The pinosylvins were converted to the dimethyl ether and degraded to benzoic and 3,5-dimethoxy benzoic acids. Where necessary, the site of the label was also established by decarboxylation of these acids. The results obtained confirm the hypothesis of Birch and Donovan, who proposed that in plants hydroxy stilbenes are derived from a phenylpropanoid precursor which links with three acetate units. The data obtained also show clearly that the fifteenth carbon atom is lost from the acetate moiety. In the experiments with acetate and glucose as precursors, considerable activity was incorporated into the glycerides and resin acids, which are formed together with the pinosylvins in the wound reaction.

INTRODUCTION

RECENTLY, one of us[1] has shown that when the cambium of red pine (*Pinus resinosa* Ait.) is wounded and the cells are allowed to die slowly under the influence of desiccation, pinosylvin (I, R = H) and its monomethyl ether (I, R = CH_3) are formed in the adjacent sapwood. Since this wound reaction can be induced artificially, it afforded an opportunity to study the biogenesis of these hydroxy stilbenes. This paper describes the results obtained when radioactive precursors, including acetate-1-^{14}C, D-glucose-G-^{14}C, L-phenylalanine-G-^{14}C, DL-phenylalanine-[COOH]-^{14}C, and cinnamic acid-β-^{14}C, were administered to wounded branches of the red pine.

Several hypotheses on the formation of hydroxy stilbenes in plants have been proposed. Thus, Robinson[2] suggested that they are formed exclusively from acetate units via the intermediate polyketo carboxylic acid II (Fig. 1, Pathway A). Such a synthesis would require a large number of reductive steps and does not explain readily the hydroxylation pattern found in the pinosylvins. Erdtman and his collaborators[3,4] have investigated the phenolic heartwood constituents of many pine species and have found the pinosylvins to be accompanied by simple flavones, like chrysin (III), pinocembrin, pinobanksin, etc. Erdtman[5] drew attention to the fact that in both groups of compounds ring A has a 1,3-hydroxylation pattern and ring B is unsubstituted, and he thought it likely that both types

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[1] E. JORGENSEN, *Can. J. Botany* **39**, 1765 (1961).
[2] R. ROBINSON, in *The Structural Relations of Natural Products*, Clarendon Press, Oxford, 1955.
[3] H. ERDTMAN, in *Progress in Organic Chemistry*, Butterworths Scientific Publications, London, 1952, pp. 22–63.
[4] H. ERDTMAN, in *Perspectives in Organic Chemistry*, edited by A. TODD, Interscience Publishers, New York, 1956, pp. 453–494.
[5] H. ERDTMAN, in *Progress in Organic Chemistry*, Butterworths Scientific Publications, London, 1952, p. 36.

of compounds were derived from the same, or similar, precursors. However, in the pinosylvins the two aromatic rings are linked by an ethylene bridge and thus have one carbon atom fewer than the flavones, a fact which has to be explained if a common origin is assumed.

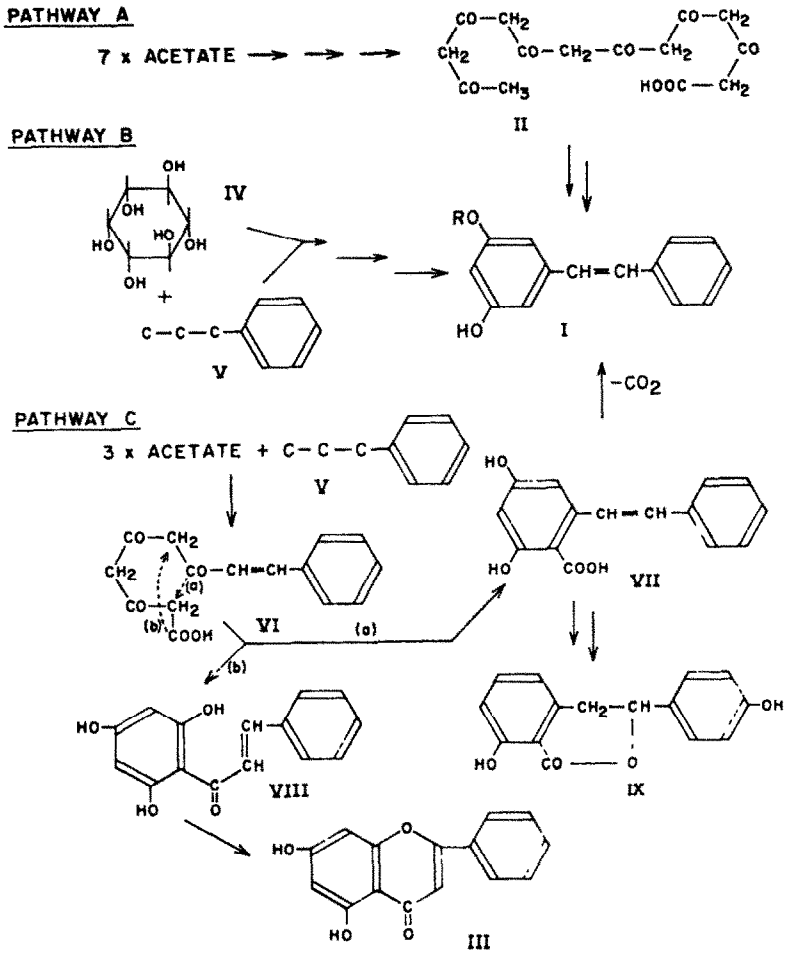

FIG. 1. PROPOSED PATHWAYS FOR THE BIOSYNTHESIS OF PINOSYLVIN.

Since pinitol is a common constituent of pine wood, Erdtman[6] thought it possible that a cyclitol of the inositol type (IV) may combine with a phenylpropanoid precursor (V) to give pinosylvin (Pathway B). Cyclitols have not been found to be precursors of aromatic plant constituents[7] and more recently Erdtman[8] has supported the views of Birch and Donovan[9,10]. These authors propose that a phenylpropanoid precursor links with three acetate units (Pathway C) to give the intermediate VI. This, in turn, could cyclize in two

[6] H. ERDTMAN, *Holz als Roh- Werkstoff* **11**, 245 (1953).
[7] A. C. NEISH, *Ann. Rev. Plant Physiol.* **11**, 55 (1960).
[8] H. ERDTMAN, *Proc. Intern. Congr. Biochem.*, 4th Congr., Vienna **2**, 1 (1958).
[9] A. J. BIRCH and F. W. DONOVAN, *Australian J. Chem.* **6**, 360 (1953).
[10] A. J. BIRCH, *Fortschritte Chem. org. Naturstoffe* **14**, 186 (1957).

different ways: (a) to the hydroxy stilbene precursor VII, which would give the pinosylvins on decarboxylation, and (b) to a chalcone (VIII) which would rearrange to give the flavonoid compounds of the chrysin series. This hypothesis can thus account for the formation of both pinosylvins and flavonoid compounds in pines. Also, the fact that it requires only three condensations, one cyclization, and one decarboxylation or isomerization step makes it attractive from a biochemical point of view.

The formation of quercetin in buckwheat[7,11,12] and of cyanidin in red cabbage seedlings[13] appears to follow the hypothesis of Birch and Donovan. More recently Ibrahim and Towers[14] as well as Billek and Kindl[15] have shown that radioactive precursors are incorporated into hydrangenol (IX) in full agreement with this hypothesis. Billek and Ziegler[16,17] have described experiments in which radioactive acetate and glucose were fed to two year old *Pinus sylvestris* seedlings. Although the incorporation into the pinosylvins was only 0·005 and 0·012 per cent respectively, their results lend further support to views of Birch and Donovan. However, experiments with cinnamic acid failed to give satisfactory results[17,18] and the question whether the phenylpropanoid moiety is incorporated into pinosylvins without loss of a carbon atom has not been proven beyond doubt. Neish[19] has shown that in the biogenesis of pungenin, the 3-glucoside of 3,4-dihydroxyacetophenone found in spruce leaves,[20] phenylpropanoid precursors were used much more efficiently than were phenylethanoid ones. Thus, it could not be ruled out that in the biogenesis of pinosylvins a phenylpropanoid precursor was decarboxylated before union with the three acetate units. Therefore, the experiments in this study were also designed to clarify this point.

Whilst this work was in progress, Hillis and Hasegawa[21] reported briefly on results obtained on feeding radioactive acetic acid, shikimic acid, and phenylalanine to twigs and leaves of two eucalyptus species. These precursors were incorporated into the hydroxy stilbene glucosides, piceid and rhapontin, in complete agreement with Birch and Donovan's theory.

RESULTS AND DISCUSSION

Initial experiments showed that 5–10 g pieces of the wounded red pine branches produced sufficient pinosylvin monomethyl ether after 5–10 days incubation to permit isolation in the crystalline state. The finely divided wood was extracted with acetone and the extract was fractionated on a column of silicic acid. Glycerides and resin acids were eluted first, followed by pinosylvin monomethyl ether and very small amounts of pinosylvin. The yield and plate count of each fraction from the experiments with different precursors are shown in Table 1. The hydroxy stilbene fractions were combined and methylated to give pinosylvin dimethyl ether. This was used for determining the per cent incorporation and, after degradation, for locating the label (see Table 2). In the experiment with cinnamic acid-β-^{14}C as precursor plate counts of the extract and of individual fractions were exceedingly high. This was found to be due to contamination with unmetabolized precursor, which was removed completely before isolation of pinosylvin dimethyl ether.

[11] J. E. Watkin, E. W. Underhill and A. C. Neish, *Can. J. Biochem. Physiol.* **35**, 229 (1957).
[12] T. A. Geissman and T. Swain, *Chem. & Ind. (London)* 984 (1957).
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[14] R. K. Ibrahim and G. H. N. Towers, *Can. J. Biochem. Physiol.* **38**, 627 (1960); and **40**, 449 (1962).
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[16] G. Billek and W. Ziegler, *Ostmärk. milchwirtsch. Ztg.* **62**, 310 (1961).
[17] G. Billek and W. Ziegler, *Monatsh. Chem.* **93**, 1430 (1962).
[18] G. Billek, private communication.
[19] A. C. Neish, *Can. J. Botany* **37**, 1085 (1959).
[20] A. C. Neish, *Can. J. Biochem. Physiol.* **35**, 161 (1957).
[21] W. E. Hillis and M. Hasegawa, *Chem. & Ind. (London)* 1330 (1962).

TABLE 1. ANALYTICAL DATA OF CRUDE AND FRACTIONATED RED PINE WOOD EXTRACTS

Precursor added	Acetate -1-^{14}C	Glucose -G-^{14}C	Cinnamic acid-β-^{14}C	L-Phenyl-alanine -G-^{14}C	DL-Phenyl-alanine -[COOH]-^{14}C
Weight of wood (air dried, g)	5·58	4·73	8·15*	4·53	8·37†
Weight of acetone extract (mg)	99·7	81·1	200·8	58·7	162·8
Plate count (c.p.m./mg)	270	1075	2220‡	390	320
SiO_2 Chromatography§	mg (c.p.m./mg)	mg (c.p.m./mg)	mg (c.p.m./mg)	mg (c.p.m./mg)	mg (c.p.m./mg)
1: mainly glycerides	7·1 (140)	5·8 (305)	20·7 (30)	6·7 (25)	38·4 (25)
2: mixture	9·4 (480)	14·3 (1790)	79·0 (270)	9·2 (50)	21·4 (55)
3: mainly resin acids	7·3 (400)	15·6 (1455)	18·1 (4770)	5·5 (125)	16·1 (160)
4: mixture	1·9 (230)	2·6 —	4·85 (2810)	0·9 —	1·2 (180)
5: pinosylvin monomethyl ether	2·95 (280)	13·6 (945)	11·3 (1455)	3·85 (445)	26·0 (450)
6: mixture	8·45 (305)	0·4 —	4·75 (6870)	0·4 —	6·5 (430)
7: pinosylvin	3·5 (300)	4·0 (985)	6·15 (5550)	4·4 (435)	3·2 (410)

* 3 pieces of wood.

† 2 pieces of wood.

‡ Contaminated with unmetabolized precursor.

§ Fractions 1 to 4 eluted with chloroform, then 3 per cent, 5 per cent and 10 per cent 2-butanone added.

Oxidative degradation of pinosylvin dimethyl ether with permanganate[22] gave a mixture of benzoic and 3,5-dimethoxy benzoic acids in about 70 per cent yield. The two degradation products could be separated from each other by fractional sublimation, the recovery being about 30 and 60 per cent respectively. After further dilution with inactive material, these acids were decarboxylated by the copper–quinoline method and the yield of carbon dioxide (as barium carbonate) was 80–90 per cent of theory. The recovery of benzene from benzoic acid was very poor (less than 5 per cent) and that of 3,5-dimethoxy benzene about 40 per cent of theory.

TABLE 2. SPECIFIC ACTIVITY OF PINOSYLVIN DIMETHYL ETHER AND ITS DEGRADATION PRODUCTS

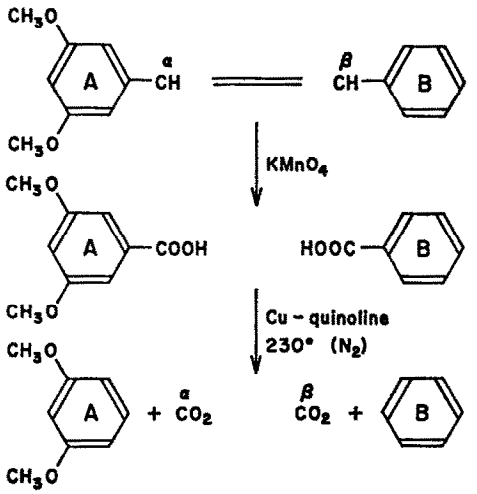
Precursor added	Acetate -1-^{14}C	Glucose -G-^{14}C	Cinnamic acid-β-^{14}C	L-Phenyl-alanine -G-^{14}C	DL-Phenyl-alanine -[COOH]-^{14}C
Weight (mg)	0·1075	1·01	3·00*	0·375	0·60†
Specific activity (μc/mmole)	7·0	10·4	18·6	5·95	14·2
Pinosylvin dimethyl ether (mg)	14·0	7·6	14·3	14·8	13·2
Specific activity (mμc/mmole)	254	1136	104	256	580
Per cent incorporation	0·21	1·70	0·03	0·26	0·22
Benzoic acid (mμc/mmole)	11	636	105	207	6
Derived CO_2 (mμc/mmole)	—	—	95	—	—
3,5-Dimethoxy benzoic acid (mμc/mmole)	234	432	6	59	480
Derived CO_2 (mμc/mmole)	—	—	—	30	0
Derived 3,5-dimethoxy benzene (mμc/mmole)	—	—	—	—	324

* 3 pieces of wood fed with 1·00 mg (6·2 μc/mmole) each.

† 2 pieces of wood fed with 0·30 mg (7·1 μc/mmole) each.

[22] H. ERDTMAN, *Ann. Chem., Justus Liebigs* **539**, 116 (1939).

In Table 2 the amounts and specific activities of the precursors administered and those of the isolated pinosylvin dimethyl ether are shown. From these data the per cent incorporation was calculated. The specific activities of the degradation products are also listed in Table 2. The radioactivity found in 3,5-dimethoxy benzoic acid corresponds to that in ring A and the α-carbon of the ethylene bridge, whereas that found in benzoic acid corresponds to the activity in ring B and the β-carbon atom (see Fig. 2). Decarboxylation of these two acids localized the label further as shown:



With acetate-1-^{14}C as precursor about 95 per cent of the radioactivity was found in the 3,5-dimethoxy benzoic acid. This lends good support to the view that ring A of the pinosylvins is a product of the acetate pathway. The small amount of activity found in the ethylene bridge carbons and ring B can be explained by some of the acetate-1-^{14}C having been decarboxylated and the radioactive carbon dioxide entering the general metabolic pool. These findings are in good agreement with those obtained by Billek and Ziegler,[15,16] and less strictly so with those described by Hillis and Hasegawa,[21] who used acetate-2-^{14}C.

The results obtained with phenylalanine and cinnamic acid confirm not only that ring B and the ethylene bridge may be derived from a phenylpropanoid precursor, but also that such a moiety is incorporated intact. In the experiment with cinnamic acid-β-^{14}C practically all of the activity was found in the carboxyl group of benzoic acid (Table 2). With generally labelled L-phenylalanine 22 per cent of the activity was found in 3,5-dimethoxy benzoic acid and 78 per cent in benzoic acid, i.e. a ratio of 2 to 7 which is exactly as predicted if the phenylpropanoid skeleton enters into the pinosylvins unaltered. This aspect was confirmed even further when the activity from DL-phenylalanine-[COOH]-^{14}C was found almost exclusively in the benzene ring portion of 3,5-dimethoxy benzoic acid. This finding also shows conclusively that the fifteenth carbon atom of the pinosylvin precursor is lost from the acetate-derived portion of the molecule.

It is now generally accepted that phenylpropanoid units are derived from glucose via the shikimic acid pathway.[7] However, when radioactive D-glucose was used as precursor a considerable amount of the activity was found in 3,5-dimethoxy benzoic acid, i.e. in that portion which appears to be formed from acetate units. A similar result was obtained by Billek and Ziegler,[15,16] leaving little doubt that a considerable amount of the glucose enters

the acetate cycle, presumably via the Emden-Meyerhof scheme.[16] Mixing of pool metabolites was reported by Geissman and Swain[12] in the biosynthesis of quercetin and caffeic acid from glucose, and by Watkin and Neish[23] in that of rutin. Glucose, being removed from shikimic acid by a fairly large number of steps, is thus not an ideal precursor for the study of the biosynthesis of aromatic nuclei. However, since it is so readily metabolized, it offers a criterion of the efficiency of incorporation of other precursors. Thus, in this study it was incorporated about eight times as well as acetate or phenylalanine. Since comparatively large amounts of cinnamic acid were fed, and because this acid was incompletely metabolized, a comparison of the degree of its incorporation with that of glucose (about fifty times better) is of limited value only.

The degree of incorporation obtained in this study is similar to that reported by Hillis and Hasegawa[21] for the twigs and leaves of eucalyptus, and it was about fifty times as good as by infusion through the cut taproot of pine seedlings.[15,16] Thus the wound reaction encountered in the cambium and sapwood of pines appears to be a relatively efficient system for incorporating radioactive precursors. Since it was found in the experiments with acetate-1-^{14}C and glucose-G-^{14}C that an appreciable amount of radioactivity was located in the glyceride and resin acid fraction (Table 1), this technique may possibly be used advantageously in a study of the biogenesis of these compounds.

EXPERIMENTAL

Melting points were determined on a Leitz hot-stage microscope. Infrared spectra were recorded with a Perkin–Elmer model 21 double beam spectrophotometer (solids as KBr disks, liquids as films between sodium chloride plates). Plate counts were carried out with 2–3 mg of material, spread as a thin and even film by evaporation from solution, on stainless steel planchets. The radioactivity (c.p.m.) was measured using an end window counter (Nuclear-Chicago Model D44 fitted with an automatic sample changer). Specific activities were determined by wet combustion[24] of accurately weighed samples ($\pm$0·002 mg) with Van Slyke reagent[25] to carbon dioxide, which was counted in the gas phase with the aid of a dynamic condenser electrometer (Nuclear Chicago Dynacon).

Feeding of precursors

Internodes of freshly collected live red pine branches (2–4 cm thick) were cut into 20-cm sections. Both ends were sealed immediately by being dipped into melted paraffin. A hole (9 mm diameter, 6 mm deep) was drilled through the bark and cambium at the middle of each section and aqueous solutions (0·25–0·5 ml) of the precursors were added to the wounds. After infusion, the wound was treated three times with solid carbon dioxide[1] and the sections were kept in a desiccator over anhydrous silica gel at 22° for 10 days.

Extraction and fractionation

The wounded section of the branch (2–5 cm long), free from residual bark, was cut into small chips, and the air-dried material was extracted with acetone in a soxhlet apparatus for 36 to 48 hr. The solvent was evaporated on a rotary evaporator (water-pump vacuum) and the residue was weighed and a plate count was taken (see Table 1). The total extract was then chromatographed on fifty times its weight of anhydrous silicic acid. The column

[23] J. E. WATKIN and A. C. NEISH, *Phytochemistry* **1**, 52 (1961).
[24] D. L. BUCHANAN and A. NAKAO, *J. Am. Chem. Soc.* **74**, 2389 (1952).
[25] D. D. VAN SLYKE and J. FOLCH, *J. Biol. Chem.* **136**, 509 (1940).

was made up with the silicic acid slurried in petrol (b.p. 60–80°) and elution (100 ml fractions) was carried out with chloroform (400 ml), followed by chloroform containing 3% (100 ml), 5% (100 ml) and 10% (200 ml) 2-butanone. The weights of each fraction obtained and the respective plate counts are shown in Table 1. Infrared spectra of all fractions were recorded as films, those of fractions 1 and 3 corresponding to spectra of glycerides and resin acids respectively. Saponification gave long chain fatty acids in the C_{16} and C_{18} range, the latter predominating. Fraction 5 tended to crystallize in nearly all experiments and in the initial blank experiment (inactive phenylalanine) pure pinosylvin monomethyl ether was obtained after recrystallization from petrol–chloroform and benzene; m.p. 120–122°, undepressed in admixture with authentic pinosylvin monomethyl ether. The infrared spectrum of fraction 7 resembled closely that of pinosylvin, when that of the latter was recorded as a film. Fraction 6 was a mixture of pinosylvin and its methyl ether.

Methylation

Fractions 5 to 7 were dissolved in 2N NaOH solution and combined. To this solution (5–10 ml) was added dimethyl sulphate (0·1 ml) with shaking, which was continued intermittently for 1 hr. The mixture was heated on a steam bath for 0·5 hr. If it did not give a strong alkaline reaction, more 2N NaOH was added and heating was continued to ensure complete reaction. The methylation product was extracted with ether and worked up in the usual manner to give the crude dimethyl ether. A concentrated solution in ether (1–2 ml) was filtered through neutral alumina (Woelm, activity Grade I) and the alumina washed with ether (10 ml). The combined filtrate was evaporated to give sirupy pinosylvin dimethyl ether, which crystallized slowly, in low yield, from ethyl acetate on seeding with authentic pinosylvin dimethyl ether (m.p. 54·5–55·5°, Literature[28] m.p. 56–57°). Paper chromatography[26] or thin-layer chromatography[27] using chloroform as solvent, showed only a single spot and the infrared spectrum was superimposable with that of authentic pinosylvin dimethyl ether. The specific activity of the crystalline product was practically the same as that of the sirupy residue from the mother liquor. Therefore, the per cent incorporation of radioactive precursors (see Table 2) was calculated from the weight of sirupy dimethyl ether. The sirupy material was diluted with its own weight of inactive dimethyl ether (m.p. 54·5–55·5°) and was recrystallized from ethyl acetate and methanol to give a product of m.p. 53–55·5°.

In the experiment with cinnamic acid-β-^{14}C the methylation and saponification was repeated twice to remove all of the unmetabolized precursor. In a blank experiment with inactive pinosylvin monomethyl ether (10·0 mg) and radioactive cinnamic acid (0·1 mg) no radioactivity was detected in the isolated pinosylvin dimethyl ether (9·5 mg) after repeating methylation and saponification three times.

Degradation

Pinosylvin dimethyl ether (10 mg) was dissolved in acetone (2–3 ml) and was shaken at room temperature with powdered potassium permanganate (20 mg) in water (1–2 ml) for 0·5 hr. The mixture was extracted with ether to remove unoxidized starting material (3 mg) and was then filtered. After acidification with 10% HCl the acids were isolated by

[26] G. Lindstedt and A. Misiorny, *Acta Chem. Scand.* **5**, 121 (1951).
[27] E. von Rudloff and A. Sato, *Can. J. Chem.* in press.

extraction with ether. The mixed acids were transferred to a long glass tube (6 mm I.D. × 30 cm) which was sealed at one end, and fractionally sublimed. Under a pressure of 10–15 mm (Hg) benzoic acid (m.p. 121–122°) sublimed readily and yields could be improved by removing it before continuing the sublimation at 1–2 mm pressure, when 3,5-dimethoxy benzoic acid (m.p. 180–184°) was obtained. The melting point of both acids was undepressed in admixture with authentic specimens.

After determination of the specific activities the residual acids were diluted two to five times with inactive acid and decarboxylated by heating with copper powder (10 mg) in quinoline (0·5 ml) at 230° in a slow stream of nitrogen for 2–3 hr. The evolved carbon dioxide was trapped in a sidearm in 2N NaOH solution (1 ml) and precipitated as barium carbonate. The neutral moiety was recovered by extracting the reaction mixture and NaOH solution with ether and distilling the residue after removal of the ether. The recovery of benzene from benzoic acid was less than 5 per cent of theory, but 3,5-dimethoxy benzene (b.p. 80–90° at 14 mm Hg) was obtained in about 40 per cent yield.

The specific activities of the degradation products are shown in Table 2.

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