122. *Mould Tropolones: Biosynthesis and Alkali-isomerization of Stipitatic and Decarboxylated Stipitatic Acid.*

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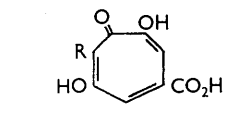
Penicillium stipitatum Thom has been grown in the presence of sodium [1-^{14}C]acetate and the labelled stipitatonic acid produced has been degraded by a new scheme. This has shown that C-2 of this metabolite is derived from the carboxyl-carbon of acetate, as in a current theory of biosynthesis. The relation of mould tropolone biosynthesis to that of some benzenoid metabolites is discussed.

Alkali-isomerization of stipitatic and decarboxylated stipitatic acid leads to the exclusive extrusion of C-1 as carboxyl. This is explained in terms of nucleophilic attack only at C-1 of the anions resulting from the mesomeric effects of the substituents.

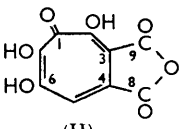
STIPITATIC acid (Ia; R = H) from *Penicillium stipitatum* and puberulic acid (Ib; R = OH) and puberulonic acid (II) from *P. puberulum* and other *Penicillia* were the first mould tropolones to be described.[1] Later, stipitatonic acid [2,3] (III) and ethyl stipitatate [4] were isolated from *P. stipitatum* culture fluid.

A study [3] of their rates of production has shown that stipitatonic is the precursor of stipitatic acid, and this has been confirmed [5] by partial decarboxylation of stipitatonic and puberulonic acid by a crude enzyme preparation from *P. stipitatum* mycelium.

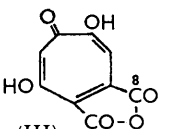
Biosynthetic studies with [^{14}C]-labelled stipitatic acid (by Bentley [6]) and puberulonic acid (by Richards and Ferretti [7]) have demonstrated the participation of acetate and formate groups. Bentley's degradation of labelled stipitatic acid involved alkali-isomerization of the metabolite and he assumed that C-1 and C-2 are equally extruded to form a carboxyl group of 5-hydroxyisophthalic acid. Richards and Ferretti's degradation



(Ia; R = H) (Ib; R = OH)



(II)



(III)

of labelled puberulonic acid involved only decarboxylation and oxidation. The enolone system of puberulic acid is mobile and the molecule is therefore essentially symmetrical [numbering throughout this paper is as in (II)]; consequently the skeletal carbons were assayed as three pairs: C-1 + C-7; C-2 + C-6; and C-3 + C-5; C-4 was obtained by difference. The activity of C-2 + C-6 was found to be 76 ± 12% of the activity of the [1-^{14}C]acetate-derived-puberulonic acid, and Richards and Ferretti therefore concluded that both C-2 and C-6 are derived from the carboxyl of the acetate. These workers questioned the validity of the assumption that C-1 and C-2 are extruded equally in the alkali-isomerization of stipitatic acid and suggested that C-1 preferentially (but not necessarily exclusively) becomes a carboxyl group in the isomerization product. More recently Ferretti and Richards [8] accounted for the preferential extrusion of C-1 in terms of the

[1] (*a*) Birkinshaw and Raistrick, *Biochem. J.*, 1932, **26**, 441; (*b*) Birkinshaw, Chambers, and Raistrick, *ibid.*, 1942, **36**, 242; (*c*) Nozoe, *Fortschr. Chem. org. Naturstoffe*, 1956, **13**, 232; (*d*) Pauson, *Chem. Rev.*, 1955, **55**, 9; Cook and Loudon, *Quart. Rev.*, 1951, **5**, 99.

[2] Segal, *Chem. and Ind.*, 1957, 1040; 1958, 1726; Doi and Kitahara, *Bull. Chem. Soc. Japan*, 1958, **31**, 788.

[3] Segal, *J.*, 1959, 2847.

[4] Divekar, Brenneisen, and Tanenbaum, *Biochim. Biophys. Acta*, 1961, **50**, 588.

[5] Bentley and Thiessen, *Nature*, 1959, **184**, 552.

[6] Bentley, *Biochim. Biophys. Acta*, 1958, **29**, 666.

[7] Richards and Ferretti, *Biochem. Biophys. Res. Comm.*, 1960, **2**, 107.

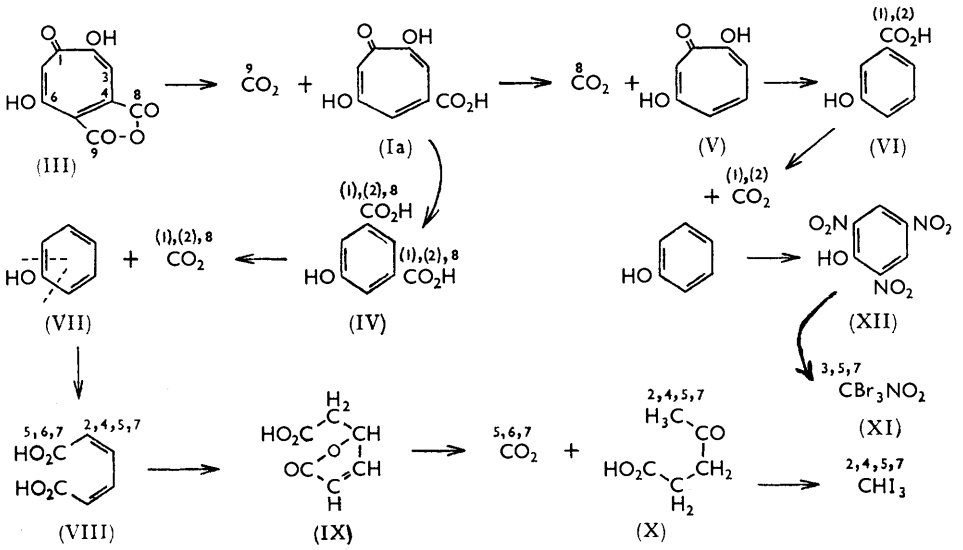
[8] Ferretti and Richards, *Proc. Nat. Acad. Sci. U.S.*, 1960, **46**, 1438.

effect of the carboxylate group hindering attack by hydroxyl ion at C-2 relative to attack at C-1. This explanation is inadequate in that alkali-isomerization of decarboxylated stipitatic acid (derived from [1-^{14}C]acetate) affords *m*-hydroxybenzoic acid, the carboxyl group of which is devoid of activity.[9]

Methylation of stipitatic [1b] and of decarboxylated stipitatic acid [9] by diazomethane gives mixtures of isomers indicating α-ketol tautomerism. Similarly, 4-hydroxytropolone affords an oily mixture of methyl ethers which gives two picrates.[10]

On the basis of their results with puberulonic acid and the assumption that C-2 of stipitatonic acid is derived from the carboxyl of acetate, Ferretti and Richards [8] proposed a biosynthetic scheme which accounts for both metabolites and has been related to the biosyntheses of some benzenoid mould metabolites. Bentley [11] proposed a scheme differing only in minor details.

In order to determine the origin of C-2 unambiguously and to compare the degrees of labelling of C-2, C-4, and C-6, which current theory [8,11] requires should be equally derived from the carboxyl-carbon of acetate, the degradation schemes (III) ⟶ (XI) and (III) ⟶ (X) were applied to [1-^{14}C]acetate-biosynthesized stipitatonic acid (III). This determination of C-2 depends on (i) the fact that C-3, C-5, and C-7 in stipitatonic acid are not derived from the carboxyl of acetate, and (ii) Bentley's result [6] for C-2 + C-6 in [1-^{14}C]-acetate-derived stipitatic acid, which is independent of assumptions concerning the course of alkali-isomerization. The scheme involves oxidation of phenol (VII) by peracetic acid to *cis-cis*-muconic acid (VIII). Elvidge, Linstead, and their co-workers [12] described the isomerization of this acid to 4-carboxymethylbut-2-enolide (IX) and partial decarboxylation of the latter to lævulic acid (X). With minor modifications these reactions have



been applied to our muconic acid (VIII). There is no assumption regarding alkali-isomerization of the tropolones (I) and (V). [In formula (IV) the designation (1), (2), 8 denotes primary derivation of the carboxyl-carbon from C-8 but that C-1 or C-2 or both may contribute to this, and similarly in other formulæ.] Decarboxylation of the lactone (IX) produces carbon dioxide derived from C-5, C-6, and C-7, the contribution of C-6 being 50%. Carbon atoms C-5 and C-7 are devoid of activity as shown by assay of the

[9] Segal, unpublished experiments, 1958—1959.
[10] Kitahara, *Sci. Reports Tohôku Univ.*, First Ser., 1956, **39**, 258 (*Chem. Abs.*, 1957, **51**, 12,873).
[11] Bentley, *Biochem. Biophys. Res. Comm.*, 1960, **3**, 215.
[12] Elvidge, Linstead, Orkin, Sims, Baer, and Pattison, *J.*, 1950, 2228.

bromopicrin (XI) derived from the picric acid (XII). Barium carbonate obtained from the carboxyl group of the lactone has 97·5% of the specific activity of the barium carbonate derived from quantitative Van Slyke–Folch [13] oxidation of the lactone itself. When account is taken of the relative contribution of C-6 to the lactone-carboxyl group, the inactivity of C-5 and C-7, and the numbers of carbon atoms involved, C-6 is found to possess 32·5% of the activity of the lactone.

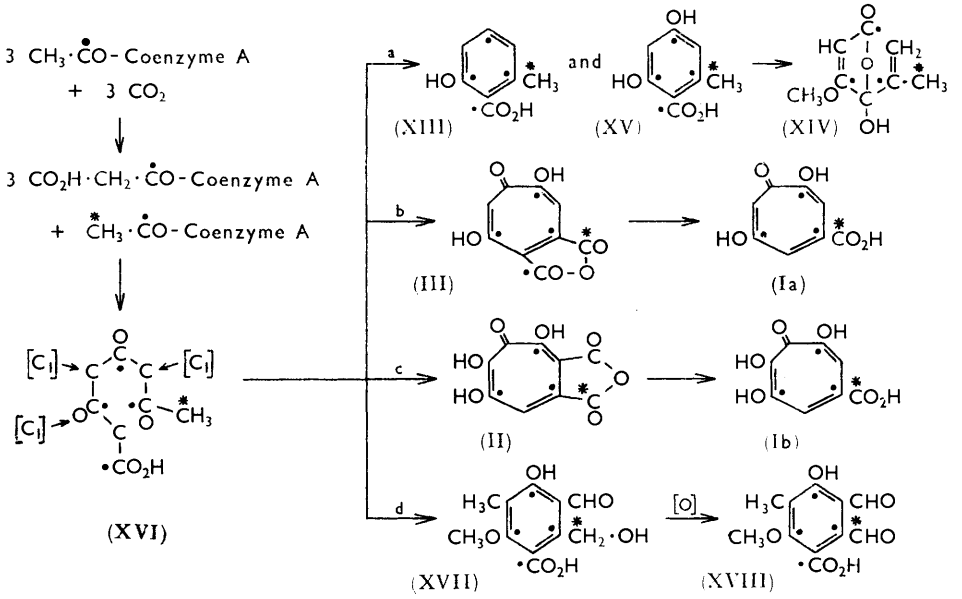
By oxidative degradation of stipitatic acid, Bentley [6] obtained aconitic acid.[14] This was oxidized to malonic and oxalic acid, thus showing by difference that C-2 + C-6 possessed 56% of the activity when derived from [1-^{14}C]acetate-biosynthesized stipitatic acid. Consequently, C-2 in this acid must bear 20—25% of the activity (this range is given because decarboxylation [6,9] of [1-^{14}C]acetate-derived stipitatic acid suggests that C-8 has 3% of the activity of the metabolite). It is thus confirmed that C-2 of stipitatonic acid is derived from the carboxyl of acetate and that in the alkali-isomerizations of stipitatic (Ia; R = H) and decarboxylated stipitatic acid (V), C-1 *alone* is extruded to become a carboxyl group in 5-hydroxyisophthalic (IV) and *m*-hydroxybenzoic acid (VI), respectively. The effects of the hydroxyl and carboxyl groups (as anions) on the course of these rearrangements are considered in the Addendum of this paper.

In order to assay C-2 + C-4, the lævulic acid (X) was converted into iodoform and this was eventually counted as barium carbonate after Van Slyke–Folch oxidation.[13] The specific activity of the iodoform-derived barium carbonate was 95·4% of that of the lactone-derived barium carbonate. At this stage it is established that C-2, C-4, C-5, and C-7 make equal contributions to the iodoform-carbon atom. As C-5 and C-7 are unlabelled, and in view of the number of carbon atoms involved, C-2 and C-4 have an average activity of 31·8% of that of the lactone. Bentley [6] found C-4 to possess 41% of the activity of the [1-^{14}C]acetate-derived stipitatic acid. This figure is essentially compatible with that determined above and demands that C-2 contributes ~25% of the activity of the skeletal carbons of stipitatic acid. On the other hand, Richards and Ferretti [7,8] found that C-2 + C-6 had 76 ± 12% and C-4 24 ± 14% of the activity of [1-^{14}C]acetate-derived puberulonic acid while C-8 and C-9 were unlabelled.

The values obtained above for C-2, C-4, and C-6 may, of course, be interpreted in terms of equality of labelling of these three positions. This is in accord with the equality of labelling of the corresponding positions in [1-^{14}C]acetate-derived 6-methylsalicylic acid (XIII) studied under a variety of conditions by Birch *et al.*[15] Birch and his co-workers [16] have also shown that the three labelled positions of [1-^{14}C]acetate-derived penicillic acid (XIV) are equally labelled; thus the three corresponding positions in the nucleus of orsellinic acid (XV) must be equally labelled, Mosbach [17] having established that orsellinic acid is the precursor of penicillic acid in *P. barnense* cultures. Further, Mosbach [18] has demonstrated the participation of malonate (malonyl-coenzyme A) and acetate (acetyl-coenzyme A) in the biosynthesis of orsellinic acid as in fatty acid biosynthesis. More recently, Birkinshaw and Gowlland [19] have shown that in [1-^{14}C]-acetate-derived orsellinic acid from *P. madriti* the labelling is equally distributed among four of the eight carbon atoms, consistently with its production by the head-to-tail linkage of four acetyl-coenzyme A units. Their results with [1-^{14}C]butyrate show breakdown to C_2 units before incorporation, as required for participation [18] of malonyl-coenzyme A. Bentley [20] has demonstrated a similar role of malonate in stipitatonic acid biosynthesis.

[13] Van Slyke and Folch, *J. Biol. Chem.*, 1940, **136**, 509; Calvin, Heidelberger, Reid, Tolbert, and Yankwich, "Isotopic Carbon," J. Wiley and Sons, Inc., New York, 1949, p. 93.
[14] Corbett, Johnson, and Todd, *J.*, 1950, 147.
[15] Birch, Cassera, and Rickards, *Chem. and Ind.*, 1961, 792.
[16] Birch, Blance, and Smith, *J.*, 1958, 4582.
[17] Mosbach, *Acta Chem. Scand.*, 1960, **14**, 457.
[18] Mosbach, *Naturwiss.*, 1961, **15**, 525.
[19] Birkinshaw and Gowlland, *Biochem. J.*, 1962, **84**, 342.
[20] Bentley, *Fed. Proc.*, 1961, **20**, 80.

The current theory of biosynthesis of mould tropolones involves condensation of active C_1 unit(s) with an acetate (or malonate)-derived benzenoid nucleus of the orsellinic acid type. After condensation, oxidative ring fission is postulated and one C_1 unit is incorporated into an expanded ring which becomes the substituted tropolone.[8,11] The



a, No C_1 unit. b, 1 C_1 unit, ring expansion. c, 2 C_1 units, ring expansion. d, 3 C_1 units.

polyoxomethylene chain (XVI) or a pre-benzenoid compound may, however, act as a recipient of the C_1 unit(s). This theory requires that C-2, C-4, and C-6 of the mould tropolones be equally derived from the carboxyl of acetate, as has been shown for the corresponding positions in (XIII),[15] (XIV),[16] and (XV).[19] The labelling pattern of [1-^{14}C]-acetate-derived stipitatonic acid (III) determined by our degradations therefore supports the theory. Such a hypothesis regarding the location of added C_1 units is in accord with the structures [21] of cyclopolic (XVII) and cyclopaldic acid (XVIII) and with the partial degradation [22] of [1-^{14}C]acetate-derived cyclopaldic acid. The latter degradation is essentially the basis of the current theory of biosynthesis of mould tropolones. Further, the biosynthetic relationship of the metabolites shown in the chart is further substantiated in that strains of a single species (*P. cyclopium*) elaborate puberulic (Ib; R = OH) and puberulonic (II) acid,[23] as well as orsellinic [24] (XV), penicillic [25] (XIV), and cyclopolic (XVII) and cyclopaldic (XVIII) acid.[21] It is worthy of note that Robinson [26] and, independently, Seshadri [27] had proposed theories of tropolone biosynthesis which involved condensation of formaldehyde or its biological equivalent with polyhydric phenols [26] or with 3,5-dihydroxyphthalic acid,[27] which is a metabolite of *P. brevicompactum* [28] and represents an oxidation product of orsellinic acid (XV). Oxidative ring fission and ring enlargement by incorporation of the C_1 unit was postulated.

[21] Birkinshaw, Raistrick, Ross, and Stickings, *Biochem. J.*, 1952, **50**, 610.
[22] Birch and Kocor, *J.*, 1960, 866.
[23] Oxford, Raistrick, and Smith, *Chem. and Ind.*, 1942, **61**, 485.
[24] Bentley and Keil, *Proc. Chem. Soc.*, 1961, 111.
[25] Oxford, Raistrick, and Smith, *Chem. and Ind.*, 1942, **61**, 22.
[26] Robinson, *Chem. and Ind.*, 1951, 12; *Proc. Roy. Soc.*, 1951, *A*, **205**, 15.
[27] Seshadri, *J. Sci. Ind. Res. India*, 1955, **14**, *B*, 248.
[28] Oxford and Raistrick, *Biochem. J.*, 1932, **26**, 1902.

There appear to be two anomalies in the labelling pattern of stipitatic acid (Ia; R = H) which are absent from 6-methylsalicylic (XIII), penicillic (XIV), orsellinic (XV), and puberulic acid (Ib; R = OH). One is the apparent small incorporation of the carboxyl of acetate into the carboxyl group of stipitatic acid (3%), which has been determined [6,9] by decarboxylation [1b] with copper chromite at 220°. On the basis of the current theory this carboxyl group corresponds to the methyl substituents of 6-methylsalicylic, penicillic, and orsellinic acid and to the carboxyl group of puberulic acid, and in these cases there is no contribution of the carboxyl of acetate to the corresponding positions. This decarboxylation of stipitatic acid gives excellent yields of carbon dioxide but is accompanied by further decomposition and only poor yields of 4-hydroxytropolone (V) result. Further, there is no analogous contribution of the carboxyl of acetate to the other positions in stipitatonic acid, which are derived from acetate-methyl (C-1, C-3, C-5). It is thus likely that the small activity assigned to C-8 in [1-^{14}C]acetate-derived stipitatic acid is due to a small contribution of the skeletal carbon atoms to the carbon dioxide of decarboxylation. The second anomaly is the small (4%) contribution of acetate-methyl [6] (from [1-^{14}C]-glucose experiments) to C-4 of stipitatic acid. Again, this has no published counterpart in the labelling of 6-methylsalicylic, penicillic, orsellinic, or puberulic acid; there is apparently no similar contribution from acetate-methyl to C-6 of stipitatic acid in [1-^{14}C]-glucose experiments.[6] This is possibly best explained in terms of accumulated experimental errors or residual activity assigned in order to account for the whole of the activity of the metabolite, C-4 having been determined [6] by difference.

Experimental

Radioactive Assay.—[^{14}C]Labelled compounds were derived from stipitatic and stipitatonic acid grown in the presence of sodium [1-^{14}C]acetate. Radioactivity was assayed by end-window counting at infinite thickness. As these experiments were carried out at different institutions, there was some variation in counting conditions.

Method (i). Powdered specimens were spread uniformly on nickel planchets (1·5 cm. diam.) and compressed. *Method* (ii). Specimens were filtered under suction on to filter paper circles (effective diam. 1·5 cm.) fitted on a perforated stainless-steel base which eventually became the support for the specimen after a flat, flanged, stainless-steel annular cover had been placed over the paper and base. The geometry in counting methods (i) and (ii) was different. Samples were counted for sufficient time to register at least 10^4 counts, so that the statistical counting error was less than 3%. Count rates were corrected for background and, where applicable, for dead time of the Geiger tube. Bentley [6] and Ferretti and Richards [7,8] expressed the activities of individual and pairs of carbon atoms of the mould tropolones as percentages of the activities of the metabolites; this procedure has been adopted for C-8 and C-9 of stipitatic and stipitatonic acid, respectively. The activities of C-2, C-4, and C-6, the only skeletal atoms labelled in the present case, are expressed as percentages of the activity of the residual carbon atoms after removal of C-1, C-8, and C-9.

[^{14}C]*Stipitatonic Acid.*—The Czapek–Dox liquid medium (350 ml.) in each of two 1-l. conical flasks was inoculated with a suspension of *P. stipitatum* Thom (L.S.H.T.M. Cat. No. P. 199) spores in sterile water. After 5 days' growth, sodium [1-^{14}C]acetate (0·1 mc.) in sterile water (5 ml.) was introduced below the mycelial mat of each flask. After a further 28 days' growth, the combined culture filtrates and mycelial washings were acidified with sulphuric acid to pH 2·0 and exhaustively extracted with ether in a continuous liquid–liquid extractor, yielding 1·732 g. of crude metabolic products. The count rate of this material (method i) was 3336 counts/min. Inactive stipitatonic acid was added to a portion of the crude product and then isolated and purified by high-vacuum sublimation and crystallization from acetone. Count rate (method i) was 1536 counts/min.

Assay of C-9.—The above diluted sample of stipitatonic acid was further diluted and the mixture crystallized from acetone to constant m. p. [237° (decomp.)] and constant radioactivity. The count rate (method i) was 395 counts/min. The diluted acid (136 mg.) was decarboxylated to stipitatic acid [3] by refluxing it for 5 hr. in air-free distilled water (15 ml.) in an apparatus swept by nitrogen which carried the carbon dioxide to standard aqueous barium

hydroxide. To avoid contamination of the barium carbonate by atmospheric carbon dioxide, the residual alkali was back-titrated before filtration. Back-titration showed the absorption of 0·985 mol. of carbon dioxide. The barium carbonate was filtered off, washed with air-free distilled water and dry distilled acetone, and dried (yield 128 mg. Theor., 129 mg.). The count rate (method i) (97 counts/min.) indicated that C-9 of the stipitatonic acid bears 23% of the activity. Bentley [11] found 26% for similarly derived stipitatonic acid. The biosynthetic scheme (XVI) ⟶ (III) requires that C-9 possesses 25% of the activity.

Assay of C-8.—The crude mixed metabolites isolated by ether-extraction of the culture fluid contained stipitatic acid as well as its precursor [3,5] stipitatonic acid. A portion of the crude metabolite mixture was therefore boiled together with inactive stipitatic acid in a system to absorb carbon dioxide evolved, and the stipitatic acid crystallized from water to constant m. p. [302° (decomp.); count rate (method i) 1740 counts/min.]. This acid (288 mg.) was decarboxylated [1b] by heating it with copper chromite (120 mg.) in quinoline (15 ml.). The carbon dioxide was swept into standard barium hydroxide solution by nitrogen and after 5 hr. at 220° back-titration showed that 0·995 mol. of carbon dioxide had been absorbed. The barium carbonate collected after back-titration possessed a count rate (method i) of 43 counts/min., indicating that C-8 bears 3% of the activity of the stipitatic acid. Bentley [6] found 4% for similarly derived stipitatic acid. The biosynthetic scheme (XVI) ⟶ (III) requires that C-8 bears zero activity. This decarboxylation was accompanied by extensive decomposition and only very poor yields of 4-hydroxytropolone (V) resulted. Birkinshaw, Chambers, and Raistrick [1b] obtained a carbon dioxide yield of 98% and their yield of 4-hydroxytropolone was $<30\%$.

Alkali-isomerization of 4-Hydroxytropolone (V).—4-Hydroxytropolone resulting from a number of small-scale [^{14}C]stipitatic acid decarboxylations was purified by high-vacuum sublimation and crystallization from acetone as described.[1b] Alkali-isomerization was effected by molten potassium hydroxide at 310° for 15 min. in conditions described [1b] for stipitatic acid. The product was isolated by acidification with hydrochloric acid to pH 4·5; the oxalic acid produced by degradation removed as calcium oxalate after addition of an excess of calcium acetate. The filtrate, adjusted to pH 2·0, was extracted exhaustively with ether in a liquid–liquid extractor. Removal of solvent afforded *m*-hydroxybenzoic acid (VI) which after sublimation in a high vacuum had m. p. and mixed m. p. 201°. Dilution with inactive material in solution and recovery of the acid afforded *m*-hydroxybenzoic acid possessing a count rate (method i) of 1056 counts/min.

Decarboxylation of m-*Hydroxybenzoic Acid.*—Our *m*-hydroxy[^{14}C]benzoic acid (194 mg.) was decarboxylated in boiling quinoline (10 ml.) in the presence of copper chromite (75 mg.) while nitrogen swept the evolved carbon dioxide into standard barium hydroxide solution for 4 hr. The barium carbonate, collected in the usual way, possessed a count rate (method i) identical with that of background. On the basis of the biosynthetic scheme (XVI) ⟶ (III), extrusion of C-1 alone in the alkali-isomerization requires zero activity; extrusion of C-2 alone requires that the barium carbonate possess 33% of the activity of the *m*-hydroxybenzoic acid.

Assay of C-3, C-5, *and* C-7.—After decarboxylation of the *m*-hydroxybenzoic acid the reaction mixture was acidified to pH 2 with sulphuric acid and exhaustively steam-distilled. The distillate was rendered alkaline, concentrated to a small bulk, acidified to Congo Red, and extracted with ether exhaustively in a liquid–liquid extractor. Removal of the solvent at room temperature, addition of carrier phenol, and nitration after preliminary sulphonation afforded picric acid (from aqueous ethanol), m. p. and mixed m. p. 122° [count rate (method i) 620 counts/min.]. This picric acid (0·5 g.) was converted into the calcium salt by suspension in water (5 ml.), addition of freshly ignited calcium oxide (60 mg.), and warming to dissolution. The cooled solution was added dropwise to a water-cooled stirred mixture of water (15 ml.), calcium oxide (3 g.), and bromine (3·9 g.) and stirring continued for a further hour. Steam-distillation carried over the bromopicrin which was extracted with ether. Removal of the dried solvent (Na_2SO_4) at room temperature afforded bromopicrin (1·66 g., 85%). The bromopicrin was counted on a nickel planchet (1·5 cm. diam.), the geometry being as in method (i). It was found to be devoid of activity [Found: C-3 = C-5 = C-7 inactive, as required by (XVI) ⟶ (III)].

Alkali-isomerization of Stipitatic Acid and Decarboxylation of the Isomerization Product.—The conditions used by Birkinshaw, Chambers, and Raistrick [1b] were used with [^{14}C]stipitatic acid isolated from crude, highly active metabolite residues by addition of inactive stipitatic acid and

crystallization from water. A sample of the isomerization product, 5-hydroxyisophthalic acid, to be decarboxylated was quantitatively converted into barium carbonate by Van Slyke–Folch oxidation.[13] The count rate (method ii) was 368 counts/min. The 5-hydroxyisophthalic acid was heated (455 mg.) with copper chromite (250 mg.) and quinoline (12 ml.) at 236°, carbon dioxide being collected by barium hydroxide in the usual way. After 6 hr. decarboxylation was complete. The barium carbonate (method ii) had a count rate of 41 counts/min., indicating that the carboxyl groups bear 2·8% of the activity of the 5-hydroxyisophthalic acid. Bentley's publication [6] implies that the extruded carbon of 5-hydroxyisophthalic acid obtained by the alkali-isomerization of [1-^{14}C]acetate-derived stipitatic acid bears no activity.

cis-cis-*Muconic Acid* (VIII).—The phenol from the above decarboxylation was obtained as a dilute aqueous solution by steam-distillation after addition of an excess of 8N-sulphuric acid. The distillate (700 ml.) was treated with sodium hydroxide (4 g.) and distilled to a small bulk. The phenol was isolated by acidification with 4N-sulphuric acid and extraction with ether in a liquid–liquid extractor. The extract was dried (Na_2SO_4) and on removal of the solvent at room temperature phenol (210 mg.) resulted. Carrier phenol (1·29 g.) was added and the whole treated with peracetic acid (30 g., 13·2%) according to the method of Elvidge *et al.*[12] After 14 days in the dark at room temperature the crystalline deposit (0·543 g.) was collected and washed with ice-cold water.

4-*Carboxymethylbut*-2-*enolide* (IX).—The muconic acid (514 mg.) was not further purified but was isomerized [12] to the lactone (IX) by treating at room temperature with a cold mixture of sulphuric acid (2·1 ml.) and water (0·7 ml.) for 32 hr., the mixture becoming homogeneous. The whole was poured on ice (10 g.), neutralized with concentrated aqueous ammonia, made acid to Congo Red with 4N-sulphuric acid, and extracted with ether in a liquid–liquid extractor (20 hr.). Removal of the solvent and crystallization from benzene containing a trace of ethanol afforded the lactone (IX) as prisms, m. p. 111° (110 mg.) (lit.,[12] 110·5—111·5°). Van Slyke–Folch [13] oxidation of the lactone (7·8 mg.) afforded barium carbonate [63·5 mg. Theor., 64·9 mg. Count rate (method ii) 32·3 counts/min.].

Assay of C-6.—In order to obtain better yields of the hydrolysis products, the lactone was decarboxylated in less drastic conditions than those described.[12] The lactone (44·8 mg.) was heated under reflux in 2% carbonate-free sodium hydroxide (3 ml.) for 45 min., the apparatus having been initially swept with nitrogen. The carbon dioxide was liberated by addition of a slight excess of dilute hydrochloric acid and absorbed in barium hydroxide during refluxing (20 min.). Back-titration indicated the absorption of 0·70 mol. The barium carbonate (collected after back-titration), washed and dried in the usual way, had a count rate (method ii) of 31·5 counts/min. (Found: C-6, 32·5% of the activity of the lactone. (XVI) ⟶ (III) requires 33·3%).

Assay of Average of C-2 *and* C-4.—Lævulic acid (X) was shown [12] to be present in the lactone hydrolysis mixture by isolation of its semicarbazone (m. p. and mixed m. p.). The lactone hydrolysis mixture (above) was made just alkaline with 2N-sodium hydroxide and treated dropwise alternately with iodine (15 g.) and potassium iodide (15 g.) in water (30 ml.), and with 2N-sodium hydroxide at room temperature as described by Tollens.[29] These reagents were added until precipitation of the iodoform was complete. The latter was steam-distilled from the alkaline mixture, collected on a chromic acid-cleaned sintered-glass semimicro-funnel, and air-dried (yield, 60 mg.). The lactone hydrolysis was repeated and a total of 132 mg. of iodoform obtained. The sintered-glass funnel with its adhering iodoform was introduced into the apparatus described by Calvin *et al.*[13] and subjected to Van Slyke–Folch oxidation. The barium carbonate collected in the usual way was found to possess a count rate (method ii) of 30·8 counts/min. [Found: C-2 and C-4 (average) 31·8% of the activity of the lactone. (XVI) ⟶ (III) requires 33·3%].