ACTA CHEMICA SCANDINAVICA 19 (1965) 1724–1732

The Biosynthesis of Flavipin

II. Incorporation of Aromatic Precursors

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Orsellinic acid (II) and 2,4-dihydroxy-5,6-dimethylbenzoic acid (III) have been isolated from flavipin (I) producing cultures of *Aspergillus flavipes*. The methyl group in position 5 of 2,4-dihydroxy-5,6-dimethylbenzoic acid was found to be derived from the C_1-pool. Radioactive tracer studies showed that the above phenolic acids, as well as orcylaldehyde and 2,4-dihydroxy-5,6-dimethylbenzaldehyde, were directly converted into flavipin (5–10 % incorporation of label) when added to *A. flavipes* cultures. The mould thus appears to be capable of introducing a C_1-unit into an aromatic intermediate (such as orsellinic acid), and flavipin is proposed to be biosynthesized by the pathway shown in Fig. 1; the observed incorporation of orcylaldehyde into flavipin probably involved the intermediate formation of orsellinic acid, into which orcylaldehyde was found to be converted by the mould.

In a recent investigation the biogenetic origin of the different carbon atoms of flavipin (I) from *Aspergillus flavipes*[1] was determined by observing the pattern of incorporation of ^{14}C-labelled acetate and methionine.[2] The results obtained were consistent with the formation of a C_8 carbon precursor by the acetate-polymalonate pathway, followed by the introduction of a C-methyl group from the C_1-pool (*cf.* Fig. 1), and it was pointed out that orsellinic acid (II) might function as a precursor of flavipin. During the experimental work routine paper chromatographic examinations of culture filtrates

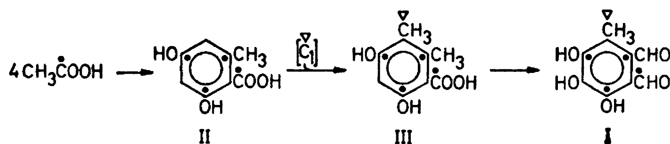

Fig. 1. Suggested pathway for the biosynthesis of flavipin (I) in *Aspergillus flavipes*. Orsellinic acid (II) and 2,4-dihydroxy-5,6-dimethylbenzoic acid (III) are present as metabolic products of the mould.

Table 1. Concentration (μM) of metabolic products in the medium of *A. flavipes* cultures at different stages in the development of the mould.

	Product	Age of culture in days						
		2	3	5	7	10	14	21
I	Flavipin	0.0	10	70	200	410	480	280
II	Orsellinic acid	0.6	2.2	3.7	4.0	3.8	0.0	0.0
III	2,4-Dihydroxy-5,6-dimethyl-benzoic acid	0.8	1.6	1.8	1.7	2.0	0.0	0.0

of the mould were undertaken, which showed that flavipin production was accompanied by the formation of small amounts of two phenolic acids. The latter compounds could, in fact, be detected 1—2 days before flavipin production started, and were found to attain a maximum concentration in the culture medium during the linear phase of flavipin formation (see Table 1). The two phenolic acids were, therefore, regarded as possible intermediates in the biosynthesis of flavipin, and have now been isolated and identified as orsellinic acid (II) and 2,4-dihydroxy-5,6-dimethylbenzoic acid (III). The identities were established by mixed melting-point determinations, spectrophotometry, and chromatographic studies of the natural products, as well as of their decarboxylated derivatives (see experimental section). The total yield of phenolic acids was generally lower than 1 mg/l culture medium.

Orsellinic acid has been recognized as a metabolic product of several mould species (*Chaetomium cochliodes*,[3] *Penicillium griseo-fulvum*,[4] *P. baarnense*,[5] *P. cyclopium*,[6] *P. madriti*,[7] *P. spinulosum*,[8] *Aspergillus fumigatus*,[9] *A. terreus*[10]), and is known to be formed by the acetate-polymalonate pathway.[5,6] 2,4-Dihydroxy-5,6-dimethylbenzoic acid, which previously has been isolated from *A. terreus*[10] and *Gliocladium roseum*,[11] must for structural reasons be expected to have the same biogenetic origin, the methyl group in position 5 probably being an "introduced" C_1-unit. Confirmatively, the addition

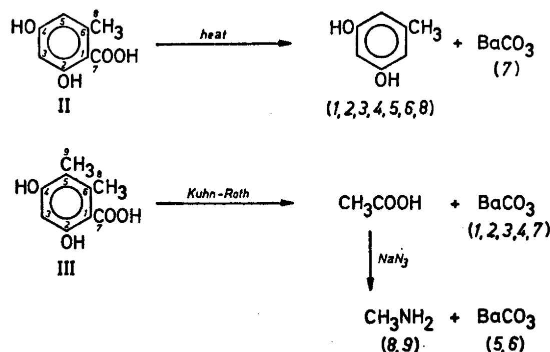

Fig. 2. Chemical degradation of orsellinic acid (II) and 2,4-dihydroxy-5,6-dimethylbenzoic acid (III). Numbers within brackets indicate carbon atoms isolated.

Table 2. Distribution of radioactivity in 2,4-dihydroxy-5,6-dimethylbenzoic acid derived from $^{14}CH_3$-L-methionine (1.0 % incorporation of label).

Material	Carbon atoms isolated	Specific activity*	Total activity	Relative total activity
Total combustion	all	1980	17 820	1.00
Kuhn-Roth oxidation, carbon dioxide	1, 2, 3, 4, 7	60	300	0.02
Kuhn-Roth oxidation, methyl group of acetic acid	8, 9	8430	16 860	0.95
Kuhn-Roth oxidation, carboxyl group of acetic acid	5, 6	40	80	0.00

* counts per min and mg $BaCO_3$

of $^{14}CH_3$-L-methionine to 2 days old cultures of *A. flavipes* led to a rapid incorporation of activity into 2,4-dihydroxy-5,6-dimethylbenzoic acid, whereas orsellinic acid remained non-radioactive (*cf.* Ref. 10). Furthermore, Kuhn-Roth oxidation of the radioactive 2,4-dihydroxy-5,6-dimethylbenzoic acid obtained yielded methyl labelled acetic acid, containing all of the activity incorporated into the phenolic acid (see Fig. 2 and Table 2). It, therefore, appears to be well established that the methyl group in position 5 of 2,4-dihydroxy-5,6-dimethylbenzoic acid is derived from the C_1-pool.

The fact that 2,4-dihydroxy-5,6-dimethylbenzoic acid only has been isolated from moulds that produce orsellinic acid (*A. terreus*, *A. flavipes*), or where the presence of orsellinic acid as a biogenetic intermediate is strongly indicated (*G. roseum*), suggests that 2,4-dihydroxy-5,6-dimethylbenzoic acid is formed by C-methylation of orsellinic acid. The two phenolic acids may, in fact, function as the natural precursors of flavipin, as shown in Fig. 1; this pathway is consistent with the results of previous tracer studies on the biosynthesis of flavipin (see above). However, it is also possible that the C-methylation process takes place on the pre-aromatic (acetate-polymalonate) level, in which case 2,4-dihydroxy-5,6-dimethylbenzoic acid would be produced without the intermediate formation of orsellinic acid. Furthermore, it cannot be excluded that both the phenolic acids and flavipin are biosynthesized independently of each others. In order to test the proposed biogenetic pathway (Fig. 1), orsellinic acid and 2,4-dihydroxy-5,6-dimethylbenzoic acid (specifically ^{14}C-labelled by chemical synthesis) were added to selected 5 days old shaken-flask cultures of *A. flavipes*. Samples of the cultures were then withdrawn at fixed intervals during a period of 24 h, and were examined paper chromatographically with respect to the presence of labelled compounds. After 24 h flavipin was isolated from the cultures, and the distribution of the activity incorporated was determined by chemical degradation as outlined in Fig. 3.

Excellent utilizations of the radioactive precursors were obtained in these experiments. The addition of 2,4-dihydroxy-5,6-dimethylbenzoic acid, ^{14}C-labelled at the methyl group in position 5, to *A. flavipes* cultures thus resulted in the production of flavipin containing 4.5 % of the activity added,

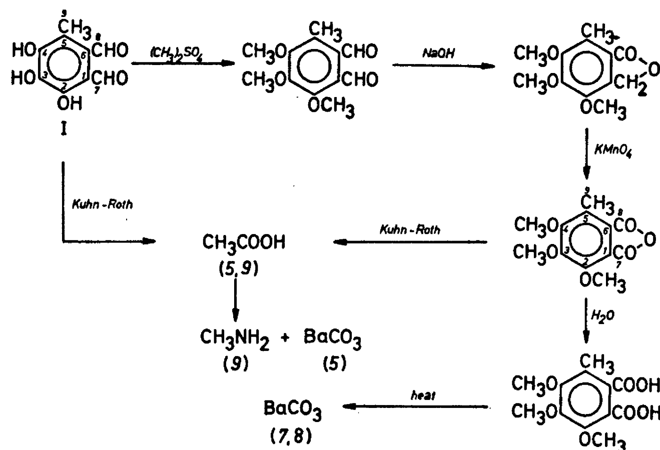

Fig. 3. Chemical degradation of flavipin (I). Numbers within brackets indicate carbon atoms isolated.

and with isotope located exclusively at the methyl group (see Table 3). Similarly, almost the whole of the radioactivity incorporated into flavipin from carboxyl labelled orsellinic acid (6.5 % incorporation of label) was found as expected (Table 4), in the carbon dioxide obtained on decarboxylation of 3,4,5-trimethoxy-6-methylphthalic acid (*cf.* Fig. 3). The same result was obtained on degradation of flavipin derived from carboxyl labelled 2,4-dihydroxy-5,6-dimethylbenzoic acid (5.7 % incorporation of activity); the radioactivity measurement data of the latter experiment are summarized in Table 5. These observations clearly show that the two phenolic acids can be directly (without primary degradation to labelled acetate or C_1-units) converted into flavipin by *A. flavipes*.

Unfortunately, the experiments gave no direct evidence for the conversion of orsellinic acid into 2,4-dihydroxy-5,6-dimethylbenzoic acid; carboxyl labelled orsellinic acid (as well as radioactive 2,4-dihydroxy-5,6-dimethyl-

Table 3. Distribution of radioactivity in flavipin derived from 2,4-dihydroxy-5,6-dimethylbenzoic acid, ^{14}C-labelled at the methyl group in position 5 (4.5 % incorporation of label).

Material	Carbon atoms isolated	Specific activity *	Total activity	Relative total activity
Flavipin	all	130	1170	1.00
Kuhn-Roth acetic acid:				
Total combustion	3, 9	605	1210	1.03
Methyl group	9	1130	1130	0.97
Carboxyl group	3	15	15	0.01

* counts per min and mg $BaCO_3$

Table 4. Distribution of radioactivity in flavipin derived from carboxyl labelled orsellinic acid. The labelled flavipin was converted into 3,4,5-trimethoxy-6-methylphthalic anhydride, which was submitted to the degradation reactions listed below (*cf.* Fig. 3).

Degradation reaction	Carbon atoms isolated	Specific activity *	Total activity	Relative total activity
Total combustion	all	8.54	102.5	1.00
Kuhn-Roth acetic acid, total combustion	5,9	2.72	5.4	0.05
Decarboxylation	7,8	47.70	95.4	0.93

* counts per min and mg $BaCO_3$

Table 5. Distribution of radioactivity in flavipin derived from carboxyl labelled orsellinic acid and 2,4-dihydroxy-5,6-dimethylbenzoic acid, as well as formyl labelled orcylaldehyde and 2,4-dihydroxy-5,6-dimethylbenzaldehyde.

Precursor	Incorporation of label (%) into flavipin	Fraction of the total activity present in carbon atoms C-5 + C-9	C-7 + C-8
Orsellinic acid	6.5	0.05	0.93
2,4-Dihydroxy-5,6-dimethylbenzoic acid	5.7	0.05	0.91
Orcylaldehyde	9.8	0.03	0.98
2,4-Dihydroxy-5,6-dimethylbenzaldehyde	7.5	0.01	0.96

benzoic acid) yielded flavipin as the only detectable labelled product. On the other hand, the observation that orsellinic acid was directly converted into flavipin appears to provide strong evidence that a C-methyl group can be introduced from the C_1-pool into an aromatic intermediate. Furthermore, considering the excellent incorporation yields obtained with both of the phenolic acids, and the established presence of the two acids as metabolic products of flavipin producing cultures of *A. flavipes*, it seems very likely that flavipin is biosynthesized by the pathway shown in Fig. 1.

The reduction of a carboxyl group to a formyl group is, obviously, involved in the conversion of orsellinic acid and 2,4-dihydroxy-5,6-dimethylbenzoic acid into flavipin. The above experiments were, therefore, repeated with formyl (^{14}C-)labelled orcylaldehyde and 2,4-dihydroxy-5,6-dimethylbenzaldehyde as the precursors. These studies showed that the latter two compounds were readily and directly converted into flavipin by *A. flavipes* (see Table 5); the aldehydes were, in fact, found to be much more rapidly incorporated than the corresponding phenolic acids, which might be due to different rates of penetration of the mycelial cell membranes (the precursors were added to the medium of the cultures). 2,4-Dihydroxy-5,6-dimethylbenzaldehyde yielded flavipin as the only detectable labelled product (7.5 % incorporation of activity), whereas orcylaldehyde was found to be converted into flavipin (9.8 % incorporation yield) and a second radioactive product, chromatographically

identical with orsellinic acid. After addition of carrier orsellinic acid to a culture of *A. flavipes*, that had been grown for 2 h in the presence of formyl labelled orcylaldehyde, radioactive orsellinic acid was isolated and recrystallized to constant specific activity (0.3 % incorporation of label). Chemical degradation (see Fig. 2) of this orsellinic acid established that isotope was located exclusively at the carboxyl group (Table 6).

Table 6. Distribution of radioactivity in orsellinic acid derived from formyl labelled orcylaldehyde (0.3 % incorporation of label).

Material	Number of carbon atoms	Specific activity *	Total activity	Relative total activity
Orsellinic acid	8	9 300	74 400	1.00
Orsellinic acid, carboxyl group	1	76 900	76 900	1.03
Orcinol	7	240	1 680	0.02

* counts per min and g $BaCO_3$

It may thus be concluded that *A. flavipes* is capable of oxidizing a nuclear formyl group to a carboxyl group, which might indicate that the enzyme system responsible for the reductive formation of the carboxyl derived formyl group (C-7) of flavipin is reversible. Anyhow, the results obtained with formyl labelled orcylaldehyde are, obviously, consistent with the pathway shown in Fig. 1; the latter precursor may have been incorporated into flavipin *via* orsellinic acid. Similarly, it is possible that the observed conversion of 2,4-dihydroxy-5,6-dimethylbenzaldehyde into flavipin involved the intermediate formation of 2,4-dihydroxy-5,6-dimethylbenzoic acid. On the other hand, it seems to be equally likely that 2,4-dihydroxy-5,6-dimethylbenzaldehyde functions as a more immediate precursor of flavipin than the corresponding phenolic acid; both the possibilities are in consistence with the pathway outlined in Fig. 1. The complete elucidation of the sequence of reactions, by which the aromatic precursors tested are converted into flavipin, appears to require more detailed enzymatic studies on the cell-free level. Such experiments are in progress.

EXPERIMENTAL

Culture conditions. Aspergillus flavipes (Bainer and Sartory) Thom and Church, L.S.H.T.M. S.M. 884, obtained from the Commonwealth Mycological Institute, Kew, Surrey, England, was used throughout this work. The mould was generally grown as submerged cultures in 500 ml Erlenmeyer flasks holding 150 ml portions of a Raulin-Thom medium according to Raistrick and Rudman,[1] on a rotary shaker (300 rpm) at 25°. For larger-scale isolation of the different metabolic products the mould was cultivated in aerated (2 l/min) fermenters (Model FS-307, New Brunswick Scientific Co., New Brunswick, N. J.) operated at a working volume of 5 l of medium. Agitation was at 150 rpm, and fermentations were carried out at 20° for 10 days. One experiment (the one in which $^{14}CH_3$-L-methionine was incorporated into 2,4-dihydroxy-5,6-dimethylbenzoic acid) involved shaken-flask cultures grown on a Czapek-Dox medium, where the phenolic

acids were found to be formed in almost the normal yields, while the rate of flavipin production was negligible (*cf.* Ref. 1).

Isolation and identification of phenolic acids. The filtered medium from a large number of 10 days old submerged cultures of *A. flavipes* was acidified by the addition of conc. hydrochloric acid (30 ml/l filtrate) and extracted with ether. The concentrated ethereal extracts were chromatographed on Whatman No. 3 MM paper with propanol-butanol-2 M ammonium hydroxide (6:1:3 by vol.) as the solvent, in which system flavipin decomposes to give a dark-brown material that remains on the start-line. For detection of the phenolic acids a strip of the air-dried chromatograms was sprayed with a 0.2 % alcoholic solution of ferric chloride, which gave a blue-violet colour with orsellinic acid (R_F 0.58) and 2,4-dihydroxy-5,6-dimethylbenzoic acid (R_F 0.69). The corresponding zones were cut out of the preparative paper chromatograms, and each phenolic acid was eluted with acetone and rechromatographed on Whatman No. 1 paper in the solvent system B of Reio [4] (orsellinic acid, R_F 0.42; 2,4-dihydroxy-5,6-dimethylbenzoic acid, R_F 0.60). The acids were then eluted with acetone and (after removal of the solvent) stored until sufficient amounts had been collected to permit a recrystallization from water to be made.

In this way orsellinic aicd was obtained as colourless needles (0.4 – 0.8 mg/l culture filtrate) of m.p. 176 – 177° (decomp.). The mixed m.p. with an authentic sample (m.p. 178°) was 178°. The behaviour on paper chromatography in the solvent systems A – F of Reio [4] and the ultraviolet absorption spectrum (bathochromic shift with alkali in ethanolic solution [3] and with aluminium trichloride in methanolic solution [12]) were identical with those of authentic material. A portion (8 mg) of the orsellinic acid was dissolved in 1 M sodium hydroxide (1 ml) and boiled in an atmosphere of nitrogen for 15 min. The product was acidified, extracted with ether and sublimed at 130° in a vacuum (1 mm Hg) to give orcinol (2.5 mg) as colourless crystals, m.p. 107°, not depressed on admixture with authentic sample (m.p. 108°). The ultraviolet absorption spectrum (bathochromic shift with alkali) and the paper chromatographic behaviour in the solvent systems mentioned above were identical with those of authentic material.

2,4-Dihydroxy-5,6-dimethylbenzoic acid was obtained as slightly yellowish needles (0.3 – 0.5 mg/l culture filtrate), m.p. 158 – 160° (decomp.); the mixed m.p. with an authentic sample (m.p. 162°) prepared according to the method of Robertson and Whalley[13] was 161 – 162°. The chromatographic behaviour and the ultraviolet absorption spectrum (bathochromic shift with alkali and with aluminium trichloride) were identical with those of authentic sample. Treatment with boiling sodium hydroxide yielded a decarboxylation product that was chromatographically and spectrophotometrically identical with authentic 1,3-dihydroxy-4,5-dimethylbenzene, prepared according to the method of Strating and Backer.[14] The latter phenol and orcinol were detected on the chromatograms by spraying the papers with a freshly prepared 0.1 % solution of 4-benzoylamino-2,5-dimethoxyaniline diazotate in acetone, followed by exposure to ammonia vapour to promote the coupling reaction; with this reagent the phenols appeared as intensely coloured red spots.

The amounts of phenolic acids present in the culture medium of *A. flavipes* (Table 1) were determined spectrophotometrically at 260 mμ after ether elution of the acids from the preparative paper chromatograms. The concentration of flavipin was estimated from measurements of the extinction at 260 mμ of ethereal extracts of the culture filtrates.

Incorporation of $^{14}CH_3$*-L-methionine into 2,4-dihydroxy-5,6-dimethylbenzoic acid.* $^{14}CH_3$-L-methionine (70 μC) was added to the medium of a 2 days old shaken-flask culture of *A. flavipes* grown on Czapek-Dox medium. Three days later 2,4-dihydroxy-5,6-dimethylbenzoic acid was isolated from the culture as described above. The radioactive 2,4-dihydroxy-5,6-dimethylbenzoic acid recovered from the preparative paper chromatogram (1.0 % incorporation of label) was diluted with carrier 2,4-dihydroxy-5,6-dimethylbenzoic acid (50 mg) and the whole was recrystallized from water to constant specific activity; the total activity of the phenolic acid (and of other compounds in the chemical degradations performed in the present work) was determined by the wet combustion technique of van Slyke and Folch.[15] 2,4-Dihydroxy-5,6-dimethylbenzoic acid was then degraded as shown in Fig. 2. The Kuhn-Roth oxidation was carried out as described by Eisenbraun *et al*;[16] a stream of nitrogen gas was passed through the reaction vessel and the evolved carbon dioxide was collected as barium carbonate. Acetic acid was recovered by steam distillation, the fractions being titrated with dilute sodium hydroxide to pH 8.7. The sodium acetate recovered on evaporation was degraded by the Schmidt reaction,

as described by Phares.[17] All determinations of radioactivity in the present work were made in a liquid scintillation counter on barium carbonate samples suspended in 10 ml of 0.5 % diphenyloxazole in toluene with the aid of 400 mg of Cab-O-Sil gel. The results of the degradation of 2,4-dihydroxy-5,6-dimethylbenzoic acid derived from $^{14}CH_3$-L-methionine are listed in Table 2; the numbering system used to refer to individual carbon atoms of the compound is shown in Fig. 2.

Incorporation of orcyladehyde into orsellinic acid. A 5 days old culture of *A. flavipes* was supplied with formyl labelled orcylaldehyde (6.2 mg) dissolved in the minimal volume of ethanol. Growth was continued for a further 2 h, when the culture was filtered and orsellinic isolated as described above. The radioactive orsellinic acid recovered from the preparative paper chromatogram (0.3 % incorporation of label) was diluted with carrier orsellinic acid and the whole was recrystallized several times from water; the specific activity remained constant after the third recrystallization. Orsellinic acid was then degraded as shown in Fig. 2. A mixture of the labelled orsellinic acid (43 mg) and 2 M hydrochloric acid (5 ml) was refluxed for 30 min in a stream of nitrogen gas. The carbon dioxide evolved was collected as barium carbonate. The residual orcinol was extracted with ether, dried and sublimed (130°; 1 mm Hg), giving colourless crystals (16 mg), m.p. 106°. The activity of the orcinol was determined by total combustion. The results of these degradations are given in Table 6.

Incorporation of aromatic precursors into flavipin. Each precursor (5—10 mg; 1—3 μC) was added to a 5 days old shaken-flask culture of *A. flavipes*, and after a further 24 h of growth the cultures were acidified by the addition of conc. hydrochloric acid (1 ml) and flavipin isolated by extraction with ether.

The radioactive flavipin obtained from 2,4-dihydroxy-5,6-dimethylbenzoic acid, ^{14}C-labelled at the methyl group in position 5, was diluted with carrier flavipin (55 mg) and the whole was recrystallized three times from acetone. The labelled flavipin was then submitted to Kuhn-Roth oxidation, and the acetic acid obtained was degraded by the Schmidt reaction. The degradation scheme and the numbering system used to refer to individual carbon atoms of flavipin are shown in Fig. 3. The degradation reactions were carried out as described above for 2,4-dihydroxy-5,6-dimethylbenzoic acid. The results obtained are given in Table 3.

The radioactive flavipin derived from carboxyl labelled orsellinic acid and 2,4-dihydroxy-5,6-dimethylbenzoic acid, as well as from formyl labelled orcylaldehyde and 2,4-dihydroxy-5,6-dimethylbenzaldehyde, was diluted with carrier flavipin (40 mg) and the whole was dissolved in acetone (10 ml). Flavipin was then converted into 3,4,5-trimethoxy-6-methylphthalic anhydride as described by Raistrick and Rudman.[1] Anhydrous potassium carbonate (0.6 g) and dimethyl sulphate (0.4 ml) were added to the acetone solution, and the mixture was refluxed for 1 h, when the initial orange colour had changed to pale yellow. The mixture was filtered, and the acetone and residual dimethyl sulphate were removed by evaporation in vacuum. The residual crude oily methyl ether of flavipin was refluxed for 30 min with aqueous sodium hydroxide (3 ml of 40 %) to yield 4,5,6-trimethoxy-7-methylphthalide. The cooled alkaline solution was extracted with ether to remove unchanged starting material, and was acidified to Congo red with hydrochloric acid and kept overnight at 4°. The colourless crystals (about 30 mg) which separated were collected, washed with water and dried. They were mixed with carrier 4,5,6-trimethoxy-7-methylphthalide (prepared as described by Raistrick and Rudman [1]) and the whole was refluxed for 10 min with 0.03 M sodium hydroxide (25 ml). Potassium permanganate (0.4—0.5 g in 10 ml of water) was added to the cooled solution, which was kept at room temperature for 24 h. The residual permanganate was destroyed by the addition of methanol and manganese dioxide was separated by filtration. The filtrate was strongly acidified with conc. hydrochloric acid, saturated with sodium chloride, and thoroughly extracted with ethyl acetate. Removal of the solvent from the extract gave a colourless product (50—70 mg) which was purified by crystallization from light petroleum (b.p. 80—100°) and sublimation (120°; 1 mm Hg), giving 3,4,5-trimethoxy-6-methylphthalic anhydride as colourless needles, m.p. 109—112°.

The radioactive 3,4,5-trimethoxy-6-methylphthalic anhydride obtained in this way was degraded as indicated in Fig. 3, which also shows the numbering system used. The total activity of the compound was determined by the wet combustion technique of van Slyke and Folch,[15] and the activity of C-5 + C-9 was obtained by total combustion of the acetic acid recovered after Kuhn-Roth oxidation (which was carried out as described

above); the latter carbon atoms were isolated since it was suspected that the carboxyl labelled precursors could be decarboxylated by the mould to give radioactive carbon dioxide, which might be incorporated into the methyl group (C-3) of flavipin *via* the C_1-pool. In order to determine the activity of C-7 + C-8 3,4,5-trimethoxy-6-methylphthalic anhydride was refluxed for 30 min with copper chromite (100 mg), quinoline (10 ml) and water (0.1 ml). The carbon dioxide obtained was collected as barium carbonate, and the amounts obtained corresponded excellently with those calculated for a quantitative decarboxylation of the two carboxyl groups of 3,4,5-trimethoxy-6-methylphthalic acid. The results of the degradation of flavipin, biosynthesized in the presence of carboxyl labelled orsellinic acid, are listed in Table 4. Similar results were obtained on degradation of flavipin derived from carboxyl labelled 2,4-dihydroxy-5,6-dimethylbenzoic acid and the two formyl labelled aldehydes tested; these radioactivity measurement data are not given in detail, but are summarized in Table 5.

Preparation of ^{14}C-labelled precursors. $^{14}CH_3$-L-methionine and $K^{14}CN$ were obtained from The Radiochemical Centre, Amersham, Buckinghamshire, England. The synthesis of carboxyl labelled 2,4-dihydroxy-5,6-dimethylbenzoic acid started with anhydrous orcinol, which was converted into orcylaldehyde by means of the Gatterman reaction.[18] Clemmensen reduction of orcylaldehyde yielded 1,3-dihydroxy-4,5-dimethylbenzene,[19] into which isotope was introduced by the Gatterman reaction, using equimolar amounts of $Zn(^{14}CN)_2$ prepared from 1 mC of $K^{14}CN$ by the method of Adams and Levine.[18] The synthesis, which was carried out in the 1 g scale, yielded 0.83 g of formyl labelled 2,4-dihydroxy-5,6-dimethylbenzaldehyde (0.5 mC). The latter compound (0.5 g) was then converted into carboxyl labelled 2,4-dihydroxy-5,6-dimethylbenzoic acid (0.21 g) as described by Robertson and Whalley.[13]

Formyl labelled orcylaldehyde (0.4 μC/mg) was prepared by the Gatterman reaction from anhydrous orcinol (2 g) and $Zn(^{14}CN)_2$.[18] One part of the radioactive orcylaldehyde was converted into carboxyl labelled orsellinic acid (0.4 μC/mg) according to the method of Hoesch;[20] another part was submitted to Clemmensen reduction to give 1,3-dihydroxy-4,5-dimethylbenzene, ^{14}C-labelled at the methyl group in position 4. The latter compound was then converted into 2,4-dihydroxy-5,6-dimethylbenzoic acid, ^{14}C-labelled at the methyl group in position 5, by the method of Robertson and Whalley.[13]

REFERENCES

1. Raistrick, H. and Rudman, P. *Biochem. J.* **63** (1956) 395.
2. Pettersson, G. *Acta Chem. Scand.* **19** (1965) 35.
3. Mosbach, K. *Z. Naturforsch.* **14b** (1959) 69.
4. Reio, L. *J. Chromatog.* **1** (1958) 338.
5. Mosbach, K. *Acta Chem. Scand.* **14** (1960) 457.
6. Bentley, R. and Keil, J. G. *Proc. Chem. Soc.* **1961** 111.
7. Birkinshaw, J. H. and Gowlland, A. *Biochem. J.* **84** (1962) 342.
8. Pettersson, G. *Acta Chem. Scand.* **19** (1965) 1016.
9. Pettersson, G. *Acta Chem. Scand.* **18** (1964) 1202.
10. Curtis, R. F., Harries, P. C., Hassall, C. H. and Levi, J. D. *Biochem. J.* **90** (1964) 43.
11. Pettersson, G. *Acta Chem. Scand.* **19** (1965) 414.
12. Nakagawa, Y., Shetlar, M. R. and Wender, S. H. *Analyt. Biochem.* **7** (1964) 374.
13. Robertson, A. and Whalley, W. B. *J. Chem. Soc.* **1949** 3038.
14. Strating, J. and Backer, H. J. *Rec. Trav. Chim.* **62** (1943) 57.
15. van Slyke, D. D. and Folch, J. *J. Biol. Chem.* **136** (1940) 509.
16. Eisenbraun, E. J., McElvain, S. M. and Aycock, B. F. *J. Am. Chem. Soc.* **76** (1954) 607.
17. Phares, E. F. *Arch. Biochem. Biophys.* **33** (1951) 173.
18. Adams, R. and Levine, I. *J. Am. Chem. Soc.* **45** (1923) 2373.
19. Clemmensen, E. *Ber.* **47** (1914) 51.
20. Hoesch, K. *Ber.* **46** (1913) 886.

Received June 9, 1965.