Biosynthesis of Chlorflavonin in *Aspergillus candidus*. ^{13}C- and ^{14}C-Labelling Evidence for a New Route to the Flavonoid Structure

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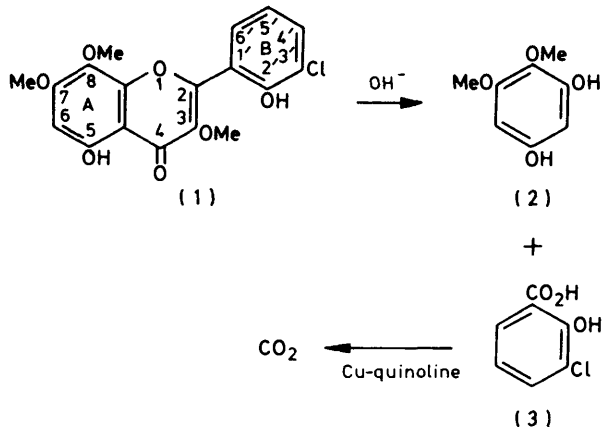
Aspergillus candidus growing in a glucose–leucine–salts medium synthesized chlorflavonin (1) from isotopically labelled phenylalanine, cinnamate, benzoate, or acetate. Radioactivity from [β-^{14}C]cinnamic acid and [α-^{14}C]-benzoic acid was incorporated less efficiently than that from L-[β-^{14}C]phenylalanine but decarboxylation of 3-chlorosalicylic acid (3) formed by alkaline degradation of the chlorflavonin samples located all of the radioactivity at C-2 in the flavonoid. 4,5-Dimethoxyresorcinol (2), obtained by alkaline degradation of chlorflavonin labelled from sodium [1-^{14}C]acetate and [α-^{14}C]cinnamic acid, accounted for only part of the radioactivity. The label from both precursors was distributed between ring A and one or more of the C-3, C-4, and C-3 methoxy-carbon atoms. ^{13}C N.m.r. spectrometry of chlorflavonin labelled from sodium [1-^{13}C]acetate showed ^{13}C incorporation into C-4, C-5, C-7, and C-8a. Sodium [1-$^{13}C_{0;1;1}$,2-$^{13}C_{1;0;1}$]acetate was incorporated intact into (C-3,C-4) and all adjacent pairs of ring-A carbon atoms. The results indicate a pathway of flavonoid biosynthesis differing from that of higher plants in that a C_6-C_1 precursor unit is condensed with four C_2 units. In the route proposed, the heterocyclic ring is formed before ring A is substituted at C-8 and while it is free to rotate at the enzyme surface.

CHLORFLAVONIN (1) is the only fungal metabolite for which a flavonoid structure has been firmly established.[1,2] It is produced *de novo* from glucose by *Aspergillus candidus*, and an investigation of its biosynthesis with ^{14}C-labelled compounds has identified phenylalanine as a precursor.[3] Chemical degradation of chlorflavonin labelled from L-[β-^{14}C]phenylalanine located all the radioactivity at C-2. Since the amino-acid was incorporated with relatively low isotopic dilution, it was assumed to be a specific precursor of the flavonoid carbon



(1) (2) (3)

SCHEME 1

skeleton. [*Me*-^{14}C]Methionine was also an efficient precursor, presumably serving as the source of the three methoxy-groups in the antibiotic.

Flavonoid secondary metabolites are ubiquitous in the plant kingdom and much is now known about the route by which they are biosynthesized.[4] Ring B and three carbon atoms of the heterocyclic ring are derived from a phenylpropanoid amino-acid, either phenylalanine or tyrosine, and ring A originates by head-to-tail condensation of three acetate units. The first step in the biosynthetic pathway is elimination of ammonia from the amino-acid to form cinnamic or *p*-coumaric acid, which is then activated to provide the chain initiator for polyketide formation with malonyl chain-extending units. To determine whether the biosynthesis of chlorflavonin in *A. candidus* follows a similar route, Marchelli and Vining [3] examined the incorporation of [1-^{14}C]acetate and [α-^{14}C]cinnamate. Both compounds yielded radioactive chlorflavonin but with extensive dilution of the isotope. DL-*o*-[α-^{14}C]tyrosine, a potential intermediate if ring B hydroxylation preceded deamination, was incorporated with similarly low efficiency while the corresponding deaminated compound, *o*-[α-^{14}C]coumaric acid, did not label the antibiotic. Although these results suggested that *A. candidus* might have a unique biosynthetic route to the flavonoid structure, they were incomplete since the location of the radioactivity that entered chlorflavonin from the poorly incorporated compounds was not determined. Moreover, the access of labelled substrates to the biosynthetic enzymes was uncertain. The timing of chlorflavonin synthesis during culture development was not known; substances introduced into cultures early in growth might have been extensively metabolized and unavailable as precursors. The permeability to cinnamic acids of the *A. candidus* cell membrane was not established and the use of complex nutrients in these experiments introduced uncertainty about the extent of precursor dilution by media constituents.

We have now developed a defined nutrient solution, consisting of D-glucose, L-leucine, and salts, in which *A. candidus* produces chlorflavonin. Several amino-acids supported higher yields than the complex nitrogen source, corn-steep liquor, used in previous work [1,3,5] and the relationship between growth, utilization of nutrient, and production of antibiotic in such cultures indicated that limitation of nitrogen enhanced production of chlorflavonin. The evidence that *A. candidus* can produce chlorflavonins in a simple medium devoid of

plant extractives is consistent with the biosynthetic labelling studies [3] which indicated that these compounds are true mould metabolites.

The extent to which sodium [1-^{14}C]acetate, L-[β-^{14}C]phenylalanine, [β-^{14}C]cinnamic acid, [α-^{14}C]benzoic acid, and [α-^{14}C]salicylic acid were incorporated into chlorflavonin is recorded in Table 1. Degradation of

TABLE 1

Specific incorporation of ^{14}C-labelled compounds into chlorflavonin [a]

Supplement	μCi mmol^{-1}	Chlorflavonin μCi mmol^{-1}	% [b]
L-[β-^{14}C]Phenylalanine	17.6	1.74	9.9
Sodium [1-^{14}C]acetate	53.1	1.34	2.5
[β-^{14}C]Cinnamic acid	39.3	0.941	2.3
[α-^{14}C]Benzoic acid	35.1	0.749	2.1
[α-^{14}C]Salicylic acid	41.8	0.027	0.06

[a] Labelled phenylalanine, sodium acetate, cinnamic acid, and benzoic acid were each added on the 4th day after inoculation to 0.5 l cultures at a broth concentration of 1 mmol l^{-1}. Salicylic acid was added on the 5th day to a 1 l culture at a broth concentration of 0.5 mM. [b] Specific incorporation = 100 × specific activity of product divided by specific activity of precursor.

TABLE 2

Distribution of radioactivity in degradation products of chlorflavonin [a]

Supplement	4,5-Dimethoxy-resorcinol	3-Chloro-salicylic acid (%)	Barium carbonate
L-[β-^{14}C]Phenylalanine		102	102
[α-^{14}C]Benzoic acid	0.13	97.6	96.3
[β-^{14}C]Cinnamic acid		103	103
[α-^{14}C]Cinnamic acid	48.2	3.7	
DL-*o*-[α-^{14}C]Tyrosine	54.0	1.74	
Sodium [1-^{14}C]acetate	66.5	1.35	

[a] Calculated from specific activities as a percentage of the specific activity of chlorflavonin.

chlorflavonin (Scheme 1) from cultures supplemented with L-[β-^{14}C]phenylalanine and [β-^{14}C]cinnamic acid (Table 2) showed all the radioactivity to be at C-2, as predicted if these compounds were incorporated with the ring and adjacent carbon atoms intact. Degradation of samples labelled as reported earlier [3] from [α-^{14}C]cinnamic acid or DL-*o*-[α-^{14}C]tyrosine showed the radioactivity to be distributed between ring A and the three unaccounted-for carbon atoms (C-3, C-4, and the methoxy-substituent) of the heterocyclic ring (Table 2). Thus the phenylpropanoid skeleton was not incorporated intact. The evidence that these two substrates are taken up and metabolized by *A. candidus* is supported by measurements of the radioactivity remaining in cultures when they were harvested. Only 5.2 and 10.6% of the amounts administered as [α-^{14}C]cinnamic acid and DL-*o*-[α-^{14}C]tyrosine, respectively, were accounted for in the culture filtrate and mycelium extracts. The recoveries were similar to those from comparable feedings of [1-^{14}C]acetate and suggest that much of the label had been oxidized *via* the Krebs Cycle to carbon dioxide.

Degradation of chlorflavonin labelled from sodium [1-^{14}C]acetate established that two-thirds of the radioactivity was associated with 4,5-dimethoxyresorcinol (2). Since the 3-chlorosalicylic acid (3) fragment contained only 1.35%, the remaining radioactivity must have been incorporated with considerable specificity in the unaccounted-for carbon atoms of chlorflavonin. The result is consistent with four, rather than three molecules of the precursor having been incorporated, with the fourth located at C-3 and C-4 in the product. The proportion of radioactivity found in ring A was less than the 75% predicted for uniform incorporation of four two-carbon units. In the experiments where [α-^{14}C]cinnamic acid and DL-*o*-[α-^{14}C]tyrosine were used, the amount of radioactivity recovered in 4,5-dimethoxyresorcinol was even lower with approximately 50% remaining unaccounted for. This might be attributed to preferential labelling of a (C-3,C-4) unit and, therefore, considered to be evidence for the presence at this position of a chain-initiating unit introduced from acetyl coenzyme A without conversion into malonyl coenzyme A. The expectation that the labelled cinnamate and tyrosine would both give [^{14}C]acetyl coenzyme A directly and thus might, in some manner, selectively enrich the pool of this intermediate to a greater extent than achieved by exogenous sodium [1-^{14}C]acetate, lends some support to this possibility. However, the 4,5-dimethoxyresorcinol recovered from alkaline degradation of chlorflavonin samples could not be rigorously purified to constant specific activity. In view of the failure of sodium [1,2-^{13}C]acetate to label the (C-3,C-4) unit to a greater extent than the carbons of ring A (see later) the ^{14}C distributions are not considered to provide clear evidence for participation of a separate eight-carbon polyketide chain.

It is apparent from the specific isotope incorporation from [α-^{14}C]benzoic acid into C-2 of chlorflavonin that phenylpropanoid precursors such as phenylalanine and cinnamic acid can enter the biosynthetic pathway after being first degraded to a C_6–C_1 intermediate. This intermediate is probably benzoyl coenzyme A, serving as a chain-initiating unit for the polyketide intermediate. If the possibility were entertained that C-3,C-4 in chlorflavonin represents a polyketide starter unit and thus that the polyketide is a discrete preformed intermediate, the C_6–C_1 component could be at the aldehyde equally as well as at the coenzyme A ester level of oxidation. The low efficiency with which benzoic acid is incorporated might then reflect a requirement for prior reduction, although this could also be attributed to rate-limiting uptake and activation in competition with endogenously formed activated ester. Since little radioactivity was incorporated from [α-^{14}C]salicylic acid into chlorflavonin, hydroxylation of the ring B precursor probably occurs after its condensation with C_2 units.

The evidence from radioisotopic tracer experiments that the chlorflavonin carbon skeleton is assembled from a C_6–C_1 and four C_2 precursors was supported by ^{13}C n.m.r. analyses of samples from cultures supplemented with sodium [1-^{13}C]acetate and with a mixture of sodium-[1-$^{13}C_{0;1;1}$,2-$^{13}C_{1;0;1}$]acetate (hereafter called sodium [1,2-^{13}C]acetate) and sodium [1-^{14}C]acetate. Resonances in

the ^{13}C n.m.r. spectrum of chloroflavonin were assigned from chemical-shift trends and known substituent effects for benzene rings,[6] ^{13}C–H and ^{13}C–^{13}C spin–spin coupling-information (Table 3), and the use of vitexin[7] and quercetin[8] as well as flavone[9] and its 7- and 8-methoxy-derivatives[10] as model compounds.

TABLE 3

^{13}C N.m.r. data for chlorflavonin labelled by [1,2-^{13}C]acetate

Carbon atoms	δ_C(Me_4Si)/ p.p.m.[a]	$^nJ_{CH}$/ Hz[b]	$^1J_{CC}$/ Hz[b]	E (%)[c]
C-3	139.44 (q)	3.8	63.3	0.70
C-4	178.63 (d)	1.0		
C-8a	148.75 (d)	0.8	64.2	0.37
C-4a	105.21 (dd)	5.3, 5.9	63.0	0.34
C-5	156.55 (dd)	4.7, 4.7	72.2	0.33
C-6	95.77 (dd)	162.8, 6.6	67.8	0.31
C-7	158.23 (dq)	3.7, 3.7	79.8	0.34
C-8	128.42 (dq)	7.0, 3.9	84.2	0.38
C-8a				

[a] δ_C in p.p.m. relative to ($^{13}CH_3$)$_4$Si, solvent (C^2H_3)$_2$SO. Multiplicity: d = doublet, q = quartet, m = multiplet. For carbons not labelled by sodium [1,2-^{13}C]acetate, δ_C and $^nJ_{CH}$ (Hz, $n \geqslant 1$) are: C-2 δ_C 155.6 (d), ^nJ 3.8; C-1′ δ_C 119.88 (m); C-2′ δ_C 150.97 (ddd), ^nJ 8.6, 7.1, 1.4; C-3′ δ_C 121.66 (ddd), ^nJ 11.2, 3.6, 1.6; C-4′ δ_C 132.15 (dd), ^nJ 166.7, 8.9; C-5′ δ_C 120.14 (d), ^nJ 167.2; C-6′ ^nJ 163.86, 9.0; methoxy-carbon atoms (not assigned individually) δ_C 60.91 (q), ^1J 144.7, δ_C 60.11 (q), ^1J 145.4; δ_C 56.51, ^1J 145.8. [b] Digitization error ±0.3 Hz, but estimated error reduced to *ca.* ±0.1 Hz by interpolation between data points. [c] ^{13}C enrichment calculated using $E(\%) = 1.1\ I_{d(1)}/(I_C - fI_{d(2)})$ (for definitions and derivation, see J. L. C. Wright, L. C. Vining, A. G. McInnes, D. G. Smith, and J. A. Walter, *Can. J. Biochem.*, 1977, **55**, 678; $I_m = 0$ for this case) from integrals of the singlet and doublet components of resonances. The value shown is the average for each ^{13}C–^{13}C coupled pair.

Identification of two of the ^{13}C resonances was trivial as the carbonyl carbon atom, C-4, is expected and observed to occur at lowest field, and C-6 is the most shielded of the aromatic methine carbon atoms because of the adjacent hydroxy- and methoxy-groups. Matching ^{13}C–^{13}C couplings derived from the sample of chlorflavonin enriched by [1,2-^{13}C]acetate thus permitted unequivocal assignment of C-3 and the remaining aromatic carbon atoms in ring A. The ^{13}C–H couplings obtained from a high resolution spectrum of the natural ^{13}C-abundance material confirmed the assignments. Thus C-7 and C-8 are coupled to methoxy-hydrogen atoms and to different extents with H-6, whereas C-3 is coupled to methoxy-hydrogen atoms only. Also, C-4a and C-5 are both typically coupled to H-6 and the intramolecular hydrogen-bonded hydroxy-hydrogen atom but C-8 is coupled only to H-6. The signal at δ_C 155.6 could be assigned to C-2 as the adjacent methoxy-group shields it relative to the corresponding carbon atom in flavone (δ_C 163.0) and it is coupled to H-6′.

Signal assignments for carbons in ring B were also straightforward. The chemical shifts could be rationalized from known substituent effects and each carbon atom was characteristically coupled as follows: C-5′ to H-5 only, C-4′ to H-4′ and H-6′, C-6′ to H-6′ and H-4′, C-3′ to H-5′, H-6′ and H′-4 or the hydroxy-hydrogen, C-2′ to H-4′, H-6′ and H-5′ or the hydroxy-hydrogen, and C-1′ to H-5′, H-4′ and the hydroxy-hydrogen. The methoxy-carbon resonances were not assigned individually.

Sodium [1-^{13}C]acetate enriched the following positions (errors ± 0.2%): C-4, 0.4%; C-5, 0.5%; C-7, 0.4%, and C-8a, 0.3% ^{13}C. The labelled carbon atoms are thus at the positions predicted if C-3 and C-4 as well as ring A of chlorflavonin are formed by a head-to-tail condensation of four two-carbon units, the methyl group of the first unit being attached to a C_6–C_1 moiety.

Experiments in which a mixture of sodium [1,2-^{13}C]- and [1-^{14}C]-acetate was administered so that 2 l cultures received a total of 10 mmol of 90% ^{13}C-enriched acetate added in 3 portions on the 4th, 5th, and 6th days of growth gave an overall ^{14}C specific incorporation of 9.66% in chlorflavonin. The ^{13}C n.m.r. spectrum of this product was complex and indicated a high rate of incorporation over short intervals, with subsequent dilution by material containing ^{13}C at natural abundance. It is apparent that the labelled acetate supplement is very rapidly taken up and metabolized by *A. candidus* under the growth conditions used. Presumably also the pool of acetyl coenzyme A is small and is, for a brief time, composed predominantly of the labelled material.

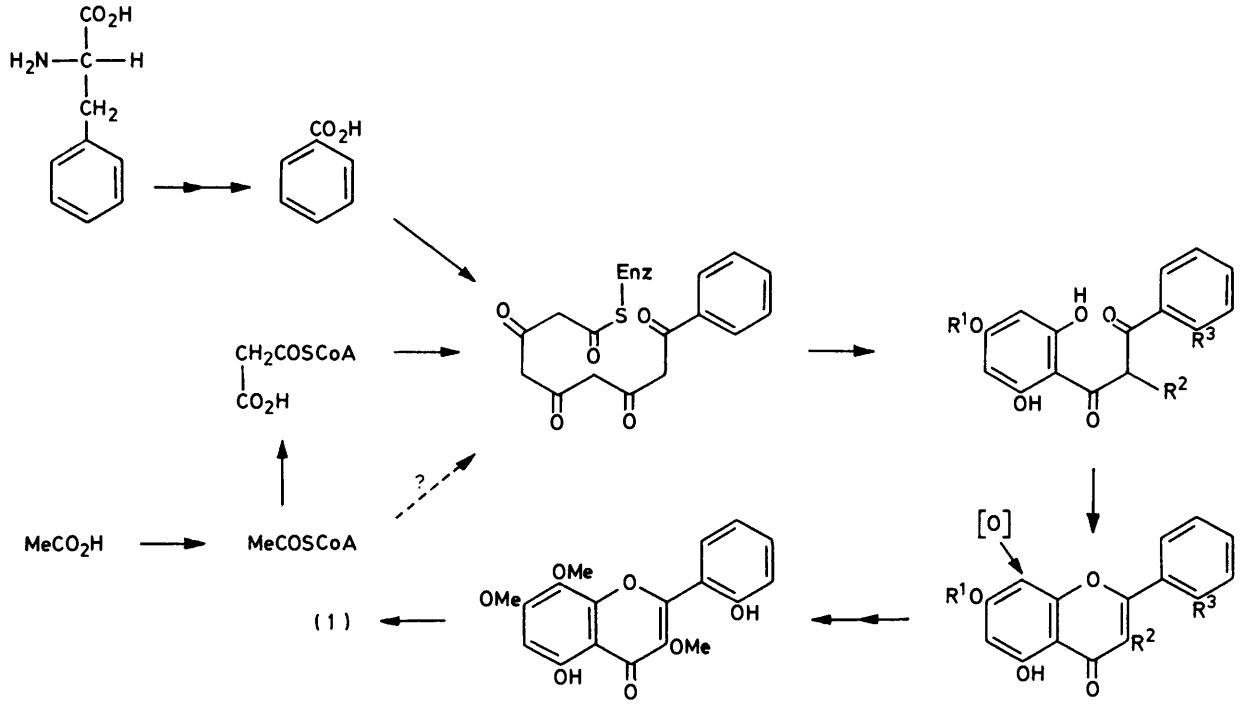
To reduce the extent of coupling between adjacent ^{13}C–^{13}C pairs and at the same time to retain an adequate degree of overall ^{13}C enrichment, sodium [1,2-^{13}C]acetate was diluted with sodium [1-^{14}C]acetate and natural abundance acetate to 22.5% ^{13}C-enrichment; a total of 20 mmol was fed in equal portions to a 2 l culture at intervals of 8 h between the 4th and the 10th days of growth. The course of chlorflavonin and dechlorochlorflavonin production over this interval indicated that synthesis of the metabolites was unaffected by the supplement and also demonstrated the precursor–product relationship for these two metabolites expected from previous results.[3] The overall specific incorporation of ^{14}C into chlorflavonin was 18.3%.

The ^{13}C n.m.r. spectrum of chlorflavonin labelled from sodium [1,2-^{13}C]acetate in the second experiment showed satellite doublets, due to intact incorporation of ^{13}C–^{13}C pairs, about the singlet resonances (arising from isolated ^{13}C atoms) of all carbon atoms except C-1′—6′ and the three methoxy-carbon atoms (Table 3). The results confirm previous evidence for the polyketide derivation of ring A and the adjacent two carbon atoms of the heterocyclic ring. No preferential enrichment of the (C-3,C-4) pair occurred. Since the conditions under which *A. candidus* was grown seem conducive to rapid labelling of the acetate pool by exogenous sodium acetate, this provides strong evidence that the indication from radiotracer experiments of polyketide chain initiation at (C-3,C-4) is spurious.

The resonances of the ring-A carbon atoms showed two sets of satellites of equal intensity but different $^1J_{CC}$ values, indicating an equal probability of each carbon atom coupling to either neighbouring carbon atom. As it is biosynthetically impossible for any carbon atom to originate from more than one ^{13}C–^{13}C unit, the distribu-

tion of the enrichment in ring A must have arisen by rotation about a two-fold axis of symmetry existing in this ring during the biosynthetic process. This rotation interchanges carbon atoms on opposite sides of the axis and can occur only if the intermediate is not rigidly bound to an enzyme surface. Since it implicates a symmetrically substituted *o*-dihydroxybenzoyl derivative in the pathway to chlorflavonin, hydroxylation and methylation at C-8 must follow the ring closures that give both ring A and the heterocyclic system. In including an *o*-dihydroxybenzoyl intermediate capable of free rotation, the pathway to chlorflavonin resembles that of revenelin [11] and the secalonic acids [12] which contain a xanthone ring system.

Scheme 2 presents a plausible biosynthetic sequence that accommodates all the information now available on the formation of chlorflavonin. The pathway is distinctly different from that by which higher plants synthesize the flavonoid skeleton and appears to represent a successful alternative, independently acquired by one of the lower fungi. In this respect it differs from



SCHEME 2 R^1 = H or Me; R^2 = H, OH, or OMe; R^3 = H or OH

those leading to gibberellins [13] and ergot alkaloids [14] which are similar enough in plants and fungi to suggest that a transfer of genetic information may have taken place.

EXPERIMENTAL

Cultures.—Procedures for maintaining *Aspergillus candidus* CMI 16046 and for preparing a vegative inoculum have been described.[3] To obtain a more uniform inoculum for media studies the initial culture after 4 days incubation was homogenized in a Waring blender and a portion (2 ml) was transferred to fresh inoculum medium (50 ml). The secondary culture was grown under the same conditions as the first but was used after incubation for 2 days.

Cultures for production of chlorflavonin were grown in 250 ml Erlenmeyer flasks containing the medium (50 ml) and the secondary inoculum (2 ml). They were incubated at 26 °C on a platform rotating at 220 revolutions per minute with an eccentricity of 3.8 cm. The complex medium used in this and earlier experiments [3] contained 5% D-glucose, 5% (v/v) corn-steep liquor, and 0.2% calcium carbonate, adjusted to pH 6.6 before autoclaving. The defined medium contained (per l): D-glucose (54.5 g), L-leucine (6.56 g), KH_2PO_4 (0.45 g), $MgSO_4 \cdot 7H_2O$ (0.45 g), a salt solution (9 ml), and a trace mineral solution (4.5 ml). The salt solution was a mixture of 1% NaCl and 1% $CaCl_2$; the trace mineral solution contained (mg l^{-1}) $CuSO_4 \cdot 5H_2O$ (39.3), H_3BO_3 (5.7), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (3.7), $MnSO_4 \cdot 4H_2O$ (6.1), and $ZnSO_4 \cdot 7H_2O$ (880).

Chemicals and Isotopes.—Chloroflavonin used as a carrier in chemical degradations was obtained from Dr. A. E. Bird, Beechams Research Laboratories, Betchworth. 3-Chlorosalicylic acid was prepared from 3-chloro-2-nitrobenzoic acid. Reduction with tin and hydrochloric acid gave 3-chloroanthranilic acid, m.p. 187 °C (72%), which was diazotized in 20% H_2SO_4 and added to boiling concentrated copper sulphate solution. 3-Chlorosalicylic acid was extracted from the reaction mixture with diethyl ether and recrystallized from methanol as needles, m.p. 182 °C (lit.,[15] 180; lit.,[16] 180—182 °C), in an overall yield of 52%. Bactopeptone, yeast extract, soytone, and casamino acid were products of Difco Laboratories, Detroit, Michigan; corn-steep liquor of the Canadian Starch Company, Cardinal, Ontario, and L-leucine of Sigma Chemical Company, St. Louis, Missouri. The silicic acid used for chromatography was purchased from J. T. Baker Chemical Company, Phillipsburg, New Jersey; Sephadex G-25 (fine) from Pharmacia (Canada) Ltd., Montreal, Quebec; silica gel

F 254 for thin-layer chromatography from E. Merck, Darmstadt, Germany; and Fast Bordeaux BD from Chemical Developments of Canada Ltd.

Sodium [1-^{14}C]acetate and [α-^{14}C]benzoic acid were obtained from New England Nuclear Corporation, Boston, Massachusetts; L-[3-^{14}C]phenylalanine and [3-^{14}C]cinnamic acid were gifts from the late Dr. A. C. Neish of the National Research Council of Canada; [α-^{14}C]salicylic acid, sodium [1-^{13}C]acetate, and sodium [1,2-^{13}C]acetate were supplied by Merck, Sharp, and Dohme, Point Claire, Quebec. The correct I.U.P.A.C. designation for the last-named compound is sodium [1-$^{13}C_{0;1;1}$,2-$^{13}C_{1;0;1}$]acetate.

Biosynthetic Experiments.—Cultures were grown in the defined medium. Each radioactive supplement was distributed in 10 flasks (500-ml culture volume); for experiments with ^{13}C-labelled acetate 2 l culture volumes were used. Sodium acetate and phenylalanine solutions were sterilized by autoclaving; cinnamic, benzoic, and salicylic acids were neutralized with an equivalent of sodium hydroxide and sterilized by filtration. Times at which supplements were added and amounts of isotope used are noted with each experiment, along with the period for which the cultures were incubated.

Cultures were harvested by filtering the acidified broth and extracting the filtrate and mycelium separately. The mycelium was leached with boiling acetone and the solution concentrated. The filtrate was extracted with chloroform and the extract combined with the concentrate from the mycelium. The chloroform solution was washed with water, evaporated, and dried thoroughly. In the radiotracer experiments the residue was leached with five 5 ml portions of the upper phase of a light petroleum (b.p. 60—80 °C)–benzene–acetic acid–water (5 : 3 : 6 : 2 v/v) mixture and the leachates applied successively to a column (1.6 × 90 cm) of cross-linked dextran (Sephadex G-25, fine) equilibrated with the same solvent mixture.[17] The column was developed with organic phase while 10 ml fractions were collected. Chlorflavonin was collected as a yellow zone with peak intensity near fraction 25. In larger scale experiments with ^{13}C-labelled acetate the dried extract was dissolved in chloroform and applied to a column (2.5 × 30 cm) of silicic acid. Chloroflavonin was eluted with chloroform–ethyl acetate (9 : 1 v/v). It was purified by crystallization from methanol and sublimation *in vacuo* at 140 °C.

Estimation of Chlorflavonins.—In experiments where the effects of medium composition or culture age was determined, analyses for chlorflavonins measured the total amount of chlorflavonin and dechlorochlorflavonin and were performed on duplicate cultures. The metabolites were extracted by the procedure described above for isolating chlorflavonin and were separated from interfering products by chromatographing the crude extract on a short (1.6 × 5 cm) column of silicic acid. Samples were applied in benzene–chloroform (2 : 1 v/v); the yellow zone containing chlorflavonins eluted with chloroform–ethyl acetate (9 : 1 v/v) was collected and its absorbance at 348 nm was measured.

To measure separately the amount of chlorflavonin and dechlorochlorflavonin in cultures the extract prepared as above was dissolved in acetone and a measured portion was applied along the origin of a 5 × 20 cm chromatography plate spread with a thin layer of silica gel F 254. The chromatogram was developed with benzene–methanol (19 : 1 v/v). Chlorflavonin and dechlorochlorflavonin were located as yellow, fluorescence-quenching zones at R_F 0.50 and 0.43, respectively. The zones were scraped from the plate and eluted with ethanol. The concentrations of chlorflavonin and dechlorochlorflavonin were estimated from the absorbance at 348 nm.

The radiochemical purity of chlorflavonin was checked by t.l.c. on silica gel F 254 with chloroform–diethylamine (9 : 1 v/v) as solvent. Chlorflavonin (R_F 0.13) was located with a chromogenic spray reagent as well as by scanning for radioactivity.[3]

Alkaline Degradation of [^{14}C]*Chlorflavonin.*—Samples were diluted with carrier to 100 mg and heated under reflux with KOH (3 g) in water (7.5 ml) for 12 h. The reaction mixture was acidified and extracted with diethyl ether. 3-Chlorosalicylic acid, removed from the ether with 1% aqueous $NaHCO_3$ solution, was crystallized from aqueous methanol to constant m.p. (180 °C) and specific activity. The yield averaged 21 mg (46%). The non-acidic ether-extractable fraction from the reaction mixture was chromatographed on a thin layer (20 × 20 cm) of silica gel F 254 using chloroform–acetic acid–water (50 : 4 : 1 v/v) as the solvent system. Phenolic compounds were located by spraying both edges with Fast Bordeaux BD. The zone of 4,5-dimethoxyresorcinol, identified by the immediate orange-red colour produced in the marker regions, was scraped from the plate. The compound was immediately eluted with acetone; the eluate was evaporated under a stream of nitrogen and dried thoroughly. The residual gum (average yield 12 mg) crystallized from chloroform–hexane as plates, m.p. 90—92 °C.

Decarboxylation of 3-[^{14}C]*Chlorosalicylic Acid.*—Samples were diluted with carrier to approximately 30 mg and heated for 1 h with copper chromite (30 mg) in quinoline (11 ml) under reflux. Carbon dioxide was swept from the reaction through a trap containing 1M-aqueous NaOH (10 ml) with a stream of nitrogen. The carbonate formed was precipitated with 1M-aqueous $BaCl_2$ (3 ml) after adding 4M-aqueous NH_4Cl (1 ml).

Measurement of Radioactivity.—Chorflavonin and its degradation products were dissolved in ethanol and mixed with scintillation fluid containing 2,5-diphenyloxazole (4%) in toluene–ethanol (7 : 3 v/v). Barium carbonate was dispersed by sonic oscillation in toluene containing 2,5-diphenyloxazole (0.4%) and kept in suspension with the aid of a thixotropic gelling agent (Cab-O-Sil, Packard Instrument Co. Ltd., LaGrange, Illinois). Radioactivity was measured with a liquid-scintillation counter. Chromatograms were scanned for radioactivity with a gas-flow Geiger-Mueller detector.

^{13}C *N.m.r. Spectrometry.*—Spectra were recorded with a Varian XL-100/15 Fourier-transform spectrometer having a Varian 620L computer and Diablo disc accessory, under the following conditions: frequency 25.16 MHz, temperature 29 °C, ^{2}H internal lock to [$^2H_6$]DMSO, ^{1}H-decoupling frequency 100 MHz, $\gamma H_2/2\pi$ *ca.* 3 800 Hz, broadband irradiation by 0—180° phase modulation at 150 Hz. Other conditions follow in the order sample, concentration, sample tube diameter, solvent, mode of decoupling, spectral width SW, acquisition time AT, flip angle FA (90° pulse 44 μs), delay between acquisition times PD: chlorflavonin, natural abundance or labelled with [1-^{13}C]acetate, *ca.* 80 mg ml^{-1}, 5 mm, [$^2H_6$]DMSO with 20 mg ml^{-1} $Cr(acac)_3$ added, continuous broadband, SW 5 120 Hz, AT 0.8 s, FA 60°, PD zero; chlorflavonin, natural abundance, 30 mg ml^{-1}, 12 mm, [$^2H_6$]DMSO without $Cr(acac)_3$, high-resolution spectrum decoupler off during AT, on during PD, SW 5 120 Hz,

AT 3.2 s, FA 47°, PD 3.2 s; chlorflavonin labelled with [1,2-^{13}C]acetate, *ca.* 150 mg ml^{-1}, 5 mm, [$^{2}H_{6}$]DMSO without Cr(acac)$_3$, continuous broadband SW 5 120 Hz, AT 3.2 s, FA 35°, PD zero; same sample with 10 mg ml^{-1} Cr(acac)$_3$ added, decoupler on during AT, off during PD, SW 5 120 Hz, AT 1.6 s, FA 55°, PD 1.6 s.

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