THE BIOGENESIS OF COLCHICINE AND FUSARIC ACID

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Robert Durward Hill

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ABSTRACT

Radioactive colchicine was obtained when various C^{14}-labelled substrates were administered to Colchicum autumnale L. plants. Colchicine derived from cinnamate-3-C^{14} was found to have substantial activity at C-5. The O-methyl groups were labelled in colchicine derived from methanol-C^{14}. Activity in colchicine derived from acetate-2-C^{14} was found mostly in the N-acetyl group. There was no appreciable activity in colchicine derived from ornithine-2-C^{14}.

Acetate-1-C^{14} and acetate-2-C^{14} were supplied to Gibberella fujikuroi (Saw.) Wr. cultures and the radioactive fusaric acid isolated. Acetate-1-C^{14} contributed activity mainly to carbons 4, 6, 7, 8 and 10 of fusaric acid, whereas acetate-2-C^{14} contributed activity to carbons 2, 3, 5, 9 and 11 of fusaric acid. These results are consistent with the hypothesis that fusaric acid is formed from a polyacetate unit and aspartic acid or closely related metabolites.

ACKNOWLEDGMENTS

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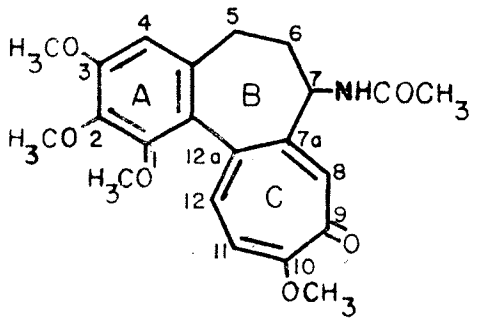
PART I

COLCHICINE

INTRODUCTION

Colchicine (I) is an alkaloid found principally



I

in the corm, seed and flowers of Colchicum autumnale and some other members of the plant family Liliaceae. Excellent summaries of the history, biology, and chemistry of colchicine may be found in a book by Eigsti and Dustin (1) and in the reviews of Cook and Loudon (2), and Wildman (3).

The alkaloid was first isolated in a relatively pure form in 1820 by Pelletier and Caventou who believed it to be veratrine. Geiger and Zeisel obtained crystalline colchicine containing firmly bound chloroform of crystallization in 1883. Analysis of the compound by Zeisel gave a molecular formula of $C_{22}H_{25}O_6N$. Pure, crystalline colchicine was obtained in 1915 as pale yellow needles, m.p. 155-157° by Clewer, Green, and Tutin (4). Chemical synthesis of colchicine was accomplished in 1959 (5,6).

At the outset of this study in 1962, the precursors of the O-methyl groups and N-acetyl group of colchicine

had been established (7,8) as well as the precursor contributing to ring A and C-5 (9). However, there was no positive evidence concerning the biological origin of the tropolone ring of colchicine. The objectives of the present work were (1) to determine the precursors of this ring and carbons 6 and 7 of the B ring; (2) to propose a mechanism for the biological formation of colchicine from its precursors. These objectives have recently been achieved by other workers (10,11,12,13).

LITERATURE REVIEW

The Chemical Structure of Colchicine

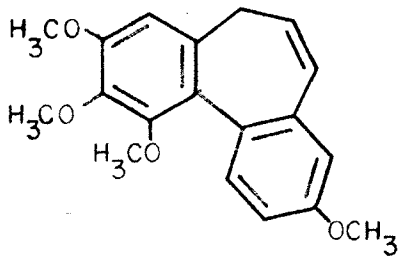
Mild acid hydrolysis of colchicine yields methanol plus colchiceine, a compound containing an enolic hydroxyl group as indicated by an intense green color produced with ferric chloride (14). A Zeisel methoxyl determination (15) on colchicine indicates the presence of four methoxyl groups in the molecule.

Prolonged acid hydrolysis of colchiceine results in the formation of acetic acid and trimethylcolchicinic acid, a compound containing a free amino group (16). The remaining oxygen is unresponsive to carbonyl reagents both in colchicine and colchiceine (17). Hydrogenation of colchicine (18) with Adam's catalyst yields tetrahydrodemethoxycolchicine and hexahydrodemethoxy colchicine. The tetrahydro derivative has ketonic properties, while the hexahydro compound behaves like an alcohol. It thus follows that the sixth oxygen of colchicine is ketonic in nature. Hexahydrocolchicine also reacts with perbenzoic acid (17) and monoperphthalic acid (19) indicating the presence of a third, more resistant, ethylenic bond.

Windaus has summarized (17,20) a series of investigations performed by himself concerning the nature of the ring system of colchicine. He concluded that three

rings existed in the colchicine molecule. He found that oxidation of colchicine with alkaline permanganate yielded trimethoxyphthalic anhydride (20). On the basis of additional reactions (21), he proposed that the anhydride produced was 3,4,5-trimethoxyphthalic anhydride.

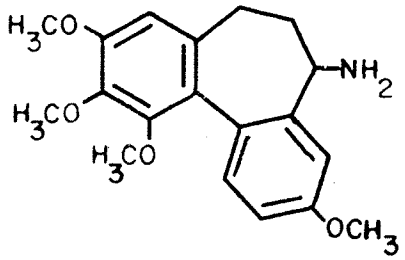
The nature of ring B was determined through a series of reactions starting with deaminocolchinol methyl ether (II) which is obtained by treatment of colchiceine



II

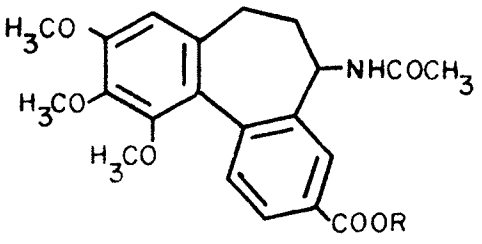
with alkaline hypoiodite followed by reduction with zinc dust to yield N-acetylcolchinol methyl ether (22,23). Treatment of this compound with phosphoric oxide in boiling xylene yields II (24). Barton, Cook, and Loudon (25) found that treatment of II with osmium tetroxide followed by oxidation with lead tetraacetate yielded a dialdehyde which cyclized in alkali to a monoaldehyde. Oxidation with permanganate yielded the corresponding acid which was found to be identical with 2,3,4,7-tetramethoxy-10-phenanthrene carboxylic acid. This series of reactions could only be explained if ring B of colchicine

was seven-membered. This was conclusively proven by the synthesis of colchinol methyl ether (III) by Cook, Jack, and Loudon (26) and Rapoport, Williams, and Cisney (27).



III

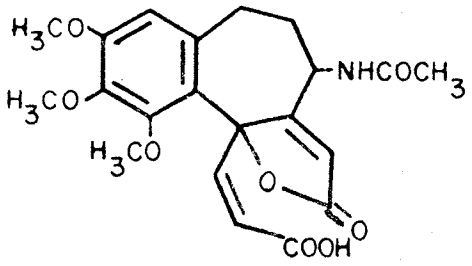
Although the C ring is not a benzene ring, it is readily converted to one by such treatments as alkaline hypoiodite (22,23), alkaline hydrogen peroxide (28), or sodium methoxide in methanol (29,30). The compounds formed with sodium methoxide in methanol are allocolchicine (IV, R=CH_3) and allocolchiceine (IV, R=H). The study of



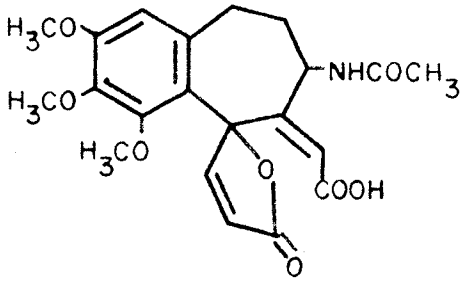
IV

the oxidation product obtained by treating colchiceine with periodic acid (31) by Ahmad, Buchanan and Cook (32) indicated the presence of α,β unsaturated acid and

γ-lactone groups in the product. The structures proposed for the product were VI and V which would indicate that the C-ring was a tropolone ring, as previously proposed



V



VI

by Dewar (33).

Synthesis of I by Eschenmoser and co-workers (5) and van Tamelen and co-workers (6) and its identification as colchicine successfully ended the quest for the chemical structure of colchicine.

Hypotheses of Colchicine Biogenesis

The benzene ring of biologically derived aromatic compounds is generally acknowledged to arise from either the shikimic acid pathway or the condensation of acetate units to ultimately form the desired ring.

The formation of shikimic acid and eventually phenylalanine and tyrosine from phosphoenolpyruvate and D-erythrose-4-phosphate in biochemical mutants of *Escherichia coli*, is well documented (34,35). In higher plants it is not possible to work with auxotrophic mutants and

it has therefore been necessary to utilize tracer techniques and enzyme isolation to establish the existence of this pathway. Nandy and Ganguli (36) have shown that phosphoenolpyruvate and D-erythrose-4-phosphate are optimal substrates for 5-dehydroshikimate formation in extracts of mung bean seedlings and have also partly purified 5-dehydroshikimate reductase from the same source (37). The same enzyme has also been purified from pea epicotyls by Balinsky and Davies (38), who also report purification of dehydroquinase from cauliflower buds (39). The conversion of quinic acid-C^{14} to shikimic acid, phenylalanine and tyrosine has been demonstrated in young rose blooms (64), while the conversion of shikimic acid-C^{14} to phenylalanine and tyrosine has been demonstrated in three different plant families (40,41).

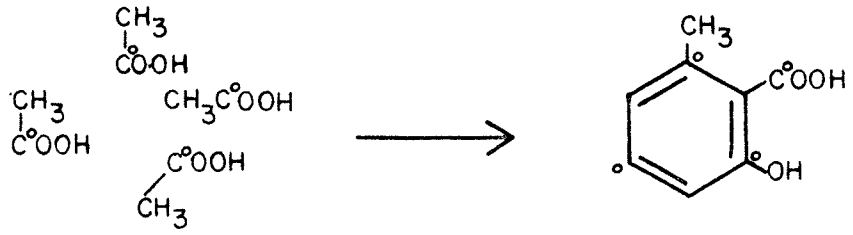
The work of Mc Calla and Neish (40) and Brown, Wright, and Neish (42) concerning lignin precursors is significant in the establishment of the phenylpropanoid (C_6-C_3 unit) precursors of aromatic compounds. The important features to be noted, with reference to the present problem are;

1. Phenylalanine and tyrosine were not interconvertible in 9 out of 10 plant families studied.

2. Hydroxylation of the benzene ring to form the lignin precursors sinapic and ferulic acid occurs after formation of cinnamic acid from phenylalanine and not from tyrosine derived precursors such as

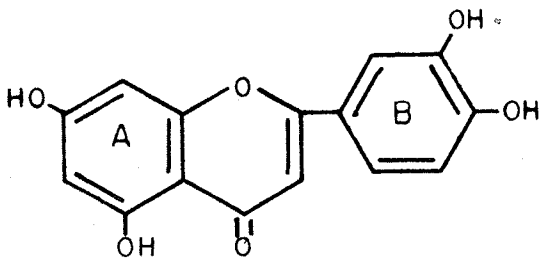
p-hydroxyphenylpyruvic acid.

Instances of the biosynthesis of aromatic compounds from the linear condensation of acetate units followed by cyclization have been found in microorganisms, fungi and higher plants. For example, feeding of acetic-1-C14 acid to Penicillium griseofulvum (43) led to 6-methylsalicylic acid (VII) in accord with the following scheme:



VII

In higher plants, a number of flavonoids have been shown to be derived from a combination of a C_6-C_3 unit and a benzene ring derived from acetate. Compounds derived in this manner are known as C_6-C_3-C_6 compounds. An example of this type of condensation is the flavone, quercetin (VIII). Ring A of this compound is derived from acetate,

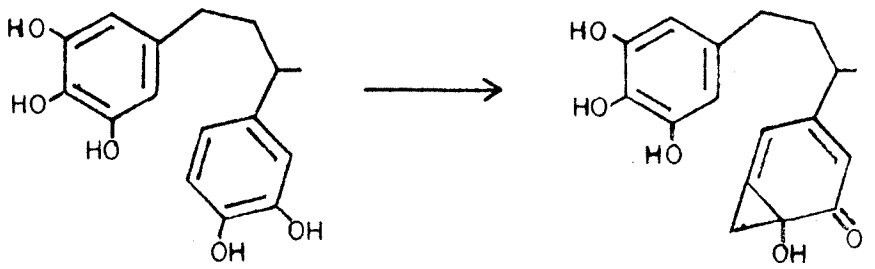


VIII

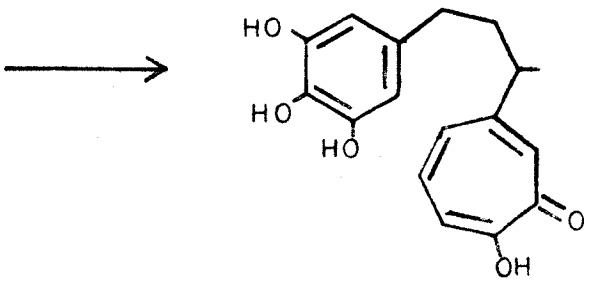
while the remaining carbon atoms in the molecule are derived from a C_6-C_3 unit (44,45).

With the establishment of the basis of the biogenesis of the benzene ring in biologically derived aromatic compounds, it is now possible to proceed to a consideration of hypotheses proposed for the biological formation of the colchicine molecule.

Robinson (46,47) suggested that colchicine may be related to the flavones with the tropolone ring derived from a ring enlargement of a 1,2-diphenol as follows:

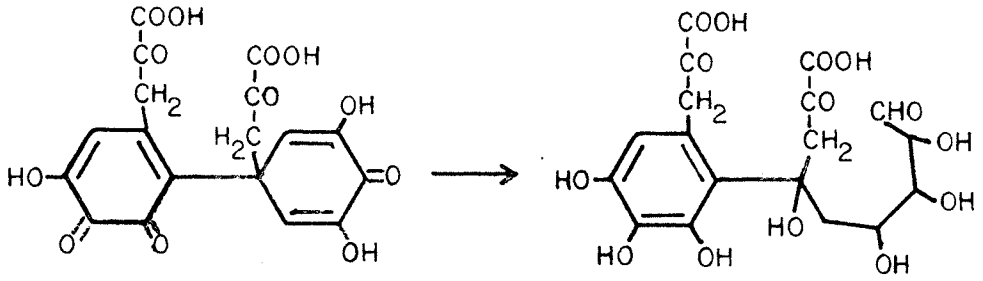


IX X

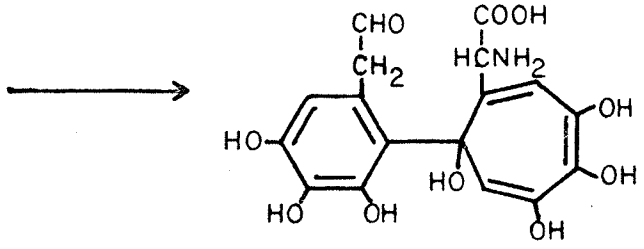


XI

Belleau (48) proposed the oxidative coupling of two moles of 3,4,5-trihydroxyphenylpyruvate as the biosynthetic pathway to colchicine via the following compounds:

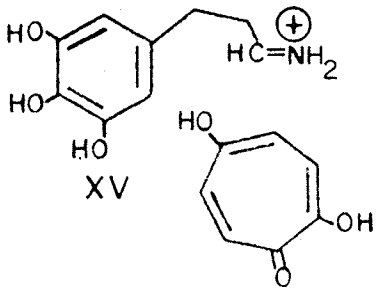


XII XIII



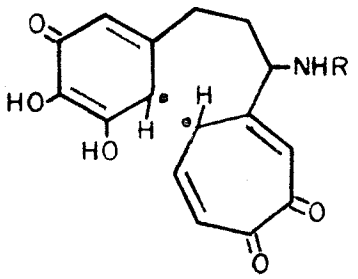
XIV

Wenkert (49) proposed a condensation involving a protonated Schiff base (XV) formed from prephenic acid and 5-hydroxytropolone (XVI) to form colchicine. Scott (50),



XVI

using Robinson's theory, has suggested a phenolic coupling process to join rings A and C analogous to the ring closures which give the morphine skeleton (51). The reactive intermediate would have a structure such as XVII.



XVII

Experiments on Colchicine Biogenesis

Administration of acetate-1-C^{14} to C. autumnale or C. byzantinum led to the formation of radioactive colchicine labelled almost exclusively in the N-acetyl group, while the O-methyl positions of the molecule were labelled as a result of methionine-methyl-C^{14}

feedings (7,8). The negligible activity found in the ring structure indicated that the rings were probably not derived by a condensation analogous to the flavonoid compounds, and, in addition, that ring C was not synthesized in a manner similar to stipitatic acid (52), a tropolone found in Penicillium stipitatum.

Other experiments showed that the A-ring and carbon atoms 5 and 6 of the B-ring were derived from phenylalanine (9,10) but not tyrosine (8). The non-equivalence of phenylalanine and tyrosine suggested that ring A and carbons 5, 6, and 7 were derived from a C_6-C_3 fragment via the cinnamic acid pathway. The results of Battersby and co-workers (11) with phenylalanine-1-C^{14} and cinnamate-3-C^{14} have shown this hypothesis to be correct. In the same paper, the incorporation of tyrosine-3-C^{14} into the tropolone ring of colchicine is reported. Additional experiments with tyrosine-3-C^{14} (12) indicated that colchicine elaborated under these conditions was labelled specifically at position 12 of the colchicine molecule. Battersby and co-workers (13) have described their complete experiments on colchicine biosynthesis and have proposed a mechanism for its formation in the plant.

EXPERIMENTAL

Plant Culture

Colchicum autumnale L. corms (Skinner's Nursery, Dropmore, Manitoba) were obtained in the fall, allowed to flower and then placed in a cold room at 3°C for a period of from four to six months. The plants were then placed in a greenhouse for a two to three day period before the commencement of feeding experiments.

Attempts were made to use seedling plants for the biosynthesis experiments. The *Colchicum* seed appeared to be viable since positive tests were obtained with 2,3,5-triphenyltetrazolium chloride. But it was not possible to germinate the seed, in spite of a variety of treatments. Seeds, sterilized with 4% calcium hypochlorite, were left on moistened filter paper in petri dishes for up to 3 months with no evidence of sprouting. Scarification with concentrated sulfuric acid for 10 minutes, or with sandpaper; stratification in moist vermiculite at 3°C for up to 6 weeks; gibberellic acid treatment; 1% hydrogen peroxide treatment; 100% oxygen atmosphere; or hot (30°C) and cold (3°C) temperature treatments were all entirely non-effective in promoting germination.

The results of these tests are consistent with earlier experiments of Butcher (53) who reported: "Seed was left on damp filter paper in a petri dish for over

six months, but no change occurred. In March, 1951, however, three seedlings came up in a pot sown with normal seed in the spring of 1949."

Radioactive Materials and their Methods of Administration

Methanol-C^{14} and acetate-2-C^{14} were obtained from Atomic Energy of Canada Limited. Cinnamate-3-C^{14} was obtained from Merck, Sharpe and Dohme of Canada, Limited; while ornithine-2-C^{14} was obtained from Tracerlab, Waltham, Mass.

The radioactive tracers were fed to the plants by one of the following methods:

1. Topical Application.-The material to be fed was dissolved in a 1% aqueous solution of Tween 20. Appropriate amounts of the solution were then placed on the surface of the leaves to be absorbed through the leaf surface.

2. Injection.-Known volumes of an aqueous solution of the radioactive tracer were injected with a hypodermic syringe into or near the vegetative apex of the plant.

3. Wick feeding.-Six strands of cotton thread were inserted through the young corms by means of a surgical needle. The thread was treated with 1% Tween 20 solution to thoroughly wet it and the ends then placed in a small beaker containing a solution of the tracer.

Assay of Radioactivity

The radioactivity of a sample was determined by one of two methods:

1. Radioactive samples were combusted to $C^{14}O_2$ using the persulfate method as described by Katz, Abraham and Baker (54). An aqueous solution of the sample, to yield at least 10 mg barium carbonate was placed in a 50 ml Erlenmeyer flask, acidified with 2N sulfuric acid (6 drops), and the system then frozen. Potassium persulfate (700 mg) and 4% silver nitrate solution (1 ml) were then added to the frozen contents of the flask. A vial containing 0.25 ml carbon dioxide-free 20% sodium hydroxide was added; the flask sealed with a serum stopper and evacuated. The system was placed in an oven at 75-80°C until such time as the contents of the flask cleared (2-3 hours). After the flask had cooled, the vial containing the sodium hyroxide solution was transfered to a centrifuge tube containing 8 ml of a 10% barium chloride-1% ammonium chloride solution. The barium carbonate was collected by centrifugation, resuspended in carbon dioxide-free water and plated uniformly on microporous porcelain planchets. The planchets, after complete drying at 110°C and weighing to determine the amount of bariumcarbonate present, were assayed for radioactivity using a mylar window continuous gas flow automatic counter. Appropriate

corrections were made for background, self-absorption, and variation in efficiency.

2. The weighed sample was dissolved in a known volume of a suitable solvent. An aliquot of this solution was transferred to a glass counting vial and a solution containing organic scintillators (5-15 ml) added. The composition of the scintillator solution was either;

a) 2,5-diphenyloxazole (PPO) (4 g) plus 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) (50 mg) in toluene (1 liter).

b) napthalene (50 g), PPO (4 g) and POPOP (100 mg) in dioxane:ethylcellosolve (5:1) (1 liter).

The solution of sample and scintillator was capped, shaken, and counted in a Nuclear Chicago Model 724 liquid scintillation spectrometer. Background corrections were made automatically and the observed count rate was converted to an absolute value (i.e., disintegrations) using the Channel Ratios Method (55).

Sample activities were determined in duplicate with a counting error of 5% or less in each determination.

Radioactive Tracer Experiments

Two experiments to determine the level of $C^{14}O_2$ production after administration of tracer were performed. In one case, sodium acetate-2-C^{14} (5μc; 4μc/mmole) was

administered in a single dose by topical application to a C. autumnale plant. In the second case, daily applications of DL-ornithine-2-C^{14} (15 μc; 0.79 μc/mmole) were topically administered at the rate of 1 μc/day. The CO_2 was collected and analyzed as follows:

> The plant was placed in a bell jar through which a slow stream of air was passed. The exit gas was passed through a solution of 1N sodium hydroxide to trap the carbon dioxide. The system was placed in a fume hood and exposed to continuous fluorescent light. The carbon dioxide trap was changed daily. Aliquots of the sodium hydroxide solutions were taken and the carbon dioxide precipitated as barium carbonate with a 10% barium chloride-1% ammonium chloride solution. The barium carbonate was assayed with a continuous gas flow counter.

The carbon dioxide from the acetate-2-C^{14} experiment was collected over a six day period beginning the day after administration of the tracer. The carbon dioxide from the ornithine-2-C^{14} experiment was collected over an 18 day period, the first sample being taken five days after the first administration of label.

Feeding experiments performed in the greenhouse under normal growing conditions are listed in Table I.

TABLE I

TRACER EXPERIMENTS WITH C. AUTUMNALE
UNDER NORMAL GROWTH CONDITIONS

Compound	Wt, mg	Activity, d.p.m. X 10^{-8}	Rate[a]	No. of Plants
A. Cinnamate-3-C^{14}	9.9	2.1	2.0[b]	10
B. Methanol-C^{14}	0.4	2.0	20.0[c]	1
C. Acetate-2-C^{14}	1.0	1.0	4.0[c]	2
D. a) Ornithine-2-C^{14}	12.8	1.7	2.0[c,d]	3
b) Ornithine-2-C^{14}	14.9	2.0	2.0[b]	10

[a] D.p.m./ plant/ day X 10^{-6}.

[b] Wick feeding.

[c] Topical Application.

[d] Injection.

Isolation of the Colchicine

Colchicine was isolated from the plant material using a modification of the method described by Leete (10). The plants were macerated in a Waring blendor with methanol (1 liter per 300 g plant material). After standing 24 hours, the slurry was filtered on a Buchner funnel and the filtrate reduced to one-tenth its volume on a rotary evaporator. This solution was filtered through Celite and the filtrate extracted with petroleum ether (b.p. 60-80°; 3 X 100 ml). The aqueous layer was made basic with sodium bicarbonate and extracted with chloroform (4 X 100 ml). Evaporation of the dried chloroform extract yielded the crude alkaloids. A chromatographic column (15 X 1 cm) was prepared with neutral Fisher alumina (Brockman activity III) (15 g) using a slurry in benzene. The alkaloids, dissolved in benzene:chloroform (1:1) were added to the column and eluted with the same solvent (100 ml). This was followed by elution with 50 ml each of 60, 70, 80, and 90% chloroform in benzene; chloroform (100 ml); 50 ml each of 2, 4, 8, 10, 20 and 40% ethyl acetate in chloroform, and finally 10% ethanol in chloroform (200 ml). The eluate was collected in 20 ml fractions. Colchicine was detected by infra red spectra of the various fractions and was normally found in the ethanol:chloroform eluate. The colchicine fractions were bulked, crystallized from ethyl acetate-petroleum

ether and assayed for radioactivity. Depending on the amount of colchicine obtained, the radioactive material was diluted with cold material before or after recrystallization to a constant specific activity. The melting point of the final product was 156-157°C.[1]

Colchicine Degradations

A. Cinnamic-3-C^{14} acid Feeding Experiment

The radioactive colchicine (114 mg) isolated from the plants was diluted with an equal weight of unlabelled alkaloid.

3,4,5-Trimethoxyphthalic Anhydride (9).-Colchicine (228 mg; 0.58 mmoles) was added to a solution of potassium hydroxide (9 g) and potassium ferricyanide (50 g) in water (200 ml) and the solution heated with stirring for 12 hours. A further quantity of potassium hydroxide (9 g) and potassium ferricyanide (50 g) was added at this time. A similar addition was made after 36 hours, and after 60 hours the solution was cooled and filtered. The filtrate was acidified with concentrated sulfuric acid and the solution (200 ml) extracted with ether on a continuous extractor for 24 hours. The ether extract was evaporated to near dryness and the residue sublimed at 160°C under reduced pressure. The sublimate (37 mg; 142-144°C) was

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[1]All melting points are uncorrected.

crystallized from acetone to yield colorless plates of 3,4,5-trimethoxyphthalic anhydride (29 mg; 21%) m.p. 146-147°C.

3,4,5-Trimethoxybenzoic Acid.-3,4,5-Trimethoxyphthalic anhydride (29 mg) was refluxed for 12 hours with concentrated hydrochloric acid (1 ml). The solution was filtered hot and on cooling yielded 3,4,5-trimethoxybenzoic acid (9.4 mg; 37%), m.p. 166-168°C.

Decarboxylation of 3,4,5-Trimethoxybenzoic Acid.-3,4,5-Trimethoxybenzoic acid (9.4 mg; 0.044 mmole) was refluxed with copper chromite (10 mg) in quinoline (2 ml) in a current of carbon dioxide-free nitrogen for one hour. The exit gases were passed through 0.125 N barium hydroxide to yield barium carbonate (7.5 mg; 89%).

B. Methanol-C^{14} Feeding Experiment

The radioactive colchicine (25 mg) was diluted with unlabelled alkaloid (75 mg).

Colchiceine.-Colchicine (100 mg) was dissolved in water (5 ml) containing concentrated hydrochloric acid (0.03 ml) and refluxed for 3 hours. The solution was filtered hot, and on cooling yielded pale yellow needles of colchiceine hydrate, m.p. 150-153°C.

The colchiceine was oxidized as in Part A to yield 3,4,5-trimethoxphthalic anhydride (3.3 mg), m.p. 165-167°C.

Gallic Acid.-3,4,5-Trimethoxyphthalic anhydride (18 mg)

was refluxed with 50% hydriodic acid (1 ml) for three hours. The solution was extracted with ether (3 X 5 ml) and the ether extract washed with sodium thiosulfate solution. The dried ether extract yielded a compound (3.3 mg; m.p. 225°C dec.) which had an infra-red spectrum consistent with that of gallic acid.

C. Acetate-2-C^{14} Feeding Experiment

Radioactive colchicine (109 mg) was diluted with unlabelled alkaloid (103 mg).

Trimethylcolchicinic Acid.-Colchicine (212 mg) was refluxed for 5 hours in a solution of water (3 ml) containing concentrated sulfuric acid (0.75 ml). The solution was neutralized with sodium carbonate and the precipitate collected by filtration. The precipitate was dissolved in water (15 ml), the solution extracted with chloroform (4 X 20 ml) and the dried chloroform extract evaporated to dryness under vacuum. The residue was crystallized from ether to yield trimethylcolchicinic acid (214 mg), m.p. 156-158°C.

Sodium Acetate.-The aqueous filtrate obtained from filtration of the crude trimethylcolchicinic acid was acidified with sulfuric acid and distilled. The distillate was titrated to pH 8.0 with sodium hydroxide and evaporated to dryness under vacuum. The residue was dissolved in water and analyzed for carbon content using the persulfate

method as previously described.

D. Ornithine-2-C^{14} Feeding Experiments

Allocolchiceine (29,30).-Colchicine (52 mg) was refluxed for 2 hours with 2 N methanolic potassium hydroxide (25 ml). The solution was neutralized with 5 N hydrochloric acid, the inorganic salt removed by filtration and the filtrate reduced in volume on a flash evaporator. The resulting solution was acidified and the precipitate collected by filtration. The filtrate was extracted with chloroform and the chloroform extract evaporated to dryness under vacuum. This residue, containing unreacted colchicine, was reacted again with 2 N methanolic potassium hydroxide and the crude allocolchiceine collected by precipitation with acid as described above. The crude allocolchiceine (45 mg) was crystallized from benzene to yield allocolchiceine, m.p. 262-264°C.

Decarboxylation of Allocolchiceine.-Allocolchiceine (45 mg; 0.12 mmoles) was refluxed for 3 hours with copper chromite (15 mg) in quinoline (3 ml) in a stream of carbon dioxide-free nitrogen. The exit gases were passed through a 0.125 N solution of barium hydroxide yielding barium carbonate (7.8 mg; 33%).

RESULTS AND DISCUSSION

A portion of the results presented here have been reported elsewhere (56).

Radioactive Tracer Experiments

The results of the experiments to determine the level of $C^{14}O_2$ production after administration of tracer are presented in Table II.

The results of the acetate-2-C^{14} experiment indicate that over 60% of the applied activity is lost as $C^{14}O_2$ four days after the application of the tracer. In addition, the loss reaches a maximum value within the first day of application. In the ornithine-2-C^{14} experiment, the per cent of the total accumulated dose lost as $C^{14}O_2$ remains relatively constant resulting in a continual increase in radioactivity within the plant during the course of the experiment. The portion of the accumulated dose lost as carbon dioxide throughout the measurement period was 42%.

From these measurements, no information regarding colchicine biosynthesis can be gathered. But they do indicate that the radioactive compounds are effectively absorbed when applied topically to the plant and are extensively metabolized by the plant.

Data concerning the incorporation of label into colchicine from various C^{14}-labelled compounds, and the distribution of label in the molecule, are presented in Tables III and IV.

TABLE II

RATE OF PRODUCTION OF $C^{14}O_2$ AFTER ADMINISTRATION OF TRACER TO *C. AUTUMNALE* PLANTS

A. Acetate-2-C^{14} Experiment

Days after Feeding	Activity of CO_2, cpm X 10^{-5}	Per cent of Applied Dose[a]
1	2.49	18.3
2	2.11	15.5
3	2.14	15.7
4	1.81	13.3
5	0.47	3.45
6	0.48	3.52

B. Ornithine-2-C^{14} Experiment

Days after First Feeding	Accumulated Dose, cpm X 10^{-5}	Activity of CO_2 cpm X 10^{-5}	Per cent of Accumulated Dose
5	13.2	0.93	7.0
6	15.8	1.30	8.25
8	21.1	2.88	12.6
9	21.1	1.59	7.5
10	23.8	1.43	6.0
11	26.4	1.75	6.65
12	29.0	1.96	6.75
13	31.7	1.55	4.90

[a]Applied Dose = 1.36 X 10^6 cpm.

TABLE III

THE INCORPORATION OF VARIOUS C^{14}-LABELLED COMPOUNDS INTO COLCHICINE BY *C. AUTUMNALE*

Compound Administered	Colchicine Isolated		Incorporation
	Wt, mg	Specific Activity, d.p.m./mmole	%
A. Cinnamate-3-C^{14}	114	2.2×10^5	0.1
B. Methanol-C^{14}	25	2.0×10^{6}[a]	1.0
C. Acetate-2-C^{14}	109	7.0×10^3	0.01
D. a) Ornithine-2-C^{14}	122	5.7×10^{3}[a]	0.002
b) Ornithine-2-C^{14}	327	6.9×10^3	0.003

[a]Counted with a mylar window continuous gas flow counter and converted to d.p.m./mmole of compound.

TABLE IV

DISTRIBUTION OF C^{14} IN COLCHICINE PRODUCED IN THE PRESENCE OF VARIOUS C^{14}-LABELLED COMPOUNDS

Compound	Specific Activity, d.p.m./mmole
A. Cinnamate-3-C^{14} Experiment	
Colchicine	1.1×10^5
3,4,5-Trimethoxyphthalic anhydride	0.92×10^5
3,4,5-Trimethoxybenzoic acid	0.81×10^5
Barium carbonate	0.88×10^5
B. Methanol-C^{14} Experiment	
Colchicine	5.0×10^5
Colchiceine	2.9×10^5
3,4,5-Trimethoxyphthalic anhydride	2.7×10^5
Gallic acid	0.0
C. Acetate-2-C^{14} Experiment	
Colchicine	3.57×10^3
Trimethylcolchinic acid	1.04×10^3
Sodium acetate	2.0×10^3
D. Ornithine-2-C^{14} Experiment	
Colchicine	5.7×10^3
Barium carbonate (from allocolchiceine)	0.0

A. Cinnamate-3-C^{14} Experiment

This experiment was performed independently of Battersby *et al.* (11). The percentage incorporation of label obtained by them was a factor of ten higher than that presented here (Table III). The distribution of label in colchicine, however, is comparable (Table IV). The decarboxylation of trimethoxybenzoic acid demonstrated that over 80% of the label is located in C-5 of colchicine. Battersby and co-workers reported 100% of the activity from cinnamate-3-C^{14} feedings in trimethoxyphthalic anhydride.

The results of the cinnamate-3-C^{14} feeding experiments indicate that the pathway leading to the A-ring and C-5 of the B-ring is similar to the one which forms the immediate precursors of the lignins (40,42). In addition, they also indicate that ring A, C-5, C-6 and C-7 are probably derived from an intact C_6-C_3 unit.

B. Methanol-C^{14} Experiment

The percentage incorporation of label from methanol-C^{14} is identical with the incorporation data of Leete and Nemeth (9) for L-methionine-methyl-C^{14}. It would thus appear that methanol rapidly forms an intermediate which is at least equivalent to L-methionine. Cossins (57) has reported that methanol-C^{14} is rapidly metabolized by various plant tissues with serine; methionine, methionine sulfone, and methionine sulfoxide being the most important labelled

compounds in the amino acid fraction. The most probable pathway for incorporation of methanol into colchicine is the one-carbon pathway involving L-methionine and S-adenosylmethionine (58).

The label in colchicine from methanol-C^{14} feeding was concentrated solely in the methoxyl carbons, with 40% in the C-ring methoxyl group as shown by the conversion of colchicine to colchiceine. A Zeisel determination converting trimethoxyphthalic acid to gallic acid demonstrated that the remaining 60% was present in the methoxyl groups attached to the A ring. The distribution of label from this precursor is not consistent with feeding experiments with L-methionine-methyl-C^{14} (7) where only 10% of the label in colchicine was found in the C-ring methoxyl group. Since the O-methyl group of the tropolone ring is more labile than the other methoxyl groups of the molecule, it would be expected that this group would exchange more readily with methyl donors after formation of colchicine. Thus, in the L-methionine experiment, where a single dose of tracer was applied followed by one month of metabolism by the plant, considerable exchange of this group with unlabelled methyl donors could occur. In the present experiment, however, the plants were administered daily doses of label over a ten day period and were harvested after fourteen days of total contact with tracer. Assuming that methanol is equivalent to L-methionine as a methyl donor, the results

presented here would appear to be reasonable.

C. Acetate-2-C^{14} Experiment

The similarity between incorporation of acetate-2-C^{14} in the present work (Table III) and that of Leete (7) with acetate-1-C^{14} would indicate that the utilization of the carbons of acetate for colchicine biogenesis are similar, or more probably, that the acetate unit is incorporated intact into colchicine. The dilution factors however, (i.e., the ratio of the specific activity of the administered tracer to the specific activity of the isolated product) for acetate-1- and -2-C^{14} do not confirm this hypothesis. The dilution factor for acetate-1-C^{14} is 5.4×10^4 while for acetate-2-C^{14} the dilution factor is 1.18×10^6. With other factors being equal, the dilution factors should have roughly the same order of magnitude. The "other factors" in this case, are most certainly not equal. The acetate-1-C^{14} experiment was performed on flowering C. byzantinum corms using a single application of tracer via a wick; while the acetate-2-C^{14} experiment was performed on C. autumnale corms during the vegetative stage of the plant using multiple, topical applications of the tracer. It is therefore not possible to directly correlate the two experiments.

Sodium acetate, derived from the N-acetyl group of the labelled colchicine, contained 56% of the activity in

the molecule, with 29% in trimethylcolchicinic acid (Table IV). There was therefore 15% of the activity in the tropolone O-methyl group. The precise location of the C^{14} remaining in trimethylcolchicinic acid was not further investigated.

D. Ornithine-2-C^{14} Experiment

The basis for the ornithine-2-C^{14} experiments was the possibility that the C-ring of colchicine may be derived in a manner similar to the tropane alkaloids (59,60). These alkaloids are believed to be derived from glutamic semialdehyde, formed by oxidation of ornithine, and acetoacetic acid in a Mannich-type condensation. Figure 1 demonstrates a plausible mechanism in which this type of condensation could be utilized in the formation of colchicine. Condensation of glutamic semialdehyde with a phenylpropanoid unit, followed by cyclization, yields XVIII. Condensation of XVIII with acetoacetic acid, followed by decarboxylation, yields XIX (65). A reaction analogous to exhaustive methylation transforms XIX to XX. Hydroxylation of XX yields XXI, which on tautomerization forms XXII. Condensation of the tropolone moiety of XXII with the benzene ring yields XXIII, which on elimination of a hydroxl group forms XXIV. Methylation, reductive amination, and acetylation yields colchicine.

Formation of tropolones from substituted tropinones is known to occur readily. For example, γ-tropolone methyl

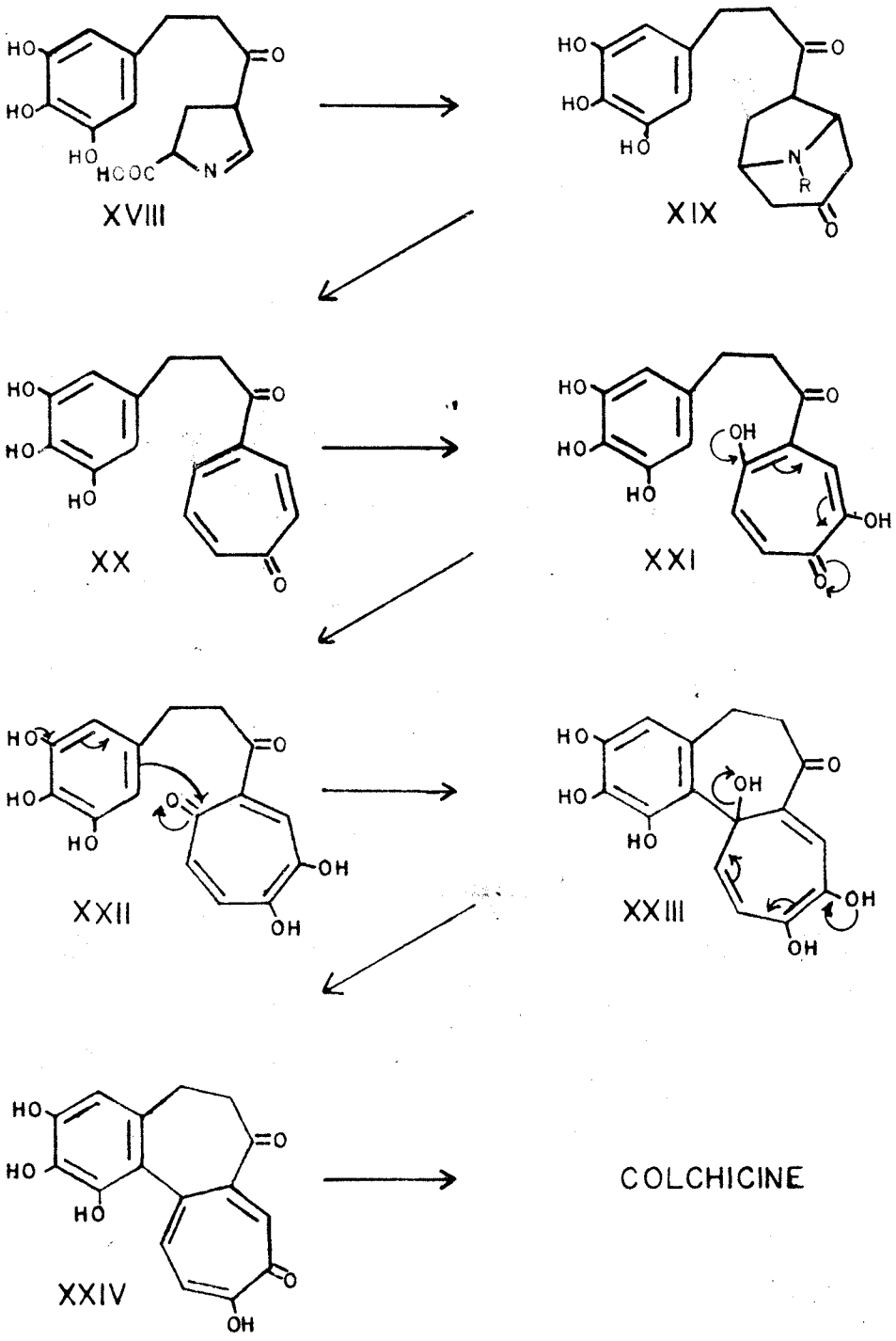
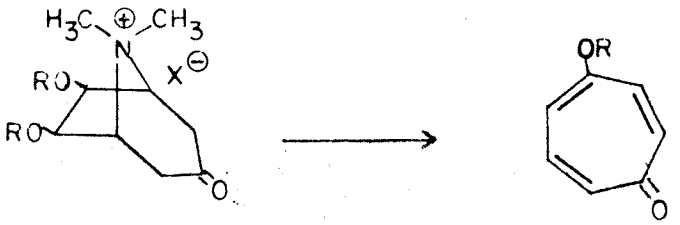


Fig. I.

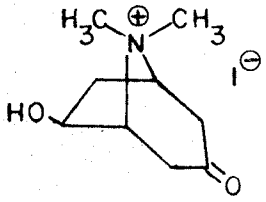
A Possible Scheme For The Biogenesis Of The C-Ring Of Colchicine From Ornithine.

ether (XXVI) is formed in 100% yield from XXV (R = CH_3; X = Br) by heating on a steam bath with 1 mM sodium bicarbonate solution (61). Treatment of XXVII with dilute aqueous sodium bicarbonate at 100°C yields 82% tropone (62).



XXV

XXVI



XXVII

The low incorporation of ornithine-2-C^{14} into colchicine (Table III) and the proof that tyrosine-3-C^{14} is incorporated into the C-ring (12,13) conclusively establishes that the pathways to colchicine via ornithine do not exist.

A degradation experiment with the colchicine from experiment D (a) was performed, however, to see if the small amount of label incorporated was concentrated in a portion of the C-ring of colchicine. The barium carbonate derived from decarboxylation of allocolchiceine in the ornithine feeding experiment represents C-9 of colchicine (29,30). The results (Table IV) indicated that there was no specific labelling in this position from ornithine-2-C^{14}. It is probable that the activity from ornithine-2-C^{14} was generally distributed throughout the colchicine molecule.

Biogenesis of Colchicine

Battersby and co-workers (13) have presented a scheme for colchicine biogenesis based on their results which, with possible minor variations, is probably the pathway to colchicine. Their scheme is shown in Figure 2. The ring expansion (XXXI⟶XXXII⟶Colchicine) is derived from the proposed mechanism for the formation of simple tropolones from methoxy substituted 1,4-dihydrobenzyltosylates (63) and requires that "X" be a good leaving group.

There are some points in Battersby's theory which are left unexplained. Firstly, how does the C_6-C_3 unit condense with the C_6-C_1 unit? In the condensation to form XXVIII, the logical mechanism is an electrophilic attack by C-7 on the

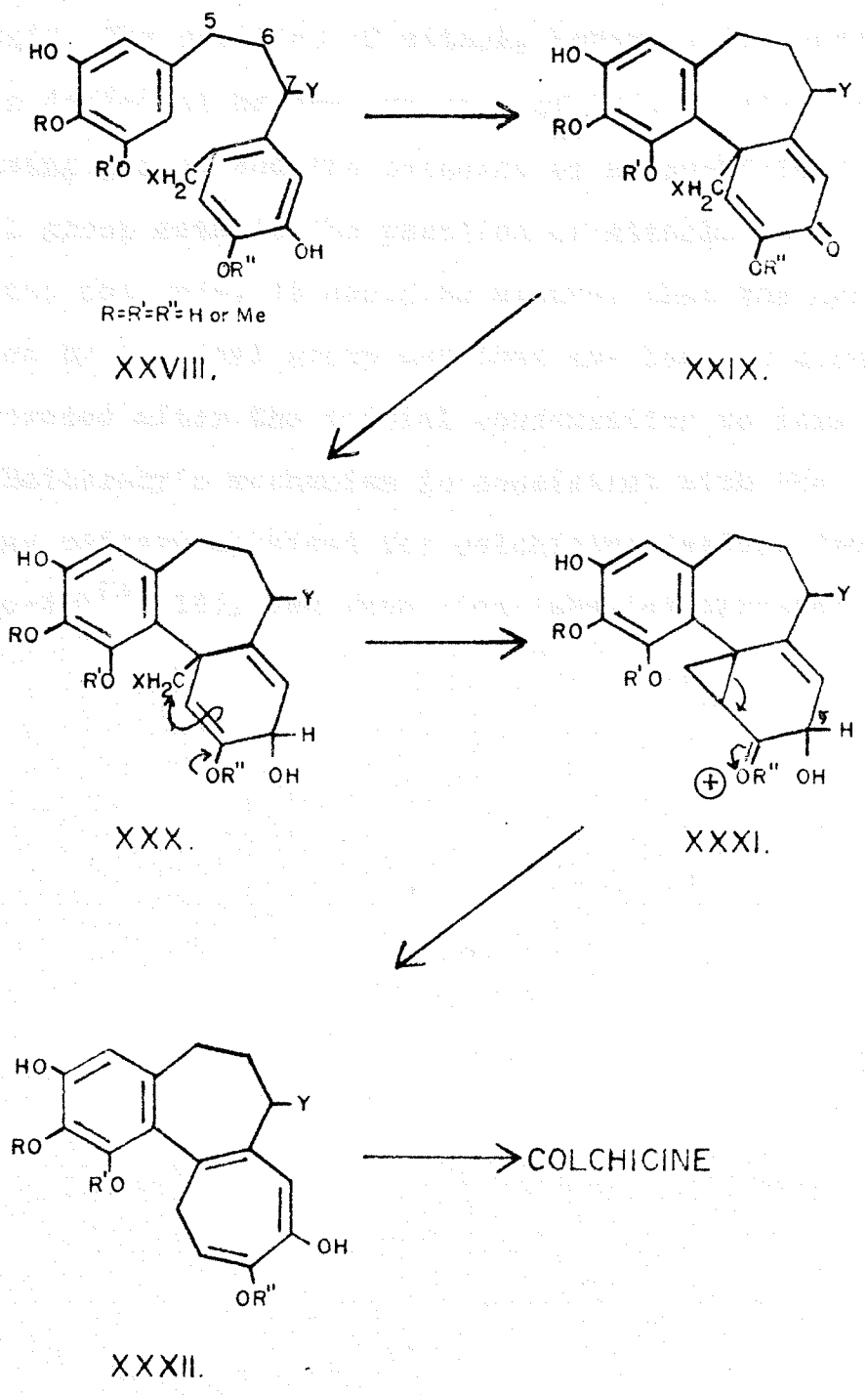


Fig. 2.

Battersby's Mechanism For The Biogenesis Of Colchicine.

C_6-C_1 unit. The position of attack, however, is rendered electron deficient by the presence of "X", an electron withdrawing group, and the presence of an unshielded hydroxyl group meta to the position of attack. To compensate for this, it could be assumed that the hydroxyl is masked by a methyl group and that the leaving group "X", is elaborated after the initial condensation to form XXVIII.

Battersby's mechanism is consistent with the labelling pattern obtained for colchicine derived from tyrosine-3-C^{14} (13), and from ring-labelled tyrosine (66).

SUMMARY

Various C^{14}-labelled substrates were administered to *Colchicum autumnale*, L. plants. After a period of time, colchicine was isolated from the plants and the distribution of activity in the colchicine determined. The following results were obtained:

1. Cinnamate-3-C^{14} was incorporated into colchicine and specifically labelled position 5 of the molecule.

2. Methanol-C^{14} was incorporated into colchicine. Forty per cent of the activity present in the colchicine was found in the O-methyl group of the tropolone ring with the remaining 60% in the O-methyl groups of the A ring.

3. Acetate-2-C^{14} was incorporated into colchicine. Fifty-six per cent of the activity in colchicine was found in the N-acetyl group of the molecule; 15% in the tropolone O-methyl group; and 29% in trimethylcolchicinic acid.

4. Ornithine-2-C^{14} was not appreciably incorporated into colchicine.

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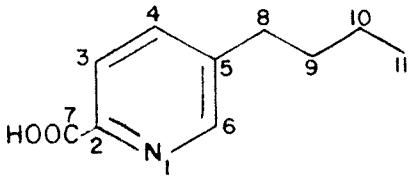
PART II

FUSARIC ACID

INTRODUCTION

Fusaric acid (I) was first isolated by Yabuta, Kambe, and Hayashi (1) as a metabolic product of Fusarium heterosporum Nees, a facultative parasite of wide distribution. It was later recognized to be an important wilt toxin produced by Fusarium lycopersici, F. vasinfectum Atk., and Gibberella fujikuroi (Saw.) Wr. by Gaumann and co-workers (2), and has been found in no fewer than 6 members of the family Hypocreaceae (3).



I

Chemically, fusaric acid is 5-n-butyl picolinic acid with an empirical formula of $C_{10}H_{18}O_2N$ and a molecular weight of 179 (1). The compound was first synthesized in 1954 by Plattner, Keller and Boller (4) and has since been synthesized by other workers (5).

There have been two previous studies (6,7) dealing specifically with the biogenesis of fusaric acid, but no conclusive proof regarding the precursors of the molecule was obtained. The present study was undertaken (1) to establish the precursors of fusaric acid; (2) to determine

the distribution of activity in fusaric acid derived from the precursors; (3) to test two theories regarding its biogenesis.

LITERATURE REVIEW

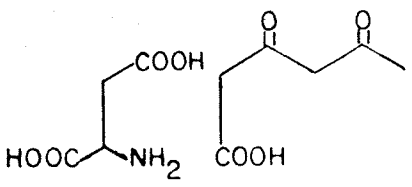
Previous Studies on the Metabolism and Biogenesis of Fusaric Acid

Sanwal (8), in a study of the metabolism of F. lycopersici, reported that fusaric acid can be produced in culture media with glycine as the sole carbon source. He suggested that amino acids play a significant role in the biogenesis of fusaric acid, with alanine and citrulline being possible precursors.

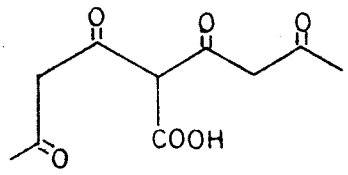
Sandhu (6) performed some studies on the biogenesis of fusaric acid in F. lycopersici and Gibberella fujikuroi using isotope competition techniques and C^{14}-labelled amino and organic acids. He found that G. fujikuroi was far superior for studies of this type, since it produces about 20 times more fusaric acid in a given time than F. lycopersici. Isotope competition experiments with glucose-U-C^{14} demonstrated that inactive γ-amino butyric acid, alanine, serine and acetate produced significant dilutions in the radioactivity of the synthesized fusaric acid, while aspartic and succinic acids produced smaller dilutions. Alanine-U-C^{14} and serine-U-C^{14} were readily incorporated into fusaric acid. Acetate-1-C^{14} was utilized to a lesser extent, while glycine-2-C^{14} and γ-aminobutyric acid were only slightly incorporated. Radioactivity from

fumarate-2,3-C^{14} did not appear in fusaric acid. No chemical degradations were performed on the radioactive fusaric acid.

The work of Dobson and Vining (7) was the first study in which C^{14}-labelled fusaric acid derived from feeding experiments was partially degraded. Growing cultures of Fusarium orthoceras App. and Wr. incorporated 16% and 30% respectively of the radioactivity from acetate-1-C^{14} and acetate-2-C^{14} into fusaric acid. Aspartate-1-C^{14}, aspartate-4-C^{14}, malonate-2-C^{14}, and glycerol-1,3-C^{14} each contributed about 3% of their C^{14} to fusaric acid. The fusaric acid obtained from the feeding experiments was degraded to determine the per cent distribution of radioactivity in the pyridine ring, the butyl side chain, and the carboxyl group. The authors concluded that the side chain is derived from acetate and that the pyridine ring may be derived from a condensation of aspartate and acetate or aspartate and malonate. Vining (9) has suggested that the molecule is elaborated through condensation of a polyacetate unit and aspartic acid, in the following manner:

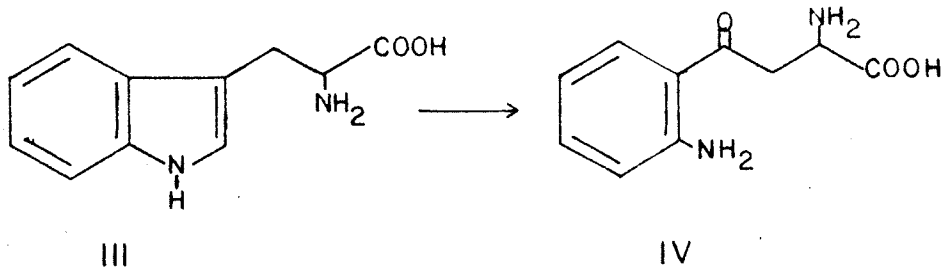


Leete (10) has proposed a slightly different polyacetate condensation to form fusaric acid, as shown below.

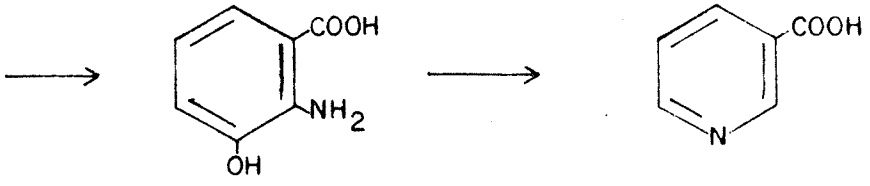


Pyridine Ring Biogenesis

It is now well established that more than one route exists for the biological formation of the pyridine ring. In mammals and in the fungus Neurospora crassa, nicotinic acid (II) is synthesized from tryptophan (III) via kynurenine (IV) and 3-hydroxyanthranilic acid (V) (11).



III IV

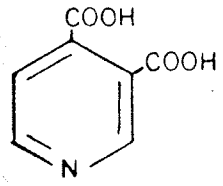


V II

In higher plants (12,13,14,15,16) and certain bacteria (17), however, experiments with C^{14} or H^3-labelled tryptophan have failed to show any transformation to nicotinic acid.

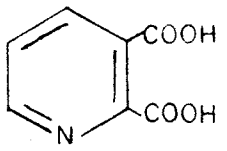
In Escherichia coli it has been shown (18) that a three carbon compound, such as glycerol, and a four carbon dicarboxylic acid are capable of supplying the carbon atoms of nicotinic acid. Experiments with either glycerol-1,3-C^{14} and unlabelled succinate, or succinate-2,3-C^{14} and unlabelled glycerol, have shown that only the pyridine ring of nicotinic acid becomes labelled (18). Administration of succinate-1,4-C^{14} and unlabelled glycerol yielded nicotinic acid labelled only in the carboxyl group (19).

When a nicotinic-acid-deficient mutant of E. coli was grown in media free of nicotinic acid, growth was exhibited only in the presence of cinchomeronic acid, (VI) (20) suggesting that this acid was the true precursor



VI

of nicotinic acid in the organism. Other workers (21), however, have reported the formation of nicotinic acid mononucleotide from quinolinic acid (VII) by E. coli.

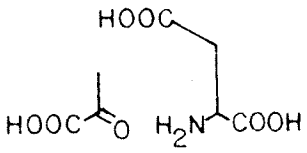


VII

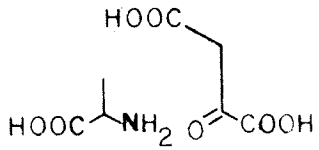
Quinolinic acid has also been implicated as a precursor of nicotinic acid in mammals (22) and plants (23). This has led to the suggestion (21) that it may be a universal intermediate in the biosynthesis of pyridine ring compounds.

Rio-Estrada and Patino (24), using non-radioactive precursors, found that aspartate and alanine were good precursors of nicotinic acid in Mycobacterium tuberculosis. They also found that glycerol-2-C^{14} was not incorporated into nicotinic acid. Administration of aspartate-4-C^{14} to M. tuberculosis resulted in the formation of radioactive nicotinic acid with all the activity in the carboxyl group (25). Aspartate-1,4-C^{14}-N^{15} feeding (26) resulted in carboxyl-labelled nicotinic acid with a C^{14}/N^{15} ratio consistent with the incorporation of one molecule of aspartic acid minus the α-carboxyl group into nicotinic acid.

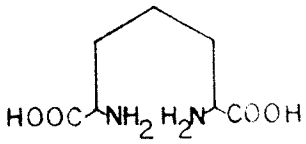
In Bacillus megaterium it has been suggested (27) that dipicolinic acid may be formed from a condensation of aspartic and pyruvic acids (Scheme 1) or alanine and oxalacetic acid (Scheme 2). In addition, 2,6-diaminopimelic



Scheme 1



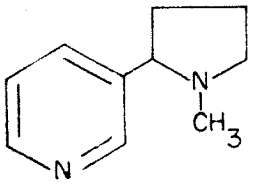
Scheme 2



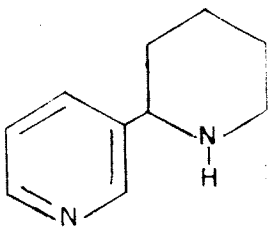
VIII

acid (VIII) has been shown to be a precursor of dipicolinic acid in B. megaterium (28) and Penicillium citreo-viride (29).

There has been considerable interest in the biogenesis of the plant alkaloid nicotine (IX) and the related compound, anabasine (X). Nicotinic acid has been shown to



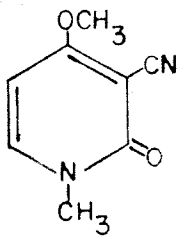
IX



X

be a precursor of the pyridine ring in both nicotine (30) and anabasine (31). The experiments of Leete (32) and Byerrum and Griffith (33) established that acetate-2-C^{14} and compounds that could be metabolized to acetate-2-C^{14} were incorporated into the pyridine ring of nicotine and anabasine. Glycerol was found to be an even more efficient precursor of the pyridine ring of these alkaloids (34). Griffith et al. (34) showed that about one-half of the radioactivity in the pyridine ring of nicotine derived from acetate-2-C^{14} was located at C-3. More recently (35) they have shown that nicotine derived from aspartate-3-C^{14} had one-half of the activity of the pyridine ring at C-3. The experiments of Friedman and Leete (36,37) have demonstrated that acetate-2-C^{14} labels C-2 and C-3 of the pyridine ring of anabasine equally; while glycerol-2-C^{14} labels C-2, C-3, and C-5 equally with little activity in C-4 and C-6. They have interpreted these results as an indication that the pyridine ring of anabasine is formed from glycerol and succinic acid or closely related metabolites.

The pyridone ring compound, ricinine (XI), from



XI

Ricinus communis L. is apparently derived in a similar manner to the pyridine rings of nicotine and anabasine. Like these alkaloids, nicotinic acid is a precursor of ricinine (38,39). Juby and Marion (40) supplied acetate-2-C^{14} to Ricinus communis L. plants and found that the pyridone ring of the resultant radioactive ricinine was labelled exclusively and equally at C-2 and C-3. Succinate-2,3-C^{14} gave almost an identical pattern of labelling. Waller and Henderson (41) found that succinate-1,4-C^{14} afforded radioactive ricinine, in which most of the activity was located on the nitrile carbon, a pattern consistent with the direct participation of succinic acid or a closely related metabolite. Glycerol-1,3-C^{14} and glycerol-2-C^{14} were also incorporated into the pyridone ring (42,43) the pattern of labelling strongly suggesting that the three-carbon chain of glycerol formed C-4, C-5 and C-6 of ricinine.

Sequential Degradation of the Pyridine Ring

Until 1963, studies on the biogenesis of the pyridine ring were hampered by the lack of a suitable method for sequential degradation of the ring. In that year, sequential degradation of the pyridone ring of ricinine was accomplished (42,43) and a method for isolation of C-2 and C-3 of the pyridine ring of nicotinic acid was presented (36). Subsequent experiments (37,44)

established complete carbon-by-carbon degradations of nicotinic acid.

The method for sequential degradation of nicotinic acid according to Friedman and Leete (36,37) is presented in Figure 1. Nicotinic acid (II) is reduced via its methyl ester (XII), with lithium aluminum hydride to 3-hydroxymethyl pyridine (XIII). The alcohol is converted with thionyl chloride to chloromethylpyridine (XIV), which is then hydrogenated in the presence of palladium on barium carbonate to yield 3-methylpyridine (XV). Reaction of XV with phenyllithium yields 3-methyl-2-phenylpyridine (XVI) which is converted to its methiodide (XVII). Hydrogenation of XVII in the presence of Adam's catalyst yields 1,3-dimethyl-2-phenylpiperidine (XVIII), which is the key compound in the degradation. Chromic acid oxidation of XVIII yields benzoic acid, representing C-2 of nicotinic acid, and acetic acid, representing C-3 and C-7. A Schmidt degradation on the sodium acetate yields C-3 as CO_2. Conversion of XVIII to its methiodide (XIX) followed by an Emde reaction using sodium in liquid ammonia, preferentially cleaves the piperidine ring between C-2 and the nitrogen to yield XX. A Hoffmann exhaustive methylation reaction yields XXI, which is then reacted with osmium tetroxide and sodium periodate to produce 3-methyl-4-phenybutanal (XXII) and formaldehyde. The formaldehyde, collected as the dimedone derivative, represents C-6 of the pyridine ring.

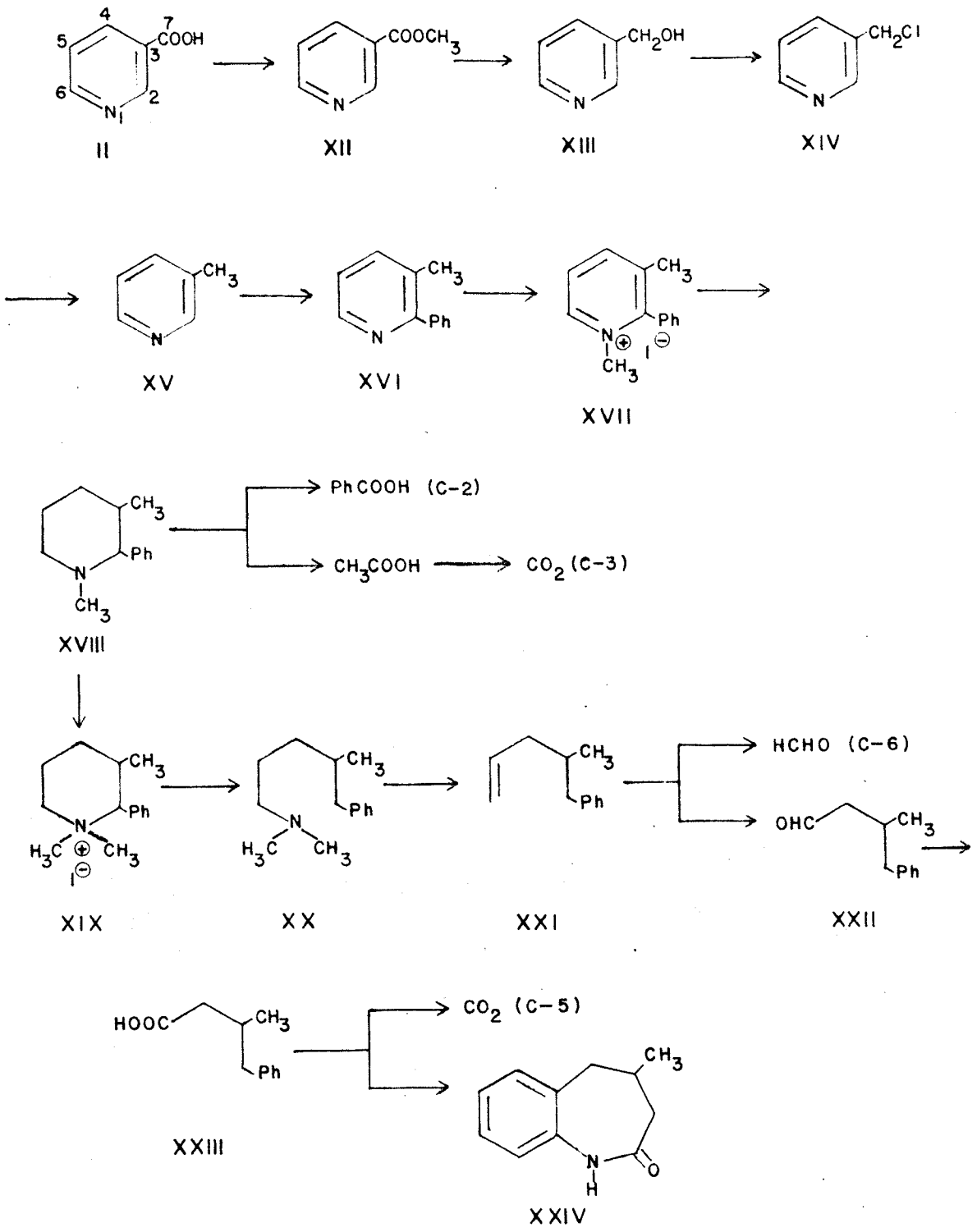


Fig. 1. Sequential Degradation Of Nicotinic Acid.

Oxidation of the butanal with a stoichiometric amount of chromic acid yields the corresponding acid XXIII, which may then be reacted with sodium azide in sulfuric acid to yield carbon dioxide from C-5 of nicotinic acid. The authors found that the final Schmidt reaction did not go to completion and they isolated a by-product which they assigned structure XXIV. As a result of the incomplete reaction C-4 of nicotinic acid was determined by difference.

EXPERIMENTAL

Conditions of Culture

Gibberella fujikuroi (Saw.) Wr., strain ETH M 82 was obtained from the Department of Special Botany, Swiss Federal Institute of Technology, Zurich, Switzerland. The fungus was maintained on malt agar or Difco potato-dextrose agar slants at 4°C with regular subculturing every two months.

Inoculum was prepared by transferring a solution of the mycelium to 125 ml Erlenmeyer flasks containing sterile, water-saturated, rice. After 5 days in the dark, the conidia were harvested by shaking with sterile distilled water (60 ml). This spore suspension was transferred to a 250 ml Erlenmeyer flask and incubated on a shaker in the dark for 24 hours. Ten ml of this inoculum was added to 250 ml of Czapek's medium in 1 liter Erlenmeyer flasks and the fungus was grown in the dark in shake culture.

Czapek's medium has the following composition; sucrose (4%), sodium nitrate (0.3%), monopotassium phosphate (0.1%), magnesium sulfate (0.05%), potassium chloride (0.05%), ferrous sulfate (0.001%) in distilled water. The pH of the medium was adjusted to 5.5 before autoclaving.

Administration of Labelled Compounds

The fungus from three day old shake cultures was

collected by low-speed centrifugation. The harvest from two culture flasks was resuspended in Czapek's medium (50 ml) and used to inoculate fresh medium (200 ml). Six flasks were normally used for each tracer experiment.

Aqueous solutions of sodium acetate-1-C^{14} or -2-C^{14} (Atomic Energy of Canada, Ltd.) were prepared and divided equally between the six flasks. After 24 hours shaking in the dark, the flasks were removed and the fusaric acid extracted from the culture filtrate.

Assay of Radioactivity

Samples were counted in a Nuclear Chicago Model 725 liquid scintillation spectrometer as described on p. 17 of Part I.

Carbon dioxide from a degradation procedure was assayed by flushing the reaction vessel with carbon dioxide-free nitrogen and passing the exit gases through either:

(a) a solution of ethanolamine:methyl cellosolve (1:2) (5 ml). The solution was diluted to a known volume and aliquots were taken for radioactive assay and quantitative determination of carbon dioxide. The carbon dioxide content was determined by acidification of an aliquot in a closed system swept with carbon dioxide-free nitrogen. The carbon dioxide was trapped as barium carbonate in 0.125 N barium hydroxide (5 ml). The barium carbonate was collected by centrifugation, plated on microporous

porcelain planchets, dried at 110°C for 1 hour, and weighed.

(b) 0.125 N barium hydroxide (5 ml). The barium carbonate was quantitatively determined as in (a). The planchet containing the barium carbonate, was placed, along with a counting vial containing 80% lactic acid (15 ml), in a wide mouth 250 ml Erlenmeyer flask. A second counting vial, suspended on a string, and containing ethanolamine:methyl cellosolve (1:2) (1 ml) was placed in the flask and the flask stoppered so that this vial was suspended in the flask. The vial containing the lactic acid was tipped, and the system allowed to stand overnight. The vial containing ethanolamine:methyl cellosolve and trapped carbon dioxide was removed, toluene scintillator solution added (ca. 10 ml), and the radioactivity assayed.

Extraction and Isolation of the Fusaric Acid

Celite was added to the culture flasks and the slurry filtered. The filtrate was reduced to one-quarter of its volume on a rotary evaporator and filtered. The filtrate (ca. 400 ml) was adjusted to pH 4.0 and extracted with ethyl acetate (3 X 300 ml). The ethyl acetate fraction was evaporated to dryness, the residue dissolved in water (50 ml), and the pH adjusted to 8.0 with 2 N sodium hydroxide. After extraction with ether (25 ml), the aqueous phase was

adjusted to pH 4.0 with dilute hydrochloric acid and extracted with ether for 36 hours in a continuous liquid/liquid extractor. The ether extract was dried with anhydrous magnesium sulfate, filtered, and evaporated to dryness on a rotary evaporator. The residue was extracted with petroleum ether (5 X 100 ml) and the extract evaporated to dryness. The residue was crystallized three times from petroleum ether affording colorless needles of fusaric acid, m.p. 101-102°C.

Degradation of the Fusaric Acid

The weights indicated in the following reactions are, with the exception of three reactions, those obtained from the degradation of fusaric acid derived from an acetate-1-C^{14} feeding experiment. Dilutions with non-radioactive intermediates were made whenever the amounts available were too small for subsequent degradation steps.

Kuhn-Roth Oxidation of Fusaric Acid.-Fusaric acid (123 mg) in water (2 ml) was added to a refluxing solution of chromium trioxide (0.8 g) in 10% sulfuric acid (14 ml). Distillation was started immediately. The volume of the reaction mixture was maintained by addition of distilled water through a dropping funnel. The distillate (130 ml) was titrated to pH 8.0 with 0.03227 N sodium hydroxide solution (20.1 ml) and evaporated to dryness. The residue was dissolved in water (5 ml), acidified (pH < 1) with

sulfuric acid and extracted with ether (4 X 5 ml). The ether extract, containing acetic and propionic acids, was dried, filtered and evaporated to ca. 0.5 ml in an air stream at room temperature.

The mixture of acetic and propionic acids was separated by gas chromatography, using a copper column (6 ft. X 0.25 in. O.D.) packed with 20% neopentyl glycol succinate on 60/80 mesh firebrick treated with 2% phosphoric acid. The column temperature was 128°C while the helium flow rate was 50 ml per minute. The pure acids were collected by bubbling the exit gas through distilled water and then titrated to pH 8.0 with 0.03227 N sodium hydroxide solution (4.56 ml for propionic acid; 3.32 ml for acetic acid). The solutions were each evaporated to dryness <u>in vacuo</u>, dissolved in absolute ethanol (10 ml) and evaporated to dryness again. Treatment with absolute ethanol was repeated twice, followed by final treatment with absolute ether (10 ml) and evaporation to dryness to yield the dry sodium salts of acetic and propionic acids.

<u>Schmidt Reaction on Sodium Acetate or Sodium Propionate</u>.- The flask containing the dry sodium salt of the acid (0.1-0.15 mmole) was cooled in an ice bath, 100% sulfuric acid (0.1 ml) added and the contents dissolved by rotating the flask. Sodium azide (15 mg) was added and the flask was connected to a gas train containing a 5% potassium permanganate in 5% sulfuric acid scrubber (to remove sulfur

dioxide). The flask was flushed for 2 minutes with carbon dioxide-free nitrogen, and a carbon dioxide trap of ethanolamine or barium hydroxide attached following the permanganate scrubber. The nitrogen flow was stopped and the reaction flask was heated on a water bath at 75-80°C for one hour. The system was flushed with nitrogen for 15 minutes, after which the carbon dioxide trap was removed and analyzed for carbon dioxide and radioactivity.

Degradation of Methylamine from Schmidt Reaction on Sodium Acetate.-A solution of 3% potassium permanganate (5 ml) was added to the residue remaining from the Schmidt reaction, followed by the addition of 40% sodium hydroxide solution (0.2 ml). The flask was connected to the gas train, containing a fresh carbon dioxide trap, and was heated for 15 minutes on a boiling water bath. The system was flushed with nitrogen, the water bath removed, and the reaction mixture acidified with sulfuric acid to release carbon dioxide. After flushing the system with nitrogen for 15 minutes, the carbon dioxide trap was removed and the contents analyzed for radioactivity and carbon dioxide.

3-n-Butylpyridine.-A flask, containing fusaric acid (2.95 g), was connected to a reflux condenser and flushed with carbon dioxide-free nitrogen. A gas train containing a dilute sulfuric acid scrubber and a carbon dioxide trap was connected following the condenser. The nitrogen flow was

stopped, and the reaction flask heated at 200°C on an oil bath. After 5 hours the oil bath was removed and the system flushed with nitrogen for 10 minutes. The carbon dioxide trap was removed and the contents analyzed as before. The condenser was set for distillation and the flask contents distilled under reduced pressure yielding 3-n-butylpyridine (2.04 g, 94%).

Nicotinic Acid.-3-n-Butylpyridine (2.04 g), mixed with water (50 ml), was reacted with potassium permanganate (4 g). The potassium permanganate was added in 0.5 g portions over a 4 hour period to the stirred solution. The temperature of the reaction mixture was raised to 40°C and additional portions of permanganate (4 g) added after 12 and 24 hours. After 48 hours, the excess permanganate was decomposed by the addition of methanol. The manganese dioxide was filtered off and thoroughly washed with hot water. The combined filtrates were evaporated to dryness, redissolved in water (25 ml) and the pH of the solution adjusted to 8.0 with dilute hydrochloric acid. A saturated aqueous solution of cupric acetate (50 ml) was added and the resultant precipitate of cupric nicotinate filtered off and washed with water. The copper salt was suspended in water and decomposed with hydrogen sulfide. The mixture was filtered and the filtrate evaporated to dryness. The residue was crystallized from ethanol, yielding nicotinic acid (862 mg, 47%).

Decarboxylation of Nicotinic Acid.-Nicotinic acid (35 mg) was refluxed with quinoline (1 ml) and copper chromite (40 mg) for 20 minutes in a stream of nitrogen. The liberated carbon dioxide was passed through a solution of 2 N sulfuric acid (to remove pyridine vapors) and thence trapped in ethanolamine:methyl cellosolve or barium hydroxide solution. The yield of carbon dioxide in the reaction was 91% of the theoretical yield.

Methyl Nicotinate.-Nicotinic acid (2.8 g) was refluxed with thionyl chloride (15 ml) for 4 hours. The excess thionyl chloride was removed *in vacuo* and methanol (15 ml) added cautiously to the residue. The mixture was warmed at 60°C for 20 minutes and then the excess methanol removed *in vacuo*. The residue was dissolved in water, made basic with sodium carbonate, and extracted with ether. Evaporation of the ether extract afforded methyl nicotinate (3.0260 g, 96%), m.p. 38-40°C.

3-Chloromethylpyridine.-Methyl nicotinate (3.0260 g) in dry ether (25 ml) was added over a period of 30 minutes to a stirred solution of lithium aluminum hydride (2 g) in dry ether (50 ml) maintained at 0°C. After 2 hours, water was added to decompose the excess lithium aluminum hydride. Sodium hydroxide solution (20%, 2 ml) was added and the precipitate of sodium aluminate was removed by filtration and washed with ether. The combined filtrates were dried

with magnesium sulfate, and evaporated to dryness *in vacuo* yielding 3-hydroxymethylpyridine (1.74 g). This alcohol was added dropwise to thionyl chloride (10 ml) cooled to -80°C. The mixture was allowed to warm to room temperature and stirred for 17 hours. The excess thionyl chloride was removed by evaporation *in vacuo*; final traces were removed by dissolving the residue in benzene and evaporating to dryness. Crystallization from ethanol and ether yielded 3-chloromethylpyridine (1.76 g, 63%), m.p. 125-127°C.

3-Methylpyridine.-3-Chloromethylpyridine (1.76 g) dissolved in absolute ethanol (50 ml) was hydrogenated in the presence of 5% palladium-on-calcium carbonate catalyst (5.0 g) for 90 minutes at a hydrogen pressure of 2 atmospheres. The mixture was filtered, and concentrated hydrochloric acid (1 ml) added to the filtrate. The filtrate was evaporated to dryness, the residue dissolved in a small amount of water, and made strongly alkaline with potassium hydroxide. The solution was extracted with ether, the ether layer was dried over potassium hydroxide and evaporated to near dryness to yield 3-methylpyridine. The 3-methylpyridine was dissolved in ether (25 ml) and mixed with a solution of oxalic acid (0.60 g) in acetone (5 ml) resulting in the precipitation of the acid-oxalate salt of 3-methylpyridine (1.83 g), m.p. 119-120°C. The oxalate salt was mixed with freshly prepared calcium oxide (1.5 g), and heated under reduced pressure to distil dry 3-methyl pyridine (806 mg,

63%).

3-Methyl-2-phenylpyridine (45,46).-3-Methyl pyridine (806 mg, 8.7 mmoles) in dry ether (25 ml) was added dropwise to a stirred solution of phenyllithium (87 mmoles) in dry ether (150 ml) in a nitrogen atmosphere. The reaction mixture was maintained below 24°C throughout the preparation. After one hour, dry oxygen was bubbled through the mixture until a white suspension formed. The mixture was treated carefully with water, made strongly alkaline with potassium hydroxide, and extracted with ether. The ether extract was evaporated to a smaller volume (50 ml) in vacuo and extracted with 2 N hydrochloric acid (4 X 25 ml). The combined acid fractions were made strongly alkaline with potassium hydroxide and extracted with ether. The ether was removed on a rotary evaporator and the residue distilled at reduced pressure and 160°C to yield 3-methyl-2-phenyl-pyridine (188 mg, 12.8%).

3-Methyl-2-phenylpyridine Methiodide.-3-Methyl-2-phenyl pyridine (188 mg) was dissolved in ethyl acetate (20 ml) and methyl iodide (2 ml) added. The solution, on standing, afforded 3-methyl 2-phenylpyridine methiodide, m.p. 185-186°C. (Friedman and Leete (34) report m.p. 178-179°C. for this compound.)

1,3-Dimethyl-2-phenylpiperidine.-3-methyl-2-phenyl-pyridine methiodide (1.21 g) was dissolved in methanol (50 ml)

and hydrogenated at atmospheric pressure in the presence of platinum oxide (200 mg) for 10 hours. Evaporation of the filtered reaction mixture and distillation of the residue at reduced pressure yielded 1,3-dimethyl-2-phenylpiperidine (560 mg, 76%).

Kuhn-Roth Oxidation of 1,3-Dimethyl-2-phenylpiperidine.-1,3-Dimethyl-2-phenylpiperidine (70 mg) dissolved in 10% sulfuric acid (2 ml) was added to a refluxing solution of chromic oxide (5.0 g) in 10% sulfuric acid (12 ml). Distillation was started immediately. The volume of the reaction mixture was maintained at about 14 ml by the addition of water. The distillate (40 ml) was extracted with ether (2 X 30 ml). The combined ether extracts were extracted with water (2 X 30 ml). These two water extracts were then re-extracted with ether (2 X 30 ml). The combined ether extracts were dried with magnesium sulfate, filtered, and the filtrate evaporated to near dryness, in vacuo. The residue was sublimed, yielding benzoic acid (5.7 mg), m.p. 119-121°C. The combined aqueous extracts were titrated with 0.03227 N sodium hydroxide (10.95 ml) and evaporated to dryness. The residue was treated three times with absolute ethanol, the ethanol being removed each time by evaporation, in vacuo. The residue was finally treated with absolute ether and the ether evaporated to yield dry sodium acetate. Decarboxylation of the sodium acetate was performed using the Schmidt reaction as described previously.

1,3-Dimethyl-2-phenylpiperidine Methiodide.-The piperidine derivative (490 mg) was dissolved in ethyl acetate (20 ml) and methyl iodide (2 ml) added. On standing overnight, 1,3-dimethyl-2-phenylpiperidine methiodide (819 mg), m.p. 176-177°C precipitated.

1-Dimethylamino-4-methyl-5-phenylpentane Methiodide.-1,3-Dimethyl-2-phenylpiperidine methiodide (1.02 g) was dissolved in liquid ammonia (50 ml) and sodium metal (0.5 g) added slowly. After the ammonia had evaporated, fresh ammonia (50 ml) and sodium (0.25 g) were added, and the ammonia was allowed to evaporate. Water (25 ml) was added cautiously, to dissolve the residue. The solution was extracted with ether (4 X 25 ml), the ether layer dried with potassium hydroxide and the ether evaporated, in vacuo. Gas chromatography of the residue on a column (5 ft. X 0.25 in. O.D.), packed with 20% SE-30 on 60/80 mesh firebrick, at 185°C and a helium flow rate of 60 ml per minute gave a single peak with an emergence time of 12 minutes. The residue was dissolved in ethyl acetate (10 ml) and methyl iodide (3 ml) added. On standing overnight, 1-dimethylamino-4-methyl-5-phenylpentane methiodide (1.0147 g, 95%), m.p. 115-117°C precipitated.

4-Methyl-5-phenyl-1-pentene.-

(a) 1-Dimethylamino-4-methyl-5-phenylpentane methiodide (1.015 g) was dissolved in water (5 ml) and placed on a Dowex 1 X 10 (OH^-) column (6.5 cm X 1 cm). The column was

eluted with water (10 ml) and the eluate evaporated to dryness *in vacuo*. The residue was distilled at 150°C and reduced pressure to yield 4-methyl-5-phenyl-1-pentene (200 mg, 39.4%).

(b) The methiodide[1] (826 mg, 2.39 mmoles) was dissolved in water (10 ml) and shaken for 20 minutes with freshly prepared silver hydroxide (from 0.8 g of silver nitrate). The mixture was filtered rapidly and the filtrate evaporated to dryness *in vacuo*. The residue was distilled at 150°C and reduced pressure to yield the pentene derivative (225 mg, 54.3%).

Oxidation of the 4-Methyl-5-phenyl-1-pentene.-The pentene derivative (87 mg) was dissolved in ether (5 ml) and water (5 ml) added. The solution was cooled to 0°C and a crystal of osmium tetroxide (30 mg) added. Sodium metaperiodate (350 mg) in water (5 ml) was added over a period of 30 minutes to the rapidly stirred solution. The stirred solution was allowed to react overnight at room temperature. Sodium iodate was removed by filtration and the filtrate extracted with ether. The aqueous fraction was distilled into a saturated aqueous solution of dimedone (75 ml), from which on standing overnight, the formaldehyde dimedone derivative (87.5 mg, 50%) crystallized. Evaporation of the dried ether extract afforded 3-methyl-4-phenylbutanal.

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[1]Prepared from fusaric acid derived from an acetate-2-C^{14} feeding experiment.

3-Methyl-4-phenylbutanoic Acid.[1]-3-Methyl-4-phenylbutanal (ca. 200 mg, 1.2 mmole) was dissolved in acetone (10 ml) and a solution of chromium trioxide (1.2 mmole) in 10% sulfuric acid (1 ml) added rapidly with stirring. After 3 minutes, water (20 ml) was added, the solution saturated with sodium chloride and extracted with ether (4 X 25 ml). The combined ether extracts were extracted with aqueous 5% sodium bicarbonate, which was then acidified with sulfuric acid and extracted with ether. The dried ether extract was evaporated to dryness, dissolved in water and titrated to pH 8.0 with 0.03227 N sodium hydroxide solution (34.1 ml). The solution was evaporated to dryness, yielding the sodium salt of 3-methyl-4-phenylbutanoic acid (160 mg, 0.81 mmole).

Schmidt Reaction on 3-Methyl-4-phenylbutanoic Acid.[1]-The sodium salt of the acid (160 mg, 0.81 mmole) was reacted with sulfuric acid and sodium azide as described previously, (p.61) except that the temperature of the reaction mixture was maintained at 45°C for 1 hour. The evolved carbon dioxide was trapped in a barium hydroxide solution yielding barium carbonate (53.0 mg, 0.27 mmole).

Oxidation of 2-Methyl-3-phenylpropylamine.[1]-The residue from the Schmidt reaction on 3-methyl-4-phenylbutanoic acid was dissolved in water (5 ml), made basic with saturated sodium hydroxide solution and extracted with ether. The ether extract was dried with magnesium sulfate, filtered, and the

filtrate evaporated to near dryness *in vacuo*. The residue was treated with 5% potassium permanganate solution (5 ml) for 3 hours. Excess permanganate was decomposed by the addition of methanol and the manganese dioxide removed by filtration. The filtrate was acidified with sulfuric acid, extracted with ether, and the dried ether evaporated *in vacuo*. The residue was dissolved in water and titrated with 0.03227 N sodium hydroxide solution (9.4 ml). The solution was evaporated to dryness and treated with absolute ethanol and ether, as described previously, to yield the sodium salt of 2-methyl-3-phenylpropanoic acid (58.1 mg, 0.31 mmoles).

Schmidt Reaction on 2-Methyl-3-phenyl-propanoic Acid.[1]-The sodium salt of the acid (58.1 mg) was treated with 100% sulfuric acid (0.2 ml) and sodium azide (40 mg) and reacted as described previously (p.61) to yield barium carbonate (19.4 mg, 32%).

RESULTS AND DISCUSSION

Acetate-1-C^{14} and acetate-2-C^{14} were efficient precursors of fusaric acid in G. fujikuroi (Table I). The per cent of added C^{14} found in fusaric acid from the two precursors is almost identical, both on the basis of overall incorporation, and incorporation per gram of fusaric acid produced.

Distribution of Activity in Fusaric Acid from Acetate-C^{14} Feedings

If fusaric acid is derived from a head to tail condensation of five acetate units as proposed by Leete (10), then carbons 2, 4, 6, 8 and 10 of the molecule should be derived from the carboxyl group of acetate, with the remaining carbons formed from the methyl group of acetate. Since five acetate units are required, each labelled position from either acetate-1-C^{14} or acetate-2-C^{14} should contain one-fifth, or 20% of the total activity present in the molecule.

A condensation of aspartic acid, or a closely related metabolite, with three acetate units would produce a similar pattern of labelling, with the exception of carbons 2 and 7 of fusaric acid. Aspartic acid and oxalacetic acid are readily interconvertible through transamination, while oxalacetate is formed from acetate, via the citric acid cycle or the glyoxylate cycle (47). It is known (48) that

TABLE I

INCORPORATION OF ACETATE-C^{14} INTO FUSARIC ACID BY *G. FUJIKUROI*

Compound Supplied	Fusaric Acid			
	Yield, g	Specific Activity, d.p.m./mmole X 10^{-6}	Dilution of Specific Activity	Incorporation %
Acetate-1-C^{14}[a]	1.084	1.07	810	12.9
Acetate-2-C^{14}[b]	1.427	2.0	275	15.1

[a] 5×10^7 d.p.m.; 8.5×10^8 d.p.m./mmole.

[b] 1.06×10^8 d.p.m.; 5.5×10^8 d.p.m./mmole.

the carboxyl group of acetate ultimately forms the carboxyls of oxalacetate, while the methyl group of acetate forms, predominantly, carbons 2 and 3 of oxalacetate. The carboxyl group of acetate would therefore form carbons 4 and 7 of fusaric acid, with the methyl group of acetate forming carbons 2 and 3.

A. Acetate-1-C^{14} Feeding Experiment

The actual distribution of activity in fusaric acid derived from acetate-1-C^{14} is shown in Table II. The specific activity values reported are for undiluted material.

In this experiment, 76.4% of the activity in fusaric acid has been accounted for by direct assay of the individual carbons. There was not sufficient material to determine the per cent activity in C-4 directly, and, therefore, this value was calculated by subtracting the sum of the distribution of activities of carbons 2, 3, 5, 6 and 8 from the distribution of activity determined for nicotinic acid. The total activity accounted for is then 95%. The per cent of the activity in the carboxyl group of fusaric acid may be low, which would then account for the remaining 5%.

The distribution of activity in fusaric acid derived from acetate-1-C^{14} indicates that carbons 7, 4, 6, 8, and 10 are derived from the carboxyl group of acetate.

B. Acetate-2-C^{14} Feeding Experiment

The distribution of activity in fusaric acid derived from acetate-2-C^{14} is shown in Table III. There is 65% of the activity in fusaric acid accounted for by direct assay of the individual carbons. An attempt to determine C-11 directly by oxidation of the methylamine, formed from a Schmidt reaction on sodium acetate derived from C-10 and C-11 of fusaric acid, was not successful. The activity in C-11 was therefore determined by subtracting the distribution of activity obtained for C-10 from the distribution of activity obtained for the sodium acetate.

The per cent activity in the pyridine ring and C-8 was calculated on the basis that 50.2% of the activity present in fusaric acid is found in the 3-methylpyridine moiety. This value may be low because, if the radioactivity in this portion is calculated by subtracting the radioactivity in carbons 7, 9, 10 and 11 from 100%, a value of 57.8% is obtained for 3-methylpyridine. The distribution of activity in carbons 2, 3, 4, 5 and 6 then becomes 12.1, 11.4, 2.54, 19.2 and 0.5% respectively, which is closer to the expected distribution.

If carbons 7, 2, 3 and 4 are formed from a four-carbon dicarboxylic acid and contain 40% of the activity of fusaric acid derived from acetate-2-C^{14}, then the per cent contribution of each carbon would be 6.65, 13.3, 13.3 and 6.65 respectively. The reasons for expecting these values are:

TABLE III

DISTRIBUTION OF ACTIVITY IN THE DEGRADATION PRODUCTS OF FUSARIC ACID SYNTHESIZED FROM ACETATE-2-C^{14} BY G. FUJIKUROI

Compound	Carbon No.	Specific Activity, d.p.m./mmole X 10^{-5}	Distribution of Activity %
Fusaric acid	all	20.0	100
Barium carbonate[a]	7	0.89	4.46
Sodium acetate[b]	10,11	3.6	18.0
Barium carbonate[c]	9	3.94	19.7
Barium carbonate[d]	10	0.12	0.6
(by difference)	11	-	17.4
Barium carbonate[e]	8	0.0	0.0

[a]Obtained from decarboxylation of fusaric acid.

[b]Obtained from Kuhn-Roth oxidation of fusaric acid.

[c]Obtained from a Schmidt reaction on sodium propionate.

[d]Obtained from a Schmidt reaction on sodium acetate.

[e]Obtained from the decarboxylation of nicotinic acid.

TABLE III (continued)

Compound	Carbon No.	Specific Activity, d.p.m./mmole X 10^{-5}	Distribution of Activity %
3-Methylpyridine	2,3,4, 5,6,8	10.0	50.2
Benzoic acid[f]	6	0.08	0.44
Barium carbonate[g]	5	3.34	16.7
Formaldehyde dimedone	2	2.1	10.5
Barium carbonate[h]	3	2.0	10.0
Barium carbonate[i]	4	0.44	2.21

[f]Obtained from Kuhn-Roth oxidation of 1,3-dimethyl-2-phenylpiperidine.

[g]Obtained from a Schmidt reaction on sodium acetate derived from Kuhn-Roth oxidation of 1,3-dimethyl-2-phenylpiperidine.

[h]Obtained from a Schmidt reaction on the sodium salt of 3-methyl-4-phenylbutanoic acid.

[i]Obtained from a Schmidt reaction on the sodium salt of 2-methyl-3-phenylpropanoic acid.

1. The glyoxylate cycle enzymes are probably non existent or at least inoperative in this organism, with glucose being supplied as a carbon source (49).

2. The equilibrium distribution of label in oxalacetate derived from acetate-2-C^{14} via the Kreb's cycle is (50);

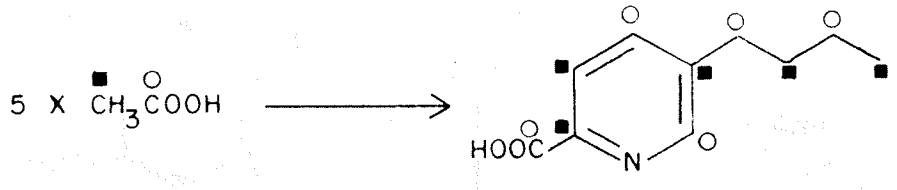
$$\underset{16.7\%}{HOOC}\text{——}\underset{33.3\%}{CH_2}\text{——}\underset{33.3\%}{CO}\text{——}\underset{16.7\%}{COOH}$$

The labelling pattern in fusaric acid derived from acetate-2-C^{14} indicates that carbons 2, 3, 5, 9 and 11 are derived from the methyl group of acetate.

Biogenesis of Fusaric Acid

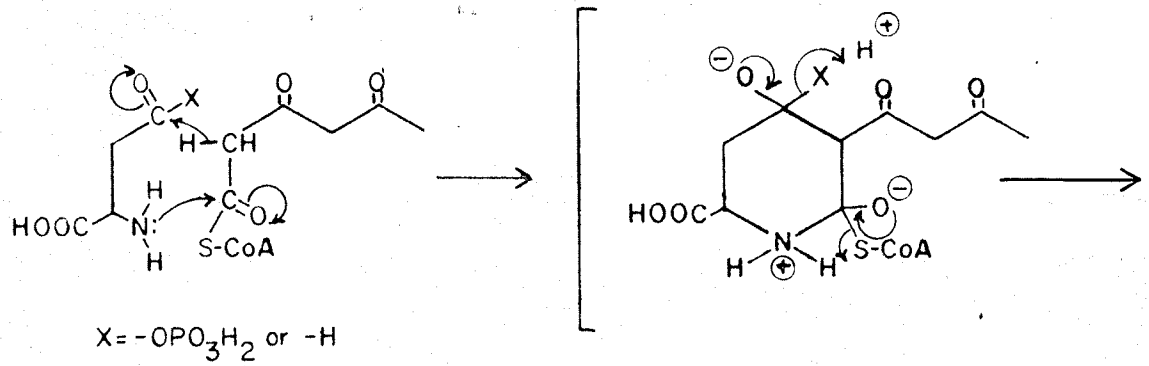
The evidence presented in this work indicates that all the carbons of fusaric acid are derived from acetate or closely related metabolites. The pattern of labelling also suggests that the immediate precursor of positions 1, 2, 3, 4 and 7 of fusaric acid may be aspartic acid. The role of aspartate is further strengthened by the fact that aspartate-1-C^{14} was found to contribute activity only to the carboxyl group and the pyridine ring of fusaric acid (7).

The contribution of acetate to the formation of fusaric acid is summarized in the following diagram:



The experimental results are consistent with the aspartate-polyacetate hypothesis (9) for fusaric acid formation and not with the polyacetate hypothesis of Leete (10).

As an extension to the polyacetate-aspartate condensation, it is possible that aspartate is activated, prior to condensation, to β-aspartyl phosphate or aspartic-β-semialdehyde, which are known intermediates in the formation of homoserine in Neurospora crassa (51) and yeast (52). It is also probable that the polyacetate unit is in the form of a Coenzyme A (CoA-SH) ester at the time of condensation. The condensation step may then take place as described below:



X= $-OPO_3H_2$ or -H

SUMMARY

A study of the biogenesis of fusaric acid was performed by supplying acetate-1-C^{14} and acetate-2-C^{14} to culture media containing Gibberella fujikuroi (Saw.) Wr. After 24 hours incubation with the C^{14}-labelled substrates, fusaric acid was isolated from the media, and the distribution of activity in the molecule determined. The following results were obtained:

1. Thirteen per cent of the added C^{14} from acetate-1-C^{14} was present in fusaric acid; while 15% was found in fusaric acid from acetate-2-C^{14}.

2. The per cent distribution of activity in fusaric acid from the acetate-1-C^{14} feeding was as follows (distribution shown in parentheses): C-2 (2.1); C-3 (2.0); C-4 (18.6); C-5 (0); C-6 (19.3); C-7 (14.2); C-8 (19.4); C-9 (0); C-10 (13.1); C-11 (7.3).

3. The per cent distribution of activity in fusaric acid from the acetate-2-C^{14} feeding was as follows (distribution shown in parentheses): C-2 (10.5); C-3 (10.0); C-4 (2.2); C-5 (16.7); C-6 (0.4); C-7 (4.5); C-8 (0); C-9 (19.7); C-10 (0.6); C-11 (17.4).

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