The Biosynthesis of Citrinin in *Penicillium citrinum*. I. Production and Degradation of Citrinin*

O. R. Rodig, L. C. Ellis, and I. T. Glover

ABSTRACT: A study of the production of citrinin by *Aspergillus niveus* and *Penicillium citrinum* Thom is described. Plots of citrinin production *vs.* time show that the *A. niveus* strain investigated produces a higher yield of the antibiotic than does the *Penicillium sp.* A stepwise chemical degradation of citrinin which is readily adaptable to radioactive tracer techniques is reported. The process involves the degradation of this substance to the known 3-(2-methyl-3,5-dimethoxyphenyl)-2-butanone which is then degraded further by two pathways. One method leads through the ozonization of 3-(2-methyl-3,5-dimethoxyphenyl)-2-methyl-2-butene which in turn is prepared by the action of methylmagnesium iodide on the 2-butanone derivative followed by dehydration. The other process involves the controlled oxidation of the 2-butanone to 3,5-dimethoxyphthalic acid, which is successively mono- and then didecarboxylated. Demethylation of the product yields resorcinol which is degraded further by the bromopicrin method. These schemes permit the radioactivity counting of a larger number of individual carbon atoms than was heretofore possible.

Recent years have seen the widespread acceptance of the polyacetate hypothesis (Birch, 1957; Birch and Donovan, 1953; Robinson, 1955) as a means to explain the biosynthesis of numerous natural substances. Major support for the hypothesis stems from the fact that exogenous acetate added to the growth media is often incorporated into the metabolic products, and this method has, in turn, frequently been used to determine whether the polyacetate scheme is operative in a particular metabolic process. Of basic importance, however, is a question which has received only limited study: namely, if exogenous acetate is incorporated by a metabolite, is it a requirement that the product be built up from acetate, or can in some cases the biosynthetic process take place by some other, perhaps more direct, route.

We have attempted to answer this question with respect to the biosynthesis of citrinin (I), a relatively easily isolated antibiotic produced by the mold *Penicillium citrinum* when grown on a glucose medium. Furthermore, it has been shown by partial degradation studies that citrinin incorporates exogenous acetate in a manner predicted by the polyacetate scheme (Birch *et al.*, 1958; Schwenk *et al.*, 1958). In this case, however, it seemed quite possible to us that citrinin production may not require the complete breakdown of glucose to acetate, particularly in view of its structural similarity to kojic acid (II) which has been shown to arise by the direct conversion of intact glucose (Arnstein and Bentley, 1950, 1953; Denison *et al.*, 1954). Such a genesis would require the introduction of a C_1 unit in the kojic acid structure at the position shown, and the C-13 methyl group of citrinin does indeed arise from C_1 donors, as shown by labeling experiments (Birch *et al.*, 1958; Schwenk *et al.*, 1958). An additional point of interest is that kojic acid is produced by several *Aspergillus sp.*, as also is citrinin (Raistrick and Smith, 1935; Raper and Thom, 1949). The purpose of this work was to study the biosynthesis of this antibiotic from [1-^{14}C]glucose and [6-^{14}C]glucose.

Before undertaking such a study, however, it was necessary to develop a degradative sequence for citrinin more complete than any heretofore reported. The sequence employed is hereby described, along with some additional studies on the conditions for the production of this fungal metabolite.

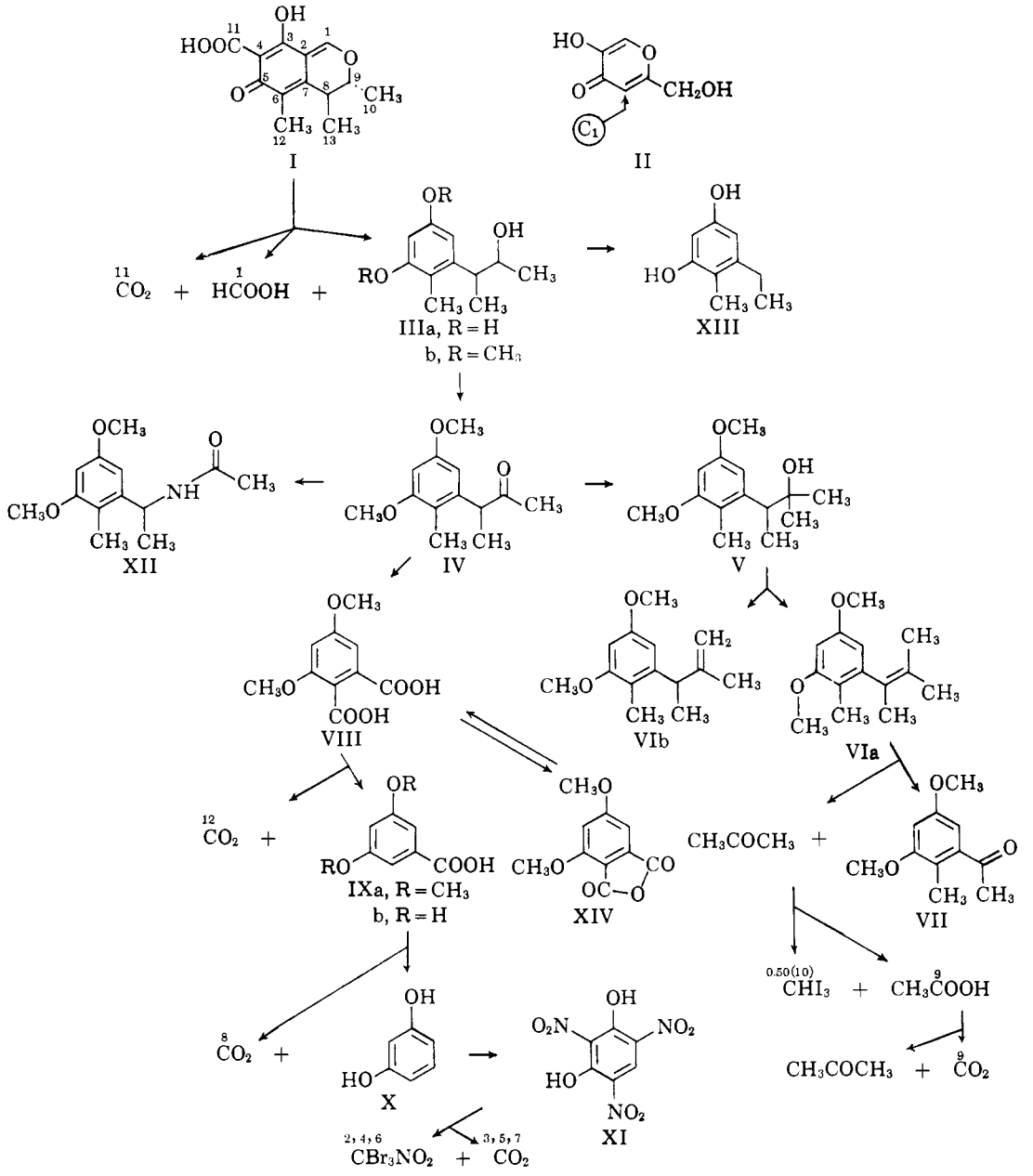
Experimental Procedures and Results

Materials. We are grateful to the Microbiology Section of Smith Kline and French Laboratories, Philadelphia, Pa., for spores of the *P. citrinum* Thom used. A culture of *Aspergillus niveus* NRRL No. 1955 was purchased from the U. S. Department of Agriculture, Agriculture Research Service, Peoria, Ill. Two series of Sabouraud agar slants were inoculated with *P. citrinum* and *A. niveus*, respectively, and incubated from 2 to 3 weeks at room temperature. The tubes were then filled with sterilized mineral oil and refrigerated until needed to inoculate metabolism solutions for citrinin production. Some spores from the *A. niveus* culture were also lyophilized in Pyrex bulbs and sealed off at reduced pressure, but the use of sterile mineral oil and refrigeration proved to be a simpler and quite satisfactory method for preserving the cultures.

Methods. The metabolism solutions were prepared according to Timonin (1942; Timonin and Rouatt,

* From the Department of Chemistry, University of Virginia, Charlottesville, Virginia. *Received March 7, 1966.* This investigation was supported in part by a grant from the National Science Foundation (NSF G6280). A brief account of this work was presented at the International Symposium on the Chemistry of Natural Products, Kyoto, Japan, April 1964.

SCHEME I: The Degradation of Citrinin.



1944) except that twice the quantity of glucose was employed. The medium contained 3.0 g of $NaNO_3$, 1.0 g of KH_2PO_4, 0.5 g of KCl, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.005 g of $Fe_2(SO_4)_3 \cdot 7H_2O$, 0.005 g of $ZnSO_4$, and 80.0 g of anhydrous D-glucose/l. of distilled water. For each growth cycle, 1-l. erlenmeyer flasks were filled with 500 ml of metabolism solution, fitted with paper-covered cotton plugs, and autoclaved for 15 min at 255°F and 19–25 psi. After being cooled, each flask was inoculated with a small quantity of inoculum by means of a sterilized platinum loop. The flasks were incubated at 40° with *A. niveus* and at room temperature with *P. citrinum*, and samples of citrinin were harvested in the usual manner (Schwenk *et al.*, 1958) at different time intervals.

The microanalyses were carried out by Mrs. W. E. Coyne. All melting points were taken in capillary melting point tubes in a silicone oil bath with a calibrated thermometer. Infrared spectra were taken on the Perkin-Elmer Infracord spectrophotometer, Model 21, fitted with a sodium chloride prism. Ultraviolet spectra were taken in 95% ethanol on the Perkin-Elmer spectrophotometer, Model 4000A. The proton magnetic resonance spectra were taken in carbon tetrachloride solution (unless specified otherwise) on a Varian nmr[1] spec-

[1] Abbreviation used: nmr, nuclear magnetic resonance.

trometer Model A-60, with tetramethylsilane as an internal standard.

Chemical Degradations. The degradation scheme used is shown in Scheme I. Superscripts by carbon atoms in the degradative products represent the carbon atoms bearing the same number in the parent molecule, I.

STEPS I–IIIB, ISOLATION OF C-1 AND C-11. The citrinin, 10.27 g, was hydrolyzed in 10% NaOH solution by heating at reflux for 4 hr under an atmosphere of nitrogen (Timonin, 1942; Timonin and Rouatt, 1944). The addition of a saturated solution of $Ba(OH)_2$ to the reaction mixture precipitated $BaCO_3$ (representing C-11 from citrinin), which was filtered, washed with water and acetone, and dried overnight at 102°. The yield of $BaCO_3$ was 6.41 g (79%).

The filtrate was adjusted to pH 7.5–8.0 with 6 N HCl and extracted with ether. The ether extracts were dried over anhydrous Na_2SO_4, concentrated to 150 ml by evaporation under reduced pressure, and filtered through a 12-cm column (i.d. 1.8 cm) of Florisil. Evaporation of the solvent left a residue which crystallized when treated with toluene, giving 7.49 g (93%) of the phenol IIIa. This material was recrystallized from toluene to a constant melting point of 132–134°. The nmr spectrum (CF_3COOH) showed two aromatic protons, 6.57 (multiplet), one proton adjacent to OH group, 4.19 (slightly perturbed quintet), one tertiary proton, 3.28 (slightly perturbed quartet), one aromatic methyl group, 2.20 (singlet), two aliphatic methyl groups, 1.41 (doublet) and 1.23 ppm (doublet).

Anal. Calcd for $C_{11}H_{16}O_3$ (196.2): C, 67.32; H, 8.22. Found: C, 67.29; H, 7.98.

The remaining aqueous layer was treated with an excess of saturated $Ba(OH)_2$ solution, filtered to remove the $BaCO_3$, acidified with 6 N HCl, and heated at reflux for 2 hr with 10.0 g of HgO (Schwenk *et al.*, 1958). Carbon dioxide arising from the oxidation of formic acid (representing C-1 from citrinin) was trapped as $BaCO_3$ and purified as described above. The yield was 0.23 g (3%).

The phenol IIIa (7.49 g) was methylated in good yield when it was heated at reflux for 24 hr with 15.0 g of K_2CO_3 and 15.0 g of methyl iodide (an additional 3.0 g of methyl iodide was added after 12 hr) in 300 ml of acetone (Timonin, 1942; Timonin and Rouatt, 1944). The reaction mixture was cooled and filtered, and the acetone was removed at 45–50° under reduced pressure. The residue was dissolved in 200 ml of ether, washed with 10% NaOH solution, and dried over anhydrous Na_2SO_4.

The NaOH washings were acidified with 6 N HCl, extracted with ether, and evaporated to give unchanged phenolic material which was recycled through the methylation procedure and the product combined with that obtained above. Removal of the ether under reduced pressure gave 7.26 g (85%) of the methylated product IIIb as an oil, bp 152–155° (3.2 mm) [lit. (Hetherington and Raistrick, 1931) bp 136–138° (1 mm)]. The nmr of this compound showed two aromatic protons, 6.39 (doublet) and 6.23 (doublet), two methoxy methyl groups, 3.69 (singlet), one proton adjacent to OH group, multiplet centered at about 3.8 and partially masked by the methoxy methyl peak, one tertiary proton, 3.00 (quintet), one alcoholic proton, 2.58 (br singlet), one aromatic methyl group, 2.10 (singlet), and two aliphatic methyl groups at 1.12 (doublet) and 1.10 ppm (doublet).

ATTEMPTS TO ISOLATE C-9 AND C-10 FROM IIIB. (A) The iodoform reaction was unsuccessful with the alcohol IIIb (and the ketone IV). The failure of this reaction might be due to the activated hydrogen atom positioned between the aromatic ring and the potential carbonyl group, or to preferential attack on the benzylic methyl group activated by the two aromatic methoxyl groups. (B) Dehydration of the secondary alcohol IIIb, under conditions which avoid rearrangement,[2] followed by ozonolysis, would provide a two-carbon fragment of C-9 and C-10 from citrinin as acetaldehyde or acetate. Attempts to dehydrate IIIb in pyridine with thionyl chloride, tosyl chloride, or phosphorus oxychloride under mild conditions gave back starting material, while more vigorous conditions yielded products containing halogen (Beilstein test) and which lacked the hydroxyl group and extended conjugation as indicated by infrared and ultraviolet absorption spectroscopy. However, attempted dehydrohalogenations of these substances under a variety of basic conditions gave back the starting material (infrared spectrum), accompanied by little or no olefin.

STEP IIIA–XIII. It was possible to effect the simultaneous removal of C-9 and C-10 from IIIa by fusing this alcohol with pulverized potassium hydroxide (Hetherington and Raistrick, 1931) whereby 4-methyl-5-ethylresorcinol (XIII) was obtained. Attempts to isolate C-9 and C-10 as acetic acid were unsuccessful, the potassium acetate formed in the reaction possibly decomposing to a large extent to potassium carbonate and methane (Oakwood and Miller, 1950). The fusion was carried out essentially by the method described previously (Hetherington and Raistrick, 1931) except that the reaction mixture was acidified with dilute HCl and the product was obtained by ether extraction and purified by recrystallization from water. In a typical run, in which the mixture was heated at 305–310° for 55 min, 0.57 g of the alcohol IIIa (as the chloroform solvate) (Birch *et al.*, 1958) yielded 0.25 g (67%) of the resorcinol XIII hydrate, mp 65–66° [lit. (Brown *et al.*, 1949) mp 66–68°].

STEP IIIB–IV, ATTEMPTS TO ISOLATE C-9 AND C-10 FROM IV. The oxidation of the alcohol IIIb was carried out with CrO_3 in pyridine according to the procedure described by Poos *et al.* (1953). Chromium(VI) trioxide (30.5 g) was added portionwise with stirring to 320 ml of pyridine cooled in an ice bath. The temperature was kept below 20° for the first 20 g of CrO_3 added and below 30° for the remaining 10.5 g. A solution of 7.26

[2] The demonstration that the 3-phenyl-2-butanol system is racemized in acid solution *via* phenyl migration (Cram, 1950) precludes the use of acidic catalysts for this dehydration because of the possibility that radioactive carbon atoms might scramble in the process.

g of the alcohol IIIb in 73 ml of pyridine was added, and the mixture was allowed to stand at room temperature for 24 hr. The solution was poured into 300 ml of water which was extracted with a 1:1 mixture of benzene–ether. The combined extracts were washed with 6 N HCl until the washings were acid to congo red paper, washed once with water, and dried over anhydrous Na_2SO_4. Removal of the solvent *in vacuo* gave 7.12 g of a dark brown oil which was dissolved in 250 ml of dry ether and percolated through a 12-cm column of Florisil (i.d. 1.8 cm). Removal of the ether under reduced pressure gave 6.73 g (94%) of the ketone as a pale yellow oil.

The infrared spectrum of this material was void of hydroxyl absorption and showed a prominent carbonyl band at 1715 cm^{-1}. The nmr spectrum contained peaks indicative of two aromatic protons, 6.33 (doublet) and 6.15 (doublet), one tertiary proton, 3.89 (quartet partially masked by the methoxy methyl peaks), two methoxy methyl groups, 3.77 (singlet) and 3.70 (singlet), one aromatic methyl group, 2.13 (singlet), a methyl group adjacent to a carbonyl, 1.90 (singlet), and an aliphatic methyl group, 1.26 ppm (doublet). The semicarbazone of the ketone IV was prepared from the product obtained from a similar run, mp 198–8.5° [lit. (Frye *et al.*, 1949) 194.8°].

Two other general procedures were studied to effect this oxidation, but both were rather less satisfactory. Frye *et al.* (1949) reported carrying out this oxidation with aluminum isopropoxide and acetone, but did not specify the yield of the product they obtained. In our hands, this procedure gave back impure starting material containing a small carbonyl peak in the infrared spectrum which in every case was estimated to betoken less than a 10% yield of the ketone. The yield of ketone was improved (29%) by using potassium *t*-butoxide as the base and 3-pyridyl phenyl ketone (Villani and King, 1957) as the hydrogen acceptor.

The second method employed a CrO_3–H_2SO_4 mixture in acetone (Bowden, 1946) as the oxidizing agent, whereby the ketone was obtained in yields up to 42%. None of these procedures, however, approached that of Poos *et al.* (1953) in yields or simplicity of operation.

Another approach centered on the Beckmann rearrangement of the oxime of the ketone IV which should give the corresponding acetamide derivative XII. Hydrolysis of this material would then afford C-9 and C-10 as acetate. The oxime (mp 125–126°) was obtained in good yield when IV was treated with $NH_2OH \cdot HCl$ in an aqueous solution of NaOAc. However, attempts to rearrange this ketoxime with concentrated H_2SO_4 (Donaruma and Heldt, 1960), or with concentrated H_2SO_4 in anhydrous ether (Campbell and Kenyon, 1946), produced noncrystalline material from which only the original ketoxime could be isolated.

The amide XII was obtained in 29% yield, however, when 3.42 g of the ketone IV was treated with a 0.85 N solution of hydrazoic acid in chloroform under the conditions of the Schmidt reaction (Wolff, 1946). When the products were chromatographed on Fisher activated alumina, an oil was eluted with toluene–ether (1:1) which crystallized when treated with petroleum ether (bp 30–60). Recrystallization of this product from cyclohexane yielded 1.04 g (29%) of the amide, mp 112.5°.

Anal. Calcd for $C_{13}H_{19}NO_3$ (237.3): C, 65.80; H, 8.07. Found: C, 65.51; H, 7.75.

Although the microanalysis and infrared spectrum of this material indicated that the product was the desired amide, XII, attempts to hydrolyze the compound with aqueous or alcoholic sodium or potassium hydroxide consistently failed. On the other hand, when XII was heated at reflux with 60% H_2SO_4 for 1 hr or concentrated HCl for 30 hr, a basic fraction was obtained whose infrared spectrum showed characteristic amine absorption, as well as an acidic fraction which had the odor of acetic acid. Yields of these materials were quite low, however, and the isolation and purification of definite compounds was not possible. Because of these unpromising results, this degradative route was discontinued.

STEPS IV–VII, ISOLATION OF C-9 AND C-10. The difficulties encountered with the above described degradation attempts prompted the investigation of yet another scheme. The ketone IV was converted to the alcohol V, which could then be dehydrated under *acidic* conditions since a rearrangement,[2] if it did occur, would probably be readily discernible from the products obtained.

High yields of the tertiary alcohol were obtained by the familiar Grignard synthesis with methylmagnesium iodide. Methyl iodide (3.91 g) in 10 ml of anhydrous ether was added dropwise with vigorous stirring to 0.66 g of Mg turnings at such a rate as to cause gentle refluxing. The mixture was then heated at reflux for an additional 0.5 hr and cooled, and a solution of 3.0 g of ketone IV in 10 ml of anhydrous ether added from a dropping funnel at a rate which maintained gentle refluxing. When the addition was complete, the mixture was refluxed for 2 hr with vigorous stirring, accompanied by the occasional addition of anhydrous ether to replace that lost during the heating period.

The mixture was cooled and poured onto a mixture of ice and 6 N HCl, and the layers were separated. The aqueous layer was extracted with ether, and the combined ether extracts were washed with a saturated solution of $Na_2S_2O_3$ and water and dried over anhydrous Na_2SO_4. Removal of the ether under reduced pressure gave a product, the infrared spectrum of which showed a prominent hydroxyl stretching band at 3420^{-1}, and a slight shoulder at 1715 cm^{-1}, indicative of a small amount of unchanged ketone. The proton magnetic resonance spectrum of the alcohol is in accord with structure V, showing two aromatic protons, 6.47 (doublet) and 6.23 (doublet), two methoxyl groups, 3.72 (singlet) and 3.70 (singlet), one tertiary proton, 3.09 (quartet), one aromatic methyl group, 2.09 (singlet), and three aliphatic methyl groups, 1.24 (doublet, partially masked under a singlet at 1.19), 1.19 (singlet) and 1.08 ppm (singlet).

This material, 2.94 g (93%, without further purification), was dehydrated in a solution of 6 ml of con-

centrated H_2SO_4 and 9 ml of water by heating to gentle reflux in a wax bath (bath temperature 148–151°) for 2 min. After cooling, ice was added, and the mixture was extracted with ether. The combined ether extracts were washed with 10% NaOH solution, then with water, and dried over anhydrous Na_2SO_4, and the ether was removed under reduced pressure. The residue was percolated with 300 ml of low-boiling petroleum ether through a 12-cm column (i.d. 1.8 cm) of Florisil beneath 2 cm of neutral alumina. Evaporation of the eluent solvent gave an oil, the infrared spectrum of which showed no hydroxyl or carbonyl bands. The proton magnetic resonance spectrum of this material showed it to be a mixture of 3-(2-methyl-3,5-dimethoxyphenyl)-2-methyl-2-butene (VIa) and 3-(2-methyl-3,5-dimethoxyphenyl)-2-methyl-1-butene (VIb) in the ratio of approximately 3:1, respectively. The nmr spectrum showed the following: aromatic protons, 6.2, vinyl protons, 4.85, methoxyl protons, 3.7, methyl protons, between 1.2 and 2.2 ppm. The integrated ratio of aromatic protons to vinyl protons was 4:1, respectively.

Elution of the column with 300 ml of dry ether gave an oil which proved to be starting material (infrared spectrum). This material was dehydrated as described earlier, giving additional VIa and b. The total yield of the mixed alkenes was 2.30 g (85%).

Ozonolysis of the alkenes was conducted according to the procedure of Cram (1949). Ozone was passed through an ice-cooled solution of 2.30 g of the mixed alkenes, VIa and b, in 60 ml of ethyl acetate for 90 min, and the solution then added dropwise to a boiling mixture of 3.0 g of zinc dust in 150 ml of water containing a trace of silver nitrate and hydroquinone. Acetone and formaldehyde from the decomposition of the mixed ozonides distilled over with the solvent and were collected in an ice-cooled flask containing 30 ml of a saturated solution of $NaHSO_3$. The aqueous layer was removed, the remaining ethyl acetate layer was extracted with saturated $NaHSO_3$ solution, and the extracts were added to the aqueous layer.

Solid iodine and KI were added in approximately equal amounts to the $NaHSO_3$ solution until no further decolorization of the iodine occurred. This solution was treated with 40% NaOH solution until distinctly basic, followed by a solution of 10.0 g of iodine and 20.0 g of KI in 100 ml of water until no further decolorization of iodine occurred, and then heated at 50–60° for 2 hr. After cooling, the precipitated iodoform was separated by filtration and recrystallized from an ethanol–water mixture. The yield of iodoform was 0.94 g (23%), mp 119–120.5° [lit. (Heilbron and Bunbury, 1936) 119°].

The filtrate from the iodoform reaction was reduced to 150 ml on a steam bath, and concentrated H_3PO_4 was added until the solution was acid to congo red paper. Solid $Na_2S_2O_3$ was added to remove molecular iodine, and the solution steam distilled until 500 ml of distillate had been collected. Mercuric oxide (5.0 g) and 20 ml of 50% H_3PO_4 were added, and the mixture was boiled until no more carbon dioxide was evolved. The solution was steam distilled until 500 ml of distillate had been collected, made basic to phenolphthalein by the addition of small portions of solid LiOH, and the solvent was evaporated at 60–70° under reduced pressure. The residue was pyrolyzed at 360–380° for 45 min (Calvin *et al.*, 1949), and after being cooled, 10 ml of concentrated HCl was added from a dropping funnel. The evolved carbon dioxide (representing C-9 from citrinin) was collected in a $Ba(OH)_2$ trap. The $BaCO_3$ was filtered, washed with water, then with acetone, and dried at 102°. The yield was 0.35 g (34%).

The original decomposition solution, containing nonvolatile ketones IV and VII, was extracted with ether and the ether extracts were dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The oily residue was chromatographed on neutral alumina by elution with 100-ml portions of petroleum ether, a 1:1 mixture of petroleum ether–benzene, and benzene, 20-ml fractions being taken. Ketone IV was obtained on evaporation of the last 40 ml of the petroleum ether–benzene eluent, mixed ketones IV and VII from the first 20 ml of benzene eluent, and ketone VII from the remaining benzene fractions. The mixed ketone fraction was rechromatographed, giving a total yield of 0.62 g (33%) of ketone VII, obtained as a noncrystallizable oil.

The structure of ketone VII was established by its proton magnetic resonance spectrum and by the microanalysis of the semicarbazone (mp 170–170.5°) which was crystallized to analytical purity from ethanol.

Anal. Calcd for $C_{12}H_{17}N_3O_3$ (251.3): C, 57.35; H, 6.82; N, 16.72. Found: C, 57.41; H, 6.88; N, 16.44.

The nmr spectrum showed two aromatic protons, 6.55 (doublet) and 6.45 (doublet), two methoxy methyl groups, 3.80 (singlet) and 3.77 (singlet), two methyl groups, 2.44 (singlet) and 2.16 ppm (singlet).

STEPS IV–IXA, ISOLATION OF C-12. A major problem encountered in the degradative sequence was the oxidation of the alcohol IIIb or the ketone IV to 3,5-dimethoxyphthalic acid (VIII). Oxidation of IIIb with $KMnO_4$ has been described in the literature (Brown *et al.*, 1949), but no yields were recorded for the process. In our hands this method gave poor yields of mixtures of the diacid VIII and a previously reported lactone (Brown *et al.*, 1949; Hetherington and Raistrick, 1931). One run yielded 39% of the diacid, but later attempts could not duplicate these results. The reaction was conveniently followed by ultraviolet absorption spectroscopy because the alcohol IIIb, the diacid VIII, and the lactone all absorb at different wavelengths in this region of the spectrum. It was found that the characteristic peak of the diacid soon reached a constant low extinction value while that of the starting material continually diminished, indicating that the reaction was proceeding to a point where the diacid was being destroyed (by oxidation of the aromatic ring) at the same rate that it was being produced. Because of this unpromising result, a more efficient oxidizing agent was sought.

Oxidation with alkaline $K_3Fe(CN)_6$ was found to give reproducible yields of the diacid VIII from 26 to 37% and consequently was adopted for this step of the degradation. The procedure is exemplified by the fol-

lowing run. A mixture of 1.42 g of ketone IV, 76.5 g of KOH, 475 g of $K_3Fe(CN)_6$, and 2.0 l. of water was heated with stirring on a steam bath (Leete and Nemeth, 1960). Water was added occasionally to replace that lost by evaporation, and further amounts of 76.5 g of KOH and 475 g of $K_3Fe(CN)_6$ were added at the end of 12, 24, and 36 hr. After 72 hr, the reaction mixture was cooled, the precipitate was removed by filtration and washed with cold water, the washings were combined with the filtrate, and the volume was reduced to 2 l. on a steam bath. The solution was acidified in a well-ventilated hood with 50% H_2SO_4, heated on a steam bath (in hood) to remove HCN, and after being cooled, filtered to remove the precipitated Prussian blue. The filtrate and the precipitate were continuously extracted for 48 hr with ether, the ether extracts evaporated, and the residue was sublimed at 148–150° (0.9 mm) to give the anhydride of VIII which was further purified by recrystallization from a benzene–petroleum ether mixture, mp 149–150.5° [lit. (Brown *et al.*, 1949) 149°]. This product (XIV) was identical with that obtained by the oxidation of IIIb with potassium permanganate (Ellis, 1962). The yield was 0.366 g (28%). By a similar procedure, the ketone VII also yielded the same product.

The anhydride, 0.366 g, was heated at reflux for 1 hr with 5 ml of 10% aqueous NaOH, acidified with 6 N HCl to pH 1–2, and refluxed for an additional 1.5 hr. The evolved carbon dioxide (representing C-12 from citrinin) was trapped as $BaCO_3$, which was filtered and washed with water, then with acetone, and dried at 102°. The yield was 0.318 g (92%). The reaction mixture was refrigerated overnight and filtered to give 0.309 g (96%) of 3,5-dimethoxybenzoic acid (IXa), mp 184–185° [lit. (King *et al.*, 1953) 185°].

STEPS IXA TO BROMOPICRIN AND CARBON DIOXIDE. ISOLATION OF C-2,4,6 AND C-3,5,7. The demethylation of 3,5-dimethoxybenzoic acid (IXa) has been carried out in 40–73% yields by refluxing this compound in glacial HOAc and HI mixtures (Ellis, 1962); however, the product was quite difficult to purify because of the production of molecular iodine during the reaction. The demethylation proceeded readily and gave a purer product when carried out with these reagents under a nitrogen atmosphere with an added trace of $NaH_2PO_2 \cdot H_2O$.

3,5-Dimethoxybenzoic acid (287 mg) was heated at reflux under nitrogen for 5 hr with 5 ml of 47% HI in 5 ml of glacial HOAc containing a pinch of $NaH_2PO_2 \cdot H_2O$. The solvent was removed by distillation under reduced pressure, and 5 ml of benzene was added and distilled from the residue to remove molecular iodine. The remaining crude product was recrystallized from a benzene–ether mixture giving 174 mg (61%) of the dihydroxy acid, IXb, as the 1.5 hydrate, mp 229–232° [lit. (Weston and Suter, 1941) 231–232°].

The decarboxylation of 3,5-dihydroxybenzoic acid has been reported to occur in 100% yield when this substance is heated in water or aniline (Hemmelmeyr and Meyer, 1926). The latter reaction was subsequently reported to be unsuccessful (Beilstein, 1949).

In the present study, several attempts to decarboxylate the acid in either of these media resulted in little or no reaction. Thus, when the acid was heated at reflux for 1 hr in water or for 14 hr in 1 N HCl or 5% aqueous KOH, no resorcinol was obtained, and the starting material was recovered in 80–88% yields. When the acid was heated at reflux for 4 hr in aniline, a 15% yield of 3,5-dihydroxybenzanilide was obtained, along with 60% recovery of starting material. The benzanilide derivative was recrystallized from ethanol–water for analysis, mp 219–220°.

Anal. Calcd for $C_{13}H_{11}NO_3$ (229.2): C, 68.11; H, 4.84; N, 6.11. Found: C, 68.09; H, 5.21; N, 6.07.

The decarboxylation was successfully accomplished by fusing the acid with NaOH (Barth and Schreder, 1879). When recovered starting material was recycled, an over-all conversion as high as 81% was realized by the following procedure. 3,5-Dihydroxybenzoic acid (0.374 g) was pulverized with 3.7 g of NaOH and the mixture was added to a nickel crucible with 0.45 ml of distilled water. The reaction vessel was placed in a Woods metal bath at 260° and heated rapidly to 350–365° for 2 hr. After cooling the mixture to room temperature, 10 ml of distilled water was added, and the resulting solution was acidified to Congo red paper with 6 N HCl and extracted with ether. (The carbon dioxide arising from C-8 of citrinin was not trapped in this process because of the difficulty of excluding atmospheric carbon dioxide from the fusion mixture.) The ether extracts were washed with a saturated solution of $NaHCO_3$ and dried over anhydrous Na_2SO_4.

The $NaHCO_3$ washings were acidified with 6 N HCl and extracted with ether. Removal of the ether *in vacuo* left a brown residue which was pulverized with 1.0 g of NaOH and fused as before. The ether extracts containing the phenolic portion were dried and combined with that obtained previously. Evaporation of the ether from the combined extracts under reduced pressure left a colorless oil which solidified when treated with petroleum ether. Recrystallization of this product from benzene gave 0.130 g (57%) of resorcinol (X), mp 108–109.5° [lit. (Andrews *et al.*, 1926) 109.7°].

The resorcinol was nitrated according to the procedure of Borsche and Feske (1928). Concentrated H_2SO_4 (1.0 ml) was added to 0.130 g of resorcinol, and the mixture was heated at 50–60° in a wax bath with vigorous stirring until the contents had changed to a gray paste. The reaction mixture was maintained at 95–100° on a steam bath for 2.5 hr, and then cooled to 0° in an ice bath. Concentrated HNO_3 (15 drops), followed by 25 drops of fuming HNO_3, was added dropwise to the stirred reaction mixture (addition rate: 1 drop/10 sec with a 15-min interval between the concentrated and fuming HNO_3). The solution was allowed to warm slowly to room temperature overnight and the reaction mixture was then added to 1.5 ml of ice water. The solid which separated was collected by filtration, washed with a small amount of chloroform, and recrystallized from benzene, giving 0.171 g (59%) of styphnic acid (XI), mp 175–176.5° [lit. (Borshe and Fescke, 1928) 175°].

The styphnic acid (0.171 g) was mixed with 8 ml of water, and the system was swept for 5 min with carbon

dioxide free air. The reaction vessel was cooled to 0°, and a cold $Ba(OBr)_2$ solution (prepared by mixing 1.4 ml of bromine with 3.6 g of $Ba(OH)_2$ in 50 ml of water at 0°) was filtered into the vigorously stirred mixture. The reaction mixture was allowed to warm to room temperature and stand for 1 hr. Concentrated HCl (12 drops) was added while the temperature was raised to 40°, and the liberated carbon dioxide (representing C-3,5,7 from citrinin) was trapped as $BaCO_3$, and purified as previously described. The yield was 0.137 g (33%).

The reaction mixture was made basic with 10% aqueous NaOH and steam distilled (Werbin and Holoway, 1956). The aqueous upper layer of the distillate was removed with a pipet, and the bromopicrin which remained was washed three times with water. Methylene chloride (10 ml) was added, and the solution was dried over anhydrous Na_2SO_4, filtered, and concentrated by evaporation at 60° from an open, tared test tube. The bromopicrin (representing C-2,4,6 from citrinin) was allowed to stand in the open test tube for 4 hr at room temperature before reaching a constant weight of 0.282 g (45%).

Discussion

Since considerable variation in the efficiency of citrinin production has been noted, particularly in the case of *A. niveus* (Timonin, 1942; Timonin and Rouatt, 1944),[3] a study was conducted with our *A. niveus* and *P. citrinum* strains to determine the incubation time for an optimum citrinin yield. Growth temperatures of 40 and 25°, respectively, were selected because these are reported to be the most favorable for citrinin genesis (Timonin, 1942; Timonin and Rouatt, 1944; Hetherington and Raistrick, 1931).[3] The *A. niveus* produced the higher yields of the antibiotic, reaching a maximum of about 2.6 g/l. after 14–16 days. These yields collate favorably with those obtained from several strains of this organism[3] investigated by Timonin and Rouatt (1944). In comparison with our results, a time study carried out by these authors revealed that a maximum citrinin yield was attained after approximately 21 days at 40° for the strain they investigated. The yield of citrinin from our *P. citrinum* strain, on the other hand, reached a maximum of about 1.2 g/l. after 35–40 days in the most favorable cases. The efficiency of citrinin production was found to vary somewhat with different generations of the *P. citrinum* mold. This was disclosed in studies with continuing cultures where the mycelial spores obtained at the time of a culture harvest were used to inoculate fresh culture media, the process being repeated through several successive growth cycles.

The described degradation scheme has the advantage that most of the reactions and manipulations are simple to perform, the scheme is readily adaptable to study by tracer techniques, and a number of the products have been previously characterized in the literature. Except for the oxidation of 3-(2-methyl-3,5-dimethoxyphenyl)-2-butanone (IV) the yields of products are generally good. Some of the carbon atoms may be isolated individually: C-1, C-9, C-11, and C-12 as $BaCO_3$: C-10 as iodoform, while alternating carbon atoms of the aromatic ring are obtained simultaneously: C-2,4,6 as bromopicrin and C-3,5,7 as $BaCO_3$. The molar radioactivity of C-13 is conveniently obtained as the difference between the molar activities of VII and VIII, while that of C-8 as the difference between IXa (or IXb) and X (C-8 can be obtained as $BaCO_3$, if particular care is taken to exclude atmospheric carbon dioxide during the fusion of 3,5-dihydroxybenzoic acid with NaOH).

[3] The citrinin-producing organism reported by Timonin (1942) and Timonin and Rouatt (1944) was identified as *A. candidus*, but according to Raper and Thom (1949), the mold used in this study was actually *A. niveus*.

References

Andrews, D. H., Lynn, G., and Johnson, J. (1926), *J. Am. Chem. Soc. 48*, 1276.

Arnstein, H. R. V., and Bentley, R. (1950), *Nature 166*, 948.

Arnstein, H. R. V., and Bentley, R. (1953), *Biochem. J. 54*, 493.

Barth, L., and Schreder, J. (1879), *Ber. 12*, 1258.

Beilstein, F. K. (1949), Handbuch der Organischen Chemie, Vol. X, 2nd Suppl., Richter, F., Ed., Berlin, Springer-Verlag, p 266.

Birch, A. J. (1957), *Fortschr. Chem. Org. Naturstoffe 14*, 186.

Birch, A. J., and Donovan, F. W. (1953), *Australian J. Chem. 6*, 360.

Birch, A. J., Fitton, P., Pride, E., Ryan, A. J., Smith, H. and Whalley, W. B. (1958), *J. Chem. Soc.*, 4576.

Borsche, W., and Feske, E. (1928), *Ber. 61B*, 690.

Bowden, J., Heilbron, I. M., Jones, E. R. H., and Weedon, B. C. L. (1946), *J. Chem. Soc.*, 39.

Brown, J. P., Cartwright, N. J., Robertson, A., and Whalley, W. B. (1949), *J. Chem. Soc.*, 859.

Calvin, M., Heidelberger, C., Reid, J. C., Tolbert, B. M., and Yankwich, P. F. (1949), Isotopic Carbon, New York, N. Y., Wiley, p 248.

Campbell, A., and Kenyon, J. (1946), *J. Chem. Soc.*, 25.

Cram, D. J. (1949), *J. Am. Chem. Soc. 71*, 3883.

Cram, D. J. (1950), *J. Am. Chem. Soc. 72*, 1001.

Denison, F. W., Carson, S. F., and Foster, J. W. (1954), *Bacteriol. Proc.*, 99.

Donaruma, L. G., and Heldt, W. Z. (1960), *Org. Reactions 11*, 57.

Ellis, L. C. (1962), Ph.D. Dissertation, University of Virginia, Charlottesville, Va.

Frye, A. H., Wallis, E. S., and Dougherty, G. (1949), *J. Org. Chem. 14*, 397.

Heilbron, I. M., and Bunbury, H. M., Ed. (1936), Dictionary of Organic Compounds, Vol. II, New York, N. Y., Oxford University, p 377.

Hemmelmeyr, F., and Meyer, T. (1926), *Monatsh. 46*, 143.

Hetherington, A. C., and Raistrick, H. (1931), *Phil.*

Trans. Roy. Soc. London 220B, 269.
King, F. E., Cotterill, C. B., Godson, D. H., Jurd, L., and King, T. J. (1953), *J. Chem. Soc.*, 3693.
Leete, E., and Nemeth, P. E. (1960), *J. Am. Chem. Soc. 82*, 6055.
Oakwood, T. S., and Miller, M. R. (1950), *J. Am. Chem. Soc. 72*, 1849.
Poos, G. I., Arth, G. E., Beyler, R. E., and Sarett, L. H. (1953), *J. Am. Chem. Soc. 75*, 427.
Raistrik, H., and Smith, G. (1935), *Biochem. J. 29*, 606.
Raper, K. B., and Thom, C. (1949), Manual of the Penicillia, Baltimore, Md., Williams and Wilkins, p 353.
Robinson, R. (1955), The Structural Relations of Natural Products, Oxford, Clarendon.
Schwenk, E., Alexander, G. J., Gold, A. M., and Stevens, D. F. (1958), *J. Biol. Chem. 233*, 1211.
Timonin, M. I. (1942), *Science 96*, 494.
Timonin, M. I., and Rouatt, J. W. (1944), *Can. J. Public Health 35*, 80, 396.
Villani, F. J., and King, M. S. (1957), *Org. Syn. 37*, 6.
Werbin, H., and Holoway, C. (1956), *J. Biol. Chem. 223*, 651.
Weston, A. W., and Suter, C. M. (1941), *Org. Syn. 21*, 29.
Wolff, H. (1946), *Org. Reactions 3*, 327.

The Biosynthesis of Citrinin in *Penicillium citrinum*. II. Tracer Studies on the Formation of Citrinin*

O. R. Rodig, L. C. Ellis, and I. T. Glover

ABSTRACT: The following observations were made concerning the production of citrinin by *Penicillium citrinum* Thom. (1) Radioactive citrinin from [1-^{14}C]glucose exhibits a labeling pattern indicative of its biosynthesis from C-2 units, probably *via* an acetate–polymalonate pathway. (2) The use of [6-^{14}C]glucose as substrate produces radioactive citrinin with a labeling pattern identical with that from [1-^{14}C]glucose, illustrating the equivalence of C-1 and C-6 of glucose in this biosynthesis. (3) Labeling of the extraskeletal carboxyl and methyl groups by [methyl-^{14}C]methionine, [1-^{14}C]glucose, and [6-^{14}C]glucose suggests that C-1 and C-6 of glucose contribute to these groups *via* the C_1 metabolic pool involving glucose degraded to methionine or species associated with it. All of these observations are in accordance with the degradation of glucose by the Embden–Meyerhof–Parnas pathway. The unequal labeling of alternating carbon atoms in the citrinin skeleton is interpreted as being due to limited scrambling of the radioactivity and by its dilution with endogenous nonradioactive acetyl- or malonyl-CoA. The decrease in radioactivity of the extraskeletal groups derived from C_1 units likewise reflects a dilution effect; present evidence supports the time sequence of attachment of these groups to the citrinin skeleton as C-11, C-12, and C-13, respectively.

Many fungal metabolites are now viewed as being formed through the polycondensations of acetate, or acetate plus malonate units (Birch and Donovan, 1953; Birch, 1957; Bentley and Keil, 1961; Bu'Lock and Smalley, 1961) while others have been shown to arise from shikimate (Davis, 1951; Sprinson, 1960) or even by the direct conversion of glucose (Arnstein and Bentley, 1950, 1953; Denison *et al.*, 1954). Tracer studies on the biosynthesis of citrinin by *Penicillium citrinum* (Schwenk *et al.*, 1958) and *Aspergillus niveus* (Birch *et al.*, 1958a)[1] employing a partial degradation of the metabolite were in accord with a biosynthesis *via* the "polyacetate scheme" and showed that the extraskeletal methyl groups arise from C_1 donor systems (formate, methionine).

As was brought to notice in paper I of this series (Rodig *et al.*, 1966), the studies of previous workers do not examine the question of whether the breakdown of glucose to acetate is a necessary requirement for the production of citrinin and it was felt that an alternate biosynthetic pathway *via* a conversion of intact glucose could be operating as well. The use of appropriately labeled glucose as substrate should provide an answer to this question and give information regarding the

* From the Department of Chemistry, University of Virginia, Charlottesville, Virginia. *Received March 7, 1966.* This investigation was supported in part by a grant from the National Science Foundation (NSF G6280). A brief account of this work was presented at the International Symposium on the Chemistry of Natural Products, Kyoto, Japan, April 1964.

[1] Birch *et al.* (1958a) reported the mold used in their work as *Aspergillus candidus;* however, Raper and Thom (1949) state that *A. candidus* does not produce citrinin, and that the true identity of the mold is *A. niveus.*