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

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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

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[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.*

metabolic fate of glucose in these mycological systems. Accordingly, the biosynthesis of citrinin and aspects of glucose metabolism in *P. citrinum* were studied by ^{14}C tracer techniques and are herewith described.

Experimental Procedure

Materials. [1-^{14}C]Glucose and [6-^{14}C]glucose were purchased from New England Nuclear Corp., Boston, Mass., and Nichem, Inc., Bethesda, Md., respectively. Spores of *P. citrinum* Thom were obtained through the courtesy of the Microbiology Section of Smith Kline and French Laboratories, Philadelphia, Pa.

Methods. Citrinin production, isolation, and media were described in paper I of this series (Rodig *et al.*, 1966). The radioactive citrinin in this work was produced from *P. citrinum* so as to parallel previous work with this mold (Schwenk *et al.*, 1958). For the production of radioactive material, radioactive glucose was either introduced at the time of initial inoculation or administered after about 1 week of growth. Comparable results were obtained by both methods and a typical procedure follows.

Two successive generations of *P. citrinum* were used as inoculum in this case because the maximum yield of citrinin was found to vary with different generations of the mold (Rodig *et al.*, 1966). Flasks (23, 1 l, each containing 500 ml of metabolism solution) were inoculated, 12 with first generation mold, and 11 with second generation mold, and the cultures were allowed to grow at room temperature. After 7 days, a solution of 19.5 mg (0.2 mc) of [6-^{14}C]glucose in 50 ml of distilled water was autoclaved under the conditions described previously (Rodig *et al.*, 1966) and 10 ml (0.04 mc) of this solution was introduced into each of two flasks of the first generation series and into each of three flasks of the second generation series by means of a sterilized pipet.

From one of the second generation flasks, the respiratory CO_2 evolved during the growth cycle was trapped and collected as $BaCO_3$ at periodic time intervals by slowly sweeping air through an Ascarite column into the flask and from there into a $Ba(OH)_2$ trap. The $BaCO_3$ was washed with water and acetone, and was dried at 102° overnight. Samples of 100 mg each were then pressed into planchets (1-cm^2 area) and counted to 10,000 counts.

The flasks containing nonradioactive citrinin of the first and second generations of mold were harvested at various time intervals by the usual procedure (Schwenk *et al.*, 1958). When the yield of citrinin approached a maximum, the flasks containing the radioactive material were worked up. In each case, the crude product was recrystallized from an alcohol–water mixture.

The second generation mold yielded 1.395 g of radioactive citrinin which was monitored at roughly 9000 cpm (planchet). This was mixed with 9.605 g of nonradioactive citrinin and the whole dissolved in 220 ml of boiling absolute ethanol. The solution was filtered while hot and an equal volume of cold water was added to the filtrate which effected immediate crystallization. After refrigerating the mixture for 2 hr, the citrinin was collected by filtration and dried in a vacuum desiccator. The recovery of citrinin by this procedure was 10.27 g [93%, mp 171–173° (dec)] and its radioactivity was 819 cpm (planchet) (relative molar activity = 3.42×10^5) and 9.36×10^7 dpm/mole (scintillation).

The procedure was repeated with 0.773 g of radioactive citrinin obtained from the first generation series and 9.23 g of nonradioactive citrinin, affording diluted material having 710 cpm (planchet). The degradation was accomplished by the methods described in paper I of this series (Rodig *et al.*, 1966) usually starting with about 10 g of radioactive citrinin, an amount more than sufficient to carry out the complete degradation sequence.

In experiments with both [1-^{14}C]glucose and [6-^{14}C]-glucose, the respiratory carbon dioxide evolved during the metabolism of the substrate was found to be radioactive. To examine whether this carbon dioxide could constitute a complicating source of radioactivity in the citrinin which was isolated, several runs with nonradioactive glucose were made where $^{14}CO_2$ was bubbled through the culture medium during the growth cycle. In none of these runs was radioactive citrinin isolated.[2,3]

Two different counting methods were employed for the determination of the radioactivity of the degradation products. Solids were pressed into planchets and counted in a Nuclear-Chicago low-background gas flow counter, Model 115, while both liquids and solids were counted in a Nuclear-Chicago scintillation counter, Model 701. The radioactivity assays of the degradation products appear in Table I where they are expressed as relative molar activities (rma) (Birch *et al.*, 1958b) and also as the per cent molar radioactivities of the parent molecule, citrinin. In the case of scintillation counting, the radioactivity was determined in terms of disintegrations per minute per mole (dpm/mole) and converted to the corresponding relative molar activities values by counting several samples by both methods and establishing the ratio of disintegrations per minute per mole to relative molar activity for the instruments used. The efficiency of the scintillation counting was obtained by counting a standard toluene sample (Nuclear-Chicago Corp., Des Plaines, Ill.), and the counting efficiency for each sample was determined from Baillie quenching curves (Baillie, 1960). Scheme I shows a comparison of the distribution of radioactivity found for both [1-^{14}C]glucose and [6-^{14}C]glucose, and the average per cent activities found for the various carbon atoms is given in Table II.

[2] It is of course possible that externally added $^{14}CO_2$ is not incorporated in the citrinin because of membrane impermeability. However, the distinct labeling patterns observed in the citrinin isolated from these runs further tend to discount malevolent intervention of radioactive respiratory carbon dioxide in the biosynthesis of this metabolite.

[3] The authors are indebted to Mr. Robert F. Brady, Jr., for carrying out these experiments.

TABLE 1: Radioactivity of Degradation Products of Citrinin.

Compd[a]	[1-^{14}C]Glucose			[6-^{14}C]Glucose		
	Run[b]	Rma[c] ($\times 10^{-5}$)	Act.[c] (%)	Run[b]	Rma[c] ($\times 10^{-5}$)	Act.[c] (%)
Citrinin I	A	2.18		E	3.42	
	B	1.80		F	4.66	
	C	3.84				
	D	4.30				
Phenol IIIa	A	1.87	85.8	E	2.91	85.1
	B	1.52	84.4	F	3.99	85.6
	C	3.50	91.1			
	D	3.96	92.1			
$BaCO_3$ C_{11}	A	0.20	9.2	E	0.34	9.9
	B	0.17	9.4	F	0.49	10.5
	C	0.35	9.1			
	D	0.43	10.0			
$BaCO_3$ C_1	A	0.04	0.2	E	0.13	3.8
	B	0.003	0.02	F	0.06	1.3
	C	0.04	0.1			
	D	0.10	2.3			
Phenol XIII	A	1.45[d]	66.5			
	B	1.17[d]	65.0			
	C	2.72[d]	70.8			
	D	2.96	68.8			
Ketone IV				E	2.44[e]	71.3
				F	3.05[f]	65.5
CHI_3 C_{10}	A	(0.42)[g]	19.3	E	0.439	12.8
	B	(0.35)[g]	19.4	F	0.635	13.6
	C	(0.78)[g]	20.3			
$BaCO_3$ C_9	D	(1.00)[g]	23.3	E	0.031	0.9
				F	0.024	0.5
Anhydride XIV	A	1.36	62.4	E	2.23	65.2
	B	1.11	61.7			
C_{13}	A	(0.10)	(4.6)	E	(0.21)	(6.1)
	B	(0.06)	(3.3)			
	C	(0.30)	(7.8)			
Acid VIII	C	2.42	63.0			
$BaCO_3$ C_{12}	A	0.19	8.7	E	0.27	7.9
	B	0.14	7.8			
	C	(0.32)	(8.3)			
Acid IXa	A	1.11	50.9	E	1.81	52.9
	B	1.01	56.1	F	2.40	51.5
	C	2.10	54.7			
Acid IXb				E	1.82	53.2
Resorcinol X				E	1.46	42.7
				F	1.86	39.9
C_8	A	(0.31)	(14.2)	E	(0.36)	(10.5)
	C	(0.58)	(15.1)	F	(0.54)	(11.6)
Styphnic acid XI	A	0.80	36.7	E	1.46	42.7
	C	1.52	39.6	F	1.85	39.7
$BaCO_3$ $C_{3,5,7}$	A	0.02	0.9	E	0.035	1.0
	C	0.04	1.0	F	0.037	0.8
Br_3CNO_2 $C_{2,4,6}$	A	0.24[h]	11.0	E	0.33[e]	9.6
	C	0.38[h]	9.9	F	0.58[e]	12.4

[a] Roman numerals refer to structures in the first paper of this series, (Rodig et al., 1966). [b] In run B, radioactive glucose was added after 8 days of growth; in runs E and F, after 7 days of growth. [c] Parentheses indicate values obtained by difference only. [d] Counted as the hydrate. [e] Values obtained by scintillation counting. All those unspecified were obtained by planchet counting. [f] Counted as the semicarbazone. [g] Combined values for C_9 and C_{10}, obtained as the difference between the relative molar activity values of IIIa and XIII. [h] Bromopicrin burned to CO_2 and counted as $BaCO_3$.

SCHEME I: Observed Labeling in Citrinine.

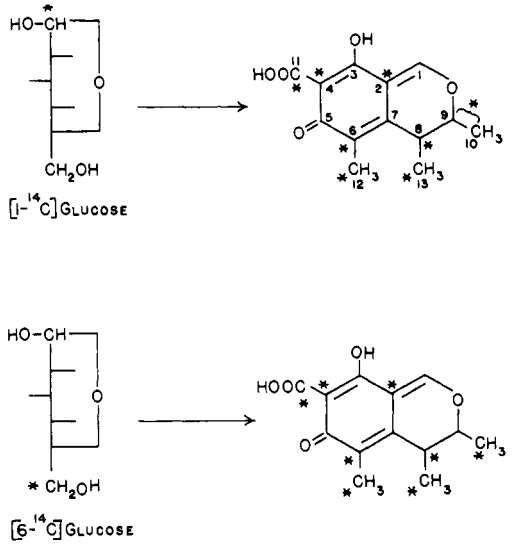


TABLE II: Observed Labeling in Citrinin. Per Cent Activity at Each Carbon (Average Values).

Carbon	[1-^{14}C]-Glucose	[6-^{14}C]-Glucose
1	0.7	2.6
2	10.5	11.0
3	1.0	0.9
4	10.5	11.0
5	1.0	0.9
6	10.5	11.0
7	1.0	0.9
8	(14.6)[a]	(11.1)
9	(20.6)	0.7
10		13.2
11	9.4	10.2
12	8.2	7.9
13	(5.2)	(6.1)

[a] See footnote *c* of Table I.

Results and Discussion

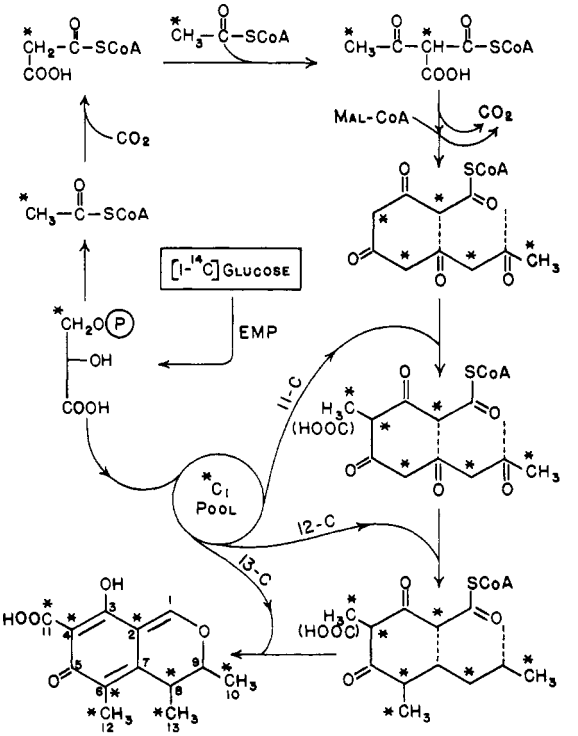
The alternation of radioactive carbon atoms from C-1 to C-10 in the labeling pattern of the citrinin skeleton is clearly evident in Table II and indicates that the biosynthesis of the metabolite originates from two-carbon fragments. The per cent activity values also demonstrate the equivalence of C-1 and C-6 of glucose, which is in accord with an Embden–Meyerhof–Parnas degradation of this substrate.

It is of particular interest that the C-9–C-10 moiety contains the most active carbon, C-10, suggesting that this fragment is added in a fashion different from the others contained in the skeleton of the compound.[4] The process probably involves the now familiar acetate–polymalonate pathway, and preliminary experiments with [2-^{14}C]malonate support this postulate.[3,5]

The C_1 methyl donors, formate (Birch *et al.*, 1958a; Schwenk *et al.*, 1958) and methionine (Schwenk *et al.*, 1958), have been demonstrated to give rise to the extra-skeletal groups (C-11, C-12, and C-13) in citrinin. Birch and co-workers (1958a) observed an inequality in the labeling of C-12, C-13 (determined together) and C-11 in their work with citrinin obtained from the *Aspergillus sp.* fed with [^{14}C]formate, which they suggested may indicate a sequential rather than a simultaneous attachment of these groups. On the other hand, from studies with *P. citrinum* Raper (ATCC 1843) and [methyl-^{14}C]methonine, Schwenk and co-workers (1958) reported that the three carbon atoms arising from C_1 units exhibited essentially the same radioactivity and thus concluded that these carbon atoms probably attach themselves at approximately the same time. The degradative scheme used by both groups did not allow a differentiation between C-12 and C-13.

In the present study, both [1-^{14}C]glucose and [6-^{14}C]-glucose yielded citrinin having these three carbon atoms

SCHEME II:[6] Proposed Biosynthesis of Citrinine.



[4] Several similar observations have been reported. (*cf.* Birch and Smith, 1958; Birch *et al.*, 1958b, 1959).

[5] From the results observed with other metabolites known to undergo biosynthesis by an acetate–polymalonate pathway, the values obtained for C-8 in those runs employing [1-^{14}C]glucose would be expected to be approximately the same as those observed for C-2, C-4, and C-6. Although it is presently not clear why the C-8 values were found to be about 4% higher, any error embodied therein may be at least partly ascribed to the fact that they were not obtained by direct measurement.

[6] Dotted lines and parentheses indicate that there are currently no criteria to suggest a time sequence for cyclization of the polyketomethylene intermediate or oxidation of the C-11 methyl group to a carboxyl function.

unequally labeled (Tables I and II). Thus, both C-1 and C-6 of glucose contribute to the C_1 pool, the radioactivity of which is time dependent.[7] Since this radioactivity would be expected to decrease with time due to normal dilution effects, the evidence on hand favors the sequence of attachment for these groups as C-11, C-12, and C-13, respectively.

In accordance with these results, Scheme II is suggested for the biosynthesis of radioactive citrinin from [1-^{14}C]-glucose. A similar sequence would apply to [6-^{14}C]-glucose. The glucose, degraded by the Embden–Meyerhof–Parnas pathway, gives rise to [2-^{14}C]acetate which undergoes carboxylation with carbon dioxide and condenses according to the process outlined by Lynen (1959) and Lynen and Tada (1961) to produce the citrinin skeleton labeled in the 2, 4, 6, 8, and 10 positions. The biosynthesis of a radioactive C_1 donor, most likely [methyl-^{14}C]methionine (Mattoon, 1963; Adams, 1962), from [1-^{14}C]glucose (or [6-^{14}C]glucose) gives rise to the radioactive extraskeletal groups, C-11, C-12, and C-13. The observed differences in radioactivity of alternating skeletal carbon atoms are presumed to be due to dilution and scrambling effects and possibly reflect the time sequence of addition of the C-2 units. Likewise, the time sequence of addition of the extraskeletal carbon atoms is disclosed by their nonuniform radioactivities because of a dilution factor arising from the addition of endogenous nonradioactive intermediates to the C_1 pool. There are currently no criteria to indicate the time of cyclization of the suppositional polyketomethylene intermediate or of the oxidation of C-11 to the carboxyl level.

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[7] It has been suggested that methionine may be the C_1 unit source in the biosynthesis of citrinin because of the high incorporation of radioactivity from [methyl-^{14}C]methionine as compared to other potential C_1 donors (Schwenk *et al.*, 1958).

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