The Biosynthesis of Kojic Acid

3. THE INCORPORATION OF LABELLED SMALL MOLECULES INTO KOJIC ACID

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It was shown in an earlier paper (Arnstein & Bentley, 1953) that the biosynthesis of kojic acid from glucose by two species of *Aspergilli* is, in the main, a direct conversion without splitting of the carbon chain of glucose. However, the results, particularly with labelled dihydroxyacetone, also indicated that in addition to the direct conversion of glucose there was at least one other pathway of kojic acid formation. In an attempt to study this and possibly other pathways in more detail and to obtain further information about the general metabolism of these moulds, a number of ^{14}C -labelled compounds, chiefly pyruvate, acetate and glycine, have been added to *Aspergillus* cultures grown initially on glucose. Kojic acid was subsequently isolated and if the sample was sufficiently radioactive the distribution of ^{14}C in the molecule was determined. Since a number of small molecules can function as sole carbon source for growth and kojic acid formation in *Aspergilli*, it seemed possible that in this way information about the nature of the suspected subsidiary pathways of kojic acid biosynthesis could be obtained.

The method of numbering the kojic acid carbon atoms throughout this paper corresponds with that adopted in the first paper of this series.

EXPERIMENTAL

The organisms used in these experiments were the strains of *A. oryzae* and *A. flavus-oryzae* described previously (Arnstein & Bentley, 1953). Details of the experimental conditions are given in Table 1. The moulds were grown on 200 ml. of the Czapek-Dox medium (containing 10% (w/v) glucose monohydrate and 1% (w/v) yeast extract) in a 1 l. conical flask plugged with cotton wool. At a given time a sterile solution of the labelled compound was added to the culture fluid below the mycelium with the aid of a long sterile pipette and the flask was then fitted with a sterile bung carrying aeration and sampling tubes. After gentle swirling to mix the contents, the flask was replaced in the hot room (28–29°). Aeration was started and the emergent gas was

passed through wash bottles containing NaOH to absorb any $^{14}\text{CO}_2$ produced. After the desired length of time, kojic acid was isolated from the culture medium as described in Part 1 of this series of papers (Arnstein & Bentley, 1953).

Fixation of CO_2 into kojic acid (Table 1, Exp. 15). The following is a typical experiment: a surface culture of *A. oryzae* was grown in a 1 l. conical flask on the usual 10% (w/v) glucose Czapek-Dox medium (200 ml.) for 9 days. On the ninth day $^{14}\text{CO}_2$ (approx. 0.08 m-mole), generated from $\text{Ba}^{14}\text{CO}_3$ (specific radioactivity = 37.4 $\mu\text{C}/\text{m-mole}$) with perchloric acid, was introduced into the gas phase above the mycelium with the aid of a specially designed gas burette. The system was then closed and the mould exposed to the $^{14}\text{CO}_2$ overnight (14 hr.). On the next day, the CO_2 was swept out by passing sterile air (3 l.) over the mycelium for 30–40 min. The $^{14}\text{CO}_2$ in the emergent gas stream was absorbed in NaOH, converted to BaCO_3 and assayed for ^{14}C . Aeration of the flask was continued for the remainder of the day (10 hr.), the CO_2 being collected as above. Fresh $^{14}\text{CO}_2$ was then introduced into the flask which was again kept closed overnight. The above procedure was repeated five times till the 16th day and kojic acid was isolated on the 18th day. The amount of $^{14}\text{CO}_2$ added daily varied slightly; the total amount of $^{14}\text{CO}_2$ introduced was 12.6 μC . Details of the radioactivity of the CO_2 collected during aeration are given in Table 2.

Kojic acid was isolated in the usual way (yield, 0.22 g.). Most of its radioactivity (0.064 $\mu\text{C}/\text{g.}$) was due to an impurity since purification and conversion to the dimethyl ether afforded an almost completely non-radioactive product (0.008 $\mu\text{C}/\text{g.}$).

Labelled compounds. Sodium [*carboxy- ^{14}C*]acetate, sodium [*Me- ^{14}C*]acetate, [*1:3- $^{14}\text{C}_2$*]acetone and DL-[γ - ^{14}C]valine were obtained from the Radiochemical Centre, Amersham. [*carboxy- ^{14}C*]glycine was prepared from K^{14}CN (Sakami, Evans & Gurin, 1947), [α - ^{14}C]glycine from [*2- ^{14}C*]bromosuccinic acid (Olsen, Hemingway & Nier, 1943), succinic [^{14}C]formate from K^{14}CN by hydrolysis (Grant & Turner, 1950) and L-[α - ^{14}C]lysine from ethyl [*2- ^{14}C*]malonate (Arnstein, Hunter, Muir & Neuberger, 1952), the starting material being obtained from the Radiochemical Centre in each case. [*2- ^{14}C*] and [*3- ^{14}C*]pyruvate were prepared from the correspondingly labelled pyruvamide, as described by Anker (1948).

In early experiments, kojic acid dimethyl ether was degraded; as this method is suitable for the isolation of $\text{C}_{(1)}$ and $\text{C}_{(4)}$ only, complete degradations were carried out on the 2-ethyl 6-methyl ether of kojic acid. The details of the degradations and of the measurements of radioactivity have been described previously (Arnstein & Bentley, 1953).

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Table 1. *Conversion of labelled precursors to kojic acid*

(The labelled compounds were added at the stated time to a surface culture of the mould which had been grown on a 10% (w/v) glucose medium (200 ml.) as described earlier (Arnstein & Bentley, 1953).)

Exp. no.	Mould	Details of precursors added		Radio-activity (μc.)	Age of culture at time of addition of precursor (days)	Interval between addition of precursor and isolation of kojic acid (days)	Yield of kojic acid (g.)	Specific radio-activity of crude kojic acid (μc./mole)	Specific radioactivity of purified kojic acid derivative (μc./mole)
		Compound	Wt. (mg.)						
1	<i>A. oryzae</i>	CH ₃ ¹⁴ CO ₂ Na	202	58.5	6	4	0.06	175	59.1*†
1a	<i>A. oryzae</i>	CH ₃ ¹⁴ CO ₂ Na	151	45	6	4	0.24	100	77.9*†
2	<i>A. flavus-oryzae</i>	CH ₃ ¹⁴ CO ₂ Na	183	44	4	3	1.21	25.6	11.2‡
3	<i>A. oryzae</i>	¹⁴ CH ₃ CO ₂ Na	186	49	6	4	0.18	182	167*†
4	<i>A. flavus-oryzae</i>	¹⁴ CH ₃ CO ₂ Na	182	34	4	3	1.16	108	95‡
5	<i>A. oryzae</i>	CH ₂ NH ₂ ¹⁴ CO ₂ H	771	30	6	4	0.20	66.8	8.9*
5a	<i>A. oryzae</i>	CH ₂ NH ₂ ¹⁴ CO ₂ H	104	46	6	4	0.37	61.2	22.3*†
6	<i>A. flavus-oryzae</i>	CH ₂ NH ₂ ¹⁴ CO ₂ H	93	41	4	3	1.73	6.5	4.7‡
7	<i>A. oryzae</i>	¹⁴ CH ₂ NH ₂ CO ₂ H	102	53	6	4	0.12	140	35.5*
8	<i>A. flavus-oryzae</i>	¹⁴ CH ₂ NH ₂ CO ₂ H	128	66	4	3	1.35	71.1	53.7‡
9	<i>A. flavus-oryzae</i>	CH ₃ ¹⁴ COCO ₂ H	§	§	4	3	0.85	26.3	17.8‡
10	<i>A. flavus-oryzae</i>	¹⁴ CH ₃ COCO ₂ H			4	3	1.96	22.7	18.1‡
11	<i>A. flavus-oryzae</i>	¹⁴ CH ₃ CO ¹⁴ CH ₃	533	181	4	3	0.73	38.2	5.2*
12	<i>A. oryzae</i>	H ¹⁴ CO ₂ Na	117	26	6	4	0.17	5.5	3.5*
13	<i>A. oryzae</i>	L-[α- ¹⁴ C]lysine	550	8.4	6	4	1.07	14.9	3.6*
14	<i>A. oryzae</i>	DL-[γ- ¹⁴ C]valine	117	6.4	6	4	0.21	19.9	—
15	<i>A. oryzae</i>	¹⁴ CO ₂	¶¶	12.6	9	¶¶	0.22	9.1	1.4*

* Determined on kojic acid dimethyl ether.

† Calculated value, based on assay of material diluted with carrier and recrystallized.

‡ Determined on kojic acid 2-ethyl ether.

§ Added in solution obtained by acid hydrolysis of [2-¹⁴C] pyruvamide (243 mg., 33 μc.) (Anker, 1948), after adjusting pH to 7 with NaOH.

|| Added in solution obtained by acid hydrolysis of [3-¹⁴C] pyruvamide (166 mg., 27.5 μc.) (Anker, 1948), after adjusting pH to 7 with NaOH.

¶¶ See text.

RESULTS

The yield and specific radioactivity of the kojic acid obtained from various labelled precursors are given in Table 1. As isolated, kojic acid was always accompanied by a radioactive impurity which was difficult to remove. However, conversion to either kojic acid 2-ethyl ether or kojic acid dimethyl ether resulted in satisfactory purification and the radioactivity of the kojic acid was therefore calculated from the specific radioactivity of these derivatives. On this basis, the amount of ¹⁴C recovered in the kojic acid has been calculated (Table 3). It will be seen that, in general, somewhat better recovery of ¹⁴C was obtained with *A. flavus-oryzae* than with *A. oryzae*. In the experiments with labelled glucose (Arnstein & Bentley, 1953) a similar difference between the two moulds was

Table 2. *Incorporation of ¹⁴CO₂ into kojic acid*

(¹⁴CO₂ was added daily for 6 days to a 9-day-old culture of *A. oryzae*, as described in the text. On the day following each addition, the CO₂ was swept out in two fractions by aeration of the culture flask for 30 to 40 min. (fraction I) and for the following 10 hr. (fraction II). The CO₂ was absorbed in NaOH and assayed for ¹⁴C after conversion to BaCO₃.)

Age of culture (days)	Radioactivity of CO ₂ (μc./g. BaCO ₃)	
	Fraction I	Fraction II
10	0.005	0.002
11	0.19	0.066
12	1.31	0.17
13	0.33	0.064
14	1.33	0.20
15*	1.02	0.083

* Kojic acid present in culture fluid (red colour with FeCl₃).

Table 3. Recovery of ^{14}C in kojic acid after adding various labelled compounds to cultures of *A. oryzae* or *A. flavus-oryzae*

Exp. no.	Mould	Precursor added	^{14}C recovered in kojic acid	
			($\mu\text{c.}$)	(%)
1	<i>A. oryzae</i>	$\text{CH}_3^{14}\text{CO}_2\text{Na}$	0.025	0.04
1a	<i>A. oryzae</i>	$\text{CH}_3^{14}\text{CO}_2\text{Na}$	0.13	0.29
2	<i>A. flavus-oryzae</i>	$\text{CH}_3^{14}\text{CO}_2\text{Na}$	0.095	0.22
3	<i>A. oryzae</i>	$^{14}\text{CH}_3\text{CO}_2\text{Na}$	0.22	0.45
4	<i>A. flavus-oryzae</i>	$^{14}\text{CH}_3\text{CO}_2\text{Na}$	0.78	2.3
5	<i>A. oryzae</i>	$\text{CH}_2\text{NH}_2^{14}\text{CO}_2\text{H}$	0.12	0.40
5a	<i>A. oryzae</i>	$\text{CH}_2\text{NH}_2^{14}\text{CO}_2\text{H}$	0.06	0.13
6	<i>A. flavus-oryzae</i>	$\text{CH}_2\text{NH}_2^{14}\text{CO}_2\text{H}$	0.06	0.15
7	<i>A. oryzae</i>	$^{14}\text{CH}_2\text{NH}_2\text{CO}_2\text{H}$	0.03	0.06
8	<i>A. flavus-oryzae</i>	$^{14}\text{CH}_2\text{NH}_2\text{CO}_2\text{H}$	0.51	0.77
9	<i>A. flavus-oryzae</i>	$\text{CH}_3^{14}\text{COCO}_2\text{H}$	0.11	0.33
10	<i>A. flavus-oryzae</i>	$^{14}\text{CH}_3\text{COCO}_2\text{H}$	0.25	0.91
11	<i>A. flavus-oryzae</i>	$^{14}\text{CH}_3\text{CO}^{14}\text{CH}_3$	0.03	0.02
12	<i>A. oryzae</i>	$\text{H}^{14}\text{CO}_2\text{Na}$	0.005	0.02
13	<i>A. oryzae</i>	$\text{L-}[\alpha\text{-}^{14}\text{C}]\text{lysine}$	0.02	0.24
15	<i>A. oryzae</i>	$^{14}\text{CO}_2$	0.002	0.02

observed, although the recovery of ^{14}C in kojic acid from labelled glucose was considerably higher (2–5 % with *A. oryzae*, 5–20 % with *A. flavus-oryzae*) than in any of the present experiments.

and 75 % of the total radioactivity was located at $\text{C}_{(4)}$, while $\text{C}_{(3)}$ contained only about 15 % of the total radioactivity (Table 6).

Table 4. Comparison of the radioactivity of kojic acid and glucose isolated at the end of the fermentation

(The glucose was isolated from the fermented medium as described in Part I (Arnstein & Bentley, 1953). The specific radioactivity of the kojic acid was determined after purification and conversion to kojic acid 2-ethyl ether.)

Exp. no.	Precursor	Specific radioactivity of kojic acid ($\mu\text{c./mole}$)	Specific radioactivity of recovered glucose ($\mu\text{c./mole}$)
2	$\text{CH}_3^{14}\text{CO}_2\text{Na}$	11.2	0.5
4	$^{14}\text{CH}_3\text{CO}_2\text{Na}$	95	1.6
6	$\text{CH}_2\text{NH}_2^{14}\text{CO}_2\text{H}$	4.7	0.2
8	$^{14}\text{CH}_2\text{NH}_2\text{CO}_2\text{H}$	53.7	1.2

In some cases, the glucose remaining in the culture fluid at the end of the fermentation was isolated and its radioactivity compared with that of the kojic acid (Table 4). The low radioactivity of the glucose indicated that resynthesis from the added compounds was quantitatively unimportant.

Table 5 gives the results of the degradation of the labelled kojic acid derived from the different precursors. It will be seen that usually there is a good agreement (within 5–10 %) between the radioactivity of the kojic acid derivative used for the degradation and the total activity recovered in the separate carbon atoms. In all cases, carbon atoms 4, 5 and 6 of kojic acid contained more radioactivity than carbon atoms 1, 2 and 3. This is particularly marked in the experiments with [*carboxy*- ^{14}C]-acetate and [*carboxy*- ^{14}C]-glycine when between 55

DISCUSSION

The experiments described in this paper confirm the results obtained earlier with labelled glucose and dihydroxyacetone (Arnstein & Bentley, 1953) which demonstrated that the major pathway of kojic acid biosynthesis from glucose does not involve preliminary splitting of the glucose carbon chain. If kojic acid were synthesized extensively from C_2 or C_3 molecules derived from glucose, the incorporation of ^{14}C into kojic acid from the labelled compounds used in this work would be expected to be considerably greater than was observed. Since the maximum recovery of isotope in kojic acid was only 2.3 % (using $^{14}\text{CH}_3\text{CO}_2\text{Na}$) and was usually less than 1 % (Table 3), the metabolic pathways of these small molecules are not important in the conversion of glucose to kojic acid. In fact, the major fate of most of these compounds was oxidation and incorporation into the components of the mycelium.

In all of the experiments glucose was present in the culture medium at the time of addition of the labelled compounds. The unfermented glucose recovered from the medium at the end of the experiment was always virtually non-radioactive (Table 4). Although this result may suggest that the added substrates were converted to kojic acid by pathways not involving glucose as an intermediate, the possibility exists that glucose in the medium is not in equilibrium with intracellular glucose or polysaccharide of the mycelium. A conversion of the labelled compounds to glucose and thence to kojic acid within the cell could therefore occur without

Table 5. *Distribution of ^{14}C in kojic acid derived from various ^{14}C -labelled precursors*

(The labelled compounds were added to a culture of *A. oryzae* or *A. flavus-oryzae* grown on a 10% (w/v) glucose medium (cf. Table 1).)

Specific radio-activity of kojic acid, as determined on derivative ($\mu\text{c./mole}$)		Specific radioactivity of kojic acid carbon atoms ($\mu\text{c./mole}$)						
Exp. no.	Precursor	$\text{C}_{(1)}$	$\text{C}_{(2)}$	$\text{C}_{(3)}$	$\text{C}_{(4)}$	$\text{C}_{(5)}$	$\text{C}_{(6)}$	Total ($\text{C}_{(1)}-\text{C}_{(6)}$)*
1	$\text{CH}_3^{14}\text{CO}_2\text{Na}$	9.5†	0.3	—	7.3	—	—	11.7
1a	$\text{CH}_3^{14}\text{CO}_2\text{Na}$	24.1†	0.3	—	22.8	—	—	30.2
2	$\text{CH}_3^{14}\text{CO}_2\text{Na}$	11.2‡	1.3	1.0	8.3	0.2	0.5	13.1
3	$^{14}\text{CH}_2\text{CO}_2\text{Na}$	48.8†	2.5	—	7.9	—	—	50.9
4	$^{14}\text{CH}_2\text{CO}_2\text{Na}$	95.1‡	10.5	10.5	16.4	26.2	32.1	101.0
5	$\text{CH}_2\text{NH}_2^{14}\text{CO}_2\text{H}$	3.1†	0.2	—	1.6	—	—	2.9
5a	$\text{CH}_2\text{NH}_2^{14}\text{CO}_2\text{H}$	10.0†	0.2	—	8.4	—	—	12.9
6	$\text{CH}_2\text{NH}_2^{14}\text{CO}_2\text{H}$	4.7‡	0.2	0.4	3.3	0.3	0.4	5.4
7	$^{14}\text{CH}_2\text{NH}_2\text{CO}_2\text{H}$	35.6†	2.5	—	7.0	—	—	33.5
8	$^{14}\text{CH}_2\text{NH}_2\text{CO}_2\text{H}$	53.7‡	4.9	5.6	3.3	7.9	14.5	49.2
9	$\text{CH}_3^{14}\text{COCO}_2\text{H}$	17.8‡	1.3	2.3	1.4	5.6	4.7	19.0
10	$^{14}\text{CH}_3\text{COCO}_2\text{H}$	18.1‡	2.1	1.7	1.0	2.5	5.2	20.3
11	$^{14}\text{CH}_3\text{CO}^{14}\text{CH}_3$	5.2‡	0.7	0.5	0.3	1.2	1.4	6.1
13	L-[$\alpha\text{-}^{14}\text{C}$]lysine	3.7†	0.2	—	1.4	—	—	2.6

* Except in the experiments where the radioactivity of $\text{C}_{(3)}$, $\text{C}_{(5)}$ and $\text{C}_{(6)}$ was determined separately, the radioactivity of the silver methoxyacetate obtained by the degradation of kojic acid dimethyl ether was used for calculating the radioactivity in $\text{C}_{(2)} + \text{C}_{(3)} + \text{C}_{(5)} + \text{C}_{(6)}$.

† Determined on kojic acid dimethyl ether used for degradation (cf. Arnstein & Bentley, 1953).

‡ Determined on kojic acid 2-ethyl ether, which was subsequently converted to kojic acid 2-ethyl 6-methyl ether and degraded (cf. Arnstein & Bentley, 1953).

—, value not determined.

Table 6. *Percentage distribution of radioactivity in kojic acid obtained from various labelled compounds*

(Details of the experiments are given in Table 1. The percentage radioactivity (x) in the separate carbon atoms of kojic acid is derived from the results given in Table 5, using the equation $x = 100a/b$, where a is the radioactivity in $\mu\text{c./mole}$ at the carbon atom and b is the total radioactivity located in all six carbon atoms (Table 5, col. 10).)

Exp. no.	Precursor	Distribution of radioactivity in kojic acid carbon atom (%)					
		$\text{C}_{(1)}$	$\text{C}_{(2)}$	$\text{C}_{(3)}$	$\text{C}_{(4)}$	$\text{C}_{(5)}$	$\text{C}_{(6)}$
1	$\text{CH}_3^{14}\text{CO}_2\text{Na}$	2.5	—	—	62.5	—	—
1a	$\text{CH}_3^{14}\text{CO}_2\text{Na}$	1.0	—	—	75.5	—	—
2	$\text{CH}_3^{14}\text{CO}_2\text{Na}$	10.0	7.6	13.8	63.5	1.5	3.8
3	$^{14}\text{CH}_2\text{CO}_2\text{Na}$	4.9	—	—	15.5	—	—
4	$^{14}\text{CH}_2\text{CO}_2\text{Na}$	10.4	10.4	5.3	16.3	26.0	31.8
5	$\text{CH}_2\text{NH}_2^{14}\text{CO}_2\text{H}$	6.9	—	—	55.2	—	—
5a	$\text{CH}_2\text{NH}_2^{14}\text{CO}_2\text{H}$	1.6	—	—	65.1	—	—
6	$\text{CH}_2\text{NH}_2^{14}\text{CO}_2\text{H}$	3.7	7.4	14.8	61.1	5.6	7.4
7	$^{14}\text{CH}_2\text{NH}_2\text{CO}_2\text{H}$	7.5	—	—	20.9	—	—
8	$^{14}\text{CH}_2\text{NH}_2\text{CO}_2\text{H}$	10.0	11.4	6.7	16.1	29.5	26.4
9	$\text{CH}_3^{14}\text{COCO}_2\text{H}$	6.8	12.1	7.4	29.5	24.8	19.5
10	$^{14}\text{CH}_3\text{COCO}_2\text{H}$	10.4	8.4	4.9	12.3	25.6	38.4
11	$^{14}\text{CH}_3\text{CO}^{14}\text{CH}_3$	11.5	8.2	4.9	19.7	23.0	32.8
13	$\text{L-}[\alpha\text{-}^{14}\text{C}]\text{lysine}$	7.7	—	—	53.8	—	—

—, value not determined.

the glucose of the medium becoming labelled and this consideration cannot at present be excluded.

Whatever the added substrate, the distribution of ^{14}C in the kojic acid was always rather widespread (Tables 5 and 6). Usually there appeared to be some ^{14}C present in each carbon atom, although in some cases the radioactivity was too low to be measured accurately. Moreover, the radioactivity of two carbon atoms (C_2 and C_6) had to be determined by difference and it is possible that some carbon atoms

were in fact non-radioactive. Values less than 5% of the total activity are therefore not considered significant.

In every experiment the sum of radioactivity in carbon atoms 4, 5 and 6 was very much greater than that in carbon atoms 1, 2 and 3; the ratio of radioactivity in these two moieties of kojic acid was almost always strikingly constant, namely 3:1. This result is very similar to that obtained previously with $[2\text{-}^{14}\text{C}]\text{dihydroxyacetone}$ in the

presence of glucose, when there was considerably more ^{14}C in position 5 of kojic acid than in position 2 (Arnstein & Bentley, 1953). A comparison of the ^{14}C distribution in kojic acid and in rat-liver glycogen derived from the same small molecules (Lifson, Lorber, Sakami & Wood, 1948) shows that a conventional reversal of the triosephosphate isomerase and aldolase reactions is not an important step in kojic acid formation from small molecules during the simultaneous fermentation of glucose. Thus, glycogen synthesis from [carboxy- ^{14}C] acetate resulted in equal incorporation of ^{14}C into positions 3 and 4 of glucose, but in kojic acid derived from the same compound 60–75 % of the total radioactivity was located in one carbon atom (position 4). With methyl-labelled acetate the differences were even more pronounced; in the glycogen, carbon atoms 1, 2, 5 and 6 contained most of the ^{14}C and were labelled almost equally; in kojic acid there was more radioactivity in carbon atom 4 than in carbon atoms 1 and 2 (which were equally labelled) and carbon atoms 5 and 6 (also equally labelled) contained about 60 % of the total radioactivity. It is probable, therefore, that under these conditions, condensation of two C_3 units in complete equilibrium is not a major pathway of kojic acid biosynthesis. In addition, the uneven distribution of radioactivity in the two halves of kojic acid shows that the ultimate C_6 precursor is unlikely to be a symmetrical molecule.

It is still possible that both halves of the kojic acid molecule are derived from a common or nearly related C_3 unit, and some evidence may be adduced in support of this hypothesis. If the same C_3 unit is used in both halves, the ratio of activities $\text{C}_{(1)}/\text{C}_{(3)}$ should equal $\text{C}_{(6)}/\text{C}_{(5)}$, and similarly $\text{C}_{(1)}/\text{C}_{(3)}$ should equal $\text{C}_{(6)}/\text{C}_{(4)}$. These ratios have been calculated in those cases where a complete degradation was carried out and are given in Table 7. In two cases (methyl-labelled acetate and glycine) excellent agreement is obtained and in two other cases (carboxyl-labelled acetate and glycine) there was so much ^{14}C in one carbon atom that small errors in the analysis of the others, or small incorporations by other pathways, would lead to gross inconsistencies

in calculations of this nature. In the other cases, the agreement is less precise but is sufficient to suggest that for a particular small molecule the same (or closely related) C_3 unit is used for both halves of the kojic acid molecule, though to a different extent.

The evidence suggests further that in the case of acetate, glycine, acetone and pyruvate the added substrate is first converted to the same C_3 intermediate which is then converted to kojic acid by a common pathway. The striking similarity of the ^{14}C distribution patterns obtained from labelled glycine and acetate suggests that either the glycine is first deaminated to acetate or that the metabolic pathways of these compounds are closely similar and deamination occurs at a later stage. Kojic acid arising from methyl-labelled acetone (Table 6, Exp. 11) has a ^{14}C -distribution pattern very similar to that from methyl-labelled acetate, and it seems likely, therefore, that the utilization of acetone involves its initial conversion to acetate, a reaction which is known to take place in animal tissues (Borek & Rittenberg, 1949; Price & Rittenberg, 1950).

The similarity of the ^{14}C distribution in kojic acid observed with methyl-labelled acetate and methyl-labelled pyruvate further suggests that the initial C_3 intermediate in each case is pyruvate, formed by the known reactions of either the tricarboxylic or dicarboxylic acid cycles. Such a reaction sequence would convert methyl-labelled acetate and methyl- or carbonyl-labelled pyruvate to pyruvate labelled in all three carbon atoms. In all of these cases the triply-labelled pyruvate gives rise to kojic acid with ^{14}C distributed predominantly in carbon atoms 4, 5 and 6. The labelling in these atoms is not equal and the ^{14}C pattern in kojic acid derived from carbonyl-labelled pyruvate is markedly different from that of the methyl-labelled compounds; these differences are probably due to the reaction rates involved and to the number of 'turns' of the di- or tri-carboxylic acid cycles necessary for the random distribution of the ^{14}C . A second type of labelling is that which results from the use of carboxyl-labelled acetate and glycine. Here, only carboxyl-labelled pyruvate can be formed and the labelling is predominantly in one

Table 7. *Comparison of the ratio of specific activities of kojic acid carbon atoms derived from labelled small molecules*

(The radioactivity ratios have been calculated from the values for specific radioactivity given in Table 5.)

Exp. no.	Precursor	Ratio of radioactivities			
		$\text{C}_{(1)}/\text{C}_{(3)}$	$\text{C}_{(6)}/\text{C}_{(5)}$	$\text{C}_{(1)}/\text{C}_{(3)}$	$\text{C}_{(6)}/\text{C}_{(4)}$
2	$\text{CH}_3^{14}\text{CO}_2\text{Na}$	1.3	2.5	0.72	0.06
4	$^{14}\text{CH}_3\text{CO}_2\text{Na}$	1.0	1.2	1.95	1.95
6	$\text{CH}_2\text{NH}_2^{14}\text{CO}_2\text{H}$	0.5	1.3	0.25	0.01
8	$^{14}\text{CH}_2\text{NH}_2\text{CO}_2\text{H}$	0.87	0.9	1.5	1.6
9	$\text{CH}_3^{14}\text{COCO}_2\text{H}$	0.56	0.79	0.91	0.66
10	$^{14}\text{CH}_3\text{COCO}_2\text{H}$	1.2	1.5	2.0	3.1
11	$^{14}\text{CH}_3\text{CO}^{14}\text{CH}_3$	1.4	1.4	2.3	1.65

position (carbon atom 4) of the kojic acid. It is interesting to note that a C_4 - or C_6 -acid cycle of the type just discussed has recently been demonstrated in a strain of *A. niger* (Carson, Mosbach, Phares, Long & Gwin, 1951). Further, α -ketoglutaric acid, which is an intermediate of the tricarboxylic acid cycle, has recently been identified during the metabolism of reserve material of *A. niger* in the presence of 0.01M-potassium arsenite (Walker, Hall & Hopton, 1951).

It is not possible at this time to give a precise explanation for the greater incorporation of ^{14}C from the labelled small molecules into positions 4, 5 and 6 compared with 1, 2 and 3 of kojic acid. The possibilities discussed earlier in connexion with the similar results obtained with [2- ^{14}C]dihydroxyacetone (Arnstein & Bentley, 1953) are also valid in the present instance and will not be repeated here. Since the unequal labelling was obtained when glucose was being fermented at the time of addition of the labelled substrates, the simplest explanation may be that kojic acid is formed from a labelled C_3 intermediate, such as pyruvate or dihydroxyacetone, and an unlabelled triose or triosephosphate derived from the glucose. The incorporation of some radioactivity into carbon atoms 1, 2 and 3 in these experiments would be accounted for by a slow inter-conversion of the labelled and unlabelled C_3 units.

The postulated C_3 unit from glucose may arise either by the reactions of the Embden-Meyerhof glycolytic pathway or by the Warburg-Lipmann-Dickens (WLD) oxidation of glucose. In cultures of *A. flavus-oryzae* utilizing [1- ^{14}C]glucose the expired carbon dioxide had a higher specific radioactivity at the beginning of the fermentation than during the later stages, but with [3:4- $^{14}C_2$]glucose the initial radioactivity was low and increased to a maximum at about the fourth day (Arnstein & Bentley, 1953). This indicates a preferential oxidation of carbon atom 1 of glucose, at least during the earlier stages of the fermentation, and

suggests that the C_3 fragment may be formed by the WLD oxidation reactions rather than the Embden-Meyerhof scheme. Although the WLD mechanism may thus be of importance in glucose oxidation by *A. flavus-oryzae*, no evidence could be found to suggest that kojic acid was synthesized by a reversal of this mechanism or by a similar $C_1 + C_6$ condensation. Neither $^{14}CO_2$ nor labelled formate were converted to kojic acid in a number of experiments; furthermore, the distribution of ^{14}C in the kojic acid in the experiments with labelled C_3 compounds was also not in agreement with such a possibility.

It is concluded that kojic acid biosynthesis may involve three metabolic processes:

(a) Direct formation from glucose without splitting of the carbon chain.

(b) Simultaneously, a secondary pathway involving formation of triosephosphates, which leads to the incorporation of ^{14}C into position 6 when [1- ^{14}C]glucose is fermented.

(c) A third pathway involving the incorporation of ^{14}C predominantly into carbon atoms 4, 5 and 6 of kojic acid when labelled small molecules are added during the fermentation of unlabelled glucose.

SUMMARY

1. The biosynthesis of kojic acid from ^{14}C -labelled acetate, glycine, pyruvate and other small molecules by *A. oryzae* and *A. flavus-oryzae* has been investigated.

2. Acetate, glycine and pyruvate appear to be converted to a common 3-carbon intermediate, which gives rise mainly to carbon atoms 4, 5 and 6 of kojic acid, whilst carbon atoms 1, 2 and 3 contain much less radioactivity.

3. The mechanism of the incorporation of the above small molecules into kojic acid is discussed.

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