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The Biosynthesis of Kojic Acid

1. PRODUCTION FROM [1-¹⁴C] AND [3:4-¹⁴C₂] GLUCOSE AND [2-¹⁴C]-1:3-DIHYDROXYACETONE

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Kojic acid was first isolated by Saito (1907) from cultures of *Aspergillus oryzae* on steamed rice. It was shown to be 5-hydroxy-2-hydroxymethyl- γ -pyrone by Yabuta (1924). During the last 30 years kojic acid has been obtained as a metabolic product of many other fungi (mainly *Aspergilli*) and certain bacteria (for reviews of early work see Barham & Smits, 1934; Foster, 1949). As early as 1934 it was reported that kojic acid inhibited the growth of Gram-negative more strongly than that of Gram-positive bacteria (Reed & Bushnell, 1934). This property was rediscovered much later and the antibiotic action of culture filtrates of several fungi was shown to be due to the presence of kojic acid (Foster & Karow, 1945).

A striking feature of kojic acid biosynthesis is the wide variety of substrates which can be utilized for its production. Thus kojic acid is formed from C₂ compounds (e.g. ethanol, glycollic acid), C₃ compounds (e.g. glycerol, dihydroxyacetone), pentose and hexose sugars, C₇ compounds (quinic and shikimic acids), disaccharides and more complex polysaccharides (e.g. starch, dextrin).

Although the way in which kojic acid is formed from so large a number of substrates has been widely discussed, the precise nature of the biosynthetic process has remained virtually unknown. Several

authors (Yabuta, 1924; Kinoshita, 1927; Haworth, 1929) have pointed out the close structural similarity of kojic acid and the pyranose structure of glucose; since both compounds have the same number of carbon atoms and the hydroxymethyl groups at position 2 of the pyrone ring, it is formally possible that kojic acid may be produced from glucose by dehydration and oxidation. A chemical synthesis of kojic acid from acetobromoglucose has been described (Maurer, 1930; Stacey & Turton, 1946) but the theory that the biosynthesis proceeds directly from glucose without rupture of the carbon chain has in general found little support.

The main objection to this theory has been the fact that kojic acid can be formed from compounds having fewer carbon atoms than glucose. Thus, arabinose is converted to kojic acid (Challenger, Klein & Walker, 1929) and not, as might be expected, to 2-hydroxy- γ -pyrone, or alternatively, 3-hydroxy- γ -pyrone (pyromeconic acid). A possible explanation, however, was provided by Birkinshaw, Charles, Lilly & Raistrick (1931) who suggested that all carbon sources were first converted to a 'reserve carbohydrate' which after hydrolysis to a monosaccharide would yield kojic acid.

The following theory has received wider support: kojic acid is formed from these widely different compounds by primary splitting to a common, small, reactive molecule (e.g. C₂ or C₃ fragments) followed by condensation directly to kojic acid

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or to a C_6 precursor of kojic acid. Corbellini & Gregorini (1930) first suggested a condensation of two C_3 fragments ($HOCH_2.CHOH.CHO$ and $CHO.CHOH.CHO$), but experimental evidence was lacking. Later, it was found that, using dihydroxyacetone as the sole carbon source, the yield of kojic acid could be almost as high as when glucose was used (Challenger, Klein & Walker, 1931) and good yields of kojic acid have also been obtained from glycerol (see review by Barham & Smits, 1934). These observations and the importance of dihydroxyacetone phosphate as an intermediate in anaerobic glycolysis suggested strongly that dihydroxyacetone would be the common precursor of kojic acid.

In attempting to decide whether or not glucose was split to a triose, Barnard & Challenger (1949) used a number of glucose derivatives as substrates for a strain of *A. oryzae*. The compounds chosen (e.g. pentaacetylglucose, glucal) were considered unlikely to split to dihydroxyacetone. However, in some cases the mould was unable to utilize the substrates for growth, and in others the compounds were metabolized without formation of kojic acid. Only 2-deoxyglucose was converted to kojic acid and no definite conclusions were possible.

In the work to be reported now, a fresh attempt has been made to elucidate the mechanism of kojic acid biosynthesis. Three approaches to the problem have been made: first, kojic acid has been produced from media containing $[1-^{14}C]$ glucose, or $[3,4-^{14}C_2]$ glucose, or $[2-^{14}C]$ -1,3-dihydroxyacetone by the action of two moulds, *A. oryzae* and *A. flavus-oryzae*. The isolated kojic acid was degraded by a method which made it possible to determine the incorporation of ^{14}C into each carbon atom. Secondly, in the following paper, experiments are described which show the presence, in extracts of mycelia, of those enzymes (aldolase and triosephosphate isomerase) which are known to be responsible for the production and isomerization of the triosephosphates. In a third paper the incorporation of small molecules, other than dihydroxyacetone, into kojic acid will be discussed. A preliminary account of part of this investigation has been published (Arnstein & Bentley, 1950).

(Throughout this and the following papers in this series the kojic-acid carbon atoms are numbered from 1 to 6, i.e.

in order to facilitate reference to the corresponding carbon atoms of glucose. However, when reference is made to

γ -pyrone or its derivatives the conventional numbering for γ -pyrones is followed, i.e.

EXPERIMENTAL AND RESULTS

Methods of incubation, etc. The two moulds used in this work were *A. oryzae* (IMI 17299, obtained from the Commonwealth Mycological Institute) and *A. flavus-oryzae* (kindly supplied by Prof. F. Challenger). Stock cultures of *A. oryzae* were maintained on agar slopes prepared as follows. Potato starch (20 g.) in a little water was added rapidly to boiling distilled water (1 l.); when the solution had cooled to 60°, malt extract (50 g.) was added. After 5 min., yeast extract (2 g.) and agar powder (25 g.) were added and the warm solution distributed into tubes prior to sterilization. *A. flavus-oryzae* was maintained on slopes of the following composition: beer wort was adjusted to a sp.gr., of 1.100 and pH 5.8-6.0; after addition of agar (to concentration of 1.5%, w/v) the material was autoclaved for 2 hr., filtered into tubes and re-autoclaved.

Unless otherwise stated, inoculations from subcultures were made into 200 ml. of culture medium (autoclaved at 15 lb./sq.in. for about 20 min.) contained in 1 l. Pyrex conical flasks. The basal medium had the following composition (w/v): $NaNO_3$, 0.2%; K_2HPO_4 , 0.1%; KCl, 0.05%; $MgSO_4 \cdot 7H_2O$, 0.05%; $FeSO_4 \cdot 7H_2O$, 0.001%; Difco yeast extract, 0.1%. In all cases, the medium contained 10% D-glucose (either radioactive or not, as stated). All cultures were grown at 28-30°, forming a surface mycelium.

Experiments were carried out in one of three ways.

(a) The culture medium contained radioactive glucose at the time of inoculation; the labelled substrate was therefore present during all stages of growth. The flask was fitted with a bung and glass inlet tubes through which a slow stream of air was continuously supplied. In addition, the bung carried a sampling tube so that small samples of the culture medium could be withdrawn at intervals for testing. CO_2 produced during growth could be collected daily and was converted to $BaCO_3$ for determinations of radioactivity.

(b) A surface mycelium was grown from non-radioactive glucose or dihydroxyacetone in a wool-plugged flask in the usual way. At a given time (usually when production of kojic acid was just beginning) a sterile solution of radioactive glucose or dihydroxyacetone was added to the culture medium by means of a long pipette pushed through the mycelium. The cotton-wool plug was then replaced by the bung and glass inlet tubes and aspiration commenced as described previously.

(c) After growth of a surface mycelium on non-radioactive glucose or dihydroxyacetone in the usual way, the culture fluid was completely removed using a large sterile pipette. The remaining mycelium was carefully washed several times by the introduction and removal of sterile water. Finally, a completely new culture medium containing radioactive substrate was introduced, and a bung with glass inlet tubes used to close the flask. Aspiration and collection of CO_2 were then carried out as before. Such cultures will be referred to as 'replacement cultures'.

Table 1. Details of culture media and labelled precursors used for kojic acid production
(The basal medium used in all the experiments, unless otherwise stated, is described in the text.)

Exp. no.	Mould	Labelled precursor	Vol. of culture medium (ml.)	Carbon source used for growing mycelium	Age of culture at time of addition of labelled precursor (days)	Interval between addition of labelled precursor and isolation of kojic acid (days)	Amount of kojic acid isolated (g.)
1	<i>A. oryzae</i>	[1- ¹⁴ C]Glucose { (20-32 g., 10-1 μc.) (1-5 g., 7-8 μc.) (7-5 g., 2-6 μc.)*	200	Glucose (20 g.)	0	17	0-35
2		(7-5 g., 2-6 μc.)†	200		8	8	0-25
3		(7-5 g., 2-6 μc.)†	200		8†	5	0-24
4		(3-0 g., 15-5 μc.)‡	200		8†	5	0-13
5		(2-04 g., 10-5 μc.)	200		7	4	0-85
6		(3-0 g., 15-5 μc.)	200		5	—	0-24
7	<i>A. flavus-oryzae</i>	[3-4- ¹⁴ C ₂]Glucose { (10-15 g., 6-7 μc.) (2-0 g., 10-3 μc.) (10-0 g., 6-2 μc.)	200	Dihydroxyacetone (10 g.) Glucose (10 g.) Glucose (10 g.)	7	4	0-83
8		(10-0 g., 6-2 μc.)	200		7	4	0-91
9		{ (0-628 g., approx. 3 μc.) (5-62 g., approx. 3 μc.)	100§		0	6	1-00
10		[2- ¹⁴ C]Dihydroxyacetone { (600 mg., 3-1 μc.) (600 mg., 3-1 μc.)	100		0	8	2-09
11			200		5	6	0-28
12			200		5	6	0
13			200		7	4	1-52
14			200		7	4	0-91

* Replacement culture using medium of the following composition: H₃PO₄ (d, 1-75) (0-286 ml.), KH₂PO₄ (1-86 g.), glucose monohydrate (7-0 g.), [1-¹⁴C]glucose (0-5 g.), water (150 ml.).
† Medium gave positive FeCl₃ colour test for kojic acid.
‡ Replacement culture using medium of the following composition: glucose monohydrate (7-0 g.), [1-¹⁴C]glucose (0-5 g.), basal salt medium (Czapak-Dox) (150 ml.).
§ Contained 84 mg. sodium fluoride (0-002 mole).
|| Added as replacement culture in medium of the following composition: MgSO₄, 0-4 g.; KH₂PO₄, 0-2 g.; NH₄NO₃, 0-1 g.; water, 200 ml.

Details of the labelled compounds and experimental conditions used are given in Table 1.

Production of kojic acid was qualitatively followed using the intense blood-red coloration produced when a few drops of 1% (w/v) FeCl_3 solution were added to a little of the culture medium. After growth for the desired time the fluid was decanted from the mycelium, usually after previous sterilization. The mycelium was washed with 50 ml. portions of water until the washings gave only a feeble reaction with FeCl_3 . The combined culture medium and washings were extracted continuously with ether for up to 48 hr., the ether extract was evaporated to dryness and the crystalline residue of crude kojic acid was sublimed *in vacuo* on to a cold-finger condenser (10^{-3} mm. Hg, bath temperature 120 – 130°). A little oily material sublimed as a forerun; this was usually more radioactive than the kojic acid itself, and was removed by washing the cold finger with a few ml. of ether. Such samples of kojic acid had m.p.'s in the range 146 – 150° . A sample of material isolated in this way gave a satisfactory analysis. (Found: C, 51.0; H, 4.6. Calc. for $\text{C}_6\text{H}_6\text{O}_4$: C, 50.7; H, 4.3%.)

Recovery of glucose from fermented medium. The fermented medium was freed from inorganic salt by passage through columns of Amberlite IR 100 (H) and Deacidite E ion-exchange resins. The effluent was then concentrated *in vacuo* almost to dryness and the glucose was precipitated by addition of ethanol and recrystallized from aqueous ethanol.

Measurements of radioactivity. All measurements of radioactivity were carried out with a helium-filled bell-shaped Geiger-Müller counter, using 'infinite thickness' samples mounted on 1 sq. cm. Polythene disks (Popják, 1950). All counts were compared with a subsidiary standard (^{14}C]glycine). All radioactivity measurements carried out directly on barium carbonate or iodoform samples were corrected for back-scattering, which had been found experimentally to cause errors of +5% and +10%, respectively. The final combined assay errors, due to variation in sample preparation, small differences in disk size and statistical errors in the counting, were about $\pm 5\%$.

Synthesis of labelled compounds

D-[^{14}C]Glucose. The preparation of D-[^{14}C]glucose was carried out substantially as described by Sowden (1949). The initial conversion of [^{14}C]methyl iodide to [^{14}C]nitromethane was carried out in the presence of unlabelled nitromethane as diluent (Arnstein & Bentley, 1951*a*).

[^{14}C]Dihydroxyacetone. The preparation of this compound has been described by Arnstein & Bentley (1951*a*).

[^{14}C]Glucose. The biosynthetic D-[^{14}C]glucose used for the kojic acid fermentation was prepared as follows: six rats (aggregate body weight approx. 1.5 kg.) were fasted for 24 hr. Each animal was then given 6 ml. of a 20% (w/v) glucose solution by stomach tube, and 1 hr. later 2 ml. of an aqueous solution of $\text{NaH}^{14}\text{CO}_3$ were injected. This solution was prepared by absorbing the $^{14}\text{CO}_2$ liberated from 5 mc. $\text{Ba}^{14}\text{CO}_3$ (200 mg.) in a slight excess of *N*-NaOH (1.5 ml.), diluting with water to about 10 ml. and adding 0.5*M*-citric acid to pH 7.5; the final volume was 12 ml. After 2 hr. the animals were anaesthetized with Nembutal, the livers extirpated and immediately placed in hot 30% (w/v) KOH (80 ml.). When the solution had become homogeneous, ethanol (160 ml.) was added. After cooling at 0° overnight the crude glycogen (2 g.) was centrifuged off, washed twice with ethanol, once with ether and dried.

Further purification by the method of Somogyi (1934) yielded 1.1 g. (12.7 μc .) of a white product.

In a second experiment glycogen was prepared by two slightly different methods and the [^{14}C]glucose was degraded in order to establish the amount of ^{14}C in carbon atoms 1–3 and 4–6, respectively. A solution of $\text{NaH}^{14}\text{CO}_3$ was prepared from 4.55 mc. $\text{Ba}^{14}\text{CO}_3$ (1.343 g.) as before, the $^{14}\text{CO}_2$ being absorbed in *N*-NaOH (8 ml.); 6.4 ml. of this solution were adjusted to pH 7 with 0.5*M*-citric acid (approx. 0.5 ml.) and diluted with water to a final volume of 15.4 ml. Two groups of four female albino rats of the Institute strain (body wt.: group I, 215, 185, 168, 146 g.: group II, 195, 180, 180, 158 g.), previously starved for 48 hr., were treated as follows. Each rat was fed 4 ml. of a 30% (w/v) glucose solution, and 1 hr. later each animal of group I was injected intraperitoneally with 2 ml. of the above $\text{NaH}^{14}\text{CO}_3$ solution. After a further 2 hr. the animals were anaesthetized as before with Nembutal and the livers extirpated and immediately placed in hot 50% (w/v) KOH (30 ml.). Each animal of group II was injected intraperitoneally with 1.85 ml. of the $\text{NaH}^{14}\text{CO}_3$ solution in four equal doses, the first injection being given 1 hr. after the glucose, the remainder at half-hourly intervals. The animals were anaesthetized 0.5 hr. after the last injection, i.e. 3 hr. after the glucose had been fed, and the livers removed as for group I.

The glycogen from each group was precipitated by adding ethanol (60 ml.) to the homogeneous KOH digest. The solution was cooled at 0° overnight and the crude glycogen was centrifuged off, washed three times with a solution of 20% (w/v) NaOH (2 vol.)/ethanol (1 vol.) and once with ethanol. The glycogen was then dissolved in water (10 ml.) and the insoluble residue was removed by centrifugation. The supernatant solution was acidified to pH 4 with 2*N*-HCl and ethanol (4 ml.) was added. The precipitate was centrifuged off and the glycogen was isolated from the supernatant solution by addition of more ethanol (4 ml.).

A portion of the above glycogen (201.8 mg. from group I; 56.6 mg. from group II) was hydrolysed for 3 hr. at 100° with *N*- H_2SO_4 (10 ml.) in the presence of carrier glucose (1 g.). The acid was removed by passing the solution through a column of Deacidite E ion-exchange resin and the glucose obtained by evaporating the effluent was crystallized from ethanol by adding acetone.

Degradation of biosynthetic [^{14}C]glucose. Degradation of the glucose samples was carried out by periodate oxidation of the phenylosazone (Topper & Hastings, 1949) which had been crystallized to constant radioactivity. The oxidation product, mesoxaldehyde bisphenylhydrazone, arising from carbon atoms 1, 2 and 3 of the glucose was recrystallized and converted to the trisphenylhydrazone (Chargaff & Magasanik, 1947). As a control of the degradation, a sample of [^{14}C]glucose, recovered from the fermented metabolism medium of Exp. 9, was degraded by the same method. This material was found to contain 98.5% of the radioactivity in carbon atoms 1–3.

The results of the estimations of radioactivity are given in Table 2. The percentage of ^{14}C in carbon atoms 1, 2 and 3 of the [^{14}C]glucose was 47.7% (group I) and 48.6% (group II). Although it seems possible, therefore, that the labelling in the two halves of the [^{14}C]glucose was not exactly equal, the accuracy of the radioactivity estimation

Table 2. *Degradation of [3:4-¹⁴C₂]glucose, prepared from liver glycogen of two groups of rats which had been injected with sodium [¹⁴C] bicarbonate, and of [1-¹⁴C]glucose recovered from fermented medium*

(The degradation method used was that described by Topper & Hastings (1949). Details of the preparation of the [3:4-¹⁴C₂] glucose are given in the text. The animals of group I were killed 1.5–2 hr. after the [¹⁴C]bicarbonate injection, those of group II received the [¹⁴C]bicarbonate in divided doses, as described in the Experimental section. The [1-¹⁴C]glucose was re-isolated from the fermentation medium of Exp. 9 (see Table 1). The radioactivity determinations are accurate to about $\pm 2-3\%$.)

Glucose used for degradation	Radioactivity (μ c./mole) of glucose derivative and degradation products			Radioactivity in C ₁ + C ₂ + C ₃ of glucose (%)
	Phenylglucosazone	Mesoxaldehyde bisphenylhydrazone	Mesoxaldehyde trisphenylhydrazone	
[3:4- ¹⁴ C ₂]glucose (group I)	464	224	219	47.7
[3:4- ¹⁴ C ₂]glucose (group II)	58.9	29.0	28.2	48.6
Recovered [1- ¹⁴ C]glucose (Table 1, Exp. 9)	63.8	62.1	63.4	98.5

($\pm 2-3\%$) does not permit a definite conclusion to be drawn.

Degradation of phenylglucosazone isolated after fermentation of [3:4-¹⁴C₂]glucose to kojic acid. Because of the small amount of residual sugar in this fermentation, glucose could not be isolated as such but was converted to the phenylglucosazone, which crystallized as needles after 7 min. (radioactivity = 141.3 μ c./mole) when a portion of the glucose solution was heated at 100° with phenylhydrazine hydrochloride (0.4 g.) and anhydrous sodium acetate (0.6 g.). A second preparation of the osazone afforded material of similar radioactivity (135.3 μ c./mole). The two preparations (approx. 70 mg.) were combined and recrystallized from methanol, giving material of somewhat lower radioactivity (130.2 μ c./mole); owing to the small amount available this fraction could not be recrystallized further and was oxidized with periodate (Topper & Hastings, 1949). A second degradation was carried out on material isolated from the mother liquors of the above recrystallization and diluted with authentic phenylglucosazone.

Table 3. *Degradation of phenylglucosazone isolated from fermented culture medium of A. flavus oryzae grown on [3:4-¹⁴C₂]glucose*

(The radioactivity determinations are accurate to $\pm 5\%$.)

Compound	Radioactivity (μ c./mole)	
	1st degradation	2nd degradation
Phenylglucosazone	130.2	6.4*
Mesoxaldehyde bisphenylhydrazone, recryst.	52.8	2.9
Mesoxaldehyde trisphenylhydrazone, recryst.	54.8	2.95
Formaldehyde dimedon	Inactive	Inactive

* Calculated from dilution with carrier.

The results of the determinations of the radioactivity of the degradation products, which are given in Table 3, indicate that approx. 44% of the ¹⁴C was located in carbon atoms 1, 2 and 3 of the recovered phenylglucosazone.

Isolation of C₆₀ of recovered [1-¹⁴C]glucose samples by periodate oxidation (cf. Table 8). The recovered glucose

(72 mg.) was dissolved in water (10 ml.) and oxidized with sodium metaperiodate (Reeves, 1941). The formaldehyde, arising from carbon atom 6 of glucose, was isolated and counted as the dimedon derivative. One recrystallization from ethanol gave material whose radioactivity was unchanged by further recrystallizations.

Experiments to test separations and degradations

Separation of formic and methoxyacetic acids. H¹⁴CO₂Na (50 mg., 5.34 μ c./mg.) and unlabelled sodium formate (950 mg.) were dissolved in water (10 ml.). A portion of this solution (1.5 ml.), methoxyacetic acid (200 mg.), 5N-H₂SO₄ (10 ml.) and water (5 ml.) were refluxed in a stream of CO₂-free N₂ for 3 min. After cooling, red mercuric oxide (5 g.) was added and the solution refluxed again for 10 min. in the CO₂-free gas stream; the exit gas was passed through Ba(OH)₂ solution and the precipitated BaCO₃ was centrifuged off and washed with boiled-out water and acetone. (Radioactivity: 1800 counts/min./mg. C; the radioactivity of the sodium formate recovered from the 'diluted' starting material was 1735 counts/min./mg. C.)

A further quantity of red mercuric oxide (1 g.) was added to the mixture and refluxing continued for 10 min. to complete oxidation of the formic acid. The solution was filtered and the filtrate extracted continuously with ether. The ether solution was extracted with dil. Ba(OH)₂ until the aqueous solution was slightly alkaline; it was then separated and concentrated *in vacuo* to 2 ml. A little BaCO₃ was centrifuged down, and an excess of ethanol added to the clear solution. The crystalline precipitate of barium methoxyacetate (200 mg.) was completely non-radioactive.

Preparation of [carboxy-¹⁴C]methoxyacetic acid. This compound was prepared from [carboxy-¹⁴C]bromoacetic acid (obtained from the Radiochemical Centre, Amersham) by reaction with sodium methoxide using standard methods.

Bromine degradation of silver methoxyacetate. Silver [carboxy-¹⁴C]methoxyacetate (150 mg., 234 μ c./mole) suspended in dry CCl₄ (35 ml.) was refluxed with excess dry Br₂ (1 ml.) in a stream of CO₂-free N₂. Br₂ was removed from the gas stream by bubbling through CCl₄, and CO₂ by bubbling through Ba(OH)₂. The specific radioactivity of the BaCO₃ was 216 μ c./mole.

Separation of methoxy- and ethoxy-acetic acids. Hyflo Super-cel (50 g.) was mixed with 0.5N-H₂SO₄ (25 ml.) and suspended in CHCl₃. (All CHCl₃ used in this experiment was first washed with water and equilibrated with 0.5N-H₂SO₄.)

It was packed into a glass tube, using a perforated plunger, and the column was washed through with CHCl_3 . A mixture of methoxyacetic acid (25 mg.) and ethoxyacetic acid (25 mg.) in CHCl_3 (2 ml.) was put on the top of the column which was then eluted with CHCl_3 . 12 ml. fractions were collected and titrated with 0.02N-methanolic KOH.

Fig. 1. Separation of methoxy- and ethoxyacetic acids on Hyflo Super-cel. For details see text.

A typical separation of ethoxyacetic acid from methoxyacetic acid is shown in Fig. 1. The completeness of separation was further tested with a mixture of [*carboxy- ^{14}C*]methoxyacetic acid and unlabelled ethoxyacetic acid, when the isolated silver ethoxyacetate was completely non-radioactive.

Degradation of kojic acid

Method A: degradation of the dimethyl ether of kojic acid. A typical methylation and degradation will be described. Kojic acid (0.5 g.) in hot methanol (10 ml.) was treated with dry, finely powdered Ag_2O (2 g.) and CH_3I (2 ml.). When the vigorous reaction had subsided, a second portion of Ag_2O and CH_3I was added. The reaction was less vigorous and the mixture was heated in the steam bath for 15 min. A third addition of Ag_2O and CH_3I was then made and the mixture refluxed for 20 min. The solution was filtered and the solid residue washed well with boiling methanol. Evaporation *in vacuo* gave a yellow crystalline material which, after washing with a little dry ether, had m.p. 86–89° (0.446 g., yield 75%). The compound could be recrystallized (m.p. 90°) by dissolving it in CHCl_3 and adding first a little ether and finally light petroleum (b.p. 40–60°), but it was best purified by sublimation on to a cold-finger condenser (10^{-3} mm. Hg, bath temp. 75–80°).

The hydrolysis was carried out as follows. Kojic acid dimethyl ether (314 mg.) in water (6 ml.) was heated on a steam bath for 20 min. with $\text{Ba}(\text{OH})_2 \cdot 8\text{H}_2\text{O}$ (585 mg.) in water (12 ml.). After standing overnight, the neutral solution was steam-distilled, a total of 90 ml. distillate being collected. A portion (20 ml.) was treated with 2:4-dinitrophenylhydrazine in 6N-HCl. Methoxyacetone 2:4-dinitrophenylhydrazone (m.p. 146°; 82 mg.; hence calc. yield of methoxyacetone = 75%) separated and was recrystallized from ethanol (m.p. 162°). The bulk of the distillate was made alkaline with NaOH (0.5 g.) in water (5 ml.) and treated with a solution of I_2 (1 g.) and KI (1.4 g.) in water (75 ml.). After 1 hr., iodoform was filtered off (202 mg.; m.p. 123°). The filtrate was acidified with H_2SO_4 and extracted with small portions of ether until the excess I_2 was removed. It was then continuously extracted overnight with ether; evaporation of solvent yielded a mixture of oil and a small amount of

crystalline material, probably oxalic acid. The oil was dissolved in light petroleum (b.p. 40–60°) containing a little ether. After removal of solvent, the oil was treated with water and excess solid Ag_2CO_3 . The mixture was filtered and the aqueous filtrate evaporated almost to dryness *in vacuo* (bath temp. 40°). Addition of acetone gave a crystalline precipitate of silver methoxyacetate.

The residue from the steam distillation was filtered and treated with conc. H_2SO_4 (1.5 ml.) in water (20 ml.). BaSO_4 was removed by centrifugation and the solution boiled out in a stream of CO_2 -free N_2 . It was then treated exactly as described previously to separate formic and methoxyacetic acids. (Yield of BaCO_3 , 230 mg.; 63.5%. Yield of silver methoxyacetate, 192 mg.; 54%.) On decomposition of the silver methoxyacetate with excess Br_2 in dry CCl_4 there were obtained 52 mg. of BaCO_3 .

Method B: degradation of the 2-ethyl 6-methyl ether of kojic acid (5-ethoxy-2-methoxymethyl-pyrone). During the course of this work it became apparent (Arnstein & Bentley, 1951b) that the alkaline hydrolysis of kojic acid dimethyl ether gives rise to a symmetrical intermediate, $\text{MeOCH}_2 \cdot \text{CO} \cdot \text{CH}_2 \cdot \text{CO} \cdot \text{CH}_2 \cdot \text{OMe}$; thus methoxyacetic acid originates not only from carbon atoms 5 and 6, but also from carbon atoms 2 and 3 of kojic acid and similarly methoxyacetone arises from carbon atoms 4, 5 and 6 as well as from carbon atoms 2, 3 and 4, i.e.:

The hydrolysis of a number of different ethers of kojic acid (*Ia*, *b*, *c* and *d*) was therefore investigated with a view to separating the hydrolysis products derived from the relevant carbon atoms, but only the ethyl methyl compound (*Id*) was hydrolysed by $\text{Ba}(\text{OH})_2$ in satisfactory yield.

(a) R = methyl, R' = phenyl; (b) R = methyl, R' = nitrophenyl; (c) R = benzyl, R' = methyl; (d) R = ethyl, R' = methyl.

A typical degradation was as follows: kojic acid (1.16 g.), isolated from the culture medium as already described, was dissolved in methanol (50 ml.). Ag_2O (2.5 g.) and ethyl iodide (1.4 ml.) were added and the mixture was mechanically shaken at room temp. for 4 hr. when a few drops of the solution no longer gave a red colour with FeCl_3 . The solution was then filtered and evaporated to dryness *in vacuo*. The residue was crystallized from benzene yielding kojic acid 2-ethyl ether (0.96 g.), m.p. 110–111° (cf. Yabuta, 1924). (The radioactivity of kojic acid 2-ethyl ether prepared in this way was unchanged when a sample was further purified by vacuum sublimation followed by recrystallization from benzene.) The ethyl methyl compound was prepared by treating a solution of the above material (0.71 g.) in water (20 ml.) at 0° with *n*-NaOH (9.9 ml.) and dimethyl sulphate (0.9 ml.), which were added over 3 hr. in 7 approx. equal portions. After 16 hr. at 0° the solution was concentrated *in vacuo* to about 15 ml. and extracted four times with ether (2 vol.). The ether was evaporated to dryness to give crystalline 2-*O*-ethyl-6-*O*-methylkojic acid (0.6 g.). In a preliminary experiment sublimation *in vacuo* yielded pure material, m.p. 45–46°. (Found: C, 58.1; H, 6.4%. $\text{C}_9\text{H}_{12}\text{O}_4$ requires C, 58.7; H, 6.6%.) Usually the crude 2-*O*-ethyl-6-*O*-methylkojic acid was directly hydrolysed in exactly the same way as the dimethyl ether, the formic acid being again converted to CO_2 with HgO .

The solution was steam-distilled to remove methoxy- and ethoxy-acetone. The residue was filtered, Hg ions were removed with H_2S , and after filtration the solution was concentrated *in vacuo* to approximately 10–20 ml. Methoxy- and ethoxy-acetic acids were then isolated by continuous ether extraction. The ether was evaporated and the mixed acids were dissolved in CHCl_3 and separated on a column of Hyflo Super-cel (see below).

The mixture of methoxy- and ethoxy-acetone obtained by steam-distillation was converted to iodoform and methoxy- and ethoxy-acetic acids. The iodoform was filtered off and

the methoxy- and ethoxy-acetic acids isolated by continuous ether extraction. After evaporation of the ether, the acids were dissolved in CHCl_3 and separated chromatographically on Hyflo Super-cel as already described. The appropriate fractions of effluent from the column were combined and evaporated to dryness, giving the potassium salts of methoxy- and ethoxy-acetic acid, respectively. After acidification with 2*N*- H_2SO_4 the free acids were isolated by continuous ether extraction and converted to the silver salts exactly as described for methoxyacetic acid under method A. The silver salts were assayed for ^{14}C and further degraded with Br_2 , as previously described. The methoxy- and ethoxy-acetic acids obtained directly from the hydrolysis were isolated, separated and degraded in the same way. When only a small amount of material was available, the methoxy- and ethoxy-acetic acids obtained from the ketones were combined with the corresponding acids obtained directly from the hydrolysis.

Oxidation of [1- ^{14}C]glucose and [3:4- $^{14}\text{C}_2$]glucose to carbon dioxide during fermentation

In these experiments (Table 1, no. 1, 9 and 10) the carbon dioxide evolved during the fermentation was continuously collected in sodium hydroxide and counted after conversion to barium carbonate. Table 4 shows that the carbon dioxide evolved during the early stages of the fermentation of [1- ^{14}C]glucose was most radioactive, and that the radioactivity later (5–14 days) declined to an approximately constant value. When [3:4- $^{14}\text{C}_2$]glucose was used the reverse was observed: the initial carbon dioxide had a radioactivity considerably lower than that produced on the third and fourth day. These results indicate that the primary point of oxidation of the glucose was C_1 rather than

Table 4. *Radioactivity of carbon dioxide evolved during the fermentation of [1- ^{14}C] and [3:4- $^{14}\text{C}_2$] glucose*

(For details of experimental conditions see Table 1. [1- ^{14}C]Glucose was used in Exps. 1 and 9 and [3:4- $^{14}\text{C}_2$]glucose in Exp. 10. The CO_2 was absorbed in NaOH over 24 hourly periods and converted to BaCO_3 . If all glucose carbon atoms were converted equally to CO_2 the calculated specific radioactivity of the BaCO_3 in Exps. 1, 9 and 10 would be 75.4, 93.4 and $100.0 \times 10^{-3} \mu\text{c./mg.}$, respectively. The accuracy of the radioactivity determinations is $\pm 5\%$.)

Age of culture at time of collection of CO_2 (days)	Weight of BaCO_3 collected (g.)			Specific radioactivity of BaCO_3 ($10^{-3} \mu\text{c./g.}$)		
	Exp. 1	Exp. 9	Exp. 10	Exp. 1	Exp. 9	Exp. 10
1	Trace	—	—	101.2	—	—
2	0.68	3.25	1.42	68.1	89.6	96.5
3	5.9	7.6	5.43	43.5	79.5	126.0
4	3.3	9.3*	5.72*	31.8	75.9	124.5
5	4.1	6.8	5.70	28.7	75.2	105.5
6	3.30	3.6	6.27	31.4	73.9	101.8
7	3.97	—	6.13	24.4	—	95.0
8	4.49	—	4.85	25.5	—	103.0
9	4.15	—	—	22.5	—	—
10	5.19	—	—	34.5	—	—
11	5.55	—	—	23.4	—	—
12	6.13*	—	—	19.5	—	—
13	4.35	—	—	16.0	—	—
14	3.15	—	—	25.8	—	—
15	3.05	—	—	47.9	—	—
16	2.94	—	—	46.3	—	—

* Kojic acid present in medium, as shown by red FeCl_3 colour reaction.

Table 6. *Percentage distribution of radioactivity in kojic acid obtained by fermentation of [1-¹⁴C]glucose, [3:4-¹⁴C₂]glucose and [2-¹⁴C]dihydroxyacetone*

(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 shown 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 obtained by adding the radioactivity located in all six carbon atoms of kojic acid (Table 5, col. 12). When this was not possible because of insufficient material for completing the degradation the specific radioactivity ($\mu\text{c./mole}$) of the kojic acid derivative (Table 5, cols. 3 or 4) used for the degradation was substituted for b .)

Exp. no.	Precursor used	Percentage distribution of radioactivity in kojic acid carbon atoms					
		C ₁	C ₂	C ₃	C ₄	C ₅	C ₆
1	[1- ¹⁴ C]Glucose	76.8, 72.8	—	—	1.5, 4.8	—	—
2		80.8	—	—	1.2	—	—
3		85.0	—	—	2.6	—	—
4		68.6	—	—	1.5	—	—
5		68.0	—	—	1.6	C ₅ + C ₆ = 2.4	
6		71.0	—	—	1.9	—	—
7		74.6	2.1	3.6	1.3	1.8	16.6
8		87.5	2.6	2.9	0.35	0.35	6.2
9		78.0	C ₂ + C ₃ = 4.1		2.5	C ₅ + C ₆ = 15.4	
10	[3:4- ¹⁴ C ₂]Glucose	{ 2.8 3.8	{ 8.5 10.0	{ 35.2 28.8	{ 51.0 56.5	{ 1.6 C ₅ + C ₆ = 0.9	{ 0.9 C ₅ + C ₆ = 60.4
11	[2- ¹⁴ C]Dihydroxyacetone	6.1	C ₂ + C ₃ = 30.7		2.8	C ₅ + C ₆ = 60.4	
13		5.4	12.7	9.6	1.9	66.5	3.9
14		13.4	30.1	9.6	1.6	33.8	11.5

—, value not determined.

Table 7. *Comparison of the specific radioactivity of kojic acid and [1-¹⁴C]glucose, [3:4-¹⁴C₂]glucose and [2-¹⁴C]dihydroxyacetone used as precursors*

(The experimental details are described in Table 1.)

Exp. no.	Precursor	Specific radioactivity ($\mu\text{c./mole}$) of		Time of addition of precursor after inoculation of medium (days)	Specific radioactivity of kojic acid and derivatives ($\mu\text{c./mole}$)		
		Precursor added to medium	Glucose re-isolated after fermentation		Kojic acid	Kojic acid dimethyl ether	Kojic acid 2-ethyl ether
1	[1- ¹⁴ C]Glucose	89.3	—	0	90.9, 78.7*	79.0	—
2		930	—	8	75.8	65.4, 65.5*	—
3		64.7	—	8†	38.6	35.0, 36.3*	—
4		64.7	—	8†	50.0	42.5, 43.3*	—
5		930	141.3	7	78.5	—	67.0
6		930	—	5	66.4	—	64.2
7		930	159.1	7	50.3	—	43.2
8		930	—	7	152.5	—	148.6
9		110.6	73.5	0	83.0	—	72.7
10	[3:4- ¹⁴ C ₂]Glucose	118.4	130.2	0	118.2	—	{ 120.4 115.4†
11	[2- ¹⁴ C]Dihydroxyacetone	430	—	5	46.3	—	42.3
13		464	0.7	7	31.9	—	26.5
14		464	—	7	53.4	—	34.0

* After recrystallization.

† Duplicate preparation.

† Replacement medium used (see Table 1).

—, value not determined.

presence of a highly active impurity derived mainly from the labelled carbon atoms of [1-¹⁴C]glucose and [2-¹⁴C]dihydroxyacetone.

Redistribution of ¹⁴C during fermentation of [1-¹⁴C]glucose

If resynthesis of glucose from triosephosphates by a reversal of the Embden-Meyerhof scheme were to occur during the fermentation, [1-¹⁴C]glucose

would give rise to glucose labelled in C₍₆₎ as well as in C₍₁₎. Such glucose might be released into the culture medium or deposited as cellular polysaccharide. In either event, subsequent direct conversion of this glucose to kojic acid would result in the formation of kojic acid containing an appreciable amount of ¹⁴C in C₍₆₎. The observed distribution of tracer in the kojic acid was very similar to that expected on the basis of the above considerations and

it was important therefore to establish the extent of the redistribution of ^{14}C in the glucose during the fermentation. For this purpose, the glucose recovered at the end of three fermentations (Exps. 5, 7 and 9) was degraded by periodate oxidation (Reeves, 1941). The formaldehyde, which is derived from carbon atom 6, was isolated and counted as the dimedon derivative. The results (Table 8) show that

Table 8. Amount of radioactivity in carbon atom 6 of $[1-^{14}\text{C}]$ glucose recovered from fermentations

(Details of the experiments are given in Table 1. The formaldehyde dimedon was obtained by periodate oxidation of the glucose (Reeves, 1941).)

Exp. no.	Specific radioactivity of recovered glucose ($\mu\text{c./mole}$)	Specific radioactivity of formaldehyde dimedon ($\mu\text{c./mole}$)	Radioactivity in C_6 of glucose (%)
5	141.3	2.69	1.9
7	159.1	2.34	1.5
9	73.5	2.62	3.5

the amount of ^{14}C in $\text{C}_{(6)}$ of the recovered glucose was only about 2–3 % of the ^{14}C present in the whole molecule. Although this amount is insufficient to account for the much greater radioactivity (up to 16 %) in the corresponding carbon atom of kojic acid (cf. Table 6), it is clear that some resynthesis of glucose must have taken place during the fermentation.

DISCUSSION

The conversion of glucose to kojic acid

The object of this investigation was to elucidate the mechanism of the conversion of glucose to kojic acid by *Aspergilli*. It was expected that the distribution of ^{14}C in the kojic acid obtained from cultures of the moulds grown on ^{14}C -labelled glucose or dihydroxyacetone would indicate whether glucose was converted to kojic acid without cleavage of the carbon chain or whether dihydroxyacetone was formed as an intermediate.

The distribution of ^{14}C in kojic acid which would result if either $[1-^{14}\text{C}]$ glucose or $[3:4-^{14}\text{C}_2]$ glucose were the substrate and if either dihydroxyacetone or dihydroxyacetone phosphate (formed from carbon atoms 1–3 of glucose) were an intermediate, is summarized in Table 9. These distributions are calculated for the case where the radioactive triose has been assumed to have condensed with a second non-radioactive C_3 compound and for the case where two radioactive triose molecules have undergone condensation. The possibilities of equilibrium either between dihydroxyacetone and glyceraldehyde phosphates under the influence of triosephosphate isomerase or between dihydroxyacetone phosphate and free dihydroxyacetone are also included. The

evidence for the participation of dihydroxyacetone in the formation of kojic acid has already been discussed briefly in the Introduction and has been reviewed in more detail by Foster (1949); despite the fact that dihydroxyacetone is usually formed as the 1-phosphate by cleavage of fructose 1:6-diphosphate, previous discussions of the biosynthesis of kojic acid have considered only free dihydroxyacetone as the intermediate. This distinction becomes important when the isotope experiments are considered, since dihydroxyacetone phosphate is not a symmetrical compound.

In our experiments the kojic acid obtained by fermentation of $[1-^{14}\text{C}]$ glucose had virtually the same molar radioactivity as the glucose and contained 70 to 90 % of the ^{14}C in carbon atom 1. Using $[3:4-^{14}\text{C}_2]$ glucose, the kojic acid again had the same molar radioactivity as the substrate and 90 % of the ^{14}C was located in carbon atoms 3 and 4. (The unequal distribution of radioactivity between these two carbon atoms will be discussed later.) These distributions of radioactivity do not coincide with any of those which would result (cf. Table 9) if the intermediary formation of dihydroxyacetone or dihydroxyacetone phosphate followed by self condensation or condensation with a second, non-radioactive C_3 compound were the only pathway; the one exception (3a) to this statement is considered in detail below. Moreover, the observed distribution of ^{14}C in the kojic acid derived from $[2-^{14}\text{C}]$ dihydroxyacetone (Table 6, Exps. 11, 13 and 14) shows clearly that if free dihydroxyacetone were an important intermediate in glucose utilization, the conversion of $[1-^{14}\text{C}]$ glucose to kojic acid would have resulted in more extensive incorporation of ^{14}C into carbon atoms other than C_1 of kojic acid. Further evidence against the participation of dihydroxyacetone or glyceraldehyde is that kojic acid production by *Aspergillus tamarii* was actually increased in the presence of the trapping reagents, sodium sulphite or dimedon (Gould, 1938).

The high incorporation of ^{14}C into carbon atom 1 of kojic acid formed from $[1-^{14}\text{C}]$ glucose indicates that the major pathway of kojic acid biosynthesis consists of a direct conversion of glucose. Such a mechanism is in agreement with the high yields of kojic acid (up to 78 % of the theoretical amount) obtainable from glucose either with specially selected organisms (Morton, Kocholaty, Junowicz-Kocholaty & Kelner, 1945) or in replacement cultures under carefully defined conditions (Kluyver & Perquin, 1933).

The above conclusions would be invalid if $[1-^{14}\text{C}]$ -dihydroxyacetone-1-phosphate (derived from $[1-^{14}\text{C}]$ glucose) condensed with a non-radioactive C_3 unit with which it was not in equilibrium (cf. Table 9, 3a). (On comparative grounds the most likely C_3 unit would be glyceraldehyde phosphate.)

Table 9. *Expected distribution of ^{14}C in kojic acid formed by various mechanisms from $[1-^{14}\text{C}]$ or $[3,4-^{14}\text{C}_2]\text{glucose}$*

Ref. no.	Origin and mechanism of formation of dihydroxyacetone (DHA) or dihydroxyacetone 1-phosphate (DHAP)	Expected ^{14}C distribution in kojic acid									
		Expected ^{14}C distribution in dihydroxyacetone or dihydroxyacetone 1-phosphate					Expected ^{14}C distribution in kojic acid				
		^{14}C distribution in substrate glucose (total radioactivity = 100)					A. Condensation of one molecule DHA or DHAP with a non-radioactive C_3 compound				
		$\text{C}_{(1)}$	$\text{C}_{(2)}$	$\text{C}_{(3)}$	$\text{C}_{(4)}$	$\text{C}_{(5)}$	$\text{C}_{(1)}$	$\text{C}_{(2)}$	$\text{C}_{(3)}$	$\text{C}_{(4)}$	$\text{C}_{(5)}$
1 (a)	1 molecule DHA from $\text{C}_{(1)}$, $\text{C}_{(2)}$ and $\text{C}_{(3)}$ of glucose	100	—	—	—	—	$\text{C}_{(1)}$ $\text{C}_{(2)}$ $\text{C}_{(3)}$ $\text{C}_{(4)}$ $\text{C}_{(5)}$	$\text{C}_{(1)}$ $\text{C}_{(2)}$ $\text{C}_{(3)}$ $\text{C}_{(4)}$ $\text{C}_{(5)}$	$\text{C}_{(1)}$ $\text{C}_{(2)}$ $\text{C}_{(3)}$ $\text{C}_{(4)}$ $\text{C}_{(5)}$	$\text{C}_{(1)}$ $\text{C}_{(2)}$ $\text{C}_{(3)}$ $\text{C}_{(4)}$ $\text{C}_{(5)}$	$\text{C}_{(1)}$ $\text{C}_{(2)}$ $\text{C}_{(3)}$ $\text{C}_{(4)}$ $\text{C}_{(5)}$
1 (b)		—	—	50	50	—	—	—	—	—	—
2 (a)	1 molecule DHA from $\text{C}_{(1)}$, $\text{C}_{(2)}$ and $\text{C}_{(3)}$; and 1 molecule DHA from $\text{C}_{(4)}$, $\text{C}_{(5)}$ and $\text{C}_{(6)}$ of glucose	100	—	—	—	—	—	—	—	—	—
2 (b)		—	—	50	50	—	—	—	—	—	—
3 (a)		100	—	—	—	—	—	—	—	—	—
3 (b)	1 molecule DHAP from $\text{C}_{(1)}$, $\text{C}_{(2)}$ and $\text{C}_{(3)}$ of glucose via fructose diphosphate, not in equilibrium with glyceraldehyde phosphate	—	—	50	50	—	—	—	—	—	—
4 (a)	1 molecule DHAP derived as in 3 (a), but in equilibrium with glyceraldehyde phosphate	100	—	—	—	—	—	—	—	—	—
4 (b)		—	—	50	50	—	—	—	—	—	—
5 (a)	1 molecule DHAP from $\text{C}_{(1)}$, $\text{C}_{(2)}$ and $\text{C}_{(3)}$ of glucose in equilibrium with free DHA (Aronoff, Haas & Fries, 1949)	100	—	—	—	—	—	—	—	—	—
5 (b)		—	—	50	50	—	—	—	—	—	—

Although it is not known whether fructose diphosphate is a normal intermediate in these organisms, extracts of mycelia contained enzymes (aldolase and triosephosphate isomerase) converting it to triosephosphate which was virtually all dihydroxyacetone phosphate (Arnstein & Bentley, 1953*a*). Any glyceraldehyde phosphate would, therefore, almost certainly be in equilibrium with dihydroxyacetone phosphate; the possibility under discussion (Table 9, 3*a*) must therefore be considered unlikely to be a major pathway. Additional evidence for this conclusion is that growth of *A. flavus-oryzae* on dihydroxyacetone, followed by addition of [$1-^{14}\text{C}$]glucose at the beginning of kojic acid production, gave the highest incorporation of ^{14}C into $\text{C}_{(4)}$ of kojic acid (88 %) observed in any of the experiments. It is probable that in the presence of a plentiful supply of triose (presumably needed for other metabolic purposes) splitting of glucose is reduced to a minimum and kojic acid is almost exclusively formed by a direct conversion of the glucose.

The results of the experiments with [$1-^{14}\text{C}$]glucose were confirmed in an experiment with [$3:4-^{14}\text{C}_2$]glucose, prepared from the liver glycogen of rats which had been injected with $\text{NaH}^{14}\text{CO}_3$. Glucose prepared in this way (Wood, Lifson & Lorber, 1945) or by incubation of rat-liver slices with $\text{NaH}^{14}\text{CO}_3$ (Topper & Hastings, 1949) was found by these authors to contain an equal amount of isotope in carbon atoms 3 and 4. Degradation of our material indicated that there was slightly more radioactivity in carbon atoms 4, 5 and 6 (52, 51 %) than in carbon atoms 1, 2 and 3 (48, 49 %). On fermentation, such glucose gave kojic acid containing about 90 % of the total radioactivity in carbon atoms 3 and 4 indicating, in the main, a direct conversion. Degradation of this kojic acid showed that about one-half of the total ^{14}C was located at carbon atom 4, but carbon atom 3 appeared to contain much less radioactivity than carbon atom 4 (Table 6, Exp. 10). The value for carbon atom 3 was obtained from the radioactivity of the carbon dioxide produced by bromine decarboxylation of silver ethoxyacetate (derived from carbon atoms 2 and 3); it is possible that some dilution of the $^{14}\text{CO}_2$ with extraneous carbon dioxide occurred during the degradation of this sample, although duplicate degradations both gave low values for carbon atom 3. The radioactivity of the silver ethoxyacetate accounted for only slightly less than half (43 %) of the total radioactivity and the real difference between carbon atoms 3 and 4 may therefore be rather less than indicated by the direct radioactivity determinations of carbon atoms 3 and 4. Another explanation is that the [$3:4-^{14}\text{C}_2$]glucose may have undergone some degradation and resynthesis during the fermentation, especially since glucose isolated from the

culture medium after fermentation of [$1-^{14}\text{C}$]glucose contained a small but definite amount of ^{14}C in carbon atom 6 (Table 8). Degradation of the residual glucose from the [$3:4-^{14}\text{C}_2$]glucose experiment showed that carbon atoms 1, 2 and 3 contained only slightly less than half of the total radioactivity (42, 46 %) compared with the values 48, 49 % for the starting material. The precise significance of these results is difficult to assess, especially considering a total error in counting of about 5 %, and the fact that the small amount of glucose re-isolated (as the phenyllosazone) was difficult to recrystallize to constant radioactivity.

The incorporation of ^{14}C into carbon atom 6 of kojic acid in the experiments with [$1-^{14}\text{C}$]glucose suggests that a second pathway also contributes to kojic acid formation, although to a much lesser extent. Since glucose remaining in the culture medium at the end of fermentations contained less than 4 % of the total ^{14}C at carbon atom 6 (Table 8) it is clear that the kojic acid, which in one experiment contained as much as 17 % of the total radioactivity in carbon atom 6, could not have arisen entirely from this glucose by a direct conversion. A possible explanation of this redistribution of ^{14}C predominantly into carbon atom 6 would be that a condensation of [$3-^{14}\text{C}$]phosphoglyceraldehyde arising from [$1-^{14}\text{C}$]dihydroxyacetone phosphate under the influence of triosephosphate isomerase, with dihydroxyacetone phosphate (to form a precursor of kojic acid) does take place to a small extent (cf. Table 9, 4*a*). The observation that extracts of the mycelia of these moulds do indeed contain aldolase and triosephosphate isomerase (Arnstein & Bentley, 1953*a*) is additional evidence that a condensation of this nature may be a minor pathway of kojic acid biosynthesis.

In order to gain further information about the two pathways involved in the conversion of [$1-^{14}\text{C}$]glucose to kojic acid this reaction was studied under a variety of conditions. By suitable modifications in the administration of the labelled glucose it was possible to influence the extent of redistribution of ^{14}C in the kojic acid. As was to be expected if a direct conversion were the major pathway, the percentage of ^{14}C in carbon atom 1 was higher (80 %) (Table 6, Exp. 2) when [$1-^{14}\text{C}$]glucose was added to the culture (*A. oryzae*) at the beginning of kojic acid production than when it was present throughout the fermentation (75 %) (Table 6, Exp. 1). However, in experiments involving complete replacement of the original culture medium, somewhat variable results were obtained. When the [$1-^{14}\text{C}$]glucose was added in a Czapek-Dox replacement medium, and the kojic acid isolated 5 days later, the amount of ^{14}C in carbon atom 1 was somewhat less, i.e. 70 %, than when the ^{14}C glucose was present from the beginning (for 19 days). With a replacement medium of

phosphate buffer at pH 2, the percentage of ^{14}C in carbon atom 1 of the kojic acid, again isolated 5 days later, was 85 % (Table 6, Exp. 3). Under the latter conditions the yield of kojic acid, based on the glucose used, is very high (Kluyver & Perquin, 1933) and this is apparently reflected in a diminished redistribution of ^{14}C .

With the *A. flavus-oryzae* strain, addition of $[1-^{14}\text{C}]$ glucose after initial growth for 7 days yielded kojic acid containing 75 % of the ^{14}C in carbon atom 1 (Table 6, Exp. 7), a little less than with *A. oryzae*. This value was increased slightly (to 78 %) when the culture was grown in the presence of sodium fluoride (Table 6, Exp. 9). However, when $[1-^{14}\text{C}]$ glucose was added to a culture pregrown on dihydroxyacetone as the sole carbon source, 88 % of the total radioactivity of the kojic acid was located in carbon atom 1 (Table 6, Exp. 8). Unfortunately, our strain of *A. oryzae* failed to produce kojic acid when grown on dihydroxyacetone and a complete comparison of kojic acid formation by the two strains was therefore impossible.

As previously noted, most of the redistributed radioactivity was located in carbon atom 6 of kojic acid. The amounts in carbon atoms 2–5 were relatively small and never exceeded 10 % of the total radioactivity. Owing to the errors involved in the degradation and assay of samples of low radioactivity, it is possible that some of these carbon atoms may in fact have been completely non-radioactive rather than weakly radioactive. No regular patterns of distribution of ^{14}C in these positions could be observed and these results will therefore not be discussed further.

The mechanism of the conversion of glucose to kojic acid

Whilst our results indicate that the carbon chain of glucose remains largely intact during its conversion to kojic acid and that symmetrical C_3 and C_6 intermediates are not involved, we have no evidence to show whether the pyrone ring also remains unbroken. The simplest scheme for kojic acid formation from glucose is of the following type (Yabuta, 1924; Kinoshita, 1927; Haworth, 1929), the initial reaction being either an oxidation, as shown, or a dehydration:

By analogy with the oxidation of glucose by notatin (Bentley & Neuberger, 1949) or by an ox-liver preparation (Strecker & Korkes, 1951), where gluconic

acid lactone is the primary product, and the similar oxidation of glucose 6-phosphate to 6-phosphogluconolactone (Lipmann, 1951; Cori & Lipmann, 1952), it is tempting to postulate that the first stage of kojic acid formation may be the removal of two hydrogen atoms from carbon atom 3 of glucopyranose under the influence of an oxidase. Such oxidations, i.e. $\text{CHOH} \rightarrow \text{CO}$, occur frequently in fungal and bacterial metabolism as shown by the common occurrence of ketogluconic acids, although usually only the 2- or 5-keto compounds are found and the compound (III) (3-ketoglucose) postulated as an intermediate is apparently unknown. However, some evidence for 3-keto compounds exists. 3-Ketogluconic acid 6-phosphate has been postulated as an intermediate in the formation of ribose 5-phosphate from 6-phosphogluconate by a yeast enzyme (Horecker & Smyrniotis, 1950). In addition, a ketogluconic acid which is not the usual 2- or 5-keto acid and is probably 3-ketogluconic acid has recently been isolated from *Acetobacter melanogenum* (Riedl-Tůmová & Bernhauer, 1950). This seems especially significant since the acetic acid bacteria are the only group of bacteria known to produce kojic acid.

Experiments with inhibitors also suggested the occurrence of an oxidase of the notatin type. Notatin is not inhibited by sodium iodoacetate (Walker, 1949) and the oxidation of glucose or gluconic acid to 2-ketogluconic acid by *Pseudomonas aeruginosa* is not blocked by 0.056M-sodium fluoride nor by 0.005M-iodoacetate (Campbell & Norris, 1950; Stokes & Campbell, 1951). Neither of these reagents in any way depresses the formation of kojic acid nor did the presence of sodium fluoride materially affect the isotope distribution when $[1-^{14}\text{C}]$ glucose was used (Table 6, Exp. 9).

Despite the simplicity of the above pathway, the frequent occurrence of gluconic and ketogluconic acids in fungi and other micro-organisms is striking. Gluconolactone (V) may therefore be the first step in kojic acid formation especially since gluconic acid is produced by a strain of *A. oryzae* (Sakaguchi, 1931), and gluconic acid (but not saccharic acid) and gluconolactone can be utilized for kojic acid production (Katagiri & Kitahara, 1929; Gould, 1938). If oxidation to 3-ketoglucolactone (VI)

were to occur, two alternative pathways would then be possible. (a) Reduction to 3-ketoglucose (III), followed by loss of two molecules of water. (b) Loss

of one molecule of water to give (VII), followed by reduction of the carbonyl group (to give (VIII)) and loss of a second molecule of water.

radioactivity, in agreement with the values 70 and 22 %, respectively, observed in the first experiment. The glucose isolated from the culture medium at the

There is some reason to suppose that the postulated reductions, CO → CHO, would occur. Lipmann (1951) pointed out that the glucose 6-phosphate/6-phosphogluconolactone system had a higher oxidation-reduction potential than the corresponding aldehyde/carboxylic acid system and suggested that the (phospho) lactone would be more readily reduced to the glucose level. Strecker & Korkes (1951) also pointed out that in their ox-liver preparation the reaction

should be reversible, and were able to show that this was so. (DPN⁺ = oxidized diphosphopyridine nucleotide, DPNH = reduced diphosphopyridine nucleotide.)

The conversion of [2-¹⁴C]dihydroxyacetone to kojic acid

When *A. flavus-oryzae* was grown from the beginning on dihydroxyacetone and labelled dihydroxyacetone was added soon after kojic acid production had begun, the kojic acid contained almost equal amounts of ¹⁴C in carbon atoms 2 and 5. This indicated that the major reaction was a condensation of two C₃ molecules, either dihydroxyacetone or closely related derivatives. In addition, carbon atoms 1, 3, 4 and 6 also contained a substantial amount of ¹⁴C.

When [2-¹⁴C]dihydroxyacetone was added at the beginning of kojic acid production to a culture of *A. flavus-oryzae*, grown from the beginning on glucose and still containing free glucose, the kojic acid unexpectedly contained very much more radioactivity in carbon atom 5 than in 2 (Table 6, Exp. 13; radioactivity in C₅ = 66.5, in C₂ = 12.7 %). In a second experiment (no. 11) a poor yield of kojic acid was obtained and the degradation could not be completed; however, carbon atoms 5 + 6 contained 60 % and carbon atoms 2 + 3 only 31 % of the total

end of this experiment contained only a very small amount of radioactivity and therefore was probably not an intermediate in the biosynthesis; the possibility that glucose bound as intracellular polysaccharide would be more highly labelled cannot at present be excluded.

Several explanations for the predominant incorporation of ¹⁴C into C₅ of kojic acid when [2-¹⁴C]dihydroxyacetone is used in presence of glucose are possible, although none is entirely satisfactory. They are as follows: (a) A C₃ + C₃ condensation followed by carbon dioxide fixation could lead to this result. However, in several experiments where ¹⁴CO₂ was added to the gas phase above a culture only a very small incorporation of ¹⁴C into kojic acid was observed (Arnstein & Bentley, 1953b). Similarly, kojic acid isolated from cultures to which sodium ¹⁴C formate or L-[α-¹⁴C]lysine had been added was almost completely non-radioactive, although both of these compounds were oxidized to ¹⁴CO₂ during metabolism and ¹⁴C was incorporated both into the mycelium and into the impurity which accompanied kojic acid during the preliminary extraction of the culture medium. There is, therefore, no real evidence for this pathway. (b) If the synthesis of kojic acid from [2-¹⁴C]dihydroxyacetone in the presence of unlabelled glucose involves the conventional triosephosphate isomerase equilibrium of the Embden-Meyerhof cycle (i.e. the secondary pathway previously discussed in the case of glucose), the excess of ¹⁴C in C₅ of the kojic acid may be due to differences in the rates of competing reactions and the relative amounts of triose intermediates available at the time of addition of the labelled dihydroxyacetone. The probable reactions which would have to be considered are given on p. 507. If B and C were fast relative to A and D, then [2-¹⁴C]dihydroxyacetone would yield predominantly 5-labelled kojic acid. (c) Condensation of the added

dihydroxyacetone with any non-radioactive C_3 unit would yield 5-labelled kojic acid provided that the dihydroxyacetone becomes the 'lower half' of the kojic acid molecule. The postulated C_3 intermediate might be dihydroxyacetone phosphate, especially since there is some evidence that the equilibrium between dihydroxyacetone and its phosphate is attained only slowly (Aronoff, Haas & Fries, 1949; Koshland & Westheimer, 1950). (d) A further possibility is that the added dihydroxyacetone is itself phosphorylated and then condenses with a non-radioactive C_3 unit, such as pyruvic acid, leading to a reversal of the recently observed split of 6-phosphogluconic acid by *Pseudomonas saccharophila* (Entner & Doudoroff, 1952).

Until more information is available, it is impossible to decide the precise mechanism of this incorporation of dihydroxyacetone, but it is of interest that an analogous result was obtained in a study of the biosynthesis of menthyl glucuronic acid in guinea pig liver slices (Bidder, 1952). Incubation of the slices with $[1-^{14}\text{C}]$ glucose gave glucuronic acid containing almost all of the radio-

activity in carbon atom 1. Using $[3-^{14}\text{C}]$ lactic acid the glucuronic acid contained ^{14}C in positions 1 and 6; however, the activity in carbon atom 6 was 2.5 times that of carbon atom 1.

Further discussion of secondary pathways of kojic acid biosynthesis will be deferred to a later paper where the distribution of ^{14}C in kojic acid arising from labelled small molecules will be discussed.

SUMMARY

1. The conversion of glucose to kojic acid by two species of *Aspergilli* has been studied using both $[1-^{14}\text{C}]$ and $[3,4-^{14}\text{C}]$ glucose. Between 70 and 90 % of the total radioactivity of the isolated kojic acid was found in the carbon atoms corresponding to those labelled in the glucose, indicating that the major pathway of kojic acid formation is a direct conversion of glucose without splitting of the carbon chain. Symmetrical C_3 or C_6 compounds are not intermediates in the biosynthesis.

2. A minor pathway of kojic acid biosynthesis, involving condensation of 'small' molecules, probably triosephosphates, leads to incorporation of ^{14}C into position 6 of kojic acid, when $[1-^{14}\text{C}]$ glucose is used as substrate.

3. The biosynthesis of kojic acid from $[2-^{14}\text{C}]$ -dihydroxyacetone, alone or in the presence of unlabelled glucose, has also been studied. In the latter case, ^{14}C is incorporated predominantly into position 5 of kojic acid.

4. Possible mechanisms for the conversion of glucose and dihydroxyacetone to kojic acid are discussed.

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The Biosynthesis of Kojic Acid

2. THE OCCURRENCE OF ALDOLASE AND TRIOSEPHOSPHATE ISOMERASE IN *ASPERGILLUS* SPECIES AND THEIR RELATIONSHIP TO KOJIC ACID BIOSYNTHESIS

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The experiments with ^{14}C -labelled glucose and dihydroxyacetone described in the preceding paper (Arnstein & Bentley, 1953) indicated that dihydroxyacetone is not an intermediate in the biosynthesis of kojic acid from glucose. Moreover, the distribution of ^{14}C in the kojic acid derived from [$1\text{-}^{14}\text{C}$]glucose suggested that most of the kojic acid was formed by a direct conversion of the glucose. However, the possibility that kojic acid might be synthesized by condensation of dihydroxyacetone phosphate and a C_3 unit, e.g. glyceraldehyde phosphate, not in equilibrium with each other, could not be excluded by the isotope experiments, as the expected distribution of ^{14}C in the kojic acid would be similar in both cases. It therefore became important to determine whether the organisms producing kojic acid used in this work contained the enzymes aldolase, which is concerned with the formation of dihydroxyacetone phosphate and glyceraldehyde phosphate, and triosephosphate isomerase; the presence of triosephosphate isomerase would be particularly significant as this enzyme would bring about the equilibrium between any dihydroxyacetone phosphate formed and

glyceraldehyde phosphate. In addition to experiments demonstrating the presence of both these enzymes in the mycelium of the moulds used for kojic acid production in this work, the present paper also discusses the influence of phosphate on kojic acid formation and the evidence for the participation of phosphorylated intermediates in kojic acid biosynthesis.

The method of numbering the kojic acid carbon atoms throughout this paper corresponds with that adopted in the previous paper (Arnstein & Bentley, 1953).

EXPERIMENTAL

Enzyme experiments. In experiments to determine whether the organisms contained the enzyme aldolase, extracts were prepared by grinding mycelia with sand. These extracts were incubated with hexose diphosphate and the formation of triosephosphate was observed by measuring the amount of alkali-labile and inorganic phosphate present at definite time intervals.

Material and Methods

Fructose diphosphate. Calcium hexose diphosphate (2.2 g.) was suspended in a little water and treated at 0° with 2N-HCl until almost all was dissolved. A little insoluble material was centrifuged off and solid potassium oxalate (1.58 g.) added to the solution. After shaking, the precipi-

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