DISCUSSION

The plots in Figs. 1 and 2 show that samples 5/B, 569 (induced) and 569/H(2) appear to be homogeneous in sedimentation, judged both by the closely Gaussian form of the boundary-gradient curves and by the linearity of σ^2 against t . 569/H(1), however, has a markedly skew boundary, with an extended advancing limb; its plot of σ^2 against t shows a possibly significant curvature. Both these criteria indicate some degree of heterogeneity. Tests of homogeneity from the pattern of fringes in the Gouy diffusion record confirm these conclusions.

Samples 569 (induced) and 569/H(2) do not differ significantly from each other, but sample 5/B differs significantly from both. This is consistent with their being essentially different substances.

Sample 569/H(1) differs significantly from all others and in particular from sample 569/H(2). The differences of sedimentation and diffusion coefficients and the frictional ratio indicate some difference of molecular configuration, though the material is not entirely uniform. Sample 569/H(1) was precipitated by ethanol in the course of purification, whereas 569/H(2) was not. A test made with the Spinco ultracentrifuge at the National Institute of Medical research (Kogut, Pollock & Tridgell, 1956) showed that precipitation of 569

(induced) with ethanol produced no obvious change in its rate of sedimentation or in the form of its sedimentation boundary. It therefore appears possible that the differences in the conditions of culture may have caused the difference between 569/H(1) and 569/H(2).

SUMMARY

1. The diffusion and sedimentation of four samples of penicillinase have been observed.

2. Sample 5/B differs from all others; samples 569 (induced) and 569/H(2) appear to be identical; samples 569/H(1) and 569/H(2) are different.

3. All samples except 569/H(1) appear homogeneous, by the tests applied.

REFERENCES

- Baldwin, R. L. (1953*a*). *Brit. J. exp. Path.* **35**, 255.
 Baldwin, R. L. (1953*b*). D.Phil. Thesis: University of Oxford.
 Cecil, R. & Ogston, A. G. (1948). *Biochem. J.* **43**, 592.
 Cecil, R. & Ogston, A. G. (1951). *J. sci. Instrum.* **28**, 253.
 Coulson, C. A., Cox, J. T., Ogston, A. G. & Philpot, J. St L. (1948). *Proc. Roy. Soc. A*, **192**, 382.
 Gosting, L. J. & Akeley, D. F. (1953). *J. Amer. chem. Soc.* **75**, 5685.
 Gosting, L. J. & Kegeles, G. (1947). *J. Amer. chem. Soc.* **69**, 2516.
 Kogut, M., Pollock, M. R. & Tridgell, E. J. (1956). *Biochem. J.* **62**, 391.

The Biosynthesis of Kojic Acid

4. PRODUCTION FROM PENTOSE AND METHYL PENTOSE*

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(Received 11 July 1955)

The biosynthesis of kojic acid is of interest because it seems likely that the C_6 precursor of this mould metabolite is a compound synthesized by well-known reaction sequences of carbohydrate metabolism; however, because of features unique to the micro-organisms producing kojic acid, this precursor is no longer metabolized in the usual way, ultimately to carbon dioxide. Instead, special (shunt) reaction mechanisms convert the C_6 precursor into the relatively undegraded end product. It was hoped that a study of the biosynthesis of kojic acid in *Aspergillus flavus* would therefore provide information about both the general and special reaction sequences of carbohydrate meta-

bolism in this organism. Some information has already been obtained from previous studies of kojic acid biosynthesis (Arnstein & Bentley, 1953*a-c*; Denison, Carson & Foster, 1954). In particular, it has been shown that the major pathway of kojic acid production from glucose is a direct conversion without splitting of the carbon chain. However, the experiments with labelled glucose, and also with a number of labelled small molecules (such as dihydroxyacetone, pyruvate, acetate, etc.) indicated a secondary pathway which was believed to involve triose phosphates (Arnstein & Bentley, 1953*a, c*).

It had at one time been supposed that, because the pentoses L-arabinose and D-xylose can also be converted into kojic acid by *Aspergillus* species

* Part 3: Arnstein & Bentley (1953*c*).

(Challenger, Klein & Walker, 1929; Katagiri & Kitahara, 1929; Corbellini & Gregorini, 1930; Birkinshaw, Charles, Lilly & Raistrick, 1931; Tamiya, 1932; Gould, 1938), kojic acid formation from both pentose and hexose required the formation of a small reactive molecule (possibly a C_2 or C_3 fragment). In view of the demonstration of the direct conversion of glucose into kojic acid it was apparent that information concerning other pathways of kojic acid biosynthesis would be obtained from a study of the reaction involved when a pentose was used as substrate. A possible mechanism for the production of the C_6 precursor of kojic acid from pentose was provided by the elucidation of the reactions involved in the biological conversion of ribose 5-phosphate into hexose phosphate (these reactions have recently been reviewed in detail by Racker, 1954). Indeed, it has been pointed out (Horecker, Gibbs, Klenow & Smyrniotis, 1954; Racker, 1954) that the distribution of ^{14}C in kojic acid formed from $[3\text{-}^{14}C_2]$ -glucose (Arnstein & Bentley, 1953*a*) is similar to that observed in hexose monophosphate formed from $[2\text{:}3\text{-}^{14}C_2]$ pentose 5-phosphate. This suggested that one secondary pathway was a resynthesis, in part, of a C_6 precursor of kojic acid by the transketolase and transaldolase reactions.

The present communication is a report on kojic acid formation from the eight stereoisomeric pentoses, and two methyl pentoses; a tracer study of kojic acid formation from D - $[1\text{-}^{14}C]$ ribose is also described. A preliminary account of some of these results has been given (Bentley, 1955).

EXPERIMENTAL AND RESULTS

Materials and methods

The organism used in most of these experiments was that previously referred to as *Aspergillus flavus-oryzae* which is now carried in the Northern Regional Research Laboratory culture collection as *A. flavus*, NRRL 2405. Culture conditions, colorimetric determination of kojic acid, the degradation of labelled kojic acid through the 2-ethyl 6-methyl ether and counting techniques were as previously described (Arnstein & Bentley, 1953*a-c*). The numbering of the kojic acid carbon atoms is that used previously (see also Fig. 1).

In the degradation, carbon atoms 2 and 3 are obtained as the acetic acid moiety of ethoxyacetic acid and carbon atoms 6 and 5 as the similar component of methoxyacetic acid. Decarboxylation of the silver salts of these acids with bromine gives respectively carbon atoms 3 and 5 as carbon dioxide. The activity of carbon atoms 2 and 6 is then derived by difference. Experiments described previously (Arnstein & Bentley, 1953*a*) demonstrated at most only a slight dilution of the carbon dioxide obtained on decarboxylating an authentic sample of silver $[carboxy\text{-}^{14}C]$ methoxyacetate.

The validity of this determination has now been more rigorously established by bromine degradation of silver

$[\alpha\text{-}^{14}C]$ ethoxyacetate. $[\alpha\text{-}^{14}C]$ ethoxyacetate was prepared by standard methods from $[\alpha\text{-}^{14}C]$ bromoacetic acid (kindly supplied by Dr J. R. Catch). Silver $[\alpha\text{-}^{14}C]$ ethoxyacetate (110 mg., $180\mu C/\text{mole}$) was treated with dry bromine in the usual way. The barium carbonate prepared from the carbon dioxide evolved was virtually devoid of radioactivity (counting rate as an infinite-thickness sample under the conditions previously described, 3 counts/min. above background; i.e. specific radioactivity, $0\cdot84\mu C/\text{mole}$, or about 0.45% of the radioactivity of the silver salt). It is clear that almost none of the carbon dioxide was derived from the α carbon atom.

D - $[1\text{-}^{14}C]$ Ribose and D - $[1\text{-}^{14}C]$ arabinose were obtained through the kindness of Dr H. S. Isbell, National Bureau of Standards, on allocation of the Atomic Energy Commission. We are also most grateful to Dr Isbell for a gift of L-ribose. D-Arabinose, L-fucose, D-lyxose, L-rhamnose, D-ribose and L-xylose were obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio. L-Lyxose was synthesized from L-galactonic acid (Ault *et al.* 1933) by the method used by Hockett & Hudson (1934) for the D-isomer. It was obtained as a syrup crystallizing slowly on long standing. Paper partition chromatography of the syrup, using phenol-water as the solvent, showed only one spot reacting with the aniline phthalate reagent. The spot had the same R_f value as that obtained using D-lyxose.

Experiments with D-[1- ^{14}C]ribose and D-[1- ^{14}C]arabinose

Ribose. Preliminary experiments showed that D-ribose was well utilized for growth and kojic acid production by *A. flavus*, NRRL 2405. The following experiment was therefore carried out. Unlabelled D-ribose (5.0 g.) and D - $[1\text{-}^{14}C]$ ribose (0.0442 g., $52\cdot5\text{ mc}/\text{mole}$) were dissolved in 25 ml. of the following medium (which will hereafter be referred to as 'salts medium'): NaNO_3 , 2.0 g.; K_2HPO_4 , 1.0 g.; KCl, 0.5 g.; $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, 0.5 g.; $\text{FeSO}_4\cdot 7\text{H}_2\text{O}$, 0.01 g.; Difco yeast extract, 1.0 g.; water, 1 l. The solution was transferred to a 125 ml. Erlenmeyer flask carrying a rubber stopper with air inlet and outlet tubes and a sampling tube. The calculated specific radioactivity of the diluted ribose is $467\mu C/\text{mole}$ and the total activity added was $15\cdot5\mu C$. The solution was steam-sterilized at 15 lb./sq.in. for 15 min. and inoculated with *A. flavus*; a slow current of air was passed through the flask and daily collections of the carbon dioxide produced during the fermentation were made with the aid of scrubbers containing 10% NaOH solution. The yield and specific radioactivities of barium carbonate samples prepared from the absorbed carbon dioxide are given in Table 1. The specific radioactivity of the samples rose to a maximum by the third day and thereafter declined progressively. The total radioactivity recovered as barium carbonate was $1\cdot35\mu C$, i.e. 8.7% of that added as ribose.

On the eighth day, the culture was sterilized; the mycelium had dry weight, 0.190 g., specific

radioactivity, $2.59 \mu\text{C/g.}$ (total radioactivity recovered in mycelium = $0.49 \mu\text{C}$, i.e. 3.2% of that added as ribose). Kojic acid was isolated by continuous ether extraction, and after sublimation of the partly oily product in vacuum the yield of purified material was 107.6 mg. (m.p. 132°). Recrystallization from ethanol-light petroleum (b.p. $30-60^\circ$) gave fine needles, m.p. 149° ; mixed m.p.

Table 1. *Radioactivity of carbon dioxide evolved during fermentation of D-[1- ^{14}C]ribose*

A. flavus grown on 25 ml. of salts medium containing 5 g. of D-[1- ^{14}C]ribose ($15.5 \mu\text{C}$). For further details see text.

Age of culture at time of CO_2 collection (days)	Wt. of BaCO_3 collected (g.)	Specific radioactivity of BaCO_3 ($10^{-3} \mu\text{C/g.}$)	Total radioactivity (μC)
1	0.86	2.8	0.002
2	1.83	77.4	0.141
3	1.59	201.0	0.319
4	1.31	186.0	0.244
5	1.58	145.0	0.229
6*	1.37	136.0	0.187
7*	1.32	100.0	0.132
8*	1.27	75.5	0.096

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

Table 2. *Distribution of radioactivity in kojic acid obtained by fermentation of D-[1- ^{14}C]ribose*

Kojic acid was isolated and degraded as described in the text (cf. Arnstein & Bentley, 1953a, c). The specific radioactivity of the isolated kojic acid was $488 \mu\text{C/mole}$. After dilution of this material (47.5 mg.) with carrier (415.1 mg.) the specific radioactivity of the diluted kojic acid and the 2-O-ethyl derivative used for degradation was 50.9 and $49.8 \mu\text{C/mole}$, respectively.

Carbon atom no.	Distribution of radioactivity in kojic acid	
	($\mu\text{C/g. atom C}$)	(%)
1	18.8	36.7
2	9.7	18.9
3	12.5	24.5
4	4.8	9.5
5	1.3	2.6
6	4.0	7.9

with authentic kojic acid, 151° ; specific radioactivity, $488 \mu\text{C/mole}$. The total radioactivity recovered as kojic acid was found to be $0.37 \mu\text{C}$, i.e. 2.4% of that added initially. The radioactivity distribution obtained by degradation of this material is shown in Table 2. Approximately 80% of the total radioactivity recovered in kojic acid was located in the first three carbon atoms. By evaporation of a measured sample of the medium remaining after ether extraction, it was calculated

that the major portion of radioactive material (about $11 \mu\text{C}$) still remained in solution, presumably as unused ribose.

Arabinose. Although L-arabinose is a fair carbon source for growth and kojic acid production by *Aspergilli*, no experiments with D-arabinose have been previously reported. It was found that while this pentose would allow growth, kojic acid was not produced. In attempts to obtain kojic acid production, training experiments were carried out in which the organism was grown first on a 1:1 mixture of L- and D-arabinose. Kojic acid was readily produced on this medium, and spores from the mycelium were used to inoculate solutions containing higher proportions of D-arabinose. Finally in one experiment using only D-arabinose (2.0 g.) in salts medium (10 ml.), a positive ferric chloride colour reaction was obtained after 14 days' growth. Ether extraction of the culture filtrate yielded a partly crystalline material which was still oily after sublimation in vacuum. The sublimed material was washed with a little ether, giving a pale tan solid, m.p. 125° (31 mg.). Recrystallization from methanol-light petroleum (b.p. $30-60^\circ$) gave fine white crystals, m.p. 154° ; there was no m.p. depression on admixture with pure kojic acid. This was the only positive test result obtained in a number of experiments. Replacement cultures were also investigated; mycelial pads were grown on a mixture of L- and D-arabinose, giving kojic acid by the fifth or sixth day. The original culture medium was then removed, the mycelium was washed with sterile water until the washings gave no colour with ferric chloride solution, and finally a fresh solution containing D-arabinose was introduced under the mycelium. No further production of kojic acid could be detected under these conditions even on prolonged standing.

In a further attempt to demonstrate kojic acid formation from D-arabinose, the following experiment was carried out using labelled material. L-Arabinose (5.0 g.), D-arabinose (5.0 g.) and D-[1- ^{14}C]arabinose (0.0276 g. , 111.3 mc/mole ; total, $20.5 \mu\text{C}$) were dissolved in salts medium (50 ml.). After sterilization, the solution was inoculated with spores obtained from material previously grown on a mixture of L- and D-arabinose. The evolved carbon dioxide was collected in the usual way; the yield and specific radioactivities of barium carbonate samples prepared from the absorbed carbon dioxide are given in Table 3. 2.39% of the added radioactivity was recovered in the evolved carbon dioxide. The mycelium (dry weight, 0.381 g.) had specific radioactivity, $0.63 \mu\text{C/g.}$ The total activity recovered in the mycelium was therefore $0.25 \mu\text{C}$ (1.2% of that added). The amount of radioactive material remaining in the solution

Table 3. Radioactivity of carbon dioxide evolved during fermentation of D-[1-¹⁴C]arabinose

A. flavus was grown on 50 ml. of salts medium containing 5 g. of L-arabinose plus 5 g. of D-[1-¹⁴C]arabinose (20.5 μ C). For further details see text.

Sample no.	Age of culture at time of collection of CO ₂ (days)	Wt. of BaCO ₃ (g.)	Specific radioactivity of BaCO ₃ (10 ⁻³ μ C/g.)	Total radioactivity (μ C)
1	1-4	4.45	22.0	0.098
2	5	2.22	27.7	0.062
3	6	2.00	36.3	0.073
4	7	1.90	28.7	0.055
5	8	3.00	22.0	0.066
6	9-11	4.50	31.6	0.136

Table 4. Formation of kojic acid from common pentoses

A. flavus grown on salts medium with substrate concentration 1 g. of sugar/10 ml. of medium in each case. Kojic acid determined colorimetrically.

Substrate	Kojic acid formed (mg./ml.)	
	Day no. 5	Day no. 8
L-Arabinose	2.0	3.4
D-Arabinose	1.7	2.3
D-Ribose	4.4	8.5
D-Xylose	3.4	9.9
D-Xylose	7.0	13.4
D-Glucose (control)	2.8	10.8
D-Glucose (control)	2.5	11.9

after ether extraction was about 17 μ C. Kojic acid was isolated in the usual way; the yield of sublimed material was 160.3 mg. After recrystallization from ethanol-light petroleum (b.p. 30-60°), fine needles were obtained, m.p. 153°. Specific radioactivity, 62.5 μ C/mole. Total recovery of radioactivity in kojic acid, 0.071 μ C, i.e. 0.35 % of that added initially. Insufficient material was available for degradation. This ¹⁴C recovery is much lower than that in the ribose experiment and confirms that D-arabinose is a poor precursor of kojic acid.

Experiments with unlabelled pentoses and methyl pentoses

Growth from spore inoculum. In these experiments a given weight of pentose was dissolved in salts medium and the sterilized solution was inoculated with spores of *A. flavus*. Samples were removed, usually at daily intervals, and the yield of kojic acid was determined colorimetrically. The production of kojic acid from the more common pentoses (L-arabinose, D-ribose, D-xylose) is compared in Table 4; experiments with rarer pentoses (D- and L-lyxose, L-ribose and L-xylose) are shown in Table 5 with the methyl pentoses (L-rhamnose and L-fucose) in Table 6. In some cases, after growth for 8-9 days the culture fluids were extracted continuously with ether; the material

extracted in this way was shown to have the same R_f value as authentic kojic acid on paper chromatography in two solvent systems. R_f of kojic acid in butanol-acetic acid-water (4:1:5, v/v) = 0.66; in 2-ethylbutan-1-ol-5M formic acid (2:3, v/v) = 0.37 (system of Jones, Dowling & Skraba, 1953). The dried papers were sprayed with 1 % ferric chloride in 0.1N-HCl to locate the kojic acid.

Replacement experiments. The formation of kojic acid from pentoses was also studied in replacement experiments using mycelial pads pre-grown on a 10 % glucose-salts medium (5 ml. portions in 25 ml. Erlenmeyer flasks). After six days, the original medium was removed with a sterile pipette and kojic acid analyses were carried out on this medium (Table 7, column 2). The mycelia were washed by addition of two 5 ml. portions of sterile water in succession. A third 5 ml. portion of water was then added and the mycelial pads were allowed to stand on this water for 2 hr. at room temperature. These washings were finally removed and analysed for kojic acid (Table 7, column 3). Sterile solutions of the various sugars (500 mg. in 5 ml. of salts medium) were added to the pads and within 30 min. 0.1 ml. portions of the media were removed for analysis (Table 7, column 4). Samples were removed from the cultures at intervals over the next 17 days (Table 7, columns 5-14).

DISCUSSION

Pentose utilization for kojic acid production

Pentoses other than L-arabinose and D-xylose have not previously been used as carbon sources for kojic acid production by *Aspergillus* species. Indeed, almost all information on pentose metabolism by fungi has been restricted to the readily accessible L-arabinose and D-xylose. Of the eight pentoses studied by us, L-ribose and L-lyxose were very poor carbon sources for growth of *A. flavus* and no kojic acid was formed from these substrates. When growth took place readily on both enantiomorphic forms of the same pentose only one enantiomer gave substantial amounts of kojic acid.

Table 5. *Formation of kojic acid from rare pentoses**A. flavus* grown on salts medium with sugars added as shown. Kojic acid determined colorimetrically.

Substrate	Wt. of substrate (g.)	Vol. of salts medium (ml.)	Kojic acid formed (mg./ml.) Day no.				Mycelium (dry wt.) (mg.)	Wt. of ether extract of culture medium (mg.)
			5	7	8	9		
D-Lyxose	1.0	10	0.4	0.7	1.3	1.5	68.7	16.8
L-Lyxose	0.5	5	0.0	0.0	0.0	0.0	49.9	—
L-Ribose	0.49	5	0.0	0.0	0.0	0.0	11.1	—
L-Xylose	1.0	10	0.8	1.2	1.1	—	92.6	15.5
D-Glucose (control)	1.0	10	6.2	14.0	18.4	—	82.4	132.3

Table 6. *Formation of kojic acid from methyl pentoses**A. flavus* grown on salts medium with sugars added as shown. Kojic acid determined colorimetrically.

Substrate	Wt. of substrate (g.)	Vol. of salts medium (ml.)	Yeast extract	Kojic acid formed (mg./ml.) Day no.						Mycelium (dry wt.) (mg.)
				5	6	7	8	9	10	
L-Rhamnose	1.0	10	+	1.4	—	3.4	6.2*	—	—	79.7
L-Rhamnose	1.0	10	+	0.4	1.1	1.2	—	—	2.8	92.2
L-Rhamnose	1.0	10	+	0.6	0.9	1.0	—	—	2.9	100.7
L-Rhamnose	1.0	10	—	0.0	0.0	0.0	—	—	0.2	32.5
L-Rhamnose	1.0	10	—	0.0	0.0	0.0	—	—	0.0	38.7
L-Fucose	0.5	5	+	No appreciable growth by 14 days						
L-Fucose†	0.45	4.5	+	No appreciable growth by 14 days						

* Experiment terminated.

† *A. oryzae* was used in this experiment.Table 7. *Formation of kojic acid from pentoses in replacement experiments**A. flavus* was grown on 10% glucose salts medium for 6 days. After washing the mycelium with water, sterile replacement medium containing various sugars was added and the culture incubated for a further 17 days. Kojic acid was determined colorimetrically.

Substrate	Kojic acid (mg./ml.)			Kojic acid formed (mg./ml.) by replacement culture in the following time (days)									
	In glucose medium	In final washing	In replacement solution after 0.5 hr.										
				1	2	3	4	6	8	10	13	15	17
D-Arabinose	5.4	0.4	0.2	0.2	0.0	0.0	0.1	0.4	0.5	0.4	0.6	0.5	0.7
L-Arabinose	5.8	0.5	0.3	0.3	0.1	0.0	0.2	0.1	0.2	0.1	0.3	0.4	0.6
D-Lyxose	4.7	0.7	0.3	0.3	0.3	0.4	0.1	0.3	0.2	0.4	0.8	0.8	0.7
L-Rhamnose	4.8	0.5	0.2	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.1	0.1	0.2
D-Ribose	4.9	0.5	0.3	0.3	0.4	0.7	1.1	1.1	1.3	1.6	1.9	2.3	2.8
D-Xylose	4.1	0.4	0.3	0.2	0.0	0.2	0.2	0.4	0.8	1.0	1.8	2.4	3.4
L-Xylose	4.7	0.5	0.3	0.0	0.0	0.1	0.1	0.1	0.0	0.0	0.0	0.0	0.1
D-Glucose	5.6	0.3	0.2	0.3	0.6	0.9	1.2	1.8	3.5	4.2	6.4	8.7	11.6
D-Glucose	5.5	0.6	0.4	0.6	1.0	1.6	2.8	3.2	4.0	4.8	6.8	7.3	9.5
No sugar	7.0	0.5	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

D-Xylose and D-ribose were the best sources for kojic acid production; L-arabinose was less effective, and D-arabinose, D-lyxose, and L-xylose were only poor precursors. Similarly, in replacement cultures, where the mycelium was pre-grown on glucose prior to replacement with the pentose substrate, only D-ribose and D-xylose gave substantial amounts of kojic acid (see Table 7), whereas L-arabinose and the other pentoses were poor precursors. Steinberg (1942) has investigated the

utilization by *A. niger* of D-lyxose and both enantiomorphs of arabinose, ribose and xylose. The best yield of mycelium was obtained with D-xylose, L-arabinose being less effective. There was very slight growth on L-ribose and L-xylose; D-lyxose, D-arabinose, and D-ribose did not support growth. The failure of the organism to grow on D-ribose seems rather surprising, but this observation has been confirmed by us in preliminary experiments. Using *A. niger* NRRL 2270, the following mycelial

yields were obtained using 0.5 g. of sugar in 5 ml. of salts medium (yeast extract omitted); D-ribose, 3.9 mg.; L-arabinose, 57.0 mg.

Of the methyl pentoses, L-rhamnose was previously reported not to be a carbon source for kojic acid production (Tamiya, 1932). It has now been shown that this sugar can be used for kojic acid production, especially when the medium is supplemented with yeast extract (see Table 6). This accords with our earlier experience that, even from glucose, kojic acid production is much slower when yeast extract is omitted from the medium. In the replacement experiments with mycelium pre-grown on glucose, L-rhamnose no longer gave a significant yield of kojic acid. Fermentation of L-fucose has been reported (Tadokoro, 1936) to yield kojinic (*sic*) acid; presumably, kojic acid is meant. The substrate used in this work was a crude syrup obtained by hydrolysis of seaweed polysaccharide. The validity of these experiments seems questionable in view of the failure to use a purified substrate; in our experiments we have been unable to obtain growth either of *A. flavus* or of *A. oryzae* (IMI 17299) on L-fucose solutions.

Mechanism of kojic acid formation from pentose

Although L-arabinose is a less effective substrate than either D-xylose or D-ribose it is noteworthy that the mould can use sugars of different configurational series to form the same compound. It is not easy to appreciate, however, why D-arabinose, which is epimeric with D-ribose, and which in *E. coli* can actually be converted into D-ribulose (Cohen, 1953), is a poor source of carbon. Before these problems are discussed further, the mechanism by which D-[1-¹⁴C]ribose is converted into kojic acid will be considered.

With D-[1-¹⁴C]ribose the observed distribution of ¹⁴C in kojic acid is in harmony with the formation of a C₆ precursor by the mechanisms now known to be involved in synthesis of hexose phosphate from pentose phosphate (see review by Racker, 1954). First, the transketolase reaction would lead to [1:3-¹⁴C₂]sedoheptulose 7-phosphate; the C₆ precursor needed for kojic acid synthesis would in turn be obtained by transfer of the first three carbon atoms of sedoheptulose phosphate to glyceraldehyde phosphate (obtained from the last three carbon atoms of D-[1-¹⁴C]ribose) under the influence of transaldolase. There would be obtained, therefore, [1:3-¹⁴C₂]fructose 6-phosphate labelled equally in carbon atoms 1 and 3. Additionally, reaction of (unlabelled) erythrose 4-phosphate, formed in the transaldolase reaction, with a further molecule of the (labelled) active glyceraldehyde complex would lead to [1-¹⁴C]fructose 6-phosphate. The net result would be the formation of a 1:3-labelled fructose 6-phosphate containing more ¹⁴C

in carbon atom 1 than 3 (Fig. 1, Scheme A). Such a labelling of hexose phosphate has been observed by Horecker *et al.* (1954) with a liver-enzyme preparation and in the fermentation of D-[1-¹⁴C]-ribose by yeast extracts (Gibbs, 1955). In the kojic acid obtained from D-[1-¹⁴C]ribose, carbon atoms 1 and 3 contain respectively 36.7 and 24.5 % of the total activity, and it seems probable, therefore, that this explanation is valid. These results are quantitatively different from the distribution observed by Horecker *et al.* with the liver-enzyme preparation, where carbon 1 of the hexose monophosphate contained 74 %, and carbon 3 24 %, of the total activity. Further, kojic acid contained an appreciable amount (18.9 %) of activity in carbon 2; this was not present in the hexose monophosphate. Since in the experiments with the liver-enzyme preparation incubation was carried out for only 17 hr., whereas in our experiment the labelled ribose was present for 8 days, it seems reasonable to postulate further oxidative reactions to account for the activity in carbon 2 of kojic acid.

It is suggested that the above [1:3-¹⁴C₂]hexose precursor is further oxidized to the corresponding 6-phosphogluconate, which in turn is converted into [2-¹⁴C]ribulose 5-phosphate. Both 1- and 2-labelled pentoses would therefore be present in the medium, which would be equivalent to a [1:2-¹⁴C₂]pentose with a higher radioactivity in carbon atom 1. Resynthesis of hexose from this material would lead to a 1:2:3-labelled hexose with a predicted order of decreasing activity, 1 > 3 > 2 (Fig. 1, Scheme B).

Evidence for the functioning of such an oxidative cycle is found in the observed radioactivities of daily samples of respiratory carbon dioxide. Using [1-¹⁴C]glucose, we have previously shown that the activity of the expired carbon dioxide was high initially, declining later to an almost constant value (Arnstein & Bentley, 1953a). In the present experiments with D-[1-¹⁴C]ribose (Table 1) and D-[1-¹⁴C]arabinose (Table 3), however, the activity in the respiratory carbon dioxide was very low initially, rising to a maximum (both in terms of specific activity and amount of carbon dioxide) by the third and fourth days. Thereafter, the radioactivity declined slowly. This strongly suggests that there is a lag period of about 3 days before the 1:3-labelled hexose intermediate is available in any quantity.

Since D-xylose and D-ribose are the two pentoses giving the highest yields of kojic acid (both in ordinary and replacement cultures) it is possible that the biosynthetic mechanism is similar in both of these cases. The much lower yields obtained from the two sugars of the L configurational series (arabinose and rhamnose), and the failure of these

compounds to yield kojic acid in replacement culture, suggest that the formation of kojic acid from these sugars may proceed by a different pathway. This seems likely on general grounds, since from L-arabinose a heptulose of the L configurational series would be obtained. In the case of

L-arabinose, fermentations are known which lead to the production of pyruvate from the last three carbon atoms (Rappoport, Barker & Hassid, 1951; Weinberg & Doudoroff, 1955). Such a process would provide a source of C₃ units for kojic acid synthesis, at the same time destroying the

Fig. 1. Formation of C₆ precursor and kojic acid from labelled pentose via transketolase and transaldolase reactions. Abbreviations:

TK = transketolase reaction } reactants being shown by the symbol ~ and cleavage
 TA = transaldolase reaction } taking place at points indicated by dotted lines.

W-L-D = Warburg-Lipmann-Dickens oxidative mechanism. Asterisks and circles are used to show the origin of labelled atoms, but have only semiquantitative significance. Radioactivity in pentoses is differentiated as follows: C* original pentoses, C° pentose formed from hexoses of Scheme A by the W-L-D mechanism. As a rough approximation C° will therefore correspond to half the radioactivity indicated by C*.

'unnatural' configuration at carbon atom 4. Similarly, a cleavage of rhamnose to two C_3 units probably occurs in the fermentation of this methyl pentose to propylene glycol (Kluyver & Schnellen, 1937). In this case, glyceraldehyde is apparently formed from the first three carbon atoms.

Pentose and heptose intermediates in kojic acid formation from small molecules and glucose

The similarity of the ^{14}C distribution in kojic acid formed from $[3:4-^{14}C_2]$ glucose, and hexose monophosphate formed from $[2:3-^{14}C_2]$ pentose 5-phosphate, suggested a partial resynthesis of the C_6 precursor of kojic acid by the transketolase and transaldolase reactions. The results reported in this paper provide strong evidence that these reaction sequences are available to *A. flavus* for the formation of kojic acid from D-ribose. On this basis, the pathways previously discussed (Arnstein & Bentley, 1953*a, c*) may be now more clearly defined and the three metabolic processes originally proposed will be reconsidered in the light of these new results.

(a) Direct formation of kojic acid, when glucose is the substrate, without splitting of the carbon chain, is still considered to be the major pathway.

(b) The secondary pathway, described as involving triose phosphates, is most probably the transketolase-transaldolase system discussed here. From 1-labelled glucose, sedoheptulose is obtained which is not radioactive. However, cleavage of glucose by aldolase, which has been identified in mycelial extracts of *A. flavus* (Arnstein & Bentley, 1953*b*), would yield glyceraldehyde phosphate labelled in carbon atom 3. From the transaldolase reaction, there would therefore be obtained $[6-^{14}C]$ hexose phosphate. This would account for our previous finding of ^{14}C in position 6 of kojic acid derived from $[1-^{14}C]$ glucose. The transketolase-transaldolase pathway would be involved to a maximum of about 10–15% when glucose is the substrate. Using pentose as a substrate (particularly ribose and probably xylose) this becomes the major pathway. Further, the transaldolase reaction accounts for our earlier observation (Arnstein & Bentley, 1953*a*) that in the presence of glucose, $[2-^{14}C]$ dihydroxyacetone is incorporated predominantly into the 4, 5 and 6 carbon atoms of kojic acid.

(c) The third pathway postulated to account for the results with added small molecules is probably an extension of (b). The small molecules all appear to give rise to a common C_3 precursor; as in the case of dihydroxyacetone, this is then incorporated mainly into the 4, 5 and 6 carbon atoms.

SUMMARY

1. The eight stereoisomeric pentoses have been used as substrates for *Aspergillus flavus*. D-Ribose and D-xylose give the highest yields of kojic acid; L-arabinose is less effective and D-arabinose, D-lyxose, and L-xylose are very poor carbon sources for kojic acid formation. *A. flavus* did not form kojic acid from L-lyxose or L-ribose, and these two pentoses barely support the growth of this organism.

2. In replacement cultures using mycelia pre-grown on glucose, only D-ribose and D-xylose are good sources for continued kojic acid production.

3. Of the two methyl pentoses tested, L-fucose does not support the growth of either *A. flavus* or *A. oryzae*. L-Rhamnose is a fairly good carbon source for kojic acid formation by *A. flavus*.

4. With D- $[1-^{14}C]$ ribose as a substrate for *A. flavus*, the radioactivity of the respiratory carbon dioxide was low initially and increased to a maximum at the third day. Of the radioactivity of kojic acid isolated from this culture, 80% was contained in carbon atoms 1, 2 and 3. This distribution pattern is interpreted to indicate a conversion of ribose into the C_6 precursor by the transketolase and transaldolase reactions. Further oxidation of the C_6 precursor is believed to account for the radioactivity in carbon atom 2 of the kojic acid.

5. The relationship of these observations to the previously postulated pathways of kojic acid formation from glucose and small molecules is discussed.

We wish to thank Clara P. Thiessen for help with some of these experiments. Grateful acknowledgement is made to the National Science Foundation for a Research Grant (NSF-G 425) to R.B. which in part supported this work.

REFERENCES

- Arnstein, H. R. V. & Bentley, R. (1953*a*). *Biochem. J.* **54**, 493.
Arnstein, H. R. V. & Bentley, R. (1953*b*). *Biochem. J.* **54**, 508.
Arnstein, H. R. V. & Bentley, R. (1953*c*). *Biochem. J.* **54**, 517.
Ault, R. G., Baird, D. K., Carrington, H. C., Haworth, W. N., Herbert, R., Hirst, E. L., Percival, E. G. V., Smith, F. & Stacey, M. (1933). *J. chem. Soc.* p. 1419.
Bentley, R. (1955). *Bact. Proc.* p. 130.
Birkinshaw, J. H., Charles, J. H. V., Lilly, C. H. & Rais-trick, H. (1931). *Phil. Trans.* **220**, 127.
Challenger, F., Klein, L. & Walker, T. K. (1929). *J. chem. Soc.* p. 1498.
Cohen, S. S. (1953). *J. biol. Chem.* **201**, 71.
Corbellini, A. & Gregorini, B. (1930). *Gazz. chim. ital.* **60**, 244.
Denison, F. W., Carson, S. F. & Foster, J. W. (1954). *Bact. Proc.* p. 99.

- Gibbs, M. (1955). *Fed. Proc.* **14**, 218.
Gould, B. S. (1938). *Biochem. J.* **32**, 797.
Hockett, R. C. & Hudson, C. S. (1934). *J. Amer. chem. Soc.* **56**, 1632.
Horecker, B. L., Gibbs, M., Klenow, H. & Smyrniotis, P. Z. (1954). *J. biol. Chem.* **207**, 393.
Jones, A. R., Dowling, E. J. & Skrabala, W. J. (1953). *Analyt. Chem.* **25**, 394.
Katagiri, H. & Kitahara, K. (1929). *Bull. agric. chem. Soc. Japan*, **5**, 38.
Kluyver, A. J. & Schnellen, Ch. (1937). *Enzymologia*, **4**, 7.
Racker, E. (1954). *Advanc. Enzymol.* **15**, 141.
Rappoport, D. A., Barker, H. A. & Hassid, W. Z. (1951). *Arch. Biochem. Biophys.* **31**, 326.
Steinberg, R. A. (1942). *J. agric. Res.* **64**, 615.
Tadokoro, T. (1936). *Bull. chem. Soc. Japan*, **11**, 239.
Tamiya, H. (1932). *Acta phytochim.*, Tokyo, **6**, 1.
Weimberg, R. & Doudoroff, M. (1955). *Fed. Proc.* **14**, 302.

The Effect of Oestrogens and Chemically Related Compounds on the Respiration of Yeast and on Oxidative Phosphorylation

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(Received 21 July 1955)

It was shown by Shacter (1953*a*) that 4:4'-dihydroxy- $\alpha\beta$ -diethylstilbene (stilboestrol) has an action on the metabolism of respiring yeast cells which shows a similarity to that of 2:4-dinitrophenol (DNP) on these cells. With increasing concentration there was at first a slight stimulation of yeast respiration, but as the stilboestrol concentration reached the value of 10^{-4} M the rate of oxygen uptake suddenly increased greatly, to a maximum value of 14 times that of the control, at 2×10^{-4} M, after which it began to fall until an inhibition was observed at concentrations above 10^{-3} M. These experiments were performed with yeast which had been washed and aerated in order to decrease the amount of oxidizable substrate in the cells. Stilboestrol was found to cause about three times the increase in respiration that was produced by 2:4-dinitrophenol. When yeast was respiring in the presence of glucose, low concentrations of stilboestrol did not increase the rate of oxygen uptake, and, in fact, as the concentration of stilboestrol was raised, respiration was gradually inhibited until it ceased completely at a concentration of stilboestrol of 10^{-4} M. Shacter (1953*b*) also found that *p*-chloromercuribenzoate, at high concentrations, stimulates the endogenous respiration of yeast in a rather similar manner. It was therefore suggested that 2:4-dinitrophenol, sulphydryl reagents and stilboestrol might affect yeast metabolism by similar mechanisms.

The following experiments were carried out in order to determine to what extent this type of action is limited to substances having oestrogenic activity, in a group of compounds which have a close chemical relationship to stilboestrol. Some powerful natural oestrogens were included.

In addition an attempt has been made to determine the effect of a similar range of compounds on hexokinase and myosin adenosine triphosphatase (ATPase) activity and on the oxidative phosphorylation in cyclophorase preparations.

MATERIALS AND METHODS

Adenosine triphosphate (ATP). The disodium salt was purchased from Sigma Chemical Co., U.S.A.

Stilboestrol and hexoestrol. These were commercial products; the other oestrogenic and related compounds were kindly given by Mr W. Lawson.

Phosphate buffer, pH 7.4. This consisted of 8 vol. of 0.1M- K_2HPO_4 and 2 vol. of 0.1M- KH_2PO_4 .

Inorganic phosphate. This was determined by the method of Fiske & Subbarow (1925), after deproteinizing with trichloroacetic acid, as described below.

Yeast suspension. Fresh baker's yeast (Distillers' Co. Ltd.) was washed three times with distilled water, suspended in approximately 100 vol. of distilled water and aerated overnight at room temperature, in order to decrease the oxidizable substrates in the yeast cells to a low value.

Myosin. This was prepared from rat skeletal muscle by the method described by Dickens & Glock (1951). After four precipitations by dilution with de-ionized water, the myosin was dissolved in 0.5M-KCl. (Dry wt. of myosin, 0.5 mg./ml.)

Hexokinase. This was prepared from baker's yeast according to the method of Berger, Slein, Colowick & Cori (1946). The method was followed as far as the second ethanol precipitation, the fraction obtained between 29 and 45% ethanol being retained. This was dissolved in 1% glucose solution and stored at -20° .

Effect of various compounds on yeast respiration. This was determined at 30° in an atmosphere of air by incubating 1 ml. of the yeast suspension (see above) (approx. 20 mg. dry weight of yeast) with 0.2 ml. of 0.67M- KH_2PO_4 and distilled water to a final volume of 2 ml. in Warburg flasks,