CXCIV.—*The Production of Kojic Acid from Pentoses by* Aspergillus oryzæ.

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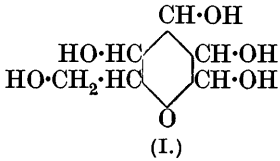
KOJIC acid (II) was obtained by Yabuta (J., 1924, **125**, 575; 8th Int. Congr. Appl. Chem. Appendix, 1912, **25**, 455) from cultures of *Aspergillus oryzæ* on steamed rice and on sugars. *A. albus*, *A. candidus* and *A. nidulans* also form this acid. Traetta-Mosca (*Ann. Chim. appl.*, 1914, I, 477; *Gazzetta*, 1921, **51**, ii, 269) observed its formation from glycerol and from sucrose, glucose, and lævulose by means of *A. glaucus*, and since then it has been obtained by several workers. There is some evidence that kojic acid is produced in traces by *A. niger*, since cultures of this mould on Raulin's sugar medium, when free from iron, occasionally give a red colour on addition of a ferric salt (Sauton, *Compt. rend.*, 1910, **151**, 241; Javillier and Sauton, *ibid.*, 1911, **153**, 1177). Kinoshita (*Acta Phytochim.*, 1927, **3**, 31) studied the production of this acid from sugars and states that the use of cobaltammine salts as source of nitrogen greatly increased the yield, but that no kojic acid was obtained from lævulose, *l*-arabinose, *l*-xylose, or rhamnose under these conditions (see also Tamiya, *Acta Phytochim.*, 1927, **3**, 51;

1928, **4**, 77). The non-formation from lævulose is surprising in view of the results of Traetta-Mosca. The close structural relation of glucose (I) to kojic acid has been pointed out by Kinoshita (*loc. cit.*) and by Haworth (" Constitution of Sugars," 1928, 38). Owing to the occurrence in nature of many other derivatives of 3-hydroxypyrone or 3-hydroxybenzopyrylium, *e.g.*, maltol (V), meconic acid (VI), flavonols such as quercitin, and anthocyanins such as callistephin (Robertson and Robinson, J., 1928, 1460), attempts have been made to obtain other simple 3-hydroxypyrones by fermentation of sugars. The synthesis of such compounds by fungi as well as by the higher plants certainly demands further investigation.

Comparatively little information exists as to the effect of moulds on pentoses. Emmerling (*Centr. Bakt.*, 1903, **10**, 273) showed that arabinose and xylose are assimilated by *A. niger*, but that no oxalic acid is produced, a result confirmed by Peterson, Fred, and Schmidt (*J. Biol. Chem.*, 1922, **54**, 19), working with various *Aspergilli* and *Penicillia*, who found no alcohol, volatile acid, or citric acid. A small quantity of a non-volatile acid was extracted with ether, but not identified. Decomposition of the pentose was complete in 4—5 days in 4% solution, and 88—98% of the total carbon consumed was recovered as carbon dioxide and mycelium.

Arguing from the behaviour of glucose, arabinose (III) might be expected to yield pyromeconic acid (IV) with *A. oryzæ*.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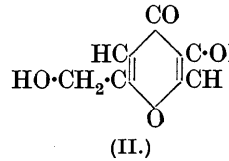
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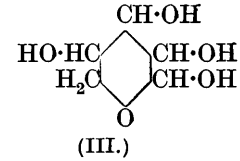
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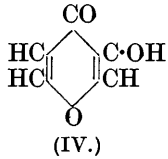
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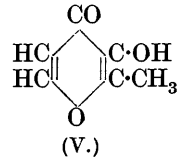
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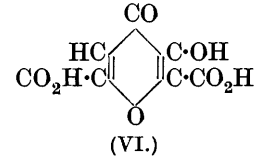
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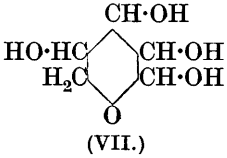
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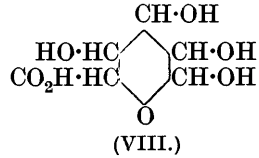
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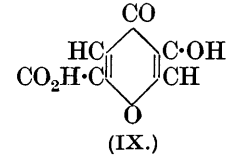
(IX.)

The mould (*Aspergillus oryzæ diastase*) grew well on this pentose and the cultures in 3 days gave the deep red colour with ferric chloride characteristic of 3-hydroxypyrones, but no trace of pyromeconic acid could be isolated. Extraction of the cultures with

ether gave kojic acid, identified by analysis, m. p. and mixed m. p. and by conversion into its diacetyl derivative. The re-sterilised culture again gave kojic acid on inoculation. The arabinose was the purest obtainable, and from its rotation could not have contained more than traces of hexoses. To remove all doubt as to the origin of the kojic acid, a considerable quantity of arabinose was converted into the diphenylhydrazone, which was recrystallised, and the arabinose regenerated by formaldehyde. The highly purified pentose again gave kojic acid in 3 days with *A. oryzæ*. The non-formation of pyromeconic acid is remarkable, since it is so closely related to arabinose, whereas the formation of kojic acid involves a breakdown of the pentose molecule and the synthesis of a 6-carbon compound. The mould would not grow upon pyromeconic acid, and the toxic action of this compound may explain the preferential formation of kojic acid, on which the mould grows well.

Kojic acid is also readily produced from carefully purified xylose (VII) by *A. oryzæ*. The mould grows well on glycuronic acid (VIII), but neither comenic acid (IX) nor kojic acid nor any hydroxypyrone derivative could be detected. Comenic acid is also toxic to *A. oryzæ*, earlier attempts to grow a strain of *A. oryzæ* differing slightly from *A. oryzæ diastase* on this acid having failed. Its formation was therefore scarcely to be expected, but it is somewhat surprising that decarboxylation to xylose and subsequent formation of kojic acid did not occur. Glycuronic acid is converted into *l*-xylose by a mixture of putrefactive bacteria (Salkowski and Neuberg, *Z. physiol. Chem.*, 1902, **36**, 261).

The production in this research of a 6-carbon compound by fermentation of pentoses finds a parallel in the observation of Amelung (*Z. physiol. Chem.*, 1927, **166**, 161), who showed that citric acid is produced when *A. niger* grows on arabinose and xylose, 10 g. of each sugar giving respectively 1·2 and 2·05 g. of citric acid, whereas glucose gave 3·63 g. These results were confirmed by Bernhauer (*Biochem. Z.*, 1928, **197**, 309), who obtained from arabinose, xylose, and glucose with *A. niger*, in presence of calcium carbonate, yields of citric acid of 5·4, 12·0 and 24·6%. Butkewitsch (*ibid.*, 1923, **142**, 195) also obtained citric acid from arabinose.

Peterson, Fred, and Schmidt (*J. Biol. Chem.*, 1924, **60**, 627) find that *B. granulobacter pectinovorum*, which produces acetone and butyl alcohol from glucose, gives the same products in almost the same proportion from xylose and arabinose, although the rate of fermentation is somewhat slower. Further evidence of the breakdown of pentoses to 3-carbon compounds is furnished by the

fermentation of *l*-xylose, but not of arabinose, to propionic and acetic acids (Virtanen, *J. Soc. Chem. Ind.*, 1924, 687B), and xylose was shown by Grimbert (*Bull. Soc. chim.*, 1896, **15**, 340) to be converted into alcohol, acetic acid and succinic acid by the pneumococcus of Friedländer.

Experimental.

The mould employed in this research was *Aspergillus oryzæ diastase* obtained through the courtesy of Professor C. Neuberg of Berlin-Dahlem. It was maintained in a young and vigorous condition by sub-culturing from time to time on solid agar slopes having the composition: maltose 4 g., peptone 1 g., agar 1·5 g., water 100 c.c., *N*-hydrochloric acid 2 c.c. The kojic acid used for comparison was kindly supplied by Messrs. Nobel, Ardeer.

The liquid medium of Kinoshita (*loc. cit.*), hereafter designated medium K, was used in most of the experiments and contained potassium dihydrogen phosphate 0·1 g., magnesium sulphate 0·05 g., and ammonium nitrate 0·04 g., in 100 c.c. of water. When *A. oryzæ diastase* was grown on this medium containing glucose, pentoses or glycuronic acid, the mycelium was at first white and after 2—3 days formed yellowish-green spores.

A morphological description of this strain of *A. oryzæ* will be published in a later communication.

The fermentations were conducted in tubes, or in flasks fitted with tubes for the aseptic removal of specimens of the cultures, and the media were sterilised at 95—100° on three successive days prior to inoculation. The results were checked by control experiments on the uninoculated media.

Fermentation of Arabinose.—Experiment 1. The pentose (2·5 g.), obtained from the British Drug Houses, dissolved in 30 c.c. of medium K was divided among seven tubes, simultaneously inoculated with *A. oryzæ diastase*, and incubated at 31—32°. On the fifth day a tube gave a cherry-red colour with ferric chloride, which was also observed in another tube on the tenth day. This was shown later to be due to kojic acid. Solutions of arabinose give no colour with ferric chloride.

Experiment 2. A solution was prepared containing arabinose 6 g., potassium dihydrogen phosphate 0·075 g., magnesium sulphate 0·038 g., and water 75 c.c. Three small tubes, each containing 5 c.c. and a trace of ammonium nitrate, were sterilised, inoculated, and incubated as before, giving a good growth after 3 days. The remaining 60 c.c. were sterilised and treated with 0·36 g. of aquopentamminecobaltic chloride, $[Co(NH_3)_5H_2O]Cl_3$, which had been

sterilised dry at 100° for 1 hour. The clear solution was then inoculated with the contents of the three tubes and incubated at 31—32°. There was a good growth on the third day and on the seventh day ferric chloride gave a faint red colour with a test-portion aseptically removed. A similar test gave a somewhat deeper colour on the eleventh day, but fainter than in experiment 1. On the sixteenth day the culture was filtered, the mycelium well washed, and the solution and washings repeatedly extracted with ether, giving 0·03 g. of a colourless crystalline solid. After draining on tile and being washed with ether, this melted at 148—149° alone and at 150—151° in admixture with kojic acid (m. p. 154°). It gave the ferric chloride reaction. The use of cobaltammine salt did not appear, from this single experiment, to present any advantage over ammonium nitrate. Further tests were not made.

Experiment 3. Pure arabinose obtained from Kerfoot & Co., Bardsley Vale, had $[\alpha]_D$ +103·2° and m. p. 149—150°. The recorded values (Beilstein, " Organische Chemie," 4th edn., vol. 1, p. 861) range from $[\alpha]_D$ + 104·4° to +105·4°, and 158·5—159·5° (corr.) is given as the m. p. The pentose (30 g.), in 300 c.c. of medium K, was divided between two small tubes containing 5 c.c., four larger tubes containing 10 c.c., and two litre conical flasks each containing 125 c.c. All were sterilised and the four larger tubes inoculated and incubated at 31—32°. A three days' growth was used to inoculate the two flasks and the two test-tubes, which were similarly incubated. The ferric chloride test was positive in the two indicator tubes on the sixth and eighth days, and accordingly on the eighth day the contents of the flasks were continuously extracted with ether, yielding 0·75 g. of almost pure kojic acid which, after crystallisation from alcohol, melted at 154°, alone or in admixture with kojic acid (Found : C, 50·8; H, 4·2. Calc. : C, 50·7; H, 4·2%). It was converted into the diacetyl derivative by boiling for 15 minutes with acetic anhydride and anhydrous sodium acetate; m. p. and mixed m. p. 101—102°. Yabuta (*J. Chem. Soc. Tokyo*, 1916, **37**, 1185, 1234; *A.*, 1922, i, 939) gives 102°.

The aqueous arabinose culture, which now gave only a very faint ferric chloride test, was concentrated in a vacuum to remove ether, and diluted to 150 c.c., of which 100 c.c. were placed in a flask and the remainder in ten tubes and sterilised as usual. Two of the tubes were again inoculated and the flask and five of the tubes were seeded with the resulting four days' growth. The mould grew fairly well and on the tenth day one of the tubes gave a ferric chloride reaction much stronger than that given by the three

remaining tubes, which had not been re-inoculated and served as controls. On the twelfth day continuous extraction of the flask culture with ether yielded a further 0·12 g. of kojic acid. This renders it extremely improbable that the kojic acid could have arisen from traces of hexose in the arabinose employed (see also p. 1504), since this if present should have been exhausted during the first fermentation.

Experiment 4. *Purification of arabinose.* In order to place the pentose origin of the kojic acid beyond all doubt, Kerfoot's arabinose (9·2 g.) was converted into the very sparingly soluble diphenylhydrazone (Neuberg and Wohlgemuth, *Z. physiol. Chem.*, 1902, **35**, 34)—a method which is recommended for removing hexoses. This was washed with alcohol and twice recrystallised from much dilute alcohol; it was then obtained perfectly white and of constant m. p. 205° (compare Muther and Tollens, *Ber.*, 1904, **37**, 312; Tollens and Maurenbrecher, *Ber.*, 1905, **38**, 500). The hydrolysis of 15 g. of the diphenylhydrazone was effected by heating with freshly distilled formaldehyde during 9 hours (Ruff and Ollendorff, *Ber.*, 1899, **32**, 3234). The mixture was extracted with ether to remove formaldehydediphenylhydrazone and evaporated repeatedly with water to expel formaldehyde. The liquid was then concentrated to a syrup, which crystallised on being seeded with pure arabinose and kept in a vacuum desiccator for a few days. The solid was rubbed with methyl alcohol and washed with 95% alcohol; it was then colourless and melted at 158—160°. The arabinose, when finally recrystallised from 90% alcohol, was unchanged in m. p. and had $[\alpha]_D^{15°}$ + 104·5°. The purification had therefore increased the rotation by about 1%.

Fermentation of the purified arabinose. The pure pentose (3·5 g., corresponding to 9·2 g. of the original arabinose) was dissolved in 35 c.c. of medium K and divided between eight test-tubes, which were sterilised, inoculated, and incubated at 31—32°. On the seventh day the filtered mycelium was well washed with hot water, and the filtrate and washings were continuously extracted with ether, giving 0·05 g. of kojic acid, m. p. 152—153°, and 153—154° in admixture with an authentic specimen (m. p. 154°). After recrystallisation from alcohol the m. p. and mixed m. p. were 154°. The extracted culture was almost neutral to litmus.

Fermentation of Xylose.—Experiment 5. The mould was grown under the usual conditions on a solution, prepared from 5 g. of xylose (British Drug Houses) in 50 c.c. of medium K, divided among 11 tubes. The ferric chloride test was positive on the third day, and on the seventh day eight of the tubes were treated as

before, yielding 0·2 g. of kojic acid, m. p. 151—152°, and m. p. and mixed m. p. on recrystallisation, 154°.

Experiment 6. 10 G. of xylose, having $[\alpha]_D$ + 18·8° and m. p. 151—153° (Beilstein, *op. cit.*, gives + 18·6°, + 19·22°, + 19·67°, and for the m. p. 143°, 144—145° and 153—154°), in 100 c.c. of medium K were similarly fermented for 16 days. The usual treatment yielded 0·21 g. of kojic acid (m. p. and mixed m. p. 154° on recrystallisation), a much poorer yield than was obtained in experiment 5. This may be due to the fermentation having been allowed to proceed too far. The kojic acid was further identified as the diacetyl derivative, m. p. and mixed m. p. 101—102°.

Concentration of the extracted culture in a vacuum, dilution, and re-inoculation gave only a very poor growth and a faint ferric chloride reaction after 6 weeks. Citric and oxalic acids were absent and apparently carbonyl derivatives also, since 2 : 4-dinitrophenylhydrazine gave no precipitate. The solution may have contained products derived from the ether which hindered growth, or more probably most of the xylose was oxidised during the sixteen days of the first cultivation. The rapid oxidation of pentoses by moulds has been already mentioned (p. 1499).

Experiment 7. About 30 g. of xylose (B.D.H.) were twice recrystallised from 90% alcohol, about 8 g. being finally obtained having $[\alpha]_D^{15°}$ + 20·1°. 8 G. of this product in 80 c.c. of medium K in eight tubes were sterilised, inoculated, and incubated for 8 days and kojic acid (0·3 g.) was obtained as usual, m. p. and mixed m. p. 154°. The diacetyl derivative after crystallisation from alcohol had m. p. and mixed m. p. 101—102°. The extracted culture was only very faintly acid to litmus, contained no oxalic or citric acid, and gave no precipitate with dinitrophenylhydrazine.

A further 8 g. of the same B.D.H. xylose were successively recrystallised from 50, 70 and 90% alcohol, giving finally 0·7 g. of a specimen which, when sterilised and inoculated as usual, gave the ferric chloride test on the third day.

Fermentation of Glucose.—Experiment 8. In view of the relatively small yields of kojic acid obtained from the pentoses an experiment was made to determine the amount obtainable from glucose by the strain of *A. oryzæ* used in this research.

A solution of 25 g. of glucose in 250 c.c. of medium K was sterilised, inoculated, and fermented for 7 days. Continuous ether-extraction gave 0·95 g. of kojic acid, m. p. 151—152° (crude) and m. p. and mixed m. p. after recrystallisation from alcohol 154°. The yield was therefore comparable with that obtained from the pentoses and affords further evidence that traces of hexoses in arabinose and xylose are not responsible for the production of kojic acid.

The yields of kojic acid obtained in the foregoing experiments are tabulated below, being calculated on 100 g. of the sugar :

Expt.	Sugar.	Incubation.	Yield.
3	Arabinose	8 days	2·5 g.
4	,,	7	1·4
5	Xylose	7	5·0
6	,,	16	2·1
7	,,	8	3·8
8	Glucose	7	3·8

Negative Results of Fermentation Experiments.—No growth of *A. oryzæ diastase* took place on a 1% solution of pyromeconic acid in medium K even after 30 days at 31—32°.

The same mould grew well in 3 days on a 1% solution of glycuronolactone in medium K. The ferric chloride test was negative even at 27 days. Tests for citric and oxalic acids were also negative.

A fair growth of the same mould occurred on a 2% potassium hydrogen adipate solution in medium K. Tests for kojic and citric acids were negative up to the sixteenth day.

Another strain of *A. oryzæ* kindly supplied by Dr. Schoen of the Pasteur Institute refused to grow on 1% solutions of dimethylpyrone or of sodium comenate in Molliard's medium (see J., 1927, 202).

The cost of this investigation has been in part defrayed by a grant from the Government Grant Committee of the Royal Society. The Department of Scientific and Industrial Research has made grants for the development of the research which have secured the participation of one of the authors (L. K.). To both of these bodies the authors wish to express their thanks.

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THE UNIVERSITY, MANCHESTER. [*Received, June 4th*, 1929.]