24. *The Formation of Kojic Acid from Ethyl Alcohol by* Aspergillus oryzæ, *and the Action of this Mould on Some Carbohydrate Derivatives.*

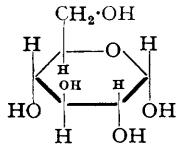
By Douglas Barnard and Frederick Challenger.

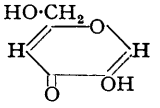
Formation of kojic acid (II) from pentoses by *Aspergillus oryzæ* requires preliminary fission. There is no evidence for the hypothesis (A) that kojic acid is formed by loss of water from hexose (I) accompanied by oxidation of >CH·OH to >CO. More probably (B) hexoses and pentoses undergo fission, two trioses then giving kojic acid by oxidation and loss of water. 2-Deoxyglucose (V) should yield by mechanism (A) 2-hydroxymethyl-γ-pyrone (VII), but kojic acid is formed (B). Glucal (III) should also give (VII) according to (A), but *A. oryzæ* does not grow on this compound or on 6-methyl glucose (VI) which by (A) should give 5-hydroxy-2-methoxymethyl-γ-pyrone

(VIII), whereas by (B) kojic acid, 3-hydroxy-5-methoxymethyl-γ-pyrone (IX), and (VIII) might result. A medium containing 1·3—2·1% of ethyl alcohol as sole source of carbon, with spores or washed mycelia of *A. oryzæ*, gives kojic acid and acetaldehyde.

CHALLENGER, KLEIN, and WALKER, (*J.*, 1929, 1498; 1931, 16) showed that *Aspergillus oryzæ* forms kojic acid (II) not only from glucose but also from arabinose and xylose. Birkinshaw, Charles, Lilly, and Raistrick (*Phil. Trans.*, 1931, *B*, **220**, 134) observed the formation from pentoses several years earlier, but publication was delayed. See also Yabuta (*J.*, 1924, **125**, 575) and footnote by Barger (*ibid.*, p. 587).

The similarity in structure between glucose (I) and kojic acid (II) suggests that the conversion of (I) into (II) involves simple loss of two molecules of water and oxidation of >CH·OH to >CO; this so-called " carving-out " process would be effected without ring fission. On this view, however, the pentoses should yield pyromeconic acid (II*a*). As kojic acid is actually formed, ring-fission must first occur yielding probably a triose and a diose. Glycerol readily gives kojic acid in cultures of *A. oryzæ*. Challenger, Klein, and Walker (*J.*, 1931, 17) showed that dihydroxyacetone gives a 20% yield of kojic acid in cultures of the same strain of *A. oryzæ*, and pointed out that this may arise directly from two molecules of dihydroxyacetone by oxidation and loss of two molecules of water without condensation to a hexose. They then stated: " It is probable, though definite proof is so far lacking, that the conversion of glucose into kojic acid may also be preceded by ring-fission with formation of a triose, and that the hydroxy-γ-pyrone nucleus is not directly carved out of the pyranose ring ". A similar conclusion was reached by Corbellini and Gregorini (*Gazzetta*, 1930, **60**, 244) and has found fairly general acceptance, but a final decision has not been possible. It was hoped by a study of the growth of *A. oryzæ* upon suitable derivatives of glucose to decide whether, in certain cases, at any rate, the occurrence of a " carving-out " process could be definitely established. Using Kinoshita's basal salt medium (K) (*Acta Phytochim.*, 1927, **3**, 31; see p. 114 for composition) the following substrates were employed as sole source of carbon; penta-acetyl glucose, glucal (III), tetra-acetyl hydroxyglucal (IV), 2-deoxyglucose, (V), 6-methyl glucose (VI), and 1-methyl glycerol.


(I.)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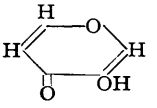
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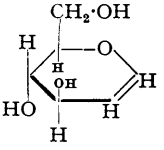
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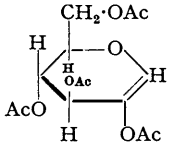
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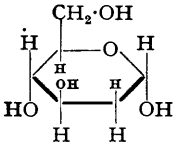
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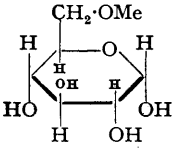
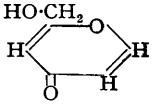

(VI.)

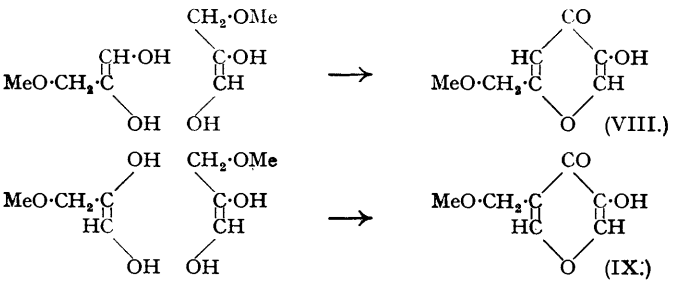

(VII.)

The mould did not grow readily upon penta-acetyl glucose, but was trained to do so by cultivation for several generations upon a medium containing glucose and the acetate, the glucose being gradually reduced to zero concentration. Fission of this completely substituted derivative to a triose or triose acetate was not to be expected. It seemed possible that a process of type (A), not involving ring fission but rather the elimination of acetic acid from positions 1 : 2 and 4 : 5 and hydrolysis of the acetyl group on 3, followed by oxidation to >CO, might yield 2 : 6-diacetyl kojic acid. Other possibilities were (*a*) hydrolysis of the acetyl groups giving glucose and then kojic acid, and (*b*) complete metabolism of the penta-acetate. In a series of experiments the reaction followed course (*b*); neither diacetyl kojic acid nor any other metabolite could be detected and only traces of the penta-acetate remained. These observations are confirmed by those of Walker and Bond (Bond, Thesis, Manchester, 1930). Similar results were obtained with tetra-acetyl hydroxyglucal (IV) which is an intermediate stage in the unrealised conversion of penta-acetyl glucose into diacetyl kojic acid. Ready-formed mycelia were used, but almost complete metabolism occurred and no γ-pyrone derivative could be isolated.

Glucal (III) cannot undergo fission to a triose, and by mechanism (A) could yield 2-hydroxymethyl-γ-pyrone (VII). No growth was observed in K-medium either on inoculation or with

ready-formed mycelia. It is certainly not in favour of mechanism (A) that three compounds which from their structure would not be expected to react by mechanism (B) should be attacked with difficulty by *A. oryzæ* and when growth is established should give no γ-pyrone derivative.

The case of 2-deoxyglucose (V) is specially interesting; here fission to glyceraldehyde and β-hydroxypropaldehyde might be expected, and kojic acid was readily formed. From the high yield (20%) it seemed unlikely that any 2-hydroxymethyl-γ-pyrone (VII) had been produced. Examination of the medium confirmed this view. On the other hand Bergmann and Schotte (*Ber.*, 1922, **55**, 158) found that 2-deoxyglucose is not attacked by yeast. 6-Methyl glucose might be expected to give by process (A) 5-hydroxy-2-methoxymethyl-γ-pyrone (VIII), whereas, if fission to glyceraldehyde and its 3-methyl ether should occur, kojic acid would arise from the first-named compound. The second is isomeric with methyl dihydroxyacetone, two molecules of which might react with the loss of one methyl group by oxidation to give 5-hydroxy-2-methoxymethyl-γ-pyrone (VIII) or by a similar process giving 3-hydroxy-5-methoxymethyl-γ-pyrone (IX), thus:



Elimination of both methyl groups would lead to kojic acid or possibly to 3-hydroxy-5-hydroxymethyl-γ-pyrone. Rather surprisingly 6-methyl glucose would not support the growth of *A. oryzæ* and was almost completely recovered from the medium.

In continuation of the earlier work on dihydroxyacetone (*J.*, 1931, 16) the behaviour of its methyl ether as a substrate for *A. oryzæ* was of interest. It was decided to study first 1-methyl glycerol which yields methyl dihydroxyacetone in cultures of *B. xylinum* (Neuberg, *Biochem. Z.*, 1932, **255**, 1) and might undergo a similar reaction with the mould. No growth was obtained, however, from spore inoculations or from washed mycelia on K-medium containing this ether. Neither kojic acid nor its 6-methyl ether, nor methyl dihydroxyacetone, could be detected after three weeks, and the methyl glycerol was recovered.

Spore inoculations of K-medium containing 1% of methyl alcohol gave no growth, and no kojic acid was produced in presence of ready-formed mycelia. With ethylene glycol growth was very poor, and washed mycelia soon deteriorated. No kojic acid was formed. This is somewhat surprising, as oxidation to glycollaldehyde and condensation to a tetrose or hexose followed by formation of some kojic acid might have been expected. Tamiya (*Acta Phytochim. Tokyo*, 1932, **6**, 1) obtained similar negative results. Walker and Kaushal (*Nature*, 1947, **160**, 572), however, find that a strain of *Acetobacter acetigenum* readily metabolises ethylene glycol giving a cellulose pellicle. Glycollaldehyde was isolated from the medium in the form of its 2 : 4-dinitrophenylosazone.

Birkinshaw, Charles, Lilly, and Raistrick (*loc. cit.*) suggested that *A. oryzæ* might break down various substrates to acetaldehyde, which by a series of (unspecified) reactions might be condensed to kojic acid. They found that all the moulds which produce kojic acid also form ethyl alcohol and acetaldehyde.

A study of the behaviour of ethyl alcohol in cultures of *A. oryzæ* was therefore begun. A survey of the literature yielded very little relevant information. Tamiya (*loc. cit.*) found that ethyl alcohol alone among the simpler aliphatic alcohols supported the growth of *A. oryzæ*. He determined the respiration coefficient for this substrate but did not detect any kojic acid (see below).

Katagiri and Kitahara (*Mem. Coll. Agric. Kyoto*, 1933, **26**, 1; *Chem. Abs.*, 1933, **27**, 3236) who studied the behaviour of several aliphatic compounds in cultures of *A. oryzæ* state that ethyl alcohol plays no important rôle in the formation of kojic acid.

Takahashi and Asai (*Zentr. Bakt., Par.* II, 1933, **88**, 286) state that ethyl alcohol yields kojic acid with *A. oryzæ*, and cite Sakaguchi (*J. Agric. Chem. Soc. Japan*, 1932, **8**, 268, Showa 7) as the authority for this observation but give no further details. Prescott and Dunn (" Industrial

Microbiology ", McGraw Hill Book Co., 1940, pp. 408, 409) also attribute to Sakaguchi (*loc. cit.*) the statement that kojic acid formation is stimulated by ethyl alcohol. His paper is in Japanese and is not contained in the usual libraries. Abstracts (*A.*, 1933, 535, 637; *Chem. Abs.*, 1933, 27, 1379) make no mention of either of these statements. It was clear that in any case details could only have been scanty and it was decided to investigate the matter.

The action of ready-formed mycelia of *A. oryzæ* (see p. 114) upon the basal salt medium of Kinoshita (*loc. cit.*) containing 1·3% of ethyl alcohol as sole source of carbon was first studied. Such mycelia could be entirely freed from kojic acid by careful washing, and the fact that they produced no kojic acid when kept on the basal salt medium alone during 6 weeks obviated the possibility of kojic acid production from reserve carbohydrate. Gould (*Biochem. J.*, 1938, **32**, 797) found that the washed, dried, and ground mycelium of *A. tamarii*—an organism which is allied to *A. oryzæ* and readily forms kojic acid from carbohydrate—when used as a substrate for this mould gave no kojic acid, indicating the absence of a suitable reserve carbohydrate.

The mycelium of *A. oryzæ* readily produced kojic acid when placed upon the ethyl alcohol–K-medium. Yields were about 12% of the theory assuming that all the carbon in the alcohol is available, and diminished after about 10 or 11 days, possibly because of assimilation of kojic acid by the mould (Tamiya, *loc. cit.*).

In confirmation of these results it was found that *A. oryzæ* grew fairly well at 32° after a spore inoculation of the K-salt medium containing 1·3—2·1% of ethyl alcohol, giving kojic acid in yields of 12—17%. Inoculation of a medium containing more than 3% of alcohol produced no growth. Using 1·3% of alcohol at 20° or 25° no kojic acid, or only traces, was produced.

The kojic acid was characterised as the diacetyl or dibenzoyl derivative and determined as the copper salt (Yabuta, *A.*, 1922, i, 940) or by the iodometric method of Birkinshaw *et al.* (*loc. cit.*). The two methods gave almost identical results.

All cultures of *A. oryzæ* on alcohol (1—4%) yielded on distillation acetaldehyde, which was isolated as the 2 : 4-dinitrophenylhydrazone. Blank experiments showed that this was not due to atmospheric oxidation. The aldehyde was also " trapped " in the cultures by addition of " dimedone " (5 : 5-dimethyl*cyclo*hexane-1 : 3-dione), the yield of kojic acid being reduced to 5% and its formation delayed. Sodium sulphite fixed some acetaldehyde but reduced the yield of kojic acid only slightly. Gould (*loc. cit.*) found that the presence of " dimedone " in cultures of *A. tamarii* on glucose had no inhibitory effect on kojic acid production, but often acted as a stimulus. No fixation of acetaldehyde was observed by Gould or in the similar experiments of Katagiri and Kitahara (*Bull. Agric. Chem. Soc. Japan*, 1929, **5**, 46). Acetaldehyde is poisonous to *A. oryzæ*, and in concentrations of 2% or more inhibits respiration completely (Tamiya, *loc. cit.*).

Attempts were made to find a substrate which, while yielding no kojic acid, would maintain the mould in vigorous growth and permit of the addition of possible precursors of kojic acid. A review of the literature suggested that mannitol and succinic acid might be of use. Both these compounds, however, gave traces of kojic acid and were, therefore, useless for our purpose.

Apart from kojic acid, only acetaldehyde could be identified in the alcohol medium. The cultures nevertheless had an evanescent but very definite fruity odour which was not that of acetaldehyde. This was observed both before and during kojic acid formation, and also in cultures where, owing to a high concentration (4%) of alcohol or a low incubation temperature, kojic acid was absent. Its origin is under investigation.

Tamiya (*loc. cit.*) grew *A. oryzæ* upon a large number of substances and determined the respiration figures and growth-characteristics of these substrates. The respiration coefficients Q_{O_2} and Q_{CO_2} are respectively the volume of oxygen and carbon dioxide in c.c. absorbed and evolved by 1 g. of mycelium per hour. If Q_{O_2}, $Q^{\circ}_{O_2}$ and $Q^{K}_{O_2}$ are the respiration coefficients when the mould was grown upon a carbon substrate, a basal salt medium, and sucrose respectively then $q_{O_2} = (Q_{O_2} - Q^{\circ}_{O_2})/(Q^{K}_{O_2} - Q^{\circ}_{O_2}) \times 100$. The production or non-production of kojic acid from the carbon substrate could be forecast. If $q_{O_2} > 80$ kojic acid was formed but not otherwise. Out of 123 substrates examined only 9 failed to conform to this rule. Although Tamiya did not detect kojic acid formation from ethyl alcohol, the average value of q_{O_2} for alcohol was 134, suggesting to us that this substance should yield kojic acid, as we have now shown.

Tamiya also found that for most substrates Q_{O_2} and Q_{CO_2} were approximately equal, Q_{CO_2} being generally slightly higher. The figures for ethyl alcohol were Q_{O_2}, 40·2 and Q_{CO_2}, 21·7. It therefore seemed that *A. oryzæ* might be using some of its own carbon dioxide. For instances of such utilisation see Barker, Ruben, and Kamen (*Proc. Nat. Acad. Sci.*, 1940, **26**, 418; **27**, 590), Wood and Werkmann (*Biochem. J.*, 1940, **34**, 7). Aristovskaja (*Microbiol., U.S.S.R.*, 1941, **10**, 701; *Chem. Abs.*, 1944, **38**, 2990, 5875) states that *A. oryzæ* as well as several bacteria

can utilise carbon dioxide. We made preliminary experiments to determine whether large excess or diminution in the amount of carbon dioxide present affected the production of kojic acid from alcohol, but no definite effect was detected.

Experimental.

Preparation of Washed Mycelia of Aspergillus oryzæ.—The strain of the mould was that used in earlier work (Challenger, Klein, and Walker, *J.*, 1929, 1498; 1931, 16, where full morphological details are given) and the basal salt medium was that of Kinoshita (*loc. cit.*) containing potassium dihydrogen phosphate (1 g.), magnesium sulphate (0·5 g.), ammonium nitrate (0·4 g.), and water (1000 c.c.) to which was added glucose (30 g.). A spore culture of the mould was grown until, after 10—14 days, a thick mycelium completely covered the surface. With the usual precautions the medium was removed from under the mycelium by means of a sterile pipette and replaced by sterile water (100 c.c.) from a second sterile pipette. This was repeated every 2 hours until the replaced water was free from traces of kojic acid. Six washings always proved sufficient. The mycelium was then left upon sterile water for a further 12 hours to free the cells from traces of carbohydrate and kojic acid. Any desired substrate could then be added under the mycelium by means of sterile pipettes. No contamination was observed when using this method, and with care the mycelium remained whole and undamaged.

Two other strains of *A. oryzæ* (Nos. 589 and 3876 from the National Collection of Type Cultures, Lister Institute) were also tested on Kinoshita's glucose medium but did not produce kojic acid at 32°.

Production of Kojic Acid from Ethyl Alcohol as Sole Carbon Source, using Washed Ready-formed Mycelia.—In 3 control experiments fully-grown washed mycelia of *A. oryzæ* were placed upon 300 c.c. of K-medium at 32°; no kojic acid was detected during 6 weeks by regular application of the ferric chloride test. Three experiments using similar washed mycelia and 300 c.c. of K-medium containing ethyl alcohol (1·3—2·1%) as sole carbon source readily gave kojic acid, which was detected by ferric chloride and later separated and characterised. Details are given in the table. The alcohol used was "absolute" and was redistilled through an apparatus which was previously sterilised at 140° and then well washed out by the hot vapour. No contamination was observed. Two control experiments were arranged in which ethyl alcohol (5 c.c.; 1·3% by weight) was added to sterile K-medium (300 c.c.) and incubated at 32° for 6 weeks. No trace of bacterial or mould growth was observed.

Production of kojic acid by A. oryzæ, *with ethyl alcohol as sole carbon source.*

Concn. of alcohol, %.	Time (days).[1]	Time (days).[2]	Wt. (g.).[3]	Yield, %.[4]	M. p. Alone.	M. p. Mixed.	Diacetyl comp., mixed m. p.		Dibenzoyl comp., mixed m. p.	
A. *Experiments using grown mycelia.*										
1·3	11	4·0	0·49	12	151·5—152·5°	151·5—152·5°	—	—	—	—
1·3 added at 7 day intervals; total, 5·2.	28	4·0	2·4	14·5	152—153	152—153	—	—	—	—
B. *Experiments using spore cultures.*										
1·3	18	9·0	0·51	12·4	152·5—153	153	101—102°	101—102°	132—133°	132—133°
1·3	18	9·0	0·49	12	152·5—153	152·5—153	—	—	—	—
2·1	21	13·0	1·1	16·7	152—153	152—153	101—102	101—102	—	—

The authentic specimen of kojic acid had m. p. 152·5—153°.

[1] Duration of experiment. [2] For first detection of kojic acid.
[3] Of kojic acid obtained through copper salt. [4] On C/C basis.

The kojic acid was isolated from the filtered cultures by evaporation under reduced pressure to $\frac{1}{15}$th of the original volume, acidification with glacial acetic acid to a concentration of 0·5N to prevent precipitation of copper phosphate, and addition of excess of saturated aqueous copper acetate. The mixture was then kept at 0° for 12 hours to complete the precipitation of the copper kojate. This was separated and decomposed with hydrogen sulphide, the filtered solution evaporated under dinished pressure, and the crude kojic acid recrystallised once or twice from methyl alcohol–ethyl acetate. It then had the correct m. p. but was sometimes sublimed at 150°/9 mm. to obtain a colourless specimen, m. p. 152—153°.

Production of Kojic Acid from Ethyl Alcohol as Sole Carbon Source, using Spore Cultures of A. oryzæ.—Inoculation of a glucose-free K-medium containing 1·3 or 2% of ethyl alcohol yielded a reasonably good growth of the mould. This began in 2—3 days and covered the surface in 8 days. Sporing began about the twelfth day but was much less than when glucose was used. Kojic acid, readily produced in yields varying from 12 to 16·7%, was identified by m. p. and mixed m. p. and by similar determinations carried out with the diacetyl and dibenzoyl derivatives. A specimen sublimed at 150°/9 mm. melted at 152—153° after recrystallisation from ether (Found: C, 50·5; H, 4·4. Calc. for $C_6H_6O_4$: C, 50·7; H, 4·3%). An authentic specimen melted at 152·5—153°.

Behaviour of A. oryzæ *with Higher Concentrations of Ethyl Alcohol.*—Concentrations of ethyl alcohol above 2·1% had a decided inhibitory effect on the growth of the mould. No growth occurred with spore inoculations of a medium containing 4% of alcohol. Washed mycelia were therefore used.

Duplicate experiments were made by placing glucose-free K-medium (300 c.c.) containing 4% of ethyl alcohol under well-washed mycelia, and incubating for 14 days during which time no kojic acid was formed and the mycelia began to lose their healthy appearance. The media were filtered and distilled. An odour of acetaldehyde was noticed in each case, and the first 10—15 c.c. of distillate gave a yellow precipitate, m. p. 152—153°, with 2 : 4-dinitrophenylhydrazine. After 2 crystallisations from alcohol the m. p.s were 158° and 165·5° respectively. The mixed m. p. with authentic acetaldehyde 2 : 4-dinitrophenylhydrazone, m. p. 165—166°, was 165—166°.

Control experiment. When 300 c.c. of sterile K-medium (glucose-free) containing ethyl alcohol (4%) were kept at 32° for 16 days no acetaldehyde was detected. It was then shown that acetaldehyde is produced in all cultures of *A. oryzæ* on ethyl alcohol (1·5—4%) whether using spores or washed mycelia.

Effect of (a) *Sodium Sulphite and* (b) 5 : 5-*Dimethylcyclohexane*-1 : 3-*dione* (*Dimedone*) *upon Kojic Acid Formation from Ethyl Alcohol.*—(*a*) Two cultures were made up using washed mycelia of *A. oryzæ*, glucose-free K-medium (300 c.c.) and ethyl alcohol (5 c.c.). Sterile 7% sodium sulphite solution (25 c.c.) was added to each. Growth was good and kojic acid was detected after 5 days. After 17 days each culture was filtered and boiled with calcium carbonate (10 g.), and 25 c.c. of each distillate were treated with 2 : 4-dinitrophenylhydrazine hydrochloride. In one case a precipitate was obtained. Determinations of m. p. and mixed m. p. showed this to be acetaldehyde 2 : 4-dinitrophenylhydrazone. Kojic acid undergoes no decomposition on being boiled with water and calcium carbonate, and was isolated from the medium through the copper salt in 9% yield.

(*b*) Two cultures were made up using a washed mycelium of *A. oryzæ*, glucose-free K-medium (300 c.c.), and ethyl alcohol (5 c.c.). Dimedone (1·2 g.) was added before sterilisation. On incubation at 32° crystals formed in both cultures after 5 days, increasing in amount up to the eighth day. Kojic acid was not detected until the twelfth day. After 16 days the mycelia and crystals were separated and washed with 30 c.c. of hot alcohol which gave characteristic flat plates, 0·42 g. and 0·5 g. These melted at 131—132° and after 3 recrystallisations from alcohol at 139—140° alone and in admixture with authentic "acetaldehyde-dimedone" of m. p. 140°; the culture media gave kojic acid in 5% yield, a fall in production as compared with the figures in the table. When K-medium (300 c.c.), ethyl alcohol (5 c.c.), and dimedone (1·2 g.) were kept at 32° for 30 days, no trace of the acetaldehyde derivative was formed.

Attempts to use Acetaldehyde and its Derivatives as Sources of Carbon for A. oryzæ.—The medium consisted of glucose-free K-medium (300 c.c.) with the appropriate substrate. Inoculation of the medium containing 1% of acetaldehyde sodium bisulphite failed to produce growth and two similar solutions were placed under washed mycelia as usual and incubated at 32° for 21 days. No kojic acid was produced, and the mycelium appeared to die. Similar results were observed with washed mycelia and media containing 1% and 0·5% of paraldehyde, and in two experiments with 1% acetal, both at 32° for 30 days. When 1 c.c. of a 33% solution of acetaldehyde in sterile water was added every 24 hours during 10 days to 2 well-washed mycelia of *A. oryzæ* on K-medium, no kojic acid was produced during 21 days at 32°.

Methyl Alcohol, Ethylene Glycol, and 1-*Methyl Glycerol as Substrates for* A. oryzæ.—Sterile K-medium (300 c.c.) with methyl alcohol (5 c.c.) as sole carbon source was inoculated with spores. No growth occurred at 32° during 1 month. Two experiments under similar conditions using washed mycelia showed no growth, but deterioration, and no kojic acid was detected by regular tests during a month. Growth was also negligible during 6 weeks using 2·5 c.c. of ethylene glycol in 300 c.c. of K-medium whether after spore inoculation or with washed mycelia. With 5 c.c. and a washed mycelium, negative results were obtained in 6 weeks; deterioration of the mycelium was soon apparent, and no kojic acid was formed. In 2 similar experiments with 1-methyl glycerol (5 c.c.) and a fully-grown washed mycelium of 14 days growth, deterioration occurred and the ferric chloride test was negative during 3 weeks, indicating also the absence of the 6-methyl ether of kojic acid. The concentrated medium was free from 1-methyl dihydroxyacetone as tests with Fehling's solution, phenylhydrazine, and 2 : 4-dinitrophenylhydrazine were negative. 4 C.c. of 1-methyl glycerol were recovered from the concentrated medium with ether.

A. oryzæ *and* 6-*Methyl Glucose.*—6-Methyl glucose was prepared by Bell's method (*J.*, 1936, 859) and had m. p. 145—146° which depended slightly on the rate of heating but was unchanged after 2 further crystallisations from alcohol–ethyl acetate; $\alpha_D^{20°}$ + 87·8° after 50 minutes, and + 55·8° after 24 hours. Bell (*loc. cit.*) found $\alpha_D^{20°}$ + 55° after 24 hours (Found : C, 43·4; H, 7·3. Calc. for $C_7H_{14}O_6$: C, 43·3; H, 7·3%).

Inoculation of 50 c.c. of K-medium containing 1% and 3% of 6-methyl glucose produced no growth at 32° during 4 weeks. In two experiments washed mycelia were placed on 50 c.c. of K-medium containing 3% of 6-methyl glucose. No further growth was noticed, and repeated tests with ferric chloride were negative during 3 weeks. The cultures were then filtered, the mycelium washed with water, and the filtrate and washings evaporated under diminished pressure. Extraction of each residue with methyl alcohol (50 c.c.) yielded a syrup which quickly crystallised. Of the original 1·5 g. of 6-methyl glucose, 1·45 and 1·39 g. were recovered. After 1 recrystallisation from methyl alcohol–ethyl acetate both specimens had m. p. 145—146° unchanged by admixture with an authentic sample.

Glucal and A. oryzæ.—Glucal was prepared from its triacetyl derivative (Fischer, *Ber.*, 1914, **47**, 196) by Gehrke's method (*ibid.*, 1931, **64**, 1729); m. p. 59—60°. It was too unstable for sterilisation by heat, but a specimen freshly recrystallised from ethyl acetate and dried in a vacuum was added (0·4 g. and 0·2 g.) to two 20 c.c. samples of K-medium which were then heavily inoculated with spores. No growth occurred during a month at 32°. When similar solutions were incubated with washed mycelia no growth occurred, and the ferric chloride test was always negative. After 25 days the media were filtered, treated with an equal volume of 2N-sodium hydroxide, and kept at 80° under reflux for 2 hours. Under these conditions any 2-hydroxymethyl-γ-pyrone would have been decomposed to acetone, formic acid, and glycollic acid. Neutralisation and treatment with a solution of 2 : 4-dinitrophenylhydrazine gave no precipitate until after 1—2 hours. This precipitate was obviously due to a decomposition product of the substrate, as the same result was obtained by similar treatment of a solution of glucal.

Preparation of 2-*Deoxyglucose.*—The deoxyglucose was prepared from pure glucal by the method of Bergmann and Schotte (*Ber.*, 1922, **55**, 158). A portion of the syrup gradually crystallised in a desiccator,

the bulk solidifying on inoculation. Oily material was removed by washing with alcohol–ether (1 : 3). The pale-brown, ill-defined needles were dissolved in water, but gave a syrup on evaporation. This solidified and was again washed. The product was then nearly white and had m. p. 145—146° (Found : C, 43·2; H, 7·05. Calc. for $C_6H_{12}O_5$: C, 43·8; H, 7·35%). No information could be obtained from the literature as to a suitable solvent for recrystallisation. All the common solvents and also dioxan, pyridine, and nitromethane were tried without success. It is slightly soluble in alcohol, and addition of ether to the solution precipitated white flocks; with great care one or two small crystals were obtained, but the process was very unsatisfactory. Sublimation at 0·5 mm. led to decomposition. Small, almost white needles, m. p. 148° (constant), were finally obtained by recrystallisation from amyl alcohol (Found : C, 43·8; H, 7·35. Calc. for $C_6H_{12}O_5$: C, 43·8; H, 7·35%). The compound is fairly soluble in the hot solvent but sparingly so in the cold. These details are recorded owing to the scanty information available and the necessity of obtaining a pure specimen for the cultures.

2-Deoxyglucose and A. oryzæ.—K-Medium (20 c.c.) containing 2-deoxyglucose (2·5%) was inoculated with spores of the mould and incubated at 32°. Growth was good, kojic acid being first detected on the fifth day. After 21 days the medium was filtered, the mycelium washed with hot water, and the filtrate and washings evaporated to 5 c.c. under diminished pressure. Saturated copper acetate solution (10 c.c.) and acetic acid (0·1 c.c.) gave crystalline copper kojate. After 12 hours at 0° this was separated (0·05 g.; kojic acid = 0·041 g.). Decomposition with hydrogen sulphide gave crude kojic acid (0·037 g.), m. p. 148—150°; yield 7·4%. The precipitation of copper kojate is not entirely quantitative. A second experiment using K-medium (25 c.c.) with 2-deoxyglucose (2·4%) gave after 18 days copper kojate (0·17 g.; kojic acid 0·14 g.). This gave crude kojic acid, m. p. 147—148° (0·12 g.), in a yield of 20% of the 2-deoxyglucose employed. Both preparations of kojic acid had m. p. and mixed m. p. 152—153° when recrystallised. A portion gave a diacetate which had m. p. 101—102° unchanged in admixture with an authentic specimen of the same m. p. After separation of the copper salt the filtrate was freed from copper by hydrogen sulphide; excess of hydrogen sulphide was removed by aeration, lead acetate added to precipitate any remaining kojic acid, excess of lead separated as sulphide, and hydrogen sulphide removed as before. Refluxing with an equal volume of 2N-sodium hydroxide for 2 hours gave no acetone (2 : 4-dinitrophenylhydrazine test), indicating the absence of 2-hydroxymethyl-γ-pyrone from the culture. The dark flocculent precipitate which separated after 4 hours was similar to that obtained from the uninoculated medium.

Tetra-acetyl Hydroxyglucal and A. oryzæ.—The glucal derivative was prepared by Maurer's method (*Ber.*, 1929, **62**, 332) and had constant m. p. 62—63°. Maurer gives m. p. 65—66° after repeated crystallisation. 1 G. was suspended in sterile K-medium (100 c.c.) and heavily inoculated with spores of the mould. Some unhealthy white growth formed after one week at 32° and the sparingly soluble crystals diminished slightly in amount, but, even after 6 weeks, growth was unsatisfactory, no kojic acid was produced, and much unattacked substrate remained. Two similar media were placed under washed mycelia. The acetyl derivative was then much more readily assimilated, and almost disappeared in 9 days. After 10 days, the media were examined by the procedure described under penta-acetyl glucose (see below). Evaporation under diminished pressure gave only a slight amount of residual organic matter which contained neither kojic acid nor diacetyl kojic acid.

Penta-acetyl Glucose and A. oryzæ.—The mould grew very poorly upon this substance and was therefore " trained ". Successive generations were grown in duplicate on double-strength K-medium containing 1% of penta-acetyl glucose in suspension (the solubility is only 1 in 500 parts of water) and 0·5, 0·2, 0·05, 0·01, and 0·00% of glucose. Two generations were grown on each concentration, the average period being 10 days. Even then growth was relatively slow and spore formation poor. Sterilisation was carried out at 120°, no hydrolysis occurring under these conditions. Both with the original and with the " trained " strain, no kojic acid could be detected. The two following experiments are typical. The double-strength K-medium (100 c.c.) containing penta-acetyl glucose (1%) in suspension was inoculated with the ordinary strain. Growth was slow at 32°, but after 2 months the mycelium completely covered the medium and the suspended solid was diminishing in amount. Tests with ferric chloride on sterile samples withdrawn at intervals showed no kojic acid. After 80 days the separated medium contained 0·03 g. of penta-acetyl glucose of the correct m. p. 129—130°. The filtrate had pH *ca.* 6 and gave no precipitate with 2 : 4-dinitrophenylhydrazine. Evaporation to 3 c.c., extraction with ether, and evaporation of the dried extract left a colourless syrup (1 c.c.). This would not crystallise on cooling, or in a desiccator during 14 days, or on inoculation with penta-acetyl glucose or diacetyl kojic acid. It gave a strong Molisch reaction. After being left in methyl-alcoholic ammonia at 0° for 24 hours, diluted with water, and neutralised, it gave no colouration with ferric chloride. Using the " trained " mould under similar conditions, growth was improved and a complete mycelium formed in 30 days. The medium was then replaced by 300 c.c. of fresh medium containing penta-acetyl glucose (3 g.). The crystals were almost completely assimilated in 26 days. The medium was examined as before with similar results. In addition, 20 c.c. were distilled, oxidised with acid potassium dichromate, and redistilled. Acetaldehyde was absent, indicating the non-formation of alcohol in the culture medium which was again only slightly acid (10 c.c. = 0·15 c.c. of 2N-sodium hydroxide). The evaporated medium after extraction with ether contained inorganic acetate but no formate. A duplicate experiment gave identical results.

Two similar cultures were left for 15 days after all solid penta-acetyl glucose had disappeared. Tests for diacetyl kojic acid were again negative, and continuous extraction with ether yielded a negligible amount of organic matter. Penta-acetyl glucose appears to be completely assimilated by *A. oryzæ* without giving rise to any recognisable product, thus resembling tetra-acetyl hydroxyglucal.

Effect of Carbon Dioxide on Kojic Acid Production from Alcohol.—(a) *High concentration of carbon dioxide.* A series of litre flasks each containing 300 c.c. of K-medium with either 1·3 or 2·1% of ethyl alcohol were inoculated and incubated at 32° till a moderate growth had formed (5—7 days). Three such cultures were then joined in series, and sterile oxygen–carbon dioxide mixture passed through at the rate of at least 3 l. each day. Samples were withdrawn under sterile conditions and tested for kojic acid. With 90% of carbon dioxide and 1·3% and 2·1% of alcohol, growth ceased when aspiration began,

and no kojic acid was produced in 30 days. With 30% of carbon dioxide, 25% of oxygen, 45% of nitrogen, and alcohol as before, growth was very poor, and no kojic acid was produced in 30 days. With 10% of carbon dioxide, 25% of oxygen, and 65% of nitrogen, and 1·3% of alcohol, growth approached normal, and kojic acid was detected after 14 days but in relatively small amount judging by the intensity of the ferric chloride test.

(b) *Low concentration of carbon dioxide.* Special flasks (1 l.) were used with a pocket of 1-inch diameter containing 50% potassium hydroxide solution. (25—30 c.c.) half-way up the side. Three flasks each containing 300 c.c. of K-medium and 1·86% of ethyl alcohol were inoculated and incubated at 32°. Kojic acid formation in 2 of the flasks was followed by the iodometric method of Birkinshaw, Charles, Lilly, and Raistrick (*Phil. Trans.*, 1931, *B*, **220**, 139) and in the other by the method already described. The yields were normal or above normal, in one case reaching 23% of the theoretical.

Attempted Detection of Possible Precursors of Kojic Acid.—Tests with phenylhydrazine and its 2 : 4-dinitro-derivative, Molisch's reagent, Denigès's reagent, and Fehling's solution upon the dilute or concentrated media from alcohol cultures before, and in all stages of, kojic acid formation, indicated acetaldehyde only. Continuous ether-extraction of the media yielded nothing but almost pure kojic acid.

Titration of the Alcohol Media with Sodium Hydroxide and with Potassium Permanganate before Kojic Acid Formation.—Any acid other than kojic formed by the mould should be detectable by alkali titration, provided such acid was not neutralised by ammonia produced in the cultures or potassium hydroxide arising from the KH_2PO_4 in the medium. This would seem improbable, as in glucose cultures only a slight increase in the alkalinity of the medium is ever noticed. Two well-grown and similar mycelia of *A. oryzæ* were well washed, and 300 c.c. of sterile K-medium containing 5 c.c. of ethyl alcohol added to each. Samples (10 c.c.) were removed at regular intervals and titrated with N/100-sodium hydroxide (phenolphthalein) to the first discernible pink colour. Five sets of these titrations were carried out, but only two are recorded below, as the others were of exactly the same type, the volumes of N/100-alkali being almost identical. The amount of acid produced before kojic acid formation is equivalent to no more than about 3 c.c. of N/100-alkali. After neutralisation the medium was acidified with 10 c.c. of 2N-sulphuric acid and titrated with N/10-potassium permanganate which was shown to react at once with kojic acid or with compounds such as ethyl acetoacetate. Nothing capable of oxidation by acid potassium permanganate was found until kojic acid formation occurred.

Details of titrations. From 0 to 46 hours the volume of 0·0094N-sodium hydroxide rose from 8·0 to 11·0 and then fell, until at 94 hours the value was 7·0 c.c. At this point kojic acid was first detected, and the titre rose to 16·0 c.c. at 142 hours. In the second culture the corresponding figures were 9, 11·2, 10·0, and 27 c.c. Kojic acid was first detected at 78 hours. The permanganate titrations at the same times were 0·05, 0·05; 0·05, 0·05; 12·0, and 24·0 c.c.

As the fall in acidity observed just before kojic acid formation might be due to the utilisation of acid phosphate for carbohydrate phosphate formation, two cultures similar to those used previously were prepared; one, however, contained no phosphate (300 c.c. of water, 5 c.c. of ethyl alcohol, 0·2 g. $MgSO_4$, and 0·2 g. of NH_4NO_3). In the phosphate-free medium no sharp minimum acidity was observed, but kojic acid formation, although delayed, was not prevented. During 182 hours (at which time kojic acid was first observed) the titre rose from 0·75 c.c. to 1·6 c.c., and the permanganate figures from 0·05 c.c. to 0·20 c.c.

Improved Preparation of Bromokojic Acid.—Yabuta's method (*A.*, 1922, i, 939) gave a very poor yield: Kojic acid (6 g.) in water (100 c.c.) was slowly treated with the theoretical amount of bromine, and the mixture continuously extracted with ether. Only 0·5 g. of bromokojic acid was obtained. Much red tar was formed. Increased concentration of bromine diminished the yield, and interaction in organic solvents gave no bromokojic acid.

Kojic acid (1 g.) in water (20 c.c.) was treated with the theoretical amount of sodium hypobromite in water (15 c.c.) at 0°; after 12 hours at 0° and 36 hours at room temperature, 0·23 g. of bromokojic acid separated, a yield of 14·6%. No red tar was produced, but the strong smell of bromoform indicated that hydrolytic fission of the γ-pyrone ring had occurred, probably giving acetol as the first product. Shimmin (Thesis, Liverpool 1947; Shimmin and Challenger, in the press) found that bromination in presence of a phosphate buffer gave greatly improved yields of bromopyromeconic acid. The method was equally successful in this case. Kojic acid (3 g.) was dissolved in 50% orthophosphoric acid (30 c.c.) at 0°. Bromine (4 g., 0·5 g. excess) in iced water (150 c.c.) containing potassium dihydrogen phosphate (15 g.) was slowly added during 1 hour to the stirred kojic acid solution. After 12 hours at 0°, the clear, pale orange solution deposited 3·35 g. of bromokojic acid (yield 72%). Similar experiments gave yields of over 60%. Crystallisation from ethyl acetate or ethyl acetate–benzene gave long white needles, m. p. 169—170°, becoming pink on exposure to light. Yabuta gives m. p. 159—160° (Found: C, 32·6; H, 2·4; Br, 36·0. Calc. for $C_6H_5O_4Br$: C, 32·6; H, 2·3; Br, 36·2%).

The double lead salt of acetic and bromokojic acids (Yabuta, *loc. cit.*) is best obtained by the use of at least 50% excess of lead acetate (Found: Pb, 41·3. Calc. for $C_8H_7O_6BrPb$: Pb, 42·6. Equimolecular proportions gave Pb, 39·1%). When 1·5 g. in water (30 c.c.) was saturated with hydrogen sulphide for 20 hours, extraction of the lead sulphide with hot water gave 0·07 g. of white needles which on recrystallisation from water melted at 217—218° (decomp.). Yabuta (*loc. cit.*) give m. p. 210—212° [Found: C, 45·9; H, 3·4; S, 9·9. Calc. for $(C_6H_5O_4)_2S$: C, 45·85; H, 3·2; S, 10·2%]. The sulphide gave a red colour with ferric chloride. It could not be isolated from the reaction of bromokojic acid with hydrogen sulphide in aqueous sodium acetate.

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