I.—*The Formation of Kojic Acid from Sugars by* Aspergillus oryzæ.

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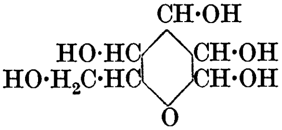
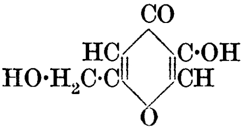
THE similarity in structure between glucose (I) and kojic acid (II) is striking and has been mentioned by many workers (see Challenger, Klein, and Walker, J., 1929, 1499; Maurer, *Ber.*, 1930, **63**, 25, 2069) who have studied the conversion of glucose and other carbohydrates into this γ-pyrone derivative by *A. oryzæ* and related moulds.


(I.)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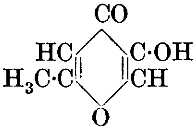
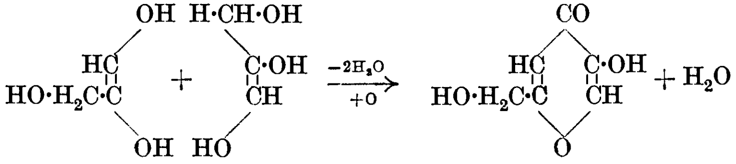

(III.)

The present authors have recently shown (*loc. cit.*) that kojic acid is also formed from arabinose and xylose by *A. oryzæ*. This may be explained by assuming a preliminary breakdown to a 3-carbon compound such as dihydroxyacetone, which then undergoes condensation to glucose or a similar aldohexose. The production of kojic acid from glycerol, which was observed by Traetta-Mosca, using *A. glaucus*, and confirmed by us with the strain of *A. oryzæ* here described, is not in disagreement with such a suggestion.

Acetobacter suboxydans, *B. xylinum*, and other organisms convert glycerol into dihydroxyacetone (Bertrand, *Ann. Chim. Phys.*, 1904, **3**, 255; Fulmer and Werkman, " Index to the Chemical Action of Micro-organisms," 1930, 131, 169), and therefore the change glycerol ⟶ kojic acid may well proceed through this triose: we have recently shown that during the growth of *A. niger* on a 5% glycerol solution containing the necessary inorganic salts the medium acquires reducing properties. Addition of phenylhydrazine causes the precipitation of the osazone of glyceraldehyde (or of dihydroxyacetone). This triose has not previously been obtained from glycerol by the action of a mould. We find that more than 30% of the theoretical yield of kojic acid may be obtained when *A. oryzæ* is cultivated in solutions containing dihydroxyacetone as sole source of carbon. No yields of kojic acid approaching this figure have been obtained by us with any other substrate. The formation of kojic acid in this way may, however, occur without any preliminary condensation to a hexose, thus :—



It is therefore unnecessary to assume the formation of a hexose during the conversion of pentoses into kojic acid, since formation of glyceraldehyde or dihydroxyacetone by loss of two atoms of carbon as glycollaldehyde may readily occur. Many examples are known where pentoses yield the same products as hexoses under the influence of micro-organisms (J., 1929, 1498; Fulmer and Werkman, *op. cit.*, 1930, 129). Consequently 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. This suggestion would fall into line with Robinson's view (*Brit. Assoc. Reports, Edinburgh*, 1921, 417; *J. Soc. Chem. Ind.*, 1925, 1182) that the formation in nature of the pyrone and the pyrylium rings of the anthoxanthins and anthocyanins involves the participation of a triose which, with the twelve carbon atoms of two hexose molecules, completes the skeleton of the colouring matter.

A. oryzæ grows well on rhamnose, but in several experiments only minute traces of a substance giving a red colour with ferric chloride could be isolated from the culture. After conversion into its copper salt and regeneration with hydrogen sulphide this substance melted at 146—148°, and at 150—151° in admixture with kojic acid (m. p. 154°). In all, about 30 g. of carefully purified rhamnose were fermented, but the result was inconclusive owing to the very poor yield. If the pyranose ring remained intact during fermentation *allo*maltol (III) (Yabuta, J., 1924, **125**, 575) should be produced, whereas if fission occurs kojic acid should arise by way of glyceraldehyde.

A study of the formation of kojic acid from organic compounds containing two and three atoms of carbon is in progress, and the behaviour of this and other γ-pyrone derivatives to micro-organisms is being investigated. The publication of the results so far obtained appears desirable in view of the work of Maurer (*loc. cit.*).

An examination of the behaviour of methylated and acetylated sugars towards moulds of the *Aspergillus* family, and other organisms, has also been commenced.

EXPERIMENTAL.

The mould used in this research, a strain of *Aspergillus oryzæ*, and the medium containing the inorganic salts, designated "medium K," were the same as those employed in previous work (J., 1929, 1498). The following details of the morphological characteristics of this strain of *A. oryzæ* were kindly supplied by Dr. C. Thom of the Bureau of Chemistry and Soils, Washington, to whom the authors' thanks are specially due.

"Mould 5060 IV, labelled 'A. oryzæ, diastase—Neuberg': The strain belongs to the flavus section of the *flavus oryzæ* series rather than to the long-stalked yellowish series identified with the original cultures of *A. oryzæ*. Colonies upon agar in Czapek's solution grow rapidly, spreading in 7 to 8 days over the whole surface of the petri dish, with marginal growing area 15—20 mm. wide consisting of radiating hyphae partly above, partly below, the surface of the agar. These are white, passing toward green with the development of stalks and heads in the central area, with the transitional area often in yellow (citrine to flavus of Saccardo's chromotaxia) or with yellow almost suppressed. The central or conidial area shows shades of yellow-green to fairly deep green (about ivy-green of Ridgway's XXXI) in the large colonies and upon deep agar, becoming brown in age. The mass of mycelium tends to become radiately wrinkled and indistinctly zonate in thin areas of the substratum. The reverse is white or mostly uncoloured; stalks from 400—500 μ in the earlier stages to 1000 μ or longer in groups developing later, from 5 μ in diameter at base to 8—10 μ or larger just below the vesicle.

"The heads (unwashed) are large and small in the same areas, up to 250—300 μ, from hemispherical to three-fourths of a spherical mass with chains radiating irregularly over three-fourths of a sphere—more than a hemisphere.

"The vesicle is an almost globose enlargement of the rather broad apex of the stalk, up to 40 μ in diameter or larger, small and large in same areas, pyriform to almost globose and fertile over the upper half to three-fourths. The sterigmata are in smaller heads and younger groups of large heads in one series, later doubled, especially near the base of the large heads, so that simple and double sterigmata are intermingled; about 10 μ by 2—3 μ, or primary about 10—12 by 3—4, and secondary about 10 by 2—3 μ. The conidia are about 5—6 μ subglobose, and are rough and very green."

Fermentation of Dihydroxyacetone.—Experiment 1. The usual precautions were adopted to ensure aseptic conditions in working and the results were checked by control experiments on the uninoculated media. The temperature of incubation was 31—32°. The dihydroxyacetone was a fresh specimen obtained from Messrs. Meister, Lucius & Brüning (Hoechst a/M., Germany) and was purified immediately before use by extraction with hot acetone, which dissolved the impurities. The substance then formed white odourless crystals, m. p. 85—86° (Found by micro-analysis: C, 40·4; H, 6·8. Calc.: C, 40·0; H, 6·7%). Nine tubes, each containing 11 c.c. of medium K, were sterilised and 0·5 g. of dihydroxyacetone was introduced into each tube. After being shaken to ensure complete solution, eight of the mixtures were inoculated with spores of *A. oryzæ*, the remaining mixture being kept as a control. Growth took place rather slowly. A tube gave an intense cherry-red colour with ferric chloride on the 12th day, and the test was still positive on the 19th day. Accordingly, on the 19th day, the contents of the remaining six tubes were filtered, the mycelia well washed with hot water, and the filtrate and washings continuously extracted with ether. The extract, dried over sodium sulphate, gave 0·75 g. of almost pure kojic acid, m. p. 145—147°, and 150—151° in admixture with an authentic specimen (m. p. 154°). After recrystallisation from alcohol, the m. p. and mixed m. p. were 153—154° (Found by micro-analysis: C, 51·3; H, 4·3. Calc.: C, 50·8; H, 4·2%).

Experiment 2. 250 C.c. of medium K were divided amongst 14 tubes, each containing 15 c.c., a control tube containing 10 c.c., and 6 small indicator tubes, each containing 5 c.c. The tubes were sterilised and dihydroxyacetone was added to each, so as to give a 5% solution. Growth was evident on the 6th day and a positive ferric chloride test was not obtained until the 9th day. On the 11th day it was found that, whilst there were good sporing growths in some of the tubes (positive ferric chloride test), others had grown badly (negative ferric chloride test). Accordingly the contents of the tubes having good growths (9 large and 1 indicator), corresponding to 7 g. of dihydroxyacetone, were worked up, and gave 0·78 g. of kojic acid, m. p. 145—147°, and 151—152° in admixture with an authentic specimen kindly supplied in 1926 by Professor H. Raistrick. After recrystallisation from alcohol, the m. p. and mixed m. p. were 154°. The kojic acid was further characterised as the diacetyl derivative, m. p. (after recrystallisation from alcohol) and mixed m. p. 101—102° (compare Yabuta, *J. Chem. Soc. Tokyo*, 1916, **37**, 1185, 1234; *A.*, 1922, i, 939).

Fermentation of Glycerol.—Although kojic acid has been obtained from glycerol by fermentation with *A. oryzæ* (Kinoshita, *Acta*

Phytochim., 1927, **3**, 31) and with *A. glaucus* (Traetta-Mosca, *Gazzetta*, 1921, **51**, ii, 269), it seemed desirable to test its production from glycerol by the strain of *A. oryzæ* used in this research. 50 C.c. of 5% glycerol in medium K were used. The ferric chloride coloration was first obtained on the 3rd day. On the 9th day, 0·05 g. of kojic acid was isolated, m. p. (after crystallisation from ether) and mixed m. p. 154°.

Negative Results of Fermentation Experiments.—No growth of *A. oryzæ* took place on 5% ethylene glycol in medium K, even after 20 days at 31—32°.

The same mould grew quite well on 2% calcium gluconate, 2% potassium hydrogen saccharate, 2% potassium citrate (containing ½% citric acid), 1% glyceric acid, and 5% trimethylene glycol, in medium K, but the ferric chloride test was negative in all these cases. The cultures in potassium hydrogen saccharate were alkaline to litmus after 19 days and contained chiefly potassium carbonate.

Attempts were made to detect intermediate products before kojic acid in cultures of *A. oryzæ* on arabinose. Tests for ketonic or aldehydic substances with 2 : 4-dinitrophenylhydrazine or Schiff's reagent were negative.

Action of Aspergillus niger *on Glycerol. Production of a Triose.*—The strain of *A. niger* was the same as that used in the research on citric acid formation (J., 1927, 200, 3044). Later it was found to have lost its power to produce citric acid from glucose, and this condition had probably been reached at the time of the experiments now to be described. Similar observations are recorded by many workers in this field. Wehmer (*Biochem. Z.*, 1928, **197**, 418) found that a strain of *Aspergillus fumaricus* which had through many generations produced excellent yields of fumaric acid finally ceased to do so and gave only gluconic acid. Bernhauer (*Biochem. Z.*, 1928, **197**, 341) reports a similar failure to produce citric acid in a mould which previously readily formed this acid. 4000 C.c. of a 5% solution of glycerol containing the mineral salts of medium M (J., 1927, 204) were sterilised and inoculated with spores of the mould. Incubation was conducted at room temperature (20°) and samples were withdrawn periodically for examination. After 21 days the acidity was negligible. Citric acid was absent, since Denigès's test (J., 1927, 3044) was negative throughout the course of the fermentation. Samples taken at this time slightly reduced Fehling's solution. After 40 days the acidity was still very slight (25 c.c. of the culture required 1·0 c.c. of $N/10$-sodium hydroxide), but the medium now reduced Fehling's solution very strongly and gave a positive Molisch reaction for carbohydrates (*Z. anal. Chem.*, 1887, **26**, 258). After 46 days these reactions still persisted. The culture was then neutralised with calcium carbonate and concentrated to 1500 c.c. : some crystalline matter which separated on standing was chiefly inorganic. No salt of gluconic or saccharic acid was found in either the filtrate or the precipitate. The dark, somewhat viscous, supernatant liquid was then decanted and examined for glyceraldehyde.

350 C.c. were diluted with an equal volume of water, boiled for a few minutes with charcoal, cooled, filtered, and treated with a solution containing phenylhydrazine hydrochloride (4·4 g.), sodium acetate (8·8 g.), glacial acetic acid (52 c.c.), and water (35 c.c.). A precipitate slowly separated during 3 days. It was removed, washed with a little water, dried in a vacuum, and recrystallised three times from benzene, forming small yellow leaflets with a bronze-like sheen (0·5 g.), m. p. 131° (Found by micro-analysis : C, 66·9; H, 6·1; N, 21·2, 20·8. Calc. for $C_{15}H_{16}ON_4$: C, 67·2; H, 6·0; N, 20·9%).

The osazone of glyceraldehyde was prepared from dihydroxyacetone and phenylhydrazine acetate; m. p. and mixed m. p. 131—132°. We are indebted to Mr. H. B. Stent, M.Sc., for carrying out this experiment.

Action of A. niger *on Glycerol. Detection of Oxalic Acid.*—Further experiments were made with glycerol and a different strain of *A. niger* (also obtained through the courtesy of Professor C. Neuberg, and designated N2) which was shown to produce citric acid in 4 days when grown on 5% glucose solution with the inorganic salts of medium M.

A 5% glycerol solution with the salts of medium M in a large number of boiling-tubes was sterilised and inoculated as usual. On the 9th day the contents were filtered, united, made faintly alkaline with ammonia, and concentrated. Precipitation with lead acetate, followed by decomposition of the lead salt with hydrogen sulphide, finally gave only oxalic acid, m. p. (after recrystallisation) 100—101° alone or in admixture with an authentic specimen. The production of oxalic acid was observed in several separate experiments and was confirmed by titration with *N*/50-sodium hydroxide and with *N*/50-potassium permanganate, and by formation of the di-*p*-bromophenacyl ester. This melted and decomposed at 230°, alone or in admixture with a known specimen. The fermented culture did not reduce Fehling's solution as in the earlier experiments with a different strain. No citric acid was obtained, although many strains of *A. niger* convert glycerol into this acid, and neither tartronic acid nor saccharic acid could be detected.

Oxalic acid was also produced as sole recognisable product by the growth of the same strain of *A. niger* (N2) on a 5% arabinose solution containing the usual inorganic salts, the cultures being worked up on the 8th day. The formation of oxalic acid from glycerol has been observed by Elfving (*Physiol. Abs.*, 1919, **6**, 162), and by Amelung (*Z. physiol. Chem.*, 1927, **166**, 161), who used various strains of *A. niger*; Banning (*Centr. Bakt. Parasitenkunde*, II, 1902, **8**, 395—556) also obtained it, using *B. oxydans*, *B. ascendens*, *B. xylinum*, and *Termobacterium aceti*.

The strain N2, while capable of converting glucose into citric acid, failed to produce, or at least to accumulate, this acid from glycerol or arabinose in recognisable quantity. The cultures were frequently tested for citric acid. Its non-formation from glycerol and from arabinose are probably inter-related, since any conversion of arabinose into citric acid probably proceeds through a 3-carbon compound.

ADDENDUM [*January 2nd*, 1931].

After this paper had been submitted for publication the authors found that Corbellini and Gregorini (*Gazzetta*, 1930, **60**, 244) had already reached very similar conclusions. They regard fission of a hexose or pentose to a triose as a necessary preliminary to kojic acid synthesis by *Aspergilli*. It is suggested that this may be effected by a condensation of two oxidised trioses such as tartronaldehyde and glyceraldehyde in a manner analogous to the benzoin synthesis.

They regard the formation of kojic acid from fructose (from inulin) as incompatible with the persistence of the pyranose ring, since, if this be not ruptured, it should yield 3-hydroxy-2-hydroxymethyl-γ-pyrone and not kojic acid.

Sumiki (*Bull. Agr. Chem. Soc. Japan*, 1929, **5**, 10; *A.*, 1930, A, 1477) reports the formation of 2-hydroxymethylfuran-5-carboxylic acid from *A. glaucus* and glucose or sucrose. Details are not available, but this substance may theoretically arise from two molecules of dihydroxyacetone or glyceraldehyde by elimination of water, and oxidation. In the case of glucose it is doubtless unnecessary to postulate the formation of fructofuranose, although the conversion of this sugar into the furan acid can readily be formulated without ring fission.

In considering the production of furan derivatives by the agency of micro-organisms the work of Yamada (*Bull. Chem. Soc. Japan*, 1929, **4**, 31; *Bull. Agr. Chem. Soc. Japan*, 1928, **4**, 18; *Chemical Abstracts*, 1928, **22**, 2435) should be borne in mind.

Katagiri and Kitahara (*Bull. Agr. Chem. Soc. Japan*, 1929, **5**, 38), using *A. oryzæ*, were unable to obtain kojic acid from ethylene glycol and glyceric or saccharic acid. This is in agreement with some preliminary results of the present authors. Saccharic acid, unlike gluconic acid, cannot yield a triose by simple fission.

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