XXXII.—*The Mechanism of the Formation of Citric and Oxalic Acids from Sugars by* Aspergillus Niger. *Part I.*

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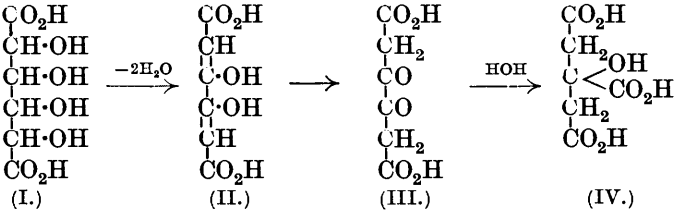
The production of citric and oxalic acids during the growth of *Aspergillus niger* and similar organisms on glucose or sucrose was first demonstrated by Wehmer (*Bot. Ztg.*, 1891, **49**, 233; *Bull. Soc. chim.*, 1893, **9**, 728; *Ber.*, 1924, **57**, 1659) and has formed the subject of various patent specifications (D.R.-PP. 72957, 91891). This transformation was further investigated by Currie (*J. Biol. Chem.*, 1917, **31**, 33) and by Butkewitsch (*Biochem. Z.*, 1923, **136**, 225; **142**, 195; 1924, **145**, 458).

Molliard (*Compt. rend.*, 1922, **174**, 881; 1924, **178**, 41, 161), Bernhauer (*Biochem. Z.*, 1924, **153**, 517; 1926, **172**, 296), and Butkewitsch (*ibid.*, 1924, **154**, 177) detected gluconic acid at an early stage of the fermentation, but were unable to convert this acid into citric acid under the conditions of their experiments. This has recently been accomplished by Wehmer (*Ber.*, 1925, **58**, 2616)

and by Schreyer (*ibid.*, 2647). Gluconic acid appears to be the first step in the conversion of sugar into citric acid, although some difference of opinion has existed upon this point (Molliard, *loc. cit.*; Falck and Kapur, *Ber.*, 1924, **57**, 920).

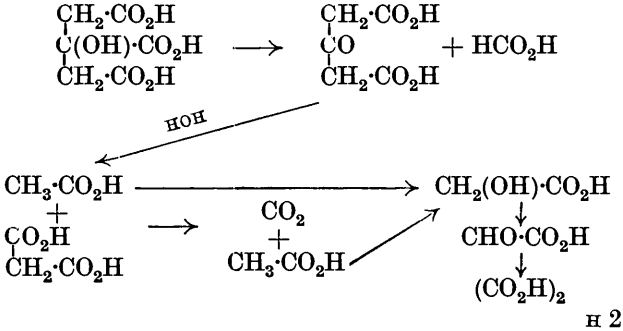
Franzen and Schmitt (*Ber.*, 1925, **58**, 222) have shown that βγ-diketoadipic acid (III) undergoes a " benzilic acid transformation " in presence of alkali-metal hydroxide, yielding citric acid (IV), and suggested that the biological formation of this acid proceeds in an analogous manner, the diketo-acid being supposed to arise from saccharic acid (I), although the production of this acid from sugar by mycological action had not been demonstrated :



(I.) (II.) (III.) (IV.)

Two of the present authors have commenced a comprehensive investigation of the production of organic acids by *A. niger*, and the present communication describes results which have been obtained from a study of the conversion of citric into oxalic acid by the mould. This stage of the oxalic fermentation has received little attention from previous workers with the exception of Raistrick and Clark (*Biochem. J.*, 1919, **13**, 329), who examined the action of *A. niger* on various possible intermediate products.

In an endeavour to discover the mechanism of this process, a hypothesis was formulated which, from purely chemical considerations, might be regarded as a possible explanation. A search has been made in various cultures for some of the intermediate products thus suggested, and conversely the behaviour of these compounds to the mould has been studied. The tentative explanation of the decomposition of citric acid is represented by the scheme :



Raistrick and Clark (*loc. cit.*) found scarcely any oxalic acid when the mould was grown upon malonic, glyoxylic, or glycollic acid. The possibility of a conversion of malonic acid into carbon dioxide by way of mesoxalic and glyoxylic acids is not excluded :

$$\begin{array}{l} CO_2H \\ | \\ CH_2 \\ | \\ CO_2H \end{array} \longrightarrow \begin{array}{l} CO_2H \\ | \\ CO \\ | \\ CO_2H \end{array} \longrightarrow \begin{array}{l} CO_2 \\ + \\ CHO \\ | \\ CO_2H \end{array} \longrightarrow H_2O + 2CO_2.$$

The culture medium used throughout the present research was that of Molliard (*Compt. rend.*, 1919, **168**, 360), in which the amount of inorganic salts is the minimum. The sugar was entirely replaced by citric or other organic acid. The *Aspergillus* strain was kindly supplied by Professor Carl Neuberg, of Berlin–Dahlem, and the solutions were incubated at 30—32°.

In three separate experiments, the formation of glyoxylic acid was observed after 15, 17, and 18 days, respectively. This was first detected by the reddish-violet colour produced with hydrochloric acid and naphtharesorcinol (1 : 3-dihydroxynaphthalene, Neuberg, *Biochem. Z.*, 1910, **24**, 436) and afterwards as the aminoguanidine derivative (Döbner and Gärtner, *Annalen*, 1901, **315**, 8; **317**, 157). Glyoxylic acid was also obtained by the action of *A. niger* on solutions containing only malonic acid and the inorganic salts of the medium. It was characterised as before.

It does not necessarily follow, although it is very probable, that glyoxylic acid is a stage in the citric ⟶ oxalic change. The quantities of the aminoguanidine derivative so far obtained are too small to warrant definite conclusions on this point. They were, however, precipitated in very dilute solution. Dakin (*J. Biol. Chem.*, 1906, **1**, 271) states that glyoxylic acid is frequently detected in cultures of moulds and bacteria, but gives no details. He also found traces of this acid in sterilised media containing glycine or creatinine. The possibility that the aminoguanidine derivative might arise from traces of glyoxylic acid in the glacial acetic acid employed by us has been excluded by blank experiments and the use of pure acetic acid.

Cultures of the mould on citric acid were next examined for the presence of malonic acid, and this was detected in several experiments by addition of benzenediazonium chloride and sodium acetate, hydrogen formazyl, $CH(N_2Ph){:}N{\cdot}NHPh$ (von Pechmann, *Ber.*, 1892, **25**, 3175), being obtained.

The production of this compound from glyoxylic acid phenylhydrazone and benzenediazonium chloride has also been observed (Busch and others, *Ber.*, 1925, **58**, 442; 1926, **59**, 1162). Its form-

ation in our experiments could not be due to the glyoxylic acid, since this cannot give a hydrazone with benzenediazonium chloride, and the reduction of the latter to phenylhydrazine appears most unlikely. No hydrogen formazyl was produced when the diazonium salt was added to mixtures of glyoxylic acid and the inorganic salts of the medium. Further, in no case during these experiments have we been able to detect the presence of glyoxylic acid before the formation of malonic acid.

The presence of malonic acid in the citric acid cultures has, however, been confirmed in a most satisfactory manner (*a*) by the method of Bougault (*J. Pharm. Chim.*, 1913, **8**, 289; *Ann. chim. anal.*, 1918, **23**, 154), cinnamylidenemalonic acid being isolated, and (*b*) by isolation of the free acid from its lead salt obtained from the evaporated culture.

Many attempts to detect acetonedicarboxylic acid in the citric acid cultures by the use of benzenediazonium chloride, with which it reacts readily (von Pechmann and Jenisch, *Ber.*, 1891, **24**, 3257; Henle and Schapp, *Ber.*, 1905, **38**, 1372), were unsuccessful. In one experiment, a reddish-purple coloration was produced on addition of ferric chloride.

Nevertheless, the transient formation of this acid seems indicated by the fact that on about the seventh or eighth day, *i.e.*, in the early stages of the fermentation, the citric acid–Molliard medium, although giving no ferric chloride reaction, contains acetone. The iodoform reaction, a red colour with alkaline sodium nitroprusside, and a white precipitate with acidified mercuric sulphate (Denigès's solution, *Ann. Chim. phys.*, 1899, **18**, 384) are obtained. Denigès (*loc. cit.*) gives the composition of the wet precipitate as $6HgSO_4,9HgO,4C_3H_6O$. This was decomposed with sodium iodide (Biilmann, *Ber.*, 1902, **35**, 2584), and the acetone characterised as the dibenzylidene derivative and as the *p*-nitrophenylhydrazone.

This appears to be the first recorded instance of the production of acetone by a mould, the well-known fermentation processes for its manufacture (Fernbach and Strange, E.P. 1912, 21073; Gill, *J. Soc. Chem. Ind.*, 1919, **38**, 273T; Speakman, *ibid.*, 155T; *J. Biol. Chem.*, 1920, **41**, 319) depending on the use of bacteria. The authors hope shortly to describe the results of some large-scale experiments. Owing to our failure to detect acetonedicarboxylic acid, it appeared possible that the acetone might arise from acetic acid. It should be mentioned, however, that, apart from an unconfirmed observation recorded by Heinze (*Annales mycologici*, 1903, **1**, 350), acetic acid has not been found in *Aspergillus niger* cultures on sugar or citric acid. Reilly, Hickinbottom, Henley, and Thaysen (*Biochem. J.*, 1920, **14**, 229) and Bakonyi (*Biochem. Z.*, 1926, **169**,

125) have shown that addition of acetic acid to the cultures increases the yield of acetone in the technical fermentation processes.

When grown on a solution of calcium acetate and the usual inorganic salts, *A. niger* gave no acetone, but formed glyoxylic, glycollic, and oxalic acids. The last acid was found by Raistrick and Clark (*loc. cit.*), the others have not previously been identified in this connexion. Dr. P. W. Clutterbuck has suggested to us that on analogy with oxidation processes in the animal organism, acetic acid is probably directly oxidised to glyoxylic acid, which, by dismutation, *i.e.*, the Cannizzaro reaction (Neuberg, *Ber.*, 1922, **55**, 3628), yields oxalic and glycollic acids.

The rapid disappearance of acetone from the citric acid cultures suggested a study of the action of the mould on this substance, but the suitable conditions for growth have not yet been ascertained.

The importance of saccharic acid in the sugar–citric acid fermentation has already been mentioned. Butkewitsch (*Biochem. Z.*, 1923, **142**, 205) was, however, unable to convert it into citric acid by *A. niger*, but this does not necessarily preclude its participation in the process. The conditions under which gluconic acid is converted into citric acid were only established after many failures (see p. 201) and it is possible that Butkewitsch was not working under the optimum conditions.

The authors find that when the mould has been grown on dipotassium saccharate for 4 to 5 weeks the culture gives a precipitate with mercuric sulphate and acidified potassium permanganate—Denigès's test—thus rendering the presence of citric acid probable. When the culture was distilled with potassium permanganate and dilute sulphuric acid, acetone was easily recognised in the distillate. Malic acid and ketonic acids give precipitates with mercuric sulphate and acidified potassium permanganate, but citric, acetoacetic, or acetonedicarboxylic acid would yield acetone on oxidation. The last two acids were shown to be absent. Aconitic acid gave no acetone on similar oxidation. Further work is necessary, however, before the production of citric acid from saccharic acid may be regarded as definitely established.

Experimental.

The medium employed throughout this research contained the following quantities (in grams) of hydrated salts per litre of water : ammonium nitrate, 0·356; potassium dihydrogen phosphate, 0·08; magnesium sulphate, 0·08; ferrous sulphate, 0·0046; zinc sulphate, 0·0046. This medium is hereafter designated solution M.

The fermentations were conducted in large flasks fitted with tubes for the withdrawal of samples under aseptic conditions. All

the mixtures were sterilised prior to inoculation, and great care was taken to prevent possible contamination by adventitious organisms. The cultures used for inoculation were all derived from Professor Neuberg's original strain, and when the work had been in progress for seven months, samples from one of the large reaction flasks were kindly examined by Miss M. Rhodes of the Lister Institute and certified as pure. All results were checked by control experiments performed simultaneously under identical conditions.

Fermentation of Citric Acid. Detection of Glyoxylic Acid.—Citric acid (50 g.) in 2500 c.c. of solution M was inoculated and incubated at 30°, and samples were tested frequently for glyoxylic acid. On the seventeenth day, a strong reddish-violet colour was obtained with naphtharesorcinol and hydrochloric acid, whereupon 2000 c.c. of the solution were withdrawn, filtered, and treated with 3 g. of aminoguanidine acetate in 100 c.c. of dilute acetic acid (1 : 1), giving after 48 hours a pale yellow solid (0·05 g.). On recrystallisation, this had m. p. 154° and melted at the same temperature in admixture with an authentic specimen of the aminoguanidine derivative of glyoxylic acid (Found by microanalysis: N, 37·4. Calc.: N, 37·9%). Döbner and Gärtner (*loc. cit.*) give the m. p. of this compound as 161°. Dakin (*J. Biol. Chem.*, 1906, **1**, 271) states that the compound does not melt very sharply, but after drying at 100° it has m. p. about 155°. The present authors find that the m. p. depends to some extent on the rate of heating.

Fermentation of Malonic Acid. Detection of Glyoxylic Acid.—Malonic acid (25 g.) in 2500 c.c. of solution M was inoculated and incubated at 30°. On the seventeenth day, a positive naphtharesorcinol test was obtained and 1000 c.c. of the filtered solution were then treated with 2 g. of aminoguanidine acetate and 50 c.c. of glacial acetic acid. After 48 hours, the deposit was separated (0·08 g.). It melted at 152·5°, and at 154·5° after recrystallisation from hot water. A mixture with a synthetic specimen (m. p. 155°) melted at 155° (Found: N, 37·5, 37·5%).

Fermentation of Citric Acid. Detection of Malonic Acid.—Citric acid (30 g.) in 2000 c.c. of solution M was inoculated and incubated at 30° and samples were tested daily with benzenediazonium chloride. On the eleventh day, a red precipitate was obtained, whereupon 600 c.c. of the fermenting liquid were filtered, cooled to 0°, and mixed with a well-cooled solution of benzenediazonium chloride (from 3 g. of aniline) in presence of excess of sodium acetate. After 24 hours, the red solid (2·9 g.) was washed with cold water, dried in a vacuum, and repeatedly crystallised alternately from aqueous methyl alcohol and benzene–light petroleum; it then

melted at 118—120°, alone or mixed with an authentic sample of hydrogen formazyl, m. p. 119°. It gave the characteristic dark green coloration with sulphuric acid (Bülow reaction) (Found : C, 69·7, 69·9; H, 5·5, 5·5; N, 25·2, 25·45. Calc. : C, 69·6; H, 5·4; N, 25·0%).

In another experiment, 2000 c.c. of the culture gave, on the tenth day, 1·3 g. of crude hydrogen formazyl.

Fermentation of Citric Acid. Isolation of Malonic Acid.—Citric acid (30 g.) in 2000 c.c. of solution M was inoculated and incubated as before. Malonic acid was detected on the tenth day, the whole of the solution (1900 c.c.) was filtered and concentrated under diminished pressure to 400 c.c., pure sodium carbonate added, and the slight excess neutralised with acetic acid. On evaporation, 43 g. remained, and 24 g. of this were well stirred with 60% alcohol in successive quantities of 25, 15, 15, and 10 c.c., and the extracts were evaporated. The residue (3·4 g.) was free from oxalate and citrate. When it was warmed with acetic anhydride and diluted with glacial acetic acid, the brown solution showed a strong greenish-red fluorescence—a reaction of malonic acid (Kleeman, *Ber.*, 1886, **19**, 2030). 1·5 G. were heated with 2 c.c. of glacial acetic acid and 30 drops of cinnamaldehyde in a sealed tube for 11 hours at 100°. Water (20 c.c.) and sodium carbonate were added, unchanged aldehyde was extracted with ether, and the alkaline solution was acidified, giving 0·6 g. of cinnamylidenemalonic acid. After recrystallisation, this melted at 207° and did not depress the m. p. (207°) of an authentic specimen. On exposure to light, it was superficially converted into a white solid (0·0214 and 0·0388 required 7·8 and 14·2 c.c. of *N*/40-NaOH. Calc., 7·85, 14·25). The alcoholic extract from the remainder (19 g.) of the evaporated culture was precipitated with aqueous lead acetate, and the lead salt decomposed by hydrogen sulphide. The white residue obtained on evaporation of the final filtrate gave, on extraction with ether, malonic acid (m. p. and mixed m. p. 132—133°, decomposing a few degrees above this temperature) (0·1002 and 0·1021 required 38·6 and 39·3 c.c. of *N*/20-NaOH. Calc., 38·55, 39·30).

Fermentation of Citric Acid. Detection of Acetone.—Citric acid (45 g.) in 2500 c.c. of solution M was fermented as usual and samples were tested daily for acetone by the iodoform reaction. This was given very faintly on the sixth day and strongly on the seventh day; 2200 c.c. of the solution were then filtered and neutralised with sodium hydroxide, and distilled. This distillate (800 c.c.) was mixed with 2000 c.c. of Denigès's reagent (50 g. of mercuric oxide in 1000 c.c. of water and 200 c.c. of sulphuric acid), and the whole heated under reflux at 100° for 40 minutes. The white precipitate was separated,

washed, dried (90 g.), and distilled, in portions of 15 g., with sodium iodide (30 g.) in 120 c.c. of water; 15 c.c. of distillate were collected in each case. A portion (55 c.c.) of the united distillates was mixed with acetone-free alcohol (60 c.c.), benzaldehyde (3 c.c.), and 10% sodium hydroxide solution (3 c.c.). The condensation product separated over-night and, after crystallisation from light petroleum, melted at 113° alone or in admixture with dibenzylideneacetone (Found: C, 86·6; H, 6·04. Calc.: C, 87·2; H, 6·0%).

The remainder of the distillate (35 c.c.) from the mercury compound was treated with *p*-nitrophenylhydrazine acetate; the product, after crystallisation from light petroleum, was identified as acetone-*p*-nitrophenylhydrazone by its m. p. (148·5°) and by the m. p., 149°, of a mixture with an authentic specimen.

Fermentation of Calcium Acetate. Detection of Glyoxylic Acid.—Calcium acetate (20 g.) in solution M (2000 c.c.) was inoculated and incubated at 32°. Glyoxylic acid made its appearance on the fifteenth day. 700 C.c. of the medium were then removed and filtered, made faintly acid to litmus with hydrochloric acid, neutralised with potassium hydroxide, and treated with 3·5 g. of aminoguanidine acetate in 50 c.c. of glacial acetic acid. After 5 days, 0·42 g. of crude condensation product was obtained. Two crystallisations from much hot water yielded 0·25 g. of m. p. 154° and mixed m. p. 154—155°.

Fermentation of Calcium Acetate. Isolation of Calcium Oxalate.—The remainder of the culture solution gradually deposited a white solid on the sides of the flask, and on the eighteenth day the mycelium was collected on a filter. The adhering solid was removed from the glass by warm 10% hydrochloric acid, which was then used for extracting the mycelium. The united extracts and washings (150 c.c.) were treated with a slight excess of ammonia, boiled for a few minutes, cooled, treated with a slight excess of 10% calcium chloride solution, and again boiled. The precipitate was well washed with much hot water and dried (Found: CaO, 43·9. Calc.: CaO, 43·75%).

Fermentation of Calcium Acetate. Isolation of Glycollic Acid.—Calcium acetate (60 g.) in 4000 c.c. of solution M showed, on the tenth day after inoculation, the presence of glycollic acid. Sulphuric acid, acetic acid, and 5% alcoholic *p*-cresol gave a dark green colour, whilst with sulphuric acid and alcoholic codeine (5%) a characteristic strong pink colour was obtained (Denigès, *Ann. Chim. phys.*, 1909, **18**, 178). The whole of the culture was filtered, treated with 60 g. of oxalic acid, this quantity being in large excess of that required to precipitate the calcium, and the deposit was separated and well washed. The filtrate, containing acetic acid,

excess of oxalic acid, and presumably glycollic acid, was treated with lead carbonate. The filtrate from the lead oxalate, containing the soluble lead salts, was treated with hydrogen sulphide, filtered, and evaporated to a syrup in a vacuum. This yielded 5·7 g. of white, transparent crystals, m. p. 68°, which were free from oxalic acid. After two crystallisations from dry ether–light petroleum, these melted at 79—80°, were indistinguishable in appearance from glycollic acid, and did not depress the m. p. (79—80°) of an authentic specimen (0·0508 required 13·40 c.c. of N/20-NaOH. Calc., 13·35 c.c. Found : C, 31·7 ; H, 5·2. Calc. : C, 31·6 ; H, 5·3%).

Fermentation of Potassium Saccharate. Detection of Citric Acid.— Potassium hydrogen saccharate (10 g.) was exactly neutralised by dilute aqueous potassium hydroxide, the usual inorganic salts were added in the proportions required by medium M, and the solution was diluted to 500 c.c., inoculated, and incubated at 31°. After being kept for 28 days at this temperature and for about a week at 20°, the solution gave a white precipitate with acidified mercuric sulphate solution and potassium permanganate, indicating the probable presence of citric acid.

200 C.c. of the culture were then acidified with sulphuric acid, slowly treated with aqueous potassium permanganate to oxidise any citric acid to acetonedicarboxylic acid, and slowly distilled. The distillate (50 c.c.), when heated with 200 c.c. of Denigès's mercuric sulphate solution (see p. 206), gave 3·8 g. of a white solid ; this was distilled with 30 c.c. of water and 8 g. of sodium iodide. The distillate (5 c.c.), treated with 5 c.c. of alcohol, 5 drops of benzaldehyde, and 5 drops of 10% sodium hydroxide solution, gave dibenzylideneacetone, m. p. and mixed m. p. 113° (Found : C, 86·5 ; H, 6·1. Calc. : C, 87·2 ; H, 6·0%).

Potassium saccharate gave no acetone on similar oxidation. Distillation of the fermented medium gave no acetone, thus excluding the presence of acetoacetic and acetonedicarboxylic acids. Aconitic acid (5 g.) in 120 c.c. of dilute sulphuric acid (1 : 5) was oxidised with 200 c.c. of 3% potassium permanganate solution ; the distillate (20 c.c.) gave only a minute trace of a precipitate when warmed under reflux with acidified mercuric sulphate.

The acetone obtained from the saccharate culture would therefore appear to have arisen from citric acid.

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