CCCCIV.—*The Mechanism of the Formation of Citric and Oxalic Acids from Sugars by* Aspergillus niger. *Part II.*

By THOMAS KENNEDY WALKER, VIRA SUBRAMANIAM, and FREDERICK CHALLENGER.

THE production of citric and oxalic acids when *A. niger* is grown upon solutions containing glucose and traces of inorganic salts has been observed by many workers. In a previous communication (this vol., p. 200), it was shown that acetone, malonic acid, and glyoxylic acid can be detected in cultures of the mould on citric acid. The formation of glycollic, glyoxylic, and oxalic acids by the action of *A. niger* on calcium acetate was also established, and the suggestion was brought forward that the change glucose —→ citric acid —→ oxalic acid takes place in stages thus: glucose —→ gluconic acid —→ saccharic acid —→ βγ-diketoadipic acid —→ citric acid —→ acetonedicarboxylic acid —→ malonic and acetic acids —→ acetic acid (2 mols.) —→ glycollic acid —→ glyoxylic acid —→ oxalic acid.

Molliard, Butkewitsch, and Bernhauer have shown that gluconic acid is readily produced in cultures of *A. niger* on glucose, while both Wehmer and Schreyer have converted gluconic acid into citric acid by the further action of this or a similar mould. The present authors (*loc. cit.*) brought forward strong evidence of the formation of citric acid when *A. niger* is grown on solutions of dipotassium saccharate. It was detected by the formation of the basic mercuric sulphate compound of mercuric acetonedicarboxylate on treatment of the culture with acidified mercuric sulphate and potassium permanganate (Denigès' test, *Ann. Chim. Phys.*, 1899, **18**, 414). The citric acid was characterised as dibenzylideneacetone.

The statement of Broeksmit (*Pharm. Weekblad*, 1917, **54**, 687)

that oxidation of malic acid by potassium permanganate gives rise to acetone, detected solely by formation of iodoform with iodine and ammonium iodide, would, if correct, render identification of citric acid by this method uncertain. It is difficult to see how malic acid could yield acetone on oxidation, and on repeating Broeksmit's test a strong odour of acetaldehyde was noticed. Acetaldehyde readily gives iodoform with iodine in ammonium iodide, such a solution being no specific test for acetone.

In the case of the cultures on dipotassium saccharate it was not possible to separate the citrate as calcium salt from the large excess of saccharate (see p. 3049). Evidence of the production of acetonedicarboxylic acid (ferric chloride test) on oxidation of the contents of the culture medium was, however, obtained. When the mould is grown on potassium hydrogen saccharate solution, potassium citrate is formed in considerable amount. Further evidence of the importance of saccharic acid in the mycological production of citric acid is afforded by its isolation in the form of the potassium hydrogen salt from cultures of *A. niger* on glucose. Apart from the observation of Grüss (*Jahrbuch. wiss. Bot.*, 1926, **66**, 155, 171, 177) that saccharic acid is formed by the action of the nectar yeasts *Anthomyces Reukaufii* and *Amphiernia rubra* on glucose, this would appear to be the only recorded instance of the production of this acid by micro-organisms. Grüss identified it microscopically as the cæsium hydrogen salt.

In cultures of the nectar yeast *Microanthomyces septentrionalis* on glucose Grüss found gluconic and oxalic acids (*Woch. Brauerei*, 1927, **44**, 233).

In view of the occurrence of gluconic acid in *A. niger* cultures, and of the results just mentioned, it was to be expected that gluconic acid would yield saccharic acid by the action of the mould. This has been shown to be the case, since growth of *A. niger* on calcium gluconate solutions gives rise to calcium saccharate and some citrate. A different type of reaction was observed by Boutroux (*Compt. rend.*, 1886, **102**, 924) and by Bertrand (*Ann. Chim. Phys.*, 1904, **3**, 279), who isolated δ-ketogluconic acid from cultures of a micrococcus and of the sorbose bacteria on gluconic acid or its calcium salt. The same result was obtained by Kluyver and de Leuuw (*Tijdschrift voor vergelykende geneeskunde*, 1924, No. 10) with *Acetobacter suboxydans* and calcium gluconate.

Seekamp (*Annalen*, 1893, **278**, 374) describes the photochemical formation of acetone from aqueous citric acid in presence of uranium oxide. In Part I of this research the formation of acetone in cultures of *A. niger* on citric acid was demonstrated and ascribed to the decarboxylation of acetonedicarboxylic acid, although proof

of its presence was lacking. By employing ammonium citrate instead of the free acid, acetonedicarboxylic acid has now been detected by the colour test with ferric chloride, and by the formation of the diphenylhydrazone of mesoxaldialdehyde on treatment of the culture with benzenediazonium chloride (von Pechmann and Jenisch, *Ber.*, 1891, **24**, 3257).

The only reference in the literature to the possible formation of this acid by a micro-organism is afforded by the observation of von Lippmann (*Ber.*, 1893, **26**, 3058; 1908, **41**, 3981) that a specimen of the calcium oxide compound of sucrose, after long keeping in a closed vessel, was converted into calcium acetonedicarboxylate. A five years old preparation had completely decomposed, giving calcium formate, acetate, oxalate and carbonate. No bacteriological examination of the products was made, but Lafar ("Handbuch der techn. Mykologie," Jena, 1908, II, 487) suggests that micro-organisms were responsible. Von Lippmann (*Ber.*, 1920, **53**, 2070) also describes the isolation of calcium malonate and calcium succinate from a solution of pure sucrose containing lime water which had become infected with bacteria, moulds, and yeasts. These observations are of interest, since malonic and acetonedicarboxylic acids have now been detected in pure cultures of *A. niger* on citric acid or a citrate. The close relations of saccharic, citric and acetonedicarboxylic acids and acetone disclosed in this research may have some bearing on the work of Wirth (*Biochem. Z.*, 1911, **33**, 49), who found that addition of gluconic, saccharic or mucic acid to blood supplied to a perfused dog's liver can lead to a decided formation of acetoacetic acid.

Oxalic acid is produced by the growth of the mould on ammonium acetonedicarboxylate, presumably by way of malonic and acetic acids, though its formation by the oxidation of "nascent" acetone produced by hydrolysis and decarboxylation is not excluded.

The tentative explanation of the mycological decomposition of citric acid suggested on p. 3044 requires the production of two molecules of acetic acid. This has not previously been detected with certainty in cultures of *A. niger* on sugars or citric acid, apart from the work of Heinze (*Ann. mycologici*, 1903, **1**, 350). This author refers to its formation in old cultures on glucose, but gives no experimental details. Acetic acid, however, may readily be isolated from ten-day cultures on citric acid, converted into acetone by dry distillation of its calcium salt, and characterised as the *p*-nitrophenylhydrazone. *B. lactis aerogenes* is stated to give rise to two equivalents of acetic acid from one of citrate (Bosworth and Prucha, *J. Biol. Chem.*, 1911, **8**, 479).

In Part I the detection of glyoxylic acid in cultures of *A. niger* on

malonic acid was described. The quantity was, however, very small, judged from the amount of the aminoguanidine derivative of glyoxylic acid which was isolated. This observation has now been confirmed, the aldehydo-acid being precipitated as the di-xanthylhydrazone (Fosse and Hieulle, *Compt. rend.*, 1925, **181**, 286), but again in very small quantity. In view of the labile nature of glyoxylic acid and its ready conversion into oxalic acid, this is perhaps not surprising. The isolation of glycollic acid from cultures of *A. niger* on calcium acetate is described in Part I. When the mould is grown upon calcium glycollate and ammonium glycollate the formation of glyoxylic and oxalic acids, respectively, may be detected, thus affording further evidence that the acetic —→ oxalic change proceeds as indicated in the scheme on p. 3044.

In Part I (this vol., 200) attention was directed to the suggestion of Franzen and Schmitt (*Ber.*, 1925, **58**, 222) that citric acid in plants is formed from saccharic acid by loss of $2H_2O$ giving rise to $CO_2H{\cdot}CH{:}C(OH){\cdot}C(OH){:}CH{\cdot}CO_2H$, which, in its keto-form, undergoes a " benzilic acid transformation," giving citric acid. The conversion of saccharic into citric acid by *A. niger* doubtless follows the same course, and is being investigated from this point of view by the authors.

Such an elimination of water from saccharic acid may possibly proceed in living organisms in a different but equally symmetrical manner, giving rise to $CO_2H{\cdot}C(OH){:}CH{\cdot}CH{:}C(OH){\cdot}CO_2H$, which by loss of carbon dioxide would yield succindialdehyde. In presence of ammonia this would form the pyrrole ring, so frequently occurring in nature. The observations of von Lippmann and of Ciskiewicz (*Ber.*, 1892, **25**, 3218; 1893, **26**, 3063) that solutions of ammonium mucate and saccharate, after spontaneous infection by atmospheric organisms, contain pyrrole are therefore of considerable interest and will be further investigated.

In view of the authors' demonstration of the biochemical significance of saccharic and acetonedicarboxylic acids, the ingredients of Robinson's synthesis of tropinone (J., 1917, **111**, 762) are seen to bear a simple relation to glucose, and the probability that similar syntheses of nitrogen compounds occur in nature (Robinson, *ibid.*, p. 880) appears to be enhanced.

Experimental.

Fermentation of Glucose. Detection of Saccharic Acid.—A medium containing glucose (300 g.), ammonium nitrate (3·2 g.), potassium dihydrogen phosphate (0·6 g.), and water (2000 c.c.) was sterilised and inoculated with a young culture of *A. niger* grown on the same

medium. A thick growth formed in 3 days at 31°. No saccharic, citric, or oxalic acid could be detected on the fourth or fifth day. On the sixth, while citric and oxalic acids were still absent, neutralisation of 150 c.c. of the culture with potassium carbonate, acidification with acetic acid, evaporation, and extraction with hot alcohol to remove glucose left a residue of about 0·2 g. of a salt giving the reactions of potassium hydrogen saccharate. Next day, traces of citric acid were detected by Denigès' test, but no oxalic acid. The whole of the solution was then neutralised and treated as before, giving 1·65 g. of a salt insoluble in alcohol, but containing much citrate. The conversion of saccharic into citric acid appears, therefore, to be very rapid after a certain phase in the development of the mould is reached. By a modification of the conditions, however, this difficulty was evaded. Twelve 300 c.c. flasks (A—L), each containing 150 c.c. of the medium, were sterilised. Six of these (A—F) were inoculated on the same day, and the remainder (G—L) after 24 hours. All were incubated at 30—32°. Growth occurred 3 days after inoculation.

Flask A was examined for saccharic, citric, and oxalic acids on the third day, flask B on the fourth day, C on the fifth, and so on. Traces of an insoluble potassium salt were isolated from C, 0·3 g. from D, and 0·8 g. from E, whilst only in E was any citric or oxalic acid to be found. After this satisfactory indication, the contents of flasks G—L were united on the sixth day after inoculation, filtered, treated with potassium carbonate and acetic acid as before, and evaporated. Repeated extraction of the residual syrup with boiling alcohol left a residue of 3·6 g. of a potassium salt. On recrystallisation from a very little hot water, this gave pure potassium hydrogen saccharate (Found: K, 15·7. Calc.: K, 15·7%. 0·0502 and 0·0461 required 20·2 and 18·5 c.c. of N/100-NaOH. Calc., 20·23, 18·57). A portion was converted into the sparingly soluble thallous hydrogen salt, and the thallium determined as thallous iodide (Found: Tl, 49·3. Calc.: Tl, 49·4%).

Fermentation of Calcium Gluconate. Detection of Saccharic Acid.—The calcium gluconate was prepared by Bert's modification of Blanchetière's method (*Bull. Soc. chim.*, 1923, **33**, 345, 733), and its purity checked by determinations of calcium (Found: Ca, 9·3. Calc.: Ca, 9·3%) and the specific rotation, $[\alpha]_D$ + 7·7°. The medium contained water (1000 c.c.), calcium gluconate (20 g.), and the usual mineral salts in the proportions contained in Molliard's solution M (this vol., pp. 202—204). Inoculation with mycelium from a 200 c.c. culture gave growths on the fourth day at 31°. On the next day the solution was filtered, concentrated to 800 c.c. under diminished pressure, and boiled; a precipitate containing calcium

citrate (1·2 g.) then separated. Further boiling produced no deposit and the filtrate was concentrated in a vacuum to 350 c.c. On cooling, a white solid (3·7 g.) separated which was free from citrate. It was recrystallised twice with difficulty from much hot water (calcium saccharate is sparingly soluble) and dried ove r sulphuric acid (Found : Ca, 16·0. Calc. : Ca, 16·1%).

0·7 G. was boiled with 0·5 g. of potassium carbonate in 10 c.c. of water, and the filtrate acidified with acetic acid. The resulting potassium hydrogen saccharate was recrystallised twice from hot water (0·0623 and 0·0712 required 25·0 and 28·7 c.c. of $N/100$-NaOH. Calc., 25·13, 28·8), and a portion of it then converted into thallous hydrogen saccharate (Found : Tl, 49·3. Calc. : Tl, 49·4%).

Fermentation of Dipotassium Saccharate. Detection of Citric Acid.—A solution containing 40 g. of the normal potassium salt in 2000 c.c. of solution M was sterilised and inoculated. After 28 days at 31° the presence of citric acid was detected by Denigès' test. 5 G. of the evaporated culture were oxidised by hot 2% potassium permanganate solution in presence of acidified mercuric sulphate solution, giving a white solid. A few grams of this were washed, suspended in water, and treated with hydrogen sulphide. The filtrate from the mercury sulphide was repeatedly extracted with ether, and the wet solvent evaporated under diminished pressure. The slight aqueous residue gave a reddish-purple coloration with ferric chloride, similar to that produced by acetonedicarboxylic acid, thus confirming the presence of a citrate (Denigès, *Ann. Chim. Phys.*, 1899, **18**, 408, 414). This reaction was not due to the presence of the keto-acid in the original culture, as this would have been decomposed during evaporation. Only a trace of a precipitate was obtained with mercuric sulphate before oxidation with permanganate, and this was removed.

Attempts were then made to precipitate calcium citrate from the culture, but the presence of the saccharate had a disturbing effect. Normal calcium saccharate is very sparingly soluble in water, but soluble in excess of calcium chloride. This solution gives no precipitate on boiling, even on evaporation to a small bulk. When citrate is present, however, the clear solution gives a precipitate when boiled which contains saccharate and citrate, no separation being effected. When a mixture of dipotassium saccharate and potassium citrate is treated in the cold with calcium chloride approximately equivalent to the saccharate, the resulting precipitate contains both saccharate and citrate. The filtrate on boiling deposits calcium saccharate.

When insufficient calcium chloride is added the initial precipitate of calcium saccharate redissolves. On boiling the clear solution,

successive deposits are obtained which contain saccharate, citrate being present in some cases.

In other experiments, the saccharic acid was partly removed as the potassium hydrogen salt by addition of acetic acid to the concentrated culture, and the filtrate was neutralised with potassium carbonate, treated with calcium chloride, and boiled. Calcium citrate was not precipitated and it was then found that excess of sodium acetate greatly retards the precipitation of this salt from hot solutions. The effect of salts of other organic acids on the precipitation of calcium citrate appears to have received very little attention.

Fermentation of Potassium Hydrogen Saccharate. Detection and Isolation of Citric Acid.—The mould grew more readily on solutions of the potassium hydrogen salt than on those of the normal salt. A mixture of solution M (2000 c.c.) and potassium hydrogen saccharate (40 g.) was sterilised, inoculated and incubated at 31°. On the fourteenth day citric acid was detected by Denigès' reagent (*loc. cit.*, p. 384), whereupon the remainder of the solution (1800 c.c.) was filtered, concentrated under diminished pressure, and evaporated, leaving 36 g. of moist residue. A duplicate experiment yielded 34 g. The residues were united and heated under reflux for 1 hour with 400 c.c. of 65% alcohol (in which potassium hydrogen saccharate is insoluble, but potassium citrate is easily soluble), and the solution was filtered and evaporated. The residue was converted into the lead salt, which was decomposed by hydrogen sulphide, and the filtrate from the lead sulphide was decolorised with charcoal and evaporated (3·6 g.). After three crystallisations from dry ether–light petroleum, its m. p., alone or in admixture with anhydrous citric acid (m. p. 153°), was 153·5° (0·1212 and 0·0560 required 37·7 and 17·5 c.c. of *N*/20-NaOH. Calc., 37·8, 17·5. Found: C, 37·5; H, 4·7. Calc.: C, 37·5; H, 4·2%). A portion was neutralised with potassium hydroxide and treated with thallous nitrate, and the resulting thallium salt was twice crystallised from hot water and analysed (Found: Tl, 76·4, 76·6. Calc.: Tl, 76·4%).

Fermentation of Ammonium Citrate. Detection of Acetonedicarboxylic Acid.—A medium containing ammonium citrate (20 g.) and citric acid (4 g.) in solution M (2000 c.c.) was inoculated from test-tube cultures, growth occurring on the third day at 31°. On the third, fourth, and fifth days 50 c.c. of the culture were removed, filtered, and treated with 3 g. of sodium acetate, and a benzenediazonium chloride solution (prepared from 0·3 g. of aniline) was added at 0°. On the fifth day, a red coloration was observed after 3 hours. A further 10 c.c. of the culture were shaken with 50 c.c. of cold Denigès' mercuric sulphate solution, a white precipitate being

obtained. (Acetone in dilute solution gives a precipitate only on warming.) These two tests were regarded as indications of the probable presence of acetonedicarboxylic acid.

At this stage 1800 c.c. of the filtered culture were treated with corresponding proportions of benzenediazonium chloride solution and sodium acetate at 0°. A red precipitate formed in 8 hours. This was separated, washed with ice-water, dried (1·3 g.), washed with light petroleum, and recrystallised from alcohol and then from ethyl acetate; it then melted at 175°, and at 175—176° in admixture with the diphenylhydrazone of mesoxaldialdehyde (m. p. 175—176°) prepared from acetonedicarboxylic acid and benzenediazonium chloride (Found: N, 21·3. Calc.: N, 21·1%). Both specimens gave a deep violet coloration with sulphuric acid (Bülow reaction).

Fermentation of Citric Acid. Detection of Acetic Acid.—The medium employed contained 3·5 g. of citric acid in 4000 c.c. of solution M. After sterilisation it was inoculated with 700 c.c. of a three days' culture of *A. niger* on the same medium. Growth was observed on the third day at 31°. 50 C.c. of the culture were then withdrawn on successive days and carefully distilled in steam and the volatile acids were determined. On the third and fourth days, 0·4 c.c. of *N*/20-sodium hydroxide was required, on the seventh 3·8 c.c., ninth 10·2, tenth 11·8, eleventh 11·9, twelfth 11·0. The whole of the culture was then filtered, neutralised with sodium carbonate, concentrated in a vacuum to 100 c.c., acidified with phosphoric acid, and carefully distilled in steam till the distillate was no longer acid (200 c.c.). This was neutralised with sodium carbonate and evaporated and the residue (2·8 g.) was extracted with 15 c.c. of 95% alcohol, in which sodium acetate is soluble. The residue (0·4 g.) contained no formate. The alcoholic extract was evaporated; the residue, which appeared to be sodium acetate, was converted into the silver salt, which was crystallised twice from hot water and dried (Found: Ag, 64·4, 64·8. Calc.: Ag, 64·7%). A further portion of the sodium salt was distilled with phosphoric acid, the distillate neutralised with calcium carbonate, and the dry calcium salt distilled, giving a few drops of liquid, which were warmed with 30 c.c. of Denigès' mercuric sulphate solution (Part I, p. 206). The resulting precipitate (2·8 g.) was separated and decomposed by distillation with 5 g. of sodium iodide and 10 c.c. of water, and the distillate was collected in a solution of *p*-nitrophenylhydrazine acetate (Part I, p. 207). Long, yellow needles separated in 3 hours which, recrystallised from alcohol, melted at 148·5° and did not depress the m. p. (148·5°) of an authentic specimen of acetone-*p*-nitrophenylhydrazone.

Malonic acid also is a product of the fermentation of citric acid

by *A. niger*. In order to exclude the possibility that the acetic acid arose by the decarboxylation of this acid (normally occurring at 140°) a mixture of 100 c.c. of solution M, 1 g. of citric acid, and 1 g. of malonic acid was distilled in steam. The distillate was not acid.

Fermentation of Ammonium Acetonedicarboxylate. Detection of Oxalic Acid.—The medium contained the ammonium salt (20 g.) in solution M (2000 c.c.). It was prepared and sterilised as in the case of the ammonium glycollate solution, inoculated, and incubated at 32°. Oxalate was found on the eighteenth day by removing 20 c.c., boiling with dilute hydrochloric acid to decompose the keto-acid (which gives a sparingly soluble calcium salt), neutralising the solution with ammonia, and adding calcium chloride.

The main bulk was then treated in a similar manner, complete decomposition of the keto-acid being indicated by the absence of a precipitate with Denigès' solution, which detects 1 part of acetonedicarboxylic acid in 5000 parts of water. The calcium oxalate was dissolved in hydrochloric acid, reprecipitated, and dried (1·6 g.) (Found: Ca, 31·2. Calc.: Ca, 31·25%. 0·1436 required 44·9 c.c. of $N/20$-$KMnO_4$. Calc., 44·87).

Fermentation of Malonic Acid. Detection of Glyoxylic Acid.—The medium contained 20 g. of malonic acid in 2000 c.c. of solution M. It was inoculated with mycelium from a smaller culture on the same medium and incubated at 31°. On the seventeenth day a positive reaction for glyoxylic acid was obtained with naphtharesorcinol. 1000 C.c. of the filtered culture were treated with 1 c.c. of 50% hydrazine hydrate and then with 4 g. of xanthhydrol dissolved in 1000 c.c. of glacial acetic acid. After addition of water and shaking, the precipitate was separated, and centrifuged with a slight excess of alcoholic N-sodium hydroxide; by this means any trixanthylhydrazine remains insoluble. The clear liquid was decanted and treated with acetic acid, and the pale pink crystalline precipitate was recrystallised twice from chloroform–light petroleum (0·5 g.). After drying in a vacuum over calcium chloride, it melted at 146·5°. In admixture with an authentic specimen of the dixanthylhydrazone of glyoxylic acid (m. p. 147°) it melted at 146·5°.

A blank experiment was carried out on the unfermented medium, but no precipitate was obtained on acidification of the alkaline alcoholic extract.

Fermentation of Malonic Acid. Detection of Oxalic Acid.—A sterilised solution containing 25 g. of malonic acid in 2500 c.c. of solution M was inoculated and incubated at 30°. On the eighteenth day oxalate was detected on addition of calcium chloride to the cold solution, whereupon 800 c.c. were withdrawn, filtered, concentrated

in a vacuum to 400 c.c., and neutralised with ammonia, and excess of 10% calcium chloride solution was added. The precipitate was redissolved in dilute hydrochloric acid and reprecipitated with ammonia (Found : Ca, 31·3, 31·2. Calc. : Ca, 31·25%. 0·0832 and 0·0976 required 25·9 and 30·10 c.c. of $N/20$-$KMnO_4$. Calc., 26·0, 30·5). (Under the experimental conditions any glyoxylic acid would be partly converted into oxalic acid.)

Glyoxylic acid or its ammonium salt in 2% aqueous solution gives no precipitate with cold calcium chloride unless the solution has been boiled and again cooled, calcium oxalate then being precipitated.

Fermentation of Calcium Glycollate. Detection of Glyoxylic Acid.— The medium contained 40 g. of calcium glycollate in 4000 c.c. of solution M. The water was first sterilised and the glycollate added. The solution gave no test for glyoxylic acid and was inoculated with mycelium from four test-tube cultures on the same medium and incubated at 22°. On the sixth day a mixture of 10 c.c. of the medium, 10 c.c. of hydrochloric acid, and a trace of 1 : 3-dihydroxynaphthalene was boiled for 1 minute, cooled to 16°, and extracted with ether, which acquired a purple colour (naphtharesorcinol test) indicating the presence of glyoxylic acid or a similar compound.

On the twenty-seventh day 1800 c.c. of the culture were treated with 7 g. of aminoguanidine acetate, giving after 3 days a yellow deposit (0·3 g.). On recrystallisation from hot water this had m. p. 155° and did not depress the m. p. (155°) of an authentic specimen of the aminoguanidine derivative of glyoxylic acid.

A further 1800 c.c. of the filtered medium were treated with 1 c.c. of 50% hydrazine hydrate and then with 4 g. of xanthhydrol in 1800 c.c. of glacial acetic acid (a blank experiment was carried out on 1800 c.c. of acetic acid) (see p. 3052). On addition of water a white precipitate was produced. This was separated after an hour, dried, and treated with alcoholic N-sodium hydroxide. The resulting solution was filtered and acidified with acetic acid, and the precipitate was washed with aqueous alcohol, dried over soda–lime, and twice crystallised from chloroform–light petroleum; it then melted at 147° and did not depress the m. p. (147°) of an authentic specimen of the dixanthylhydrazone of glyoxylic acid (Found: C, 71·8; H, 4·85. Calc. : C, 72·1; H, 4·7%). The acetic acid used as solvent was shown to be free from glyoxylic acid both by the naphtharesorcinol and by the xanthhydrol test.

Fermentation of Ammonium Glycollate. Detection of Oxalic Acid.— The medium contained 20 g. of ammonium glycollate in 2000 c.c. of solution M. It was not sterilised in the usual manner, the glycollic acid being dissolved in sterile water, almost neutralised with am-

monia and diluted with sterile water. Inoculation was effected with 200 c.c. of a smaller culture on the same medium. On the tenth day 1800 c.c. of the culture were filtered, treated with a slight excess of 10% calcium chloride solution, warmed, and filtered. The precipitate was dissolved in hydrochloric acid and reprecipitated with ammonia (2·9 g.) (Found : Ca, 31·2. Calc. : Ca, 31·25%. 0·1237 required 38·6 c.c. of $N/20$-$KMnO_4$. Calc., 38·65).

The cost of this investigation has been in part defrayed by a grant from the Government Grant Committee of the Royal Society. A State Scholarship awarded to one of us (V. S.) by the Ministry of Development, Government of Madras, has enabled him to participate in the research. To both of these bodies the authors wish to express their thanks, and also to Mr. P. D. Coppock, M. Sc., for carrying out the fermentation of calcium glycollate and the analysis of the products.

THE MUNICIPAL COLLEGE OF TECHNOLOGY AND
THE UNIVERSITY, MANCHESTER. [*Received, October 8th,* 1927.]