CII. A STUDY OF THE MECHANISM OF THE DEGRADATION OF CITRIC ACID BY *B. PYOCYANEUS*. PART I.

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THE formation of citric acid in plants and its probable participation in certain phytochemical alkaloid syntheses [cf. Robinson, 1917], together with the facts relating to its production and degradation by various species of *Aspergillus* and *Penicillium*, render of interest the study of its behaviour when submitted to the action of biological agents. Several investigations of the actions of various micro-organisms on citric acid and on citrates have been undertaken already; thus Bosworth and Prucha [1911] detected butyric and succinic acids together with hydrogen, carbon dioxide, and ethyl alcohol amongst the products of bacterial action on this acid. They also stated, without giving details, that two equivalents of acetic acid are produced from one equivalent of citric acid by *B. lactis aerogenes*. Terada [1924] fermented citric acid by a pure culture of an unclassified micro-organism isolated from an aerial infection, and obtained much succinic acid together with an appreciable quantity of acetic acid and a little aconitic acid. In the course of a quantitative investigation of the action of *B. coli communis* on various organic acids in the presence of formates, Grey [1924] found that cultures on citric acid gave rise to two equivalents of acetic acid together with some succinic acid and carbon dioxide. Brown, Duncan and Henry [1924] fermented trisodium citrate by *B. suispestifer* in the presence of peptone and concluded that the sole volatile acid formed was acetic acid (two equivalents) whilst the other products were carbon dioxide and a trace of succinic acid.

During a recent examination of the action of *Aspergillus niger* on citric acid, Challenger, Subramaniam and Walker [1927] were able to demonstrate the formation of a number of substances, including acetone and malonic acid, which, hitherto, had not been identified amongst the products of mycological degradations of citric acid. It appeared of interest, therefore, to endeavour to ascertain whether the formation of these substances from citric acid by the action of *A. niger* is to be regarded as specific to this organism.

For the purposes of this enquiry *B. pyocyaneus* was selected, since it may be grown very readily in media in which certain salts of citric acid constitute

the sole source of carbon [cf. Quastel, Stephenson and Whetham, 1925]. This organism was cultivated at 34° in solutions containing 2 % of ammonium citrate together with the requisite inorganic salts, and all the conditions were carefully standardised. By this means it proved possible to follow, stage by stage, the course of the fermentation, and to characterise intermediate substances hitherto unnoted. Proliferation was rapid, and after a period of about 27 hours from the time of inoculation small quantities of acetonedicarboxylic acid could be detected in the culture solutions by precipitation in the cold with Denigès' [1899] mercuric sulphate reagent. Brown, Duncan and Henry [1924] postulated the formation of acetonedicarboxylic acid as an essential intermediate but failed to detect its presence, most probably owing to the facts that they conducted their fermentations at a somewhat higher temperature (37°) and postponed examination of samples of the fermenting solutions until 48 hours from the time of inoculation. After fermentation for 44 hours the rate of formation of acetonedicarboxylic acid increased rapidly, as evidenced by the accumulation of acetone. The acid has merely a transitory existence under these conditions, its formation being followed immediately by decarboxylation, since it did not accumulate in the culture media. The total "acetone bodies" (i.e. acetonedicarboxylic acid plus acetone) reached maximum concentration under the conditions of the experiments usually at 56 hours from the time of inoculation, when they were found to correspond approximately to a 66 % conversion of the citric acid. Thereafter, they decreased rapidly in quantity and had disappeared entirely at 80 hours.

In another series of experiments, conducted under precisely the same conditions, production of acids during the fermentation was investigated. In the first instance, samples of the fermenting media were withdrawn at intervals of 4 hours and tested, by means of a solution of diazotised aniline, for the presence of malonic acid, which, it was anticipated, might arise from oxidation or hydrolytic fission of acetonedicarboxylic acid. This expectation was realised, since slight traces of a precipitate of hydrogen formazyl [von Pechmann, 1892] separated when samples were tested after 32 hours. The quantities of this derivative yielded by samples of equal volume increased to a maximum at 96 hours, after which progressive decrease was observed, and at 133 hours malonic acid was entirely absent.

In view of the production of succinic acid from citric acid, noted in the cases mentioned above, search was made for it in the cultures during the course of the present work, and its appearance was indicated by positive pyrrole tests after incubation had proceeded for 50 hours. Subsequently, the acid was isolated and characterised as its *p*-nitrobenzyl ester. The amount present in the cultures was then roughly estimated at intervals during the course of several separate fermentations. It was present in very small amount only, but the quantity slowly increased and reached its maximum at 167 hours, when it represented a yield of approximately 5 % on the weight of the citric acid present initially in the media.

During the course of these experiments the presence of a volatile acid or acids was noted at an early stage in each fermentation. The quantity of volatile acid present in the cultures at any one time was always small, though the amount varied during the progress of the fermentation, increasing rapidly after 44 hours from the time of inoculation. Samples of volatile acid isolated during the period 44 to 100 hours were accumulated in the course of several experiments and consisted principally of acetic acid. It was characterised as the *p*-nitrobenzyl ester. Subsequently, determinations of volatile acid performed at regular intervals of time afforded data for the construction of an acidity-time curve (see Fig. 1), the general shape of which has formed a convenient means of correlating the formation of volatile acid and the appearance, accumulation, and disappearance of the other degradation products.

Fig. 1.

Discussion of the volatile acidity-time curve.

It will be understood that the deductions to be made from consideration of the curve must be based necessarily upon the relative concentrations in which the various intermediates were found to be present together in the fermenting medium at different periods of time; for it must be assumed that every intermediate product is subject to enzymic attack from the moment of its formation, and that at no particular moment is any one stage of the whole fermentation process represented in its entirety in the culture solution.

Phase *A* to *B* shows volatile acid formation increasing at a slow uniform rate. *B* to *D* corresponds to a period during which "acetone bodies" were slowly increasing in the culture solution. At *D* rapid increase in the rate of formation of volatile acid is depicted, and this was accompanied by rapid production of "acetone bodies" until *G* was reached, at which point the culture solution contained the maximum quantity of the latter. Thereafter, "acetone bodies" diminished in quantity until at *H* none could be detected.

From *G* to *H* the quantity of volatile acid still increased, although the slope of the curve diminished.

Malonic acid, first detected at *C*, appeared in greatest concentration during the phase *G* to *H*. The disappearance of malonic acid took place gradually from *H* to *K*, and, subsequently, at an enhanced rate from *K* to *L*, at which latter point only traces remained.

The rapid disappearance of the "acetone bodies" from the cultures, which became evident after a period of approximately 60 hours, was not caused by reduction to isopropyl alcohol. The experimental data just recorded point to an entirely different explanation, namely, the probability that the acetone was oxidised, presumably by way of pyruvic acid, to form the greater part of the acetic acid which arose during the phase *D* to *H*. According to Pasteureau [1905] oxidation of acetone by a 2 % solution of hydrogen peroxide affords pyruvic acid amongst other products, whilst Fournier [1908] has recorded that pyruvic acid is produced when acetone is oxidised by alkaline potassium permanganate. Some of the acetic acid detected during the course of the present work must have had its origin, however, in the hydrolytic fission of acetonedicarboxylic acid, since in some cases the yields of malonic acid found in the cultures were as high as 51 % of those theoretically possible. It was also considered possible that some acetic acid might be produced by enzymic decarboxylation of malonic acid, and in order to test this view the organism was cultivated in a medium containing ammonium malonate as sole source of carbon, when it was found that an appreciable yield of acetic acid was obtained. In this latter experiment the medium also gave the dark green and pink colour tests typical of glycollic acid [Denigès, 1909] and a positive naphtharesorcinol test for glyoxylic acid [Neuberg, 1910] when treated with the appropriate reagents, although conditions could not be found for the isolation of these two acids in quantities which permitted of their further characterisation. It may be mentioned, however, that both glycollic acid and glyoxylic acid have been isolated recently from cultures of *A. niger* on calcium acetate [Challenger, Subramaniam and Walker, 1927], whilst of further interest in the same connection are the facts that Grey [1924] fermented malonic acid by *B. coli* in the presence of sodium formate and obtained a 34 % yield of acetic acid, and that Hopkins and Cole [1901] demonstrated the production of glyoxylic acid from acetic acid under the influence of hydrogen peroxide and an iron salt. *B. pyocyaneus* was then cultivated on a 0.5 % sodium acetate medium, when the solution gave positive colour tests for both glycollic and glyoxylic acids on the fourth day. In view of these data the cultures of the organism on ammonium citrate were examined for glycollic and glyoxylic acids, and the former was detected on the fourth day and persisted for 10 days in the media. Glyoxylic acid was also detected, its appearance being noted first when the cultures were 54 hours old. Neither acid could be isolated in more than traces.

The section *H* to *M* of the acidity-time curve proves of particular interest.

At *H* there was present a relatively large quantity of malonic acid. If, subsequently, this acid had undergone no change other than decarboxylation to acetic acid the production of volatile acid would at least have been maintained. On the contrary there occurred at *H* a sudden fall in volatile acid. This may be accounted for in part by the assumption that some of the acetic acid formed from the malonic acid underwent oxidation to glycollic and glyoxylic acids, a view which is supported by the fact that during this period the fermenting medium was found to give an intense naphtharesorcinol reaction for glyoxylic acid. It is necessary, however, to consider the falls in the concentrations of malonic acid and acetic acid in conjunction with the fact that early in the same period, namely, *H* to *K*, succinic acid (which appeared first at *E*) was found to increase rapidly in concentration, and the suggestion is put forward that the latter acid has its genesis in the decarboxylation of the malonic acid in the presence of a hydrogen acceptor. If, as suggested earlier, the acetone is oxidised to acetic acid by way of pyruvic acid, a portion of the latter substance could then act as the necessary acceptor, being itself reduced to lactic acid, and it is significant that Aubel [1922, 1923] and Aubel and Cambier [1922] detected the presence of lactic acid in cultures of *B. pyocyaneus* both on pyruvic acid media and citric acid media. The precise stages by which the decarboxylation of malonic acid is effected by the organism are not known, but it is at least evident that in the final stage of the process a hydrogen atom and the group $\text{CO}_2\text{H}-\text{CH}_2\cdots$, the latter having free affinity, must have a momentarily separate existence. In the absence of disturbing factors coalescence then takes place with formation of acetic acid, but, in the presence of a powerful hydrogen acceptor, it would appear more probable that the hydrogen atom eliminated from the carboxyl group is accepted at this stage rather than at a later stage from the activation of acetic acid. This view of the formation of succinic acid is being tested experimentally in mixed fermentations of (*a*) pyruvic and malonic acids, and (*b*) pyruvic and acetic acids. In connection with this question it should be noted that Quastel and Whetham [1925] found the values of the reducing coefficients of formic, acetic, and malonic acids, as hydrogen donors, to be 700, 0.95 and 0.4 respectively. The figure assigned to formic acid provides the explanation of the reason why Grey obtained no succinic acid by the fermentation of malonic acid in the presence of sodium formate. It is interesting to note also that the explanation of the formation of succinic acid suggested above receives further support from recent work by Quastel and Wooldridge [1927] and Quastel and Whetham [1928], in which it was shown that malonic acid is specifically effective in retarding the velocity of reduction of methylene blue by succinic acid, although malonic acid is classed by the authors as neither an acceptor nor donor of hydrogen. They find on the other hand that acetic acid cannot effect retardation of the velocity of reduction of methylene blue; whence it would appear that the formation of succinic acid should be effected more readily from malonic acid than from acetic acid.

The sudden increase in volatile acidity which occurred after 128 hours was unexpected and its explanation is not altogether clear. It has been shown by Aubel [1921] and by Quastel [1924] that ammonium succinate is fermented readily by *B. pyocyaneus* to yield mixtures of propionic, acetic and formic acids, and it is possible that in the present instance some of the increase in volatile acidity after 128 hours was due to breakdown of succinic acid in this manner. This view receives support from the observation that the acidity produced during the phase *LM* was not due entirely to acetic acid, since a sample of volatile acid collected at *M*, neutralised with sodium carbonate and treated with *p*-nitrobenzyl bromide, gave what was evidently a mixture of two or more esters. Before this question can be settled definitely, however, it will be necessary to correlate the change in volatile acidity along *HKLM* and the variation in succinic acid content during the same phase.

EXPERIMENTAL.

The strain of *B. pyocyaneus* with which the experiments were performed was Fildes III, Number 1999, and was kindly supplied by the Curator of the National Collection of Type Cultures, Lister Institute. Stock cultures were re-inoculated at 14-day intervals on broth-agar slopes. In all cases, where possible, the substance under investigation was used as a 2 % solution of its ammonium salt in Ringer's inorganic salt solution. The solutions were all sterilised and were adjusted, when necessary, to p_H 7.0–7.4. A boiling-tube containing 25 cc. of the culture solution was sterilised on three consecutive days and then inoculated from a platinum loop of culture taken from an agar slope which had been incubated for 24 hours at 34°. The boiling-tube culture was subsequently incubated for 30 hours at 34°, after which 1 cc. of the fermenting liquid was withdrawn by means of a sterile pipette and transferred to a fresh boiling-tube charged with 25 cc. of the same medium. This also was incubated for 30 hours at 34°, after which it was used as the inoculum for a flask of the same medium in which the experimental fermentation was conducted. By the adoption of this procedure it was found possible to ensure that each flask received a dose of organisms of approximately standard amount and virility. The flasks in which the experimental fermentations were carried out were all of 500 cc. capacity, and each was charged with 250 cc. of the liquid substrate under examination. They were shaken twice daily during the progress of the fermentations.

Fermentation of ammonium citrate. Detection of acetonedicarboxylic acid and of acetone.

30 flasks charged with 2 % ammonium citrate-Ringer solution were inoculated and incubated. At intervals of 4 hours the contents of one of these flasks were mixed with a large excess of Denigès' [1899] mercuric sulphate reagent in the cold. The presence of acetonedicarboxylic acid was indicated by the formation of a white precipitate, the separation of which is accelerated

by vigorous agitation. When it was ground finely, suspended in water, and treated with hydrogen sulphide, acetonedicarboxylic acid was liberated and was detected by the reddish-purple coloration obtained on addition of a drop of ferric chloride solution.

Acetone also gives a white precipitate with Denigès' reagent, but this is not obtained with dilute solutions of the ketone unless the mixture be heated on the boiling water-bath for a period of 15 to 40 min., as was the case in the present investigation. The procedure adopted for the detection of acetone was as follows. To the contents (250 cc.) of the flask to be examined were added 5 cc. of dilute hydrochloric acid and the whole was heated for 15 min. under reflux. This served to moderate the frothing which occurred in the subsequent distillation. The flask was adapted to a condenser and 100 cc. were distilled into a bolt-head flask containing 700 cc. of Denigès' reagent. The total contents of the receiver were then heated under reflux on the steam-bath for 40 min. when, if acetone was present, a white precipitate was deposited. When acetone was thus detected the precipitate was removed, washed with cold water and dried on porcelain. A quantity (17 g.) of the precipitate thus obtained was cautiously distilled with 30 g. of sodium iodide in 120 cc. of water, and 20 cc. of distillate were collected and added to an excess of a solution of 2:4-dinitrophenylhydrazine hydrochloride. The orange-yellow precipitate which formed immediately was removed, and after three recrystallisations from boiling absolute alcohol there was obtained 1 g. of pure material of M.P. 128°, alone or in admixture with an authentic specimen of acetone-2:4-dinitrophenylhydrazone (M.P. 128°).

A further 17 g. of the mercury compound was distilled similarly and the distillate was mixed with 20 cc. of ethyl alcohol (free from acetone), 1 cc. of freshly distilled benzaldehyde and 1 cc. of 10 % aqueous sodium hydroxide. The whole mixture was shaken and then allowed to remain in the cold for 12 hours when yellow leaflets separated. These were washed with a little cold alcohol and recrystallised from absolute alcohol (yield 1.2 g.). The pure material had M.P. 113° alone or mixed with authentic dibenzylideneacetone (M.P. 113°).

The yields of acetone plus traces of acetonedicarboxylic acid obtained after different intervals of time are shown in the following table:

Time of incubation (hrs.)	27	32	40	44	48	55	60	64	72
Percentage conversion of the citric acid to "acetone bodies"	0.04	0.115	0.20	14.5	16.5	67.0	66.2	0.39	0.115

Fermentation of ammonium citrate. Detection of malonic acid.

The medium consisted of citric acid (20 g., as ammonium salt) dissolved in 1000 cc. of water together with the requisite Ringer salts. Flasks containing this medium were inoculated with standard inoculum and, during the subsequent incubation, the contents of a flask (5 g. citric acid) were removed at regular intervals and treated in the following manner. To the liquid were

added 5 cc. of 10 % hydrochloric acid and the mixture was then warmed under reflux on a water-bath for 30 min. This served to facilitate the subsequent filtration. The filtrate was cooled to 0°, treated with an appropriate quantity of benzenediazonium chloride and excess of sodium acetate and allowed to stand during 10 hours in an ice-chest. When malonic acid was present the precipitation of hydrogen formazyl commenced usually within an hour from the time of mixing the reagents, and it was found advantageous to remove this product at intervals, since tar formation sometimes occurred towards the completion of the reaction. The filtered product was washed well with cold water, dried in a vacuum over sulphuric acid, and crystallised twice from methyl alcohol by addition of a little water and once from benzene by addition of light petroleum. It melted at 118° and the melting-point was not lowered when it was mixed with authentic hydrogen formazyl (M.P. 118–120°).

Found (micro-analysis): C, 69.1 %; H, 5.4 %.

Calculated for $C_{13}H_{12}N_4$: C, 69.6 %; H, 5.4 %.

The quantities of hydrogen formazyl produced in one experiment after different intervals from the time of inoculation are shown in the following table.

Time of incubation (hrs.)	48	60	72	88	96	120	133
Weight (g.) of hydrogen formazyl from 5 g. of citric acid	Traces	0.4	1.0	2.0	3.0	1.5	Traces
Percentage conversion of citric acid to malonic acid	—	6.8	17.2	34.4	51.6	25.8	—

Fermentation of ammonium citrate. Isolation and estimation of acetic acid.

Flasks charged with a 2 % solution of ammonium citrate in Ringer's medium were inoculated and incubated. At regular intervals 100 cc. of culture solution were withdrawn for examination. The sample was placed in a 2-litre bolt-head flask, 25 cc. of a 20 % solution of phosphoric acid were added, and the mixture was distilled in steam for 30 min. In every case the volume of distillate was 300 cc. ($\pm$ 5 cc.). The distillate was titrated against 0.05 *N* sodium hydroxide in the presence of phenolphthalein. A further 100 cc. of culture solution from the same flask was treated similarly and served as a check on the first estimation. Culture liquid of the same age from another flask was treated in exactly the same manner, so that, in all, at least four values were taken to establish any one point on the time-acidity curve. The maximum variation in the titre for 100 cc. of culture solution at any particular age was 0.5 cc. of 0.05 *N* sodium hydroxide. The above procedure did not serve to remove every trace of volatile acid from the culture sample, but the amounts obtained from the several samples were comparable, and indicate with fair accuracy the general course of volatile acid production as depicted by the curve. It was observed that during the first 125 hours of the fermentation the volatile acidity was due mainly to one particular acid. In one series of experiments the neutralised distillates from the samples collected during this

125 hour period were combined and evaporated to dryness (0.55 g.). This residue was dissolved in 5 cc. of water, mixed with a solution of 1 g. of *p*-nitrobenzyl bromide in 10 cc. of alcohol, and the whole boiled under reflux for 90 min. The mixture deposited white crystals on cooling; these were separated, washed with a little dilute alcohol, then with water, and re-crystallised three times from dilute alcohol. The product (0.2 g.) then had M.P. 77.5° and when it was mixed with an authentic specimen of *p*-nitrobenzyl acetate (M.P. 78°) it did not depress the M.P. of the latter.

Fermentation of ammonium citrate. Isolation of succinic acid.

The pyrrole test [Neuberg, 1901] was employed to detect succinic acid, and when it was judged that the latter was present in the culture liquid in a quantity sufficient to permit of isolation the medium was acidified with phosphoric acid and distilled in steam. The residue containing non-volatile acids was partially evaporated and treated with ether in a continuous extraction apparatus. The ether was removed from the extract and the latter taken up in 50 cc. of distilled water, the solution heated to 70° and a 2 % solution of potassium permanganate added, drop by drop, until the solution remained permanently just faintly pink. This treatment served to destroy non-volatile acids other than succinic acid, *e.g.* glycollic acid. The solution was then boiled with a little animal charcoal, filtered, and again extracted thoroughly with ether. The ethereal extract was dried over sodium sulphate, allowed to evaporate, and the crystals which first separated were removed. These possessed all the properties of pure succinic acid and were characterised by the mixed melting-point method and by conversion to the di-*p*-nitrobenzyl ester. The latter had M.P. 89° alone or mixed with authentic material. The yields of crude succinic acid isolated, in each case from 1000 cc. of 2 % ammonium citrate medium, after 72, 90, 120 and 167 hours were 0.03, 0.1, 0.4 and 0.55 g. respectively.

Fermentation of ammonium malonate. Isolation of acetic acid.

Malonic acid (19.5 g.) was dissolved in distilled water (500 cc.) and neutralised with ammonia. A further quantity (0.5 g.) of malonic acid was then added, followed by the requisite amounts of Ringer's mineral salts, and the whole was made up to 1000 cc. with distilled water. The medium was distributed in four flasks, sterilised for 15 min. at 100° on three successive days, and the p_H adjusted to 7.4 by addition of sodium hydroxide. A blank experiment showed that this treatment did not result in decarboxylation to acetic acid. The flasks were inoculated and incubated in the usual manner. After 96 hours the contents of the flasks were combined, acidified with phosphoric acid and subjected to steam-distillation. The distillate was neutralised with sodium hydroxide and evaporated, yielding 4 g. of dry residue. 1 g. of this material was treated with *p*-nitrobenzyl bromide by the

method previously described and yielded 1.5 g. of crude product. On recrystallisation of this from dilute alcohol pure *p*-nitrobenzyl acetate was obtained, and melted at 77.5° alone or mixed with an authentic specimen of M.P. 78°.

REFERENCES.

- Aubel (1921). *Compt. Rend. Acad. Sci.* **173**, 179.
 — (1922). Dissertation, Paris.
 — (1923). *Compt. Rend. Acad. Sci.* **176**, 332; see also ref. by Supniewski (1924), *Biochem. Z.* **154**, 95.
 — and Cambier (1922). *Compt. Rend. Acad. Sci.* **175**, 71.
 Bosworth and Prucha (1911). *J. Biol. Chem.* **8**, 479.
 Brown, Duncan and Henry (1924). *J. Hygiene*, **23**, 1.
 Challenger, Subramaniam and Walker (1927). *J. Chem. Soc.* 200, 3044.
 Denigès (1899). *Ann. Chim. Phys.* [vii], **18**, 408.
 — (1909). *Ann. Chim. Phys.* [viii], **18**, 179.
 Fournier (1908). *Bull. Soc. Chim.* (iv), **3**, 259.
 Grey (1924). *Proc. Roy. Soc. Lond. B*, **96**, 156.
 Hopkins and Cole (1901). *Chem. Zentr.* **1**, 797.
 Neuberg (1901). *Z. physiol. Chem.* **31**, 574.
 — (1910). *Biochem. Z.* **23**, 148; **24**, 436.
 Pasteureau (1905). *Compt. Rend. Acad. Sci.* **140**, 1591.
 von Pechmann (1892). *Ber. deutsch. chem. Ges.* **25**, 3175.
 Quastel (1924). *Biochem. J.* **18**, 365.
 — Stephenson and Whetham (1925). *Biochem. J.* **19**, 304.
 — and Whetham (1925). *Biochem. J.* **19**, 520.
 — (1928). *Biochem. J.* **22**, 689.
 — and Wooldridge (1927). *Biochem. J.* **21**, 148.
 Robinson (1917). *J. Chem. Soc.* **111**, 878.
 Terada (1924). *J. Pharm. Soc. Japan*, **511**, 697.