XVII. A STUDY OF THE MECHANISM OF THE DEGRADATION OF CITRIC ACID BY *B. PYOCYANEUS* (*PSEUDOMONAS PYOCYANEA*).

II. ACTION OF *B. PYOCYANEUS* ON SUCCINIC ACID.

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IN Part I [Butterworth and Walker, 1929] it was shown that the products of the action of *B. pyocyaneus* on ammonium citrate include malonic acid and a small quantity of succinic acid, and it was suggested that the latter arises by simultaneous decarboxylation and dehydrogenation of two molecules of the malonic acid in presence of a hydrogen acceptor. Pyruvic acid, which might well be derived from the acetone also produced in the ammonium citrate cultures, could exercise such a function, being thereby converted to lactic acid or, under certain conditions, to propionic acid. Support for this hypothesis is to be found in the experiments of Aubel and Cambier [1922], who detected lactic acid in cultures of *B. pyocyaneus* on solutions of the salts of both citric and pyruvic acids. Further, as will be evident from results described in the course of the present communication, the fact that propionic acid has been shown to be an end-product of the action of *B. pyocyaneus* on ammonium succinate [Aubel, 1921; Quastel, 1924] supports the same view. We have also obtained additional experimental evidence in favour of such a possibility by establishing the fact that addition of small quantities of acetone to cultures of *B. pyocyaneus* on calcium acetate media is followed by the formation therein of lactic acid.

In view of the occurrence of succinic acid in the citrate cultures, and of the fact that propionic acid is formed by the growth of *B. pyocyaneus* on succinate media, it was considered of interest to make a closer study of the behaviour of succinic acid under the attack of this organism. It was considered unlikely that the propionic acid originates in the simple decarboxylation of succinic acid under the influence of the bacillus, as suggested by Aubel, since such a view is not in harmony with the known facts of organic chemistry, and its formation was regarded as more probably due to the following series of changes:

In the comprehensive work of Van Niel [1928] on the chemistry of the propionic acid bacteria, the formation of propionic acid from both pyruvic acid and lactic acid was verified beyond doubt.

Adopting the above tentative scheme as a basis of enquiry, search for the presence of malic acid was made in cultures of *B. pyocyaneus* on ammonium succinate, and its formation was demonstrated in several experiments after fermentation had been in progress for about 9 days. The acid was detected by Denigès' mercuric acetate reagent [1900] and in subsequent experimental fermentations it was isolated and examined in detail, and, in every instance, it was found to consist of a mixture of the *dl*- and *l*-forms. Since *B. pyocyaneus* does not effect racemisation of the active forms of malic acid, and since we have proved by control experiments that *l*-malic acid is unaltered by the treatment adopted for its isolation from the cultures, there remain only two possible sources from which the *dl*-malic acid could have been derived, namely, (*a*) from the succinic acid by the action of activated hydrogen peroxide, for *B. pyocyaneus* is known to contain a peroxidase system, and (*b*) from oxaloacetic acid by symmetrical reduction. We regard (*a*) as the more probable alternative, for biological reduction has usually been observed to yield asymmetric compounds when structure has permitted optical activity and, further, the fact that *B. pyocyaneus* is rich in catalase is not an argument against (*a*), since it has been demonstrated clearly [Chodat and Pasmanik, 1907; Thurlow, 1925] that a partition of hydrogen peroxide between catalase and peroxidase occurs when both enzymes are present.

In order to throw light on the source of the *l*-acid the bacillus was then cultivated on media in which *dl*-malic acid constituted the sole source of carbon and, in the several experiments undertaken, such media invariably developed dextrorotatory properties. They also gave positive reactions to the test for pyruvic acid devised by Quastel [1924] and were free from lactic acid. Hence it follows that the *l*-malic acid obtained by the fermentation of ammonium succinate is not derived through resolution by the bacillus of preformed *dl*-malic acid, but must have its genesis in the asymmetric addition of water to fumaric acid arising from the dehydrogenation of succinic acid. In this connection it is to be noted that Stent, Subramaniam and Walker [1929] have recently shown that succinic acid yields a mixture of *dl*- and *l*-malic acids when submitted to the attack of *Aspergillus niger*, and that the latter organism renders solutions of *dl*-malic acid dextrorotatory, whilst Challenger and Klein [1929] have established the fact that the same strain of this mould produces high yields of *l*-malic acid from potassium fumarate, whence it would appear that the degradation of succinic acid is achieved in precisely the same manner by both *B. pyocyaneus* and *A. niger*.

Quastel [1924], Quastel and Whetham [1924] and Quastel, Stephenson and Whetham [1925], in the course of studies of the reactions induced by resting bacteria have considered the cases of the equilibria which are set up (*a*) between fumaric acid and *l*-malic acid in the presence of resting *B. coli*, and (*b*) between

succinic acid and fumaric acid in the presence of methylene blue and resting *B. pyocyaneus*. Their experimental results are later interpreted as special cases of the general theory of the mechanism of oxidation and reduction *in vivo*, postulated by Quastel [1926], in which the breakdown of succinic acid by resting bacteria is assumed to proceed by initial formation of an activated form of fumaric acid, symbolised as $\text{COOH}-\text{CH}_2-\overset{\vee}{\text{C}}-\text{COOH}$, and regarded as identical with the activated form into which it is assumed fumaric acid is first converted under similar enzymic influence. This explanation is an elaboration and to some extent a modification of Wieland's dehydrogenation hypothesis, and if it also applied to the behaviour of succinic acid when submitted to the attack of actively growing *B. pyocyaneus* under aerobic conditions, we should expect to obtain products identical with those which arise by the action of this organism on fumaric acid. Such, however, is but partially the case for, as we have just seen, the breakdown of the succinic acid in this instance is largely achieved by what appears to be a peroxidase reaction (leading to the formation of racemic malic acid), which proceeds simultaneously with the dehydrase and fumarase reactions affording respectively fumaric acid and thence *l*-malic acid.

According to Quastel [1924] the growth of *B. pyocyaneus* on media containing salts of fumaric acid is very vigorous and malic acid cannot be detected among the products, of which the first to be identified is pyruvic acid. Further, Quastel and Whetham [1924] found malic acid to be incapable of donating hydrogen to methylene blue in the presence of the resting organism. As a result of these observations they concluded that the conversion of fumaric acid to pyruvic acid probably proceeds through the intermediate formation solely of oxaloacetic acid

In a later paper, however, Quastel [1926] brings forward evidence which vitiates this conclusion, for he shows that the effect of an activating field on malic acid is to produce a certain amount of activated fumaric acid which functions as a hydrogen acceptor

in consequence of which he states that malic acid, after activation, behaves both as a hydrogen acceptor and donator, and considers that probably the donating properties are obscured in the experiments with *B. coli* by the accepting powers.

In our view the formation of *l*-malic acid in cultures of *B. pyocyaneus* on succinate media which, as we have demonstrated, can only have taken place by asymmetric addition of water to fumaric acid arising by preliminary dehydrogenation of the succinic acid, renders it more probable that the pyruvic acid produced in cultures of *B. pyocyaneus* on fumarates arises through the changes:

than by the direct oxidation of fumaric acid to oxaloacetic acid. This opinion must not be construed as belief on our part that direct oxidation of this type cannot take place, for Quastel's activation theory [1926] indicates a reasonable mechanism by which such a change could, in certain cases, be effected.

EXPERIMENTAL.

The strain of *B. pyocyaneus* employed was that used in Part I of this investigation, namely, Fildes III, No. 1999, from the National Collection of Type Cultures, Lister Institute. The substances under investigation were submitted to its action in Ringer's inorganic salt solution, the various media being adjusted to p_H 7.4 prior to inoculation.

Enzymic conversion of acetone to lactic acid.

Forty flasks of 500 cc. capacity were each charged with 5 g. of calcium acetate (Kahlbaum) and 1.0 g. of ammonium nitrate in 250 cc. of Ringer's solution. After sterilisation, 0.5 g. of pure acetone was delivered from a sterile pipette into each flask, followed by an inoculum of 1 cc. of a 24 hours old culture of the organism on the same medium. Incubation was conducted for 3 days at 25°, after which the contents of the flasks were combined and concentrated somewhat by distillation. The distillate contained no acetone. The residue was evaporated to dryness on the steam-bath. Preliminary tests having indicated that this material contained calcium lactate, a portion (20 g.) was distilled with 100 cc. of sulphuric acid (50 % concentration) until 30 cc. of distillate had passed through the ice-cooled condenser. The acetic and formic acids in this distillate were neutralised with sodium hydroxide and the solution was then distilled through the ice-cooled condenser until 20 cc. of distillate were obtained. This gave a strongly positive test for acetaldehyde by Rimini's reaction [1904]. It was mixed with a solution of *p*-nitrophenylhydrazine (5 g.) in acetic acid (40 cc. of 50 % concentration) and the precipitate was washed, dried and recrystallised from alcohol; m.p. 128° alone or in admixture with an authentic specimen of acetaldehyde *p*-nitrophenylhydrazone (m.p. 128°). Yield, 0.8 g. (found, N, 23.8; calc. N, 23.46 %).

Another portion (22 g.) of the original dry residue presumed to contain calcium lactate was heated with 50 % sulphuric acid as before, and the distillate was neutralised with sodium hydroxide and evaporated to dryness. The residue was extracted several times with methyl alcohol in order to remove sodium acetate, and the residual small quantity of insoluble solid material was taken up in 10 cc. of water and treated with 2 g. of lead nitrate dissolved in 10 cc. of water, when a precipitate formed and was removed. It was washed with cold water and recrystallised from hot water, yielding 0.8 g. of pure lead formate (found, Pb, 69.4; calc. 69.7 %).

A control experiment in which pure calcium lactate was distilled with sulphuric acid gave acetaldehyde and formic acid as above. In a blank experi-

ment no trace of lactic acid was detected in a solution of pure calcium acetate fermented in the absence of acetone.

Detection of malic acid in cultures of B. pyocyaneus on ammonium succinate.

The most delicate test for the detection of malic acid is that of Denigès [1900], and it was found that this could be combined with the use of 2 : 4-dinitrophenylhydrazine whereby a means was provided for obtaining quickly a rough approximation of the concentration of malic acid in a solution containing only a trace. Thus, dilute aqueous solutions were made up containing respectively 0.1, 0.05, 0.033, 0.025 and 0.02 % of malic acid. 25 cc. of each solution were treated with 3 to 4 cc. of Denigès's mercuric acetate solution, then boiled and filtered. Slight excess of a 2 % potassium permanganate solution was added to each, drop by drop, whilst the solutions were maintained at the boiling point. The precipitates of the basic mercury salt of oxaloacetic acid which were thus obtained were allowed to settle in test-tubes, the clear supernatant liquid was decanted and the precipitates were then each treated with 5 cc. of a saturated solution of 2 : 4-dinitrophenylhydrazine hydrochloride in 2*N* hydrochloric acid. This gave rise to slight precipitates of yellow crystalline material in all cases in which the malic acid had been present originally at a concentration of 0.025 % or higher. These crystals consist probably of a mixture of the 2 : 4-dinitrophenylhydrazones of pyruvic acid and acetaldehyde, since treatment of the basic mercury salt of oxaloacetic acid with cold dilute hydrochloric acid yields a solution which smells strongly of acetaldehyde, and which gives a positive reaction for pyruvic acid when treated with an alcoholic solution of guaiacol and strong sulphuric acid (Quastel's test [1924]). The results of the above-described experiments may be tabulated thus:

% concentration of malic acid in solution	0.1	0.05	0.033	0.025	0.020
Volume (cc.) of 2 % KMnO_4 solution equivalent to 25 cc. of malic acid solution	0.56	0.30	0.20	0.15	0.10
Quantity of 2 % KMnO_4 solution added (drops)	12	9	7	5	4
Precipitate	+			+	+
Action of 2 : 4-dinitrophenylhydrazine	Yellow precipitate and golden opacity			Golden opacity. Precipitate on standing	Opalescence only

This information was then utilised in subsequent experiments in which it was sought to prove the presence of malic acid.

Ammonium succinate (30 g.) was dissolved in Ringer's solution (1500 cc.) and this mixture was distributed equally between 6 flasks of 500 cc. capacity. After sterilisation, inoculation was performed by addition to each flask of 1 cc. of an actively fermenting culture on the same medium, and incubation was conducted at 34°. At intervals of 7 hours portions of 25 cc. were withdrawn

and tested with 2 % KMnO_4 solution in the presence of mercuric acetate solution at the boiling point. After 3 days this test for malic acid was positive, whereupon 700 cc. of the culture solution were treated with these reagents and yielded 1.5 g. of the characteristic basic mercury salt of oxaloacetic acid which, on subsequent distillation with 5 g. of sodium iodide in 25 cc. of water, gave traces of acetaldehyde (Rimini's test). After 11 days the remaining contents of the culture flasks were combined and treated as above, when 18 g. of moist mercury salt were obtained. This was distilled with sodium iodide (30 g.) in water (150 cc.) and the well-chilled distillate was conducted beneath the surface of a solution of 2 : 4-dinitrophenylhydrazine hydrochloride (250 cc.) prepared according to the particulars given by Brady and Elsmie [1926]. The resultant precipitate (0.6 g.) was collected, washed, dried and recrystallised several times from hot ethyl alcohol, when it formed glistening orange-yellow crystals, m.p. 162° . This melting point was not depressed by admixture with a pure specimen of acetaldehyde 2 : 4-dinitrophenylhydrazone (found, by micro-method: N, 24.5; calc. N, 25.0 %).

*Isolation of dl-malic acid together with l-malic acid from cultures
of B. pyocyaneus on ammonium succinate.*

Exp. 1. 2500 cc. of a 2 % solution of ammonium succinate in Ringer's solution, after adjustment to p_{H} 7.4, were equally distributed between 10 flasks of 500 cc. capacity. After sterilisation these were inoculated at the rate of 1 cc. per flask with an active culture which had grown for 24 hours on a medium of the same composition. Incubation was carried out at 34° and the flasks were carefully shaken twice daily in order to aerate the media. A positive test for malic acid was obtained on the fifth day, and increased in intensity up to the ninth day, whereupon the contents of all the flasks were combined, acidified with a little glacial acetic acid, and digested on the water-bath to coagulate the culture. The solution was filtered, further concentrated to 700 cc., treated with 140 g. of crystalline lead acetate in 200 cc. of water, and then made slightly alkaline with ammonia, after which 500 cc. of 95 % alcohol were added. Two days later the precipitate of lead salts was removed at the pump, washed with 30 % alcohol and dried. The material was triturated in a mortar and suspended in 100 cc. of water, and the lead removed by H_2S . On subsequent concentration to 75 cc. crystals of succinic acid separated on standing. These were removed and the mother liquor was then concentrated to 10 cc., when a further crop of succinic acid separated and was also removed. The residual syrupy liquid was neutralised with sodium hydroxide, diluted with water to 56 cc. and 4 cc. of glacial acetic acid were added. 25 cc. of this solution were mixed with 10 cc. of an 8 % solution of uranium acetate, and after standing in the dark showed a rotation of -2.4° (Ventzke scale). 25 cc. of a 1 % solution of authentic sodium *l*-malate plus 10 cc. of an 8 % uranium acetate solution showed, on similar treatment, a rotation of -13.7° . Assuming

the optical activity of the fermentation product to be due to *l*-malic acid, this result represents a yield of approximately 0.1 g. of the latter.

Exp. 2. This was a repetition of *Exp. 1*, with the exception that the fermentation was allowed to continue for a longer time. Malic acid was first detected on the 4th day. On the 14th day the bacteria were still active and a sample of the liquid gave a positive test for the presence of pyruvic acid when tested by Quastel's method. The whole of the culture medium was then worked up as in the previous experiment and the acid liquid was concentrated until succinic acid separated. The latter was removed and the process of concentration and removal of succinic acid was repeated until finally a viscous syrup, nearly free from succinic acid, was obtained. This was ground with anhydrous sodium sulphate and extracted with dry ether. The ethereal solution was concentrated to 20 cc. and treated with light petroleum until a faint turbidity appeared. On slow evaporation a brownish crystalline product was obtained. This sintered at 96° and a portion of it finally melted at 165°, hence it evidently still contained traces of succinic acid. The material was then shaken with 5 cc. of water and 10 cc. of ether. The aqueous extract, now entirely free from succinic acid, was almost neutralised with sodium hydroxide, evaporated to 5 cc. and heated on the water-bath with 0.8 g. of *p*-nitrobenzyl bromide in 10 cc. of alcohol. The product was recrystallised twice, when it had m.p. 114°. This value lies between that of the di-*p*-nitrobenzyl ester of *l*-malic acid (124–125°) and that of the di-*p*-nitrobenzyl ester of the *dl*-acid (109°). A mixture of the *dl*- and *l*-forms of malic acid in the ratio of 55 parts of the former to 45 parts of the latter was made from authentic materials and treated with *p*-nitrobenzyl bromide as above. The product after two recrystallisations had m.p. 115° and appeared identical with the esterified culture product.

Exp. 3. 3.5 litres of 2 % ammonium succinate medium were inoculated as before, but the fermentation was conducted at a lower temperature (30°). On the 13th day the malic acid test became strongly positive, whereupon the whole of the culture solution was concentrated to a syrup. Phosphoric acid was added and the mixture was treated with ether for several hours in a continuous-extraction apparatus. This afforded a light brown solid which was then treated with charcoal in hot aqueous solution. On evaporation of the filtered liquid there remained 27 g. of colourless crystals, m.p. 164°. These were redissolved in hot water and fractionally crystallised, when 7 g., m.p. 139–140°, were obtained. This was redissolved in water and extracted with ether. The aqueous layer on evaporation yielded 2.5 g. of slightly deliquescent crystals, m.p. 114–116°, and the ether extract gave material which, on further fractional crystallisation from hot water, afforded a small quantity of pure *dl*-malic acid, m.p. 130°. The substance of m.p. 114–116° gave tests for pure malic acid (found: equivalent, 66.4; calc. 67.0; found by micro-method: C, 35.2, 35.3; H, 4.4, 4.5; calc. C, 35.8; H, 4.5 %). A portion (0.111 g.) on polarisation in the presence of uranium acetate by Dakin's method [1924] gave a reading of – 6.3° (Ventzke scale) which indicated that 40 % of the material was *l*-malic acid. This was

confirmed by reference to a fusion-point curve of mixtures of *dl*- and *l*-malic acids which was constructed from determinations of the melting points of known mixtures. From this curve it was seen that a mixture of 60 % *dl*-acid with 40 % *l*-acid melts at 117°. A further small portion (0.1 g.) of the material of m.p. 114–116° was polarised in the presence of 10 % ammonium molybdate solution (Williamson's method [1918]), when a reading of +2.1° (Ventzke scale) was observed, a final confirmation of the presence of *l*-malic acid. The remainder of the material was then fractionally crystallised from ether-light petroleum, when a very small amount of pure *dl*-malic acid, m.p. and mixed m.p. 130°, was finally separated.

*Development of dextrorotatory activity in cultures of
B. pyocyaneus on dl-malic acid.*

Pure *dl*-malic acid (60 g.) was dissolved in 2 litres of Ringer's solution and neutralised by addition of approximately 50 cc. of concentrated ammonia solution (sp. gr. 0.88). The solution was divided between eight flasks, sterilised, and adjusted to p_H 7.4 by addition of hydrochloric acid. After inoculation, followed by incubation at 34° for 7 days, the cultures were combined and evaporated to dryness. The residue was redissolved in a little water, acidified with phosphoric acid and extracted with ether as in Exp. 3 for several hours. The ether extract was dried and evaporated to a syrup which slowly crystallised in a desiccator. The material was tested for lactic acid by distillation with sulphuric acid, but the distillate showed no reaction for acetaldehyde, hence lactic acid was absent. Two quantities (0.578 g. and 0.711 g.) of the crystals were dissolved in 80 cc. of distilled water in each case. The solutions were clarified with charcoal, neutralised with potassium hydroxide, treated with a drop of glacial acetic acid and then each made up to 100 cc. A portion (10 cc.) of one of the solutions showed a rotation of +5.4° (Ventzke scale) in the presence of uranyl acetate; a further 10 cc. showed a rotation of –7.8° (Ventzke scale) in presence of ammonium molybdate. Hence Williamson's test [1918] for the presence of *d*-malic acid was positive. The specific rotations of the two solutions were then determined by Dakin's method, when an average value of 301.5° was found, corresponding to the presence of 62.5 % of *d*-malic acid in the crystals.

The whole experiment was repeated, the fermentation being allowed in this case to continue for 14 days.

Polarimetric examination of the product demonstrated the presence of 70 % of *d*-malic acid.

During the fermentations of *dl*-malic acid described above the presence of pyruvic acid was detected in the media by Quastel's test, the reaction being positive after 21 hours' incubation, and intense colorations were obtained after 45 hours. The concentration of this acid was too low, however, to permit of its isolation, only traces of precipitate being obtained with ketonic group reagents.

SUMMARY.

1. The results described in the present paper, when considered in conjunction with those recorded by Butterworth and Walker [1929], show that the growth of *B. pyocyaneus* on salts of citric acid results in precisely the same reactions as occur during the breakdown of this acid by the mould *A. niger* [cf. Challenger, Subramaniam and Walker, 1927].

2. The action of *B. pyocyaneus* on succinates also leads to the formation of the same products, *dl*-malic acid and *l*-malic acid, as are obtained by the action of *A. niger* on succinates. The evidence indicates that the *dl*-malic acid arises by direct hydroxylation of succinic acid and the *l*-malic acid by dehydrogenation of succinic acid to fumaric acid, followed by asymmetric addition of water to the latter.

3. From these observations in conjunction with other experimental data it is rendered likely that the formation of pyruvic acid from fumaric acid under the influence of *B. pyocyaneus*, as studied by Quastel, proceeds by the stages fumaric acid $\rightarrow$ *l*-malic acid $\rightarrow$ oxaloacetic acid $\rightarrow$ pyruvic acid, and not in the more direct manner which experiments with methylene blue would appear to indicate.

4. A method, based on Denigès' mercuric acetate test, is described for the detection and approximate estimation of traces of malic acid in solution.

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