XLVIII. ON THE FERMENTATION OF THE UNSATURATED DICARBOXYLIC ACIDS. PART I. FUMARIC ACID.

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Both ammonium fumarate and ammonium succinate provide excellent sources of nutrition to a number of bacteria—particularly to *B. pyocyaneus* and *B. fluorescens liq.* Table I shows the nutritional properties of these substances for various types of bacteria.

Table I.

Organism	Ammonium succinate	Ammonium fumarate
Sarcina aur.	Practically no growth after 21 days' incubation	No growth after 21 days
B. pyocyaneus	Extensive surface growths. Thick, almost gelatinous growth after 21 days. Considerable pigmentation after this time	Extensive surface growths. Growth extremely vigorous Not so much pigmentation as with the succinate
B. subtilis	Little or no growth. No film produced	No growth observable. No film produced
B. fluorescens liq.	Thick brown growth of a jelly-like consistency after 21 days	Excellent growth
B. proteus vulg.	Slow but perceptible growth	Fairly good growth
Staphylococcus aur.	Poor growth	Poor growth
B. prodigiosus	Fairly good growth	Fairly good growth
Timothy grass bacillus	No growth observable	No growth observable
B. coli comm.	Very good growth	Very good growth

The ammonium succinate or fumarate was the sole source of carbon and nitrogen in the media provided for these observations. A concentration of about 1 % was usually employed. It was used in conjunction with a Ringer solution of the following composition: NaCl ·5 g.; K_2HPO_4 ·2 g.; $MgSO_4$ 0·2 g.; $CaCl_2$ 0·1 g.; water, 1 litre; the medium being generally at an initial p_H of 7·4. Twenty-four hour nutrient agar cultures of the organisms were used for inoculation into the medium. The extraordinarily good growths of *B. pyocyaneus* made this organism very suitable for an investigation into the products formed by its fermentative action and for determining its relative rates of utilisation of the succinate and the fumarate.

The products of fermentation of succinic and fumaric acids by B. pyocyaneus.

Aubel [1921] subjected ammonium succinate to fermentation by *B. pyocyaneus* and found that propionic acid was produced. It will be found, however, that other lower fatty acids are also formed, the actual yield of propionic

acid varying considerably with the conditions under which the fermentation is allowed to proceed. If to the ammonium succinate caseinogen be added, a much larger amount of propionic acid is formed than in its absence—the difference not being due to the fermentation of caseinogen alone, as a control experiment showed. In no case is the yield of propionic acid high, and if the fermentation be allowed to proceed too far (for instance to a jelly-like consistency) the actual amount is very small. From the products of fermentation of 30 g. succinic acid which had been neutralised with ammonia and dissolved in 3 litres of water (to which mineral salts in the concentrations given above had been added), about 2 g. of a fairly pure specimen of propionic acid were obtained. The acid was identified by an analysis of the barium salt recrystallised from water (Ba, found 45·43 %; Ba, calculated for hydrated propionate 45·58 %). This was obtained after a fortnight's growth at 37° with an initial p_H of 7·4. Analysis showed that after this time 85 % of the succinate had been utilised. In a number of other experiments on the fermentation of succinic acid the analysis of the volatile acids produced showed a variable barium figure demonstrating the existence of a varying mixture of fatty acids (chiefly lower ones). Small quantities of formic acid were usually found. Fumaric, malic, and other non-volatile acids were especially sought among the products of fermentation, but none was found.

Fumaric acid gives rise to similar products after a fermentation lasting over a fortnight, and it is itself entirely utilised by the end of that time. There seems to be generally a greater proportion of acetic acid among the fatty acids produced than in the case of succinic acid. The crude acids obtained by distillation *in vacuo* were neutralised with baryta and an analysis of the dried barium salts gave Ba 50 to 53 %. Traces of formic acid but no non-volatile dibasic acids were found among the products of fermentation.

In the cases of both succinic and fumaric acids, the fermentations are accompanied by marked increases in p_H, and ammonium carbonate is found in solution. The pigmentation in both cases is not so great as that produced by the growth of *B. pyocyaneus* in a caseinogen digest, and generally appears to be more marked in a succinate medium than in a fumarate. The amount of pigmentation, however, is affected not only by the type of medium utilised and the length of the fermentation, but by such conditions as the nature of oxygen supply, stirring of the medium, and especially by p_H.

The rates of utilisation of ammonium succinate and ammonium fumarate by B. pyocyaneus.

The growth of *B. pyocyaneus* in fumarate and succinate media appears to occur chiefly at the surface of the solution. After shaking, the scum falls below the surface and renders the solution turbid. It is very difficult to obtain a homogeneous emulsion of the organism in such a solution. Hence in determining the rates of utilisation of the media it is inaccurate to use a method

involving the analysis of aliquot parts of the medium at various intervals. Moreover, the medium evaporates to a considerable extent in the incubator, the amount of evaporation depending on the extent of the surface of the solution, so that it is difficult to apply corrections. The following method, which was found to give the most concordant results, was eventually used.

Ten cc. of approximately 2 % ammonium succinate-Ringer solution which had been brought to a p_H of 7·4 were placed in each of 20 test-tubes. These were plugged and sterilised by autoclaving. The ammonium succinate had been prepared by neutralising a strong aqueous solution of succinic acid with ammonia, evaporating, and allowing to crystallise. The dried crystals consisted chiefly of the ammonium salt, the remainder being the acid salt. A series of ammonium fumarate-Ringer tubes was prepared in a similar way.

Nutrient agar-agar slopes were sown with *B. pyocyaneus* and after 24 hours the organisms were emulsified in 0·85 % sterile saline solution, washed by centrifugalisation, and finally made up with the saline solution to a volume which showed just a slight opalescence. This appeared to be fairly homogeneous, and after several hours no deposit of the organism was formed. Into each of the tubes of media 1 cc. of the *B. pyocyaneus* suspension was run by means of sterile pipettes, and the tubes were then incubated at 37°. Each tube was well shaken every morning and at definite intervals the contents of a number of the tubes were analysed. The analyses were done in duplicate.

Estimations of succinate and fumarate in fermentation media.

A method had to be devised which would allow of a fairly rapid and accurate determination of succinic acid in the presence of products of fermentation, protein matter, phosphates, chlorides, etc. The following method proved to be the most satisfactory of a number of attempts. The medium containing the succinate is washed into a conical flask and about 1 g. lime added. The mixture is boiled gently for two minutes. It is then cooled, filtered, and the residue washed three or four times with small quantities of water. The total filtrate and washings should not occupy a volume of more than 40 cc. This solution is quite clear and apparently free from protein matter and from phosphates. A few drops of phenolphthalein indicator are added, the solution titrated with approximately $N/5$ sulphuric acid until just acid, and titrated back with a drop or two of dilute alkali until the solution is faintly pink. Silver nitrate in concentrated solution and in slight excess of that necessary to precipitate the whole of the chlorides and succinate is added, the solution allowed to stand a few minutes, filtered and the precipitate washed until free from silver. The precipitation should be carried out in a dark part of the laboratory and the washing should be done as quickly as possible. The precipitate is washed into a white porcelain basin, a little nitric acid added to dissolve the succinate, then a few drops of ferric alum indicator added and the solution finally titrated with standard potassium thiocyanate solution in the usual manner. An error of 2 % was the largest experienced in numerous

analyses of succinate solutions of known composition where the succinate varied in concentration between 5 % and 0·02 %. At a concentration of 0·01 % or less the method is less reliable, but an accuracy of 5 % can usually be obtained. Care must always be taken in precipitating and washing the silver succinate.

This method of silver precipitation may also be used with accuracy for the estimation of fumaric acid. The products of fermentation of succinic and fumaric acids by *B. pyocyaneus* are not precipitated by silver under the conditions occurring in this method of analysis.

The results of a parallel series of fermentations of succinic and fumaric acids are expressed graphically in Fig. 1.

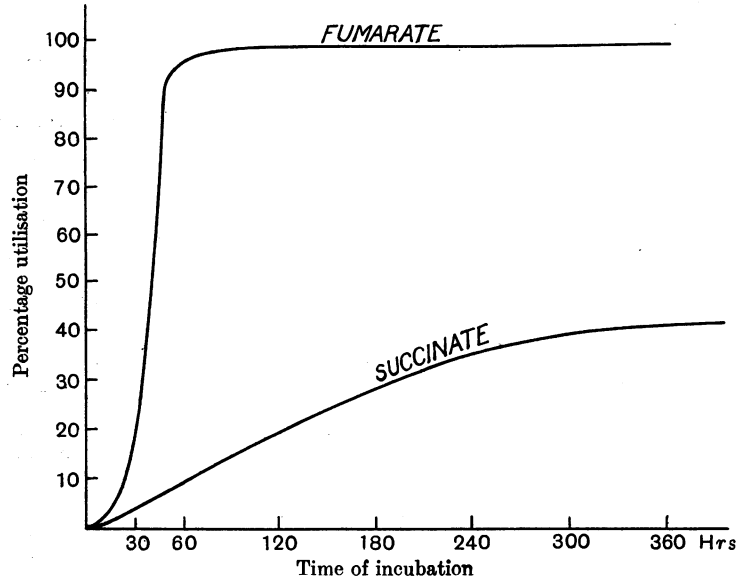


Fig. 1.

This is typical of a large number of parallel series of fermentations. The rate of fermentation of ammonium succinate varies considerably with the number of organisms inoculated and the initial p_H, but generally not more than 50 % of the succinate is utilised within the first week of fermentation. Under these conditions, however, a fumarate medium of the same percentage composition as the succinate is almost entirely utilised within two or three days. In both cases there is a rise in p_H; in the fumarate the p_H may rise to 11 or over, but in the succinate the p_H has not been observed to rise above 10. Ammonium carbonate is produced in both cases. The rates of fermentation, especially in the case of the succinate, are accelerated by bubbling air or oxygen through the medium. Under this condition an ammonium succinate medium may be utilised to the extent of 70 % within a week and a fumarate medium utilised to the extent of 95 % within 36 hours.

In spite of the wide differences in the rates of utilisation of ammonium succinate and ammonium fumarate by *B. pyocyaneus*, the actual amounts of growth of the organism in both are about the same. This was shown by comparing the volumes of centrifugalised deposits of organism. An attempt was made to count the number of organisms by the usual procedure of plating-out, but owing to the coherent nature of the bacillus this was abandoned. The pigmentation of a fumarate medium which had just been completely utilised (*i.e.* within two or three days of inoculation) was slight, but it increased considerably later. On the whole a parallel succinate fermentation showed greater pigmentation.

Properties of a fumarate medium after fermentation for 48 *hours.*

The next step in the work was to ascertain the nature of the constituents of the medium after the fumaric acid has just been utilised, *i.e.* after roughly 48 hours' fermentation. It was obvious that the fumaric acid had been replaced by a reactive material, for the solution was capable of reducing silver oxide to silver, mercuric chloride to mercurous chloride, and picric acid to picramic acid. It could reduce molybdates and tungstates to the blue lower oxides, but did not react with Fehling's solution. Colour tests capable of detecting small quantities of this reactive material were sought and two were found which were of considerable use in the early part of the work. A third was subsequently discovered which facilitated the isolation of the material. The two tests referred to were:

(1) *Tryptophan test.* If 10 cc. of a 1 % solution of ammonium fumarate in a Ringer solution containing chalk in suspension (the p_H being approximately 8·0) be inoculated with a loopful of *B. pyocyaneus* and allowed to ferment for 24 hours, the solution gives a characteristic colour reaction with tryptophan. To 1 cc. of this solution in a test-tube, a little tryptophan is added and conc. H_2SO_4 slowly poured down the side of the tube; a bluish violet coloration is produced and this slowly spreads upwards—not into the acid layer as with the glyoxylic test. If fermentation be allowed to proceed for a week or more, the solution gives a deep brown ring instead of a bluish violet. If a solution capable of giving the violet ring be boiled vigorously, it will no longer (on cooling) give this ring. If chalk be omitted from the medium, the initial p_H still being approximately 8·0, the violet ring is not always given but is replaced by a brown one. If, as was subsequently done, the fermenting medium be continually aerated or bubbled with oxygen, a violet ring is rarely given but usually a deep brown one. This test is not given by a succinate medium which has been fermented by *B. pyocyaneus*, nor is it given by a fumarate medium which has been fermented by *B. coli.*

(2) *Nitroprusside test.* If in 1 cc. of a 1 % ammonium fumarate-Ringer solution which has been fermented for 36 hours with *B. pyocyaneus*, a crystal of sodium nitroprusside be dissolved, and to the solution solid ammonium sulphate and finally strong ammonia be added, a blue or bluish-green colora-

tion gradually appears in the vicinity of the ammonium sulphate crystals. The colour becomes most intense after standing at room temperature for a quarter to half an hour. The best tests are obtained when the fumarate medium has been inoculated with a loopful of fresh culture of *B. pyocyaneus* and has been continually aerated. A medium which has been boiled will give the test provided that the medium was originally capable of giving a strong reaction. If, however, the fermentation has been carried out with an insufficient oxygen supply, the reaction is usually a weak one and after boiling the test is hardly given. The colour of this test seems to be identical with that given by pyruvic acid. It disappears on standing, as is usual with nitroprusside tests. It is readily given when the blue-violet ring of the tryptophan test has been replaced by a brown one.

The only organisms found so far which yield the tryptophan and the nitroprusside tests after fermentation on a fumarate medium are *B. pyocyaneus* and *B. fluorescens liq.* No organisms have yet been found to give these tests after fermentation on a succinate medium. The tests are not given by the pigment of *B. pyocyaneus* or by the organisms alone.

With these tests for the reactive constituent of the medium, attempts were made to extract or isolate it. It was found in the first place to be non-volatile at or below the boiling-point of water. Distillation at 100° or at low temperatures *in vacuo* failed to carry over the reactive material. Moreover, prolonged boiling destroyed it. If the medium were evaporated to dryness *in vacuo*, the residue only gave a faint nitroprusside reaction—not nearly as great as that expected had the evaporation brought about a concentration of the material without loss due to decomposition. The addition of phosphoric acid to a reactive medium did not destroy its activity but distillation in steam of the acid solution failed to carry over the material. Ether extraction of a slightly acid reactive medium carried out continuously over 36 hours failed to extract more than a trace of material giving a nitroprusside reaction. Moreover, the aqueous portion after thorough extraction with ether still yielded a fairly good test. On adding a solution of phenylhydrazine hydrochloride to a few cc. of the reactive medium saturated with sodium acetate, no precipitate of a hydrazone appeared. All attempts to precipitate the reactive material with metals failed. Other attempts at isolation by different solvents, by distillation, etc. were focussed on the residue left after evaporation *in vacuo*, and it was obvious after many failures to isolate either the reactive material or a derivative of it that a different mode of attack was necessary.

These attempts at isolation were all carried out on 1 % ammonium fumarate dissolved in Ringer solution (at a p_H varying between 7·4 and 8·0) which after inoculation had been incubated at 37° for two or three days. It was evident that this method of inoculating 500 to 1000 cc. of the medium and incubating it yielded only very small quantities of the reactive constituent. It was evident, too, that the constituent was probably a ketonic acid and that its isolation by ether extraction or as a hydrazone was being hindered not

only by its low concentration but probably by the presence of other substances in the medium.

Two courses were possible, (1) to determine the conditions which would bring about an increase in the concentration of the reactive constituent; (2) to guess the nature of the constituent from the data already available, to determine experimentally the precise conditions for its isolation from a medium made up roughly to correspond with a fermented fumarate medium, and then to apply these conditions to its isolation from the original medium.

The work at this point was considerably facilitated by the discovery of a characteristic reaction given by the above medium in the presence of guaiacol and sulphuric acid.

Guaiacol test. If to 1 cc. of a 1 % ammonium fumarate-Ringer solution which has been fermented by *B. pyocyaneus* for 36 hours, a few drops of an alcoholic solution of guaiacol be added and conc. H_2SO_4 poured cautiously down the side of the test-tube, a characteristic band of colours is formed at the surface of separation. There is a thin crimson band nearest the water layer. Underneath this is a wider band which is deep carmine and fades into pink as it penetrates the sulphuric acid layer. This test is very sensitive—more so than the nitroprusside test—and there is no indecision as to its being positive or not as there may be with a weak nitroprusside test. The intensities of the guaiacol and nitroprusside tests proceed parallel to one another as fermentation progresses. Both are most intense at the same period of fermentation and both disappear after a few days' fermentation.

The guaiacol test is very characteristic of this fermentation. It is not produced in a succinate medium fermented by *B. pyocyaneus* and the only organisms among those hitherto examined which produce it in a fumarate medium are *B. pyocyaneus* and *B. fluorescens liq.* The only substance found so far which gives a guaiacol reaction identical with that of a fermenting fumarate is pyruvic acid, but a wide range of substances has not yet been tested. Acetone gives a yellow ring. Acetaldehyde produces a white diffused layer underneath which is a scarlet band followed by a clear yellow one penetrating into the sulphuric acid. Formaldehyde gives a very thick white diffused layer over a deep claret band which penetrates into the acid. All these tests are given at low concentrations and the distinct differences in colours are best seen at these concentrations (about 1 part in 1000 and less).

These colour reactions, together with the reducing properties, exhibited by the medium after a relatively short period of fermentation, made it extremely probable that pyruvic acid was produced during the fermentation. A number of experiments were then undertaken to determine under what conditions pyruvic acid in dilute solution (approximately 0·2 %) could be isolated as such or as a known derivative from a medium made up roughly to correspond with a fermented medium. Eventually it was found that the most satisfactory method was to precipitate the pyruvic acid as the *p*-nitrophenylhydrazone, but certain precautions must be taken to ensure the precipitation.

It was necessary that bacterial matter should first be removed from the medium, that any carbonate originally in the medium should be removed by the addition of a little syrupy phosphoric acid, and that only a few drops (not excess) of a *freshly made* saturated solution of *p*-nitrophenylhydrazine in strong acetic acid should be used for the precipitation. It was advisable always to add a little syrupy phosphoric acid for this seemed to facilitate the precipitation considerably. There was, however, a limiting concentration of pyruvic acid under which no precipitation could be obtained and since the strength of the guaiacol and nitroprusside tests showed that this concentration was approximately that obtained in a fermenting fumarate medium it was necessary to find conditions to increase this concentration in the medium in order to ensure precipitation.

Isolation of pyruvic acid as the p-*nitrophenylhydrazone.*

It was finally found that under the following conditions a maximum yield of the reactive substance is formed.

1. A 1 % solution of sodium fumarate instead of ammonium fumarate should be fermented. The nitrogen is supplied in the form of ammonium chloride (0·4 %).

2. The initial p_H of the medium should be approximately 7·4 and no chalk should be added.

3. *The medium must be continually aerated.*

4. It must be initially inoculated with a loopful of a 24 hour agar-agar culture of *B. pyocyaneus*.

5. The fermentation should not be carried on for more than two days. Generally 30 hours' fermentation or even less gives the most intense reactions. When a film of the organism no longer forms at the surface of the medium, the change of the fumaric acid is generally completed.

6. Large quantities of the medium should not be fermented at a single time. 100 cc. and 50 cc. lots are most satisfactory and these are fermented in conical flasks so that as large a surface as possible is obtained.

A medium treated in this way gives most intense nitroprusside and guaiacol reactions.

To 10 cc. of this medium is added about 1 g. chalk. The solution is thoroughly mixed and filtered. The filtrate is practically clear and free from protein matter. One cc. of syrupy phosphoric acid is added to the filtrate and this is slightly warmed until CO_2 is no longer evolved. The solution is cooled and a *freshly prepared* saturated solution of *p*-nitrophenylhydrazine in glacial acetic acid is added a drop at a time, with shaking. Not more than 0·5 cc. is added. On standing a yellow precipitate appears. This is filtered on a Buchner funnel, washed with a little glacial acetic acid and subsequently with water. The precipitate is recrystallised from hot dilute alcohol, from which it separates in

sheaves of yellow needles. Its melting-point is 219° which is identical with that of the *p*-nitrophenylhydrazone of pyruvic acid [Hyde, 1899] and its melting-point is not changed by the admixture of a pure specimen of this hydrazone.

There is little doubt therefore that the substance responsible for the reactive properties of a fumarate medium which has been inoculated with *B. pyocyaneus* is pyruvic acid.

Pyruvic acid has itself been subjected to putrefactive fermentation and acetic acid has been the main product formed [Neuberg, 1914, 2]. The formation of acetic acid by lengthy fermentation of fumaric acid probably proceeds therefore through pyruvic acid.

The yield of pyruvic acid is smaller in an ammonium fumarate medium than in one containing a relatively small quantity of ammonia. There is evidence that in the former medium complexes are formed, which are probably condensation compounds of pyruvic acid with ammonia. The p_H of the medium becomes very high; in this state of alkalinity pyruvic acid readily produces condensation products with ammonia.

No substances other than pyruvic acid, the lower fatty acids, bacterial matter, and CO_2 were found in measurable quantity. Hydrogen has not yet been sought. The following were sought and not found: acetaldehyde and formaldehyde; lactic, acrylic, glycollic, malic, tartaric and malonic acids; ethyl alcohol and methylacetylcarbinol. Methods for acetaldehyde fixation in the fermenting medium have not yet been tried.

The effects of various organisms on an ammonium fumarate medium.

One loopful of a fresh culture of each of a number of organisms was inoculated into 1 cc. of a 1 % ammonium fumarate-Ringer solution at p_H 7·6, and incubated at 37°. They were examined by the guaiacol and nitroprusside tests after (1) 40 hours, and (2) six days from the time of inoculation—the fermentation tubes being well shaken each morning. The results are summarised in Table II.

Table II.

	(1) After 40 hours			(2) After 6 days		
Organism	Growth	Nitroprusside test	Guaiacol test	Growth	Nitroprusside test	Guaiacol test
B. pyocyaneus	Very good	+ + +	+ + +	Very good	Very feeble, positive	Very feeble
B. fluorescens liq.	„	+ + +	+ + +	„	–	–
B. coli communis	Good	–	–	„	–	Slight test indicative of aldehyde (not of pyruvic acid)
B. subtilis	? Growth	–	–	? Growth	–	–
B. prodigiosus	Fair growth	–	–	Fairly good	–	Slight test indicative of aldehyde (not of pyruvic acid)
B. proteus vulg.	Slight growth	–	–	Slight	–	„
Staphylococcus aur.	? Growth	–	–	„	–	„

The powerful action of *B. pyocyaneus* and *B. fluorescens liq.* compared with the other bacteria is most marked and the guaiacol test provides an excellent method for the differentiation of these organisms.

No growth of *B. pyocyaneus* (which is a facultative anaerobe) could be obtained anaerobically in an ammonium fumarate or succinate medium.

Oxygen uptake and carbon dioxide output.

Investigations were made to determine the amount of oxygen taken up and the quantity of carbon dioxide produced during the fermentation of fumaric acid by *B. pyocyaneus*.

For this purpose use was made of an apparatus designed by Stephenson and Whetham [1923]. This consisted of an incubation flask with an air condenser tube. Air, after passing through wash-bottles containing potash and conc. H_2SO_4, was aspirated slowly through the incubation flask. It passed out through the air condenser tube and then through two weighed pumice-conc. H_2SO_4 absorption tubes and a weighed potash bulb. The apparatus was disconnected at intervals and each part cooled and weighed. The gain in weight of the whole apparatus gave the total oxygen uptake. The gain in weight of the potash bulb plus that of the sulphuric acid absorption tube attached to it gave the total carbon dioxide output.

The following figures give the result of a typical blank experiment:

	Initially	After 24 hours' aeration
Wt. of incubation flask	149·7765 g.	149·7550 g.
Wt. of first H_2SO_4 absorption tube	45·3690	45·3905
Wt. of second H_2SO_4 absorption tube	43·4934	43·4945
Wt. of potash bulb	62·1096	62·1120
	300·7485	300·7520

There is generally an error of not more than 5 mg., and this may be regarded as small compared with the total oxygen uptake and carbon dioxide output in the course of a fermentation.

The following gives the result of a fermentation carried out on 50 cc. of a 1 % fumaric acid as ammonium fumarate medium, p_H 7·4.

	After 20½ hours' fermentation	After 42½ hours' fermentation	After 64½ hours' fermentation	After addition of phosphoric acid and aeration
Carbon dioxide output	·0384	·1224	·1529	·2129
Oxygen uptake	·0684	·0754	·0744	·0744

The total oxygen uptake is 0·0744 g. and the total CO_2 output is 0·2129 g.

The oxygen is absorbed to the greatest extent during the first 30 hours of fermentation. Even after 20 hours, over 90 % of the total oxygen absorbed has been taken up. After two days oxygen is taken up very slowly and in certain cases, as in the one quoted, appears to cease to be absorbed. This is partly due to the retardation of growth brought about by the increased p_H of the medium, and partly due to the fact that all the fumaric acid has been utilised. Carbon dioxide appears to be evolved at a rate similar to that of

oxygen uptake. The actual values of carbon dioxide output must be taken in conjunction with the increase in p_H. At the end of the experiment a little phosphoric acid is added to the incubation flask, which is thoroughly aerated, and the remaining carbon dioxide is thus driven over into the potash bulb which is re-weighed. The total carbon dioxide produced is estimated at the end of the experiment. The ratio $\frac{\text{carbon dioxide output}}{\text{oxygen uptake}}$ is in the example quoted above 2·86. A second example is given below (50 cc. of a 1 % fumaric acid medium were used).

	After 22½ hours' fermentation	After 47 hours' fermentation	After 98½ hours' fermentation	After addition of phosphoric acid and aeration
Carbon dioxide output	·0305	·1760	·1965	·2370
Oxygen uptake	·0822	·0915	·0870	·0870

Total oxygen uptake = 0·0870 g.

Total CO_2 output = 0·2370

Ratio $\frac{CO_2 \text{ output}}{O_2 \text{ uptake}} = 2{\cdot}72.$

A third experiment gave the ratio $\frac{CO_2 \text{ output}}{O_2 \text{ uptake}} = 2{\cdot}61$, and we have as the average of these three results $\frac{CO_2 \text{ output}}{O_2 \text{ uptake}} = 2{\cdot}73$. If we assume the early part of the fermentation of fumaric acid to follow the equation

$$\begin{matrix} CH.COOH \\ \| \\ CH.COOH \end{matrix} + O = \begin{matrix} CH_2 \\ | \\ CO.COOH \end{matrix} + CO_2,$$

we have the ratio

$$\frac{CO_2 \text{ output}}{O_2 \text{ uptake}} = \frac{44}{16} = 2{\cdot}75.$$

Moreover, since 116 g. of fumaric acid will require an uptake of 16 g. of oxygen for this reaction, the amount subjected to fermentation, *i.e.* 0·5 g. will require 0·069 g. of oxygen. Experiment shows that a little more than this amount of oxygen is utilised, and this is probably used in the oxidation of pyruvic acid to acetic acid:

$$\begin{matrix} CH_2 \\ | \\ CO.COOH \end{matrix} + O = \begin{matrix} CH_3 \\ | \\ COOH \end{matrix} + CO_2,$$

where the ratio CO_2/O_2 is the same as in the previous reaction. If this be the case, then the difference in oxygen uptake between the experimental and the theoretical should be equivalent to the amount of acetic acid produced. This may be shown to be approximately the case. In the second example quoted above, the amount of fatty acids present calculated as acetic acid was 0·077 g. The theoretical quantity was 0·071 g.

The Balance Sheet.

An effort was made to construct a balance sheet between the fumaric acid and the products of its fermentation. This involved determining the quantity of pyruvic acid present. A number of methods were tried. The most

satisfactory—in that it gave consistent results—depended on the oxidation of the pyruvic acid with silver oxide and the estimation of the acetic acid produced.

50 cc. of the medium containing the pyruvic acid were treated with 1 g. of lime, warmed and filtered. 2 g. of freshly prepared, well washed, silver oxide were added to the filtrate and the solution was boiled gently for a quarter of an hour or more. A silver mirror usually formed. The solution was filtered, the precipitate washed, and the total amount of acetic acid in the filtrate and washings determined. This was carried out by acidifying the solution with glacial phosphoric acid and distilling *in vacuo*, the distillate being passed into a known quantity of standard baryta solution. The baryta was titrated after the distillation and the amount of fatty acid distilled over thus determined. Another 50 cc. of the same medium were acidified and distilled without previous treatment with silver oxide. This gave an estimation of the original quantity of fatty acids present; the difference between this figure and the previous one gave the quantity of acetic acid derived from the pyruvic acid and a simple calculation served to determine the amount of pyruvic acid originally present.

The amount of bacterial substance was estimated roughly by filtering through asbestos on a Gooch crucible (which acted as a fairly efficient filter) and drying the bacteria in a steam oven to constant weight. They were then placed (with the asbestos) in a combustion tube, burned in a current of oxygen, and the amount of carbon dioxide produced was estimated.

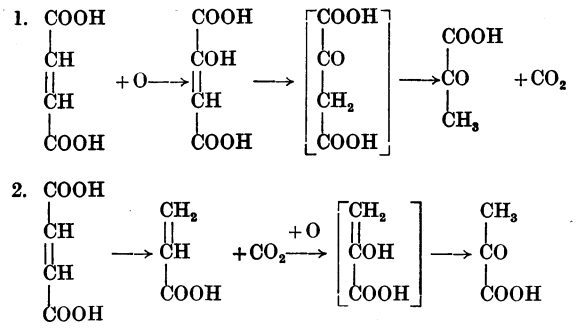
The following is an analysis of the products of 100 cc. of a 1 % fumaric acid medium (the fumaric acid being present as the sodium salt and ammonia as 0·4 % ammonium chloride). The fermentation had been allowed to proceed for 20 hours. 92 % of the fumaric acid had been utilised.

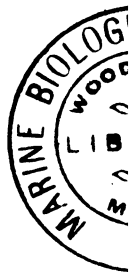
		Calculated in terms of CO_2	Fumaric acid utilised expressed in terms of CO_2
Acetic acid (being total fatty acids expressed as acetic acid) ...	=0·0922 g.	0·1352	
Pyruvic acid	=0·287	0·431	
CO_2 output	=0·550	0·740	
CO_2 from the organism	=0·190		
		1·3062 g.	1·398 g.

Thus at least 90 % of the fumaric acid utilised can be accounted for; the remainder is probably in the form of complex condensation products. Generally the yield of pyruvic acid from 1 g. of fumaric acid under the best conditions is about 0·20 g.

Discussion.

Since pyruvic acid is formed in the fermentation of fumaric acid by *B. pyocyaneus*, our first enquiry turns on the nature of the intermediate compound which is presumably formed. There are two possibilities, viz.:





The following evidence is available:

1. Oxalacetic acid is known to be easily decarboxylated by yeast [Mayer, 1913]. It is fermented by putrefying bacteria to acetic acid [Neuberg, 1914, 2]. Experiments are now in progress on the growth of *B. pyocyaneus* on oxalacetic acid.

2. The theoretical quantity of carbon dioxide required by the decarboxylation of either fumaric or oxalacetic acid is eliminated. The organism must, therefore, use the decarboxylated molecule for growth. Hence it would be expected that if decarboxylation of fumaric acid were the first step, growth would occur on acrylic acid. It has been impossible so far to obtain any growth of *B. pyocyaneus* on a medium in which acrylic acid forms the sole source of carbon; neither has acrylic acid been found among the products of fermentation of fumaric acid. Growth does occur with pyruvic acid as sole source of carbon.

3. No oxalacetic acid has been found, but experiment shows that the rate of decarboxylation (*i.e.* of CO_2 output) is practically equal to the rate of oxygen uptake, so that the oxalacetic acid is decomposed about as quickly as it is formed.

4. Oxalacetic acid has been previously postulated as the first product formed in the utilisation of fumaric acid by *Aspergillus niger* [Clark and Raistrick, 1919].

On the whole the evidence is in favour of oxalacetic acid (or its enol isomer) being the first product formed in the utilisation of fumaric acid. This phenomenon adds another example to the list of biological oxidations which we find, as yet, impossible to repeat *in vitro*. Neuberg [1914, 1] has been able to obtain acetaldehyde from fumaric acid by oxidation with hydrogen peroxide in the presence of iron. It is possible that oxalacetic acid is actually produced here as an intermediate compound, and that this then breaks down to acetaldehyde and carbon dioxide.

The actual mechanism of decarboxylation of oxalacetic acid (assuming that this is the process which occurs) is of interest. Two views are possible—that decarboxylation occurs at the carboxyl group next the ketonic group, or at the carboxyl group next the $> CH_2$ group. On this point there is at present little evidence. Experiments on substituted fumaric acids are about to be carried out and these should give data for a definite conclusion.

Our next enquiry turns on the differences exhibited by various bacteria in a fumarate-Ringer medium. *B. pyocyaneus* and *B. fluorescens liq.*, as possibly might have been expected, are closely related in their behaviour, but *B. coli*, *B. prodigiosus* and *B. proteus* show apparently considerable differences from the highly pigmented bacteria. It would be desirable to know whether the difference is due to the absence of specific enzymes which are present in *B. pyocyaneus* and *B. fluorescens liq.*, or whether it is due to differences in the relative rates of the different reactions constituting the whole process—the process being the same with all these organisms.

It will not be out of place to mention at this point the results of certain experiments which bear directly on the fermentative properties of *B. pyocyaneus*. These experiments, however, are purely preliminary, and will be discussed in detail in a subsequent communication.

If *B. pyocyaneus* be grown in considerable quantity on an ammonium fumarate medium, centrifuged, well washed with normal saline, and finally ground up thoroughly with acetone and re-washed to remove acetone, these bacteria show no power to oxidise fumaric acid to pyruvic acid. When half a gram of bacteria treated in this way were placed in a fumarate-Ringer medium and thoroughly aerated, no trace of pyruvic acid was found for at least 12 hours. A good guaiacol test then resulted but it was found that the medium after this time contained living *B. pyocyaneus*. The pyruvic acid was thus probably derived from the growth of a few bacteria which had not succumbed to the treatment with acetone.

Experiments carried out by Miss Stephenson, Miss Whetham and the author show that *B. pyocyaneus* well washed with normal saline, and dried at $-6°$ with absolute alcohol and ether, has no power of oxidising fumaric acid to oxalacetic or pyruvic acids. The dried organism, it is interesting to note, resembles muscle tissue which has been similarly treated in that it reduces oxidised glutathione with considerable rapidity. It yields, itself, a slight nitroprusside sulphydryl reaction which is not increased by warming the organism (thus differing slightly from muscle tissue). A large quantity of *fresh B. pyocyaneus* grown on a caseinogen digest, centrifuged, and well washed with normal saline, shows no signs of oxidising fumaric acid within a few hours—though the quantity of organism placed in the fumarate medium is far in excess of the amount formed from a small inoculation of the organism after complete utilisation of a similar amount of fumarate medium. Fresh *B. pyocyaneus* seems to show a distinct resemblance to fresh muscle tissue in its action on succinic acid. The rate of reduction of methylene blue by the fresh organism is considerably accelerated by the presence of sodium succinate. Sodium fumarate has no effect. (If the fresh organism be boiled, its power of reducing methylene blue is destroyed.) From analogy with muscle, we should expect the succinic acid to be oxidised to fumaric acid, the methylene blue acting as hydrogen acceptor. In other words, when succinic acid is fermented by *B. pyocyaneus*, we should expect the main course of fermentation to proceed

through fumaric acid and pyruvic acid to acetic acid—the two intermediate acids being relatively quickly dealt with by the organism. This would necessitate the uptake of a considerable quantity of oxygen (three atoms per molecule of succinic acid). One experiment shows that actually about 90 % of the quantity theoretically required is taken up.

B. coli resembles *B. pyocyaneus* in its ability to reduce oxidised glutathione to the sulphydryl form and to accelerate the reduction of methylene blue in the presence of sodium succinate but not in presence of sodium fumarate.

The apparent refusal of fresh washed *B. pyocyaneus*, or the "resting" organism, quickly to oxidise fumaric acid to pyruvic acid is unexpected. It seems possible that the actual process of growth—of protein synthesis—induces chemical changes in the nutrient medium whose velocities may differ considerably from those of the changes induced by the "resting" organism. The "resting" organism and the washed and dried organism (in the case of *B. pyocyaneus* and *B. coli*) have similar properties to fresh and to washed and dried muscle tissue respectively. It is interesting to note that in the quantitative work on the fermentation of fumaric acid an almost theoretical quantity of carbon dioxide is eliminated. It would seem that the organism does not take up the carbon dioxide produced in decarboxylation, but uses the remaining part of the decarboxylated molecule for its synthetic operations. The actual yield of pyruvic acid is much lower than that expected from a theoretical decarboxylation. The problem of the actual mechanism by which the oxidation of fumaric acid is accomplished, and of its *apparent* connection with an actively working synthetic machinery in the organism, is one whose elucidation should throw considerable light on the metabolism of *B. pyocyaneus*.

Summary.

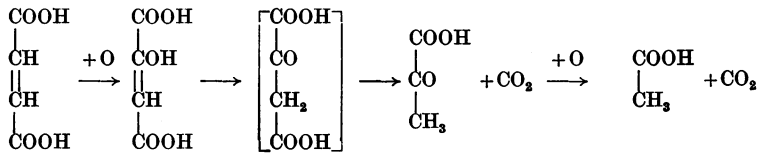
1. Both succinic and fumaric acids are fermented by *B. pyocyaneus* to give mixtures of the lower fatty acids—in the case of fumaric acid chiefly acetic acid.

2. The rate of utilisation of fumaric acid by *B. pyocyaneus* is much more rapid than that of succinic acid, a 1 % solution of ammonium fumarate being entirely utilised in 30 hours. The rate of utilisation in both cases is accelerated by aeration. Ammonium carbonate is produced with increase of p_H. Details of the methods of analysis of the succinate and fumarate media are given.

3. Pyruvic acid can be isolated in the form of the *p*-nitrophenylhydrazone from a fermenting fumarate medium and is responsible for two colour tests—with nitroprusside and with guaiacol—which are given by this medium. These two tests provide an excellent method of differentiating *B. pyocyaneus* and *B. fluorescens liq.* (both of which give the tests) from other bacteria which are capable of growing on a fumarate medium.

4. Investigations have been made on the quantity of oxygen taken up and carbon dioxide produced in the fermentation of a known amount of

fumaric acid. It has been shown that these are practically the theoretical quantities deduced on the basis that the following scheme represents the main course of the fermentation of fumaric acid.



5. A balance sheet between fumaric acid and the products of its fermentation is given, in which over 90 % of the former can be accounted for.

6. The results of a number of preliminary experiments dealing with the relative differences in fermentative properties of the (*a*) washed and dried organism; (*b*) washed fresh, or "resting" organism; (*c*) growing organism, are discussed.

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