THE DECOMPOSITION OF CITRIC ACID BY BACILLUS AERTRYCKE

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The ease with which certain pathogenic bacteria grow in media composed of simple chemical substances, such as ammonium citrate and a few inorganic salts, presents the possibility of a detailed study of the chemical changes caused by these bacteria. Since many non-pathogenic organisms also are known to decompose such media, a comparison of their action with that of the pathogens can be made. From relatively simple salts, the bacteria are able to synthesize all the complex materials which are necessary for their growth. As a result of bacterial action, the substrate is decomposed into smaller molecules, some of which can be isolated and identified.

Some results of a study of the decomposition of citric acid by a laboratory strain of Bacillus aertrycke are reported here. This bacillus is a member of the paratyphoid Type B group, pathogens which have the special ability to grow well on a synthetic citrate medium (1). This medium is not readily contaminated, and is composed only of well known chemically pure substances.

The course of the decomposition of citric acid by Bacillus aertrycke was followed by the use of a convenient procedure for the determination of citrate which is described elsewhere (2). Examination of the medium at various stages of the decomposition showed that formic and acetic acids were produced early in the process. 1 mole of citric acid through the action of the bacteria gave rise to 0.25 mole of formic acid, 1.5 moles of acetic acid, and nearly 0.75 mole of succinic acid, in addition to carbon dioxide. Study of the volatile acid fraction showed that under the conditions used, formic and acetic acids were the only volatile acids.
produced in appreciable amounts. This fraction was isolated by distillation \textit{in vacuo}, a method which, with certain precautions, has proved both rapid and reasonably accurate. Succinic acid was then isolated from the residue by extraction with ether.

The results of this study differ from those reported in the literature concerning the action of other organisms on citric acid, first, in the demonstrated presence of formic acid; second, in the rapid production of relatively large amounts of succinic acid; third, in the somewhat diminished production of acetic acid; fourth, in the absence of demonstrable amounts of acetonedicarboxylic acid or acetone resulting from its decomposition; and fifth, in a comparison of the products formed by the action of rough and smooth forms of the same organism, with the identification of lactic acid produced by the smooth form.

Studies of the products of decomposition of citric acid by other organisms have been made by a number of workers. Terada (3) obtained a rather large yield of succinic acid by the prolonged action of an unclassified organism from the air. The action of \textit{Bacillus suipestifer} on citrate in the presence of peptone has been studied by Brown, Duncan, and Henry (4) who identified acetic acid, carbon dioxide, and a trace of succinic acid as the products. Some of the volatile acid was formed from the peptone. Bosworth and Prucha (5) reported that the decomposition of citric acid in milk by \textit{Bacillus lactis aerogenes} yields 2 moles of acetic acid for 1 of citric acid. Butterworth and Walker (6) in studying the action of \textit{Bacillus pyocyaneus} on citric acid found acetonedicarboxylic acid and acetone produced by its partial decomposition; malonic and acetic acids were then isolated and a trace of succinic acid was found.

In the decomposition of citrate by \textit{Aspergillus niger}, Walker, Subramaniam, and Challenger (7) have established a series of products which likewise begins with acetonedicarboxylic acid. This is followed in turn by malonic, acetic, glycolic, glyoxylic, and finally oxalic acid. No succinic acid was reported. Wieland and Sonderhoff (8) studied the anaerobic oxidation of citric acid by yeast. They found acetic acid and a small amount of succinic acid, but were unable to show that hydrogen was a product of the decomposition, although they had reason to expect it.
EXPERIMENTAL

Synthetic Bacteriological Culture Medium

A medium similar to those suggested by Braun and Cahn-Bronner (9), Pesch (10), and Koser (1) has been prepared. Excellent growth was secured with several strains of *Bacillus paratyphosus* B and *Bacillus aertrycke* in the medium, prepared from chemically pure salts, of the following composition.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diammonium citrate, anhydrous, gm.</td>
<td>1.0</td>
</tr>
<tr>
<td>Dipotassium phosphate, &quot;</td>
<td>1.0</td>
</tr>
<tr>
<td>Sodium chloride, gm.</td>
<td>0.5</td>
</tr>
<tr>
<td>Magnesium sulfate, hydrated, gm.</td>
<td>0.1</td>
</tr>
<tr>
<td>Ferric chloride 0.01 per cent, drops</td>
<td>3</td>
</tr>
<tr>
<td>Calcium &quot; 0.01 &quot; &quot; &quot;</td>
<td>3</td>
</tr>
<tr>
<td>Distilled water, ml.</td>
<td>100</td>
</tr>
<tr>
<td>pH</td>
<td>6.0</td>
</tr>
</tbody>
</table>

The medium was filtered and sterilized by autoclaving at 120° for half an hour. For the purposes of this study, it proved desirable to subculture in the synthetic medium at least ten times in order to secure rapid and uniform growth from small inocula. Tubes of 50 ml. capacity containing 15 ml. of medium were sterilized and used for transfers, which were made with a 2 mm. platinum loop. Incubation at 37° of a 100 ml. portion inoculated in the same way resulted in a heavy turbidity in 24 hours with disappearance of nearly all of the citrate. The medium became more alkaline. Growth was particularly heavy at the surface and took place best when the medium was exposed to the air in shallow layers.

A bacteriological study of the stock culture after 233 transfers in the synthetic medium was secured through the kindness of Dr. Carl Ten Broeck. The organisms consisted of rough and smooth forms of *Bacillus aertrycke*. The former, which greatly predominated, were motile and in part greatly elongated. The latter were short motile rods, more actively motile when transferred to broth. Both forms produced gas from glucose, the rough form acting much more rapidly. Both forms were agglutinated with specific immune serum in dilutions of 1:12,000 for the smooth strain and 1:50,000 for the rough strain, in which there was a tendency to spontaneous
agglutination; this dilution was near the limit for the immune serum which was used.

Methods and Apparatus—For chemical study 1 liter of solution in a 3 liter round bottomed sterile flask plugged loosely with cotton was inoculated with the platinum loop and incubated at 37° or in some cases at 34°. At intervals 100 ml. portions were withdrawn under sterile conditions for chemical examination. Determination of residual citrate, together with formic, acetic, and succinic acids, was made.

Duplicate determination of citrate in the sample required 2 ml. and was accomplished by the use of Denigès’ reaction (2). The remaining 98 ml. were filtered through a Seitz filter with two 5 ml. washings, giving a water-clear filtrate. Volatile acidity was determined by adding 9 ml. of 20 per cent phosphoric acid and distilling in a modified Claisen flask at a water pump vacuum of about 20 mm. The distilling flask had a capacity of 250 ml. and was provided with a 20 cm. indented tubulation. When the residual material had largely solidified, two 15 ml. portions of water were added and distilled separately to sweep over any residual volatile acid. The volume of the distillate was determined and 25 ml. taken in duplicate for titration with standard sodium hydroxide. The end-point was determined in boiled solution with phenolphthalein as indicator. Phosphate was uniformly absent, but variable amounts of hydrochloric acid, produced from the sodium chloride of the medium, were present. To the neutralized sample was therefore added 1 ml. of 10 per cent potassium chromate, chloride ion was titrated with 0.05 N silver nitrate, and the total acidity was corrected for the amount of hydrochloric acid thus determined.

Formic acid was determined in 2 ml. of the distillate by adding 2 ml. of the mercuric chloride-sodium acetate reagent of Franzen and Egger (11). The solution heated at 100° for 4 hours in a centrifuge tube deposited calomel, which was washed, dried, and weighed in the same vessel.

The non-volatile residue from the distillation was transferred to a long 30 ml. test-tube by rinsing with three or four 5 ml. portions of hot water. Two short, dense plugs of absorbent cotton between which a layer of anhydrous sodium sulfate was inserted were next placed at the mouth of the tube, well above the fluid,
and around the funnel tube used in ether extraction (12). The whole was then continuously extracted with ether for 24 to 36 hours. The receiver was changed at the end of 5 hours in order to obtain some fractionation of the extract.

The procedure was the outcome of study of many cultures. It was found necessary to remove the bacteria by filtration in order (a) to avoid excessive foaming which sometimes occurred during distillation in the presence of bacterial bodies, (b) to lessen the production of emulsions during ether extraction, and (c) to avoid the introduction of impurities extracted from the bacterial bodies. Since filtration was frequently slow, the effects of using the unfiltered culture were studied. There was no significant change either in the quality or quantity of the products. This was determined by dividing a 200 ml. culture into 2 equal parts, one of which was filtered, the other not. The amounts of acetic and formic acids from each were the same. The non-volatile residue from the filtered specimen gave a nearly clear water solution, but that from the unfiltered part upon addition of water left a wax-like material which weighed less than 0.01 gm., and was readily separated by filtration. The foaming which occasionally became serious during the distillation of this sample was controlled by the addition of a drop of octyl alcohol as often as necessary. Control tests showed that this did not alter the amount of either fraction.

The formation of emulsions during ether extraction caused little difficulty with the extracting arrangement described. In spite of the absence of mechanical entrainment of phosphoric acid, after long extraction a detectable amount of phosphate was found in the residue. Since, when the decomposition of citrate is complete, the non-volatile fraction is all extracted in 5 hours, this difficulty has not in general been found serious.

In order to show the reliability of the method of volatile acid determination, studies were made of the recovery of known mixtures of the acids identified below when distilled with phosphoric acid. By the procedure described, 85 per cent of the volatile acid was recovered in one distillation, and with two washings at least 96 per cent was secured. The missing portion was largely acetic acid.

Identification and determination of the products were made as follows: The presence of formic acid in the distillate was shown not
only by reduction of mercuric chloride to calomel in the reaction used for its determination, but also by the reduction of methylene blue in the presence of sodium sulfite (13). Acetic acid was identified by the preparation of p-nitrobenzyl acetate, melting at 76-79°, from the sodium salt of the distillate and p-nitrobenzyl bromide. A mixed melting point with an authentic sample (78-80°) showed no depression. By subtracting from the standard alkali consumed by the distillate the part due to hydrochloric and formic acids, the amount used by the remaining volatile acid was obtained. This was then calculated as acetic acid.

The conclusions concerning the nature and amount of these volatile acids were confirmed by a Duclaux analysis carried out by the method of Virtanen and Pulkki (14). After 33 hours, a 100 ml. sample of culture was removed from 1 liter of medium inoculated with Subculture 153. The volatile acid distillate, including washings, was 150 ml. Of this, 50 ml. were used for determining titratable acidity, chloride, and formate. This analysis showed that the volatile acids present in 100 ml. were equivalent to 2.2 ml. of 0.1 N HCl, 22.6 ml. of 0.1 N formic acid, and 86.0 ml. of 0.1 N acetic acid. By using the known semidistillation constants for the organic acids and neglecting the hydrochloric acid, the amount of 0.1 N NaOH necessary to neutralize this distillate was calculated to be 36.7 for 50 ml. The observed value was 37.2 ml.

While less than 0.05 ml. of 0.05 N hydrochloric acid appeared in the distillate during semidistillation, the effect of this acid on the constants of the organic acids was unknown. Decreasing ionization would no doubt slightly increase the semidistillation constants. The constants for the particular apparatus used were not determined. The agreement between calculated and observed values is therefore considered to indicate, within the experimental error, that no other volatile acid was present in significant amounts.

The non-volatile residue from cultures grown under the conditions already described consisted of succinic acid in amounts varying for the most part between 0.1 and 0.3 per cent. The succinic acid obtained on evaporation of the ether extracts was remarkably pure, showing a melting point of 180-182° without further treatment. One crystallization from water sufficed to give an analytically pure sample melting at 187.5° (corrected). A titra-
tion equivalent of 59.8 was found (theory 59.0). The methyl ester, prepared by the action of a slight excess of diazomethane, melted at 20° and gave no depression with a known sample.

The quantitative relation between the decomposition products of citric acid at various stages of the growth of the culture is shown in Table I.

In many experiments, the amount of acetic acid was slightly higher than in this case, and the amount of succinic acid between 1 and 2 millimoles, with not much variation of the quantity of formic acid. Carbon dioxide was present whenever tests for it were made. It was identified by the formation of barium carbonate from the CO₂-free air used in aerating some of the cultures.

The distribution of carbon among the various decomposition products of citric acid was studied with both the rough and the pure smooth strains of Bacillus aertrycke isolated from the laboratory strain previously described, after 233 transfers on synthetic citrate medium. Table II shows the carbon distribution found in 100 ml. cultures incubated at 37° after inoculation from a suspension of these forms. The carbon dioxide was absorbed in standard barium hydroxide by aerating, acidifying, and further aerating with carbon dioxide-free air until no more barium carbonate was precipitated. The amount of bacterial bodies was estimated by filtering 10 ml. of the acidified culture through a weighed platinum Munroe crucible, which removed most of the turbidity. After washing thoroughly and drying at 100° in vacuo for 5 hours, the difference in weight multiplied by 10 was taken to be that of the bacterial bodies, for which a carbon content of 50 per cent was assumed.

The carbon balance for the rough form of the organism shows the distribution of 95 per cent of the carbon initially introduced. In

<table>
<thead>
<tr>
<th>Incubation time</th>
<th>Citric acid</th>
<th>Acetic acid</th>
<th>Formic acid</th>
<th>Succinic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>hrs.</td>
<td>mM</td>
<td>mM</td>
<td>mM</td>
<td>mM</td>
</tr>
<tr>
<td>0</td>
<td>4.43</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>19</td>
<td>3.32</td>
<td>1.6</td>
<td>0.35</td>
<td>0</td>
</tr>
<tr>
<td>33</td>
<td>0</td>
<td>6.95</td>
<td>1.02</td>
<td>3.06</td>
</tr>
</tbody>
</table>

Table I Products Formed during Growth of Bacillus aertrycke on Citric Acid
Decomposition of Citric Acid

addition, 1 per cent was used for the determination of citric acid; 96 per cent is thus accounted for. For the smooth form 92 per cent of the initial carbon is accounted for in the products, and an additional 1 per cent was used for the citric acid estimation. The chief sources of error appear to lie in the determination of the weight of the bacterial bodies and perhaps in the incomplete extraction of the non-volatile acid fraction. Lactic acid was identified regularly in smooth cultures, but not in rough cultures or in the ordinary Bacillus aertrycke culture under the conditions used.

### TABLE II

**Carbon Distribution in Synthetic Citrate Medium after Incubation with Rough and Smooth Forms of Bacillus aertrycke**

<table>
<thead>
<tr>
<th>Substance found</th>
<th>Rough form (1 loop inoculum)</th>
<th>Smooth form (0.5 ml. inoculum)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amount after</td>
<td>Carbon after</td>
</tr>
<tr>
<td></td>
<td>0 hrs</td>
<td>40 hrs</td>
</tr>
<tr>
<td>Acids</td>
<td>mg.</td>
<td>mg.</td>
</tr>
<tr>
<td>Carbonic (CO₂)</td>
<td>0</td>
<td>376</td>
</tr>
<tr>
<td>Formic</td>
<td>0</td>
<td>23</td>
</tr>
<tr>
<td>Acetic</td>
<td>0</td>
<td>199</td>
</tr>
<tr>
<td>Succinic</td>
<td>0</td>
<td>130</td>
</tr>
<tr>
<td>Lactic</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Citric</td>
<td>850</td>
<td>110</td>
</tr>
<tr>
<td>Bacteria</td>
<td>0</td>
<td>40</td>
</tr>
<tr>
<td>Total</td>
<td>319</td>
<td>302.9</td>
</tr>
</tbody>
</table>

Identification was made by the thiophene reaction and was substantiated by the guaiacol, codeine, and acetaldehyde tests (15).

A comparison of the rough and smooth strains through a number of experiments showed that the rough type regularly had a denser, more rapid growth; produced more succinic acid; formed less formic acid, in some cases none whatever; formed less acetic acid; evolved more carbon dioxide; and produced no lactic acid. A further comparison of these forms is being made.

**DISCUSSION**

The identification of formic, acetic, lactic, succinic, and carbonic acids in a synthetic citrate medium inoculated with Bacillus aer-
trycke shows that a very thorough decomposition of the citrate molecule has taken place. The precise manner in which these substances are formed is not yet clear, but important processes are evidently concerned with oxidation, since the most rapid action occurs in the aerated culture. Since acetonediacarboxylic acid can be obtained from citric acid by the action of various oxidizing reagents, this acid might be a product of the bacterial action. No evidence of its presence was found, however, nor of acetone, into which it readily decomposes. Moreover, growth on a medium composed of acetonediacarboxylic acid and the requisite salts both with and without formate was feeble, and produced no non-volatile and little volatile acid. It therefore appears improbable that this substance is an intermediate in the decomposition. In view of the identification of lactic acid, the possibility that pyruvic acid or aldehyde may be an intermediate (16, 17) deserves investigation.

The formation of succinic acid in relatively large amounts appears to require some other mechanism than that proposed by Brown, Duncan, and Henry (4), who assumed free radicals as intermediates. Grey (18) had proposed the same mechanism to explain the action of Bacillus coli on citrate in the presence of calcium formate, by which acetic and succinic acids were formed. Butterworth and Walker (6) have likewise resorted to the assumption of free radical formation. It is evident from the equations proposed by Brown, Duncan, and Henry that for every molecule of succinic acid isolated, a molecule of formic acid should be found. The data in Table I show, however, that this is not the case, for 3 times as many molecules of succinic acid as of formic acid were isolated. The separate rough and smooth forms likewise fail to produce the expected ratio (Table II). The bacteria are able to utilize succinate but not formate under these experimental conditions, so that the amount of formic acid remains nearly constant in a series of experiments, while the quantity of succinic acid varies. The mechanism proposed by these authors therefore does not appear to explain the decomposition of citric acid by Bacillus aertrycke.

The formation of succinic acid from the protein constituents of the bacteria (19) does not account for the data under discussion, since the amount of acid is far greater than the total amount of protein. Acetic acid can scarcely be the stage preceding succinic
Decomposition of Citric Acid

acid (16, 20, 21) for in synthetic media the bacteria fail to utilize acetate at hydrogen ion concentrations more acid than pH 7, whereas the optimum pH for the production of succinate lies between 5 and 6. Whether a hydroxyadipic acid (17) may be the precursor in this case, there are not sufficient data to decide. A substituted acetic acid is also worth consideration (22) in a discussion of this question.

While both the rough and smooth forms produce succinic acid, the former gives a larger yield and acts more rapidly; the inoculum of the smooth form was much larger than that of the rough in the experiments shown in Table II. The rough form produces much less formic acid than the smooth, and appreciably less acetic acid. The cultural differences between these forms are those commonly found between rough and smooth forms; the rough is more resistant, and the smooth is more sensitive to changes in environment. Both forms have maintained their cultural and biochemical characteristics in this simple medium through twenty transfers. The more selective action of the smooth form is no doubt responsible for the isolation of lactic acid. Since the substance isolated is optically inactive, it may be the result of a dismutation or other non-enzymatic reaction which has time to occur in the smooth but not the rough culture.

SUMMARY

1. A study was made of the chemical changes which occur when *Bacillus aertrycke* is cultivated on a synthetic citrate medium with ample exposure to the air.

2. The products identified were formic, acetic, and succinic acids and carbon dioxide.

3. The ratio of succinic to formic acid which was found renders improbable the assumption that free radicals play an important part in the decomposition of citrate by bacteria.

4. Rapid and convenient methods for the determination of citric and acetic acids were developed.

5. Comparison of the rough and smooth forms of *Bacillus aertrycke* showed that the smooth form produces more formic and acetic acids, less carbon dioxide and succinic acid; and in addition, small amounts of lactic acid.
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