BUTYRATE OXIDATION IN THE ABSENCE OF INORGANIC PHOSPHATE BY CLOSTRIDIUM KLUYVERI*

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Preparations of soluble, cell-free enzymes of the bacterium Clostridium kluyveri, capable of carrying out both the synthesis and the oxidation of short chain fatty acids, have recently been described in a series of reports by Stadtman and Barker (16-21). The stability and activity of these enzyme preparations offer great advantages for the study in vitro of the mechanisms by which fatty acids are synthesized and metabolized in living organisms. These properties are in striking contrast to the extreme lability of the enzyme systems which carry out the oxidation and synthesis of fatty acids in animal tissues (1, 8, 13).

In the present study, attention has been centered on the series of reactions by which fatty acids are oxidized in enzyme preparations of C. kluyveri. There is considerable evidence which warrants the belief that the mechanism by which fatty acids are oxidized in this system consists simply in the reversal of the mechanism of synthesis. For this reason, information obtained about the oxidative process should be applicable to the problem of fatty acid synthesis. Technically, it is easier to study the oxidative than the synthetic reactions. Butyric acid has been selected for special attention, since possible intermediates involved in the reaction sequence are more readily available for this acid than for higher homologues.

Methods

Enzyme preparations used in this work consisted of dried cells of C. kluyveri, or of cell-free, soluble enzymes extracted from dried cells, prepared by the method of Stadtman and Barker (16). Since the enzymes responsible for butyrate oxidation are readily inactivated by exposure to oxygen in the absence of substrate, the dried cells suspensions were prepared in vacuo. The desired quantity of dried cells was placed in a Thunberg tube and water or buffer containing 0.05 per cent Na$_2$S·9H$_2$O

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adjusted to pH 7 with H$_2$SO$_4$ was placed in the hollow stopper. The tube was evacuated and the contents were mixed thoroughly. Aliquots of the resulting suspension were added to the reaction mixture just before the start of each experiment.

Most of the experiments were performed in Warburg vessels, and the gas exchange was measured by conventional methods.

Acetoacetate was measured by the aniline citrate decarboxylation method of Edson (6) or by the method of Greenberg and Lester (9). Distribution of the radioactivity in acetoacetate was measured essentially as described previously by Stadtman and Barker (19).

Counter-current distribution studies on the 2,4-dinitrophenylhydrazone of acetoacetate and on acetic and butyric acids were made according to techniques and with the apparatus described by Craig et al. (3, 15).

The 2,4-dinitrophenylhydrazone of acetoacetate was isolated from enzyme suspensions by the following method: 6.0 ml. of the enzyme suspensions were treated with 21 ml. of a saturated solution of 2,4-dinitrophenylhydrazine in 95 per cent ethanol. The protein precipitate was removed by centrifugation. 1.2 ml. of 50 per cent citric acid were then added to the supernatant, and the solution was held at room temperature for 10 minutes. The reaction mixture was extracted with two successive 10 ml. portions of benzene. The combined benzene extracts were then extracted with 10 ml. of 0.1 M phosphate buffer, pH 7.0. The 2,4-dinitrophenylhydrazone of acetoacetate passed quantitatively into the aqueous phase. The benzene layer, containing unchanged 2,4-dinitrophenylhydrazone and neutral hydrazones, was discarded. The aqueous layer was acidified with 0.4 ml. of 50 per cent citric acid and extracted with 10 ml. of benzene. The 2,4-dinitrophenylhydrazone of acetoacetic acid passed into the benzene phase. The benzene extract was washed with distilled water and an aliquot introduced into the Craig apparatus. The distribution procedure was then carried out with mutually saturated benzene and 0.1 M phosphate buffer, pH 5.46, as solvents. At the end of the run, each tube was analyzed for total 2,4-dinitrophenylhydrazones by a modification of the method of Friedemann and Haugen (7).

Acetic and butyric acids, isolated from the enzyme digests by steam distillation, were separated in the Craig apparatus by distribution in butanol and 0.1 M citrate buffer containing 1 per cent KCl. The pH of the aqueous phase after equilibration was 4.1. Radioactive fatty acids were used in this procedure, and at the end of the run, the distribution of the components was determined by measuring the radioactivity in each tube.

Fatty acids were identified either by Duclaux distillation or by the application of paper chromatography (12).

Samples of $dl$-threo-$\alpha,\beta$-dihydroxybutyric acid, $\gamma$-butyrolactone, aconic
Acid, and isocrotonic acid were generously provided by Dr. D. E. Green. Samples of barium dl-α-hydroxybutyric acid and sodium α-ketovaleric acid were donated by Dr. Alton Meister. dl-β,γ-Dihydroxybutyrolactone and β,γ-epoxybutyric acid were synthesized by R. G. Bartsch of this laboratory, using the methods of Nef (14) and Hartenstein (11), respectively. The lactone was converted to the dihydroxybutyrate by treatment with a slight excess of sodium hydroxide at room temperature. Succinic semialdehyde was prepared by heating an aqueous solution of aconic acid (10); the pure compound was not isolated.

Other methods used in the course of this work have been previously described (16–21).

**EXPERIMENTAL**

Oxidation of Fatty Acids in Absence of Inorganic Phosphate—Enzyme preparations of C. kluyveri have been shown to oxidize butyrate to acetyl phosphate and acetate according to Reaction 1 (17). Furthermore, it was observed that the oxidation of butyrate was dependent upon the presence

\[
\text{CH}_3\text{CH}_2\text{CH}_2\text{COO}^- + \text{O}_2 + \text{HPO}_4^{2-} \rightarrow \text{CH}_3\text{COOPO}_4^{2-} + \text{CH}_3\text{COO}^- + \text{H}_2\text{O}
\]  

of inorganic phosphate; in the absence of phosphate, no uptake of oxygen occurred. However, further investigation of this point has shown that when the enzyme preparations are buffered with either tris(hydroxymethyl)aminomethane (TRIS) or bicarbonate, they catalyze a fairly rapid oxidation of fatty acids in the absence of inorganic phosphate. Data on oxygen uptake with butyrate in TRIS and in phosphate buffers are given in Fig. 1.

It will be noted that the rate of oxidation in the absence of phosphate is only about one-third that in the presence of phosphate. Since a small amount of inorganic phosphate, about 1.5 μM per 50 mg. of dried cells, was present in the enzyme preparation, the possibility was considered that the observed oxidation might depend upon the catalytic action of this phosphate. The latter could be converted to acetyl phosphate during the oxidation of butyrate and then be regenerated by the action of the acetylphosphatase known to be present in the preparation (21). By such a cyclic series of reactions, a trace of phosphate could cause the oxidation of a large amount of butyrate.

Two types of experimental evidence rule out the above hypothesis. First, the effect of the addition of barium chloride to the reaction mixture may be noted. Since barium phosphate is very slightly soluble at pH 8, it would be expected that the addition of barium chloride at this pH would minimize the effect of the inorganic phosphate present in the preparation. Actually, the rate of oxygen uptake was decreased very little if at all by
the addition of 10 μM of BaCl₂ to 2 ml. of the reaction mixture containing 50 mg. of dried cells and 20 μM of sodium butyrate at 7.05 M TRIS buffer, pH 7.8.

More conclusive evidence against a catalytic rôle of inorganic phosphate was provided by the demonstration that the oxidation of butyrate follows a different course in the presence and absence of added phosphate.

According to Reaction 1, which has been confirmed in the present study, the oxidation of butyrate in the presence of phosphate results in the formation of 1 mole of acetyl phosphate and 1 mole of acetate for each mole of oxygen consumed. Since acetyl phosphate breaks down to acetic acid under the conditions of the determination of steam-volatile acid, there is also an increase of 1 equivalent of volatile acid per mole of oxygen used. When the oxidation of butyrate is carried out in TRIS rather than in phosphate buffer, it is obvious that acetyl phosphate cannot accumulate. More significant was the finding that the steam-volatile acid titer did not increase as the reaction proceeded, as would have been necessary if the products of the reaction were 2 moles of acetic acid, formed by the breakdown of acetyl phosphate with "catalytic" regeneration of inorganic phosphate. On the contrary, a net decrease in steam-volatile acid was observed. Data illustrating this finding are given in Table I.
In order to ascertain the oxidation level of the product of butyrate oxidation in the absence of phosphate, oxygen uptake was measured in experiments in which limiting amounts of butyrate were added to the enzyme system. The results show that approximately 1 mole of oxygen (1.06 and 1.18 moles) was consumed per mole of added butyrate. Thus the extent.

**Table I**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>O₂ uptake</th>
<th>Δ steam volatile acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butyrate</td>
<td>7.4</td>
<td>-5.1</td>
</tr>
<tr>
<td>Caproate</td>
<td>5.9</td>
<td>-2.0</td>
</tr>
</tbody>
</table>

The experiments were conducted in Warburg vessels at 26° with air as the gas phase. Each vessel contained 50 mg of dried cells, Lot T, and 20 μM of the indicated substrate in 2 ml of 0.05 M TRIS buffer, pH 7.9. The values are corrected by subtracting the small oxygen uptake and volatile acid formation observed in vessels without added substrate. The duration of the experiments was 90 to 120 minutes.

**Table II**

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Substrate</th>
<th>O₂ uptake</th>
<th>Acetoacetate formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Butyrate</td>
<td>11.4</td>
<td>5.1</td>
</tr>
<tr>
<td>2</td>
<td>&quot;</td>
<td>8.3</td>
<td>5.0</td>
</tr>
<tr>
<td>3</td>
<td>&quot;</td>
<td>13.8</td>
<td>5.7</td>
</tr>
<tr>
<td>4</td>
<td>&quot;</td>
<td>8.6</td>
<td>3.8</td>
</tr>
<tr>
<td>5</td>
<td>Vinyl acetate</td>
<td>4.2</td>
<td>4.2</td>
</tr>
</tbody>
</table>

The Warburg vessels contained either 20 μM of butyrate or 88 μM of vinyl acetate as substrate in a final volume of 2.0 ml of 0.05 M TRIS buffer, pH 7.8. Other experimental conditions are the same as in Table I. Acetoacetate was determined by the aniline citrate method (6).

of butyrate oxidation is the same whether or not inorganic phosphate is added, the butyrate in either case being converted to the level of acetate or a keto acid. Since the data on volatile acid changes rule out the formation of 2 moles of acetate per mole of butyrate, it was certain that some other product (or products) must be formed. Therefore the reaction mixture was examined for the presence of acetoacetate by the method of Edson (6). The data from four experiments, given in Table II, show that considerable quantities of a β-keto acid are formed in the oxidation of butyrate in the absence of inorganic phosphate.
Since the aniline citrate method of Edson is not entirely specific for acetoacetate, other evidence was sought to identify the $\beta$-keto acid. Acetone was determined by the method of Greenberg and Lester (9) and the results were found to be in satisfactory agreement with those for carbon dioxide evolution obtained by the aniline citrate method. In addition, the 2,4-dinitrophenylhydrazone of the $\beta$-keto acid was isolated from the enzyme mixture by counter-current distribution in the Craig apparatus. The distribution coefficient of the substance isolated was 0.29, closely similar to the value of 0.32 observed for the 2,4-dinitrophenylhydrazone of synthetic acetoacetate.

The oxidation of vinyl acetate in the absence of phosphate was shown also to result in the formation of acetoacetate in about the same yield as from butyrate.

The finding that acetoacetate is a product of the oxidation of butyrate and vinyl acetate in the absence of phosphate is somewhat surprising. Acetoacetate is known not to be an intermediate in the sequence of reactions by which butyrate is oxidized to acetyl phosphate and acetate in the presence of phosphate. This fact was demonstrated in earlier studies by Stadtman and Barker (19) and was confirmed in the present investigation. Although acetoacetate is converted to acetyl phosphate and acetate by these enzyme preparations at pH 6.5, it is only very slightly reactive at pH 7.8, a pH value at which the oxidation of butyrate proceeds very rapidly (17). Furthermore, the reduction of acetoacetate in an atmosphere of hydrogen proceeds only to the stage of $\beta$-hydroxybutyrate under conditions in which acetyl phosphate and acetate are reduced completely to butyrate (19). Also, it has been shown that acetoacetate is not in isotopic equilibrium with acetyl phosphate and acetate. Finally, the oxidation of C$^{14}$-labeled butyrate in the presence of unlabeled acetoacetate does not result in the incorporation of C$^{14}$ in the latter. These considerations completely eliminate the possibility that acetoacetate is a normal intermediate in the oxidation or synthesis of butyrate.

Mechanism of Formation of Acetoacetate—Studies of the oxidation of octanoate in animal tissues by Weinhouse et al. (23), Buchanan et al. (2), and Crandall et al. (4, 5) have shown that acetoacetate is formed mainly by the recondensation of 2-carbon units derived from the fatty acid. Thus when octanoate labeled in the carboxyl position with C$^{13}$ was used as substrate, the acetoacetate was found to have C$^{13}$ distributed in both the carboxyl and the carboxyl carbons. It was of interest to determine whether the same mechanism was involved in the formation of acetoacetate from butyrate in C. kluyveri preparations. Accordingly, butyrate labeled in the carboxyl position with C$^{14}$ was oxidized in the absence of added inorganic phosphate. At the end of the experiment, the acetoacetate was
decarboxylated with aniline citrate, and the resulting carbon dioxide collected as barium carbonate. The acetone moiety of the acetoacetate was isolated as the 2,4-dinitrophenylhydrazone without the addition of carrier. The radioactivity of these two fractions was then determined. All of the radioactivity of the acetoacetate was found to be present in the carboxyl carbon. The acetone fraction was completely devoid of activity. The data of such an experiment are summarized in Table III. It is clear from these findings that in this system acetoacetate is not formed by a random recombination of 2-carbon units. Probably the C₄ chain remains intact during the conversion of butyrate to acetoacetate.

**Effect of Hydroxylamine**—The rate of oxidation of butyrate in the absence of phosphate is about one-third the rate in its presence. Also the rate in the absence of phosphate appears to fall off somewhat more rapidly with time. These results suggested that the slower rate without phosphate might be due to the accumulation of acetoacetate. This possibility was tested by observing the effects of adding acetoacetate and hydroxylamine in separate experiments. The latter was expected to remove acetoacetate from the reaction mixture by formation of the oxime.

The addition of acetoacetate in concentrations from 5 to 10 μM per ml. was found not to retard the oxidation of butyrate in TRIS buffer. However, the addition of hydroxylamine markedly stimulated the rate of oxidation in the absence of phosphate (Fig. 2). Contrary to expectation, this stimulation was not caused by the removal of acetoacetate as the oxime but by a change in the course of the reaction so as to produce acetate instead of acetoacetate. This was first indicated by an increase in steam-volatile acid, as shown in Table IV, instead of the decrease that

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**Table III**

<table>
<thead>
<tr>
<th>Substance</th>
<th>Specific activity (c.p.m. per μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial butyrate, carboxyl carbon</td>
<td>207</td>
</tr>
<tr>
<td>Acetoacetate formed, carboxyl carbon</td>
<td>190</td>
</tr>
<tr>
<td>Acetone moiety</td>
<td>0</td>
</tr>
</tbody>
</table>

The experiment was carried out in a 125 ml. Erlenmeyer flask. The final concentration of carboxyl-labeled butyrate was 0.02 M. TRIS buffer of pH 8.0 was used to maintain the pH. Barium chloride (0.005 M) was added to minimize the effect of small amounts of inorganic phosphate. The final volume of the system was 30 ml. 1.0 gm. of dried cells of Lot O was used, and the vessel was shaken at 26° in air for 2 hours. At the end of the reaction, the acetoacetate formed was decarboxylated with aniline citrate, and the carboxyl carbon recovered as barium carbonate. The acetone was isolated, recrystallized, and counted as the 2,4-dinitrophenylhydrazone.
occurs in the absence of hydroxylamine and phosphate (Table I). Micro-Duclaux distillation of the volatile acid showed that the product was acetic

![Graph](image)

**Fig. 2.** Influence of hydroxylamine on butyrate oxidation. Each vessel contained 50 mg. of dried cells of Lot T, 20 \(\mu\)M of sodium butyrate, and 40 \(\mu\)M of hydroxylamine as indicated in 2.0 ml. of 0.05 M TRIS buffer, pH 8.0.

**Table IV**

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Hydroxylamine added (\mu)M</th>
<th>Butyrate added (\mu)M</th>
<th>(\Delta)Pm uptake</th>
<th>(\Delta) volatile acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>45</td>
<td>40</td>
<td>20</td>
<td>15.1</td>
<td>5.8</td>
</tr>
<tr>
<td>46</td>
<td>400</td>
<td>200</td>
<td>10.3</td>
<td>-7.7</td>
</tr>
<tr>
<td>47</td>
<td>400</td>
<td>200</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

All experiments were performed in 0.05 M TRIS buffer, pH 7.9. The final volume of the system was 2.0 ml. in Experiment 45 and 20 ml. in Experiments 46 and 47. In Experiments 46 and 47, manometric measurements were not made, and the reactions were carried out in 125 ml. Erlenmeyer flasks, shaken in air at 26°. Details of the manometric technique in Experiment 45 were the same as in Table I. 50 mg. of dried cells of Lot T were used in each vessel in Experiment 45, and 500 mg. per vessel in Experiments 46 and 47.

acid. The identification was confirmed by making a paper chromatogram of the products (12).

The mechanism by which hydroxylamine induces the formation of ace-
tate has not been determined, but it has been shown not to involve the participation of acetoacetate. The incubation of acetoacetate and hydroxylamine with the enzyme preparation at pH 8 in the absence of phosphate does not result in the formation of a significant amount of acetate.

The above experiments lead to the conclusion that the slow rate of butyrate oxidation in the absence of phosphate is not due to an accumulation of acetoacetate. The rate-limiting reaction appears to be the conversion of the intermediate at the acetoacetate level into acetoacetate. This point is considered further in the discussion.

During experiments on the oxidation of butyrate in the presence of hydroxylamine and the absence of phosphate, it was found that an unidentified compound accumulated which could be detected by its characteristic absorption spectrum with a peak at 415 μm. The same absorption band was observed, under the above conditions, with a cell-free preparation of Lot S and a dried cell preparation of Lot T, and with either butyrate or vinyl acetate as substrate. The 410 μm absorption was not increased when acetoacetate was incubated with the enzyme under identical conditions, indicating that the compound is not the oxime or other simple derivative of acetoacetate. Furthermore, the compound was not formed when butyrate was oxidized in the presence of both hydroxylamine and inorganic phosphate, when hydroxylamine was incubated with the enzyme preparation in the absence of butyrate and phosphate, or when hydroxylamine was added to a reaction mixture immediately after a small quantity of butyrate had been oxidized to completion in the absence of phosphate.

The above results strongly suggest that the compound responsible for the 410 μm absorption band is a derivative of hydroxylamine and some intermediate in the oxidation of butyrate and vinyl acetate. It possesses several properties, such as reducibility and reactivity with phosphate and arsenate, that would be expected of an intermediate at the acetoacetate level. However, much more work will be required to establish the identity of the compound and its relation to fatty acid oxidation.

Effect of Arsenate on Butyrate Oxidation—It was shown by Stadtman and Barker (21) that arsenate is a potent inhibitor of fatty acid oxidation in enzyme preparations of C. kluyveri when these reactions are carried out in phosphate buffer. This finding was completely confirmed in the present investigation. Table V shows the relation between arsenate concentration and the degree of inhibition of butyrate oxidation in phosphate buffer. Inhibition begins at an arsenate concentration of about 10^{-4} M and becomes almost complete at about 10^{-8} M. In view of these relations we were surprised to find that, in the absence of phosphate, arsenate is markedly stimulatory in the concentration range between 0.001 and 0.002 M.
This was demonstrated by experiments in which TRIS buffer was used to maintain the pH. Upon the addition of increasing amounts of inor-

**Table V**

<table>
<thead>
<tr>
<th>Arsenate concentration</th>
<th>Per cent inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0001</td>
<td>0</td>
</tr>
<tr>
<td>0.00025</td>
<td>23</td>
</tr>
<tr>
<td>0.0005</td>
<td>40</td>
</tr>
<tr>
<td>0.001</td>
<td>88-100</td>
</tr>
</tbody>
</table>

Each Warburg vessel contained 50 mg. of dried cells, of Lot T and 100 \( \mu \text{M} \) of sodium butyrate in 2 ml. of 0.05 M phosphate buffer, pH 7.9. Inhibition is calculated on the basis of oxygen uptake in the first 25 minutes of the experiment, a control vessel without arsenate serving as standard for 100 per cent activity.

Fig. 3. Influence of phosphate and arsenate on butyrate oxidation. Each vessel contained 50 mg. of dried cells of Lot T, 100 \( \mu \text{M} \) of sodium butyrate, and the indicated amounts of phosphate and arsenate in 2.0 ml. of 0.05 M TRIS buffer, pH 7.9.

Organic phosphate in the presence of a constant amount of arsenate, an increasing potentiation of the arsenate inhibition was noted (Fig. 3). It is evident therefore that either arsenate or phosphate alone is stimulatory,
whereas both together are inhibitory. This rather complex relation is not unique, since Stumpf et al. (22) have found an essentially similar behavior of phosphate and arsenate in glutamyltransphorase that catalyzes an exchange of the amide group of glutamine with ammonia and hydroxylamine.

When the oxidation of butyrate is carried out in the presence of arsenate and in the virtual absence of inorganic phosphate, no acetoacetate accumulates. Paper chromatograms of the steam-volatile acids resulting from the reactions demonstrated that acetate is the main product under these conditions.

Possible Intermediates in Oxidation of Butyrate—Present concepts of the mechanism of biological oxidations lead to the expectation that the conversion of butyrate to acetate involves at least two distinct oxidative steps and two intermediates, one at the oxidation level of $\beta$-hydroxybutyrate, the other at the acetoacetate level. In a previous paper (20), several C$_4$ compounds at these oxidation levels were tested, but none was found to possess the necessary properties of an obligatory intermediate. In the present investigation, we have confirmed the results previously reported with vinyl acetate, $\beta$-hydroxybutyrate, crotonate, isocrotonate, and acetoacetate, and have tested several additional compounds, namely dl-$\alpha$-hydroxybutyrate, $\gamma$-hydroxybutyrate, $\gamma$-butyrolactone, dl-threo-$\alpha$, $\beta$-dihydroxybutyrate, dl-$\beta$, $\gamma$-dihydroxybutyrate, $\beta$, $\gamma$-epoxybutyrate, succinic semialdehyde, succinate, and $\alpha$-ketovalerate. The latter compound was used in place of $\alpha$-ketobutyrate, which was not available. In view of the fact that valerate is oxidized as rapidly as butyrate in the C. kluyveri system, it can be anticipated that $\alpha$-keto derivatives also would react similarly.

The reactivity of compounds at the hydroxybutyrate level was determined by testing for oxygen uptake and acetyl phosphate formation in the presence of the enzyme and phosphate buffer, pH 7.8. Controls without substrate and with butyrate were always run simultaneously. Compounds at the ketobutyrate level were tested for ability to undergo a phosphoroclastic conversion to acetate and acetyl phosphate in phosphate buffer at pH 7.8.

The results showed conclusively that none of the newly tested compounds is decomposed at a significant rate in this system. Consequently, these compounds can be eliminated from consideration as possible intermediates in the oxidation and synthesis of butyrate. The results also emphasize the great specificity of the C. kluyveri preparations as compared with enzymes derived from animal sources (8).

In addition to the experiments summarized above, attempts have been made to demonstrate the accumulation of compounds other than acetoacetate and acetate when butyrate is oxidized in TRIS buffer, pH 7.8, in the absence of phosphate. Under these conditions the rate of the reaction
is markedly slower than in the presence of phosphate and some indications had been obtained that the lower rate might be due to or might cause a limited accumulation of intermediate products. An experiment to test this possibility, repeated several times, was carried out by incubating C\textsuperscript{14}-1-butyrate with the enzyme system until it was about one-third decomposed and then examining the reaction mixture for various types of labeled compounds. Results of a typical experiment are given in Table VI. At the end of the incubation period, essentially all of the radioactivity added initially could be accounted for as acetoacetate and steam-volatile acids. No radioactivity was found in the non-volatile residues after steam distillation. The steam-volatile acid fraction contained only acetic and butyric acids, as revealed by paper chromatography and radioautography of the ammonium salts (12). It can be concluded that no stable intermediate accumulated in amounts greater than the over-all accuracy of the methods used (about 0.1 per cent), even though the rate of the oxidation was reduced to about a third by carrying out the reaction in the absence of added inorganic phosphate.

**Oxidation of Butyrate at Various pH Values**—The influence of pH on the rate of butyrate oxidation in the presence of inorganic phosphate has already been described (17). The effect of pH on the oxidation in the absence of inorganic phosphate was studied in an effort to determine whether the accumulation of products other than acetoacetate or acetate could be brought about by a differential pH inhibition of one or more enzymatic steps in the reaction.

It was found that the oxidation of butyrate can be carried out over a wide range of pH values, from 6.5 to 9.5. In Table VII are given the results of a series of such experiments. As the pH of the medium is raised above 7.9, the stimulatory effect of added phosphate diminishes. At pH

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**Table VI**

**Products of Butyrate-1-C\textsuperscript{14} Oxidation at pH 7.8 in Absence of Inorganic Phosphate**

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>Quantity</th>
<th>Total radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Initial butyrate added.</td>
<td>16.1</td>
<td>82,300</td>
</tr>
<tr>
<td>2. Steam-volatile acids recovered.</td>
<td>13.8</td>
<td>64,300</td>
</tr>
<tr>
<td>3. Acetoacetate found.</td>
<td>3.1</td>
<td>(15,900)</td>
</tr>
<tr>
<td>(2) + (3)</td>
<td></td>
<td>80,260</td>
</tr>
</tbody>
</table>

The experiment was carried out in 0.05 M TRIS buffer, pH 7.8, with 50 mg. of dried cells of Lot O and in a final volume of 2.0 ml. The radioactivity of the acetoacetate is calculated on the assumption that the specific activity per micromole is identical with that of the initial butyrate added (see Table III). The manometric details are the same as in Table I.
values above 8.8 oxygen uptake actually proceeds at a higher rate in the absence of inorganic phosphate. The amount of oxygen consumed in the first 20 minutes of the oxidation was used as an index of activity in these experiments. The interpretation of these results is complicated by the fact that at the higher pH values hydrogen peroxide is formed and is used up in side reactions not involving the oxidation of further amounts of substrate. In contrast to this, at pH 7.8, hydrogen peroxide, if formed, is utilized for the oxidation of substrate. Evidence bearing on this point will be presented below.

The yield of acetoacetate as a function of pH was next investigated.

### Table VII

**Influence of pH on Butyrate Oxidation with and without Phosphate**

<table>
<thead>
<tr>
<th>pH</th>
<th>Rate + phosphate (μl.)</th>
<th>Rate - phosphate (μl.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.5</td>
<td>166</td>
<td>51</td>
</tr>
<tr>
<td>7.9</td>
<td>225</td>
<td>70</td>
</tr>
<tr>
<td>8.5</td>
<td>192</td>
<td>100</td>
</tr>
<tr>
<td>8.8</td>
<td>175</td>
<td>102</td>
</tr>
<tr>
<td>9.4</td>
<td>43</td>
<td>137</td>
</tr>
</tbody>
</table>

Stock buffer solutions used in this experiment were made by adding sufficient concentrated NaOH to an equimolar mixture of TRIS and maleic acid to obtain the desired pH as measured with a glass electrode. Each vessel contained a final concentration of 0.1 M TRIS and maleate. 50 mg. of dried cells of Lot O were used per vessel. Inorganic phosphate (100 μM) was added as indicated. Butyrate (20 μM) was added to all vessels. The final volume of the system was 2.0 ml. The manometric procedure is described in Table I.

Data in Table VIII show that the acetoacetate-oxygen uptake ratio falls considerably as the pH of the reaction medium is raised, from a value of 0.56 at pH 7.4 to 0.11 at pH 8.8 and 0.04 at pH 9.4.

An experiment was then designed to find out what product other than acetoacetate was formed at pH 8.8 in TRIS buffer. A technique essentially similar to that described for the experiment summarized in Table VI was used. C\(^{14}\) butyrate was used as the substrate, and at the end of the reaction, an attempt was made to account for all of the radioactivity added. In this experiment, in contrast to those, at pH 7.8, 97 per cent of the radioactivity was found in the steam-volatile acid fraction, indicating a very small formation of acetoacetate or other non-volatile compounds. Radioautographs of paper chromatograms of the steam-volatile fraction indicated the presence of only two substances, acetic and butyric acids. In order to confirm this finding, the steam-volatile acid fraction was also
subjected to counter-current distribution in the Craig apparatus (15). Two fractions, corresponding in distribution coefficients to acetic and butyric acids, were isolated and analyzed by a micro-Duclaux procedure. The Duclaux constants were found to be exactly those of pure acetic and butyric acids, respectively. The results of this experiment offer convincing evidence that no stable compounds other than acetate and small amounts of acetoacetate accumulate to a significant extent when butyrate is oxidized at pH 8.8.

Peroxide Formation—Although C. kluyveri is an obligate anaerobe, enzyme preparations from this organism possess the ability to utilize atmospheric oxygen as the ultimate electron acceptor for the enzymatic oxida-

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>pH</th>
<th>O₂ uptake</th>
<th>Acetoacetate found</th>
<th>AcetoacetateO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.6</td>
<td>11.9</td>
<td>6.2</td>
<td>0.52</td>
</tr>
<tr>
<td></td>
<td>7.0</td>
<td>13.4</td>
<td>7.5</td>
<td>0.56</td>
</tr>
<tr>
<td></td>
<td>7.4</td>
<td>13.0</td>
<td>7.3</td>
<td>0.56</td>
</tr>
<tr>
<td></td>
<td>8.0</td>
<td>11.4</td>
<td>5.1</td>
<td>0.45</td>
</tr>
<tr>
<td>2</td>
<td>8.5</td>
<td>6.1</td>
<td>1.2</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>8.8</td>
<td>7.4</td>
<td>0.8</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>9.4</td>
<td>7.7</td>
<td>0.3</td>
<td>0.04</td>
</tr>
</tbody>
</table>

The experimental conditions were the same as those described in Table VII. No inorganic phosphate was added. The duration of Experiment 1 was 2 hours; Experiment 2, 1 hour. Acetoacetate was determined by the method of Edson (6), and checked by the method of Greenberg and Lester (9).
In the course of experiments at pH 8.8, some evidence was obtained for the hypothesis that hydrogen peroxide is formed, and, at pH 7.8, may be used for the further oxidation of the substrate.

When catalase (Armour and Company, crystalline) was added to the enzyme system containing butyrate in TRIS buffer, pH 7.8, little or no effect on the rate or amount of oxygen uptake was noted. However, when catalase was added to the enzyme system at pH 8.8, oxygen uptake in the initial minutes of the reaction was only a little more than one-half of that observed in the absence of catalase, as shown in Fig. 4. As the reaction proceeded, the vessel to which catalase was added showed considerably more than half the oxygen uptake of control vessels without catalase. This may be due to a protective effect of catalase as a result of the decomposition of peroxide, with a correspondingly more rapid substrate oxidation than in the control without catalase.

These results may be interpreted as indicating the presence of electron-transporting systems that oxidized reduced coenzymes and are in turn oxidized by atmospheric oxygen, with the production of hydrogen peroxide. At pH 7.8, peroxidases present in the enzyme preparation may uti-
lize the hydrogen peroxide so formed for the oxidation of further amounts of reduced coenzymes, so that in the net reaction there is good agreement between oxygen uptake and butyrate removal. However, at pH 8.8, peroxidase activity is presumably greatly impaired, and the hydrogen peroxide cannot be utilized for the oxidation of further amounts of butyrate, but instead is dissipated in unidentified side reactions. No evidence of the accumulation of hydrogen peroxide could be obtained by adding catalase to the control reaction mixture at the end of an experiment such as that shown in Fig. 4.

![Graph showing oxygen uptake in microliters over time for different conditions](image)

**Fig. 5.** Influence of aeration and BAL on butyrate oxidation. Each Warburg vessel contained 50 mg. of dried cells of Lot O in 2.0 ml. of 0.1 M phosphate buffer, pH 7.8. In the unaerated control, 40 mM of sodium butyrate were present in the main compartment and the enzyme was added from the side arm at zero time. In the aerated control vessel, the enzyme was placed in the main compartment in the absence of substrate and was shaken in air for 35 minutes at 26°; then 40 μM of butyrate were tipped in from the side arm at zero time and the oxygen uptake noted. In another vessel, treated in the same way, 40 μM of butyrate and 4 μM of BAL were tipped in after shaking the enzyme for 35 minutes without substrate. The data are corrected for the small oxygen uptake with BAL alone.

If our interpretation is correct, it can be expected that at pH 8.8, 2 moles of oxygen should be taken up for each mole of butyrate oxidized. This is approximately the ratio observed in these experiments.

*Reversible Inactivation of Butyrate-Oxidizing Enzymes—*The investigations of Stadtman and Barker (17) have shown that some of the enzymes derived from *C. kluyveri* are very sensitive to shaking in air in the absence of substrate. Enzyme suspensions shaken at 26° in air for 30 minutes lose almost completely the ability to carry out the enzymatic oxidation of butyrate. In the course of preliminary attempts to fractionate the
enzymes involved in butyrate oxidation by the use of ammonium sulfate it was found that considerable inactivation, presumably due to oxidation, occurred. It was therefore of some interest to determine whether suitable reagents might be found to reverse this oxidative inhibition. To test the effect of these reagents, the crude enzyme suspension was shaken in air for 30 minutes at 26° in the absence of substrate. Butyrate was then tipped in from a side arm, together with a variety of sulfhydryl reagents, such as sodium sulfide, cysteine, thioglycolate, and BAL (dimercapropanol). BAL was found to be most effective in reversing the inhibition. Fig. 5 shows the effect of BAL in such an experiment. Almost complete reversal of the inhibitory effect of shaking in air was noted. Other reagents tested were relatively ineffective.

DISCUSSION

In any interpretation of the experimental findings concerning the oxidation of butyrate to acetyl phosphate and acetate in enzyme preparations of C. kluyveri cognizance must be taken of at least three fundamental and, in part, rather puzzling sets of facts. The first of these is the behavior of vinyl acetate (20). This compound, like butyrate, is readily oxidized to acetyl phosphate and acetate when phosphate is present or to acetoacetate in the absence of phosphate. And like acetyl phosphate and acetate, it is readily reduced by hydrogen to butyrate. In these respects, vinyl acetate displays the properties of an intermediate between butyrate and acetate. Nevertheless, tracer experiments have demonstrated unequivocally that vinyl acetate cannot be a normal intermediate, and, furthermore, it does not appear to undergo any simple transformation into an intermediate, such as the addition of the elements of water or a shift in the position of the double bond. All of the other possible straight chain C₄ compounds at this oxidation level, i.e. the three monohydroxybutyrates and the two α,γ-unsaturated acids, have been shown to be unreactive in this system.

Secondly, one must consider the behavior of acetoacetate and related compounds. Acetoacetate can be formed by the oxidation of butyrate or vinyl acetate and it can be converted to acetate and acetyl phosphate by a phosphoroclastic reaction (19). But despite this proof of the reactivity of acetoacetate, the evidence against its participation in butyrate oxidation and synthesis is conclusive. At the pH that is most favorable for the oxidation of butyrate to acetyl phosphate and acetate, the phosphoroclastic decomposition of acetoacetate is extremely slow (19). Moreover, conditions that allow the rapid reduction of acetyl phosphate and acetate to butyrate, result only in the conversion of acetoacetate to β-hydroxybutyrate. Finally, tracer experiments have demonstrated the
complete absence of interchange between acetoacetate and the intermediates of butyrate oxidation. This indicates that acetoacetate is not itself an intermediate and, like vinyl acetate, is not readily transformed into an intermediate. No other compound at the oxidation level of acetoacetate, thus far tested, is at all reactive.

The third significant fact is that the oxidative steps can proceed at a fairly rapid rate in the absence of inorganic phosphate. This makes it very unlikely that vinyl acetate or acetoacetate is converted to a phosphorylated intermediate by addition of a molecule of inorganic phosphate. If we assume that the path of butyrate and vinyl acetate oxidation is the same in the presence and absence of phosphate, this also means that inorganic phosphate participates only in the final formation of acetyl phosphate from the oxidized product at the acetoacetate level.

To account for the above facts and for our failure to detect the accumulation of intermediates under a variety of experimental conditions, we are led to the theory that the actual intermediates in butyrate oxidation are not C₄ compounds, but are complexes of such compounds with a coenzyme, possibly Lipmann's coenzyme A. According to this hypothesis, illustrated in the accompanying scheme, the first step in butyrate oxidation is the addition of butyrate to the coenzyme (indicated by X in the scheme). The coenzyme may itself require a preliminary activation by reaction with acetyl phosphate. This is definitely indicated by the fact that acetyl phosphate is required for butyrate oxidation by dialyzed enzyme preparations. The butyrate-coenzyme complex must be readily dissociable since butyrate is formed in the synthetic reaction. The butyrate-coenzyme addition product is then oxidized to a vinyl acetate-coenzyme compound. It is at this point that vinyl acetate is assumed to enter the scheme by addition to the free coenzyme to form the same vinyl acetate-coenzyme compound. The latter is rapidly further oxidized to an acetoacetate-coenzyme compound. This product, in the presence of phosphate,
breaks down to yield acetyl phosphate and acetate (or possibly 2 molecules of acetyl phosphate) plus the free coenzyme, which is thus made available to carry out the oxidation of another butyrate molecule. The rate-limiting reaction under these conditions is presumably one of the oxidative steps, probably the initial oxidation of butyrate. In the absence of phosphate, the acetoacetate-coenzyme compound is assumed to decompose much more slowly to acetoacetate plus coenzyme. The assumption of a slow rate is necessary to account for the fact that acetoacetate is not formed in detectable amounts in the presence of phosphate (19). Since the splitting of the acetoacetate-coenzyme compound is presumably rate-making, owing to the limited supply of coenzyme, the over-all rate of butyrate oxidation in the absence of phosphate is much slower than in the presence of phosphate, even though inorganic phosphate is not involved in the oxidative reactions per se. Acetoacetate cannot be converted to either butyrate or acetyl phosphate and acetate under conditions (i.e., pH 8) favorable for butyrate oxidation and synthesis, and therefore the conversion of the acetoacetate-coenzyme complex to acetoacetate must be irreversible at pH 8. At pH 6, which is favorable for the phosphorolytic reaction, acetoacetate is presumably converted to the acetoacetate-coenzyme compound at a relatively slow rate. The latter then reacts with inorganic phosphate to give acetyl phosphate and acetate.

The above theory is based upon the idea that the components of the intermediate substrate-coenzyme compounds are joined by a chemical bond of considerable stability, rather than by weak forces such as are implied in the Michaelis-Menten theory of enzyme action. The concept of a relatively stable chemical bond appears to be necessary to account for the lack of equilibration, demonstrated by tracer experiments (20), between the intermediates and the C\textsubscript{4} compounds, vinyl acetate and acetoacetate. In this connection it may be noted that there is a marked difference in behavior between the compounds at these two oxidation levels. Both the formation and decomposition of acetoacetate are slow. With vinyl acetate, however, the decomposition is rapid, whereas only the formation appears to be slow, judging from tracer experiments. Actually, the latter reaction may also be relatively fast, but appears slow by comparison with the other two possible reactions of the vinyl acetate-coenzyme complex. The relative rates of the competing reactions, rather than the absolute rate of the formation of vinyl acetate, would determine how much C\textsuperscript{14} got into the free vinyl acetate in the tracer experiments mentioned above.

While the foregoing theory can account for most of the data so far obtained, it is by no means the only theory that could be advanced. It should be regarded as a working hypothesis until more direct experimental support has been obtained.
BUTYRATE OXIDATION BY C. KLUYVERI

SUMMARY

Butyrate and vinyl acetate are oxidized to acetoacetate by enzyme preparations of Clostridium kluyveri in the absence of inorganic phosphate. The oxidation of carboxyl-labeled butyrate yields carboxyl-labeled acetoacetate, proving that acetoacetate is not formed by a random condensation of C₂ units. Hydroxylamine accelerates the oxidation of butyrate, apparently by catalyzing the conversion of an unidentified intermediate to acetate. Arsenate can replace phosphate in butyrate oxidation, but, when arsenate and phosphate are added together, the reaction is completely inhibited. Several C₄ compounds have been excluded as possible intermediates in butyrate oxidation. Evidence is presented for the formation of hydrogen peroxide during oxygen utilization by the enzyme preparation. The significance of the above results is discussed in relation to the mechanism of synthesis and oxidation of butyrate.

BIBLIOGRAPHY

BUTYRATE OXIDATION IN THE ABSENCE OF INORGANIC PHOSPHATE BY CLOSTRIDIUM KLUYVERI
E. P. Kennedy and H. A. Barker


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