Butyribacterium rettgeri: a Role of Lipoic Acid in Anaerobic Electron Transport

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Butyribacterium rettgeri is an anaerobic bacterium that ferments glucose, pyruvate, or lactate, with the formation of acetate, butyrate, and CO₂ as the major end products (lactate may also be a major product of the fermentation of pyruvate or glucose). Extensive studies in the laboratory of Barker have shown two unusual features of these fermentations (1-8). The first is that the observed low yield of CO₂ and high yield of fatty acid, reflects a reutilization of CO₂ which is incorporated into both carbon atoms of acetate (2). Tracer studies with intact cells have served to eliminate certain possible pathways of acetate formation from CO₂ such as "reverse di- or tricarboxylic acid cycles," or CO₂ fixation mediated by incorporation of formate into serine (5, 6). However, the nature of the primary CO₂ fixation, and of the intermediary reactions leading to acetate, have remained obscure.†

† Tentative evidence against the participation of some other known possible pathways from CO₂ to acetate is provided by the failure to detect the relevant enzymes in sonic extracts of homogenates of B. rettgeri L (M. Flavin and H. A. Barker, unpublished). In these experiments, B. rettgeri preparations were compared, with appropriate additions to the reaction mixture, with companion extracts of some standard source of the enzyme in question. This approach was adopted after the preliminary observation that it was not possible to observe any CO₂ fixation into acetate with broken cell preparations of B. rettgeri.

Ribulose-di-P-carboxylase could not be detected, in an assay allowing the detection of approximately 1/1000 the activity of spinach leaf extract. With pig heart extract as reference material, allowing the detection of approximately 1/1000 the activity of that it was not possible to observe any CO₂ fixation into acetate with broken cell preparations of B. rettgeri.

The second unusual feature, and the one with which the work to be reported here has been concerned, involves the role of lipoic acid in the fermentation of lactate. Lipoic acid is required for growth of B. rettgeri on lactate as the main energy source, but is not required for growth on pyruvate or glucose (3, 4). Furthermore, the anaerobic fermentation of lactate by resting cells is inhibited by low concentrations of arsenite, whereas that of pyruvate is not (7). These results indicate that lipoic acid is not required for pyruvate oxidation, but rather may play a novel role either in the conversion of lactate to pyruvate, or in the transport of lactate into the cell (7).

The results to be reported here indicate that lipoic acid is involved in the transport of electrons originating in lactate and does not interact directly with the substrate (9).

EXPERIMENTAL PROCEDURE

Materials—All cofactors employed in this study were purchased from the Sigma Chemical Company. dl-Dihydro-α-lipoic acid and the sodium salt of dl-α-lipoic acid were prepared according to the procedure of Wagner et al. (10). Lithium lactate was prepared as described by Barker (11). dl-Lactate-1-C¹⁴ and pyruvate-1-C¹⁴ were obtained from the Volk Radiochemical Company, 5,6-dithiobis-(2-nitrobenzoic acid) from the Aldrich Chemical Company, and acetyl-1,2-C¹⁴-CoA was a gift from Dr. Donald Martin.

Growth Medium—Butyribacterium rettgeri, strain 10825 of the American Type Culture Collection, was routinely cultivated anaerobically at 37° in a complex medium of the following composition: K₂HPO₄, 1.0 g; proteose peptone (Difco), 1.0 g; yeast extract (Difco), 0.5 g; MgSO₄·7H₂O, 0.05 g; (NH₄)₂SO₄, 0.05 g; sodium lactate syrup (60%), 3.4 ml; sodium mercaptoacetate, 0.05 g; dl-α-lipoic acid, 0.007 µg; tap water to a final volume of 100 ml. The organism grown in this manner was designated as strain L. For some experiments, glucose-adapted (G) strains were used. These were obtained by repeated transfer of B. rettgeri L through a glucose-synthetic medium devoid of lipoic acid (3, 5).

Cell Preparations—Resting cell suspensions were prepared from a 24-hour, 1-liter culture. The cells were harvested by centrifugation for 20 minutes at 7000 × g, washed once with 200 ml of 0.01 M potassium phosphate buffer containing 0.001 % glutaraldehyde.

Fixation of CO₂ by B. rettgeri suspensions was stimulated by P-enolpyruvate, or by pyruvate + DPNH + TPN. Lactate + DPN + TPN could not replace the latter. The principle ether-extractable labeled product was lactate. No C¹⁴ was found in succinate, fumarate, or malate.
thione, pH 6.2, and resuspended in 15 to 20 ml of the same solution. The final cell suspension was flushed with H₂ for 5 minutes and sealed with a rubber stopper. Dried cells were prepared by lyophilization of the final suspension; the resulting cell powder was resuspended in the phosphate-glutathione solution just prior to use.

**Cell-free Extracts**—Cells were washed and resuspended as described above and then disrupted either by a 25-minute treatment in the Raytheon 10-ke sonic oscillator, or by passage through an Amino French pressure cell. Unbroken cells were removed by centrifugation at 10,000 × g for 20 minutes. The supernatant fluid was collected and stored under H₂ until used. Unincubated extracts were used in all of the experiments described.

**Analytical Procedures**—Lactate- or pyruvate-decomposing activity by cell suspensions or cell extracts was estimated in some cases from the amount of C⁴O₂ produced from the corresponding C⁴-carboxyl-labeled substrate. The ratio of specific activities of substrate added to CO₂ produced was found on several occasions to be unity, indicating that little CO₂ was formed from substrate carbon atoms other than carboxyl. However, it is to be noted that cell suspensions of *B. rettgeri* convert some of the CO₂ produced from lactate or pyruvate into acetate, so that this procedure gives only a minimal value for the amount of substrate decomposed. Reactions were effected in double sidearm Warburg flasks, which contained NaOH in the center well. The flasks were gassed with H₂ or He for 10 minutes,² after which time the reactions were started by adding substrate from one of the side arms. After a specified incubation period at 37°C, reactions were terminated by adding 6 N H₂SO₄ from the second side arm. Incubations were continued for another 30 minutes to insure complete trapping of CO₂ in the center well. The center well contents were then quantitatively removed from each flask and diluted to 1 ml with water, and 0.1-ml aliquots were assayed for radioactivity in a liquid scintillation counter.

For precise determination of the amount of substrate decomposed, and of the proportions of fermentation products other than CO₂ formed, lactate, pyruvic, acetic, and butyric acids were separated from reaction mixtures by chromatography on Celite columns (12). The acids were quantitatively determined by titration of eluted fractions with standard ethanolic KOH. When known mixtures of 10 to 70 μmoles of each acid were chromatographed, recoveries were within 3% of theoretical. Lipoic acid, when present in reaction mixtures, moved with butyric acid on these columns. In these cases, the butyric acid fractions were pooled and taken to dryness after adding excess alkali. The residue was dissolved in 0.5 ml of 3% peroxide and allowed to stand at room temperature for approximately 20 minutes. This treatment rendered lipoic acid nonsteam-volatile. A pinch of zinc dust was then added with shaking, and the pH of the sample was adjusted to approximately 2.0 with H₂SO₄. Butyric acid was recovered from the sample by steam distillation and was quantitatively determined by titration as described above.

For some studies, pyruvate was determined with DPNH and crystalline lactic dehydrogenase (Worthington). Protein was determined by the biuret method (13). Ferricyanide was determined spectrophotometrically at 420 mp and dihydrolipoic acid was assayed according to the procedure described by Ellman (14) for SH determinations.

**Enzyme Assays**—The enzymatic reduction of ferricyanide by lactate was measured spectrophotometrically at 420 μm (aₕ of ferricyanide = 985). The reaction cuvette contained 100 μmoles of potassium phosphate (pH 6.2), 0.8 μmole of K₃Fe(CN)₆, 10 μmoles of lithium DL-lactate, and 0.3 to 1.3 mg of cell extract protein in a final volume of 1 ml. The absorbancy change in a companion cuvette without lactate was subtracted from that of the reaction cuvette. The rate of reaction was proportional to protein concentration within the range specified, and 1 unit was defined as that amount of enzyme which reduced 1 μmole of ferricyanide per minute.

DPN-linked lactic dehydrogenase was assayed at 340 μm with reaction cuvettes containing 100 μmoles of potassium phosphate, pH 6.2, 0.1 μmole of DPNH, 10 μmoles of potassium pyruvate, and cell-free extract in a final volume of 1 ml. The rate of reaction was proportional to protein between 0.02 and 0.2 mg. An appreciable oxidation of DPNH without added pyruvate was subtracted. An enzyme unit was defined as the amount oxidizing 1 μmole of DPNH per minute.

**RESULTS**

The experimental approach to the problem of the role of lipoate in the fermentation of lactate by *B. rettgeri* has been largely dictated by the inability of some mediating component(s) of this process. In contrast to the pyruvate fermentation, the intact lactate fermentation is lost after any of several mild treatments of freshly harvested cells. Some insights have been gained through contrasting the remaining potentialities of those "damaged" cells with the characteristics of the pyruvate and lactate fermentations of untreated cells.

In cell-free extracts, although some components of the lactate fermentation remain, all links between lactate and lipoate are lost. Here, and elsewhere, we have made some use of glucose-adapted (G) strains (6). When cells maintained on lactate-lipoate media are transferred to glucose-synthetic medium devoid of lipoate, they first grow poorly. When, after a variable number of transfers, normal growth rate is resumed, the glucose strains are found to have a number of additional metabolic differences from the parental (L) strain. These changes are not always the same from one glucose strain to another (6). However, we have taken the absence of a metabolic component from a glucose strain as suggestive evidence that it was part of the lactate fermentation. No conclusion can be drawn from the continuing presence of a component.

The instability of the lactate fermentation has also prevented a final answer to the initial question of whether it involves an alternate carbon pathway in which pyruvate is not an intermediate. The available evidence suggests that pyruvate is an intermediate, and this conclusion will be assumed in some of the following interpretations.

**Resting Cell Experiments**—The results illustrated in Table I show that 10⁻⁴ M arsenite virtually completely inhibits the ability of *B. rettgeri* L resting cells to form CO₂ from lactate carboxyl, while the decarboxylation of pyruvate is not affected (7). In other experiments, chromatographic analysis of the fermentation products has shown that added lactate can be recovered unchanged after incubation with cells and arsenite.

Pyruvate and lactate fermentation products were determined with a number of different types of resting cell preparations.
The experiments were done under conditions similar to those described in Table I. The CO₂ liberated was determined as in Table I, and the residual substrate and the volatile acid products by Celite chromatography. The results were in general accord with previous reports (2, 5, 6), but some of them will be briefly mentioned, because of differences in the cell preparations and conditions used.

The B. rettgeri L strain used in these experiments fermented pyruvate exclusively at the expense of reduction of acetate to butyrate, and CO₂ to acetate, the proportions of the two processes being reflected in a ratio of 0.90 for the 1-carbon to 2 carbon + derived 4 carbon products. No lactate was formed. However, in the presence of arsenite some lactate did accumulate. The products of the lactate fermentation were qualitatively the same as those from pyruvate, the extra electrons generated being represented both in a relatively increased proportion of butyrate, and a decreased proportion of CO₂ (C₁:C₂ + C₄ = 0.35).

Both of the glucose-adapted strains used in these experiments (G1 and G3) fermented pyruvate mainly by dismutation to lactate, acetate, and CO₂. Butyrate accumulated in small amounts, or in some experiments not at all. Carbon recoveries were less than theoretical in these resting cell fermentations, so that the reduction of CO₂ to acetate could not be quantitatively determined. Previous work cited leaves little doubt that it was occurring. The addition of 10⁻³ m arsenite had no effect on the extent of the pyruvate fermentation, and appeared, if anything, to induce an even greater accumulation of lactate at the expense of butyrate formation.

Neither of the G strains was able to decompose lactate (7), which could be quantitatively recovered after incubation. One of them (G3) appeared to be able to decompose very small amounts of lactate when it was added together with stoichiometric amounts of lipoate, but not when supplemented with ferricyanide (see below).

Experiments with Washed or Lyophilized Cell Suspensions—When freshly harvested cells were washed several times, by centrifuging and resuspending in buffer, the ability to form CO₂ from pyruvate, the extra electrons generated being reflected in a ratio of 0.60 for the l-carbon to 2 carbon + derived 4 carbon products. No lactate was formed. However, in the presence of arsenite some lactate did accumulate. The products of the lactate fermentation were qualitatively the same as those from pyruvate, the extra electrons generated being represented both in a relatively increased proportion of butyrate, and a decreased proportion of CO₂ (C₁:C₂ + C₄ = 0.35).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>No. of cell washes</th>
<th>Substrate carboxyl converted to CO₂ μmoles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate-1-C¹⁴</td>
<td>1</td>
<td>23</td>
</tr>
<tr>
<td>Pyruvate-1-C¹⁴</td>
<td>3</td>
<td>0.4</td>
</tr>
<tr>
<td>Lactate-1-C¹⁴ + NaAsO₃</td>
<td>3</td>
<td>21</td>
</tr>
<tr>
<td>Pyruvate-1-C¹⁴ + NaAsO₃</td>
<td>4</td>
<td>24</td>
</tr>
</tbody>
</table>

The same result was obtained if un-washed cells were lyophilized. The ability to decompose lactate could be restored to either type of cell preparation by stoichiometric levels of ferricyanide and, more interestingly, of lipoate. Neither was effective at catalytic levels. Stoichiometric amounts of DPN, TPN, FAD, or flavin mononucleotide did not restore activity to dried cells. Catalytic amounts of ATP, CoA, or thiamine-PP plus magnesium ions were ineffective in the latter capacity, and also had no effect on the extent of the lipoate-dependent decomposition of lactate by dried cells. It is, of course, possible that dried cells are still impermeable to these cofactors.

Approximately 1 mole of CO₂ was evolved from lactate per mole of lipoate, and 0.5 mole of CO₂ per mole of lipoate, added. The decomposition of pyruvate was little affected by either addition. For each mole of lactate that decomposed, 2 moles of ferricyanide were reduced (Table III), the predicted stoichiometry for a terminal one-electron acceptor. Slightly less than 1 mole of dihydrolipoate accumulated for each mole of lactate that decomposed.

It was apparent that these “damaged” cells required a net electron acceptor to decompose lactate. The damage was apparently not caused by exposure to oxygen, since cells washed anaerobically with buffer containing cysteine, glutathione, or mercaptoethanol showed the same properties. Cell washings, added back after concentration by lyophilization, did not restore lactate-decomposing activity to washed cells.

The question was next investigated whether the inability of dried or washed cells to dispose of electrons from lactate involved a block only in their utilization for butyrate synthesis, or only for CO₂ reduction, or whether the block occurred closer to lactate, before a bifurcation in the electron transport pathways. The results shown in Table IV indicate that dried cells can reduce acetyl-CoA to butyrate with pyruvate as hydrogen donor, but not with lactate. Table IV also shows that dihydrolipoate can provide electrons for the reduction of acetyl-CoA to butyrate, which may account for the less than stoichiometric accumulation of dihydrolipoate shown in Table III.

In a similar experiment, washed cell suspensions of B. rettgeri L...
were incubated with 40 μmoles of NaHCO₃ and 50 μmoles of either pyruvate or lactate. In the former case, 9.3 μmoles of CO₂ were fixed; 32% of the C₄ fixed was present in acetate, and 14% in butyrate. In the lactate incubation, only 0.8 μmol of CO₂ was fixed, and none was present in acetate or butyrate. These results, and those described below, suggest that the electron transport block lies close to lactate.

Prior incubation of dried cell suspensions for 20 minutes with

**TABLE III**

**Stoichiometry of lactate decomposition and ferricyanide or pyruvate reduction by lyophilized cell suspensions of B. rettgeri L**

Reaction mixtures were set up in test tubes. Each tube contained 20 μmoles of lithium D-lactate, 100 μmoles of potassium phosphate buffer, pH 6.2, and 1 ml (150 mg, dry weight) of a lyophilized cell suspension. Where shown, 50 μmoles of K₃Fe(CN)₆ or 20 μmoles of sodium D-α-lipoate were added. Total volume was 3.0 ml. Tubes were fitted with vaccine stoppers and flushed with N₂ through syringe needles for 5 minutes. Reactions were started by adding the cell suspension with a syringe. Tubes were incubated at 37°C for 30 minutes, after which time 0.2-ml samples were withdrawn, cooled in ice, and centrifuged to remove cells. The supernatant fluids were analyzed for dihydrolipoate or ferricyanide as described in "Experimental Procedure." To each tube was then added 0.2 ml of 6 N H₂SO₄ and, after removal of cyanide as described for Table I except that flask were incubated in an atmosphere of H₂ for 20 minutes prior to starting reactions by tipping in the contents from side arm 1.

**TABLE IV**

**Conversion of acetyl-CoA to butyrate by lyophilized cell suspensions of B. rettgeri L**

Reactions were carried out in test tubes. Each tube contained 60 μmoles of potassium phosphate buffer, pH 6.2, 1 μmole of acetyl-1,2-C⁴-CoA (145,000 c.p.m.), and the additions shown. Tubes were fitted with vaccine stoppers and flushed with helium through syringe needles for 5 minutes. Reactions were started by adding 1 ml of a lyophilized cell suspension (190 mg, dry weight) to each tube with a syringe. The main compartment of each Warburg flask contained 200 μmoles of potassium phosphate buffer, pH 6.2, 1 ml of a lyophilized cell suspension (185 mg, dry weight), 5 μmoles of lithium D-lactate, and, where shown, 3 μmoles of sodium arsenite. Side arm 1 contained 50 μmoles of lactate-1-C⁴ and 50 μmoles of sodium D-α-lipoate where indicated. Other conditions were as described for Table I except that flasks were incubated in an atmosphere of H₂ for 20 minutes prior to starting reactions by tipping in the contents from side arm 1.

**TABLE V**

**Effect of arsenite on reduction of lipoate by lactate or pyruvate with lyophilized cell suspensions of B. rettgeri L**

The main compartment of each Warburg flask contained 200 μmoles of potassium phosphate buffer, pH 6.2, 1 ml of a lyophilized cell suspension (185 mg, dry weight), 5 μmoles of lithium D-lactate, and, where shown, 3 μmoles of sodium arsenite. Side arm 1 contained 50 μmoles of lactate-1-C⁴ and 50 μmoles of sodium D-α-lipoate where indicated. Other conditions were as described for Table I except that flasks were incubated in an atmosphere of H₂ for 20 minutes prior to starting reactions by tipping in the contents from side arm 1.

10⁻³ M arsenite was found to have no effect on the ferricyanide-dependent decomposition of lactate. During the prior incubations, a small amount of unlabeled lactate was added, to help maintain endogenous dithiols in a reduced state in which they could react with arsenite. Similar results were obtained with untreated resting cells. In the latter experiment, without ferricyanide, 63% of the isotope of the added lactate-1-C⁴ was recovered as CO₂; prior incubation with arsenite reduced this to 7%. With ferricyanide, 97% was recovered as CO₂, and prior incubation with arsenite reduced this amount only to 87%.

In contrast, when lipoate served as the electron acceptor, prior incubation of dried cells with arsenite essentially completely inhibited lactate decomposition and lipoate reduction (Table V). The reduction of exogenous lipoate by lactate therefore appears to be mediated by an endogenous dithiol, while that of ferricyanide does not. A smaller amount of lipoate was also reduced by pyruvate with dried cells (Table V). This reaction was insensitive to arsenite, and therefore is presumably unrelated to the reduction by lactate. It cannot be assumed, however, that all of the dihydrolipoate was formed by an arsenite-insensitive route, since a small amount formed by such a route could reverse an arsenite inhibition.

**Experiments with Cell-free Extracts**—Early experiments indicated that sonic extracts of B. rettgeri L, supplemented with pyruvate or lactate as reductants, and a variety of cofactors, did not incorporate C⁴ into acetate. It also appeared from these experiments that a small amount of labeled butyrate was formed from lactate-2-C⁴, but not from lactate-1-C⁴, in the presence of 2,6-dichlorophenolindophenol. Unsupplemented extracts do decompose pyruvate (Table VI), mainly by a lactate-acetate dismutation, although some butyrate was also formed. Extracts thus have some ability to synthesize fatty acid, but none to reduce CO₂.

Unlike the "damaged" cells discussed previously, extracts are
unable to decompose lactate at the expense of lipoate reduction. Only the ability to reduce ferriyanide by lactate is retained. As with cells, the ferriyanide reaction in extracts is insensitive to arsenite, and the products of the fermentation resemble those formed from pyruvate (Table VI). Since even a tenuous link between lipoate and lactate was thus lost in extracts, some evidence as to whether various enzyme activities of extracts were parts of the lactate fermentation was sought by determining whether they were absent from glucose-adapted strains.

Table VII shows a typical specific activity for the ferriyanide-linked lactic dehydrogenase in extracts of B. rettgeri L. No reduction of ferriyanide (or 2,6-dichlorophenolindophenol) by lactate has ever been observed in B. rettgeri G extracts under assay conditions which could allow detection of $\frac{1}{10}$ of the amount of enzyme in the L strain. DPN-linked lactic dehydrogenase activity was found in low but significant levels in B. rettgeri L extracts (Table VII). However, glucose-adapted cell extracts were found to contain approximately 8 times more activity. The reaction was arsenite-insensitive in both strains of the organism. In examining many different extracts, the difference between the two strains proved to be reproducible and leaves no doubt that lactate-adapted cells contain less enzyme, or a more labile form of the enzyme.

As previously mentioned, glucose-adapted cells form lactate as a major end product of pyruvate fermentation. As far as it goes, the above evidence suggests that the DPNH-linked reaction functions to form lactate as an end product, while the ferriyanide-linked reaction functions in the fermentation of lactate. With this consideration in mind, B. rettgeri L extracts were used to seek more direct evidence for pyruvate as an intermediate in the fermentation of lactate. The addition of a large excess of unlabeled pyruvate to a reaction mixture containing cell-free extract, ferriyanide, and a small amount of lactate-1-C¹⁴, completely inhibited the evolution of labeled CO₂ (Table VIII). An additional 1 pmoles of potassium phosphate buffer, pH 6.2, in the main compartment of the Warburg flask contained 106 cells of B. rettgeri L and B. rettgeri G extracts, according to experimental procedure noted in Table I. There was complete equilibration between lactate and pyruvate. The results so far had suggested that pyridine nucleotide was not directly reduced by lactate in the fermentative pathway, and that lipoate functioned near, but not next to, the substrate in lactate electron transport. The possibility that lipoate mediated the reduction of pyridine nucleotide by lactate hydrolase was therefore investigated, by comparing the lipoic dehydrogenase activities of extracts of lactate- and glucose-adapted strains.

Lipoic dehydrogenase has been measured by the forward reaction between DPN and dihydrolipoic in an anaerobic cuvette (the identity of DPNH was confirmed in this case by its reoxidation by pyruvate with lactic dehydrogenase). For routine assay, the back reaction was measured under the conditions described in the legend to Fig. 1. The observed rate was not proportional to protein at lower concentrations, but in a linear range (0.03 to 0.07 mg), no significant difference has been found between the specific activities (1 "unit" is the amount of enzyme catalyzing the oxidation of 1 pmoles of DPNH in 1 minute) of G and L extracts. There is considerable variation among different extracts of the same strain. As mentioned above, this result can provide

The reduction of 2,6-dichlorophenolindophenol by lactate in extracts had previously been shown to be insensitive to arsenite (H. A. Barker, personal communication).
no strong evidence for, or against, participation of lipoic dehydrogenase in the lactate fermentation.

Results of some preliminary studies on the lipoic dehydrogenase of the L strain are shown in Figs. 1 to 3. The back reaction, followed either by DPNH disappearance (Fig. 1), or by dihydro-lipoate formation (Fig. 2), was inhibited by prior incubation of the enzyme with DPNH and \(10^{-4}\) M arsenite. Fig. 3 shows that lipoamide is reduced by DPNH approximately 10 times faster than lipoate, with this enzyme preparation.

**DISCUSSION**

It has been suggested that the requirement by *B. rettgeri* for lipoate for growth only on lactate might be due to its ability to synthesize the vitamin when growing on other substrates. The present results do not add to the evidence previously reported against this possibility (7). A second possibility is that lipoate is required for the penetration of lactate into the cell (7). The observation that lactate decomposition becomes insensitive to arsenite when ferricyanide is added to untreated resting cells seems to eliminate this possibility. It also rules out any other direct interaction of lipoate with lactate, provided that it can be shown that ferricyanide captures electrons from a carrier in the physiological fermentation pathway, and not from a carrier in an alternate pathway for lactate decomposition. The loss of ability to reduce ferricyanide with lactate when cells are adapted to glucose gives some evidence that the former is correct. Lest the elimination of a substrate level role for lipoate seem superfluous, we might mention one such possible role:

\[
\text{Lactate} \rightarrow \text{methylglyoxal} \quad (1)
\]

\[
\text{Methylglyoxal} + \text{lipoate} \rightarrow \text{pyruvate} + \text{dihydrolipoate} \quad (2)
\]

The first reaction would be the reverse of that catalyzed by glyoxalase, and the second finds analogy in the possible involvement of dithiols in aldehyde dehydrogenase (18).

The same argument has been invoked in obtaining evidence that pyruvate is an intermediate in the lactate fermentation. Isotopic equilibration between lactate-1-C\(_{14}\) and pyruvate in ferricyanide-supplemented extracts of *B. rettgeri* L probably reflects an intermediary role for pyruvate in the lactate fermentation. In contrast, pyridine nucleotide-linked lactic dehydrogenase is probably not involved in the lactate fermentation. This enzyme is strikingly present only in glucose-adapted cells. Although the reaction catalyzed by it is reversible, it clearly will not function in the direction of lactate oxidation if any part of the product hydrogen can not be disposed of to cellular electron acceptors.

Washed or lyophilized *B. rettgeri* L cells are unable to ferment lactate without an added electron acceptor. Lipoate can serve in this capacity and dihydrolipoate then accumulates in an amount almost equimolar with the lactate decomposed. This reaction is sensitive to arsenite. It seems likely that the mediating dithiol is bound lipoic acid and that the "damaged" cells are unable to effect its reoxidation.
The "damage" in washed cells is to the system involved in transporting lactate hydrogens. The continuing ability of washed cells to ferment pyruvate to the usual products shows that there is no impairment of the pathways leading to the formation of the natural electron acceptors. The "damage" is also not due merely to depletion of a pool of natural electron acceptors. Such a depletion would be expected to result in delayed onset, rather than abolition of lactate fermentation, and a maximal rate of CO₂ evolution from lactate would be restored by catalytic levels of lipoate or ferricyanide. Neither of these requirements is met by the results. We have found that pyruvate can replace lipoate of lipoate or ferricyanide. Neither of these requirements is met by the results. We have found that pyruvate can replace lipoate or ferricyanide in permitting C₄O₂ evolution from lactate₁⁻C₄M. If one assumes the same rapid equilibration in cells as is found in extracts, between the carbons of pyruvate and lactate, the requirement for pyruvate appears to be roughly stoichiometric, rather than catalytic. This activity of pyruvate is not attributed to its ability to generate natural electron acceptors (acetoacetyl-CoA, etc.). The interpretation is, rather, the same as in the case of ferricyanide; however, since there is no net reaction in the transfer of lactate hydrogens to pyruvate, these results might better be described as reflecting a fermentation of pyruvate which has become labeled by exchange, with no decomposition of lactate.

Lactate cannot reduce acetyl-CoA to butyrate or CO₂ to acetate in washed or dried cell preparations. Free dihydrolipoate can accomplish the former, however, and pyruvate can accomplish both. The site of the defect(s) in washed cells is obscure, but the results suggest that lipoate functions as a carrier for lactate hydrogens and is located near, but not next to, the substrate, in the electron transport chain.

The next step in lactate electron transport might be mediated by lipoic dehydrogenase. This enzyme survives not only washing of the cells, but can be extracted. Its continuing presence in glucose-adapted cells raises some doubt about its role in lactate fermentation. The fact that lipoamide is a better substrate than lipoate suggests that bound lipoate is the natural substrate. The presence of lipoamide in the latter may represent a fortuitous survival of a component of the lactate fermentation. The arsenic sensitivity of the enzyme in B. rettgeri G cells also suggests mediation by a dithiol other than lipoate. In this respect, it would resemble lipoic dehydrogenase from other sources (19–21).

The preceding remarks can be illustrated in the notation of Scheme 1, for the lactate electron transport chain. It is far too early to identify the exact position of lipoate, or indeed of any of the carriers, in the chain.

Perhaps the only hitherto clearly established role of lipoic acid is in the dehydrogenation of α-keto acids, in which it mediates electron transport between a substrate (aldehyde-thiamine-PP) and a carrier (FAD) (17). A dithiol closely related to lipoate appears to be involved in sulfate reduction, where it again functions at the substrate level (22, 23). In isolated systems from Clostridium sticklandii, dithiols have been shown to function as electron donors in the reductive deamination of certain amino acids (24). However, the arsenite insensitivity of these reactions in intact cells raises some doubt about the physiological participation of the dithiols (25). On the basis mainly of varying degrees of arsenite sensitivity, dithiols have been less surely implicated in a number of other reactions (26, 27).

The role of lipoic in the B. rettgeri lactate fermentation seems to differ from any unequivocally described until now, in that it mediates electron transport between carriers, rather than at a substrate level.

**SUMMARY**

One of the novel properties of the anaerobic bacterium Butyribacterium rettgeri is that it requires lipoic acid to ferment lactate, but not to ferment pyruvate or glucose. Washed or lyophilized cells were found to have lost the ability to decompose lactate but not pyruvate. The ability to ferment lactate could be restored by the addition of various electron acceptors, which were stoichiometrically reduced. Reduction of lipoic acid was sensitive to arsenite, whereas that of ferricyanide was not. The presence or absence of various enzyme activities in extracts of glucose-adapted strains has been used to estimate whether they represent parts of the lactate fermentation, which itself can be studied only with intact cells. The results indicate that lipoic acid is involved in the transport of electrons originating from lactate, and does not interact directly with the substrate.

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