EFFECT OF pH AND AMMONIUM IONS ON THE PERMEABILITY OF BACILLUS PASTEURII

W. R. WILEY AND J. L. STOKES

Department of Bacteriology and Public Health, Washington State University, Pullman, Washington

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ABSTRACT

WILEY, W. R. (Washington State University, Pullman), AND J. L. STOKES. Effect of pH and ammonium ions on the permeability of Bacillus pasteurii. J. Bacteriol. 86:1152-1156. 1963.—Cell suspensions of Bacillus pasteurii require an alkaline pH (8.5 to 9.0) and NH₄⁺ for the oxidation of low concentrations (4 μM) of fumaric acid, glutamic acid, alanine, and other oxidizable substrates. In contrast, cells disrupted by a French press or by lysozyme oxidize these substrates at pH 7.2 and without NH₄⁺. Moreover, the alkaline pH and NH₄⁺ inhibit substrate oxidation by the broken cells. These striking differences between whole and disrupted cells suggest that pH and NH₄⁺ affect whole cells externally and not internally. It appears that the alkaline pH is needed to convert NH₄⁺ to free NH₃. The latter in turn is required by the cells for the transport of low concentrations of substrate across the cell membrane. At high concentrations (20 to 250 μM), substrates force entry into the cells by simple diffusion, thereby eliminating the need for a high pH and NH₄⁺ for oxidation.

Bacillus pasteurii requires unusual conditions for growth, in that it develops only in alkaline media (pH 8.0 or higher) and only in the presence of relatively high concentrations of NH₄ salts, i.e., 0.5 to 1.0% (Gibson, 1934; Bornside and Kallio, 1956). The physiological basis for these requirements was established by Wiley and Stokes (1962). The requirements for growth were shown to be needed also for the oxidative metabolism of the organism. Thus, substrates such as amino acids and tricarboxylic acid intermediates are oxidized only in the presence of NH₄⁺ at pH 8.5 or higher. The requirement for NH₄⁺ was shown to be relatively specific and the NH₄⁺ could not be replaced by a number of monovalent and divalent cations such as Na⁺, Li⁺, Mg²⁺, and others.

The present investigations were undertaken to determine the specific roles of the alkaline pH and NH₄⁺ in the oxidative metabolism of B. pasteurii. When intact cells suspended in distilled water are disrupted in a French press, the pH of the resulting cell juice is 6.8, which suggests that the internal pH of the cells is near neutrality. This implies that the alkaline pH requirement is an external one and that the internal pH is independent of the external pH. This, in turn, suggests that the high pH and NH₄⁺ may be required for substrate penetration and accumulation in B. pasteurii. To test this possibility, comparative studies were made of the oxidative capabilities of whole cells and cells disrupted by either lysozyme or the French press.

MATERIALS AND METHODS

Cells were grown and prepared as previously described (Wiley and Stokes, 1962). To disrupt the cells, suspensions were prepared in 0.01 M phosphate buffer containing 0.005 M MgSO₄ (pH 7.2) at a concentration of 15 mg of cells (dry weight) per ml. The lysozyme treatment consisted of adding 400 μg of lysozyme per ml of suspension and incubating the mixture at 30 C for 15 min. Also, similar cell suspensions without lysozyme were disrupted in the French press at 4 C and under a constant pressure of 12,000 psi. Microscopic examination indicated that both types of treatment disrupted 90 to 100% of the cells. After breakage, portions were removed and diluted in the appropriate buffer solution to give a final concentration equivalent to 7.5 mg of cells (dry weight) per ml.

Conventional manometric techniques were used to measure oxidation by whole and disrupted cells. The final concentration of whole cells in the Warburg vessels was 1.5 mg (dry weight) per ml. The buffer concentrations in the vessels with both whole and disrupted cells were 0.04 M phosphate buffer for pH 7.2 and 0.15 M tris(hydroxymethyl)aminomethane (tris) buffer.
for pH 9.0. NH₄⁺ was supplied as (NH₄)₂SO₄ in 0.03 M concentration.

**Results**

Substrate oxidation by disrupted cells. Lysozyme and the French press were used interchangeably for disrupting the cells, since both methods gave preparations of equal oxidizing activity (Table 1). For the oxidation of 4 μmoles of fumarate, there was little difference in the relative activities of the two types of cell preparations at each pH level. The activities were greater at pH 7.2 than at pH 9.0. This particular aspect will be discussed later.

The results of a typical experiment in which the oxidation of 4 μmoles of fumarate by whole and disrupted cells was compared are shown in Fig. 1. Whole cells required pH 9.0 and NH₄⁺ for the oxidation of fumarate. There was very little oxidation at pH 9.0 in the absence of NH₄⁺ or at pH 7.2 with or without NH₄⁺. In sharp contrast, disrupted cells oxidized fumarate at pH 7.2 with or without NH₄⁺, whereas the rate and extent of oxidation were considerably reduced at pH 9.0 irrespective of the presence of NH₄⁺. Thus, whole cells actively oxidize fumarate at pH 9.0 with NH₄⁺, whereas disrupted cells oxidize fumarate at pH 7.2, and this activity is uninfluenced by NH₄⁺.

Similar results were obtained with other oxidizable substrates. Data for the oxidation of L-alanine are plotted in Fig. 2. Again, whole cells required the alkaline pH and NH₄⁺ for oxidation but disrupted cells oxidized alanine at pH 7.2 in the absence of NH₄⁺. The alkaline pH and NH₄⁺ are actually inhibitory for the disrupted cells, and NH₄⁺ is inhibitory also at pH 7.2.

Similar results were obtained for the oxidation of L-glutamate by whole and disrupted cells. However, pH 9.0 was not inhibitory, but NH₄⁺ markedly reduced the rate and extent of glutamate oxidation by disrupted cells.

NH₄⁺ inhibition of amino acid oxidation by disrupted cells may involve interference with deamination. Fisher and McGregor (1960) have shown that NH₄⁺ competes with glutamate for binding by glutamic dehydrogenase. Also, the absence of NH₄⁺ inhibition in fumarate oxidation by disrupted cells supports this reasoning. With whole cells, absence of NH₄⁺ inhibition may be due to exclusion of NH₄⁺ by the cell membrane.

**Table 1. Effect of the method of cell disruption on the rate of fumarate oxidation**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Relative activity†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 7.2</td>
</tr>
<tr>
<td>Lysozyme . . .</td>
<td>53</td>
</tr>
<tr>
<td>French press . .</td>
<td>50</td>
</tr>
</tbody>
</table>

* Each Warburg vessel contained 2 ml of disrupted cells equivalent to 7.5 mg of whole cells (dry weight) in either 0.04 M phosphate buffer (pH 7.2) or 0.15 M tris buffer (pH 9.0), with or without added NH₄⁺. NH₄⁺ was added as (NH₄)₂SO₄ in a concentration of 0.03 M. Fumarate (4 μmoles), contained in 0.1 ml water, was added to the side arm. The final liquid volume in the main compartment of the vessel was 2.1 ml; 0.2 ml of 10% KOH was present in the center well to absorb CO₂. The gas phase was air, and the incubation temperature was 30 C.

† Based on 100 for the rate of oxidation of fumarate by whole cells at pH 9.0 with NH₄⁺.

The main conclusion that can be drawn from these experiments is that whole cells require a high pH and NH₄⁺ for substrate oxidation while disrupted cells do not. This strongly indicates that these two requirements for growth and oxidation by whole cells of *B. pasteurii* affect the cells externally and not internally. The most plausible explanation is that the high pH and NH₄⁺ are needed for the penetration of substrates, probably by means of active transport or facilitated diffusion across the cell membrane, and not for internal metabolism.

**Effect of substrate concentration on oxidation rates.** In addition to the functional transport of oxidizable substrate in *B. pasteurii*, which is facilitated by an alkaline pH and NH₄⁺, there exists another mechanism for substrate penetration. When the substrate concentration is increased to levels considerably in excess of the 4 μmoles normally used, rates of oxidation by whole cells are greatly increased, and the high pH and NH₄⁺ are no longer needed for appreciable oxidation. The data in Table 2 show the influence of substrate concentration on the oxidation rates for fumarate, glutamate, and isoleucine. The oxidation of 4 μmoles of fumarate at pH 7.2, with and without NH₄⁺, occurs at approximately one-third the rate of that at pH 9.0 with NH₄⁺. With 24 μmoles of fumarate,
however, the rate of oxidation at pH 7.2 is increased threefold and is comparable with the rate at pH 9.0 in the presence of \( \text{NH}_4^+ \). Moreover, the rate of oxidation of 4 \( \mu \)moles of fumarate at pH 9.0 in the absence of \( \text{NH}_4^+ \) is nil, but is increased to 39 \( \mu \)liters of \( \text{O}_2 \) consumption per hr when the concentration is increased to 24 \( \mu \)moles. As shown, similar results were obtained in the oxidation of glutamate and isoleucine. The extent to which the substrate concentration must be increased to obtain the concentration effect varies with the particular substrate.

These results suggest that the alkaline pH and \( \text{NH}_4^+ \) are necessary for oxidation only at low substrate concentrations. If the substrate concentration is increased, these requirements are partially or completely eliminated because the substrates force entry into the cell by simple diffusion, thereby eliminating the need for an alkaline pH and \( \text{NH}_4^+ \) for transport.

**DISCUSSION**

Our data indicate that the required alkaline pH and \( \text{NH}_4^+ \) in the oxidative metabolism of _B. pasteurii_ specifically affect cell permeability. The alkaline pH is required to generate free \( \text{NH}_3 \) from \( \text{NH}_4^+ \). The free \( \text{NH}_3 \), in turn, is necessary for the uptake of low concentrations of substrates. At high substrate concentrations, a gradient is established across the cell membrane...
which allows substrates to enter the cell by simple diffusion, and therefore the alkaline pH and NH$_4^+$ are not required. Thus, the organism possesses two different types of transport mechanisms.

Both mono- and divalent cations, Na$^+$, K$^+$, Mg$^{++}$, Ca$^{++}$, and others, are known to be essential cofactors for many enzymatic reactions in microorganisms. In contrast, little information is available on the role of cations in substrate transport or accumulation. Payne (1960) has shown that the uptake of glucuronate by a marine pseudomonad is dependent upon the presence of Na$^+$. MacLeod and Hori (1960) demonstrated a Na$^+$ requirement for the oxidation of tricarboxylic acid cycle intermediates by a marine bacterium and suggested that the Na$^+$ may be involved in cellular transport processes. They suggested the possibility that substrate transport is linked to a sodium pump mechanism analogous to the active transport of sugars across intestinal mucosa (Crane, 1960). Although a similar mechanism may explain the NH$_4^+$ requirement for substrate transport in *B. pasteurii*, the available data are insufficient to establish the actual operation of such a mechanism.

Our data suggest that the high pH and NH$_4^+$ are required for the growth of *B. pasteurii* simply to facilitate transport across the cell membrane of essential nutrients and growth factors which may be present in small and therefore limiting concentrations in the growth medium. Therefore, it may be possible to obtain growth at pH 7.2 if such nutrients are supplied in high concen-
TABLE 2. Influence of substrate concentration on the rates of substrate oxidation by whole cells of Bacillus pasteurii*

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Amt (µmoles)</th>
<th>pH 7.2</th>
<th>pH 9.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fumaric acid</td>
<td>4</td>
<td>39</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>96</td>
<td>90</td>
</tr>
<tr>
<td>L-Glutamic acid</td>
<td>4</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>45</td>
<td>45</td>
</tr>
<tr>
<td>L-Isoleucine</td>
<td>4</td>
<td>9</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>48</td>
<td>48</td>
</tr>
</tbody>
</table>

* Each Warburg vessel contained 2 ml of cell suspension, equivalent to 1.5 mg dry weight of cells per ml. The buffer concentrations in the vessels were: phosphate buffer, 0.04 M (pH 7.2); and tris, 0.15 M (pH 9.0). (NH₄)₂SO₄, when added, was present in a concentration of 0.03 M. The substrates (0.1 ml) were added to give the concentrations indicated; 0.2 ml of 10% KOH was added to the center well to absorb CO₂. The gas phase was air, and the incubation temperature was 30°C.

trations in the external medium. These could then enter the cell, in adequate amounts and at adequate rates, by simple diffusion to permit growth. Experiments are underway to test this possibility.

Acknowledgment

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Literature Cited


