Bacterial Inhibitory Effects of Nitrite: Inhibition of Active Transport, But Not of Group Translocation, and of Intracellular Enzymes

J. M. YARBROUGH, J. B. RAKE, AND R. G. EAGON*

Department of Microbiology, University of Georgia, Athens, Georgia 30602

Nitrite inhibited active transport of proline in *Escherichia coli* but not group translocation of sugar via the phosphoenolpyruvate:phosphotransferase system. These results were consistent with previous results that nitrite inhibits active transport, oxygen uptake, and oxidative phosphorylation in aerobic bacteria. Nitrite also inhibited aldolase (EC 4.1.2.13) from *E. coli*, *Pseudomonas aeruginosa*, *Streptococcus faecalis*, and rabbit muscle. Thus, these various data showed that nitrite has more than one site of attack in the bacterial cell. These data also indicated that nitrite is inhibitory to a wide range of physiological types of bacteria.

Sodium nitrite has been extensively used in the curing of meats and in meat products (4). It functions not only as a color fixative and flavor developer, but as a preservative as well. The inhibition of a wide variety of bacteria, including clostridia and *Staphylococcus aureus*, by sodium nitrite is well documented (1, 5, 6, 8, 9, 17, 18). The mechanism of inhibition of bacteria by nitrite, however, has not been satisfactorily explained, particularly in regard to anaerobic bacteria and facultatively anaerobic bacteria under anaerobic conditions.

We previously reported that nitrite inhibited active transport, oxygen uptake, and oxidative phosphorylation in *Pseudomonas aeruginosa* (20). The evidence strongly suggested that nitrite exerted these effects at the electron carrier(s) level in the cell membrane. Nitrite, however, had no effect on glucose transport by *Streptococcus faecalis* and *S. lactis*, organisms that lack cytochromes and that rely upon glycolysis for the generation of adenosine triphosphate when grown on glucose (10); and that transport glucose by group translocation via the phosphoenolpyruvate:phosphotransferase system (PEP:PTS) (14). Yet, other workers previously have reported that nitrite inhibited growth and energy generation in these physiological types of bacteria (11, 21). Thus, the work presented herein was carried out to determine the effect of nitrite on a representative facultative anaerobe (*Escherichia coli*) and a strictly fermentative bacterium (*S. faecalis*) and to confirm and extend our previous observations.

**MATERIALS AND METHODS**

Cultivation of organisms. The organisms used for the studies were: *E. coli* ML 308-225, *E. coli* YML, *P. aeruginosa* PAO, and *S. faecalis* 9790. *P. aeruginosa* PAO and *E. coli* ML 308-225 were grown aerobically in a basal salts-glucose medium, pH 7, containing 2.5 g of glucose per liter as previously described (7). *E. coli* YML was grown aerobically in a tryptic soy broth medium (Difco Laboratories, Detroit, Mich.) containing 0.01 g of thiamine per liter. *S. faecalis* was grown fermentatively at 37°C in KTY medium as described by Harold and Baarda (10). The bacterial cells used for the transport assays were grown, harvested, and handled as reported previously (20).

**Transport assays.** The accumulation of radioactivity from [4C]glucose (7.9 mCi/mmol), [a-methyl-14C]glucoside (a-MG) (0.33 mCi/mmol), [d-[a-methyl-14C]methyl-β-D-glucoside (β-MG) (0.56 mCi/mmol), and [14C]proline (20 Ci/mmol) was determined at 30°C as previously described (20). Nitrite was included in the experiments as appropriate. The reaction was initiated by the addition of substrate.

The initial rate of transport (i.e., that taking place within the first 15 s) of metabolizable substrates by intact cells is considered to closely approximate the true rate of transport. Thus, in experiments in which the metabolizable substrates proline and glucose were used, the rate of uptake after 15 s was considered to be an accurate measure of transport, whereas the effects due to catabolism or incorporation were considered to be minimal at this point.

**Preparation of cell extracts.** Cultures of *E. coli*, *P. aeruginosa*, and *S. faecalis* were grown to late exponential phase. The cells were washed twice in basal salts solution and suspended in the same solution at a density of 1 g (wet weight) of cells per ml of basal salts solution. The cells in these suspensions were captured by passage twice through a prechilled French pressure cell at 14,000 to 16,000 lb/in². Whole cells were removed by centrifuging for 10 min at 10,000 × g. The supernatant fluid was decanted and centrifuged for 10 min at 12,000 × g. The final supernatant fluid, i.e., "cell extract," was collected, transferred to stoppered tubes, and frozen. The temperature was maintained at 0 to 4°C during each step in the preparation of the extracts.
Enzyme assays. Hexokinase (EC 2.7.1.1) activity was measured spectrophotometrically by the method of Barnard (2). Aldolase (EC 4.1.2.13) activity was determined by the method of Lowry (13).

Protein estimation. Cell suspensions were treated with urea as described by King (12) and then analyzed for protein by the semi-microbiuret procedure (16). Cell extracts were analyzed by the semi-microbiuret procedure without the addition of urea.

Chemicals. [U-14C]glucose was purchased from Amersham-Searle Corp., Arlington Heights, Ill. [U-

14C]proline and a-MG were purchased from New England Nuclear Corp., Boston, Mass. a-MG was purchased from Calbiochem, Los Angeles, Calif. Rabbit muscle aldolase, type III, and yeast hexokinase, type III, were obtained from the Sigma Chemical Co., St. Louis, Mo. All other reagents were purchased from commercial sources in the highest available state of purity.

RESULTS

Nitrite inhibition of active transport in E. coli. Nitrite inhibited proton-dependent active transport in P. aeruginosa (20), and preliminary experiments indicated that nitrite inhibition of oxygen uptake in E. coli was similar to that in P. aeruginosa. We reasoned, therefore, that nitrite would inhibit the transport of proline in E. coli, which is by a proton-dependent active transport mechanism (3). The initial rate (15 s) of proline transport in control cell suspensions was 2.8 nmol/mg of protein per min (Fig. 1), whereas 50 mM nitrite reduced the rate to 1.2

nmol/mg of protein per min (57% inhibition). The apparent inhibition was even greater in the later phases of uptake (e.g., at 3 min, uptake in the presence of 50 mM nitrite was 10% of the control rate); however, owing to the possibility of additional effects of nitrite on proline incorporation, these later time points were not considered to reflect absolute effects on transport per se. Nevertheless, the inhibition of proline transport in E. coli by nitrite was quantitatively comparable to the inhibitory effect of nitrite on active transport of glucose and proline in P. aeruginosa (20).

Lack of nitrite inhibition of group translocation. Nitrite did not inhibit glucose uptake in S. faecalis or S. lactis (20), organisms that take up glucose by group translocation via the PEP-PTS (14). Similarly, E. coli also takes up glucose via the PEP-PTS (19). It was surprising, therefore, when nitrite inhibition of glucose uptake in E. coli ML 308-225 was observed (Fig. 2). However, even at 100 mM nitrite, the initial (15 s) rate of uptake was inhibited little relative to the control. Similar results were obtained for E. coli YML (data not shown). It was considered likely, therefore, that nitrite was inhibiting subsequent glucose catabolism or incorporation or both rather than transport per se.

It was thus necessary to determine the effect of nitrite on the uptake of the non-metabolizable analogs of glucose, a-MG and b-MG. Nitrite did not inhibit the uptake of a-MG in E. coli (Fig. 3). Similarly, the uptake of b-MG in E. coli was scarcely affected by even high concentrations of nitrite (Fig. 4). The slight inhibition that was observed was not considered to be significant. It is also noteworthy that the initial rates (15 s) of a-MG and b-MG uptake controls were quite comparable to those measured for glucose.
DISCUSSION

Our present data show that nitrite inhibited active transport of proline in *E. coli*, which confirms and extends our previous report that nitrite inhibited oxygen uptake, oxidative phosphorylation, and active transport of glucose and proline in *P. aeruginosa* (20). Our experiments did not explicitly address the question of the site of action of nitrite in active transport inhibition of *E. coli*. Preliminary experiments (data not shown) indicated that, as with *P. aeruginosa* (20), inhibition of respiration and active transport by nitrite occur coordinately. It is probable that nitrite inhibition of transport occurs via inhibition of the cytochrome chain, thus preventing formation of a proton gradient. However, it is also entirely possible that nitrite has separate inhibitory effects on the respiratory system and on active transport carriers. Furthermore, other authors recently have presented evidence that nitrite acts as an uncoupler, causing a collapse of the proton gradient across the membrane of *Paracoccus denitrificans* (15). Thus, it is now evident that one inhibitory action of nitrite is exerted at the bacterial cell membrane level.

The absence of nitrite inhibition of PEP:PTS-

**Figure 3.** Lack of nitrite inhibition of the accumulation of radioactivity from α-MG by *E. coli* ML 308-225. The control curve represents the uptake of the analog in the absence of nitrite.

**Figure 4.** Lack of nitrite inhibition of the accumulation of radioactivity from β-MG by *E. coli* ML 308-225. The control curve represents the uptake of the analog in the absence of nitrite.

**Table 1. Nitrite inhibition of aldolase activity**

<table>
<thead>
<tr>
<th>Nitrite concn (mM)</th>
<th><em>E. coli</em></th>
<th><em>P. aeruginosa</em></th>
<th><em>S. faecalis</em></th>
<th>Rabbit muscle aldolase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>139</td>
<td>45</td>
<td>153</td>
<td>1104</td>
</tr>
<tr>
<td>10</td>
<td>73</td>
<td>40</td>
<td>32</td>
<td>165</td>
</tr>
<tr>
<td>50</td>
<td>0.16</td>
<td>16</td>
<td>0.91</td>
<td>125</td>
</tr>
<tr>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0.72</td>
<td>35</td>
</tr>
</tbody>
</table>

a Activity is expressed as micromoles of fructose 1,6-diphosphate split per minute per milligram of extract protein.

b Control data represent aldolase activity in the absence of nitrite.

**Table 2. Lack of nitrite inhibition of hexokinase activity**

<table>
<thead>
<tr>
<th>Nitrite concn (mM)</th>
<th><em>E. coli</em></th>
<th><em>P. aeruginosa</em></th>
<th><em>S. faecalis</em></th>
<th>Yeast hexokinase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.057</td>
<td>0.065</td>
<td>0.102</td>
<td>2.07</td>
</tr>
<tr>
<td>10</td>
<td>0.028</td>
<td>0.061</td>
<td>0.107</td>
<td>1.94</td>
</tr>
<tr>
<td>50</td>
<td>0.031</td>
<td>0.057</td>
<td>0.129</td>
<td>2.07</td>
</tr>
<tr>
<td>100</td>
<td>0.028</td>
<td>0.050</td>
<td>0.142</td>
<td>2.07</td>
</tr>
</tbody>
</table>

a Activity is expressed as micromoles of acid produced per minute per milligram of extract protein.
b Control data represent hexokinase activity in the absence of nitrite.

**Effect of nitrite on aldolase and hexokinase.** We chose two enzymes, aldolase and hexokinase, for study. Nitrite was shown to be an inhibitor of aldolases from *E. coli*, *P. aeruginosa*, and *S. faecalis* as well as from rabbit muscle (Table 1).

Nitrite was not an inhibitor of hexokinase from *P. aeruginosa*, *S. faecalis*, and yeast (Table 2). The hexokinase activity from *E. coli*, however, was inhibited by about 50%; but, since this level of inhibition was observed at all concentrations of nitrite, it is likely that the observed inhibition was due to some factor other than nitrite per se.

**Figure 3.** Lack of nitrite inhibition of the accumulation of radioactivity from α-MG by *E. coli* ML 308-225. The control curve represents the uptake of the analog in the absence of nitrite.

**Figure 4.** Lack of nitrite inhibition of the accumulation of radioactivity from β-MG by *E. coli* ML 308-225. The control curve represents the uptake of the analog in the absence of nitrite.
linked group translocation of α-MG or β-MG in *E. coli* coupled with the presence of nitrite inhibition of glucose uptake was interpreted to indicate that the inhibitory effect of nitrite upon glucose uptake was due to inhibition of catabolic steps subsequent to group translocation rather than inhibition of the translocation system itself. This finding is in agreement with our previous findings of nitrite-insensitive PEP:PTS group translocation in *S. lactis* and *S. faecalis* (20).

Additional evidence for nitrite inhibition of catabolic steps subsequent to glucose uptake was provided by the strong inhibition by nitrite of aldolase from a variety of sources (Table 1). Hexokinase, in contrast, was nitrite insensitive (Table 2), which demonstrated that nitrite is not simply a nonspecific enzyme inhibitor. Interestingly, although aldolase is involved in the postuptake catabolism of glucose in *E. coli* via the glycolytic pathway, hexokinase is not because the product of the PEP:PTS group translocation system is glucose-6-phosphate (19). Other workers, moreover, have also previously reported nitrite inhibition of certain glycolytic enzymes of *Clostridium* (17).

Our experimental data do not provide an answer to the question of the source of PEP to *E. coli* cells whose aldolase was inhibited by nitrite. Apparently the PEP was formed by a means other than glycolysis, such as via gluconeogenesis or by the hexose monophosphate pathway.

It is interesting that nitrite did not impede the accumulation of glucose in *S. faecalis* or *S. lactis* (20) even though the streptococcal aldolase was sensitive to nitrite. These organisms, moreover, have been reported to be highly resistant to nitrite (6). These data, therefore, are suggestive that these streptococci are impermeable to nitrite.

It is now clear from recent evidence that nitrite inhibits bacteria by several different means: first, nitrite interferes with energy conservation by inhibiting oxygen uptake, oxidative phosphorylation, and proton-dependent active transport as previously reported (20) and as shown herein; second, nitrite acts as an uncoupler, causing a collapse of the proton gradient (15); and third, nitrite inhibits certain metabolic enzymes as shown herein and as reported by others (17). Thus, nitrite is an effective inhibitor of a wide range of physiological types of bacteria.

ACKNOWLEDGMENT

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LITERATURE CITED


