MECHANISMS IN THE INHIBITION OF MICROORGANISMS BY SORBIC ACID

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ABSTRACT

York, George K. (University of California, Davis), and Reese H. Vaughn. Mechanisms in the inhibition of microorganisms by sorbic acid. J. Bacteriol. 88:411-417, 1964.—Oxidative assimilation of glucose, acetate, succinate, and fumarate by washed cells of Escherichia coli, Pseudomonas aeruginosa, and Saccharomyces cervisiae was inhibited by concentrations of sorbic acid ranging from 15 to 105 mg per 100 ml. At higher concentrations, the oxidation of these substrates was inhibited. Oxidative phosphorylation by submicroscopic particles of E. coli was reduced by about 30% by 37 mg per 100 ml of sorbic acid. The sulfhydryl enzymes fumarase, aspartase, and succinic dehydrogenase were inhibited by sorbic acid. The loss of activity of sorbic acid after reacting with cysteine suggested that a thiol addition occurred, which is believed to be the mechanism of action against sulfhydryl enzymes or cofactors.

The inhibition of various enzymic reactions by sorbic acid (2,4-hexadienoic acid) has been suggested as the mechanism by which microbial growth is inhibited. Whitaker (1959) reported the inhibition of the sulfhydryl enzymes fein and alcohol dehydrogenase, and postulated that the poisoning of one or more sulfhydryl enzymes within a microbial cell would prevent growth. Melnick, Luckmann, and Gooding (1954) suggested that the dehydrogenases in fungi were the sites of inhibition. Azukas, Costilow, and Sadoff (1961) reported that sorbic acid competitively inhibited enolase and thus prevented the fermentation of glucose by Saccharomyces cervisiae.

York and Vaughn (1960) observed that, in addition to a reduction in the rates of oxidation of several substrates by washed cells, sorbic acid also decreased the oxidative assimilation of these compounds. Therefore, an attempt is made in this report to correlate the findings with isolated systems with those obtained with whole cells to gain a better understanding of the mechanisms of microbial inhibition by sorbic acid.

MATERIALS AND METHODS

Microbial growth was estimated turbidimetrically by use of a Klett-Summerson colorimeter. Oxidative assimilation was determined by the manometric method described by Siegel and Clifton (1950).

Fumarase activity was followed spectrophotometrically by a modification of the method given by Racker (1950), in which the wavelength was changed to 300 m and sorbic acid solutions were used as reference blanks. A commercial preparation of fumarase was used (Nutritional Biochemicals Corp., Cleveland, Ohio). The measurement of ammonia by acidometric titration (Hawk, Oser, and Summerson, 1947) was used to estimate aspartase activity. Aspartase was extracted from Escherichia coli and partially purified as described by Ellfolk (1953).

Cell-free extracts of E. coli were used as a source of succinic dehydrogenase, and activity was estimated by measuring the increase in absorbancy at 300 m with succinate as substrate. Sorbic acid was measured spectrophotometrically as described by Alderton and Lewis (1958).

Submicroscopic particles of E. coli were used as a source of the oxidative phosphorylation system. A suspension of 40 mg (wet weight) of cells in 40 ml of 0.017 M tris(hydroxymethyl)-aminomethane (tris) buffer (pH 7.0) was treated at maximal output of a 10-ke Raytheon sonic oscillator under hydrogen for 5 min, and was centrifuged in the cold for 20 min at 5,000 x g to remove residual cells and large debris. The cell-free extract was then centrifuged for 2 hr at 100,000 x g at approximately 2°C. The particles were washed once in cold tris buffer (pH 7.0) and suspended again in 20 ml of this buffer. Oxygen uptake was measured manometrically at 30°C, with succinate as the substrate in reaction mixtures which contained enough of the particle

411
preparation to give a change of about 3 μliters of O₂ per min. The adenosine triphosphate (ATP) formed from added adenosine diphasphate (ADP) and inorganic phosphate during the oxidation of succinate was trapped by the hexokinase reaction. The reaction was stopped after 60 min by heating for 1 min at 80°C, the sample was centrifuged, and the residual glucose was measured by use of a preparation of glucose oxidase (Worthington Biochemical Corp., Freehold, N.J.).

**RESULTS**

*Inhibition of growth.* When the reciprocals of the growth rates of several microbes were plotted against the concentrations of sorbic acid, a straight line was obtained over a portion of the curve (Fig. 1). This indicates that the rapidity of the growth rate is inversely proportional to the concentration of sorbic acid, and this ratio is constant for a specific microbe in a given environment:

\[
\frac{1}{V} = b(I) + \frac{1}{V_0}
\]

where \( V = \) rate of growth in presence of sorbic acid; \( V_0 = \) rate of growth in absence of sorbic acid; \( I = \) concentration of sorbic acid; \( b = \) a constant.

At concentrations below 1.5 mg of undissociated sorbic acid per 100 ml of medium, there was little or no inhibition of the four microorganisms tested. This may be explained by a detoxification of that amount of sorbic acid as a result of a reaction between sorbic acid and some compound or compounds present in the yeast extract-peptone medium. Evidence to support this explanation is found in the observation that the addition of cysteine and other thiols to a mineral salts medium decreases the inhibition of growth by sorbic acid.

*Inhibition of oxidative assimilation.* The effect of sorbic acid on the oxidation of acetate and growth in a basal medium containing acetate is shown in Table 1. The concentrations which essentially completely inhibit oxidative assimilation also markedly inhibited growth. The respiratory quotients (RQ) were not changed at different concentrations of the inhibitor, and only at the highest concentration tested was there an inhibition of oxidation of acetate.

Similar inhibition of oxidative assimilation was observed with pyruvate, malate, fumarate, and succinate (Table 2), and with glucose (Table 3). With glucose, a difference was observed in the behavior of *S. cerevisiae* and the other test organisms in the presence of sorbic acid. The oxidation of glucose continued nearly to completion with the bacteria in a suitable range of concentration of the inhibitor. With the yeast, however, there was a reduction in oxygen uptake, both of rate and total amount, whereas the production of carbon dioxide continued with a resulting change in the RQ (Table 3).

*Inhibition of oxidative phosphorylation.* Oxidative phosphorylation by submicroscopic particles of *E. coli* was reduced by about 30% by sorbic acid at a concentration which diminished oxidative assimilation by 79% (Tables 3 and 4). Hexokinase and adenosine triphosphatase activity of the particles was negligible.

*Inhibition of isolated enzyme systems.* Activity of both fumarase and aspartase was inhibited by sorbic acid, with the inhibition being linear over a portion of the curves (Fig. 2 and 3). The \( I_{50} \) for sorbic acid with fumarase was 0.0034 M. The addition of cysteine reduced the inhibition of fumarase (Table 5). Because fumarase follows first-order reaction kinetics only at low concentrations of fumarase (Bock and Alberty, 1953), it was not possible to determine the type of in-
TABLE 1. Effect of sorbic acid on the oxidation and assimilation of 10 μmoles of acetate by washed cells (pH 5.5)

<table>
<thead>
<tr>
<th>Organism</th>
<th>Conc of sorbic acid (mg/100 ml)</th>
<th>0.294</th>
<th>0.350</th>
<th>0.411</th>
<th>0.420</th>
<th>0.460</th>
<th>0.30</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15</td>
<td>0.300</td>
<td>0.357</td>
<td>0.400</td>
<td>0.426</td>
<td>0.458</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>0.302</td>
<td>0.358</td>
<td>0.399</td>
<td>0.400</td>
<td>0.459</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>105</td>
<td>0.302</td>
<td>0.358</td>
<td>0.399</td>
<td>0.400</td>
<td>0.460</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>210</td>
<td>0.302</td>
<td>0.358</td>
<td>0.399</td>
<td>0.400</td>
<td>0.459</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>420</td>
<td>0.302</td>
<td>0.358</td>
<td>0.399</td>
<td>0.400</td>
<td>0.459</td>
<td>0.33</td>
</tr>
</tbody>
</table>

TABLE 2. Effect of sorbic acid on the oxidation and assimilation of several substrates by washed cells in phosphate buffer (pH 5.5)

<table>
<thead>
<tr>
<th>Organism</th>
<th>Conc of sorbic acid (mg/100 ml)</th>
<th>pyruvate, 5 μmoles</th>
<th>rate (μliters O₂/min)</th>
<th>RQ</th>
<th>oxidized</th>
<th>malate, 5 μmoles</th>
<th>rate (μliters O₂/min)</th>
<th>RQ</th>
<th>oxidized</th>
<th>fumarate, 5 μmoles</th>
<th>rate (μliters O₂/min)</th>
<th>RQ</th>
<th>oxidized</th>
<th>succinate, 5 μmoles</th>
<th>rate (μliters O₂/min)</th>
<th>RQ</th>
<th>oxidized</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>3.7</td>
<td>1.35</td>
<td>62</td>
<td>3.8</td>
<td>1.48</td>
<td>70</td>
<td></td>
<td>3.1</td>
<td>1.54</td>
<td>68</td>
<td></td>
<td>3.2</td>
<td>1.19</td>
<td>72</td>
<td></td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>3.6</td>
<td>1.35</td>
<td>87</td>
<td>3.1</td>
<td>1.52</td>
<td>95</td>
<td></td>
<td>1.2</td>
<td>1.51</td>
<td>93</td>
<td></td>
<td>1.3</td>
<td>1.21</td>
<td>94</td>
<td></td>
<td>1.2</td>
</tr>
<tr>
<td>Saccharomyces cerevisiae</td>
<td>0</td>
<td>2.4</td>
<td>1.29</td>
<td>60</td>
<td>2.7</td>
<td>1.53</td>
<td>69</td>
<td></td>
<td>2.5</td>
<td>1.53</td>
<td>64</td>
<td></td>
<td>2.9</td>
<td>1.17</td>
<td>65</td>
<td></td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>2.2</td>
<td>1.32</td>
<td>82</td>
<td>2.4</td>
<td>1.50</td>
<td>89</td>
<td></td>
<td>1.0</td>
<td>1.56</td>
<td>85</td>
<td></td>
<td>1.2</td>
<td>1.21</td>
<td>87</td>
<td></td>
<td>1.2</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>0</td>
<td>3.9</td>
<td>1.31</td>
<td>71</td>
<td>4.1</td>
<td>1.51</td>
<td>73</td>
<td></td>
<td>4.1</td>
<td>1.50</td>
<td>65</td>
<td></td>
<td>4.3</td>
<td>1.22</td>
<td>70</td>
<td></td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>3.7</td>
<td>1.34</td>
<td>94</td>
<td>3.8</td>
<td>1.49</td>
<td>95</td>
<td></td>
<td>1.6</td>
<td>1.54</td>
<td>90</td>
<td></td>
<td>1.8</td>
<td>1.20</td>
<td>89</td>
<td></td>
<td>1.6</td>
</tr>
</tbody>
</table>

* Grown in mineral salts medium containing 0.3% sodium acetate and 0.05% yeast extract (pH 5.5).

Hibition by means of a plot of the reciprocal velocities versus reciprocal substrate concentrations; at these concentrations, less than 0.001 M, the initial velocities could not be determined spectrophotometrically in the presence of sorbic acid, and the fluorescent assay for malate gave erratic results if the reaction time was less than 60 sec.

The I₅₀ for aspartase was 0.00083 M, which was considerably less than for fumarase (Table 6). Reaction of sorbic acid with cysteine. The absorbance of light at 263 μ by sorbic acid was reduced in the presence of cysteine (Fig. 4). This indicates a thiol addition reaction, saturating the double bonds of sorbic acid which are responsible for its ultraviolet-light absorbancy. Identifica-
TABLE 3. Effect of sorbic acid concentration on oxidation and assimilation of glucose by washed cells of Escherichia coli, Pseudomonas aeruginosa, and Saccharomyces cerevisiae (pH 5.5)

<table>
<thead>
<tr>
<th>Conc of sorbic acid (mg/100 ml)</th>
<th>E. coli*</th>
<th>P. aeruginosa*</th>
<th>S. cerevisiae†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>O₂</td>
<td>CO₂</td>
<td>RQ</td>
</tr>
<tr>
<td></td>
<td>µlites</td>
<td>µlites</td>
<td>%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>351</td>
<td>358</td>
<td>1.02</td>
</tr>
<tr>
<td>15</td>
<td>552</td>
<td>570</td>
<td>1.01</td>
</tr>
<tr>
<td>37</td>
<td>036</td>
<td>624</td>
<td>0.98</td>
</tr>
<tr>
<td>105</td>
<td>664</td>
<td>691</td>
<td>1.04</td>
</tr>
</tbody>
</table>

* Glucose, 5 µmoles.
† Glucose, 10 µmoles.

TABLE 4. Effect of sodium sorbate on oxidative phosphorylation by a crude submicroscopic particle preparation of Escherichia coli

<table>
<thead>
<tr>
<th>Reaction system*</th>
<th>No sorbate</th>
<th>Sorbate (0.0033 M)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose</td>
<td>Oxygen (O)</td>
</tr>
<tr>
<td></td>
<td>µmoles/3 ml</td>
<td>µmoles/3 ml</td>
</tr>
<tr>
<td>Complete†</td>
<td>5.6</td>
<td>11.8</td>
</tr>
<tr>
<td>Less succinate</td>
<td>12.4</td>
<td>0.6</td>
</tr>
<tr>
<td>Less hexokinase</td>
<td>13.0</td>
<td>11.2</td>
</tr>
<tr>
<td>Less glucose</td>
<td>0</td>
<td>11.8</td>
</tr>
<tr>
<td>Less particles</td>
<td>13.3</td>
<td>0</td>
</tr>
<tr>
<td>Zero time, complete</td>
<td>13.3</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sorbate</th>
<th>Glucose (µmoles)</th>
<th>Oxygen uptake (µmoles)</th>
<th>Phosphorylation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Added</td>
<td>Less succinate</td>
<td>Final</td>
</tr>
<tr>
<td>None†</td>
<td>13.3</td>
<td>12.7</td>
<td>7.9</td>
</tr>
<tr>
<td>0.0033 M†</td>
<td>13.0</td>
<td>12.7</td>
<td>7.9</td>
</tr>
<tr>
<td>None†</td>
<td>13.0</td>
<td>12.7</td>
<td>9.8</td>
</tr>
<tr>
<td>0.0033 M‡</td>
<td>12.8</td>
<td>12.7</td>
<td>10.7</td>
</tr>
</tbody>
</table>

* Inoculated for 60 min at 30 C. Final concentration in Warburg vessels (total fluid volume of 3.0 ml): glucose, 0.004 M; K₂HPO₄, 0.01 M; tris buffer (pH 7.0), 0.017 M; MgSO₄, 0.01 M; ADP, 0.005 M in phosphate buffer; sodium succinate, 0.01 M; crystalline hexokinase, 6 mg/3 ml in tris buffer; particles, washed once, in tris buffer to give ca. 3 µlites of O₂ uptake per min. All reagents were adjusted to pH 7.0.
† Determined within 1 hr after second centrifugation at 100,000 X g.
‡ Determined after 4 days of storage of crude particles at -30 C.

Oxidative assimilation by E. coli, S. cerevisiae, and Pseudomonas aeruginosa with several substrates was inhibited by sorbic acid in a manner similar to the inhibition of assimilation observed with 2,4-dinitrophenol (Clifton, 1946). Because 2,4-dinitrophenol is an uncoupling agent in phosphorylation, it is suggested that sorbic acid inhibits assimilation by inhibition of oxidative

DISCUSSION

Oxidative assimilation by E. coli, S. cerevisiae, and Pseudomonas aeruginosa with several substrates was inhibited by sorbic acid in a manner similar to the inhibition of assimilation observed with 2,4-dinitrophenol (Clifton, 1946). Because 2,4-dinitrophenol is an uncoupling agent in phosphorylation, it is suggested that sorbic acid inhibits assimilation by inhibition of oxidative
phosphorylation. The similarity of action of sorbic acid and 2,4-dinitrophenol is observed in the relatively small but definite inhibition of oxidative phosphorylation in extracts and particulate fractions of bacterial cells by both compounds (Marr, 1960). The correlation observed between inhibition of growth and assimilation by sorbic acid suggests that the uncoupling of oxidative phosphorylation is a principal mechanism in the inhibition of microbial growth by sorbic acid.

In addition to the reduction of assimilation by sorbic acid, the marked, but not complete, decrease in the rate of oxidation of succinate and fumarate, in contrast to the slight decrease in the rate of oxidation of glucose, acetate, pyruvate, and malate, suggests that there is another site of inhibition possibly involving fumarase. The partial inhibition of fumarase activity could explain the decrease in rate of oxidation by washed cells with fumarate as the substrate. Because fumarate is an inhibitor of succinic dehydrogenase (Kearny and Singer, 1954), an accumulation of fumarate at the site of the reaction, by the inhibition of fumarase or aspartase by sorbic acid, could account for the reduction in the rate of oxidation.

**TABLE 5. Effect of sorbic acid concentration, pH, and incubation on activity of fumarase**

<table>
<thead>
<tr>
<th>Sorbic acid conc* (mm)</th>
<th>pH 7.3</th>
<th>pH 6.2, incubated 1 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Incubated 1 hr</td>
<td>Incubated 5 min</td>
</tr>
<tr>
<td></td>
<td>Activity†</td>
<td>Inhibition</td>
</tr>
<tr>
<td>0</td>
<td>208</td>
<td>0</td>
</tr>
<tr>
<td>1.0</td>
<td>154</td>
<td>24</td>
</tr>
<tr>
<td>2.0</td>
<td>143</td>
<td>31</td>
</tr>
<tr>
<td>3.0</td>
<td>119</td>
<td>43</td>
</tr>
<tr>
<td>4.0</td>
<td>101</td>
<td>56</td>
</tr>
<tr>
<td>5.0</td>
<td>58</td>
<td>72</td>
</tr>
<tr>
<td>6.0</td>
<td>312</td>
<td>85</td>
</tr>
<tr>
<td>7.0</td>
<td>17</td>
<td>92</td>
</tr>
<tr>
<td>5.0 mm sorbate + 10 mm cysteine</td>
<td>97</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>3.4 X 10^-2</td>
<td>5.0 X 10^-2</td>
</tr>
</tbody>
</table>

* Initial concentration of fumarate was 5.0 mm.
† Amount (μg per ml) of fumarate used per 10 min.
TABLE 6. Effect of sorbic acid on aspartase activity of a cell-free preparation of Escherichia coli (pH 6.8, 30 mM fumarate, 37 C)

<table>
<thead>
<tr>
<th>Concentration of sorbic acid (mM)</th>
<th>Ammonia-nitrogen* (µg/5.0 ml)</th>
<th>Activity (µg/10 min)</th>
<th>Inhibition %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.553</td>
<td>0.171</td>
<td>7.1</td>
</tr>
<tr>
<td>0.2</td>
<td>3.582</td>
<td>0.142</td>
<td>5.9</td>
</tr>
<tr>
<td>0.4</td>
<td>3.599</td>
<td>0.125</td>
<td>5.2</td>
</tr>
<tr>
<td>0.6</td>
<td>3.614</td>
<td>0.110</td>
<td>4.6</td>
</tr>
<tr>
<td>0.8</td>
<td>3.635</td>
<td>0.089</td>
<td>3.7</td>
</tr>
<tr>
<td>1.0</td>
<td>3.656</td>
<td>0.068</td>
<td>2.8</td>
</tr>
</tbody>
</table>

* Initial concentration of NH₃-N = 3.724 mg/5.0 ml.

FIG. 4. Effect of cysteine on the absorption by sorbic acid in the ultraviolet region.

oxidation of succinate. The inhibition of oxidative phosphorylation may also have a greater effect on the active transport systems for succinate and fumarate than on the systems for the other substrates tested.

If a site of respiration involving fumarate is partially inhibited by sorbic acid, the ability of microbes to respire acetate, pyruvate, malate, and glucose at higher rates than fumarate and succinate in the presence of the same concentration of sorbic acid may involve a mechanism which bypasses the oxidation of succinate and fumarate. It may be possible that a system involving the carboxylation of pyruvate or the operation of the glyoxylate acid cycle could provide such a bypass mechanism.

An increase in the ratio of CO₂ to O₂ (RQ) and a diminution of the total uptake of oxygen was observed with washed cells of *S. cerevisiae* in the presence of sorbic acid with glucose as the substrate. A similar change from a predominantly aerobic to a predominantly anaerobic metabolism in yeast was reported in the presence of 2,4-dinitrophenol (Pickett and Clifton, 1943). An explanation based on the inhibition of oxidative phosphorylation may be proposed on the assumption that the depletion of ADP by oxidative phosphorylation suppresses fermentation. In the presence of sorbic acid, the phosphate is uncoupled, resulting in higher concentrations of ADP which allow fermentation to proceed. The smaller amount of oxygen uptake may be explained by assuming that the acetaldehyde formed effectively competes with oxidative mechanisms for reduced nicotinamide adenine nucleotide.

The inhibition of anaerobic growth may be explained on the basis of the inhibition of fermentative assimilation by sorbic acid, which in turn may be caused by the uncoupling of phosphate. The inhibition of aspartase may also be an explanation for the decrease in assimilation under anaerobic conditions. Because sorbic acid has been reported to inhibit several sulfhydryl enzymes, it is also possible that other reactions such as the amination of a-ketoglutarate may be inhibited at the higher concentrations needed to inhibit anaerobic growth, but this has not been investigated.

Sorbic acid reacts with cysteine, although slowly, with a decrease of absorption in the ultraviolet region. It is believed that an addition reaction occurs between sorbic acid and the thiol group of cysteine, and that this is the mechanism of inhibition of sulfhydryl enzymes. Since there is evidence that a vicinal dithiol is involved in a primary energy-trapping reaction in oxidative phosphorylation (Fluharty and Sanadi, 1960), the mechanism of inhibition of oxidative phosphorylation by sorbic acid may involve an interaction of such a thiol-containing compound.

Because sorbic acid caused a shift from an oxidative to a fermentative metabolism in this yeast under aerobic conditions, it is possible that such a shift was involved in the development of resistance. A similar shift was observed in yeast with the petite colonies obtained in the presence of ethylene oxide (Whelton and Phaff, 1947) and acriflavine (Ephrussi et al., 1949). It is not assumed, however, that the site and mechanism of inhibition of oxidative metabolism is the same for sorbic acid, ethylene oxide, and acriflavine.
SORBIC ACID INHIBITION OF MICROORGANISMS

LITERATURE CITED


