INHIBITION OF ALCOHOLIC FERMENTATION BY SORBIC ACID[1]

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Sorbic acid (2,4-hexadienoic acid) is the most effective of a number of α,β-unsaturated monocarboxylic fatty acids reported to inhibit the fungi (Gooding, 1945). It has been studied extensively with respect to inhibitory activity on various groups of microorganisms; and its use in controlling fermentative yeasts in cucumber fermentations has been the subject of considerable investigation in this laboratory (Costilow et al., 1955, 1957). Although this acid is a very effective inhibitor of yeast fermentation, the actual enzyme(s) inhibited have not been clearly elucidated. The present study was undertaken to determine the site(s) of sorbic acid inhibition in the anaerobic metabolism of *Saccharomyces cerevisiae*.

Melnick, Luckmann, and Gooding (1954) suggested that sorbic acid inhibited dehydrogenase systems in molds, and York and Vaughn (1955) reported it to be active in suppression of fumarase action. Both of these reports were concerned with aerobic metabolism and shed no light on the activity of this compound in anaerobic systems. Recently, Whitaker (1959) demonstrated that crystalline alcohol dehydrogenase was inhibited after incubation with sorbic acid. He suggested that the action on microorganisms is similar to that of maleic acid; i.e., the forming of stable complexes with sulfhydryl containing enzymes. Data collected in these studies are not in agreement with this hypothesis.

MATERIALS AND METHODS

The yeast used in these experiments was baker's yeast purchased locally, air dried, and maintained in the freezer. The cell-free extracts were prepared by blending a mixture of 15 g of dried yeast, 45 g of Superbrite glass beads[2] (average size, 0.2 mm), and 50 ml of water in a Servall omnimixer. The mixture was cooled in an ice bath before blending and held in the bath during a 5-min blending period. The beads, intact cells, and cell debris were removed by centrifugation in the cold. This method resulted in a high percentage of cell disruption and yielded an extract with good fermentative activity. However, it was necessary to add appropriate cofactors to maintain constant fermentation rates with various substrates.

Warburg techniques were used to measure CO_2 production by the yeast extracts from glucose (0.01 M), fructose 1,6-diphosphate (0.13 M), and 3-phosphoglyceric acid (0.01 M). These were all run in 0.033 M phosphate buffer at pH 6.0. Glucose fermentation was also tested at pH 5.0 with 0.033 M phthalate buffer. A mixture containing 5 μmoles adenosine triphosphate (ATP), 2 μmoles adenosine diphosphate (ADP), 10 μmoles, $MgCl_2$, and 2 μmoles diphosphopyridine nucleotide (DPN) was added to each Warburg cup along with 0.5 ml of the cell-free extract to study glucose fermentation. With fructose 1,6-diphosphate as substrate, the ATP was omitted from the reaction mixtures; and with 3-phosphoglyceric acid, both the ATP and DPN were omitted and 2 μmoles of reduced diphosphopyridine nucleotide (DPNH) were added. The total volume of liquid in each cup was adjusted to 3 ml, and the reactions run in a nitrogen atmosphere.

Activity of yeast extract with phosphoenolpyruvate as substrate was determined by measuring the rate of DPNH oxidation spectrophotometrically at 340 mμ. The crude yeast extract was dialyzed overnight against distilled water in the cold before running the assay. Reaction mixtures contained 0.5 ml of dialyzed yeast extract, phosphoenolpyruvate (8×10^{-4} M), ADP (3.3×10^{-4} M), $MgSO_4$ (8×10^{-3} M), and DPNH (1.5×10^{-4} M) in 0.067 M phosphate buffer (pH 7.4). The volume of the liquid in the cuvette was adjusted to 3 ml with distilled water.

Possible phosphoglyceromutase inhibition was tested indirectly by blocking enolase with fluoride in dialyzed yeast extract and measuring DPNH

[1] Journal article no. 2497.

[2] Manufactured by Minnesota Mining and Manufacturing Company, St. Paul.

oxidation with 2-phosphoglyceric acid as substrate The methods used were as described in the preceding paragraph except that the reaction mixture contained 0.05 ml of dialyzed yeast extract, 2-phosphoglyceric acid (4×10^{-4} M), ATP (2×10^{-4} M), $MgSO_4$ (8×10^{-3} M), DPNH (3.6×10^{-4} M), and NaF (0.16 M) in 0.05 M imidazole buffer (pH 7.4). When the ATP was replaced with ADP in the mixture, no oxidation of DPNH occurred indicating that the fluoride blocked the enolase activity completely.

Carboxylase activity was measured by the method of Green, Herbert, and Subrahnanyan (1941) and alcohol dehydrogenase by that of Racker (1950) except that in both instances the crude yeast extract was used without purification. The effect of sorbic acid was also tested on a commercial preparation of alcohol dehydrogenase by the same method.

Possible inhibition of the aldolase or 3-phosphoglyceraldehyde dehydrogenase of the yeast extract was tested by the method of Warburg and Christian (1943) for determining aldolase activity, except that no purification procedures were used and no 3-phosphoglyceraldehyde dehydrogenase was added to the reaction mixture.

Enolase was determined by the method of Warburg and Christian (1941) as modified by Wold and Ballou (1957). With 2-phosphoglyceric acid as substrate, the appearance of phosphoenolpyruvate is followed by measuring the increase in optical density at 240 mμ. A Beckman model DU spectrophotometer was used for all spectrophotometric work.

Refined sorbic acid, obtained from the Carbide and Carbon Chemicals Company, was recrystallized three times from distilled water before use. Fresh stock solutions (1.5×10^{-2} M) were maintained in the refrigerator during the experiments. These were adjusted to the pH level desired for each experiment and diluted to give a final concentration of 5×10^{-3} M in all inhibition studies except for those with enolase. This was much too concentrated for the enolase work, and a number of lower concentrations were used.

The cofactors and glycolytic-intermediate compounds used were of commercial origin, and solutions were either prepared fresh for each experiment or maintained in the frozen state.

RESULTS AND DISCUSSION

Initial experiments demonstrated that the fermentation of glucose by resting cells of baker's yeast was greatly inhibited by sorbic acid. As expected from previous work (Bell, Etchells, and Borg, 1959), the extent of the inhibition was very dependent on the pH. At pH 6.0, the fermentation was inhibited 25 per cent, whereas 46 per cent inhibition was observed at pH 5.0. In an attempt to ascertain the reason for this effect of pH, cell-free extracts of yeast were prepared and used for inhibition studies. As shown in figure 1, glucose fermentation was inhibited 26 per cent at pH 6.0 and 29 per cent at pH 5.0.

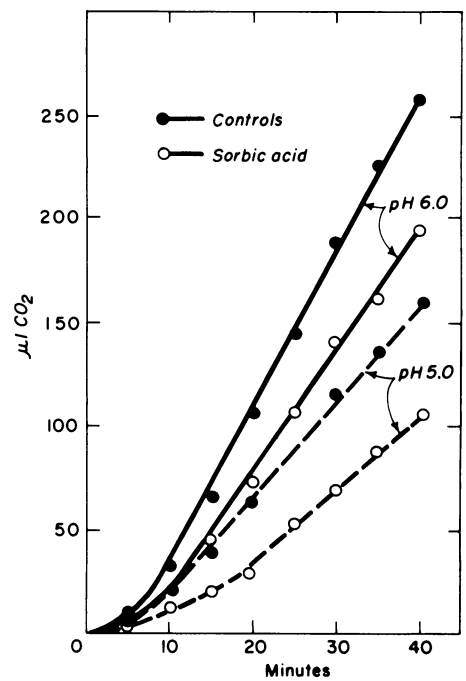

Figure 1. Effect of sorbic acid (5×10^{-3} M) on glucose fermentation by cell-free extracts of yeast at two pH levels as measured by CO_2 evolution. The main compartment of each Warburg cup contained 5 μmoles ATP, 2 μmoles ADP, 2 μmoles DPN, 10 μmoles $MgCl_2$, 0.5 ml of 0.2 M buffer (phosphate buffer used for pH 6.0 and phthalate buffer for pH 5.0), and 0.5 ml extract. Glucose, 0.5 ml of 0.06 M, was tipped in from a side arm after thermal equilibrium had been attained at 30 C. One milliliter of 0.015 M sorbic acid adjusted to the reaction pH was added to main compartments of cups for inhibition tests. The total volume was 3.0 ml. The flasks were gassed with N_2.

Thus, there was no significant difference in the per cent inhibition at the two pH levels. These data support the hypothesis of Bell et al. (1959) that the enhanced inhibitory action of this acid at low pH levels is primarily due to the greater permeability of the cell to the undissociated form.

To determine the specific enzyme(s) inhibited by sorbic acid, tests for inhibition of activity of cell-free extracts with a number of glycolytic intermediates as substrates were conducted. The rationale behind these experiments was to limit the number of possible inhibitory sites.

First, a study was conducted of the influence of sorbic acid on the rates of CO_2 production from fructose 1,6-diphosphate and 3-phosphoglyceric acid. Manometric studies demonstrated that CO_2 production was inhibited by 28 per cent with the former and by 26 per cent with the latter substrate. Since the degree of inhibition was the same as with glucose, it was indicated that the site of sorbic acid inhibition was beyond 3-phosphoglyceric acid in the Embden-Meyerhof sequence. This was substantiated by the fact that there was no inhibition of DPN reduction by these crude yeast extracts using fructose 1,6-diphosphate as substrate, indicating that neither aldolase or 3-phosphoglyceraldehyde dehydrogenase were affected greatly. The range of possible sites was further narrowed by testing the effect of sorbic acid on carboxylase and alcohol dehydrogenase activity of cell-free yeast extract. No inhibition of these two enzymes was observed. Neither was there any indication of phosphopyruvic acid transphosphorylase inhibition since sorbic acid did not influence the rate of DPNH oxidation by dialyzed crude yeast extract with phosphoenolpyruvate as substrate. Phosphoglyceromutase is probably not affected by sorbic acid since there was no inhibition of DPNH oxidation by dialyzed yeast extract with 2-phosphoglyceric acid as substrate in the presence of ATP and sufficient fluoride to inhibit enolase completely. Therefore, it was concluded that enolase was the most likely site of sorbic acid inhibition of alcoholic fermentation.

Since there are a number of the α,β-unsaturated fatty acids which are quite inhibitory to yeasts, whereas their saturated counterparts are ineffective at similar concentrations, and, since phosphoenolpyruvate is unsaturated in the α,β position, it appeared that a competition between sorbate and phosphoenolpyruvate might occur. While 2-phosphoglyceric acid is the substrate for enolase in the normal fermentation sequence, the Michaelis constants for the forward and reverse reaction are about equal according to Wold and Ballou (1957); therefore, the affinity of the enzyme for 2-phosphoglyceric acid and phosphoenolpyruvate is about the same, and competition with the product would be expected to cause inhibition.

To test this hypothesis, purified yeast enolase was obtained through the generosity of Dr. Finn Wold, Department of Chemistry and Chemical Engineering, University of Illinois, Urbana, and tested for sorbic acid inhibition. These tests were conducted using a constant enzyme concentration of 6.0×10^{-10} M and various sorbic acid and substrate (2-phosphoglyceric acid) concentrations.

Sorbic acid concentrations of 1.5×10^{-4} and higher resulted in definite inhibition of enolase activity with a substrate concentration of 2×10^{-4} M (figure 2). Concentrations of sorbic acid below 1.5×10^{-4} M resulted in very little or no inhibition of the enzyme at this substrate level; and, when the sorbic acid level was raised much above 2.5×10^{-4} M, no significant enzyme activity could be detected.

Similar experiments conducted at substrate concentrations of 4×10^{-4} and 5×10^{-5} M demonstrated that the substrate concentration had a pronounced effect on the degree of enolase inhibition by a given level of sorbic acid. This is illustrated in figure 3 where the reciprocals of the reaction velocities are plotted versus the reciprocals of substrate concentrations for the various sorbic acid concentrations used (Lineweaver-Burk plot). From this plot it appears that the inhibition is competitive at sorbic acid concentrations up to 2.0×10^{-4} M and is noncompetitive at higher inhibitor levels. Thus, the intercept is constant and the slope increases with increasing inhibitor concentrations up to 2.0×10^{-4} M, whereas both the slope and intercept change at higher concentrations. However, a plot of reciprocal velocity versus inhibitor concentration yields a curve whose slope increases with increasing inhibitor levels, which indicates that the inhibition is partially competitive and partially noncompetitive (Dixon and Webb, 1958). Therefore,

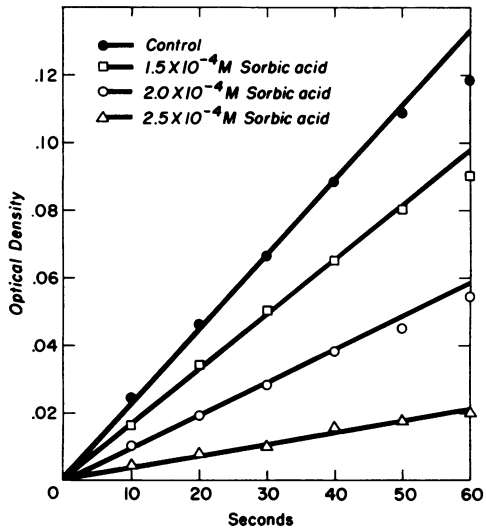

Figure 2. Inhibition of yeast enolase activity by sorbic acid. Activities were measured in a spectrophotometer at room temperature. Each cuvette contained 3 ml of a reaction mixture of the following: 6×10^{-10} M enolase, 2×10^{-4} M substrate (2-phosphoglyceric acid), 8×10^{-3} M $MgCl_2$, and 5×10^{-2} M imidazole buffer at pH 7.4. Appropriate solutions of sorbic acid adjusted to pH 7.4 were added to give the concentrations indicated in the figure. The increase in optical density was recorded at 10-sec intervals.

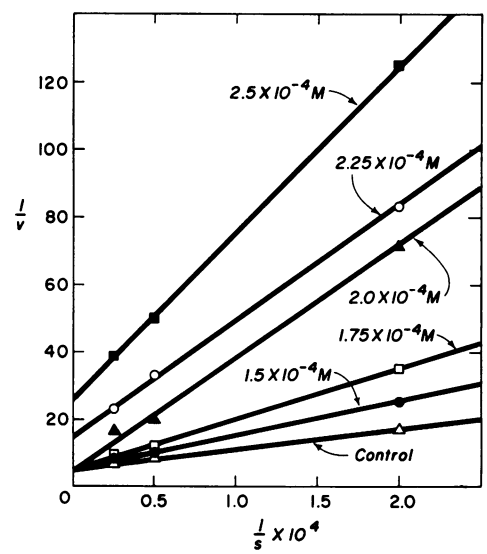

Figure 3. Lineweaver-Burk plot of sorbic acid inhibition of enolase. Inhibition studies were run as described under figure 2 with substrate concentrations of 5×10^{-5} M, 2×10^{-4} M, and 4×10^{-4} M. The reaction velocities (V) were calculated in terms of the change in optical density per min for each sorbic acid-substrate combination, and the reciprocals of the velocities plotted versus the reciprocals of the substrate concentrations for each sorbic acid concentration indicated in the figure.

it is concluded that the sorbic acid inhibition of enolase is a complex type.

Other α,β-unsaturated acids, crotonic (4×10^{-4} M) and cinnamic acid (4×10^{-4} M), were found to inhibit enolase activity. However, they were not as effective as sorbic acid.

In determining enolase activity in the presence of sorbic acid, it was noted that there was an initial decrease in optical density at 240 mμ on the addition of enolase to the reaction mixture. This masked the initial increase in optical density due to enzymatic action and created an apparent lag. The problem was eliminated by adjusting the instrument to zero 10 sec after adding enzyme to the reaction mixture. This decrease in absorbancy probably results from a combination of the sorbate with the enzyme, since the sorbate absorbs light strongly at this wavelength. Further studies showed that this drop was independent of the substrate, but was influenced by the concentrations of sorbic acid, enolase, and Mg^{++}. Up to a point, an increased level of any one of these with a given concentration of the other two, would result in a greater optical density drop. Similar losses in optical density were observed on mixing other α,β-unsaturated acids (crotonic and cinnamic acids) with enolase. Also this phenomenon was observed when phosphoenolpyruvate was used as the substrate for enolase activity in the absence of sorbic acid. Finally, it was demonstrated that the addition of individual amino acids to a solution containing Mg^{++} and either sorbic acid or phosphoenolpyruvate would result in a similar loss of optical density.

Mg^{++} is known to be important as an activator of enolase, and Malmstrom, Kimmel, and Smith (1959) concluded that the activation process results from a combination of the ion with the enzyme and not the substrate. Therefore, the substrate combines with a metal-enolase complex. Since these data indicate that α,β-unsaturated acids in general react or combine with enolase in the presence of Mg^{++}, it is proposed that this may be the basis of sorbic acid inhibition. At

lower levels, the inhibition may be at least partially competitive with the sorbic combining with a site(s) adjacent to the active site on the enzyme reducing the affinity of the enzyme for the substrate; whereas at higher levels, the number of sorbic acid molecules combining with each molecule of enzyme may be much greater, resulting in primarily a partially noncompetitive inhibition.

Since the report of Whitaker (1959) appeared during the course of these experiments, the work with alcohol dehydrogenase was repeated using both cell-free yeast preparations and crystalline alcohol dehydrogenase. Our data with the crystalline enzyme substantiated Whitaker's report; i.e., sorbic acid inhibited this enzyme preparation and the degree of inhibition was greatly enhanced by incubating the enzyme with sorbic acid prior to adding substrate. Whitaker observed considerable protection of the crystalline enzyme from the effects of sorbic acid by sulfur-containing amino acids. He, therefore, postulated that alcohol dehydrogenase was inhibited as a result of the formation of a stable complex between sorbic acid and the sulfhydryl groups of the enzyme. Since no inhibition of alcohol dehydrogenase activity in the yeast extracts was observed even after prolonged incubation of the extracts with sorbic acid, the concentration of protective materials in the yeast extracts must have been sufficient to offer complete protection. It is significant that yeast enolase contains no sulfhydryl groups according to Malmstrom et al. (1959); thus the enolase could not be affected in this manner.

It must be concluded from these data that the inhibition of alcoholic fermentation by sorbic acid is primarily the result of enolase inhibition. The question of the failure of this acid to affect lactic acid bacteria to any great extent is yet to be resolved. Preliminary experiments in our laboratory indicate that enolase activity in cell-free extracts of *Lactobacillus brevis* and *Pediococcus cerevisiae* is not inhibited by sorbic acid.

SUMMARY

The extent of sorbic acid inhibition of alcoholic fermentation by resting cells of baker's yeast is dependent on the pH, but no significant effect of pH was evident in the per cent inhibition of fermentation by cell-free extracts of yeast. Therefore, it is concluded that the pH effect observed with this acid is probably related to the greater permeability of the cell to the undissociated form of the acid.

Sorbic acid was shown to inhibit CO_2 production by cell-free yeast extracts from glucose, fructose 1,6-diphosphate, and 3-phosphoglyceric acid. The per cent inhibition was essentially the same with all three of these substrates. However, it did not inhibit the activity of the yeast extracts in the reduction of diphosphopyridine nucleotide with fructose 1,6-diphosphate as substrate, the oxidation of reduced diphosphopyridine nucleotide with phosphoenolpyruvate as substrate, the oxidation of reduced diphosphopyridine nucleotide with 2-phosphoglyceric acid as substrate in the presence of adenosine triphosphate and sufficient fluoride to block enolase activity, the decarboxylation of pyruvate, or the reduction of diphosphopyridine nucleotide with ethanol as substrate. Therefore, it was concluded that the site of sorbic acid inhibition was between 2-phosphoglyceric acid and phosphoenolpyruvate.

Studies with purified yeast enolase demonstrated that this enzyme was inhibited greatly by sorbic acid. The degree of inhibition was dependent on both the substrate and inhibitor concentrations; but the type of inhibition appeared to be complex. It is concluded that enolase is the primary site of sorbic acid inhibition of alcoholic fermentation.

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