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PRELIMINARY STUDIES OF THE MECHANISM OF YEAST INHIBITION BY SORBIC ACID

by

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A THESIS

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INTRODUCTION

Many organic acids have long been used in food industry for the control of undesirable microorganisms. Among these are some of the short-chain fatty acids, di- and tri-carboxylic acids and aromatic acids.

Considerable information has been accumulated in the past on the antimicrobial properties of these acids. However, still little is known on the mechanism by which sensitive microorganisms are inhibited by them. Elucidation of the mechanism of inhibition is essential from the standpoint of understanding the behavior of these acids and more logical application to the food industry.

Most organic acids are useless at high pH levels. Certain groups of organic acids have similarities in the spectra of sensitive microorganisms. There are relationships between the structures of organic acids and their inhibitory properties. These observations seem to suggest that organic acids, especially those in a homologous series, may owe their inhibitory action to the physiochemical properties they have in common. It is probable that the inhibitory action of various organic acids may depend, to varying extents, on the same mechanism of inhibition.

This study has been initiated for the purpose of throwing light on this problem by making systematic investigations of various organic acids. As the first approach, the present study deals with a specific organic acid, sorbic acid, which is used in food industry as a mold and yeast inhibitor. The effects of this acid on the metabolism of a sensitive microorganism have been studied. Also, an attempt has been made in determining the type of mechanism by which sorbic acid inhibits the sensitive microorganism.

REVIEW OF LITERATURE

Studies have established that sorbic acid, 2, 4-hexadienoic acid, has marked antimicrobial action against a wide variety of microorganisms; molds, yeasts, and many bacteria have been found to be sensitive to this agent (Emard and Vaughn, 1952). Among the various types of bacteria tested, lactobacilli and clostridia are much less sensitive to sorbic acid than others. Therefore, it has been suggested that the antimicrobial action of sorbic acid appears to be directed against the "catalase positive" microorganisms. Little information is available on the mechanism of action of sorbic acid but its inhibitory action on molds has been attributed to a probable inhibition of dehydrogenase systems (Melnick et al, 1954). Also, it has been reported that the principal inhibition by this agent results from suppression of fumarate oxidation (York and Vaughn, 1955).

In an attempt to throw light on the present problem, the following review has been made of the published accounts concerning the mechanism of inhibition of various organic acids and some important factors which have to be considered in this type of study. It is probable that the antimicrobial properties exhibited by certain groups of organic acids,

especially those in a homologous series such as fatty acids, may depend, to various extents , on the same mechanism due to the physicochemical properties they have in common. Attention has been concentrated on the aspects where certain groups of organic acids may be interrelated. However, it is also possible that other mechanisms may operate and that some organic acids may depend on an entirely different mechanism. Efforts have been made in formulating and summarizing the scattered information on these acids so that they will become available and more meaningful. Also, a review has been made of the published accounts concerning sorbic acid; and based on our knowledge of the action of organic acids, suggestions have been made on the various points which should be considered.

The Mechanism of Action of Organic Acids

It has long been recognized that many organic acids have a toxicity for microorganisms in excess of what could be possibly due to the hydrogen ion concentration alone.

A relatively narrow range of pH exists for the optimum growth of most bacteria, and this is usually in the neutral to slightly alkaline range. With such microorganisms, acidities may be inhibitory due to the hydrogen ion concentration alone (Degering and associates, 1940). However, this is generally not true with molds and yeasts. Common molds appear to grow over a wide range of pH and optimally in acidic media. Many

organic acids are inhibitory to yeasts and molds and to bacteria at pH levels where the hydrogen ion concentration has no inhibitory action. For instance, acetic acid has been found to be inhibitory to many microorganisms at pH levels which would normally be non-inhibitory (Kahlenberg and True, 1896; Winslow and Lockridge, 1906). In solutions of equal pH, butyric acid and acetic acid are more toxic than HCl and other strong acids (Collett, 1919; 1921).

The relation of ionization of organic acids to their inhibitory action and to cell permeability. Most organic acids with antimicrobial properties have remarkable characteristics in that their inhibitory activity is greatly influenced even by small changes in pH. The important effect of pH on the antimicrobial properties of fatty acids was demonstrated by Levine and Fellers (1940). In all cases, their inhibitory action is enhanced markedly with an increase in acidity of the media. A similar effect of pH has been demonstrated with many other organic acids. In this connection, the extensive works by Degering and his associates (1938; 1939; 1940) are of interest. These authors have measured the effect of pH on the concentration of benzoic acid, compounds related to benzoic acid, and many other antiseptics required to kill Staphylococcus aureus and Bacillus coli in 10 minutes. For most compounds tested, a smaller amount was required to kill the test microorganisms at low pH than at high pH levels.

Many theories have been proposed by various authors, in an attempt to explain the effect of pH on inhibitory properties of various compounds. The effect of pH on the inhibitory action of organic acids and other weak acids has been explained on the basis that non-ionized acid molecules rather than anions are responsible for the inhibitory action (Krahl and Clowes, 1938; Rahn and Conn, 1944).

The relation between pH of solution and degree of ionization of monobasic weak acids is given by the Henderson and Hasselbalch equation as follows (Jacobs, 1940):

$$pH = pK_a + \log \frac{(A^-)}{(HA)} \qquad \ldots\ldots\ldots (1)$$

where (HA) represents concentration of non-ionized molecules and (A^-) concentration of the corresponding anions. Therefore, the degree of ionization in terms of percent of non-ionized molecules can be expressed by the following alternative equation:

$$\text{Percent non-ionized} = \frac{100}{1 + \text{antilog } (pH - pK_a)} \qquad \ldots\ldots(2)$$

Most organic acids are ionized to a considerable extent in neutral solution, but to a lesser extent in acid solution, and are almost completely non-ionized in strongly acidic solution.

The enhanced toxicity of organic acids at low pH levels is possible if the non-ionized acid molecules rather than ions are responsible for the inhibitory action. It has been shown that Streptococcus lactis ceases to produce lactic acid when the non-ionized acid reaches 0.017 molar, regardless of pH

(Rogers and Whittier, 1928). The non-ionized molecules of lactic acid have been held responsible for the inhibitory action where the pH value was such as to be unimportant (Bach, 1932). The inhibitory concentration of benzoic acid and salicylic acid for Saccharomyces ellipsoideus has been determined at various pH levels and the inhibitory concentration of the non-ionized acid calculated. The inhibitory concentrations of non-ionized acid molecules were fairly constant for all the pH levels tested, while nearly 100 times higher concentration of total acids were required in neutral than in strongly acidic solutions (Rahn and Conn, 1944).

The view that only the non-ionized molecules of the organic acids are toxic has been subject to criticism. Simon (1950) has illustrated that the relative contribution of non-ionized molecules and the corresponding anions can be calculated from the ionization constant or pK_a of the acid and analyzed graphically. With many examples using both his own data and results by various authors, it was demonstrated that the anions do contribute, to varying extents, to inhibitory action of various compounds. The logarithm of the concentration of total acid required to produce a standard toxic effect and the corresponding concentration of non-ionized molecules were plotted against pH. Thus two curves were obtained, one representing the concentration of total acid required and the other representing the corresponding concentration of non-ionized acid molecules. The toxicity of compounds such as phenol (pK_a 10.0)

which are not ionized in solution is not affected by pH. On the other hand, the toxicity of most organic acids is dependent on pH of the medium and the pK_a of the acid. At pH's two or more units below pK_a, where the acid is almost completely non-ionized, the toxicity is not influenced by pH. The concentration of total acid required to produce a standard toxic effect increases as the pH increases; the point along the pH scale at which the rise of the curve for total acid commences is governed by pK_a of the acid. With acids where only non-ionized acid is responsible for the toxicity, the concentration of the non-ionized acid required to produce a given effect is independent of pH. However, it is significant that, in general, the extra amount of total acid required at high pH is not so large as to maintain a constant concentration of the non-ionized acid; i.e., the equi-effective concentration of non-ionized molecules decreases at pH levels where there is much ionization. Therefore, the anions do appear to contribute toxicity to varying extents. The significance of this finding has been emphasized (Simon and Blackman, 1949).

McElroy (1947) has pointed out that the ionization of a toxic agent is important from the standpoint of both the mechanism of cell penetration and the actual mechanism of inhibition. It is obvious that the factor of permeability is important wherever a particular agent must penetrate the cell membrane in order to gain access to the site of action and produce its effect. With organic acids which are generally ionized to a considerable

extent within the range of the normal physiological pH, it is important to determine whether the acid penetrates in the form of non-ionized molecules, ions, or both.

The mechanism of penetration into cells by ions and non-ionized molecules of weak acids has been discussed by Jacobs (1940). It has been pointed out that the entrance of non-ionized molecules of weak acids, particularly those with considerable non-polar CH portions, seems to take place with great rapidity according to the simple law of diffusion. However, the permeability of cells to ions in general is a decidedly complex phenomenon involving theoretical equilibria which may be approached very slowly and perhaps never be attained. The majority of cell membranes behave as though they consist of two monolayers of lipids surrounded on either side by a monolayer of protein (Davson and Danielli, 1943). Such membranes are strongly charged and it is difficult for hydrated ions of similar charge to gain access.

In the extensive studies on the effect of substituted phenols on the respiration and cell division of sea urchin eggs, it has been found that various phenols penetrate the fertilized eggs of Arbacia punctulata only in the form of non-ionized molecules (Krahl and Clowes, 1936; 1938; 1940). The equi-effective concentrations of non-ionized molecules have been found to be independent of the concentration of phenol anions in the medium. It appears, therefore, that the relation between

pH and toxicity of organic acids discussed previously may be the result only of the higher penetrability of the non-ionized acid molecules. The effect of pH on the inhibitory action could also be explained in terms of concentration of ions and non-ionized molecules at the site of action, assuming both forms have toxic action within the cells (Simon and Blackman, 1949).

Surface mode of action of long-chain saturated and unsaturated fatty acids. Recent studies have established that many antibiotic agents can affect microorganisms by altering or interfering with the structure and properties of the cell wall and properties of the adjacent protoplasm (Cooper, 1956; Newton, 1956; Lederberg, 1957). Recent views on the cell wall of bacterial cells strongly indicate that the cell wall has to be considered not only as a boundary between the living cytoplasm and surrounding, but also, as an effective part of the microorganism. Consequently, antimicrobial agents may inhibit bacteria through a mechanism which alters or prevents formation of the cell wall.

Considerable information has been accumulated in the past on the antimicrobial properties of fatty acids, among which long-chain unsaturated fatty acids have attracted the particular interest of various authors. It has been demonstrated that these acids act because of their surface properties (Kodicek, 1949).

Those unsaturated fatty acids which act as growth factors for some microorganisms in very low concentration are inhibitory to growth in higher concentrations. This combined stimulatory and inhibitory action which has been referred to as "double action" (Pollock, 1949), has been demonstrated in many cases. Oleic acid has been found to exhibit "double action" on Mycobacterium tuberculosis (Dubos and Davis, 1946); Micrococcus 'C' (Dubos, 1947); Erysipelothrix rhusiopathiae (Hutner, 1942); Corynebacterium diphtheriae (Cohen and Boggiano, 1947); and on Lactobacillus bulgaricus and Streptococcus lactis (Williams and Fieger, 1947). Linoleic and linolenic acid have the same action on Lactobacillus casei (Bauernfeind et al, 1942; Strong and Carpenter, 1942).

The antibacterial action of unsaturated fatty acids has been found to be far more extensive than the saturated ones (Baurenfiend et al, 1942; Strong and Carpenter, 1942). In general this activity increases with an increase in the number of double bonds; e.g., oleic<linoleic<linolenic (Kodicek, 1949). Naturally-occurring cis-forms of the unsaturated fatty acids appear to have more marked activity than the other forms. (Bergstrom et al, 1946; Dubos and Davis, 1946; 1947; Kodicek and Worden, 1946). One of their outstanding properties is the extremely low concentration at which they act.

Esters of the unsaturated fatty acids are inactive (Kodicek and Worden, 1946). In fact, these esters are often growth-promoting, especially in presence of proteins or other

surface active agents, such as lecithin, the sterols, tocopherol, proteins, etc.

It has been observed that gram-positive bacteria appear to be more sensitive to inhibition by unsaturated fatty acids than gram-negative bacteria (Kodicek, 1946; 1949). Non-bacterial microorganisms do not appear to show such a high sensitivity to these acids as bacteria (Benham, 1941; Rothman et al, 1946; Sprince and Kupferberg, 1947; Wyss et al, 1945).

Attempts have been made to explain these findings by postulating probable mechanisms of action. While it is generally accepted that the antibacterial action of the long-chain fatty acids is primarily concerned with the bacterial surface, the exact mechanism of action is not at all certain. The earliest explanation is that the long chain fatty acids, by virtue of their surface activity, exert inhibitory action by altering the surface tension at the cell surface (Frobisher, 1926; Lamar, 1911). Later authors suggested that the site of action is on the lipo-protein of the cell wall and explained the mechanism of action on the basis of altered permeability of the cell wall (Williams and Fieger, 1947). More recently, Kodicek (1949) has postulated a "physicochemical theory of mechanisms," based on his findings. This author found that the inhibitory action of the unsaturated fatty acids is reversible by naturally-occurring surface-active substances such as sterols, vitamins, lecithins and proteins, apparently involving a dynamic equilibrium. In addition, naturally occurring -

cis-forms of the unsaturated fatty acids have more marked activity in comparison with other forms. These findings together with other physicochemical evidence led the author to postulate a mechanism involving a change in the permeability of the cell wall, or in the rate of permeation of substances through the cell wall regulated by a mechanism more complex than diffusion.

Recently, the development of electron transparent areas (ETA) in the cells of Mycobacterium avium treated with high concentrations of oleic acid and linoleate (10^{-3}M) has been shown by electron microscopic technique (Minami, 1957). Although no significant morphological changes were noticeable at a lower concentration (10^{-5}M) which was bactericidal to the bacilli, it is probable that the ultramicroscopic changes would not be revealed by this technique.

Metabolic effects of organic acids. Certain organic acids may exert inhibitory action on microorganisms by acting as the inhibitory analogues and, thus, interfering with utilization or synthesis of metabolites. Many examples are available where slight modification in structure of a metabolite converts it into an inhibitory compound (Welch, 1945).

The inhibitory action of some short-chain fatty acids such as acetate, propionate and butyrate can be reversed by casein hydrolysate, by aspartate or glutamate, and by pantothenate (Wyss, 1946). On the other hand, the inhibitory action

of formate, valerate and fatty acids of chain length exceeding five carbon atoms is not reversed by these compounds. With E. coli, propionate has been shown to have a greater inhibitory effect than that observed with other fatty acids such as acetate and butyrate (Wright and Skeggs, 1946). The inhibitory action of propionate on E. coli which is capable of rapid growth in medium free of B-alanine or pantothenate can be reversed by B-alanine which is structurally related to propionate and pantothenate which contains B-alanine in its molecule. It has been postulated that propionate may exert inhibitory action by competitive interference with the synthesis of B-alanine by E. coli. The effect of each pantothenate moiety on the inhibitory action of propionate has been studied in Acetobacter suboxydans and Saccharomyces cerevisiae in which the ability to synthesize the vitamin is more restricted than E. coli (King and Cheldelin, 1948). Pantothenate was found to be much superior to B-alanine in reversing the inhibition. The great sensitivity of the yeast which cannot synthesize B-alanine and the ready reversal of the propionate inhibition by B-alanine indicates that the utilization of B-alanine is impaired. It has been postulated that propionate may compete with B-alanine for attachment within the yeast cell; and, thereby, prevent the coupling of B-alanine and pantoic acid to form pantothenate.

Recent studies have established the important role of CoA in fatty acid oxidation (Lipman, 1953; Lynen, 1956). The relation of pantothenate to CoA has been elucidated and the

structure of CoA established (Baddiley, 1955). Various carboxylic acids, both aliphatic and aromatic, have been reported to affect the growth of various microorganisms as previously discussed. Many carboxylic acids have also been shown to affect the fatty acid oxidation involving CoA (Avigan et al, 1955).

The effects of propionate on the acetate metabolism have been studied in some detail and the corresponding postulates for the mechanism of inhibition have been discussed (Pennington, 1957). It has been pointed out that the inhibitory effect of propionate on microorganisms cannot be solely attributed to the interference with the synthesis of pantothenate since higher levels of propionate inhibit the growth of microorganisms even in the presence of pantothenate (Wright and Skeggs, 1946; Hill, 1952). Evidence has been presented that crystalline protogen which has been identified with the acetate replacing factor and the pyruvate oxidation factor (POF), is effective in reversing the inhibitory action of propionate on Streptococcus faecalis (Stokstad and Pierce, 1952). POF has been shown to be necessary for the formation of acetate from pyruvate (O'Kane and Gunsalus, 1948; O'Kane 1950). It appears, therefore, that propionate may inhibit growth of bacteria by interfering with acetate formation. Recently, the involvement of CoA in the oxidation of pyruvate to acetate has been demonstrated (Chantrene and Lipman, 1950;

Korkes et al, 1950). Propionate has been shown to combine with CoA in the extracts of Clostridium kluyveri (Stadtman, 1952). The finding that inhibition of Streptococcus faecalis can be overcome by addition of acetate has been explained by postulating a propionate-CoA combination which prevents CoA from participating in the formation of acetate via oxidative decarboxylation of pyruvate (Hill, 1952).

The inhibitory effects of propionate on the acetate metabolism have been studied in various animal tissues and purified enzyme preparations. With liver and kidney "cyclophorase" preparations, it has been shown that oxidation of acetate is inhibited by propionate which itself is not oxidized (Grafflin and Green, 1948). With sheep-rumen epithelial tissue, propionate markedly depresses the oxygen uptake in a medium without bicarbonate but increases uptake in a medium buffered with bicarbonate. It has been suggested that CoA may be immobilized as propionyl CoA, the metabolism of which is blocked in absence of CO_2 (Pennington, 1954). With pyruvate as the substrate, propionate has been shown to suppress ketone-body formation by rumen-epithelium from pyruvate and decrease slightly the uptake of pyruvate (Pennington and Sutherland, 1956). Propionate has also been shown to inhibit the oxygen uptake of rabbit liver and kidney preparations in the presence of low levels of succinate. Also, formation of acetoacetate is depressed which has been attributed to the effective

competition by propionate for CoA (Lang and Bassler, 1953). More recently, the effects of propionate on acetate metabolism have been investigated with rat liver preparations in an attempt to throw light on the mechanism of inhibition (Pennington, 1957). Propionate suppressed the metabolism of acetate markedly; both the uptake of acetate and its conversion into CO_2 and ketone bodies were inhibited. On the other hand, the oxidation of pyruvate is not greatly affected and butyrate not at all. It has been postulated that propionate blocks the combination of acetate with CoA.

However, the mechanism by which propionate inhibits the enzyme system responsible for acetate metabolism is not at all certain. Investigation with purified enzyme from ox-heart muscle which appears to be a single enzyme responsible for conversion of acetate and propionate into their CoA derivatives seems to exclude the possibility of straightforward competition between acetate and propionate for a common enzyme. The Michaelis-Menten constant is greater with propionate (5.00×10^{-3}) than with acetate (1.42×10^{-3}) (Hele, 1954). It has been suggested as a possibility that separate acetic and propionic thiokinase may exist in liver, and the acetic enzyme may be blocked by propionate (Pennington, 1957).

Benzoate has been shown to inhibit acetoacetate synthesis from butyrate in "cyclophorase" preparations (Grafflin and Green, 1948) and in guinea pig liver preparation (Jowett and Quastel,

1935). Benzoate also inhibits the oxidation of butyrate to CO_2 in guinea pig slices (Avigan and Scholefield, 1954) and the oxidation of alkylthio fatty acids to CO_2 in rat liver slices (Brown and Scholefield, 1954).

Alkylthioacetate has been found to inhibit acetoacetate synthesis from acetate and butyrate in guinea pig slices to varying extents. It has been suggested that ethylthio-acetate and benzoate probably inhibit the acetoacetate synthesis by competition of their CoA derivatives with acetyl-CoA (Avigan and Scholefield, 1954).

Butyrate has been shown to inhibit markedly the acetate oxidation to CO_2 (Pennington, 1957). Butyrate also inhibits acetoacetate synthesis from acetate by guinea pig liver preparations which synthesize butyrl-CoA. On the other hand, little or no inhibitory effect of butyrate has been observed on the acetoacetate synthesis from acetate by pigeon liver preparations in which the necessary enzyme for butyrl-CoA synthesis is absent (Avigan et al, 1955).

The effects of various carboxylic acids on fatty acid oxidation have been investigated (Avigan et al, 1955). Short-chain fatty acids such as acetate, propionate and butyrate inhibit the acetoacetate synthesis from acetate by guinea pig preparations which are capable of forming acyl-CoA derivatives from the fatty acids. The effects of long-chain fatty acids such as palmitate and octanoate have been studied on carboxyl-C^{14} labelled butyrate oxidation in rat liver preparations.

Octanoate inhibits equally both CO_2 formation and acetoacetate synthesis from butyrate. Palmitate, on the other hand, causes much larger inhibition of acetoacetate synthesis than of CO_2 formation from butyrate. These facts indicate that other fatty acids may compete for butyrate activating enzyme systems. On the basis of these findings, it has been postulated that various reactions of acetyl-CoA and possibly of aceto-acetyl-CoA are specifically inhibited by acyl-CoA complexes and that these inhibitory effects influence the kinetics of fatty acid oxidation to acetoacetate and CO_2. In further support of this hypothesis, the effects of acyl-CoA complexes on acetylation have been shown to be reversible by acetyl-CoA prepared *in situ* from acetyl phosphate in the presence of *E. coli*.

The Mechanism of Action of Sorbic Acid

The antimicrobial properties of sorbic acid. Studies by various authors have established the marked antimicrobial properties of sorbic acid on a wide variety of microorganisms (Emard and Vaughn, 1952). These have been thoroughly reviewed by Sheneman (1954) and Ferguson (1955).

In connection with the present study, the following points are of interest. Emard and Vaughn (1952) tested the effect of sorbic acid on 299 different cultures including actinomycetes, bacteria, molds and yeasts. All cultures were greatly inhibited except for those of the genera *Lactobacillus*,

Leuconostoc and Clostridium, which, in general, do not possess catalase. Therefore it was concluded that the inhibitory action of sorbic acid is directed against the "catalase positive" microorganisms.

The effect of pH on the inhibitory action of sorbic acid has been investigated (Emard and Vaughn, 1952). At pH levels of 5.0 to 5.5, 0.07 percent sorbic acid in glucose broth effectively inhibited the growth of all the sensitive microorganisms, whereas it was inadtive at pH levels above 5.5. A similar relation has been observed with yeasts (Sheneman, 1954; Ferguson, 1955). Etchells et al. (1955) tested eight species of yeasts for the effect of sorbic acid at pH levels between 4.0 and 6.9. These authors concluded that the inhibitory concentration of sorbic acid was directly related to the concentration of the non-ionized acid molecules.

Probable mechanisms of action of sorbic acid. Little work has been done which elucidate the mechanism by which sorbic acid inhibits various microorganisms. The mechanism of mold inhibition by sorbic acid has been explained on the basis of the finding by Mukherjee (1952b) that accumulation of an intermediate product in fatty acid oxidation may inhibit the oxidation of fatty acids, probably by inactivating the dehydrogenase systems in molds (Melnick et al, 1954). Extensive works conducted by Mukherjee (1951; 1952a; 1952b) have demonstrated the occurrence of beta-oxidation of fatty acids through the

Wieland scheme in molds. Butyrate has been shown to be oxidized according to the reactions; butyrate → crotonate → β-OH-butyrate → acetoacetate (Mukherjee, 1952b). The reactions are reversible and, therefore, the addition or accumulation of an intermediate product will result in the reversion of equilibrium due to the mass action effect. In the presence of cyanide which is a selective inhibitor of crotonate oxidation, crotonate accumulates; the accumulation of crotonate beyond a certain concentration inhibits its dehydrogenation.

Evidence has been presented that sorbic acid and its corresponding saturated fatty acid are oxidized according to the above mechanism by molds and animal tissues (Witter et al, 1950; Witter et al, 1953; Deuel et al, 1954; Melnick et al, 1954). It has been postulated that sorbic acid is a normal transitory metabolite in the oxidation of saturated fatty acids and that the high initial concentration may inhibit the growth of molds by inactivating dehydrogenase system.

The inhibition of reactions of acetyl-CoA by acyl-CoA complexes, discussed previously, may have some bearing in this connection, although there is no sufficient evidence to support it at this time. It has been established that CoA derivatives instead of free acids are involved as the intermediates in the fatty acid oxidation (Lynen, 1955). Fatty acid activating enzyme, which catalyzes the formation of acyl-CoA derivatives from C_4 to C_{14} has been isolated from ox liver; it also shows activity toward sorbic acid (Mahler et al, 1953). It is

possible, then, that sorbic acid by conversion into its CoA derivative may inhibit the reactions of acetyl-CoA.

In another line of study, the inhibitory action of sorbic acid has been attributed to the suppression of fumarate oxidation (York and Vaughn, 1955). Inspection into the spectra of the sorbic acid sensitive microorganisms, which are limited primarily to the "catalase positive" microorganisms, has led these authors to believe that oxidative metabolism of microorganisms is suppressed. The results of exploratory experiments were reported to eliminate the possibility of direct involvement of the catalase, cytochrome oxidase or glucose oxidative systems. Studies with growing cultures, intact cell suspensions, and crude and purified enzyme systems indicated that the inhibitory action of sorbic acid probably results from suppression of fumarate oxidation.

EXPERIMENTAL METHODS

Five grams of commercial bakers' yeast were washed three times in a centrifuge with total volume of 600 ml. phosphate buffer solutions. The washed cells were suspended in 0.2 M KH_2PO_4 solution to give a definite density as determined by use of a spectrophotometer, and stored in a freezer at -40F. The stock suspensions of yeast prepared at different times were designated as yeast A, B, C and D.

Sorbic acid used in the experiments was refined sorbic acid (water content, 11.8 percent), provided by the Carbide and Carbon Chemicals Company, New York. Glucose used was "Difco" bactodextrose. Pyruvic acid solution was prepared by acidifying sodium pyruvate solution to pH 4.2.

The effects of sorbic acid on the metabolism of yeast was investigated exclusively by using conventional Warburg technique. The stock yeast suspension stored in the freezer was thawed just previous to each experiment and diluted with 0.2 M KH_2PO_4 solution so that 0.4 ml. of the diluted suspension would result in a certain desired activity.

Four-tenths ml. of the cell suspension was placed in the main compartment of a Warburg flask with two side arms, and the pH was adjusted to 4.2 with 1.0 ml. of 0.2 M phthalate buffer

in all experiments except the study of the effect of pH on the inhibitory action of sorbic acid, in which case 0.2 M Na_2HPO_4 solution was used. The initial pH was maintained during the period of experimentation with very little change. One-half ml. of substrate and/or sorbic acid were placed in the side arms of the Warburg flask. The concentrations of the substrate and inhibitor solutions were so adjusted that 0.5 ml. of the respective solution would result in the desired concentrations when tipped in. Finally, distilled water was added to make a total volume of 3 ml. in the flask. In studying the effect of sorbic acid on oxygen consumption, 0.2 ml. of 20 percent KOH solution was placed in the center well.

After the flasks were connected to the manometer and placed in the constant temperature bath (30° C), they were shaken until the endogenous respiration became fairly stable (usually 1 to 2 hours). In some cases yeast cells were shaken in buffer solution for 3 hours on a shaker before washing. Then substrate and inhibitor were tipped in and the manometer readings recorded. Normal response usually occurred after 20 minutes. Therefore, this was taken as zero time, and readings were taken at definite time intervals thereafter.

The activity of cells and the magnitude of the endogenous respiration may vary considerably among different stock yeast suspensions, and, therefore, may not be comparable quantitatively. Even among the results obtained using the same stock suspension

there was variation. The results reported were usually the average of results of duplicate flasks or of two sets of experiments run successively.

RESULTS

The Effects of Sorbic Acid on Glucose and Pyruvic Acid Oxidation by Baker's Yeast

Relation between pH and inhibition of glucose oxidation. The effect of 0.05 percent sorbic acid on the oxygen uptake in 0.01 M glucose solution was determined at two different pH levels during a 60-minute period. At pH 4.2, the oxygen uptake was inhibited by 78 percent, while at pH 6.2, only 56 percent inhibition occurred. It can be seen, therefore, that a given concentration of sorbic acid inhibits the glucose oxidation by yeast to a larger extent at low pH than at high pH level. All further experiments were conducted at pH 4.2.

Relation between the substrate concentration and the rate of oxidation. In the absence of inhibitor, the rate of oxygen consumption by yeast is limited by low substrate concentrations. The rate of oxygen uptake increased as the concentration of glucose was increased up to 0.01 M and as the pyruvic acid concentration was increased up to 0.06 M (Figures 1 and 2). The maximum rates of oxygen uptake were reached at these levels and further increases in the substrate concentration were found to be of no effect in increasing the rate. With

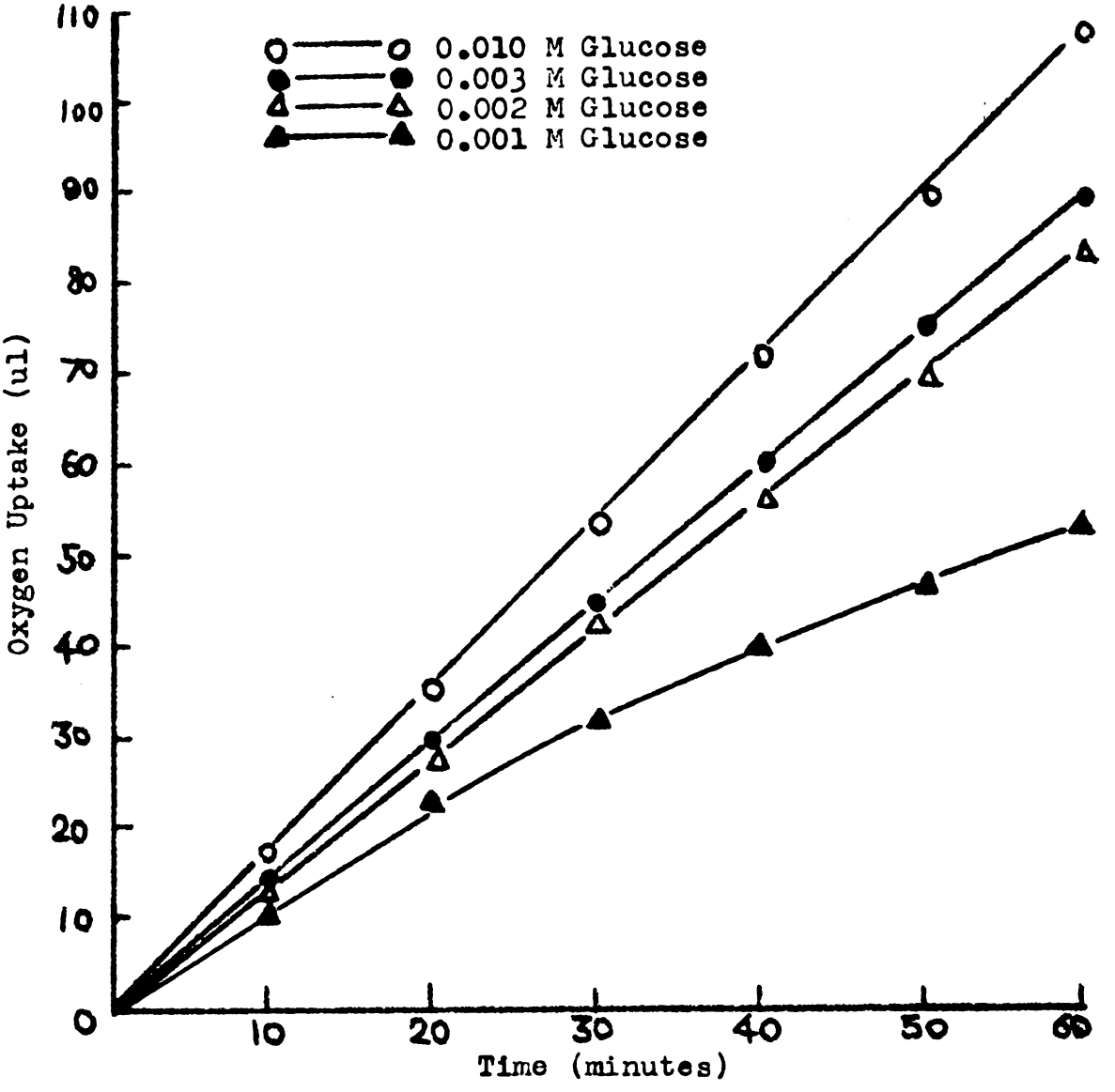

Fig. 1. Rate-glucose concentration curves in the absence of sorbic acid.

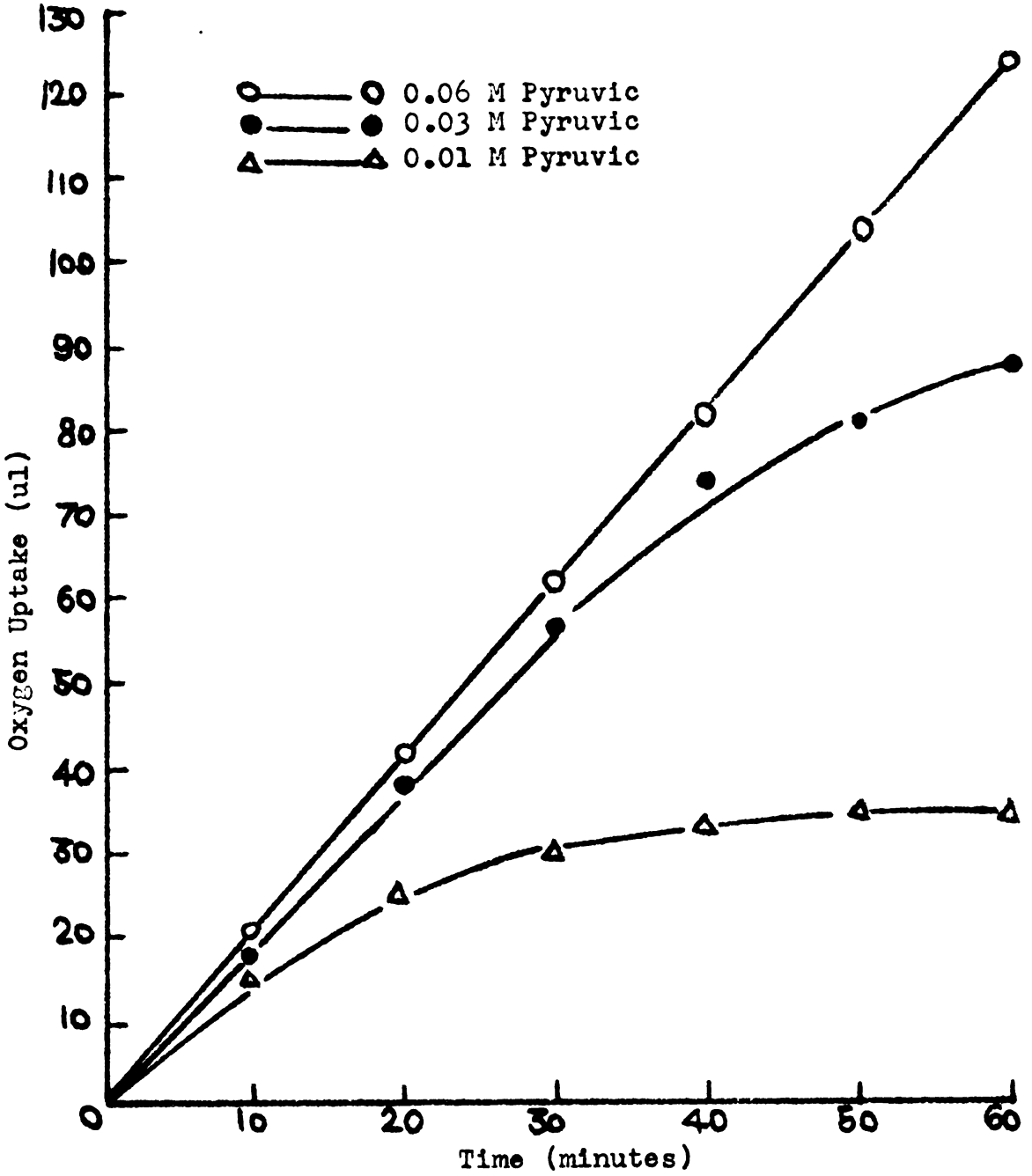

Fig. 2. Rate-pyruvic acid concentration curves in the absence of sorbic acid.

glucose concentrations of 0.002 M and above, and with 0.06 M pyruvic acid, the rates of oxidation were constant through the test period. A decline in the rate prior to the end of the 60-minute test period was noted with 0.001 M glucose and with 0.03 and 0.01 M pyruvic acid.

Relation between the degree of inhibition of glucose and pyruvic acid oxidation by sorbic acid and the substrate concentration. The effects of sorbic acid on glucose and pyruvic acid oxidation were studied by determining the effect of sorbic acid on the oxygen uptake in various concentrations of glucose, or pyruvic acid. The results of these studies are summarized in Table 1.

It is apparent that there is no appreciable difference in the percent inhibition of the initial rate of oxygen uptake with different concentrations of substrate. With glucose as the substrate, the inhibition of oxygen uptake during the first 10-minute period is in the range of 80 to 82 percent in the presence of 0.05 percent sorbic acid and 45 to 50 percent in the presence of 0.01 percent sorbic acid. With pyruvic acid as the substrate, 40 to 43 percent inhibition was noted. In any case, no greater inhibition was obtained with lower concentrations of substrate.

It may be noted that the percent inhibition tends to decrease gradually with time when low initial concentrations of substrate are used. Thus, with 0.001 M glucose, the

TABLE 1

PERCENT INHIBITION* OF OXYGEN UPTAKE BY SORBIC ACID**

Sorbic Conc. (percent)	Substrate	Substrate Conc. (molar)	Time (minutes) 10	20	30	40	50	60
0.05	Glucose	0.010	80	79	79	79	80	80
		0.003	82	80	80	78	81	81
		0.002	81	81	80	77	78	77
		0.001	81	76	74	72	75	75
0.01	Glucose	0.010	50	51	52	50	50	47
		0.003	50	48	49	48	43	40
		0.002	46	51	51	36	44	37
		0.001	45	48	31	25	15	4
0.01	Pyruvic Acid	0.06	43	38	40	39	38	37
		0.03	39	37	37	36	31	28
		0.01	40	40	33	33	32	29

*Percent inhibition = $\frac{\text{ml } O_2 \text{ Control} - \text{ml } O_2 \text{ Sorbic}}{\text{ml } O_2 \text{ Control}} \times 100$

**For detailed data see Appendix I, II and III.

percent inhibition decreased from 81 to 75 percent with 0.05 percent inhibitor and 45 to 4 percent with 0.01 percent inhibitor during the 60-minute period. With 0.03 M and 0.01 M pyruvic acid, the percent inhibition decreased from 39 to 28 and from 40 to 29 percent in the presence of 0.01 percent sorbic acid during the test period. At substrate concentrations, where rate limiting concentrations were not attained, fairly constant percent inhibition was maintained through the test period.

Relation between the concentration of sorbic acid and the degree of inhibition of glucose and pyruvic acid oxidation. The effects of various concentrations of sorbic acid on glucose and pyruvic acid oxidation were studied by determining their effects on oxygen uptake during a 60-minute period in the presence of 0.01 M glucose, or 0.06 M pyruvic acid. The results of the determinations are presented in Figure 3.

Sorbic acid has a marked inhibitory action on glucose and pyruvic acid oxidation by yeast. Sorbic acid, in concentration of 0.01 percent (ca. 0.0009 M), inhibited the glucose oxidation by 47 percent and the pyruvic oxidation by 40 percent.

It is apparent that the inhibition of oxygen uptake increases almost proportionally with the increase in concentration of sorbic acid up to 0.002 percent (ca. 0.0018 M), in both cases. Thus, by doubling the concentration of sorbic acid

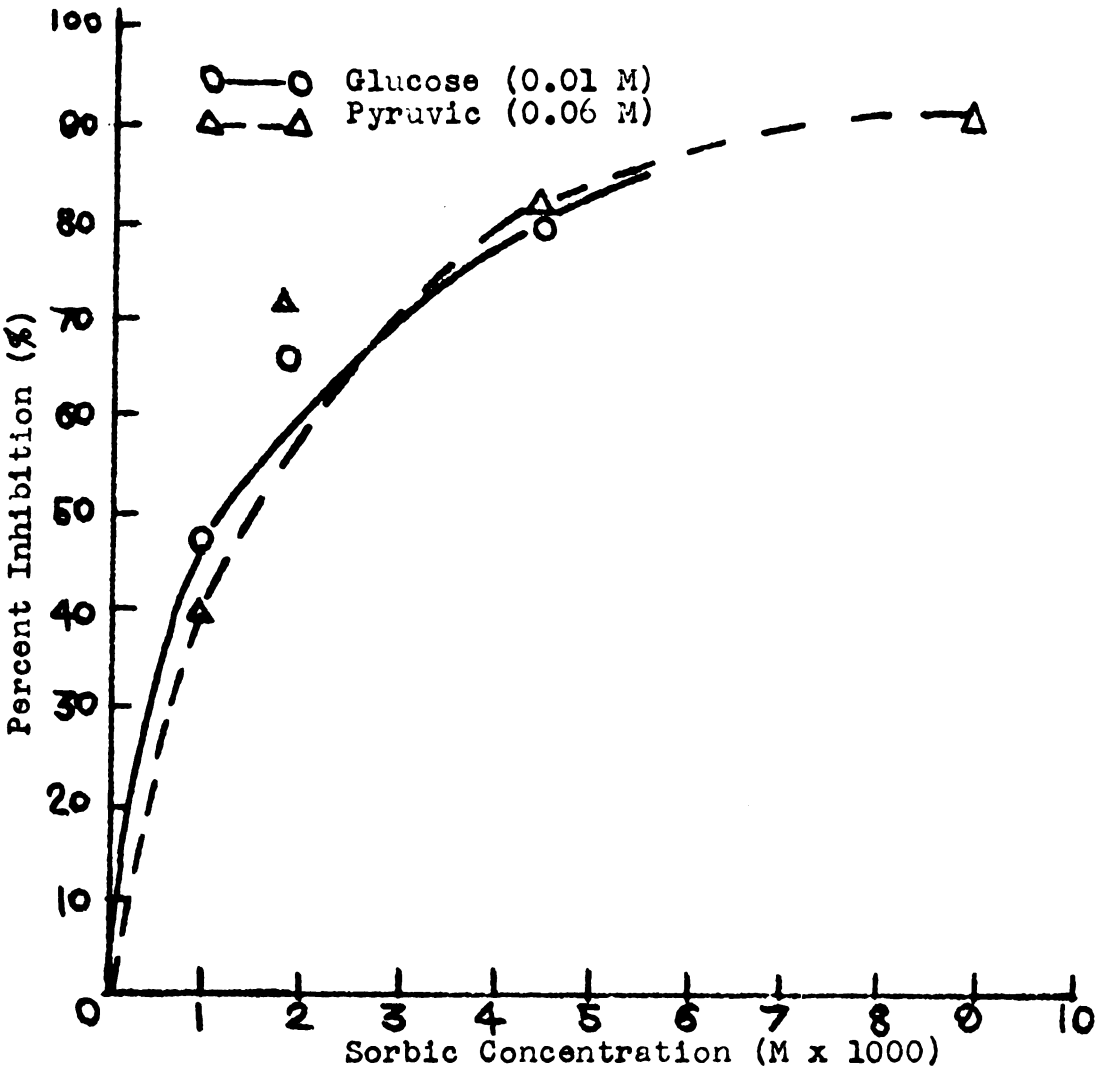

Fig. 3. Effect of various concentrations of sorbic acid on the percent inhibition of O_2 uptake in glucose, and pyruvic acid.

from 0.01 to 0.02 percent, the percent inhibition of oxygen uptake was increased from 47 to 66 percent with glucose and from 40 to 71 percent with pyruvic acid as substrate. However, beyond 0.02 percent, increases in the concentration of sorbic acid were no longer as effective.

The Effects of Sorbic Acid on the Aerobic and Anaerobic Fermentation by Baker's Yeast

Effect of sorbic acid on aerobic fermentation. The effect of 0.01 percent sorbic acid on carbon dioxide evolution under aerobic conditions was determined in 0.01 M glucose during an 80-minute period. This was calculated by subtracting the carbon dioxide evolved by the oxidative process as estimated by oxygen uptake from the total carbon dioxide evolved. The carbon dioxide evolution via the Embden-Meyerhof pathway is never very great under aerobic conditions due to the well-known "Pasteur effect." However, it is apparent that sorbic acid inhibited this process during the initial period (Figure 4, and Appendix IV). No significant inhibition occurred after this time. In fact, the calculated inhibition became less as time progressed.

Effect of sorbic acid on anaerobic fermentation. The effect of 0.01 percent sorbic acid on the carbon dioxide evolution via fermentation was determined in two concentrations of glucose, 0.01 M and 0.001 M, by measuring the carbon dioxide

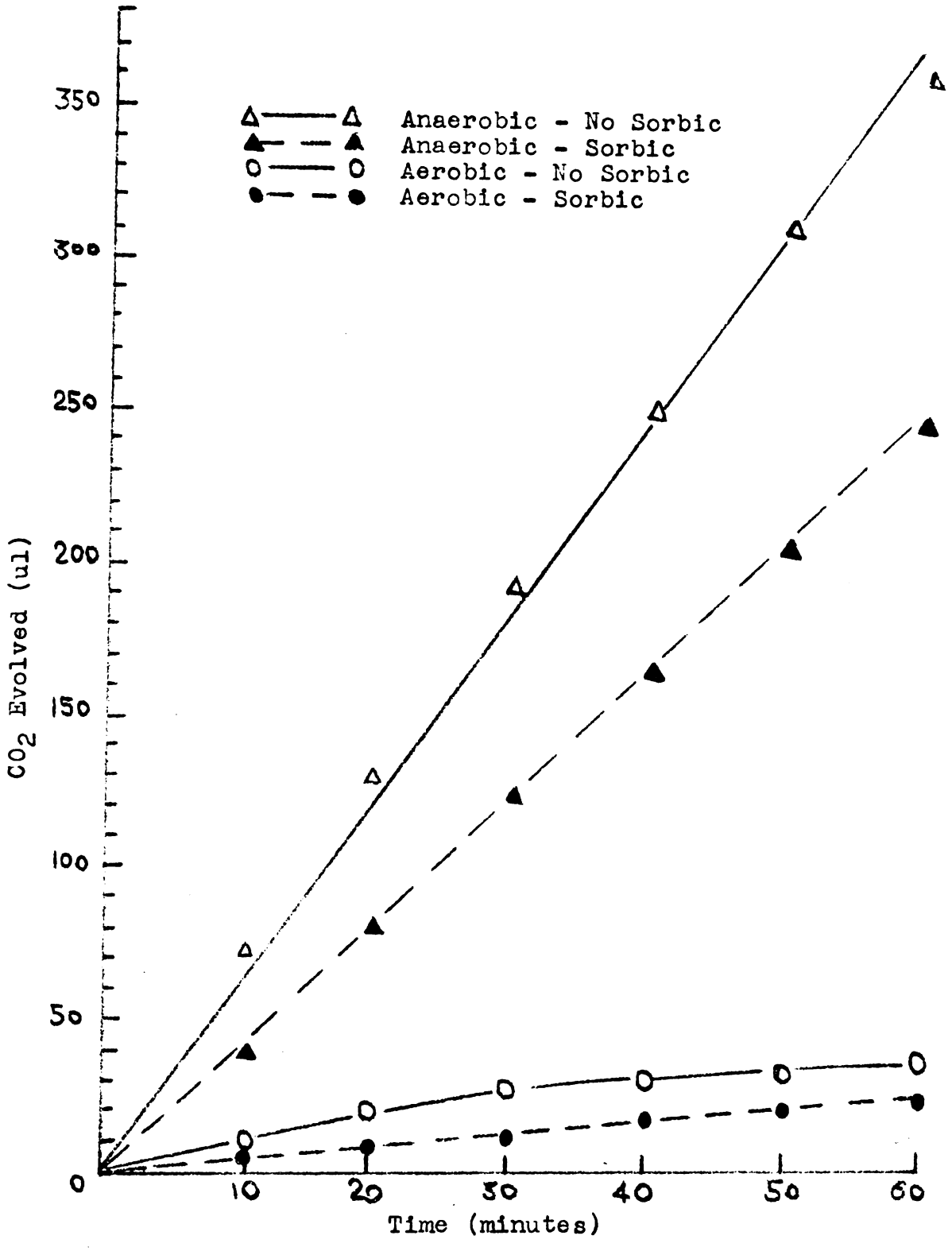

Fig. 4. Effect of 0.01 percent sorbic acid on CO_2 evolution from glucose via the Embden-Meyerhof pathway under aerobic and anaerobic conditions.

evolved in a nitrogen atmosphere. The fermentation of 0.01 M glucose was greatly inhibited (31 to 39 percent) by the sorbic acid (Figure 4 and Appendix IV). The rate of carbon dioxide evolution was fairly constant during a 60-minute period with 0.01 M glucose. However, when 0.001 M glucose was used, the substrate became limiting before the end of a 60-minute period and no significant sorbic inhibition was observed (Appendix IV).

DISCUSSION

Study of the inhibitory action of 0.05 percent sorbic acid on glucose oxidation at two different pH levels indicates that greater inhibition was obtained at low pH than at high pH level. It is apparent, therefore, that with intact cells non-ionized molecules of sorbic acid are more effective than the anions in inhibiting glucose oxidation. No serious attempt was made in determining the relative activity of the non-ionized acid molecules and anions. However, the concentration of non-ionized molecules have been shown to be directly related to the inhibitory action of sorbic acid on the growth of yeasts (Etchells et al, 1955).

The results of studies on the relatión between degree of inhibition of glucose oxidation by sorbic acid and substrate concentration indicate that the percent inhibition of the initial rate of glucose oxidation was not appreciably different with different glucose concentrations. In low concentrations of glucose, the degree of inhibition tends to decrease gradually with time. No proper explanation for this can be given at present. However, it is probable that the glucose concentration at these levels becomes limiting earlier in the absence of inhibitor than in its presence and, thus, results in a

relative slowdown of the rate of glucose oxidation in the absence of sorbic acid. In the presence of sorbic acid, a part of the glucose oxidative enzyme system may be combined with sorbic acid resulting in a relatively high degree of saturation of enzyme system with glucose in comparison with that in the absence of the inhibitor. This may have produced the constant rate of glucose oxidation in the presence of sorbic acid which was observed during the period studied.

It was concluded that the degree of inhibition of glucose oxidation was independent of the substrate concentration; i.e., sorbic acid inhibits glucose oxidation by a non-competitive type of mechanism. For a competitive type of mechanism, a higher degree of inhibition should result at low concentrations than at high concentrations of substrate.

The dissociation constant, K_s, for the rate limiting reaction in glucose oxidation may be calculated by the equation derived by Lineweaver and Burk (1934). This dissociation constant for enzyme-substrate complex, K_s, is given by an alternative form of the Michaelis-Menten equation as

$$\frac{1}{v} = \frac{1}{v_{max}} + \frac{K_s}{v_{max}} \frac{1}{(S)}$$

where v represents the measured rate of reaction, v_{max} the maximum rate of reaction, and (S) the substrate concentration. When values of $\frac{1}{v}$ are plotted against $\frac{1}{(S)}$, a straight line with a slope of $\frac{K_s}{v_{max}}$ and an intercept of $\frac{1}{v_{max}}$ results.

On plotting the data from this study (Figure 5) and fitting the straight line by the least squares method, it was found that the slope (Ks/v_{max}) was equal to 4×10^{-4} and the intercept ($1/v$) was 0.5. Therefore, the dissociation constant of the enzyme-substrate complex (Ks) was estimated approximately as 8×10^{-4}.

Equations for testing the types of enzyme inhibition have been described by Ebersole *et al* (1943). For the non-competitive type of inhibition, the ratio of the rate of reaction in the absence and in the presence of inhibitor, v_o/v_i, is expressed as the function of concentration of inhibitor (I), alone:

$$\frac{v_o}{v_i} = 1 + \frac{(I)}{K_i}$$

where K_i represents the dissociation constant for the enzyme-inhibitor complex. Therefore, if v_o/v_i is plotted against (I), a straight line independent of the substrate concentrations should result. On the other hand, v_o/v_i for the competitive type of inhibition is expressed as the function of concentrations of inhibitor and substrate:

$$\frac{v_o}{v_i} = 1 + \frac{K_i}{1 + K_s\ (S)}\ (I)$$

Therefore, the slope for the straight line should become dependent on the concentration of substrate.

In Figure 6, the ratio of the initial rate of glucose oxidation in the absence and presence of sorbic acid at various

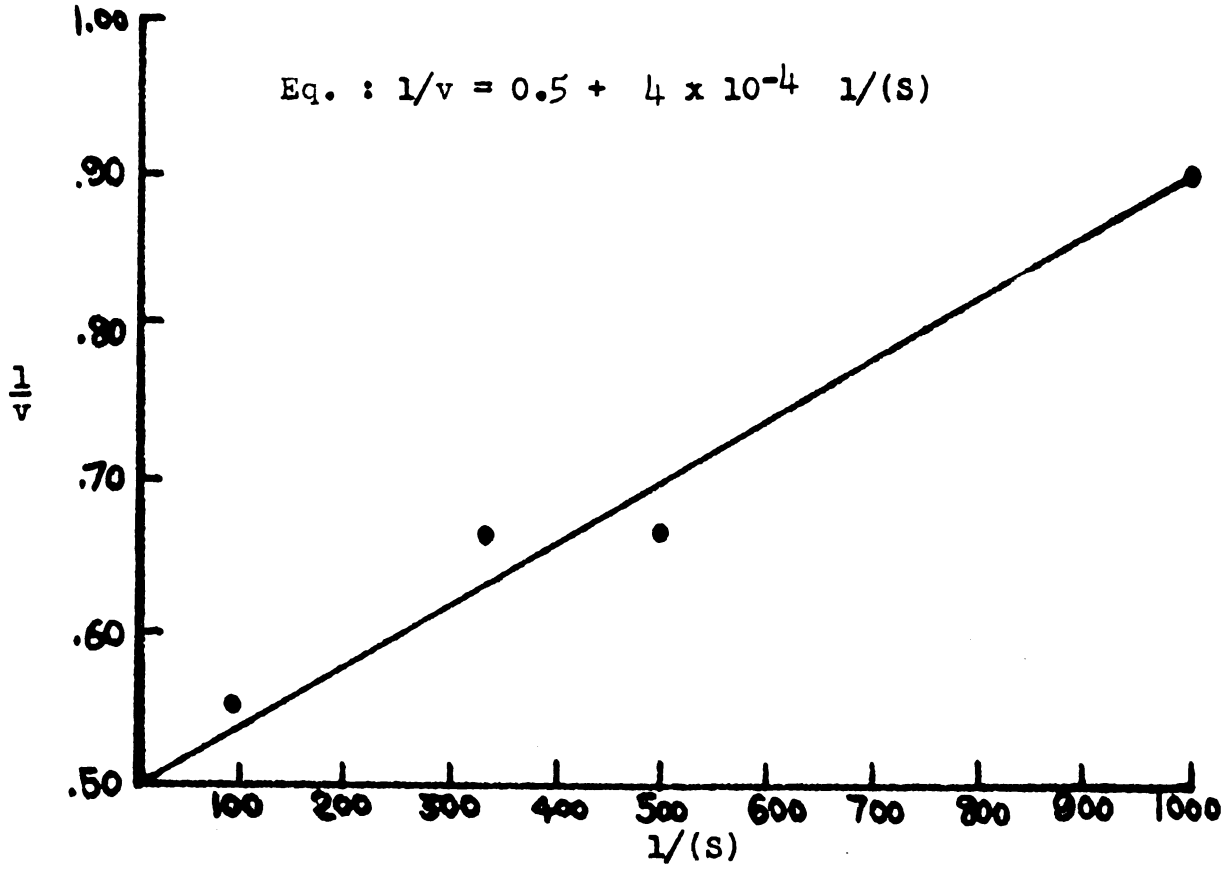

Fig. 5. Lineweaver-Burk plot for the glucose oxidation by baker's yeast.

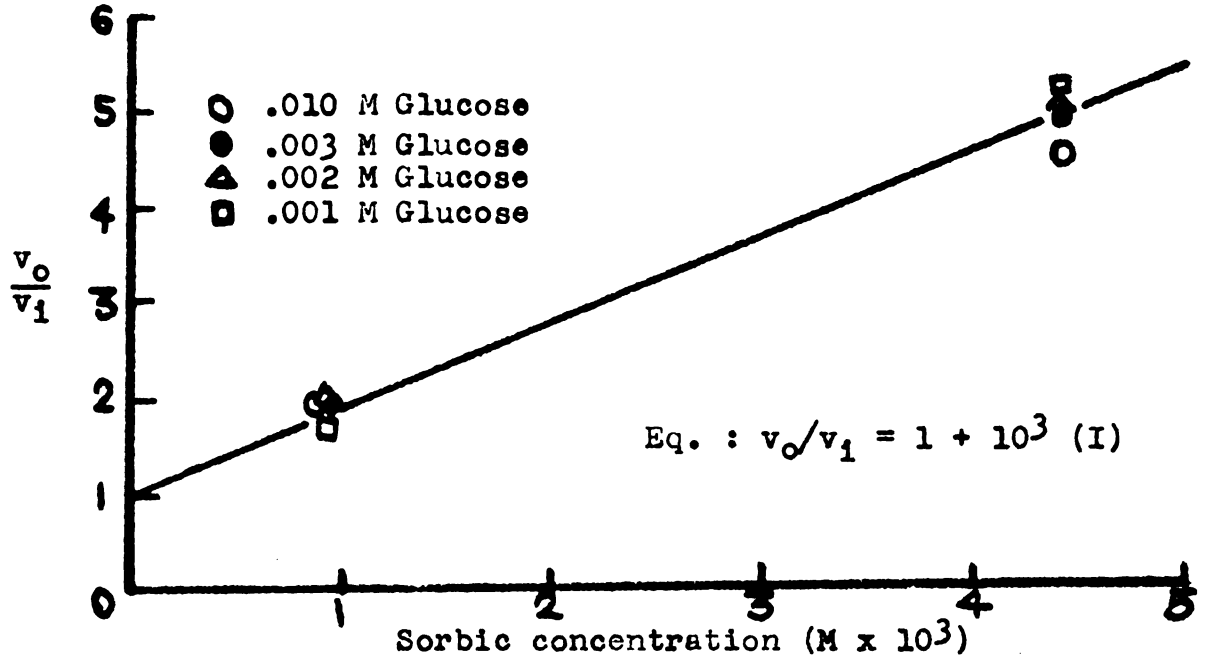

Fig. 6. Variation of v_o/v_i with concentration of sorbic acid.

concentrations of glucose (reciprocal of percent inhibition multiplied by 100) was plotted against the concentration of sorbic acid. It may be noted that there is no appreciable difference in v_o/v_i with the different concentrations of glucose either in the presence of 0.01 percent (0.009 M) or 0.05 percent (0.0044 M) sorbic acid. A straight line in accord with the given equation for the non-competitive type of inhibition was drawn between the points since the inhibition appeared to be non-competitive; i.e., the degree of inhibition was independent of the concentration of substrate. By the least squares method, the equation for the straight line was calculated as $v_o/v_i = 1 + 10^3$ (I), assuming a unit intercept. Therefore, the dissociation constant for the enzyme-inhibitor complex would be approximately 10^{-3}.

No attempt was made to determine the correlation between the inhibitory effect of sorbic acid on the glucose oxidation and its effect on viability of the yeast cells. Ferguson (1955) found that 0.05 percent sorbic acid effectively inhibited the growth of many yeasts at pH 5.0. In the present study, it was found that the same concentration of sorbic acid inhibited the glucose oxidation by Baker's yeast by 80 percent at pH 4.2.

Anaerobic and some microaerophilic microorganisms are insensitive to the action of sorbic acid while many aerobic microorganisms are inhibited by sorbic acid. One of the basic differences between these two groups of microorganisms is the

absence of the tricarboxylic acid cycle in the former. The occurrence of tricarboxylic acid cycle has been demonstrated in yeasts by various authors (Barron et al, 1950; Foulkes, 1951; Weinhouse et al, 1948). With Baker's yeast, it has been shown that the metabolism by pyruvic acid proceeds in the presence of oxygen by its initial oxidation to acetic acid and subsequent oxidation through the tricarboxylic acid cycle (Barron et al, 1950).

In the present studies, pyruvic acid oxidation was found to be inhibited by sorbic acid. The study on the relation between degree of inhibition and concentration of sorbic acid indicates that the inhibition of pyruvic acid oxidation is also independent of the substrate concentration; i.e., sorbic acid inhibits pyruvic acid oxidation according to the non-competitive mechanism of inhibition. Also, the relationship between the degree of inhibition of pyruvic acid oxidation and concentrations of sorbic acid was shown to be similar to that observed with glucose oxidation.

These findings seem to indicate that both glucose and pyruvic acid oxidation were inhibited according to an identical mechanism of inhibition. York and Vaughn (1955) attributed inhibitory action of sorbic acid to the suppression of fumarate oxidation. Melnick et al, (1954) explained the mechanism of mold inhibition on the basis of the effect of high initial concentration of sorbic acid on the dehydrogenase system responsible for the fatty acid oxidation. In another line of

study, Avigan et al, (1955) postulated that various acyl-CoA complexes inhibit the reactions of acetyl-CoA. This hypothesis is of interest because pyruvic acid is metabolized by yeast by its initial oxidation to acetate, which enters the tricarboxylic acid cycle as acetyl-CoA. Therefore, by assuming a formation of sorbate-CoA complex in yeasts, the inhibitory effect of sorbic acid on pyruvic acid and also on glucose oxidation may be explained.

The pathway of glucose fermentation was elucidated through the work of Meyerhof, Embden, Neuberg, Warburg, Cori and others; i.e., the Embden-Meyerhof pathway (Porter, 1946). Recently, some microorganisms have been shown to oxidize glucose by an alternative pathway differing from the classical Embden-Meyerhof'; i.e., hexose monophosphate shunt (Entner and Doudroff, 1952; Gibbs and DeMoss, 1954; Korkes, 1956; Wood, 1955). The relative magnitude of the shunt process versus the Embden-Meyerhof'in baker's yeast has been evaluated by Blumenthal et al (1954). Under aerobic conditions, the extent of the shunt process ranges from 0 to 30 percent. On the other hand, the Embden-Meyerhof pathway is greatly predominant under anaerobic conditions. At least 95 percent of glucose is fermented in this manner.

The effect of sorbic acid on the glucose fermentation was studied under aerobic and anaerobic conditions. Glucose fermentation was inhibited under both conditions. Therefore,

it must be assumed that the Embden-Meyerhof pathway in yeast was also inhibited by sorbic acid. This finding cannot be explained from the two published accounts discussed previously.

No proper postulate as to the exact site(s) of yeast inhibition by sorbic acid can be made from the evidence available at present. However, it is probable that there is more than one site of inhibition by sorbic acid.

SUMMARY

The effects of sorbic acid on the metabolism of baker's yeast has been investigated by the Warburg manometric technique. An attempt has also been made to determine the mechanism by which sorbic acid acts on the yeast.

A given concentration of sorbic acid inhibits the glucose oxidation by yeast to a greater extent at low pH than at a high pH level.

Glucose and pyruvic acid oxidation, and aerobic and anaerobic fermentation of glucose were inhibited by 0.01 percent sorbic acid to varying extents. Glucose oxidation was inhibited by 47 percent (yeast B), aerobic fermentation by 35 percent, and anaerobic fermentation by 32 percent (yeast D) in 0.01 M glucose during a 60-minute period. Pyruvic acid oxidation was inhibited by 37 percent in 0.06 M pyruvic acid during a 60-minute period (yeast C).

The relation between glucose concentration and the degree of inhibition of glucose oxidation has been studied in the presence of 0.05 percent and 0.01 percent sorbic acid. The percent inhibition of the initial rate of glucose oxidation was shown to be independent of the substrate concentration, indicating that sorbic acid inhibits glucose oxidation by a

non-competitive type of mechanism. A similar relationship was observed between pyruvic acid concentration and the degree of inhibition of pyruvic acid oxidation, indicating pyruvic acid and glucose are inhibited by an identical mechanism of inhibition.

The relationship between the concentration of sorbic acid and the degree of inhibition of glucose and pyruvic acid oxidation has been studied. The percent inhibition increased almost proportionally with increases in the concentration of sorbic acid up to around 0.02 percent, beyond which increases in the concentration of sorbic acid were much less effective.

Sorbic acid was also found to inhibit glucose fermentation by yeast both under aerobic and anaerobic conditions. This cannot be explained by the two published accounts on the mechanism of inhibition by sorbic acid.

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APPENDIX I

EFFECT OF 0.05 PERCENT SORBIC ACID ON O_2 UPTAKE IN VARIOUS CONCENTRATIONS OF GLUCOSE (YEAST B)

Time	0.01 M Glucose			0.003 M Glucose			0.002 M Glucose			0.001 M Glucose		
	O_2 Uptake		Percent Inhibit.	O_2 Uptake		Percent Inhibit.	O_2 Uptake		Percent Inhibit.	O_2 Uptake		Percent Inhibit.
	Control	Sorbic		Control	Sorbic		Control	Sorbic		Control	Sorbic	
(min.)	(ul.)	(ul.)		(ul.)	(ul.)		(ul.)	(ul.)		(ul.)	(ul.)	
10	22	4.5	80	19	3.5	82	18	3.5	81	16	3.0	81
20	45	9.5	79	39	8.0	80	36	7.0	81	29	7.0	76
30	68	14.	79	59	12.	80	54	11.	80	42	11.	74
40	91	19.	79	79	17.	78	71	16.	77	50	14.	72
50	113	23.	80	99	19.	81	87	19.	78	57	15.	75
60	137	28.	80	118	23.	81	101	23.	77	63	16.	75

APPENDIX II

EFFECT OF 0.01 PERCENT SORBIC ACID ON O_2 UPTAKE IN VARIOUS CONCENTRATIONS OF GLUCOSE (YEAST B)

Time	0.01 M Glucose			0.003 M Glucose			0.002 M Glucose			0.001 M Glucose		
	O_2 Uptake		Percent Inhibit.	O_2 Uptake		Percent Inhibit.	O_2 Uptake		Percent Inhibit.	O_2 Uptake		Percent Inhibit.
	Control	Sorbic		Control	Sorbic		Control	Sorbic		Control	Sorbic	
(min.)	(ul.)	(ul.)		(ul.)	(ul.)		(ul.)	(ul.)		(ul.)	(ul.)	
10	17	8.5	50	14	7.0	50	13	7.0	46	11	6	45
20	35	17	51	29	15	48	28	14	51	23	12	48
30	54	26	52	45	23	49	43	21	51	32	22	31
40	72	36	50	60	31	48	56	36	36	40	30	25
50	90	45	50	75	43	43	70	39	44	47	40	15
60	108	57	47	90	54	40	84	53	37	54	52	4

APPENDIX III

EFFECT OF 0.01 PERCENT SORBIC ACID ON OXYGEN UPTAKE IN VARIOUS CONCENTRATIONS OF PYRUVIC ACID (YEAST C)

Time	0.06 M Pyruvic			0.003 M Pyruvic			0.07 Pyruvic		
	O_2 Uptake		Percent Inhibition	O_2 Uptake		Percent Inhibition	O_2 Uptake		Percent Inhibition
	Control	Sorbic		Control	Sorbic		Control	Sorbic	
(min.)	(ul.)	(ul.)		(ul.)	(ul.)		(ul.)	(ul.)	
10	21	12	43	18	11	39	15	9	40
20	42	26	38	38	24	37	25	5	40
30	62	37	40	57	36	37	30	20	33
40	82	50	39	74	47	36	33	22	33
50	103	64	38	81	56	31	34	23	32
60	123	78	37	88	63	28	34	24	29

APPENDIX IV

EFFECT OF 0.01 PERCENT SORBIC ACID ON THE CO_2 EVOLUTION FROM GLUCOSE VIA THE EMBDEN-MEYERHOF PATHWAY UNDER AEROBIC AND ANAEROBIC CONDITIONS (YEAST D)

Time	Aerobic (air) 0.01 M Glucose			Anaerobic (N_2) 0.01 M Glucose			0.001 M Glucose		
	CO_2 Evolved		Percent Inhibition	CO_2 Evolved		Percent Inhibition	CO_2 Evolved		Percent Inhibition
	Control	Sorbic		Control	Sorbic		Control	Sorbic	
(min.)	(ul.)	(ul.)		(ul.)	(ul.)		(ul.)	(ul.)	
20	22	8	64	130	81	39	31	30	3
40	30	19	37	250	163	35	57	57	0
60	37	24	35	359	246	32	66	68	-1
80	44	34	23	485	337	31	69	76	-12