# THE INHIBITION OF ALCOHOLIC FERMENTATION BY SORBIC ACID

Thesis for the Degree of M. S.

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John Joseph Azukas

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By

John Joseph Azukas

AN ABSTRACT

Submitted to the College of Science and Arts of Michigan State University of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

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The purpose of this investigation was to determine the site(s) of sorbic acid inhibition in anaerobic metabolism. It was demonstrated that pH influenced the inhibition of intact cells by sorbic acid, however, it had no measurable influence on fermentation by cell free extracts. Actively fermenting cell free extracts for this investigation were prepared from baker's yeast by blending cells in an Omnimixer with fine glass beads and adding the necessary co-factors. Sorbic acid inhibited the fermentation of glucose, fructose 1, 6 diphosphate, and 3-phosphoglyceric acid as measured by $CO_2$ evolution. Inhibition with 3-phosphoglyceric acid as substrate was also demonstrated by measuring the rate of DPNH oxidation. There was no inhibition of carboxylase activity as determined manometrically, or of alcohol dehydrogenase as measured by DPN reduction with ethanol as substrate. Therefore, an inhibition site had to be between 3-phosphoglyceric acid and pyruvate. Due to structural similarity of sorbate with phospho-enol-pyruvate, enolase was postulated and proven to be an inhibition site. This was demonstrated to be related to both sorbic acid and substrate concentrations. An unusual and unexplained lag was observed in enolase activity in the presence of sorbic acid which was related to the sorbic acid concentration. A partially competitive type of inhibition is hypothesized.

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TABLE OF CONTENTS

LIST OF FIGURES

# INTRODUCTION

Sorbic acid, 2,4-hexadienoic acid, is used in the food industry for the control of undesirable microorganisms. Interest in the use of sorbic acid as a food preservative was stimulated by Gooding (1945) and by the report of its harmlessness as a dietary component Deuel (1945). The numerous reports that followed accumulated considerable information on the antimicrobial properties of this acid.

The action of sorbic acid appears to be directed against the catalase positive microorganisms. Among the various types of bacteria tested, lactobacilli and clostridia are much less sensitive to sorbic acid than others. However, still little is known on the mechanism by which sensitive microorganisms are inhibited. Elucidation of the mechanism of inhibition would aid in a more logical application to the food industry. This study was initiated for the purpose of throwing light on this problem by making systematic investigations of the inhibition of intact cells, cell-free preparations, and isolated enzymes.

# REVIEW OF LITERATURE

Marked antimicrobial action against a wide variety of microorganisms has been attributed to sorbic acid (Vaughn, 1952). The antimicrobial action of sorbic acid appears to be directed against the "catalase positive" microorganisms. Thus, lactobacilli and clostridia appear to be much less sensitive to it than other organisms. Little information is available on the mechanism of action or the site(s) of inhibition by sorbic acid. 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 sorbic acid and probable site(s) and also of some important factors which have to be considered in this study.

The mechanism of inhibition of sorbic acid. Many studies by various authors have established the antimicrobial properties of sorbic acid on a wide variety of microorganisms (Emard and Vaughn, 1952; York and Vaughn, 1954, 1955; Hansen and Appleman, 1955). It has also been established that it does not inhibit the catalase negative lactic acid bacteria and clostridia. However, little work has been done on the mechanism by which sorbic acid inhibits various microorganisms. Melnick et al. (1954) has theorized that since $\alpha,\beta$ unsaturated fatty acids such as sorbic acid are normal transitory metabolites in the oxidation of saturated fatty acids by molds, a high initial concentration was capable of inhibiting the dehydrogenase system in

molds. Inhibition of this enzyme system would of course result in fungicidal activity. York and Vaughn (1955) attributed the inhibitory action of sorbic acid to the suppression of fumarate oxidation. Studies involving the use of growing cultures, intact cell suspensions, and crude enzyme systems indicated that fumarase was inhibited.

Whitaker (1959) has reported the inhibition of alcohol dehydrogenase activity by sorbic acid. This was shown to be a time reaction; i.e., the inhibition became more pronounced on continued incubation of sorbic acid with the enzyme. Maleic acid has been shown to be an inhibitor of a number of sulfhydryl enzymes and to inhibit the growth and reproduction of cells. Morgan and Friedmann (1938) demonstrated that maleic acid and sulfhydryl compounds react to from stable compounds. On the basis of the similarity in the structure of sorbic acid, $CH_3$-CH=CH-CH=CHCOOH, and maleic acid, HOOC-CH=CH-COOH, and the similarity in their action on alcohol dehydrogenase, Whitaker suggested that sorbic acid inhibits this enzyme by the same mechanism as does maleic acid; i.e., it forms a thiohexenoic acid derivative, $CH_3$-CH=CHRSCH-$CH_2$COOH. He also postulated that since the living cell contains a myriad of sulfhydryl enzymes this is the mode of inhibition of the growth and reproduction of microorganisms. As for the organisms that are not inhibited, he claims a mechanism for metabolizing or detoxifying the sorbic acid.

The relation between pH and permeability of cells to organic acids. It was demonstrated by Etchells et al. (1955, 1959) that there was a relation between pH and sorbic acid inhibition of growth. There are numerous reports (Davson and Danielli, 1952; Orskov, 1945; Steinback,

1951; Teorell, 1949; Foulkes, 1955; and Giese, 1957) which show the relation between pH and permeability of cells to organic acids. The degree of ionization of a weak electrolyte is determined by the pH. By varying the pH, within limits safe for the organism, one may determine the effect that the charge on an electrolyte has upon its ability to penetrate a cell membrane. Giese (1957) demonstrated the effect of pH on the dissociation of carbonic acid. At pH 6.11, this acid is one-half dissociated. As the pH rises, the dissociation increases but the entry of $CO_2$ decreases. Conversely, with a fall in pH, the amount of dissociation decreases and the entry of carbon dioxide into the cell increases. Similar relations hold for hydrogen sulfide, auxins, dinitrophenol and other biologically active weak acids. The effect of degree of ionization on the entry of electrolytes into cells is further illustrated by comparing the rates of entry of members of a homologous series of weak organic acids. It appears that in spite of the increase in molecular size, the entry of the acids is more rapid the smaller their degree of dissociation. Presumably, permeation is proportional to the number of undissociated molecules, and a change in pH in the direction which increases the proportion of undissociated molecules will enhance penetration. The data indicate that the presence of a charge on an ion decreases its chance of entry.

According to Jacobs (1940) and Anderson (1945), in the case of weak acids, within the physiological pH range, significant quantities of both HA and BA are typically present, but these two substances differ greatly in their ability to enter cells. BA is a salt, assumed to be completely ionized, and it is now generally believed that the permeability

of cells to ions is a decidedly complex phenomenon, more or less limited in its extent, and involving theoretical equilibria which may be approached very slowly and perhaps never be attained. The entrance of most undissociated weak acids and bases, on the other hand, particularly those of organic origin whose molecules contain considerable non-polar hydrocarbon portions, seems to take place quite rapidly according to the simple laws of diffusion. It follows that the entrance of weak acids into cells, other things being equal, will be favored by low pH values of the external medium.

Rahn and Conn (1944) demonstrated that the toxic principle of sodium benzoate is the undissociated benzoic acid molecule which increases with the acidity of the medium. The test organism, a wine yeast, was completely suppressed whenever the concentration of undissociated benzoic acid reached 25 mg. per 100 ml. They also used salicylic acid and found that the antiseptic power of it depended entirely upon the undissociated fraction. The different efficiency of undissociated molecules and ions of the same acid may be explained by the fact that it is difficult for ions to permeate living cell membranes.

Cowles (1941) tested the germicidal action of a number of unbuffered acids such as acetic, propionic, butyric, valeric, caproic, and caprylic. The germicidal action of these acids seemed to be due almost entirely to the undissociated fraction.

Samson et al. (1955) working with yeast, demonstrated that the inhibitory action of acetate is increased by a decrease in pH and that a stimulation of rate appears at the higher pH used. It seems that

the undissociated molecule, rather than the acetate ion, is the effective agent since the pH change is accompanied by a large change in the concentration of undissociated acid and a small change in the anion concentration. The strong inhibitory action of the undissociated acid as compared with its ion is probably due to the different rate at which they enter the cell.

The preparation of cell-free enzymes from microorganisms. During the conduction of these experiments, considerable time was devoted to the preparation of cell-free extracts of yeasts capable of fermenting glucose at a constant rate. Therefore, it appears desirable to include some review of the previous work in this area.

The disruption of bacterial and yeast cells to give the cell-free enzymes and other soluble cell constituents is a problem which presents many practical difficulties. Buchner (1897) in a classical experiment prepared a cell-free enzyme from a yeast, capable of fermenting sucrose, by grinding the yeast cells with quartz sand. Macfadyen and Rowland (1901) disintegrated the typhoid "bacillus" by mechanical grinding with silver sand. Stevens and West (1922) described the preparation of a lipase, invertase and peptase from a strain of hemolytic streptococcus by grinding the cells with powdered glass in an agate mortar in the presence of phosphate buffer. Wiggert, et al. (1939) extended this technique to the preparation of enzymes from other organisms. Kalnitsky (1945) used a mechanical method for grinding with glass. The cell-glass mixture was forced between a pair of concentric glass cones; the outer cone was static, the inner cone was rotated. Because the preparation of powdered glass of uniform

size is a difficult task, McIlwain (1948) suggested the use of a commercial grade of aluminum oxide as a substitute. Using this modification, McIlwain described the preparation of a cell-free extract from a hemolytic streptococcus. The alumina used for this work may be recovered and used again. Carlson, et al. (1953) employed powdered pumice for the preparation of dextransucrase from Leuconostoc dextranicum by hand grinding. A process which has the special feature of combining low temperatures and a short period of mechanical treatment has been described by Hughes (1951). The microorganism together with appropriate abrasive is placed in a cylinder hollowed in a stainless steel block previously cooled. An accurately machined piston is driven by means of a fly press at a force of 12-15 tons per square inch, on to the cells, and the latter are forced from the cylinder into a reservoir channel cut in the block. Crushing has also been done without the use of abrasives.

Booth and Green (1938) designed a wet crushing mill for disruption of bacteria. The cell suspension was fed between the roller and the race of a modified roller bearing and could be recirculated through the mill if necessary by means of a pump. Muys (1949) described an automatic bacterial mill in which cells could be crushed anaerobically if necessary. A rounded cylinder of glass is made to revolve in a horizontal plane inside a thick walled glass tube. The revolving cylinder is arranged to touch at its end the rounded end of the tube, and it is across this point of contact that the bacteria are forced by gravity to fall in a suitable receiver.

Disintegration has also been accomplished by agitation with small

particles. Mickle (1948) designed an apparatus for the rapid mechanical shaking of bacterial or yeast cells with glass beads.

Sound waves are used quite frequently for the disruption of bacterial cells. However, they have not proved very successful with yeasts. Chambers and Flosdorf (1936) used a magnetostriction oscillator of 8.900 c/sec. to prepare labile antigenic material from Salmonella typhi and Streptococcus haemolyticus. Many other methods of disruption and preparation of cell-free extracts have been used, most of which may be useful only for special instances. Some of these methods are: disintegration by alternate freezing and thawing; disintegration by forcing through a small orifice; disruption by release of gas pressure; autolysis; use of enzymes; use of bacteriophage; and use of chemicals.

Hochster and Quastel (1951) have made an interesting contribution to the preparation of active enzymes from yeast. When fresh baker's yeast is crushed, the fermenting ability of the extract is impaired because the cozymase is decomposed by diphosphopyridine nucleosidase. They found that if the cells were crushed in the presence of nicotinamide, yeast extracts of high activity were obtained as DPN-ase activity was inhibited.

## EXPERIMENTAL METHODS

The yeast used was baker's yeast. The yeast was air dried and frozen for storage. The cell-free extracts were prepared from this dried and frozen yeast. Fifteen grams of dried yeast and 45 grams of glass beads were combined with 50 ml. of water and blended in a Serval omnimixer for 5 minutes in an ice bath to disrupt the cells. The beads were pavement marking beads, average size 0.2 mm., made by the Minnesota Mining and Manufacturing Co. Before blending in the omnimixer, the mixture of cells and beads was cooled to a few degrees above $0^{\circ}C$. The cup was filled to the top to prevent foaming and therefore surface denaturation. The beads, cell debris, and intact cells were removed after blending by centrifugation in the cold. The supernatant contained the enzymes of glycolysis.

The successful disruption of the bacterial or yeast cell does not necessarily mean that cell-free enzymes will result with biochemical activities of the same range or order as those exhibited by the intact cells. The glucose molecule passes anaerobically through about twelve stable intermediary stages before becoming alcohol and $CO_2$; at least three dissociable organic coenzymes, twenty or more enzyme proteins, and some bivalent metals are necessary for the breakdown. Since every single reaction concerned may be varied in speed according to the dilution of the coenzymes and other reactants and to the activity of the enzyme proteins, it is small wonder that many variations occur in

the preparation of yeast extracts. Many variations did occur in the preparation of the extracts used in this study. It should be noted that organisms vary considerably in their resistance to disruption, the mycobacteria and yeasts being particularly difficult to disrupt. Enzyme preparations may need to be activated by addition of cofactors. Sometimes the activity of the preparation may be enhanced by carrying out the disruption in the presence of a reducing agent such as cysteine or in an inert atmosphere or in the presence of an agent which prevents the decomposition of coenzymes by other enzymes liberated from the cell. For example, fluoride inhibits the action of adenosine triphosphatase, whereas nicotinamide inhibits diphosphopyridine nucleosidase (Hochster and Quastel, 1951). Considerable time was spent during this study in obtaining extracts of high and constant metabolic activity. The use of nicotinamide gave constant metabolic activity in most cases but since it was acting as an inhibitor, it was thought it would be better if it could be eliminated. Finally, by much experimentation, the proper ratio of cells to beads and the time of mixing along with the addition of a mixture of cofactors always gave active preparations of fairly constant activity. The mixture of cofactors contained $MgCl_2$, ATP, ADP, and DPN.

The sorbic acid used in the experiments was refined sorbic acid (water content, 5.5%) provided by the Carbide and Carbon Chemicals Co., New York. For each type of experiment, the desired amount of sorbic acid was dissolved in water and adjusted to the desired pH. In all experiments except those involving crystalline enolase, the concentration of sorbic acid was $5 \times 10^{-3}$M. The glucose used was "Difco"

Bacto dextrose. Pyruvic acid solution was prepared by dissolving sodium pyruvate and adjusting the pH. Fructose-1,6-diphosphate was made from the barium salt by dissolving it in 1.0 N HCl and then adding sodium sulfate equivalent to the barium ion. The precipitate of barium sulfate was removed by centrifugation, the supernatant made up to volume, and the pH adjusted. The other intermediates used, 3-phosphoglyceric acid and 2-phosphoglyceric acid, were also prepared from their respective barium salts by the procedure just described.

The effects of sorbic acid on the fermentation of glucose by intact cells and cell-free extracts were measured by the conventional Warburg technique. Cells or extract, buffer, sorbic acid, cofactors, and water were placed in the main compartment of the Warburg flask. The substrate was put in one of the side arms. The total volume after tipping in of the substrate was 3.0 ml. After the flasks were connected to the manometer and placed in the constant temperature bath at 30°C., they were flushed with nitrogen that had been passed over hot copper shavings in order to remove any oxygen present in the nitrogen. They were shaken until thermal equilibrium was reached, readings taken and the substrate tipped into the main compartment. After this, readings were taken at definite time intervals. The effect of sorbic acid on the fermentation of fructose-1,6-diphosphate, and 3-phosphoglyceric acid and on carboxylase activity was measured in the same way by use of the Warburg.

To test the effect of sorbic acid on aldolase activity, the optical method described by Christian in Methods in Enzymology, Vol I (Colowick and Kaplan, 1955) was used. This involves the coupling of

glyceraldehyde-3-phosphate dehydrogenase and measurement of DPN reduction spectrophotometrically at 340 mu.

The effect of sorbic acid on alcohol dehydrogenase was also measured optically by the method of Racker given in Methods in Enzymology, Vol. I. In one instance, the cell-free extract was used while in another experiment crystalline alcohol dehydrogenase was employed. Also, in one of the experiments, the procedure described was deviated from by incubation of enzyme and sorbic acid for one hour before addition of substrate.

Enolase activity as affected by sorbic acid was measured optically by the method employed by Wold (1957). This is based on the absorption of phospho-enol-pyruvate at 240 mu. The crystalline enolase was kindly supplied by Dr. Finn Wold, Department of Chemistry and Chemical Engineering, University of Illinois, Urbana, Illinois.

# RESULTS

Relation between pH and inhibition of glucose fermentation by sorbic acid. The effect of $5 \times 10^{-3}$ M sorbic acid on glucose fermentation was determined at two pH levels. At pH 6.0, sorbic acid inhibited glucose fermentation to the extent of 25 per cent, while at pH 5.0, the inhibition increased almost twofold to 46 per cent. This is illustrated in Figure 1. This is in agreement with the findings that have been reported by Etchells, et al. (1955, 1959) with respect to growth inhibition.

Since it has been proposed that there is a great difference in cell permeability to dissociated and undissociated molecules, it was decided to test the pH effect on fermentations by cell-free extracts. The effect of $5 \times 10^{-3}$ M sorbic acid on glucose fermentation by cell-free extracts was determined at two pH levels. In this case, the co-factors ATP, ADP, $MgCl_2$ and DPN were added to the reaction mixture. With the cell-free extracts, the inhibition of glucose fermentation by sorbic acid did not increase with a decrease in pH as was the case with the intact cells (Figure 2). At pH 6.0, glucose fermentation was inhibited 26 per cent and at pH 5.0 the amount of inhibition was 29 per cent. These data indicate that the pH effect is related to the relative permeability of the cell to the two forms of the acid.

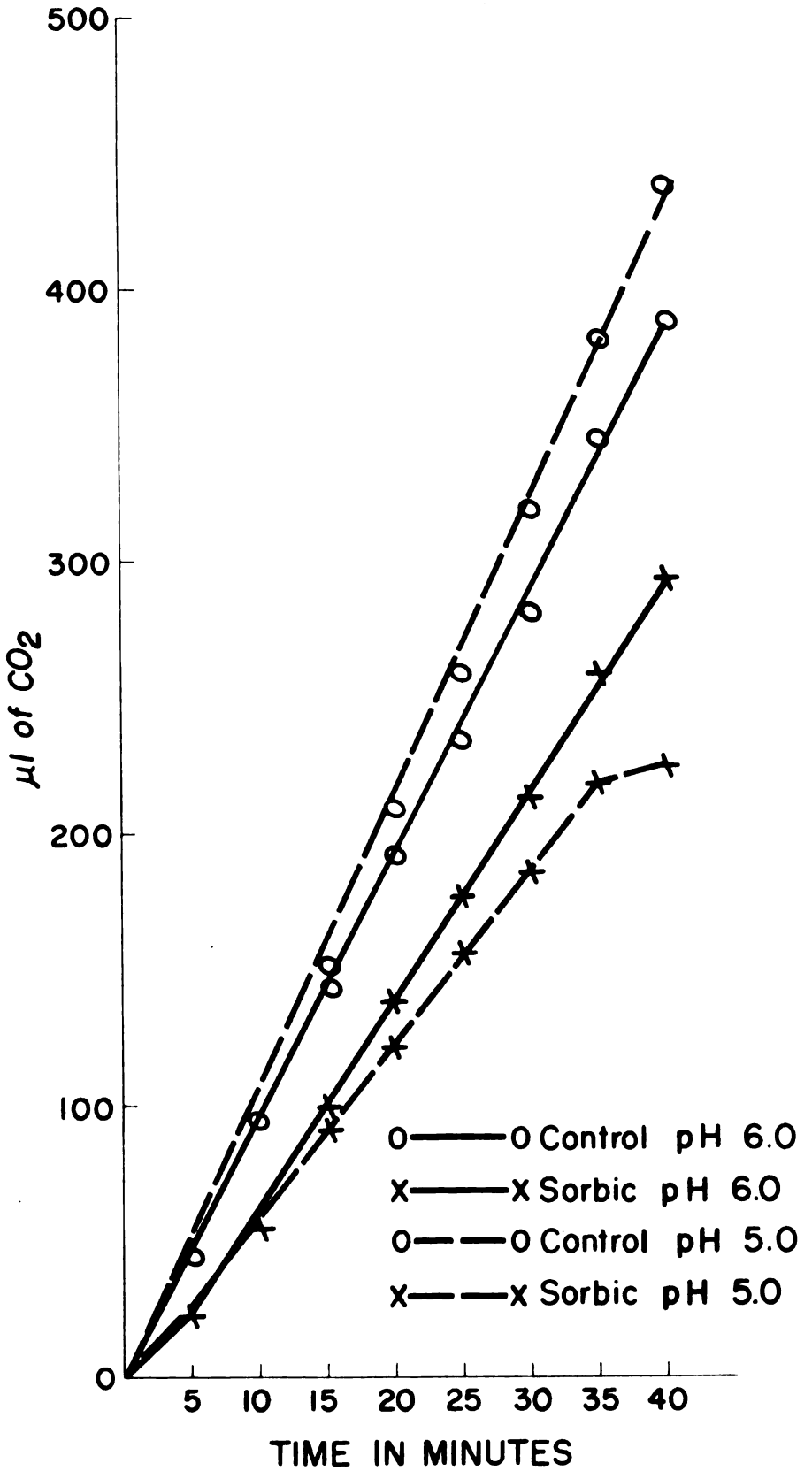


Figure 1. Inhibition of glucose fermentation.

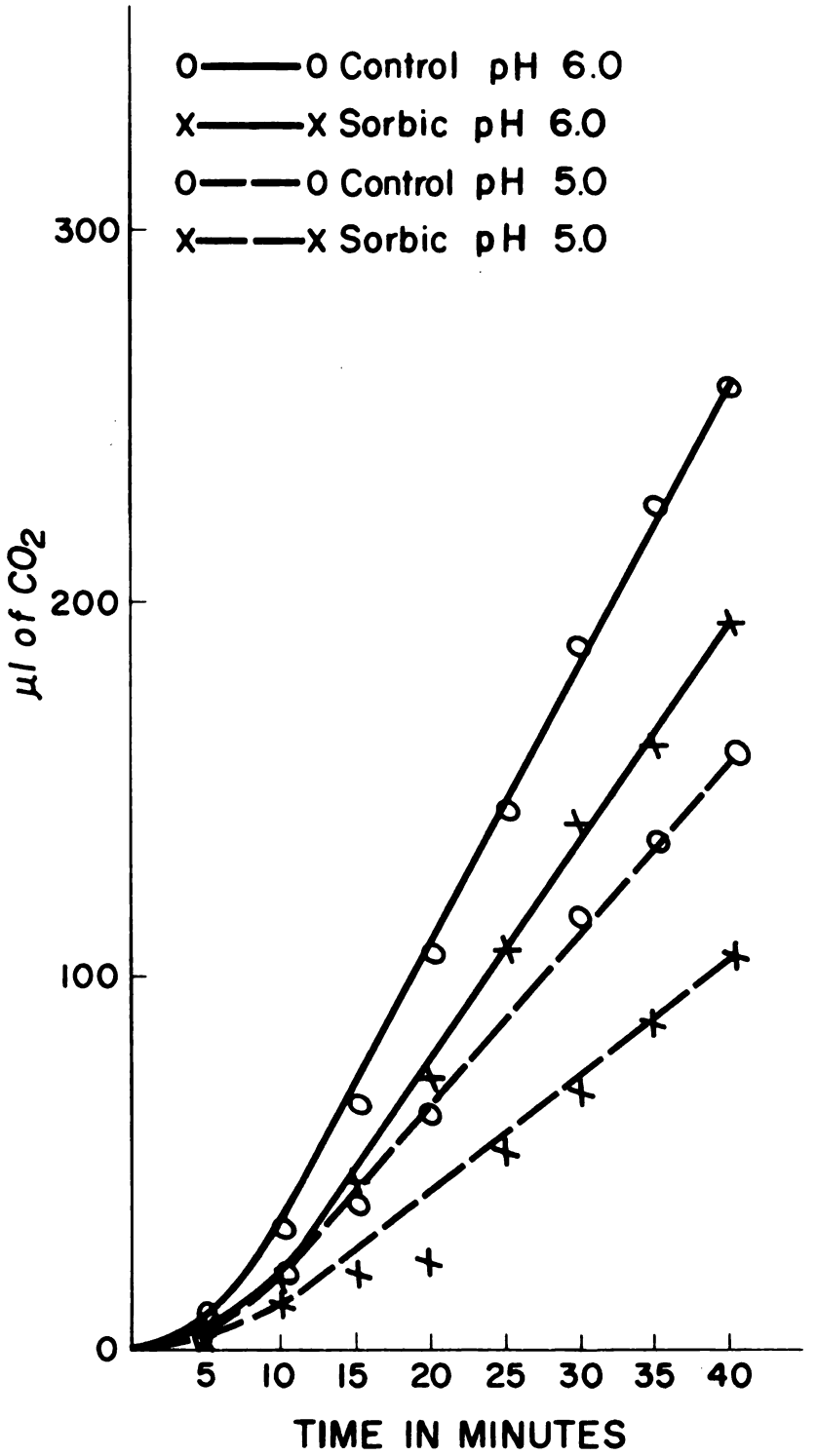


Figure 2. Effect of sorbic acid on glucose fermentation by cell-free extracts.

The effect of sorbic acid on the fermentation of intermediates in the Embden-Meyerhof pathway. In an attempt to find the exact site of inhibition, the effect of sorbic acid on the fermentation of the various intermediates of glycolysis was tested. This was done as a screening process to narrow down the possible sites. The first intermediate tested was fructose-1,6-diphosphate. With the use of the Warburg, the fermentation of this substrate by cell-free extracts was measured by $CO_2$ evolution. A mixture of cofactors containing ADP, DPN, and $MgCl_2$ was added to the fermentation vessel. Sorbic acid inhibited the fermentation of this intermediate to the extent of 28 per cent as shown in Figure 3. Therefore, if only one site of sorbic acid activity is presumed, it has to be beyond fructose-1,6-diphosphate.

The next intermediate to be tested in the screening process was 3-phosphoglyceric acid. This was also measured in the Warburg by $CO_2$ evolution. The concentration of sorbic acid was the same as before, $5 \times 10^{-3}$ M. A mixture of cofactors containing ADP, DPNH and $MgCl_2$ was added with the yeast cell extract. This fermentation was inhibited by sorbic acid to the extent of 26 per cent (Figure 4). The inhibition with 3-phosphoglyceric acid as substrate was further tested by measuring the rate of DPNH oxidation at 340 mu in the Beckman DU Spectrophotometer, and sorbic acid inhibition was again demonstrated (Figure 5).

The effect of sorbic acid on alcohol dehydrogenase activity. Next, as a part of the screening process, the problem was attacked from the other end of the pathway at alcohol dehydrogenase. The reaction was measured in reverse from the normal pathway with ethanol

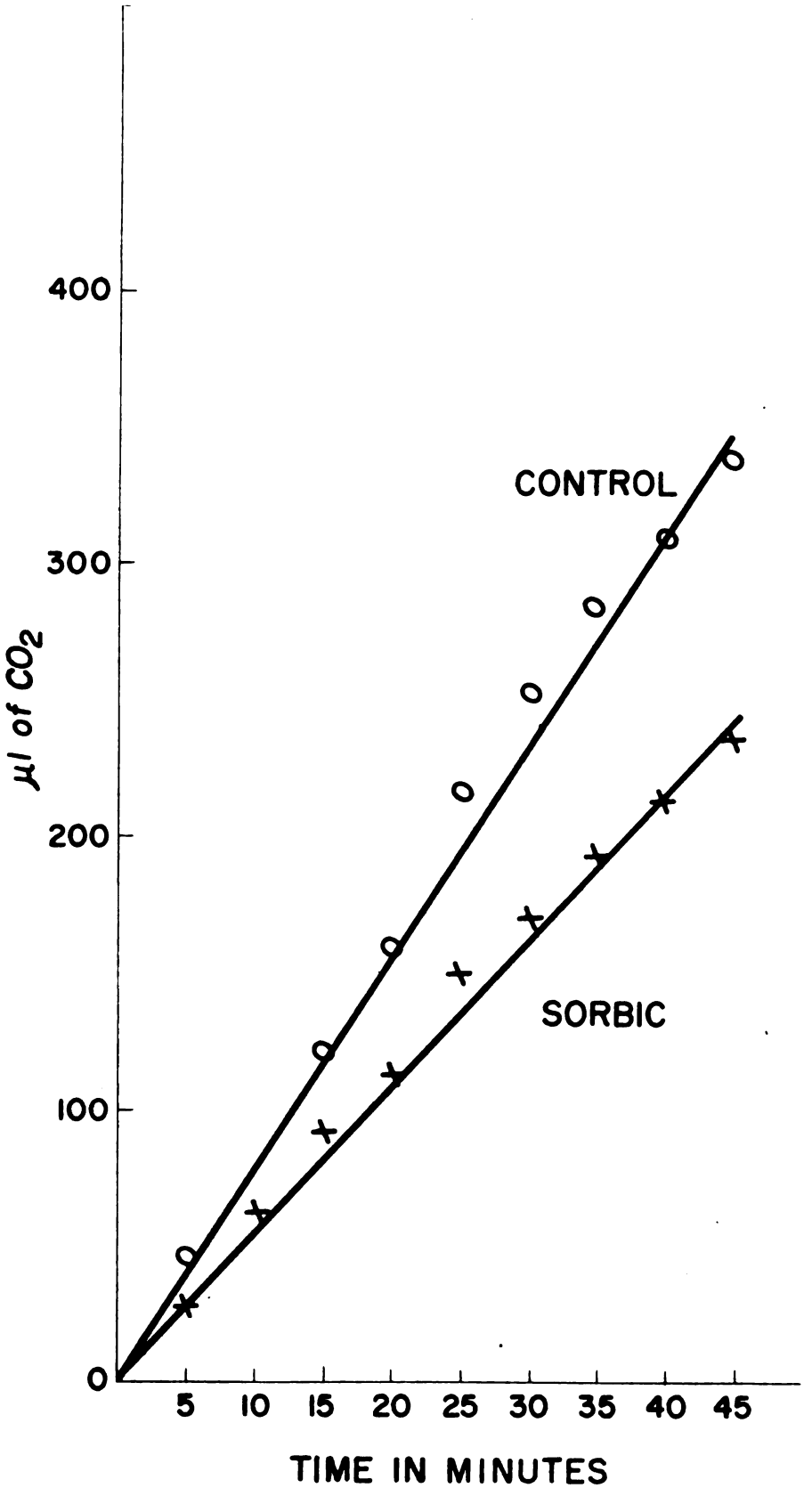


Figure 3. Inhibition of fructose- 1,6-diphosphate fermentation.

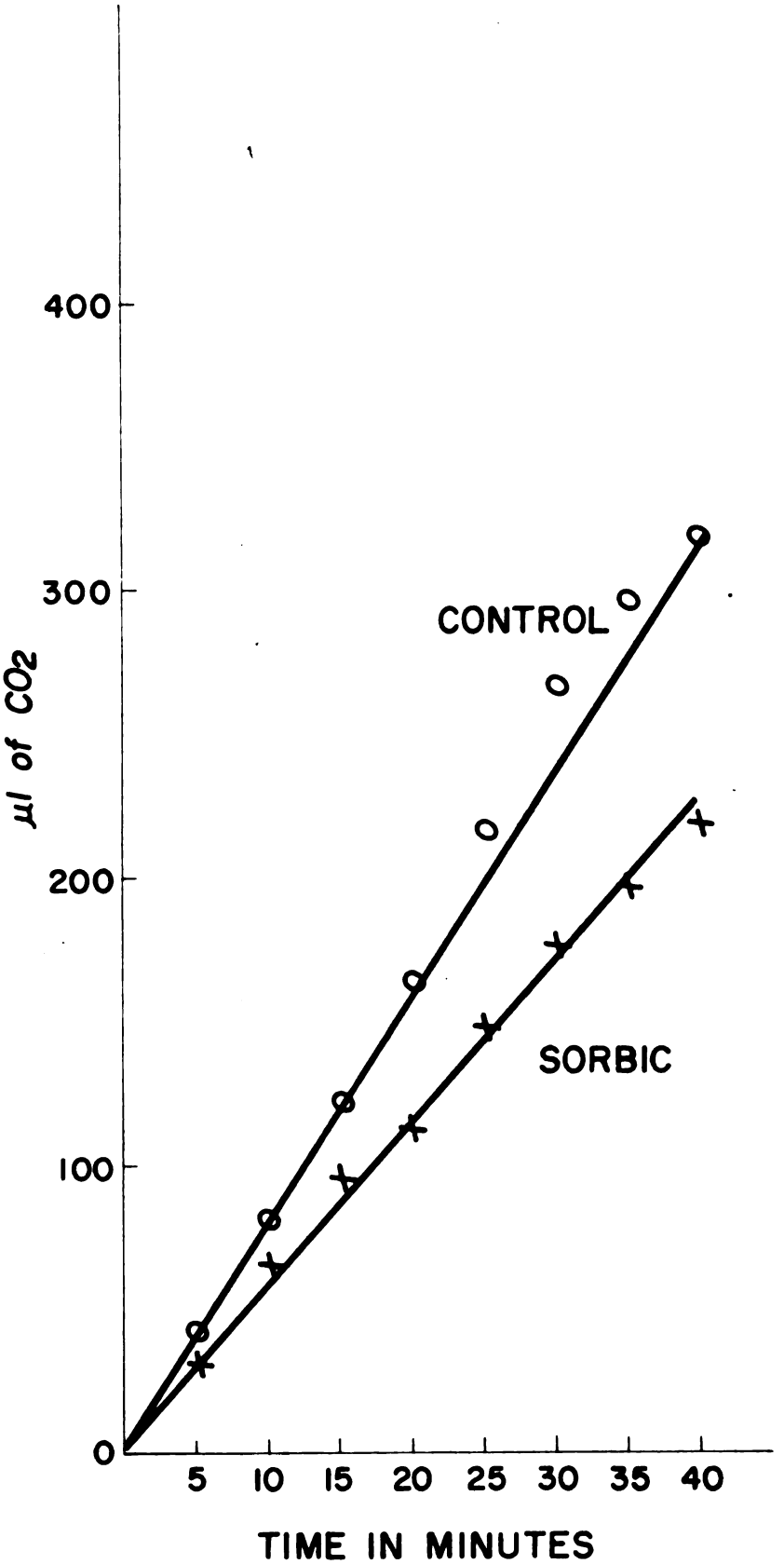


Figure 4. Inhibition of 3-phosphoglyceric acid fermentation.

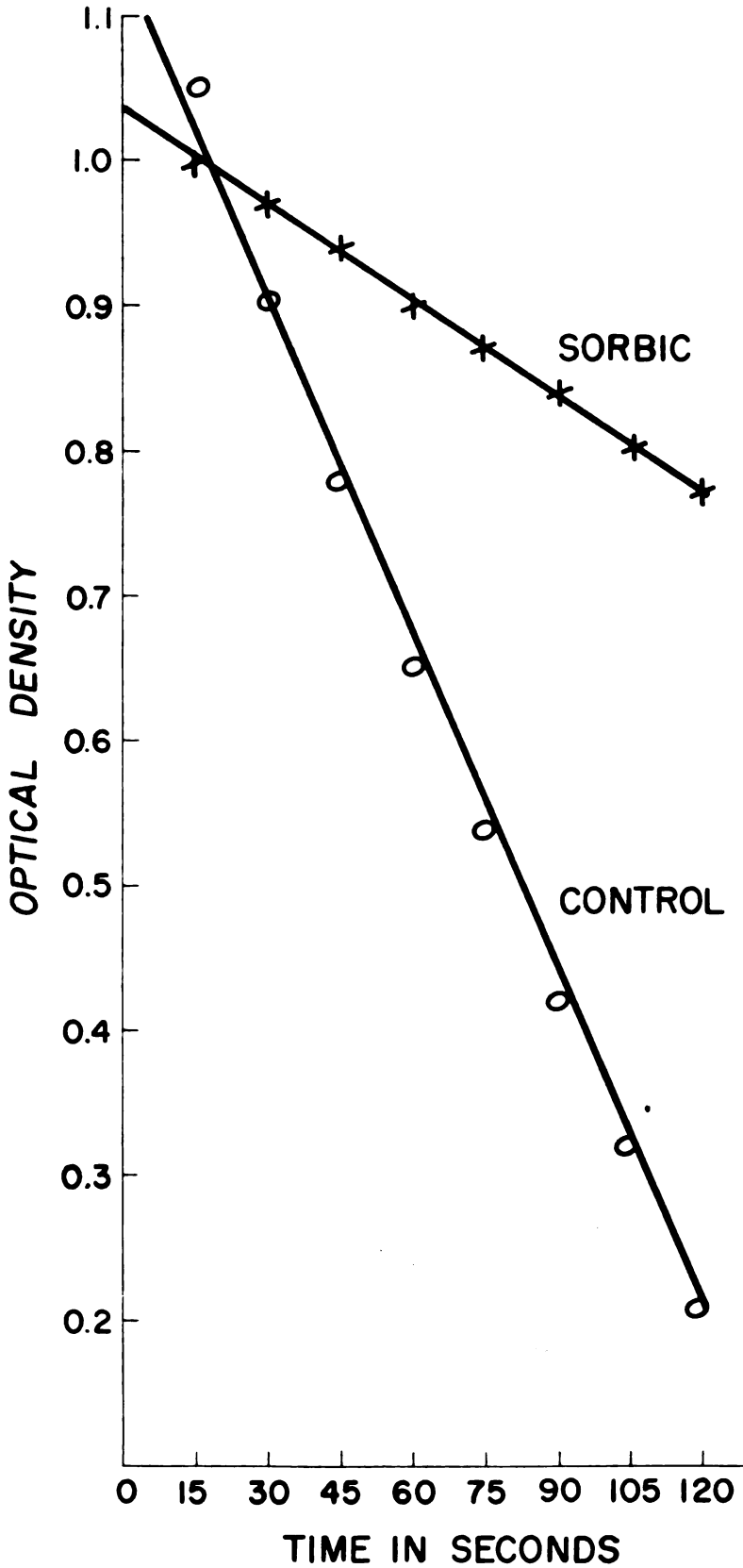


Figure 5. Inhibition of 3-phosphoglyceric acid fermentation as measured by oxidation of DPNH.

as substrate. The method used was that of Racker (Colowick and Kaplan, 1955) using cell-free extract as the enzyme preparation. In the one instance, the rate of DPN reduction was measured immediately after combining enzyme and substrate, and there was no measurable difference in DPN reduction with or without sorbic acid (Figure 6). In the other experiment, the rate of DPN reduction was measured after incubation of enzyme with sorbic acid for one hour before addition of substrate. However, this treatment did not result in any significant inhibition of the rate of DPN reduction (Figure 6). The lines of the graph do not coincide but are parallel so the rates were the same. With this system, using the crude cell-free extract with or without incubation, there was no inhibition by sorbic acid.

Inhibition studies using crystalline alcohol dehydrogenase were run in the same manner as with the cell-free extracts. Using the crystalline enzyme without incubation, there was no inhibition (Figure 7). However, after incubation of the enzyme with sorbic acid for one hour before addition of substrate a definite inhibition was observed (Figure 8).

The effect of sorbic acid on carboxylase activity. To further narrow down the site, the effect of sorbic acid on carboxylase was tested. Carboxylase activity was measured by the conventional Warburg technique with pyruvate as substrate. Based on the measurement of the $\mu$l of $CO_2$ evolved at the end of a 3-minute period, there was no evidence of any inhibition by sorbic acid. Therefore, the site of inhibition had to be between 3-phosphoglyceric acid and pyruvate.

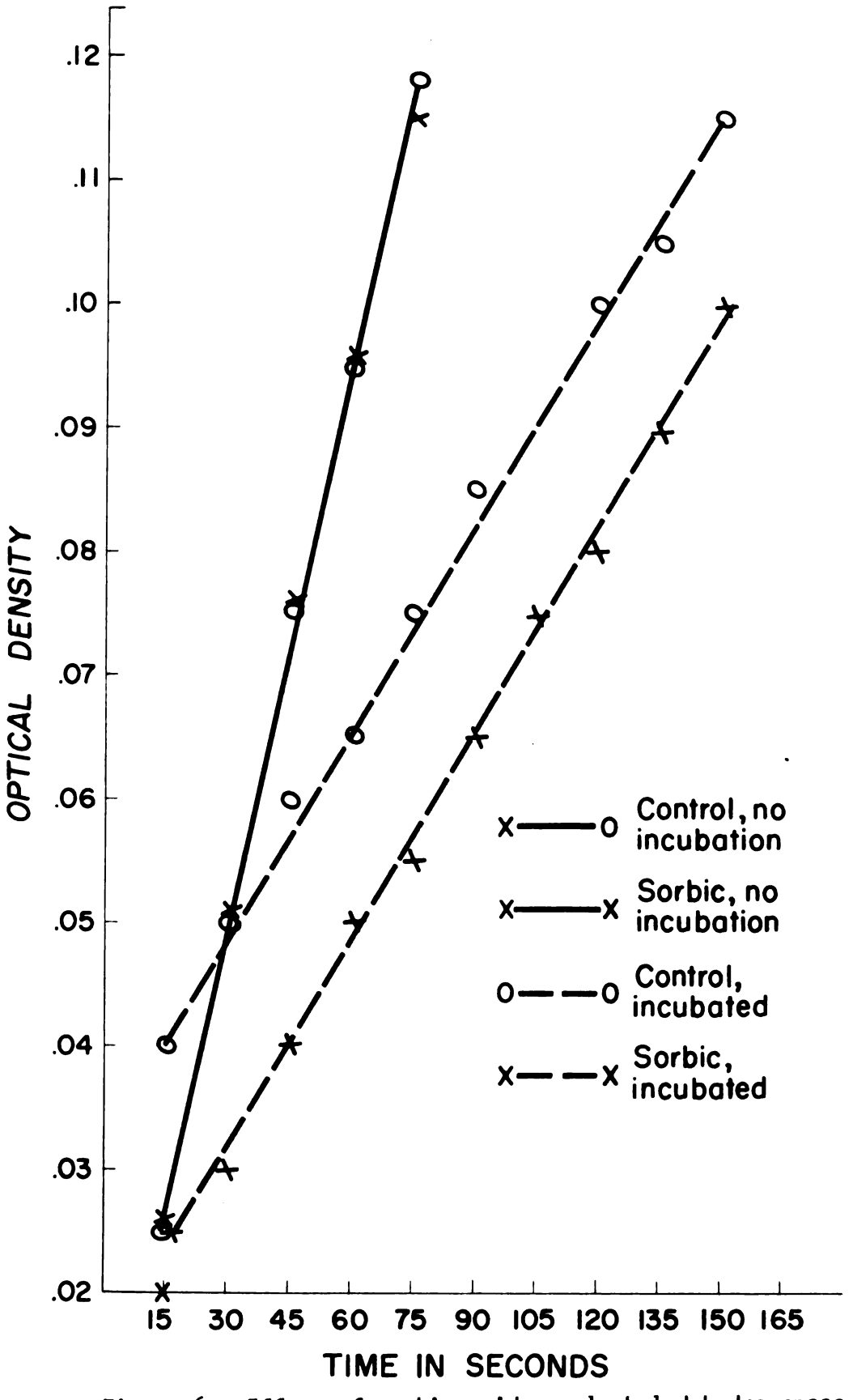


Figure 6. Effect of sorbic acid on alcohol dehydrogenase activity.

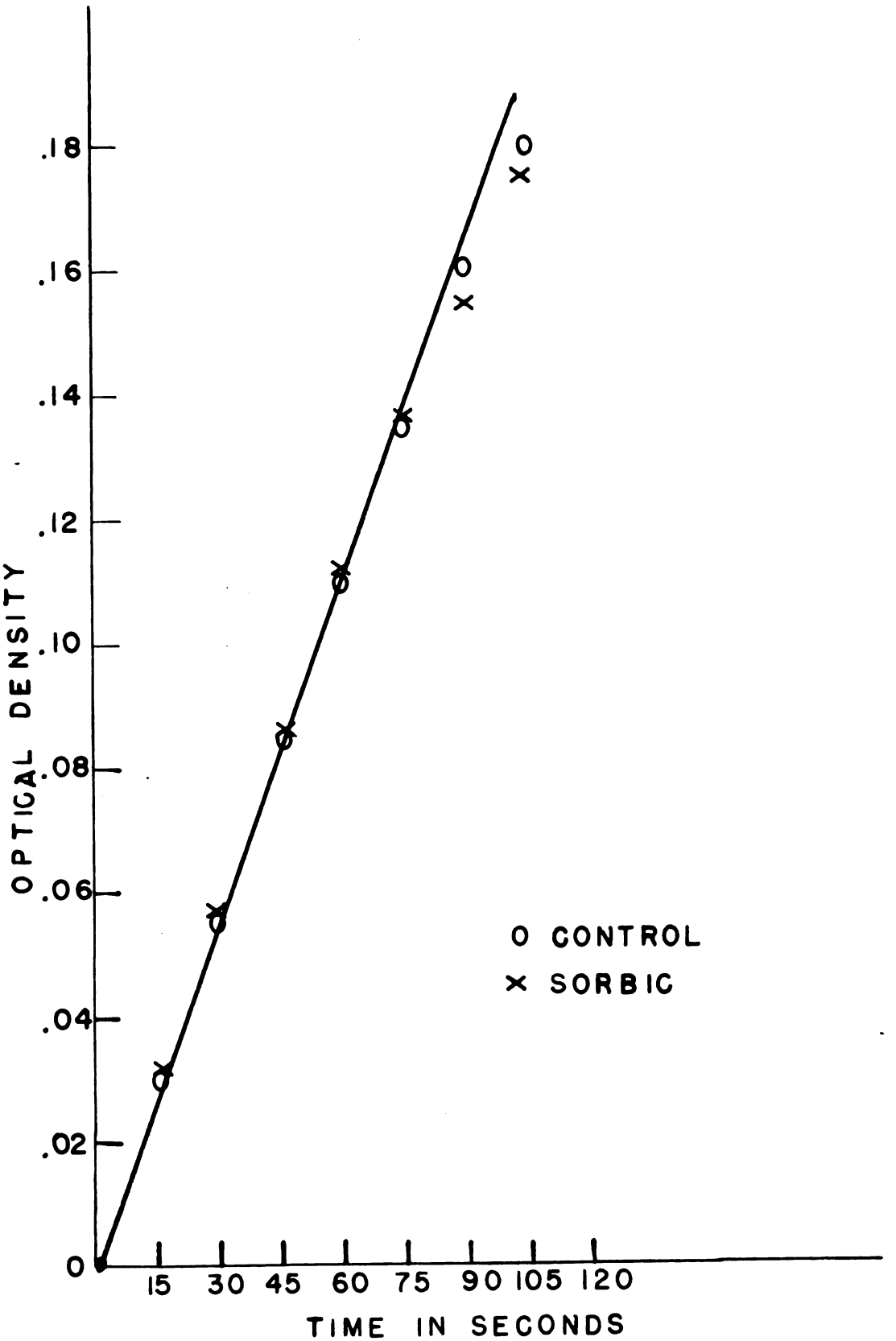


Figure 7. Effect of sorbic acid on alcohol dehydrogenase using crystalline enzyme.

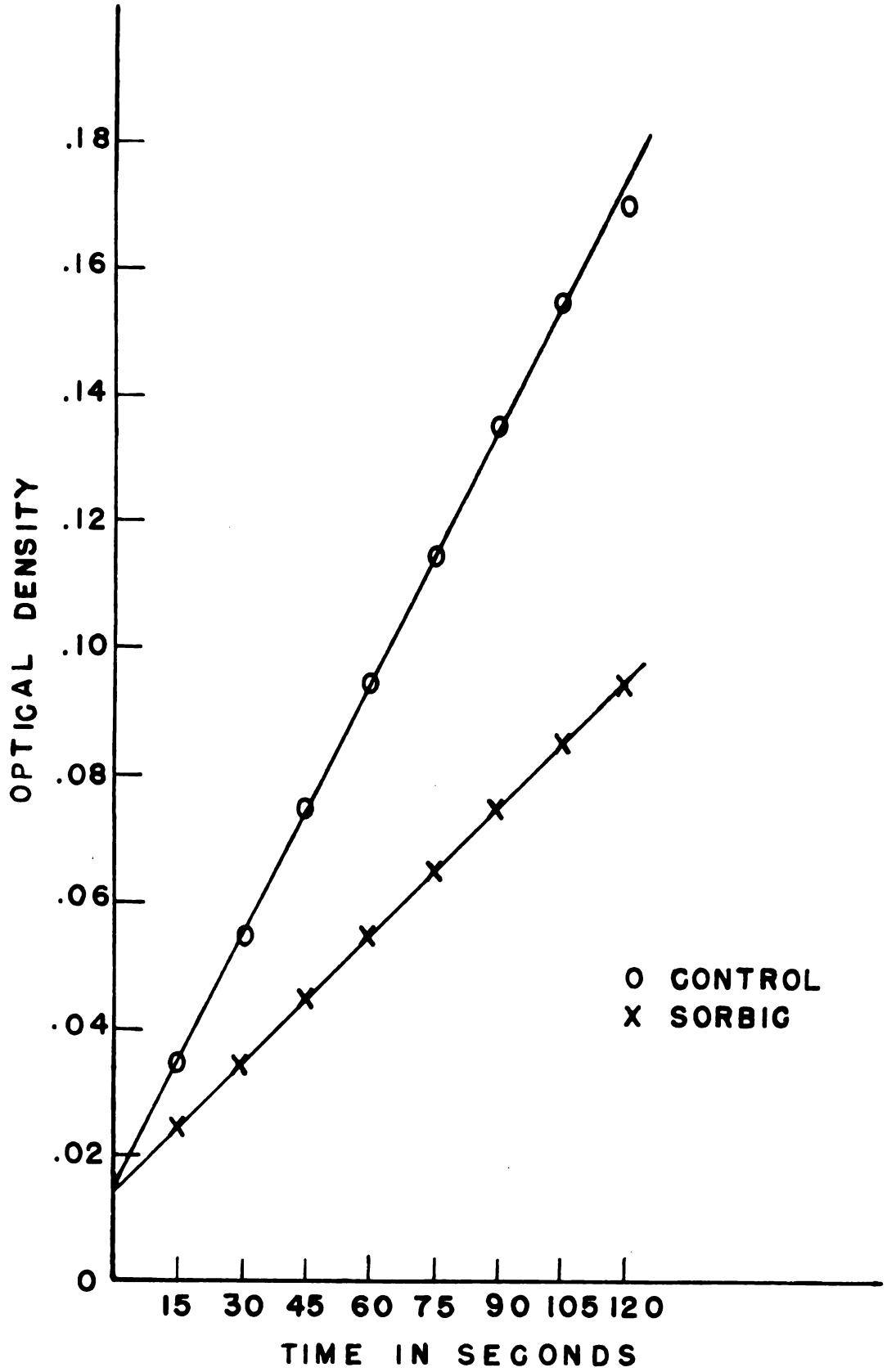


Figure 8. Effect of sorbic acid on alcohol dehydrogenase using crystalline enzyme with incubation.

The conversion of 2-phosphoglyceric acid to phospho-enol-pyruvate as affected by sorbic acid. Taking into consideration the structural similarity between phospho-enol-pyruvate and sorbic acid, enolase was picked as the site to be tested. The method used was that employed by Wold (1957). This method is based on the absorption of phospho-enol-pyruvate at 240 mu. These tests were conducted using a constant enzyme concentration, sorbic acid concentrations of $5 \times 10^{-5}$ to $1.5 \times 10^{-4}$ M and substrate levels from $1 \times 10^{-4}$ to $2 \times 10^{-3}$ M. Enolase was inhibited by sorbic acid. This, then, was the site or one of the sites of inhibition of glycolysis by sorbic acid. As the concentration of sorbic acid increased, so did the inhibition of enolase (Figure 9). The only peculiarity was the presence of a lag phase which was not related to substrate concentration. As the sorbic acid concentration increased, so did the lag. No explanation can be given for this at the present time.

An attempt was made to determine the type of inhibition by plotting the reciprocal of velocity against the reciprocal of substrate concentration. The plot indicates a purely competitive type of inhibition (Figure 10). However, a plot of the reciprocal of velocity against inhibitor concentrations did not give a straight line as it does in the fully competitive or non-competitive cases. This indicates that the inhibition is partially competitive, a mixture of competitive and non-competitive effects, or a rare case of an inhibitor acting in some unusual manner.

Conversion of fructose-1,6-diphosphate to 1,3-diphosphoglyceric acid. There still existed the possibility that inhibition of either

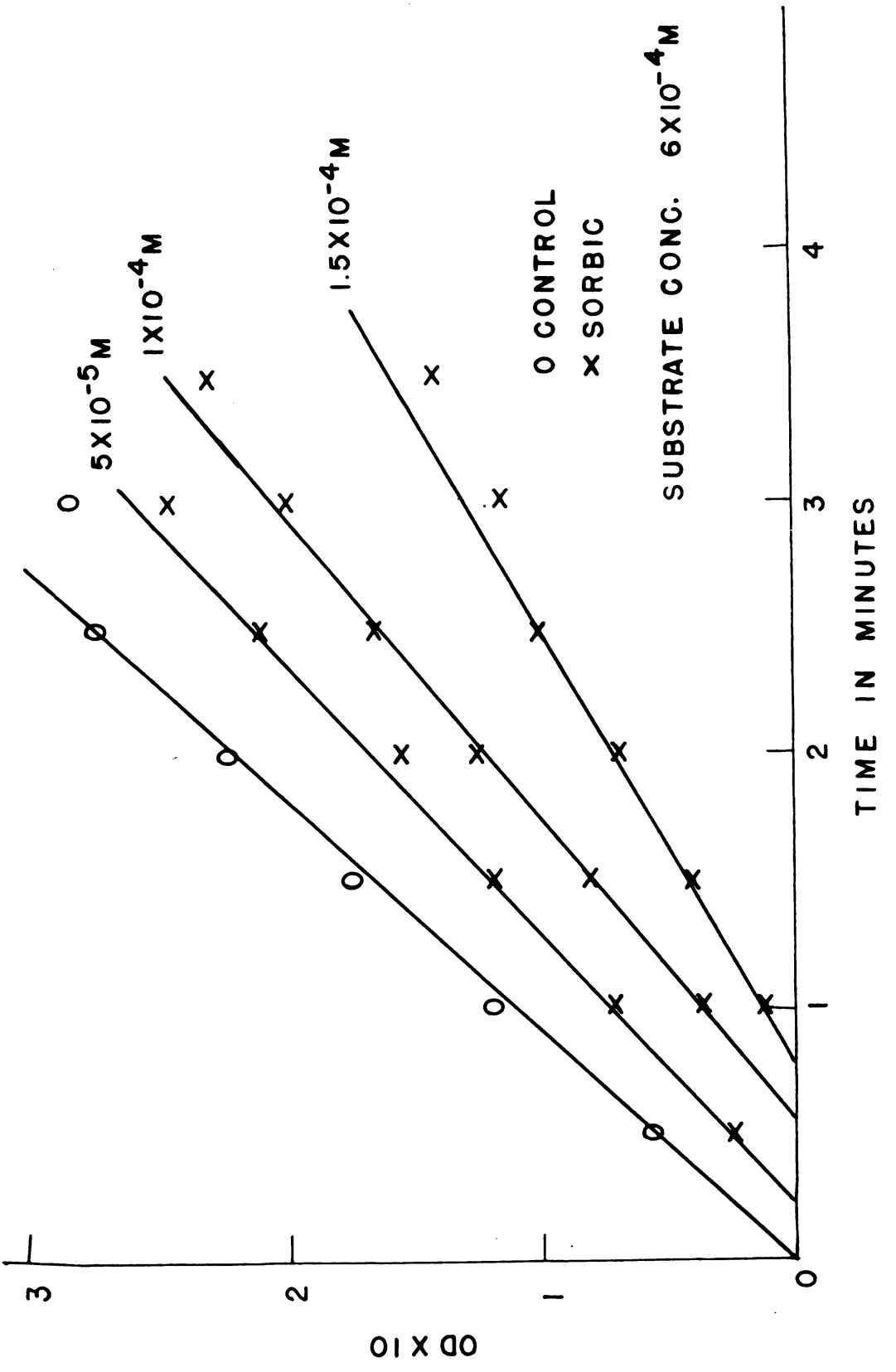


Figure 9. Effect of sorbic acid on enolase activity.

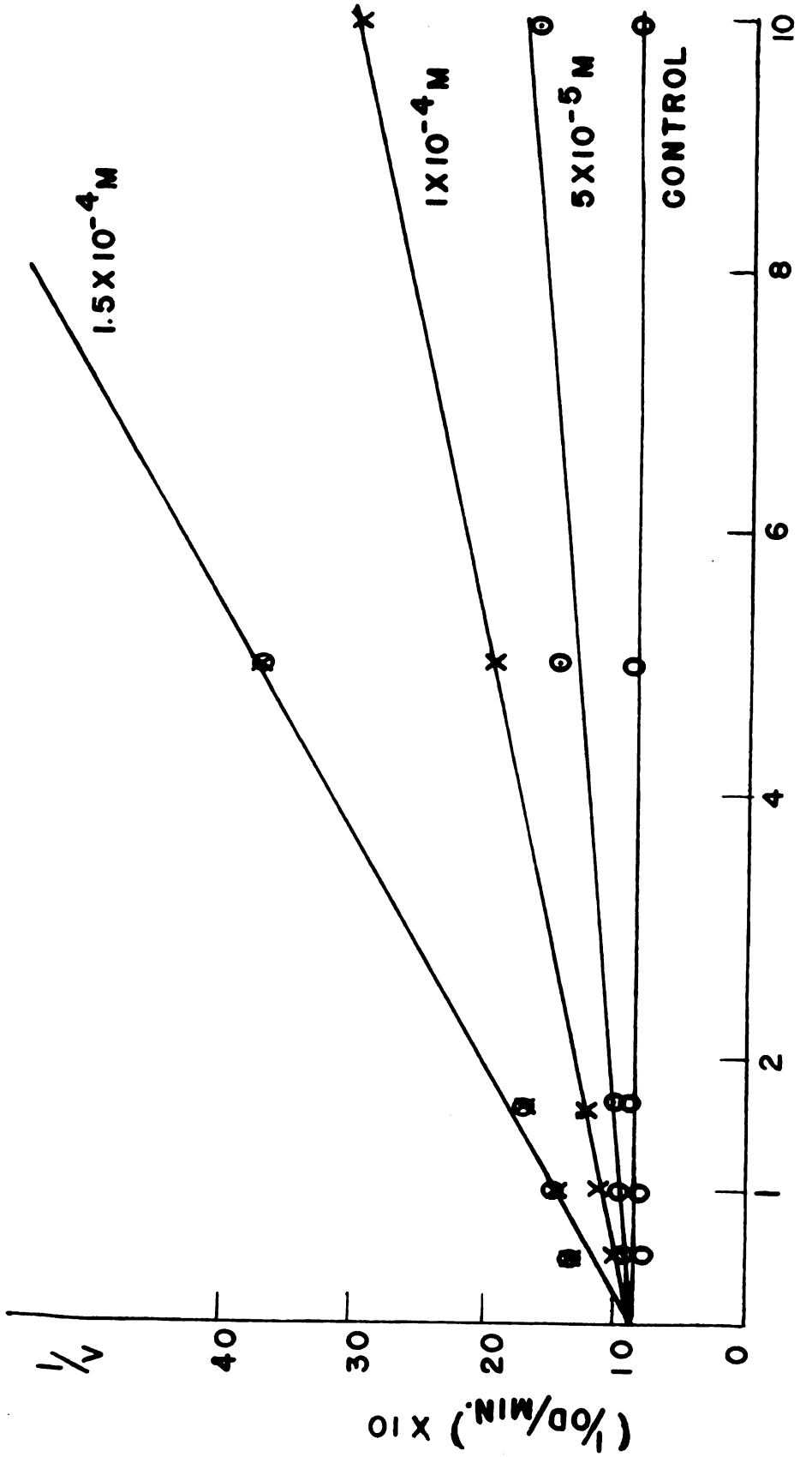


Figure 10. Lineweaver Burk plot of enolase inhibition studies.

aldolase or glyceraldehyde-3-phosphate dehydrogenase might occur. This was tested by the optical method described by Colowick and Kaplan (1955) for aldolase, except that no glyceraldehyde-3-phosphate dehydrogenase was added. As evident in Figure 11, no inhibition was observed. This conversion did not eliminate the possibility of one of these enzymes being inhibited since one could have been in great excess, but it did eliminate either as the inhibitory site with the cell-free extracts used.

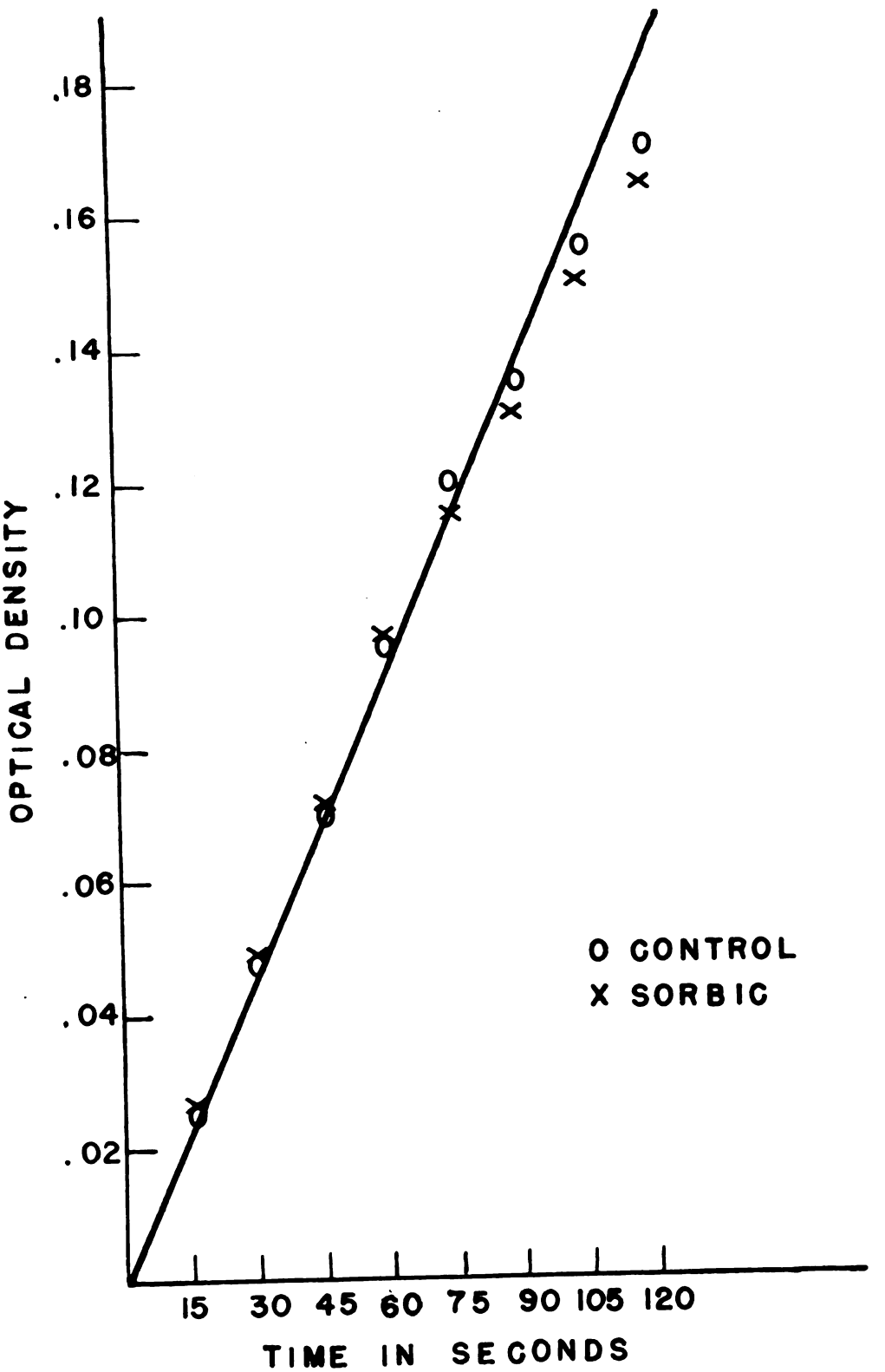


Figure 11. Effect of sorbic acid on aldolase activity.

# DISCUSSION

It is obvious that the factor of permeability is important wherever a particular inhibitory 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. A study of the inhibitory action of $5 \times 10^{-3}$ M sorbic acid on the fermentation of glucose at two different pH levels showed that greater inhibition was obtained at low pH than at a higher pH level. This effect of pH on the effectiveness of sorbic acid has been demonstrated a number of times (Etchells, et al. , 1955, 1959; Beneke and Fabian, 1955). The relation between pH of a solution and degree of ionization of monobasic weak acids is given by the Henderson and Hasselbach equation; $pH = pK_a + \log \frac{(A^-)}{(HA)}$ (Jacobs, 1940), where (HA) represents the concentration of non-ionized molecules and ($A^-$) the concentration of the corresponding anions. The degree of ionization in terms of per cent of non-ionized molecules can be expressed by the following equation:

$$\text{Per cent non-ionized} = \frac{100}{1 + \text{antilog } (pH - pK_a)}$$

Therefore, 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. Therefore, if the

undissociated molecule has greater penetrability, the entrance of organic acids into the cell will be favored by low pH values of the external medium at which (HA) is increased at the expense of ($A^-$).

Let us now calculate the concentration of the two forms of sorbic acid existing at the two pH levels used in these experiments. Using the formula given above, we find that at pH 6.0 there is 5.4 per cent undissociated while at pH 5.0 it increases to 36.5 per cent. Since the sorbic acid concentration was $5 \times 10^{-3}$ M, the amounts of dissociated and undissociated forms were as follows:

| pH | Undissociated | Dissociated |
|---|---|---|
| 6.0 | $2.7 \times 10^{-4}$ M | $47.3 \times 10^{-4}$ M |
| 5.0 | $18.2 \times 10^{-4}$ M | $31.8 \times 10^{-4}$ M |

It can be seen that with a decrease of pH from 6.0 to 5.0, the concentration of undissociated acid increases almost seven times while the dissociated acid decreases about one third. With the disruption of the cell membrane both the undissociated and dissociated molecules have the same chance to reach the site of inhibition. Due to the large difference in concentration of the undissociated forms with change in pH, if this were the toxic agent there would still be a great effect of pH with cell-free extracts. In comparison, the concentration of the dissociated form changes very little with change in pH. Since, with cell-free extracts, the degree of inhibition was essentially the same at both pH 5.0 and 6.0, it is indicated that either both the undissociated molecule and the anion or the anion alone is the toxic agent rather than the undissociated molecule alone.

Since glucose fermentation was inhibited, the site(s) of inhibition had to be in the pathway of glucose to $CO_2$ plus $CH_3CH_2OH$. The pathway of glucose fermentation was elucidated through the work of Meyerhof, Embden, Neuberg, Warburg, Cori, and others; i.e., the Embden-Meyerhof pathway (Fruton, 1958). 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 Doudoroff, 1952; Gibbs and DeMoss, 1954; Korkes, 1956; and 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 per cent. On the other hand, the Embden-Meyerhof pathway is greatly predominant under anaerobic conditions. At least 95 per cent of glucose is fermented in this manner.

To attack the problem of determining the site(s) of inhibition, it was proposed to determine the effect of sorbic acid on the various intermediates of the Embden-Meyerhof pathway. The reason being that it would help to narrow down the site(s) of inhibition. For example, if compound D is an intermediate in the pathway of A going to Z and A to Z is inhibited by sorbic acid, then if D going to Z is also inhibited, a site of inhibition lies between D and Z. On the other hand, if D going to Z is not inhibited, a site of inhibition lies between A and D. Using this approach, it was demonstrated that a site of inhibition of glycolysis by sorbic acid was between 3-phosphoglyceric acid and pyruvate.

Whitaker upon showing that crystalline alcohol dehydrogenase was inhibited by sorbic acid with time, proposed this as a site of inhibition.

On the basis of similarity in structure of sorbic acid, $CH_3$-CH=CHCH=CHCOOH, and maleic acid, HOOC-CH=CH-COOH, and the similarity of their action on alcohol dehydrogenase, he suggested that sorbic acid inhibits this enzyme by the same mechanism as does maleic acid; i.e., it forms a thiohexenoic acid derivative, $CH_3$-CH=CHRSCH-$CH_2$COOH. He also suggested that the inhibition of the sulfhydryl enzyme systems of microorganisms could explain why many of these organisms cannot grow and reproduce in the presence of sorbic acid. However, in using the crude cell-free extracts, we found no inhibition of alcohol dehydrogenase with or without incubation. And since the use of crude cell-free extracts is more closely allied to what happens in the intact cell than the use of crystalline alcohol dehydrogenase, it seems reasonable to assume that this is not the site of inhibition. Furthermore, the inhibition of glucose fermentation by cell free extracts is immediate and does not increase with time. Apparently, there are some substances in the crude cell-free extract that protect the sulfhydryl enzymes from inhibition by sorbic acid.

Based on the screening program used and taking into consideration the structural similarity between phospho-enol-pyruvate, $HOOC-\overset{OPO_3H_2}{\overset{|}{C}}=CH_2$, and sorbic acid, $CH_3$-CH=CHCH=CHCOOH, enolase was postulated and proved to be a site of inhibition. The equilibrium constant of the enolase reaction is 1.4 (Colowick and Kaplan, 1955). Therefore, it is freely reversible for enolase and the affinity of the normal product, phospho-enol-pyruvate, is very near that of the normal substrate, 2-phosphoglyceric acid. Enolase appears to be specific for 2-phosphoglyceric acid and the substrates must be in the totally ionized forms for the reaction to take place. It therefore, is postulated that the undissociated

molecules of sorbic acid penetrate the cell, are dissociated due to the pH inside the cell; and the anion, which is structurally similar to phospho-enol-pyruvate, is the toxic agent. The peculiarity of a lag in enolase activity which was not related to substrate concentration but directly related to sorbic acid concentration cannot be explained at the present time. However, further work will be done in this area as it may uncover a different type of inhibition from the types already known.

An attempt was made to determine the type of inhibition by plotting 1/v (velocity) against 1/s (substrate concentration). The effect of a competitive inhibitor is to increase the slope of the line without a change in the intercept. In other words, if the substrate concentration is large enough, the effect of the inhibitor can be overcome. The effect of a non-competitive inhibitor is to increase both the slope and the intercept of the line. The difference in the nature of the Lineweaver-Burk plot for competitive and non-competitive inhibition thus provides a quantitative means of distinguishing between the two (Dixon, 1958; Umbreit, 1957). This plot indicated that the enolase inhibition was indeed of a competitive nature as postulated. However, it does not seem to be the fully competitive type since a plot of 1/v against i (inhibitor concentration) did not give a straight line, as it does in the fully competitive or non-competitive cases. This may be a type of partially competitive system where the inhibitor affects the affinity of the enzyme for the substrate, although the inhibitor and substrate combine with different groups. This system is completely indistinguishable from the fully competitive type merely by varying the

substrate concentration at fixed inhibitor concentrations, as in the Lineweaver and Burk graphical method. It may, however, be distinguished by varying the inhibitor concentration at fixed substrate concentration, for the inhibition does not increase indefinitely with increase of inhibitor concentration, as in the fully competitive case, but increases to a definite limit where all the enzyme is combined with inhibitor, and can then increase no further.

It is not possible in this case to determine equilibrium constant ($K_i$) for the enzyme-inhibitor reaction by simple graphical methods. There is not sufficient data at the present time to determine exactly what type of inhibition this is. However, one can say that it is not purely competitive or purely non-competitive. Further research is needed in this area and it may uncover a different type of inhibition from the types already known. Further work will be done on this and also an attempt will be made to find an explanation for the lag mentioned earlier. It will also be interesting to find the site of inhibition in aerobic metabolism of yeast. Aconitase seems like a good guess based on structural similarity. Future attempts will also be made to study the effects of other similar organic acids on enolase and in this way possibly find a better inhibitor than sorbic acid. It is hoped that this study has shed some light on the inhibition of organic acids and specifically sorbic acid and that future studies will elucidate this problem still more clearly.

## SUMMARY

Studies on sorbic acid inhibition of alcoholic fermentation by baker's yeast demonstrated that while the pH influenced the inhibition of intact cell activity greatly, it had no measurable influence on fermentation by cell-free extracts. Therefore, it is postulated that the influence of pH is related primarily to the low permeability of cells to ionized molecules. Thus, sorbic acid in the undissociated state could penetrate the intact cells but would dissociate more extensively at the relatively high pH occurring within the cell. Conversely, in a high pH medium, the sorbic acid anion would not be able to penetrate the cell to reach a site of inhibitory activity.

Sorbic acid inhibition of the fermentation of fructose-1,6-diphosphate and 3-phosphoglyceric acid by cell-free yeast extracts was demonstrated. However, there was no inhibition of alcohol dehydrogenase, carboxylase, or of the conversion of fructose-1,6-diphosphate to 3-phosphoglyceric acid by cell-free extracts. It was postulated that enolase was the most likely site of action between 3-phosphoglyceric acid and pyruvate, and this was proved to be the case. The inhibition of purified enolase was demonstrated to be related to both the sorbic acid and the substrate concentrations. In fact, when the reciprocal of the velocity was plotted against the reciprocal of the substrate concentration, the inhibition appeared to be strictly competitive. However, the plot of the reciprocal of the velocity versus the inhibitor

concentration proved that this was not the case. Also, there was an unusual lag in enolase activity in the presence of sorbic acid that was independent of substrate concentration. Therefore, the type of inhibition occurring must be somewhat unusual and remains to be elucidated.

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