# SORBIC ACID INHIBITION OF ENOLASE FROM YEAST AND LACTIC ACID BACTERIA

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ABSTRACT

SORBIC ACID INHIBITION OF ENOLASE FROM YEAST AND LACTIC ACID BACTERIA

by John J. Azukas

This investigation was conducted to determine the kinetics of sorbic acid inhibition of enolase from yeast, and the reason for the failure of this acid to inhibit lactic acid bacteria. The inhibition of yeast enolase is believed to be dependent on the alpha, beta unsaturation in the acid, since crotonic and cinnamic acids were also found to be effective inhibitors. It appears that a complex is formed consisting of acid, enzyme, and $Mg^{++}$. This is indicated by a loss of absorbancy of sorbic acid solutions at a wavelength of 250 mu on addition of either enzyme or amino acids along with $Mg^{++}$. A similar phenomenon was observed when phosphoenolpyruvate was used in place of sorbate. The enolase inhibition by sorbate as indicated by a Lineweaver-Burk plot was unusual in nature in that it appeared competitive at lower sorbate concentrations and non-competitive at higher sorbate concentrations. Further analysis indicated that the innibition was actually partially competitive and partially non-competitive.

Glucose fermentations by intact cells and by acetone dried cells of Lactobacillus plantarum were not inhibited by sorbic acid. Furthermore there was no innibition of enolase activity in crude extracts of L. plantarum, Lactobacillus brevis, and Pedicoccus cerevisiae as measured by the rate

of formation of phosphoenolpyruvate and of acid labile phosphate from 2-phosphoglyceric acid or by the rate of oxidation of reduced diphosphopyridine nucleotide with 2-phosphoglyceric acid as substrate. However, when the enolase from L. plantarum was partially purified by organic solvent fractionation and passage through a phosphorylated cellulose column charged with $Mg^{++}$, it was greatly inhibited by sorbic, crotonic, and cinnamic acids. Thus, it was postulated that the lactic acid bacteria contain a factor or factors which protect the enolase from inhibition. Fractions containing both sorbate sensitive and insensitive enolase were separated from L. plantarum extracts by starch block electrophoresis and by ammonium sulfate fractionation, and protection was demonstrated by addition of insensitive fractions to a sensitive fraction. All of the sorbate insensitive fractions contained high levels of lactic dehydrogenase. Also, the addition of a solution of a commercial preparation of crystalline lactic dehydrogenase to a fraction containing enolase that was inhibited by sorbate, resulted in a significant degree of protection. Therefore, it is postulated that the lack of sorbate inhibition of the lactic acid bacteria is due to the protection of enolase by lactic dehydrogenase.

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by

John J. Azukas

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# INTRODUCTION

Sorbic acid, 2,4-hexadienoic acid, is used in the food industry for the control of undesirable microorganisms. It has been studied extensively with respect to inhibitory activity on various groups of microorganisms, and is the most effective of a number of alpha, beta-unsaturated monocarboxylic aliphatic acids reported to inhibit the fungi (Gooding 1945). Its effective action against different microorganisms, harmlessness as a dietary component, ease of quantitative determination, and chemical stability are among the main reasons for its rapid adoption in the food industry.

The action of sorbic acid appears to be directed against the catalase positive microorganisms. Of the various types of bacteria tested, the lactobacilli and clostridia are much less sensitive to this agent than others. This acid is a very effective inhibitor of yeast fermentation and this has been shown to be due to the inhibition of enolase by Azukas, Costilow and Sadoff (1961).

In earlier studies with pure yeast enolase and sorbic acid, a lag was encountered which was independent of substrate concentration but directly related to enzyme, sorbate and magnesium concentration. Also it was not understood why the lactic acid bacteria were not affected by sorbic acid

since they also contain an enolase which performs the same catalytic function as yeast enolase. The present work is concerned with elucidation of the lag encountered previously, the kinetics of inhibition of purified yeast enolase and the lack of inhibition of lactobacilli by sorbic acid.

## REVIEW OF LITERATURE

Mechanism of inhibition by sorbic acid. The antimicrobial action of sorbic acid appears to be directed against the "catalase positive" microorganisms, and sorbate has been incorporated in media for the selection of catalase negative lactic acid bacteria and clostridia by Emard and Vaughn (1952). Until recently there have been many hypotheses but very little data on the mechanism(s) of action or the site(s) of inhibition by sorbic acid.

Melnick, Luckmann, and Gooding (1954) suggested that sorbic acid inhibited dehydrogenase systems in molds, and York and Vaughn (1955) reported it to be active in suppression of fumarase action.

Palleroni and DePritz (1960) reported on the influence of sorbic acid on acetate oxidation by Saccharomyces cerevisiae var. ellipsoideus. They found that the inhibitory effect of sorbate on the respiration is competitive as shown by a Lineweaver-Burk plot where the reciprocal reaction velocity is plotted against the reciprocal substrate concentration. This conclusion is not believed to be valid, however, since a number of reactions are involved in the oxidation of acetate, and this could greatly influence the type of data obtained. They did not determine the mechanism of inhibition, but they did hypothesize that sorbate

interferes with the synthesis of citric acid in the cells by way of formation of sorbyl coenzyme A (sorbyl-CoA). This is theoretically possible in light of the work of Wakil and Hubscher (1960) in which they quantitatively determined coenzyme A (CoA) by sorbyl-CoA formation. However, Palleroni and DePritz (1960) presented no data to support their hypothesis. They also claimed that there was no appreciable effect of sorbate on the oxidative phosphorylation by whole cells in the presence of acetate and the inhibitor. Finally they concluded that since their manometric experiments were performed under conditions which rule out any significant assimilation through the Krebs cycle (absence of carbon dioxide and of combined nitrogen in the medium), it is possible that sorbate could inhibit the synthesis of higher fatty acids by the resting cells.

All of the above reports mentioned were concerned with aerobic metabolism and shed no light on the activity of this compound in anaerobic systems. However, Whitaker (1959) demonstrated that crystalline alcohol dehydrogenase was inhibited after incubation with sorbic acid, and suggested that the action on microorganisms is similar to that of maleic acid; i.e., the forming of stable complexes with sulfhydryl containing enzymes. This is in some doubt since Azukas, Costilow and Sadoff (1961) found no inhibition of alcohol dehydrogenase in crude extracts and demonstrated

that the inhibition of yeast fermentation was due primarily to the inhibition of enolase by sorbic acid. This was shown through the use of intact cells, cell-free extracts, and the purified enzyme. This is not in agreement with Whitaker's hypothesis as enolase is not a sulfhydryl containing enzyme according to Malmstrom, Kimmel, and Smith (1959). Thus the enolase could not be affected in the manner that he suggests.

Types of enzyme inhibition. The inhibition of any single enzyme involved in a main metabolic chain will render the whole chain inoperative and will have a profound or even fatal effect upon the organism. Some have called such an inactivation of an essential enzyme a biochemical lesion. One of the most obvious examples of the fatal effects which may be caused by the specific inhibition of a single enzyme is the toxicity of cyanide. This is primarily due to the inhibition of cytochrome oxidase, resulting in a cessation of the aerobic oxidation processes and death in a very short time. Destruction of enzymatic activity by denaturation of protein as in the case of denaturation by heat, strong acid, or other means is not usually regarded as an inhibition. Inhibitors in the usual meaning work by a definite chemical action and may be either reversible or irreversible. Reversible implies that the activity returns on merely removing free inhibitor by dialysis or other means; thereby showing

that there is an equilibrium between free inhibitor and enzyme. On the other hand, with the irreversible inhibitors no such equilibrium is set up and the activity does not return on dialysis. All cases of inhibition do not have to involve a direct combination of the inhibitor with the enzyme; but inhibition may be produced by an agent which combines with the substrate, coenzyme or metal activator thereby rendering them unavailable for the enzyme. However, addition of sufficient substrate or cofactor will abolish the inhibition.

Dixon and Webb (1958) classify the effects of inhibitors in the following manner:

a. Purely competitive
b. Partially competitive
c. Purely non-competitive
d. Partially non-competitive
e. Mixed
f. Uncompetitive

In the case of the purely competitive enzyme (E) inhibition, the inhibitor (I) combines with the substrate (S)-binding site and the following reactions occur:

$$(1)\quad E + S \overset{k_1}{\rightleftharpoons} ES$$

$$(2)\quad E + I \overset{k_2}{\rightleftharpoons} EI$$

$$(3)\quad ES \overset{k_3}{\rightarrow} E + P$$

In such an inhibition the EI undergoes no further reaction and an EIS complex cannot be formed.

In the case of the partially competitive inhibition, the inhibitor affects the affinity of the enzyme for the substrate, although the inhibitor and substrate combine with different groups. The following reactions occur in addition to reactions (1), (2), and (3):

$$(4)\quad EI + S \rightleftharpoons EIS$$

$$(5)\quad ES + I \rightleftharpoons EIS$$

$$(6)\quad EIS \xrightarrow{k_3} EI + P$$

Here it is assumed that the inhibition is purely an effect on affinity, so that the complexes ES and EIS break down at the same rate. Therefore, the same velocity constant ($k_3$) applies to the breakdown of both, and the overall velocity will be the sum of these two reactions. Thus, the effect of the inhibitor is on the rate of formation of ES and EIS complexes. If this were not the case the inhibitor would be acting in two ways, affecting the velocity (V) as well as the Michaelis Constant ($K_m$). This system is indistinguishable from the fully competitive type merely by varying the substrate concentration at fixed inhibitor concentrations and plotting the reciprocals of the reaction velocities (V) versus the reciprocals of substrate concentrations (S) (Lineweaver-Burk plot). However, a plot of 1/v against inhibitor concentrations (i) will not give a straight line as it does in the fully competitive or non-competitive cases, and the

inhibitor constant ($K_i$) which is the dissociation constant of the enzyme-inhibitor complex (the reciprocal of the affinity of the enzyme for the inhibitor) cannot be determined by this method.

In fully non-competitive inhibition, the inhibitor does not affect the combination of the substrate with the enzyme but affects only the maximum velocity (V). Reactions (1) through (5) occur, but not reaction (6). Thus, the complex EIS does not break down at all and the velocity is entirely that of the breakdown of ES. The effect of the inhibition is equivalent to a reduction in the amount of active enzyme.

In the partially non-competitive case, the complex EIS may break down at a different velocity from ES and the velocity is the sum of the two reactions. The inhibitor constant ($K_i$) again cannot be determined graphically.

In the mixed type of inhibition, the inhibitor affects both the maximum velocity (V) and the Michaelis constant ($K_m$), giving a mixture of competitive and non-competitive or partially competitive and partially non-competitive. It is not possible to combine fully competitive with either fully non-competitive or partially non-competitive; since with a fully competitive inhibitor no EIS complex can be formed. A general equation for such mixed types was derived by the application of steady-state kinetics by Botts and Morales (1953) but it is too complicated to be of much

practical value. The Lineweaver-Burk plot showing the effect of such an inhibitor is distinguished from the plots for the fully competitive or non-competitive types by the fact that both intercepts change on addition of the inhibitor. Plots of 1/v against i will not be linear.

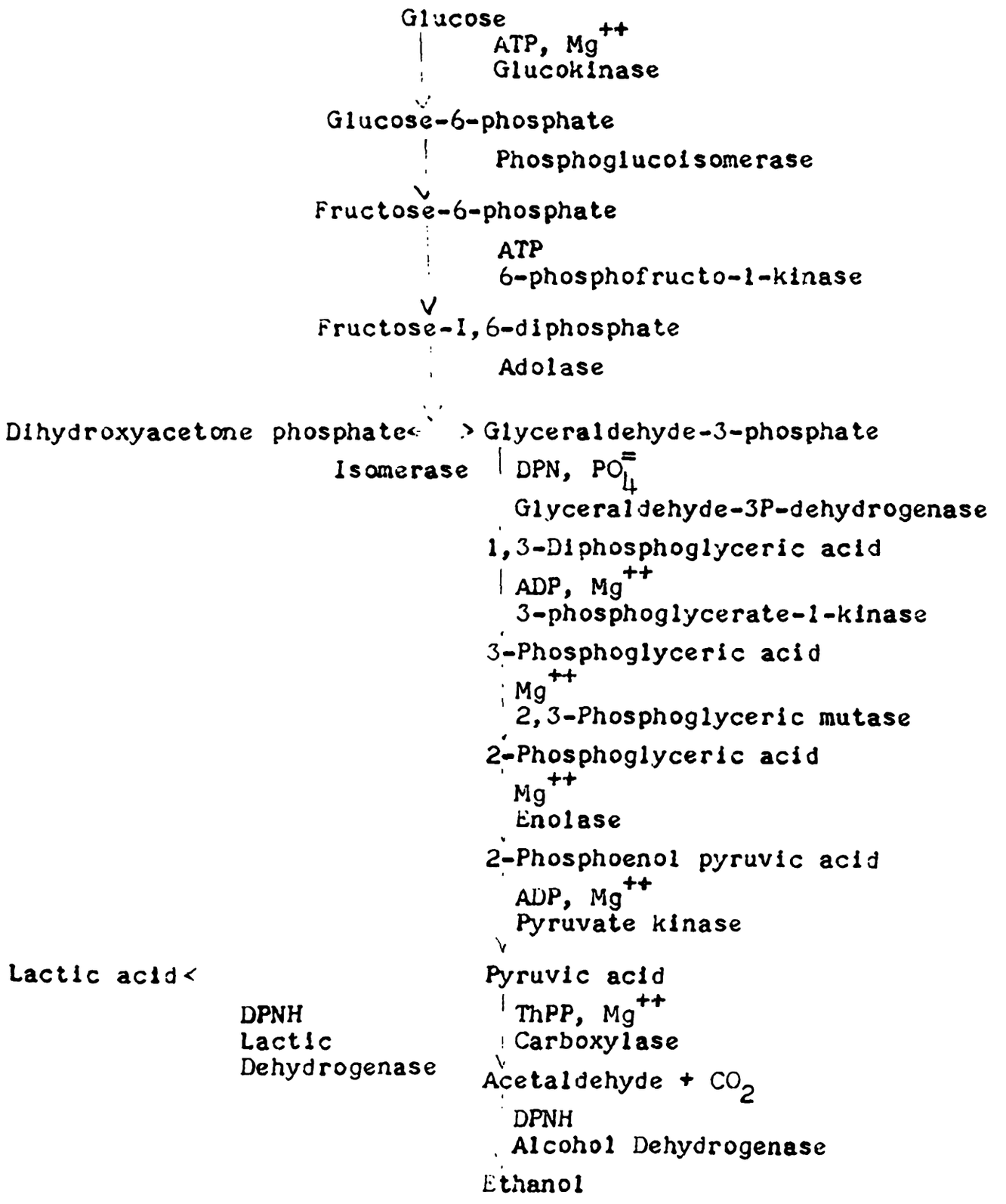
Finally there is an uncompetitive type of inhibition. In rare cases where $K_m = k_3/k_1$ (see reactions (1) and (3)), an inhibitor which affects only $k_3$ will produce the same proportional changes in V and $K_m$. Therefore, the reciprocal plots 1/v versus 1/S in the presence of various amounts of inhibitor will all be straight lines parallel to that obtained in the absence of inhibitor. This is typical of the "so-called" uncompetitive inhibition. It is not as originally suggested by Ebersole, Guttentag and Wilson (1943), who said that the inhibitor can only combine with the ES complex. This new explanation was put forward by Dodgson, Spencer, and Williams (1956) for the inhibition of arylsulphatase by cyanide or hydrazine and seems to be a far more plausable explanation for those cases of uncompetitive inhibition which have been observed.

Protection from antibacterial activity. Antibacterial properties of some saturated fatty acids have been demonstrated with pure cultures of many microorganisms. In many cases it has been demonstrated that the antibacterial action of fatty acids can be overcome by certain substances. Lamar (1911) and Walker (1926) used serum to neutralize the

growth inhibiting action of unsaturated fatty acids. It later became evident that this property of serum was due to the presence of albumin. Dubos (1947) used serum albumin to protect Mycobacterium tuberculosis and Micrococcus "C" from inhibition by oleic acid, and Hutner (1942) overcame the fatty acid inhibition of Erysipelothrix rhusiopathiae with bile salts. Kodicek and Worden (1945) found that the growth inhibition of Lactobacillus helveticus by linoleic acid could be reversed by cholesterol, lecithin, calciferol, lumisterol, and alpha-tocopherol. However, it should be emphasized that these investigations were concerned with protection of cells and not specific enzymes. No data were found with respect to the protection of enzymes by such agents.

Comparison of anaerobic glucose metabolism of yeast and lactic acid bacteria. According to Cook (1958) yeast has the pathway of anaerobic glucose metabolism shown in Scheme I.

The lactic acid fermentations are of two types, the homofermentative and the heterofermentative. According to Gunsalus, Horecker, and Wood (1955) in the homofermentative type the only product is lactic acid. This differs from the yeast alcoholic fermentation in that all the pyruvate is reduced to lactic acid by lactic dehydrogenase in the presence of DPNH. Thus the homofermentative lactic acid organism is dependent on all of the same enzymes active in




Scheme I. Pathway of anaerobic glucose metabolism in yeast.

alcoholic fermentation but it does not have the enzymes carboxylase and alcohol dehydrogenase.

The heterolactic fermentation by Leuconostoc mesenteroides was elucidated by DeMoss, Bard and Gunsalus (1951) and by Gunsalus and Gibbs (1952) upon the following evidence. Equimolar quantities of $CO_2$, ethanol, and lactate were produced and the ratio of these was constant under varying conditions. Aldolase could not be demonstrated. Also, $CO_2$ arises from carbon 1 of glucose, the lactate from carbons 4, 5, and 6 and the methyl carbon of ethanol from carbon 2 of glucose. In this fermentation glucose goes to glucose-6-phosphate and then to 6-phosphogluconic acid. The phosphogluconic acid is oxidized and decarboxylated to yield $CO_2$ and a phosphorylated five-carbon sugar, ribulose phosphate. Ribulose phosphate is then split to yield one molecule of triose phosphate and a two-carbon fragment that becomes reduced to alcohol. Triose phosphate is oxidized via the Embden-Meyerhof pathway (EMP) to pyruvic acid and the pyruvate goes to lactic acid. In this pathway only one molecule of lactic acid is formed from the hexose substrate, while $CO_2$ and alcohol are also formed. More recently, Eltz and Vandemark (1960) demonstrated a similar system in Lactobacillus brevis. Thus, the heterofermentative lactic acid bacteria are also dependent on the enzymes of the lower fraction of the EMP scheme.

Comparative enzyme biochemistry. Are the preparations of an enzyme obtained from different sources identical? Before this question can be answered, one must define what is meant by identical. Is it identity merely of the active center and its immediate surroundings, so that the name of an enzyme is merely the name of a particular active center, which may be attached to different proteins; or does it mean that the whole protein is identical? If it is the latter, a study of only the catalytic properties will not answer the question. One must study the protein itself, and in particular the amino acid composition and sequence.

Jolles and Fromageot (1956) prepared lysozymes from the spleen and kidney of dog and rabbit. They were all similar in properties, but not identical even when prepared from two tissues of the same animal. Henion and Sutherland (1957) found that phosphorylases from different organs in the same animal as well as from different animals were immunologically different; and Antoni and Keleti (1957) found quantitative immunological differences between alcohol dehydrogenase from closely related yeast species.

The observation that a single organism may contain more than one enzyme catalyzing the same biochemical reaction is not uncommon. Kornberg and Pricer (1951) found yeast to contain two isocitric dehydrogenases. One was DPN and the other TPN dependent. Ebesuzaki and Barron (1957) found yeast to contain two alcohol dehydrogenases. Stadtman

*et al.* (1961) demonstrated that cell-free extracts of *Escherichia coli* contain two different aspartokinases that catalyze the phosphorylation of aspartate by ATP. These enzymes have been separated from each other by ammonium sulfate precipitation. It is postulated that the biological significance of the presence of more than one enzyme catalyzing the same biochemical reaction is part of a cellular regulatory control mechanism.

## EXPERIMENTAL METHODS

The sorbic acid used was refined sorbic acid (water content, 5.5%) provided by the Carbide and Carbon Chemicals Co., New York. This was recrystallized three times from distilled water before use. Fresh stock solutions ($2.0 \times 10^{-2}$ M) were maintained in the refrigerator during the experiments. These were adjusted to the pH level desired for each experiment and diluted to give the final concentration necessary for the specific study.

Enolase activity was determined by the method of Warburg and Christian (1941) as modified by Wold and Ballou (1957). With 2-phosphoglyceric acid (2PG) as a substrate, the appearance of phosphoenolpyruvate (PEP) is followed by measuring the increase in optical density (OD) at 240mu. A Beckman model DU spectrophotometer was used for all spectrophotometric work. The crystalline enolase used was prepared from yeast and kindly supplied by Dr. Finn Wold, Department of Chemistry and Chemical Engineering, University of Illinois, Urbana, Illinois.

The glucose used was "Difco" Bacto-dextrose. Pyruvic acid solution was prepared by dissolving sodium pyruvate and adjusting the pH. The 2-phosphoglyceric acid solution was made from the barium salt by dissolving it in 1N 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. 3-Phosphoglyceric acid (3PG) was also prepared from its barium salt by the procedure just described. Phosphoenolpyruvate was prepared from its silver barium salt. The barium was removed as previously described and the silver was removed as silver chloride.

The cultures Lactobacillus plantarum, Lactobacillus brevis, and Pediococcus cerevisiae used in this study were grown in a medium of the following composition: 1% yeast extract, 0.5% dextrose, and 0.5% dibasic potassium phosphate. They were stock cultures which had been originally isolated from cucumber fermentations. Twenty liters of medium minus the dextrose were sterilized in carboys. The dextrose was sterilized separately and added aseptically. The inoculum was started in a test tube, and was transferred 18 hours later to a 1 liter flask, which in turn was transferred to a 3 liter flask after 18 hours. This was transferred to the carboys. The carboys were incubated for 18 hours at 30C, the cells harvested with a Sharples Super Centrifuge (the Sharples Specialty Co., Philadelphia, Pa.) and washed twice with distilled water. These cells were used for the intact cell Warburg studies, the acetone dried cell Warburg studies, the cell-free extract studies, and the enzyme purifications.

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.

The effects of sorbic acid on the fermentation of glucose by intact yeast and lactic acid bacteria were measured by the conventional Warburg technique. Cells, buffer, sorbic acid, 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. The flasks were flushed with nitrogen in experiments with yeast cells, while in those with lactic acid bacteria they were flushed with a mixture of 5% $CO_2$ and 95% 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. In both instances $CO_2$ evolution was measured, but in the case of the lactic acid organisms this was an indirect measurement of lactic acid production. The homofermentative lactic organism produces two moles of lactic acid per mole of glucose. One mole of acid will liberate one mole of $CO_2$ from a bicarbonate buffer system.

The acetone dried cells were prepared in the following manner from cells grown in carboys. The aqueous cell suspension was added slowly with stirring to ten volumes of acetone previously cooled to -20 C. After stirring briefly, the cells were allowed to settle, the solvent removed by

filtration, and the filter cake of cells washed with 2 to 5 volumes of -20 C acetone. The filter cake was then transferred to a desiccator in the presence of paraffin to absorb the solvent. The resulting powder was used for Warburg studies.

For preparing cell-free extracts, 15g (wet weight) of lactic acid bacteria or air dried yeast cells and 45g of glass beads were combined with 50ml of water and blended in a Servall Omnimixer (Ivan Sorvall, Inc., Norwalk, Conn.) for five minutes in an ice bath to disrupt the cells. The beads were pavement marking beads, average size 0.2mm., manufactured by the Minnesota Mining and Manufacturing Co., Minneapolis. Before blending in the Omnimixer, the mixture of cells and beads was cooled to a few degrees above O C. The cup was filled to the top to prevent foaming and the resulting surface denaturation. The beads, cell debris, and intact cells were removed after blending by centrifugation in the cold. The supernatant contained the enzymes of interest. The extracts were dialyzed for 24 hrs against distilled water before use in the various assays.

The protein concentration in the extracts was determined by the turbidimetric trichloroacetic acid method of Stadtman, Novelli, and Lipmann (1951). The extract containing 0.1 to 2.0 mg of protein was made up with water to a volume of 2 ml, and 3 ml of 5% trichloroacetic acid added. The resulting suspension was allowed to stand for 30 sec and

the turbidity measured in a colorimeter at a wavelength of 540 mu. Crystalline egg albumin was used as a standard.

Enolase activity in the cell-free extracts of lactic acid bacteria was measured in several different ways. One of these ways was by coupling enolase to pyruvate kinase and lactic dehydrogenase. With 2-phosphoglyceric acid as substrate in the presence of $MgSO_4$ and adenosine diphosphate (ADP), the oxidation of reduced diphosphopyridine nucleotide (DPNH) was followed spectrophotometrically at 340 mu. Enolase activity in cell-free extracts was also measured by acid labile phosphate (that which is hydrolyzed completely in 1N acid at 100 C in seven minutes). With 2-phosphoglyceric acid as substrate and $MgSO_4$ as cofactor, the amount of acid labile phosphate formed in three minutes was measured. The total volume of the reaction mixture was 3.0 ml. The reaction was started by the addition of substrate and stopped at the end of three minutes by the addition of 3 ml of 10% trichloroacetic acid. This also served to deproteinize the reaction mixture. The mixture was then centrifuged to remove the precipitated protein. Sulfuric acid was added to the supernatant fluid to a concentration of 1N, the tubes heated in a boiling water bath for seven minutes, and cooled. Phosphate was determined by the procedure of Fiske and SubbaRow (1925). This procedure was slightly modified by use of monomethyl-para-aminophenol sulfate in place of 1-amino-2-naphthol-4-sulfonic acid in the reducing reagent.

The estimation of free sulfhydryl (SH) groups was accomplished by use of the p-chloromercuribenzoate procedure based on the method of Boyer (1954). This is based on the fact that p-chloromercuribenzoate reacts stoichiometrically with SH groups to form mercaptides which can be quantitated by measurement of their strong light absorption at 255mu. Crystalline glutathione was used as a reference standard.

Enolase in extracts of lactic acid bacteria was partially purified by the following procedure. The cell-free extracts were prepared as previously described and then were combined into one batch. The extract was cooled in an ice bath and cold acetone was added slowly with stirring until a 33 1/3% acetone concentration was reached. The precipitate formed was centrifuged and discarded. Additional cold acetone was added to the supernatant fluid until a concentration of 50% acetone was reached. The precipitate was centrifuged and the supernatant fluid discarded. The precipitate was then taken up in distilled water and the insoluble material was centrifuged and discarded. The solution was then brought to pH 4.8 with 1N acetic acid, cold ethanol added cautiously with stirring until 33 1/3% concentration of ethanol was reached, and the precipitate centrifuged and discarded. Additional cold ethanol was added to the supernatant fluid until 50% ethanol concentration was reached, the precipitate centrifuged, and the supernatant fluid discarded. The precipitate was then suspended

in distilled water and dialyzed overnight against distilled water. After dialysis, insoluble material was centrifuged, and the solution put through a column that was prepared in the following manner. Twenty-five grams of cellex-P cation exchange cellulose (California Corporation for Biochemical Research, Los Angeles, Calif.), exchange capacity 1.0 milliequivalents per gram, which consists of phosphoric acid exchange groups on a Whatman cellulose lattice, were made into a slurry with distilled water and packed into a column 3 cm in diameter and 16 cm long. The column was charged with magnesium by passing 100 ml of 1M $MgSO_4$ through it. It was then washed with distilled water. The solution containing the enzyme was then applied to the column and washed in with distilled water. The enzyme was then eluted with 1M KCl. Five ml fractions were collected with an automatic fraction collector and these were assayed for activity of enolase spectrophotometrically at 240 mu with 2-phosphoglyceric acid as substrate.

A Shandon electrophoresis (Consolidated Laboratories, Inc., Chicago Heights, Ill.) apparatus was used for the starch block electrophoretic separation of the extracts of cells of lactic acid bacteria. The buffer used was tris-(hydroxymethyl)aminomethane (tris) 0.05M, pH 8.6. The time of the run was three hours at 100 volts, 15 milliamps. A well was cut in the center of the starch block and the sample placed in it and then the starch was replaced. After the

completion of the run, the starch block was cut into strips about 1 cm in width. These were placed in 3 ml of 0.05M imidazole buffer, pH 7.4, and the protein eluted from the starch by shaking and allowing to settle. The eluates were assayed for enolase and lactic dehydrogenase. Enolase activity with 2-phosphoglyceric acid as substrate was measured spectrophotometrically. Lactic dehydrogenase was determined by the method of Kubowitz and Ott (1943). With pyruvate as substrate the oxidation of DPNH was followed spectrophotometrically at 340 mu.

Extracts of cells of L. plantarum were fractionated with the addition of solid ammonium sulfate in the following manner. The solid ammonium sulfate was added to the extract slowly with stirring to the point of desired saturation. The precipitate was then centrifuged and redissolved in distilled water. The supernatant fluid was used for further fractionation. The mixture was maintained throughout the fractionation process at 4 C. The assays for enolase and lactic dehydrogenase were run on the precipitates of the particular fractions that were redissolved in distilled water.

Crystalline lactic dehydrogenase was purchased from General Biochemicals, Chagrin Falls, Ohio. It was originally isolated from animal muscle tissue and had been crystallized 2x.

# RESULTS

## Mechanism of Yeast Enolase Inhibition by Sorbic Acid

In determining enolase activity in the presence of sorbic acid, it was noted that there was an initial decrease in optical density at 240 mu on the addition of enolase to the reaction mixture. This masked the initial increase in optical density due to enzymatic action and created an apparent lag. In the inhibition studies this was eliminated by adjusting the instrument to zero 5 sec after adding enzyme to the reaction mixture. This decrease in absorbancy probably results from a combination of the sorbate with the enzyme, since the sorbate absorbs light strongly at this wavelength. The extent of the decrease in optical density (OD) was directly related to sorbic acid concentration when the enzyme concentration was constant at $6.0 \times 10^{-10}$M (Fig.1). The same relationship was observed when the sorbate concentration was held constant and the enzyme concentration varied. However, the lag was independent of substrate concentration (Table 1).

Similar results were obtained when phosphoenolpyruvate was added to the enzyme and the OD loss measured at a wavelength of 240 mu. Since the sorbate and phosphoenolpyruvate

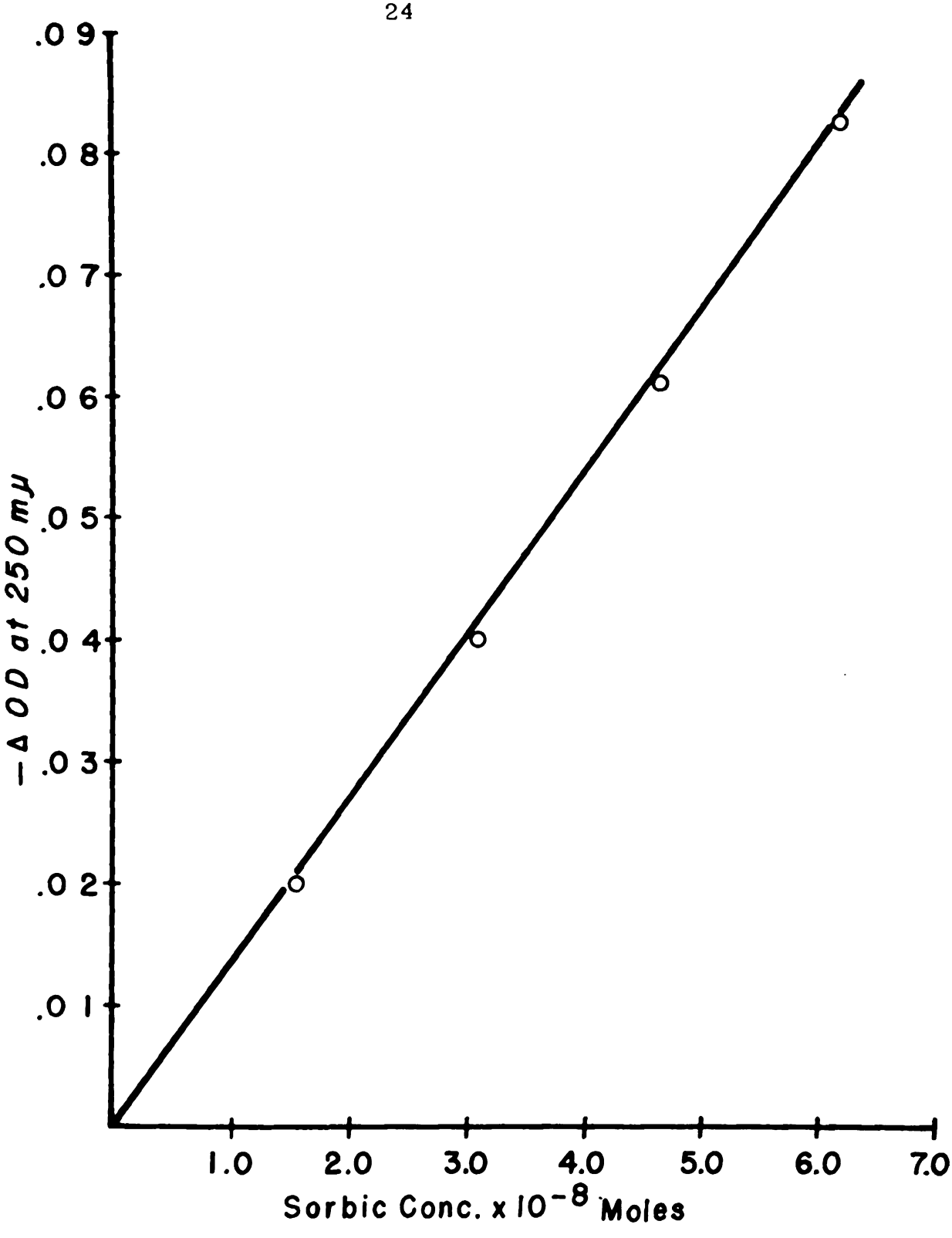



Figure 1. The change in OD due to addition of sorbate to enolase and magnesium. Cuvette contents: $6.0 \times 10^{-10}$ M enolase, $8.0 \times 10^{-3}$ M $MgSO_4$.

TABLE 1

EFFECT OF SUBSTRATE CONCENTRATION ON TIME BETWEEN ADDITION OF SUBSTRATE AND APPARENT ENZYMATIC ACTIVITY (LAG)

| Substrate (2-phosphoglyceric acid) conc. | Time of lag in sec |
|---|---|
| $2 \times 10^{-3}$ M | 30 |
| $2 \times 10^{-4}$ M | 30 |
| $6 \times 10^{-4}$ M | 30 |

must be bound to amino acid residues on the enzyme, a number of amino acids were added to these two compounds along with $Mg^{++}$ and the loss in optical density measured. It was found that the addition of individual amino acids to a solution containing magnesium and sorbic acid, and also the addition of an amino acid to a solution containing magnesium and phosphoenolpyruvate would result in a similar loss of optical density (Table 2).

Similar losses in optical density were observed on mixing other alpha, beta-unsaturated acids (crotonic and cinnamic acids) with magnesium and enolase.

The inhibition of yeast enolase activity by sorbic acid was measured in a spectrophotometer at room temperature. As can be seen in Fig. 2 sorbic acid concentrations of $1.5 \times 10^{-4}$ M and higher resulted in definite inhibition of enolase activity with a substrate concentration of $2 \times 10^{-4}$ M.

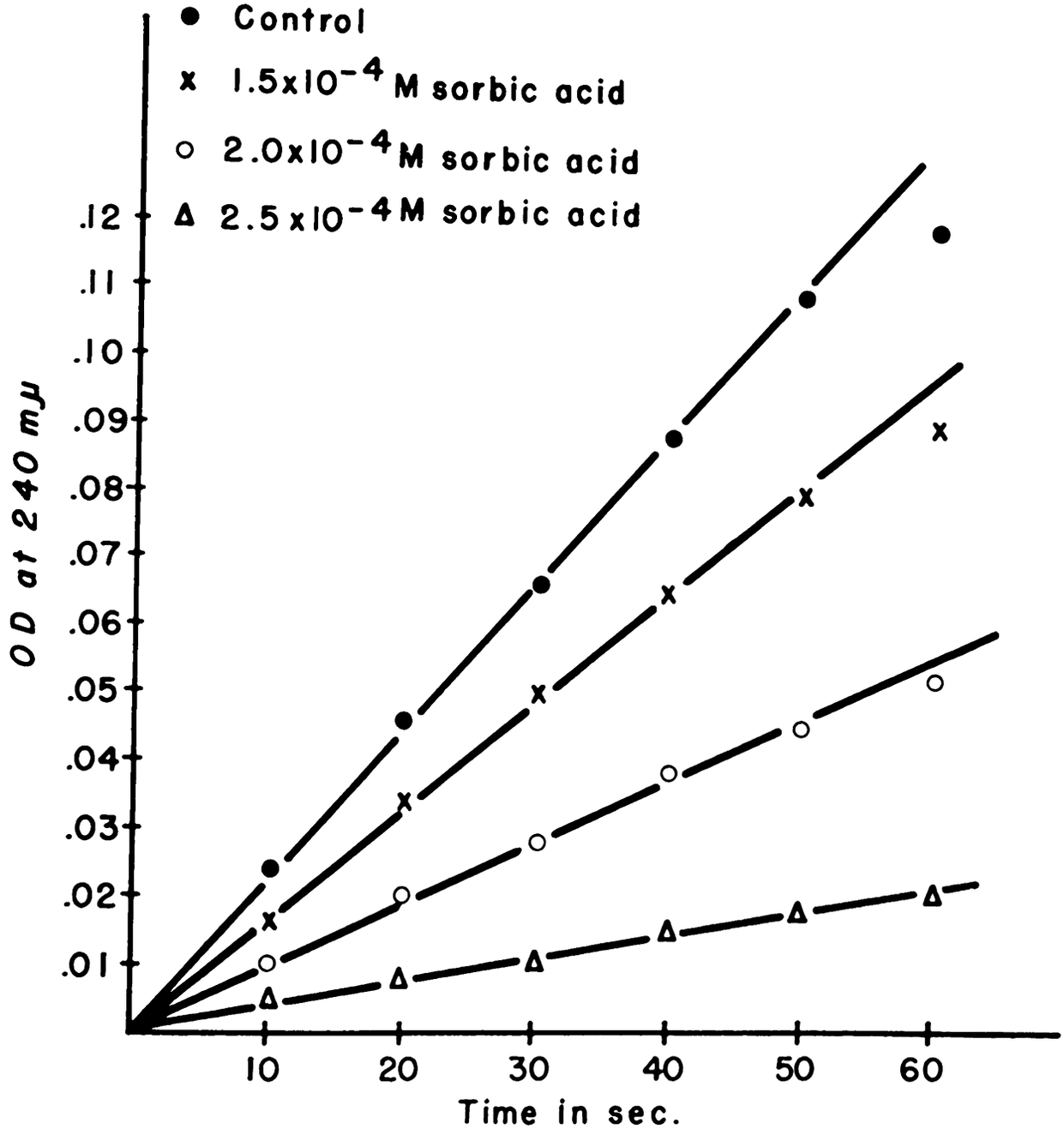



Figure 3. Inhibition of yeast enolase activity by sorbic acid. Each cuvette contained 3 ml of a reaction mixture of the following: $6 \times 10^{-10}$ M enolase, $2 \times 10^{-4}$ M substrate (2-phosphoglyceric acid), $3 \times 10^{-3}$ M $MgSO_4$, and $5 \times 10^{-2}$ M imidazole buffer at pH 7.4. Appropriate solutions of sorbic acid adjusted to pH 7.4 were added to give the sorbate concentrations indicated.

Concentrations of sorbic acid below $1.5 \times 10^{-4}$ M resulted in very little or no inhibition of the enzyme at this substrate level, and when the sorbic acid level was raised much above $2.5 \times 10^{-4}$ M no significant enzyme activity could be detected.

TABLE 2

REDUCTION IN OPTICAL DENSITY ON ADDING VARIOUS AMINO ACIDS TO SOLUTIONS OF SORBATE AND PHOSPHOENOLPYRUVATE

| Amino Acid Added (0.1 M) | Net Loss in OD[a] of solutions of: Sorbate | Phosphoenolpyruvate |
|---|---|---|
| Glycine | .150 | .090 |
| L-Histidine | .145 | .080 |
| L-Arginine | .150 | .085 |
| L-Alanine | .145 | - |
| L-Lysine | .150 | - |
| L-Methionine | .145 | - |

[a]The losses in OD with sorbate ($1 \times 10^{-4}$ M) and phosphoenolpyruvate ($2 \times 10^{-4}$ M) were measured at 250 and 240 mu respectively.

The inhibition of enolase was also demonstrated with phosphoenolpyruvate as substrate. The same procedure was followed as previously described except that phosphoenolpyruvate ($1 \times 10^{-3}$ M) was used as substrate and the decrease in optical density at 240 mu followed. As illustrated in Fig. 3, $1.0 \times 10^{-4}$ M and $2.0 \times 10^{-4}$ M sorbic acid concentrations significantly inhibited enolase activity.

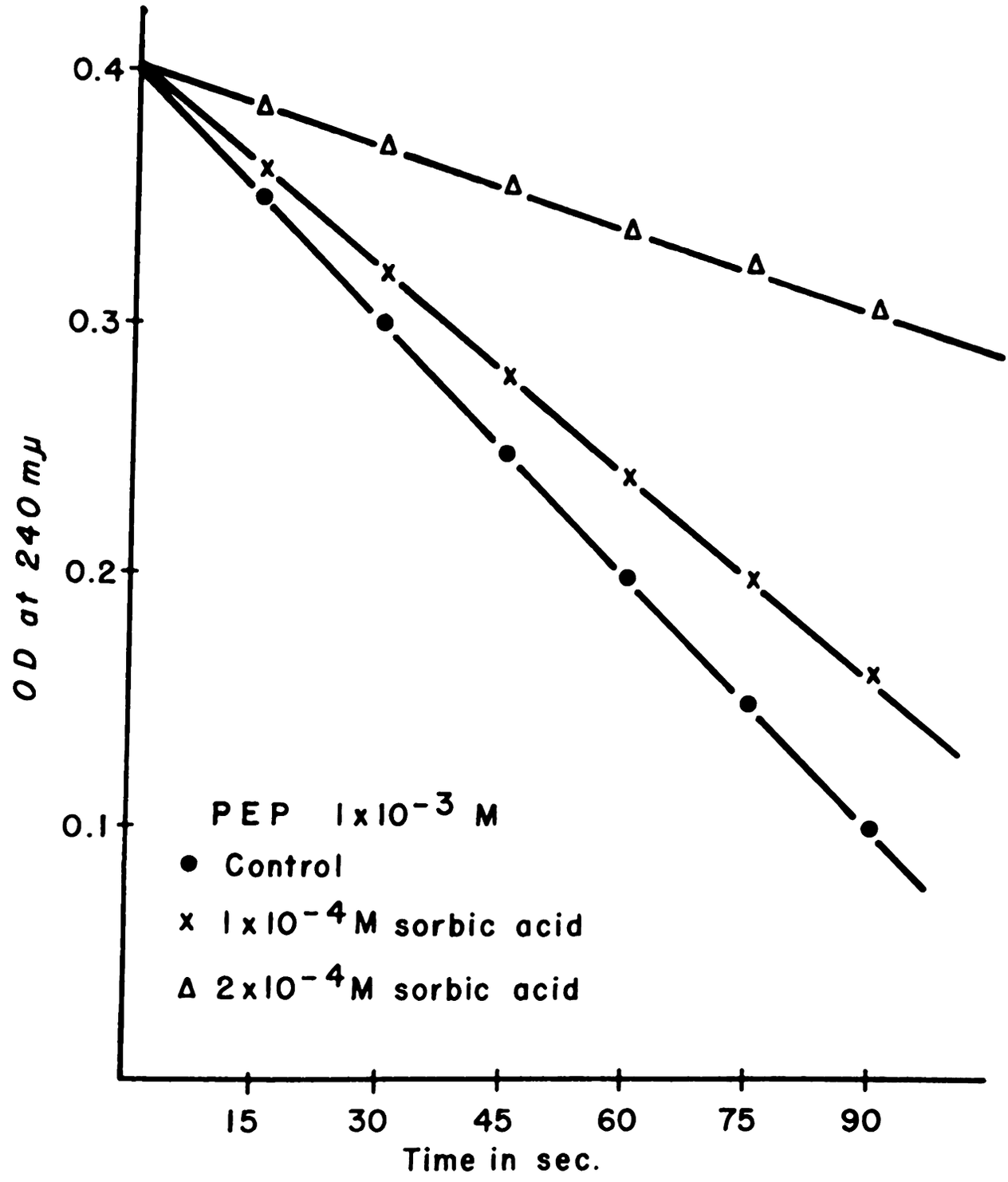



Figure 3. Inhibition of yeast enolase activity by sorbic acid with phosphoenolpyruvate as substrate. Reaction mixtures were as detailed in Fig. 2 except for the substrate.

Crotonic and cinnamic acids also inhibited enolase activity (Fig. 4). However, $4 \times 10^{-4}$ M cinnamic acid was no more effective than $2 \times 10^{-4}$ M sorbic, and crotonic acid was even less effective. Thus, the chain length may influence the relative effectiveness of these acids. The presence of the unsaturated bond is important since the addition of $6.6 \times 10^{-3}$ M of either glycine, acetic or butyric acid had no effect on the purified yeast enolase.

The results of inhibition studies run as described in Fig. 2 utilizing substrate concentrations of $5.0 \times 10^{-5}$ M, $2.0 \times 10^{-4}$ M, and $4.0 \times 10^{-4}$ M were used in analyzing the type of inhibition occurring by use of a Lineweaver-Burk plot. The reaction velocities were calculated in terms of the change in optical density per min for each sorbic acid-substrate combination, and the reciprocals of the velocities plotted versus the reciprocals of the substrate concentrations for each sorbic acid concentration indicated in Fig. 5. It can be seen that the substrate concentration had a pronounced effect on the degree of enolase inhibition by a given level of sorbic acid. From this plot it appears that the inhibition is competitive at sorbic acid concentrations up to $2.0 \times 10^{-4}$ M and is non-competitive at higher inhibitor levels. Thus, the intercept is constant and the slope increases with increasing inhibitor concentrations up to $2.0 \times 10^{-4}$ M, whereas both the slope and intercept change at higher concentrations. However, plots of reciprocal

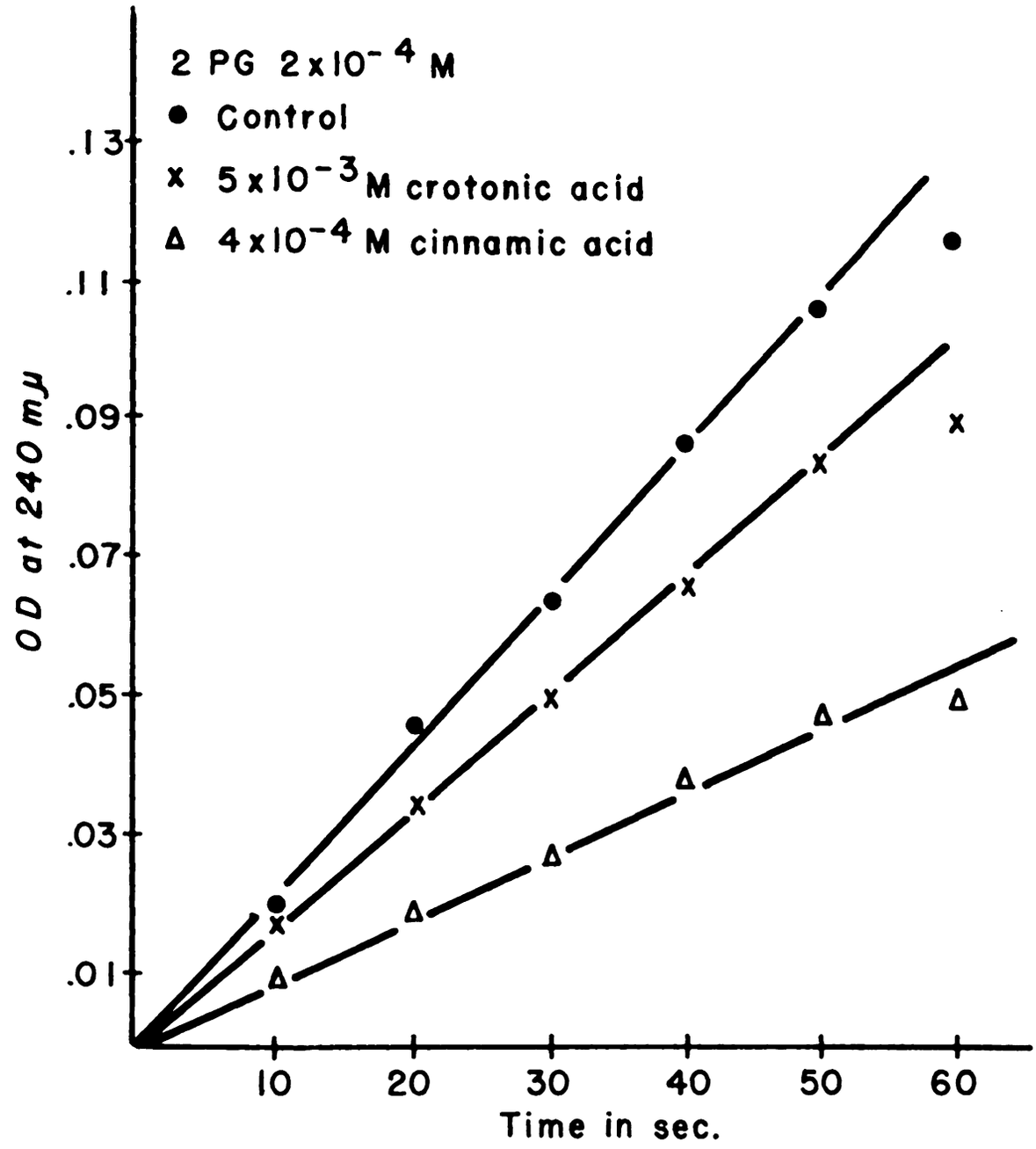



Figure 4. Inhibition of yeast enolase activity by crotonic and cinnamic acids. See Fig. 2 for composition of reaction mixtures. 2PG = 2-phosphoglyceric acid.

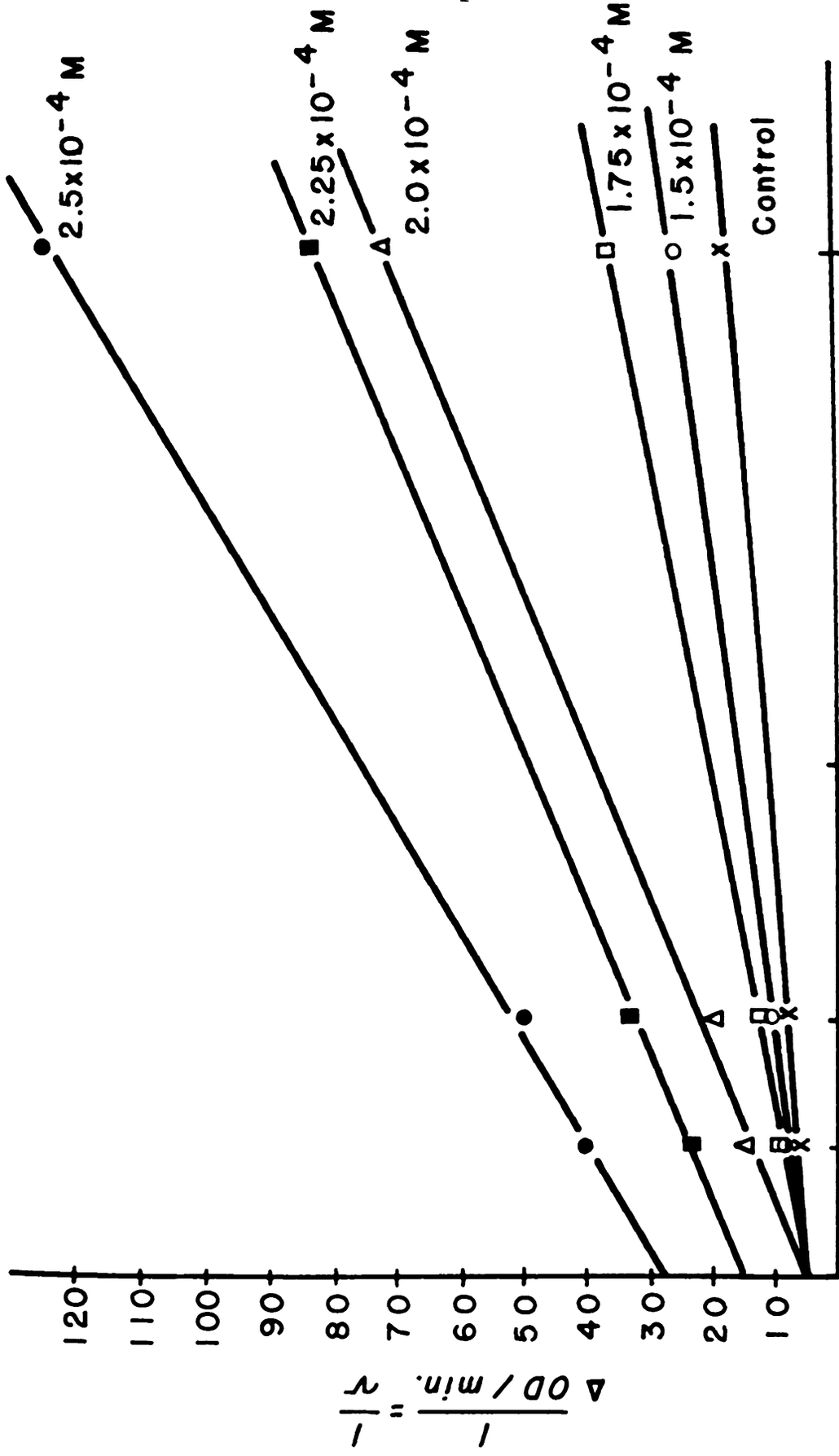



Figure 5. Lineweaver-Burk plot of enolase inhibition by sorbic acid at the concentrations indicated.

velocities versus inhibitor concentrations (Fig. 6) for the various substrate concentrations do not yield straight lines. This indicates that the inhibition is partially competitive and partially non-competitive (Dixon and Webb, 1958).

## Effect of Sorbic Acid on Enolase From Lactic Acid Bacteria

The effect of sorbic acid on fermentation by intact yeast cells was compared to its effect on fermentation by intact cells of L. plantarum. The conventional Warburg technique was used with glucose as the substrate. At pH 6.2 the yeast was significantly inhibited by $6.0 \times 10^{-3}$ M sorbic acid, but L. plantarum was not affected by this concentration of sorbic acid (Fig. 7).

Since the lactic fermentation by intact cells of L. plantarum was not inhibited by sorbic acid, the question arose as to whether the sorbate was getting to the site of action. In order to eliminate the permeability barrier acetone dried cells were prepared as described in Materials and Methods. With glucose as substrate, the production of lactic acid was measured in the Warburg from a bicarbonate buffer pH 6.2. As can be seen from Fig. 8, sorbic acid at a concentration of $6.0 \times 10^{-3}$ M had no effect on the lactic acid production of the acetone dried preparation of L. plantarum.

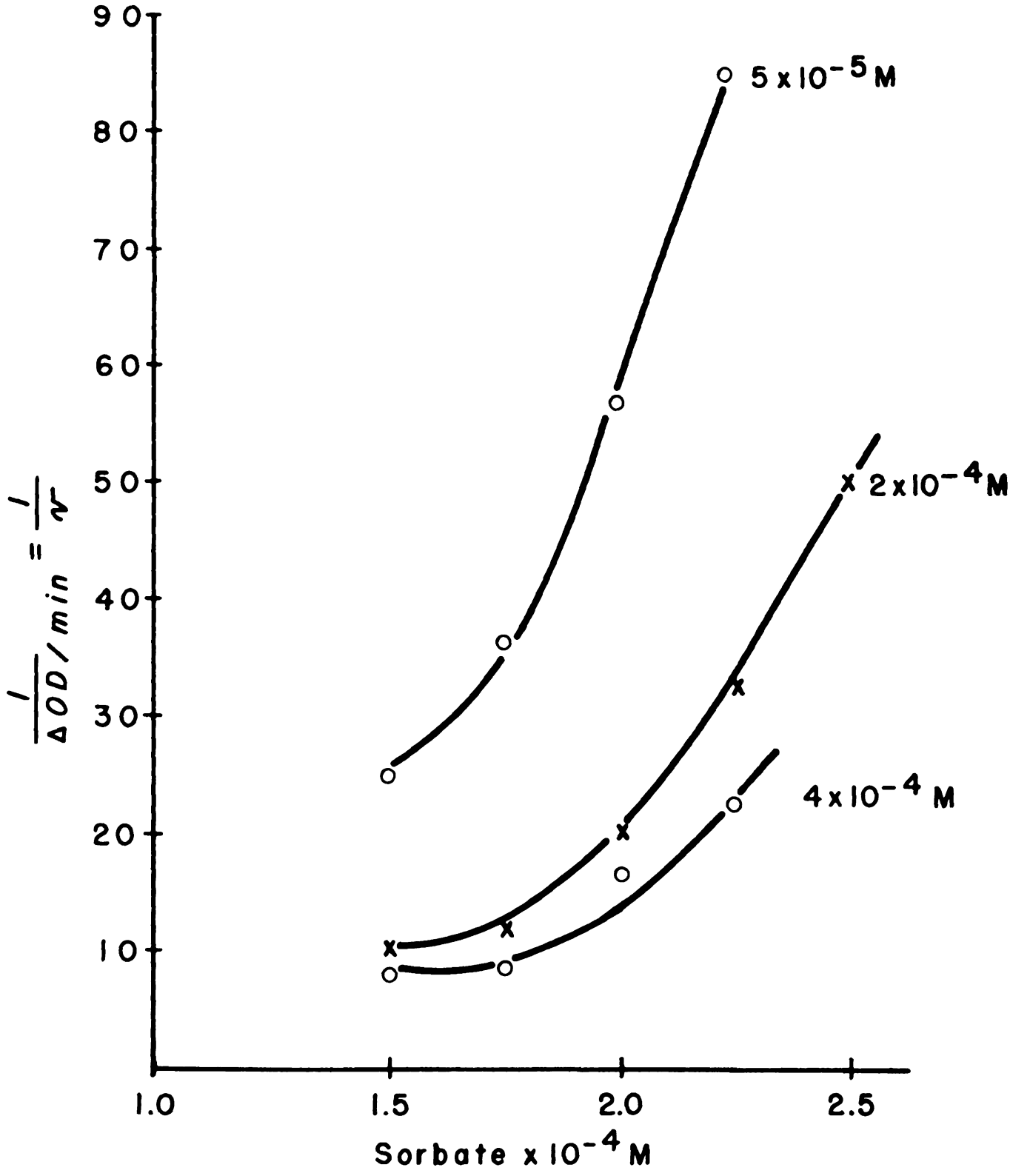



Figure 6. Plot of reciprocal velocities versus inhibitor concentrations with the substrate levels indicated.

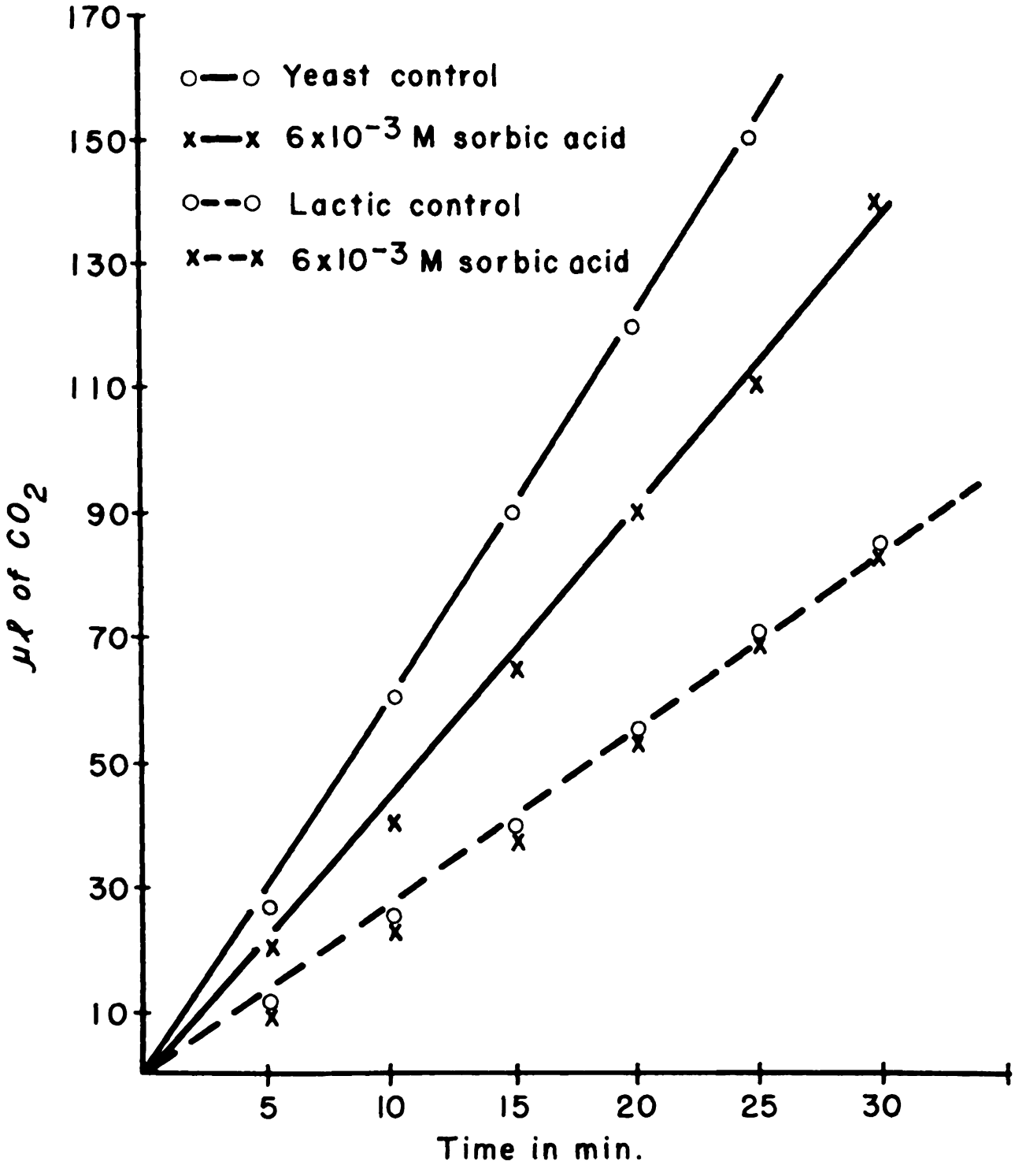



Figure 7. Effect of sorbic acid on glucose fermentation by intact cells of yeast and L. plantarum. The cups contained the following mixture: 0.5 ml of 0.1 M glucose, 0.2 ml of 0.033 M $NaHCO_3$, 20 mg of cells.

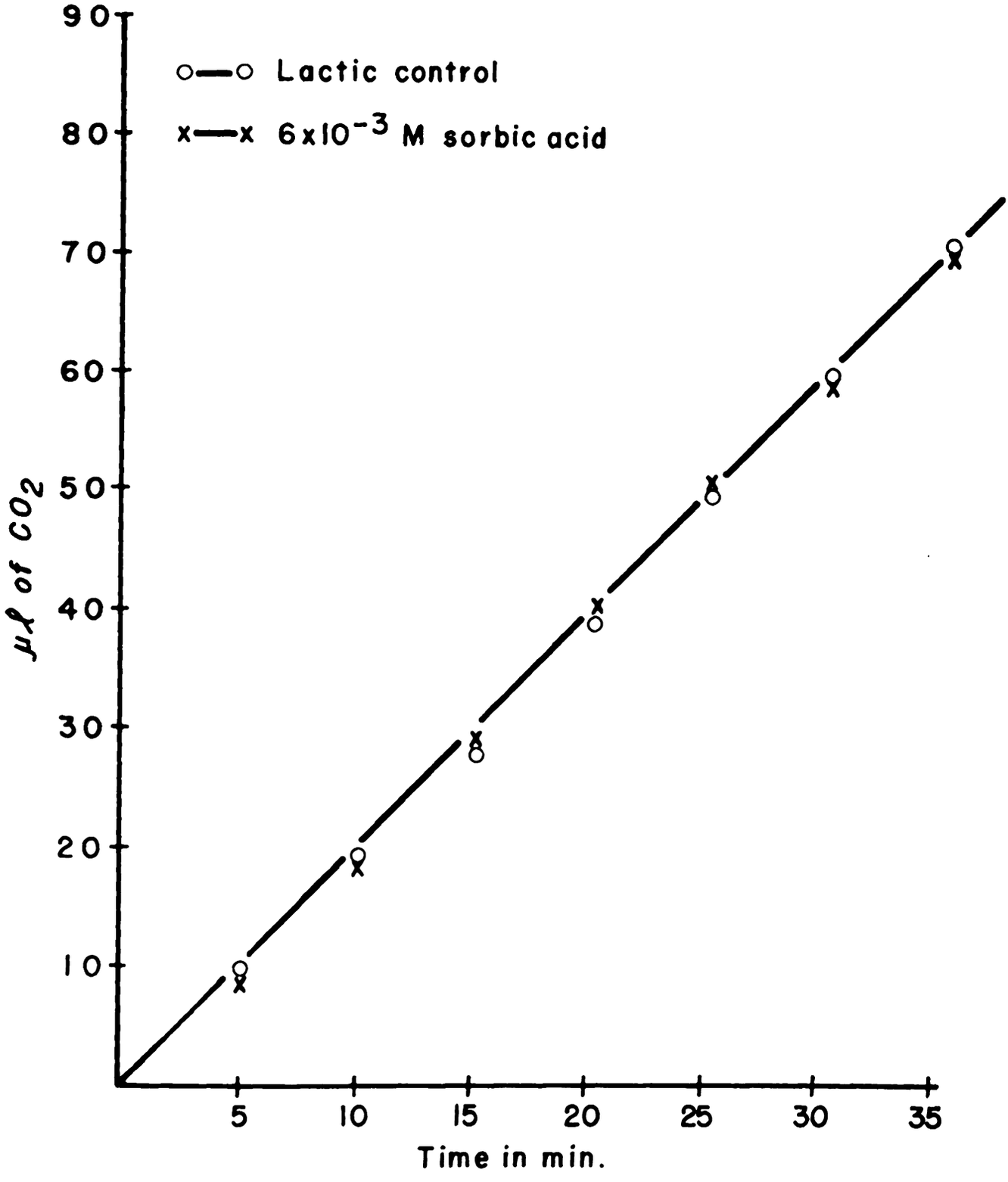



Figure 8. Effect of sorbic acid on glucose fermentation by acetone dried cells of L. plantarum. Reaction mixtures were the same as described in Fig. 7.

In order to completely remove the permeability barrier, cell-free extracts were prepared from L. plantarum, L. brevis, and P. cerevisiae as described in Materials and Methods. These cell-free extracts were assayed for enolase activity by several different methods. One of these methods consisted of coupling enolase to pyruvic kinase and lactic dehydrogenase and measuring DPNH oxidation at 340 mu with 2-phosphoglyceric acid as substrate. It is evident in Fig. 9 that sorbic acid concentrations of $2.5 \times 10^{-3}$ M, $5.0 \times 10^{-3}$ M, and $1.0 \times 10^{-2}$ M had no significant effect on DPNH oxidation by these extracts with 2-phosphoglyceric acid as substrate.

Enolase in the cell-free preparations was also assayed directly after much dilution of the extract. With 2-phosphoglyceric acid as substrate the appearance of product was followed at 240 mu. The yeast and the extracts of lactic acid bacteria were diluted to give the same protein concentration ($2.9 \times 10^{-2}$ mg) in the assay. As can be seen in Fig. 10, the activity in the yeast extract was inhibited while the bacterial extract was not affected by $1 \times 10^{-4}$ M sorbic acid. Due to the absorbancy of the extract and sorbate at this wavelength it was difficult to run the assay using higher sorbate levels. However, higher sorbate levels were tested by further dilution of the extracts. These assays were at the very limit of this procedure, but the results indicated some inhibition of the enolase in extracts of lactic acid bacteria at a concentration of $4 \times 10^{-4}$ M.

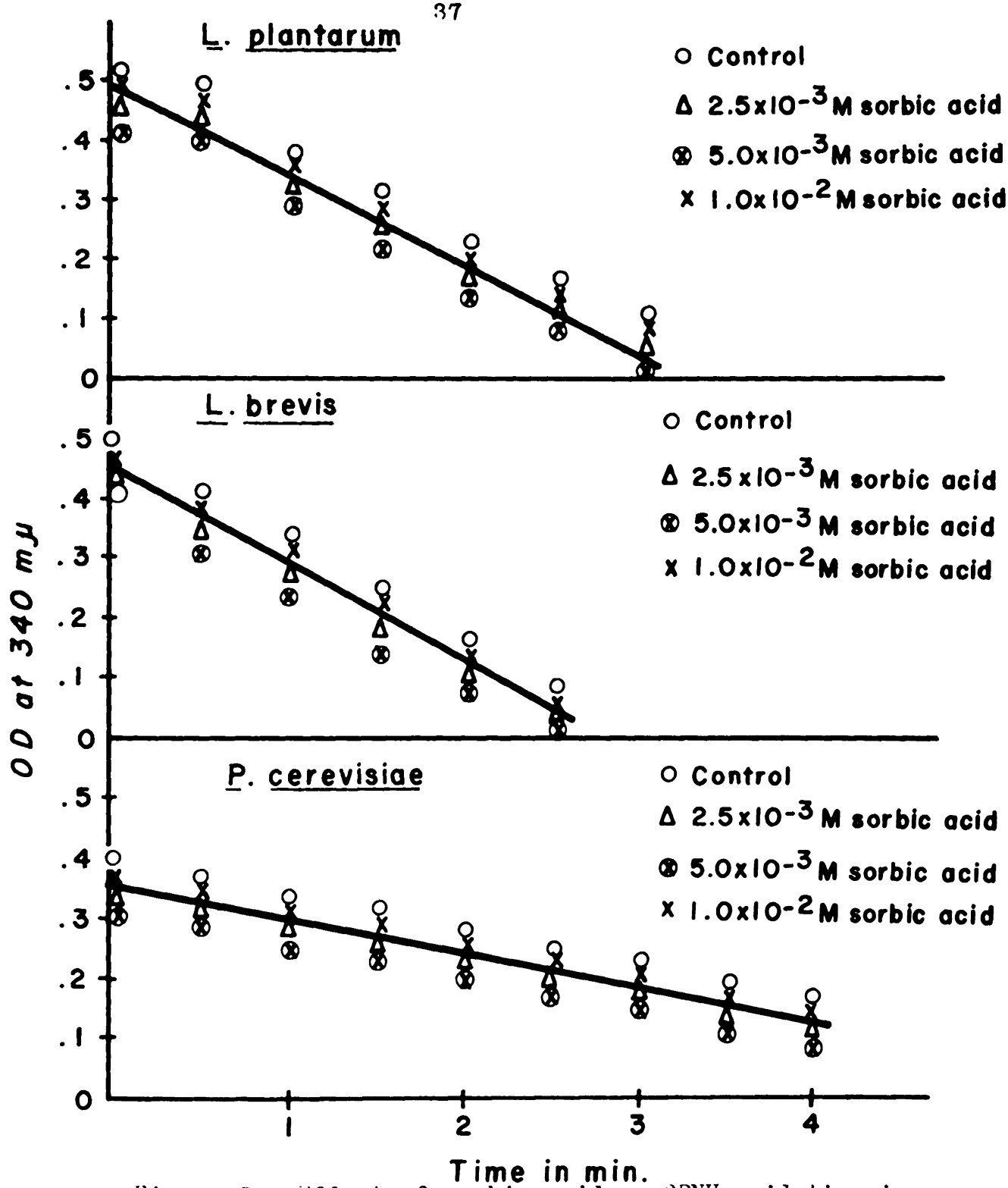



Figure 9. Effect of sorbic acid on DPNH oxidation in cell-free extracts of L. plantarum, L. brevis, and P. cerevisiae with 2-phosphoglyceric acid as substrate. The reaction mixture contained the following: DPNH 2 x $10^{-4}$ M, ADP 3 x $10^{-4}$ M, $MgSO_4$ 8 x $10^{-3}$ M, 2-phosphoglyceric acid 6 x $10^{-4}$ M, extract 0.5 ml to a final volume of 3 ml.

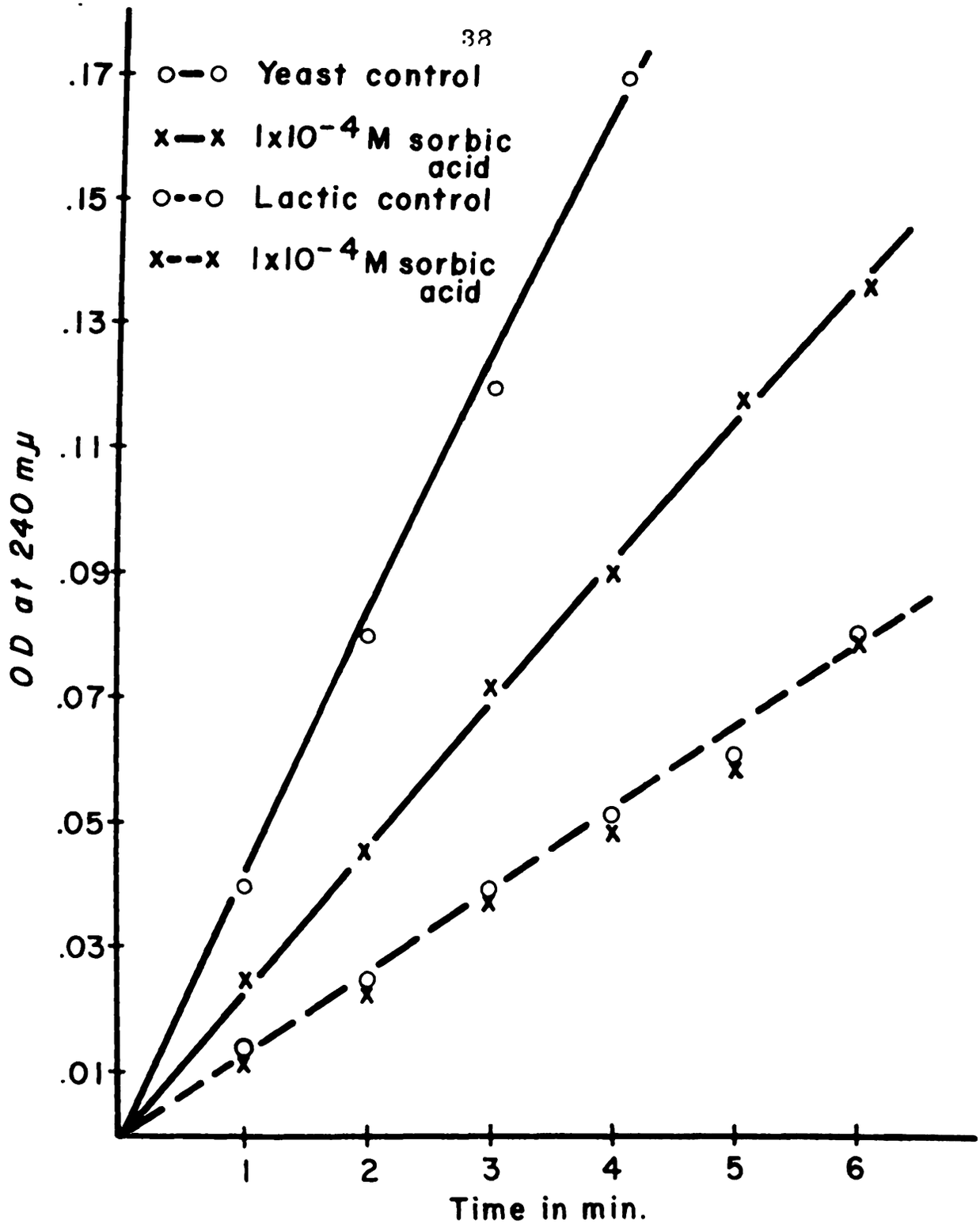



Figure 10. Effect of sorbic acid on enolase activity of yeast and L. plantarum extracts measured at 240 mμ. Reaction mixture contained: 2 x $10^{-4}$ M 2-phosphoglyceric acid, 5 x $10^{-2}$ M imidazole buffer pH 7.4, 8 x $10^{-3}$ M $MgSO_4$.

To obtain a more reliable measure of the effect by high sorbate concentrations on the enolase activity in the cell-free extracts, they were assayed by determination of the acid labile phosphate produced on conversion of 2-phosphoglyceric acid to 2-phosphoenolpyruvate. A concentration of $5 \times 10^{-3}$ M sorbic acid inhibited the activity of the enolase in the crude extract of yeast by over 50%, but failed to affect the reaction rate observed with the extract of L. plantarum cells (Table 3).

TABLE 3

THE EFFECT OF SORBIC ACID ON ENOLASE ACTIVITY OF YEAST AND L. PLANTARUM CELL-FREE EXTRACTS AS MEASURED BY ACID LABILE PHOSPHATE

| Preparation[a] | Acid Labile P OD at 660 mu | Minus endogenous |
|---|---|---|
| Yeast extract endogenous | .035 | -- |
| Yeast extract control | .080 | .045 |
| Yeast extract + sorbic ($5 \times 10^{-3}$ M) | .055 | .020 |
| Lactic extract endogenous | .040 | -- |
| Lactic extract control | .085 | .045 |
| Lactic extract + sorbic ($5 \times 10^{-3}$ M) | .085 | .045 |

[a]The reaction mixture contained the following: extract 1.0 ml, $8 \times 10^{-3}$ M $MgSO_4$, $5 \times 10^{-2}$ M imidazole buffer (pH 7.4), $1 \times 10^{-3}$ M substrate 2-phosphoglyceric acid, and water to a total volume of 3.0 ml. The reaction was started by the addition of substrate and stopped at the end of 3 min by the addition of 3 ml of 10% trichloroacetic acid. After hydrolysis of the acid labile phosphate the total phosphate was measured by the procedure of Fiske and SubbaRow (1925) modified as described in Materials and Methods. The color formed was measured at 660 mu.

The enolase from L. plantarum was partially purified by organic solvent fractionation and passage through a phosphorylated cellulose column, and then tested for sensitivity to sorbic acid. Fig. 11 shows that it was inhibited by $1.0 \times 10^{-4}$ M sorbic acid, $7.0 \times 10^{-4}$ M crotonic acid and $3.0 \times 10^{-4}$ M cinnamic acid. The order of effectiveness of these acids was the same as observed with the yeast enolase; sorbic being the most effective followed by cinnamic and then crotonic. These data indicate the presence of a factor(s) in extracts of L. plantarum which protect the enolase from inhibition by alpha, beta-unsaturated acids. Thus, a search was initiated for such factors.

## Protection of Enolase in Extracts of Lactic Acid Bacteria from Sorbic Acid Inhibition

Sorbic acid has been reported to combine with free sulfhydryl (SH) groups (Morgan and Friedmann, 1938). This suggested that an abundance of free SH groups might be responsible for the protection of the lactic extract. Therefore, the free SH levels in extracts from both yeast and L. plantarum cells were analyzed for free SH by use of the p-chloromercuribenzoate method. The results indicated that the yeast extract contained 1.2 microequivalents per mg of protein, and the Lactobacillus extract contained 1.1

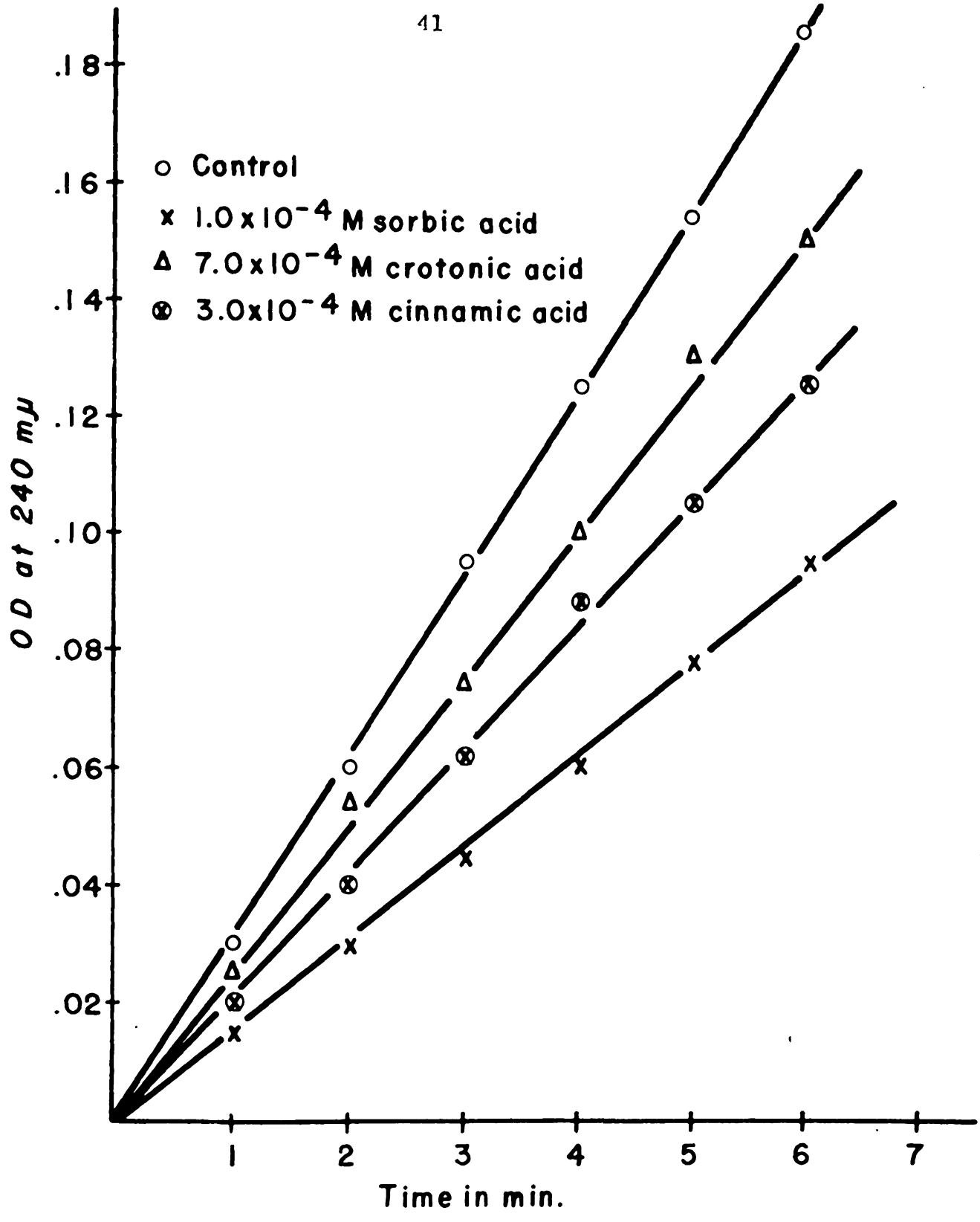



Figure 11. The effect of sorbic, crotonic, and cinnamic acids on activity of enolase partially purified from extracts of L. plantarum. Reaction mixture as in Fig. 10.

microequivalents per mg of protein. While these values are believed to be unrealistically high due to impurities in the glutathione standard, they are satisfactory for comparative purposes. Certainly, the extract from the lactic acid bacterium did not contain high levels of free SH as compared to the yeast extract. The addition of either cysteine or crystalline alcohol dehydrogenase did not protect yeast enolase from sorbic acid inhibition; in fact high levels of cysteine were inhibitory. Therefore, the protection of the enolase in the extract of the lactic acid bacteria from sorbate did not seem to be due to an unusually high concentration of free SH groups.

Attempts were made to demonstrate protection of purified yeast enolase by addition of crude extracts from cells of L. plantarum but without success. When the crude extract of L. plantarum was added to pure yeast enolase in the presence of sorbic acid it was found to be additive to the inhibition of sorbic acid, and the bacterial extract by itself was inhibitory to the yeast enolase. This is probably due to free SH groups since iodoacetate reduced the inhibition. The protection of the enolase is not due to nonspecific protein since the addition of albumin had no effect.

Next an attempt was made to find a protective factor by adding back various fractions from a $Mg^{++}$ activated phosphorylated cellulose column to a fraction containing sorbic acid sensitive enolase. The enolase in the bacterial

extracts was separated from its protective factors by a direct passage of the crude extract through the phosphorylated cellulose column. Then the other fractions were checked for protective activity by addition back to the unprotected enolase. No protection could be demonstrated by any of the fractions from the column. Either the material(s) responsible for the protection did not come through the column, were too dilute in the eluates, or the process is irreversible.

The above work led to a different approach to the fractionation of the extracts. The crude extract was subjected to starch block electrophoresis, various eluates tested for enolase activity, and active fractions tested for sensitivity to sorbic acid. This technique yielded both sorbic acid sensitive and protected enzyme fractions (Table 4).

Since the lactic acid bacteria in general are relatively insensitive to sorbic acid and since high lactic dehydrogenase activities are common to this group of organisms, the possibility of this enzyme protecting the enolase system was considered. This experiment offered some support of this hypothesis. Thus, the fraction in tube 9 (Table 4) was protected from inhibition and also contained the largest amount of lactic dehydrogenase activity. Also, the addition of the contents of tube 9 to the contents of tube 8 resulted

TABLE 4

SEPARATION OF ENOLASE IN L. PLANTARUM EXTRACTS FROM THE PROTECTIVE FACTOR(S) BY STARCH BLOCK ELECTROPHORESIS

| Tube Number[a] | Enolase activity[b] $\Delta$ $OD_{240}$ | Lactic dehydrogenase activity[c] $\Delta$ $OD_{340}$ | Inhibition by $1.0 \times 10^{-4}$ M sorbic |
|---|---|---|---|
| 1 | --- | --- | --- |
| 2 | --- | --- | --- |
| 3 | --- | --- | --- |
| 4 | --- | --- | --- |
| 5 | .005/min | --- | yes |
| 6 | .030/min | .050/min | yes |
| 7 | .030/min | .060/min | yes |
| 8 | .020/min | .100/min | yes |
| 9 | .020/min | .250/min | no |
| 10 | --- | .080/min | --- |
| 11 | --- | .030/min | --- |
| 12 | --- | --- | --- |
| 13 | --- | --- | --- |
| 14 | --- | --- | --- |

[a]The tubes contained $5 \times 10^{-2}$ M imidazole buffer pH 7.4, and the protein in that particular fraction.

[b]Enolase activity was assayed by taking 0.5 ml of each fraction, adding $8 \times 10^{-3}$ M $MgSO_4$, 2-phosphoglyceric acid as substrate and measuring the increase in OD at 240 mu.

[c]Lactic dehydrogenase was assayed with pyruvate as substrate, and measuring DPNH oxidation at 340 mu.

in the protection of the enolase activity in tube 8 from innibition by sorbic acid.

Fractions of crude extracts of L. plantarum cells in which enolase was inhibited by sorbic acid and other fractions in which no inhibition was evident were also obtained by fractionation with ammonium sulfate (Table 5). The fractions with the highest levels of lactic dehydrogenase were those in which the enolase was protected from sorbic acid inhibition. However, the specific activity of enolase was quite low in the protected fractions.

Finally the effect of purified lactic dehydrogenase (obtained from General Biochemicals Corporation, Chagrin Falls, Ohio, from muscle) was tested on the inhibition of enolase from L. plantarum by sorbic. The 70% ammonium sulfate fraction was used as a source of enolase. As can be seen in Fig. 12 the enolase in this fraction was inhibited by $1.0 \times 10^{-4}$ M sorbic acid, but the addition of purified lactic dehydrogenase protected it from inhibition.

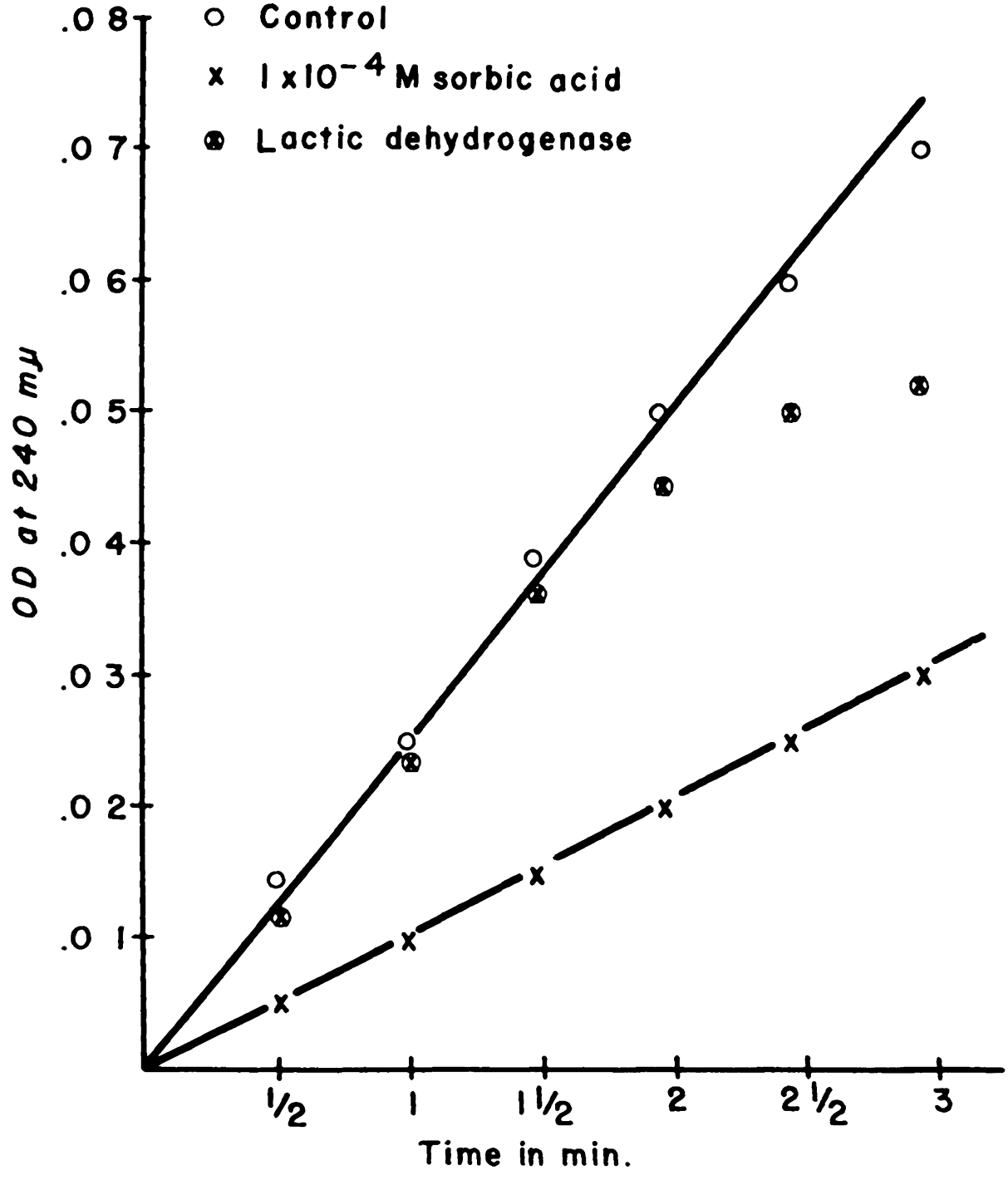



Figure 12. The effect of pure lactic dehydrogenase on the inhibition of enolase from L. plantarum by sorbic acid. Reaction mixture as in Fig. 10.

TABLE 5

SEPARATION OF ENOLASE IN L. PLANTARUM EXTRACTS FROM THE PROTECTIVE FACTOR(S) BY AMMONIUM SULFATE FRACTIONATION

| % $(NH_4)_2SO_4$ saturation[a] | Enolase activity[b] △ OD/min | Lactic dehydrogenase[b] △ OD/min | Specific activity of enolase △ OD/min/mg | Protection |
|---|---|---|---|---|
| 10 | --- | --- | --- | --- |
| 20 | --- | --- | --- | --- |
| 30 | --- | .050 | --- | --- |
| 40 | .015 | .100 | .013 | yes |
| 50 | .015 | .150 | .013 | yes |
| 55 | .015 | .200 | .008 | yes |
| 60 | .100 | .060 | .046 | no |
| 65 | .150 | .050 | .150 | no |
| 70 | .030 | --- | .025 | no |
| 80 | --- | --- | --- | --- |

[a]The precipitate obtained at each point of saturation was redissolved in distilled water and the various assays performed on this protein solution.

[b]See Table 4 for procedures.

## DISCUSSION

An initial decrease in optical density occurs when either sorbic acid or phosphoenolpyruvate is added to a solution containing enolase and $Mg^{++}$. When sorbic acid is added, this expresses itself as a lag in enzyme activity by masking the optical density increase due to enzymatic action. This drop is probably due to the formation of a complex that does not absorb at this wavelength and similar complexes are probably formed with alpha, beta-unsaturated aliphatic acids in general. Also, complexes are probably formed with amino acids, magnesium, and sorbic acid. Magnesium is known to be important as an activator of enolase and Malmstrom, Kimmel, and Smith (1959) concluded that the activation process results from a combination of the ion with the enzyme and not the substrate. Therefore, the substrate combines with a metal-enolase chelate. Smith (1949) proposed that the mechanism of enzyme action is basically the same irrespective of the enzyme. He assumed that the enzyme-substrate combination was of a chelate character and that the lowering of the free energy of activation is due in each instance to electronic deformation. In instances where no metal is needed, he claimed that the protein itself performed the same function as the metal supplying the necessary electronegative and electropositive groups. Since these results

indicate that alpha, beta-unsaturated acids in general react or combine with enolase in the presence of $Mg^{++}$, it is proposed that this may be the basis of sorbic acid inhibition.

The actual type of enolase inhibition indicated by the Lineweaver-Burk plot was different from any of the individual types of inhibitions summarized in Dixon and Webb (1958). This plot indicated that the enolase inhibition was competitive at sorbic acid concentrations up to $2.0 \times 10^{-4}$ M and then changed to non-competitive at higher sorbate levels. However, a plot of reciprocal velocities versus inhibitor concentrations yielded curves whose slopes increased with increasing inhibitor levels. This, according to Dixon and Webb (1958) indicates that the inhibition is partially competitive and partially non-competitive. This system is completely indistinguishable from the fully competitive or the fully non-competitive merely by varying the substrate concentration at fixed inhibitor concentrations, as in the Lineweaver-Burk method. It is not possible in this case to determine the equilibrium constant ($K_i$) for the enzyme-inhibitor reaction by simple graphical methods. At lower levels, the inhibition may be partially competitive with the sorbic combining with a site(s) adjacent to the active site on the enzyme thereby reducing the affinity of the enzyme for its substrate; while at higher levels the number of sorbic acid molecules combining with each molecule of enzyme may be greater, resulting in a partially non-competitive

inhibition. Crotonic and cinnamic acids probably inhibit enolase in a similar way.

Comparing the pathways of anaerobic glucose metabolism in yeast and a homofermentative lactic acid bacterium such as L. plantarum one wonders why sorbic acid does not inhibit the lactic organism. They both contain an enolase that performs the same catalytic function. As was demonstrated in the results with intact cells at pH 6.2, the glucose fermentation by yeast is significantly inhibited while that by the lactic acid organisms is not affected by sorbic acid. This difference cannot be explained on the basis of cell permeability, since fermentations by acetone dried preparations of L. plantarum were not inhibited. Also, it was demonstrated with cell-free extracts of L. plantarum, L. brevis, and P. cerevisiae that sorbic acid even to the concentration of $1.0 \times 10^{-2}$ M had no significant effect. This was demonstrated by three different methods of assaying for enolase activity in the crude extracts.

Now the question arises as to whether the enolase in lactic acid bacteria is so different that it is not affected by sorbic acid, or whether there is some material in the bacterial extract that protects it from inhibition by sorbic acid. In order to differentiate between these two possibilities enolase from lactic acid bacteria was partially purified. It was found that this partially purified enolase was inhibited by sorbic acid with concentrations of the same

magnitude as the pure yeast enolase. This seems to exclude the possibility that the enolase is so different that this is the reason it is not affected by sorbate and leaves the other alternative of the presence of a protective material in the extract.

What high molecular weight material is present in the cells of the lactic organisms that is not present in yeast to the same extent? It is very attractive and logical to hypothesize lactic dehydrogenase since the lactic acid bacteria in general have high lactic dehydrogenase activities, and are relatively insensitive to sorbic acid. The data presented seem to bear this out although they are not conclusive. By partially separating the lactic dehydrogenase from enolase by electrophoresis and ammonium sulfate fractionation, fractions were found having different ratios of enolase and lactic dehydrogenase activity. The enolase was relatively insensitive to sorbic acid in the fractions with the highest levels of dehydrogenase. Also, lactic dehydrogenase was the only purified protein of three tested that was found to partially protect enolase from sorbic inhibition.

Another possible explanation of the data referred to above is based on enzyme multiplicity. It is possible to have two enolases in a cell; one that is inhibited by sorbate and one that is not. However, in this case one should not get protection from a fraction not containing enolase activity when added to the enolase that is sensitive to

sorbate. On the other hand the possibility exists that in the intact cells of lactic acid bacteria these two mechanisms may be acting in conjunction with each other.

The basic question still remains as to whether information derived from in vitro experiments can be projected precisely into its exact place in the complex mechanics of the living cell. It must be remembered that in the preparation of cell extracts, there is a distortion of the ratios of the various cellular components. The intact cell is a compact, closely knit, organized system whereas the extract is a conglomeration of different agents. Thus, in the homogenate some reactions which normally occur at a certain velocity may decrease or even disappear while others which ordinarily would not predominate due to the inherent composition of the intact cell will now come to the fore. One should not lose sight of the ultimate goal of all in vitro studies: to fit the facts into their plausible place of action within the living cell. However, sometimes nature in her various ways makes it difficult for the experimenter to attain this goal.

## SUMMARY

Studies with pure yeast enolase demonstrated that this enzyme was inhibited by sorbic acid. Other alpha, beta-unsaturated acids (crotonic and cinnamic) were also found to be inhibitory to enolase. A loss in the optical density of alpha, beta-unsaturated fatty acids was observed in the presence of $Mg^{++}$ and enolase which is believed to be due to the formation of a complex.

When the reciprocal of the velocity of enolase activity was plotted against the reciprocal of the substrate concentration, the inhibition appeared to be competitive at the lower levels of sorbate and non-competitive at the higher levels. However, the plot of the reciprocal of the velocity versus the inhibitor concentration indicated that sorbate inhibition of enolase is partially competitive and partially non-competitive.

The comparison of intact cells of yeast and lactic acid bacteria showed that the same concentration of sorbate inhibited glucose fermentation by yeast but had no effect on that by lactic acid bacteria. Also, there was no effect of sorbate on fermentation by acetone dried preparation of L. plantarum.

The enolases in cell-free extracts of L. plantarum, L. brevis, and P. cerevisiae were not affected by sorbic

acid concentrations that inhibited yeast extracts. However, on partial purification of these enolases, they became extremely susceptible to sorbic acid inhibition. Fractions containing enolase which was inhibited by sorbic acid and others with enolase which was not inhibited could be separated from the crude extracts of cells of lactic acid bacteria by starch block electrophoresis or ammonium sulfate fractionation. Lactic dehydrogenase activity was consistently high in the protected fractions. The addition of a protected fraction to an unprotected one resulted in the protection of enolase activity from sorbate inhibition. The addition of pure lactic dehydrogenase to an unprotected fraction demonstrated protection. Conversely the addition of albumin or alcohol dehydrogenase to enolase preparations was ineffective. It is, therefore, postulated that the reason for the lack of inhibition of lactic acid fermentation by sorbic acid is due to the protection of the enolase by lactic dehydrogenase.

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