THE PRIMARY SITE OF INHIBITION OF YEAST RESPIRATION BY SORBIC ACID

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ABSTRACT

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by Theodore E. Anderson

Sorbic acid selectively inhibits the growth of catalase positive microorganisms. Preliminary studies in this laboratory suggested that the inhibition of metabolic pathways unique to catalase positive microorganisms was responsible for this selective inhibition. The present research was conducted to locate the primary inhibitory site of respiration of catalase positive microorganisms by sorbic acid.

Baker's yeast was chosen as a typical sorbic acid-sensitive microorganism. Initial studies indicated that a variety of cyanide- and azide-sensitive substrate oxidations by intact yeast were markedly inhibited by sorbic acid. The rates of oxidation of pyruvate, acetate, ethanol, acetaldehyde, reduced diphosphopyridine nucleotide (DPNH), reduced triphosphopyridine nucleotide (TPNH), ascorbate and lactate were sharply lowered.

Several possible theories were investigated. First, sorbate could effect a general inhibition of substrate permeation; but this theory appears unlikely as sorbate appreciably lowered endogenous O_2 uptake, and anaerobic pyruvate decarboxylation was not inhibited. Second, the cell semipermeable membrane might be disorganized; but sorbate does not possess the surface active characteristics usually associated with inhibitors having this mode of action, nor did it lower endogenous CO_2 evolution anaerobically. Third, the electron transport system (ETS) or associated oxidative phosphorylation could be influenced by sorbate. The latter possibility is unlikely since there was no change in the phosphate/oxygen ratio when sorbic acid was used to inhibit respiration of liver mitochondria, nor was the sorbate inhibition of respiration by liver mitochondria or of endogenous respiration of intact yeast reversed by 2,4-dinitrophenol. Inhibition of ETS was indicated by the observation that about 3×10^{-2} M sorbate inhibited O_2 uptake at least 50% by liver mitochondria with pyruvate, β-hydroxybutyrate, or succinate as substrate. Such a high level of sorbate would be expected at the active site since intracellular sorbate accumulation occurs (Oka, Bull. Agr. Chem. Soc. 24:59, 1960). Inhibition of succinate oxidation by liver mitochondria was

demonstrated only when fumarase was blocked with 1 X 10^{-1} M KI. In the absence of KI, succinate oxidation was stimulated and the sorbate level decreased during the reaction. Similar results were obtained with α-ketoglutarate as substrate. These findings suggest that sorbate is metabolized by liver mitochondria when sufficient tricarboxylic acid cycle activity occurs.

Sorbate did not inhibit substrate oxidation by either acetone-dried or dry ice-treated yeast cells nor by crude yeast mitochondria. In fact, DPNH oxidation by the extracts was stimulated by sorbate, although oxygen uptake was not significantly affected. Possible explanations for such results are that (a) the treatments result in an activation of an alternate electron transport system or in an alteration of the system used, (b) that only cell membrane electron transport in yeast cells is inhibited, or (c) that a material(s) is released during treatment which neutralizes the inhibitory action of sorbate.

It is concluded that the cytochrome c oxidase system is the primary site of inhibition of respiration since with liver mitochondria this system was markedly inhibited while the DPNH-methylene blue reductase system of intact yeast and the DPNH-cytochrome c reductase activities of liver and

yeast mitochondrial preparations were sorbate-insensitive. Caproate, the saturated analogue of sorbate, demonstrated negligible inhibition at comparable concentrations in these studies. Cytochrome c oxidase inhibition can explain the generally observed selective inhibition of catalase positive organisms by sorbic acid.

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By

Theodore E. Anderson

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INTRODUCTION

Sorbic acid, 2,4-hexadienoic acid, has found use in the food and pharmaceutical industries as an antimicrobial agent. This compound and its salts are the most widely used of a series of alpha, beta-unsaturated aliphatic monocarboxylic acids and their salts which were patented by C. M. Gooding (1945) as mold inhibitors. Sorbic acid has found widespread acceptance in the food industry because it possesses such attributes as chemical stability and high effectiveness; yet it is relatively tasteless, odorless and harmless as a dietary component.

While a considerable amount of data has been accumulated on highly utilitarian aspects of microbial inhibition by organic acids, much less information is available on the mechanism(s) of inhibition, especially in the alpha, beta-unsaturated aliphatic monocarboxylic acid series of compounds.

Hsu (1957), in his preliminary studies of the effect of sorbic acid on yeast metabolism, observed that the inhibition of respiration was approximately the same with both glucose and pyruvate as substrates. He conjectured, therefore, that the site of inhibition was identical for both

substrates. The present study was initiated in an attempt to find this site of inhibition.

REVIEW OF LITERATURE

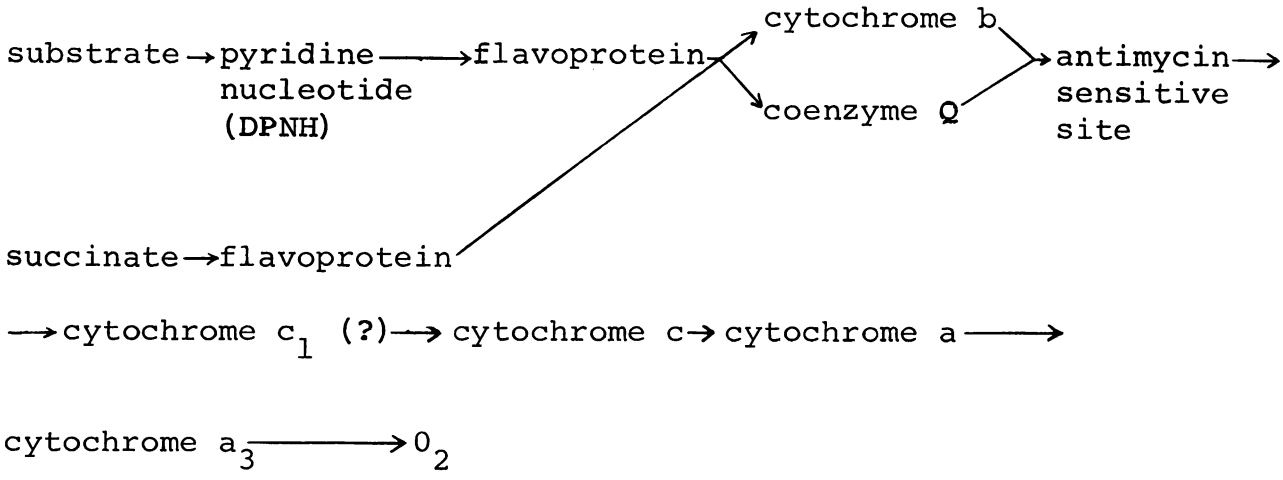
The object of this study was to find the primary site(s) of inhibition of yeast respiration by sorbic acid. Such a study requires discussions of metabolic pathways, permeability and mechanisms of inhibition by several classes of inhibitors. Therefore, a review of the pertinent aspects of these subjects is presented.

Yeast respiration. Saccharomyces cerevisiae (baker's yeast) was chosen as a typical sorbic acid-sensitive microorganism in the present study. Yeast employ primarily the Embden-Meyerhof pathway in the first stages of glucose breakdown. Like the filamentous fungi (molds) against which sorbic acid is usually directed, S. cerevisiae utilizes the Tricarboxylic Acid Cycle (De Moss and Swim, 1957) and a "classic" cytochrome system (Cochran, 1958) in terminal oxidation of substrates. To link the Embden-Meyerhof pathway to the Tricarboxylic Acid Cycle, and other pathways, acetyl-coenzyme A (acetyl-CoA) must be formed. Two pathways exist in yeast to form acetyl-CoA from pyruvate. The first is the enzyme complex pyruvate oxidase which is located in mitochondria only and which has the same cofactor requirements as animal and bacterial pyruvate oxidases. The second is an enzyme system occurring

only in the soluble fraction of yeast and which consists of carboxylase, acetaldehyde dehydrogenase and aceto-CoA-kinase (Holzer and Goedde, 1957). Reduced diphosphopyridine nucleotide (DPNH) is generated during the operation of these pathways and is reoxidized via the electron transport system.

An alternate pathway of catabolism employed by baker's yeast is the oxidative pentose phosphate cycle (Entner and Doudoroff, 1952; Gibbs and De Moss, 1954; Korkes, 1956; and Wood, 1955). Blumenthal et al. (1954) determined that this pathway is utilized aerobically to the extent of 0 to 30%. Reduced triphosphopyridine nucleotide (TPNH) is generated during the operation of the cycle. Three enzymes catalyzing TPNH oxidation in animal tissue are known: (1) one catalyzing the reduction of oxidized cytochrome c (Horecker, 1950), (2) one catalyzing TPNH reaction with oxidized glutathione (Rall and Lehninger, 1952), and (3) pyridine nucleotide transhydrogenase which reduces oxidized diphosphopyridine nucleotide (DPN). The last enzyme, which is located in the mitochondria, appears to be quantitatively the most important in mitochondrial respiration (Slater, 1958).

The probable scheme of the electron transport system in S. cerevisiae is suggested by Crane and Glenn (1957):



Green (1959) points out that the electron transport system may be much more complex than that outlined above, but that the sequence from cytochrome c to O_2 seems irrefutable.

Yeast lactic dehydrogenase has been well established as being flavin linked (Bach et al., 1946; Boeri and Tosi, 1956; Nygaard, 1960). Antimycin A does not inhibit the enzyme and so it appears that the "antimycin sensitive site" is bypassed and that the enzyme reduces cytochrome c directly. Ascorbic acid and p-phenylenediamine also rapidly reduce cytochrome c directly. Heart muscle preparations do not possess a pathway for the oxidation of p-phenylenediamine additional to the cytochrome c oxidase system (Slater, 1949).

Cytochrome c peroxidase catalyzes the reduction of H_2O_2 by accepting electrons from cytochrome c. Yeast respiration is inhibited by CO which inhibits cytochrome a_3, in the same manner that cytochrome c oxidase is antagonized. CO does not inhibit cytochrome c peroxidase so nearly all

of the O_2 uptake in yeast appears to be via cytochrome c oxidase (Smith, 1954a).

"Typical" cytochrome c oxidases (cytochrome a_3) are inhibited by cyanide, azide and CO (Chance and Williams, 1956; Smith, 1954b); and yeast contains a cytochrome of the classic a_3 type (Smith, 1954b). Therefore, cyanide and azide inhibit yeast respiration at the cytochrome a_3 level. Catalase is also inhibited by cyanide (Dolin, 1961a).

Intact yeast probably carry out oxidative phosphorylation at the same sites as do isolated rat liver mitochondria (Chance and Williams, 1956). The sites of oxidative phosphorylation have been definitely established as existing between pyridine nucleotide and flavoprotein, cytochrome b and cytochrome c_1, and cytochrome c and cytochrome a (Chance, 1959). However, it has not yet been possible to obtain P/O ratios greater than one with cell-free yeast preparations (Utter et al., 1958). The P/O ratio is the number of atoms of inorganic phosphorus incorporated into organic phosphate, primarily adenosine triphosphate, per atom of oxygen consumed.

In the presence of the appropriate catalyst, electron flow in the electron transport system will take place from the system of lower potential (more negative) to the system of higher potential. Methylene blue has an E'_o (volts at pH 7)

of 0.011 and would therefore be expected to accept electrons from cytochrome b and flavins which have lower potentials, but not from cytochromes c, a or a_3 which have higher potentials (Dolin, 1961a). Methylene blue has been shown to act as an oxidant for an enzyme having flavin adenine dinucleotide (FAD) as a prosthetic group (Savage, 1957) and from cytochrome b (Singer and Kearney, 1957).

Endogenous respiration by yeast is not a completely defined phenomenon, but appears to be nonidentical to metabolism resulting from exogenous substrate. Kotyk (1961) conjectures that endogenous metabolism "appears to be qualitatively different from that proceeding from glucose and produces high energy sources at the expense of other energy sources (physicochemical state of proteins, nucleic acids ?) than those involved in oxidative phosphorylation known heretofore." This may be reflected in the observation by Potter and Reif (1952), that although antimycin A completely prevented the oxidation of α-ketoglutarate, fumarate, malate, pyruvate, citrate and cis-aconitate, the endogenous oxidative rate in rat liver mitochondria appeared to be largely unaffected.

Cell Permeability. Advances in two areas of permeability research are of interest in the present discussion. The first is the recent advances in the knowledge

of the compartmentalization of mammalian cells, e.g., mitochondria are now known to have two permeability barriers. Secondly, it is no longer believed that natural membranes are relatively inert lipoprotein films which act mainly as osmotic barriers through which a chemical may or may not diffuse according to its charge, size and lipid solubility. Rather it is now thought that such membranes contain enzymes and carriers, and act as highly specific links through which chemical and osmotic contact is regulated and maintained by movement of substrates between the phases on either side (Mitchell, 1958).

The passive permeability characteristics of the plasma membranes of microorganisms and most mammalian cells do not differ fundamentally. Low molecular weight solutes generally permeate either type cell with difficulty if they carry more than four water molecules. Positively charged solutes permeate somewhat more readily than negatively charged ones. In general, lipid solubility is the property which allows a chemical to permeate membranes of bacteria and mammalian cells alike (Mitchell, 1958). Although early workers have shown a correlation between the rate of absorption of organic solutes and their lipid solubility, a number of cases have been found where the rate of absorption is much greater than would be expected on this basis. In

these cases it has been assumed that additional energy has to be supplied to the system and this rapid uptake has been termed "active" uptake. Transport against a concentration gradient is frequently termed active uptake (Taylor, 1960).

The bacterial cell wall also plays a role in permeability in that it functions as a molecular sieve, preventing hydrophilic solutes of molecular weight 10,000 or above from leaving the protoplast or reaching its surface from outside the cell wall (Mitchell, 1959).

Very little is known of mitochondrial membranes, but Tedeschi and Harris (1955) report that one or both of the membranes behave similarly to bacterial and mammalian plasma membranes. In agreement with the observations of Tedeschi and Harris, it appears that streptomycin-sensitive systems in mitochondria are protected from the antibiotic by the mitochondrial membrane; streptomycin is a highly polar and water soluble compound of very low lipid solubility (Umbreit and Tonhazy, 1949; Umbreit, 1955).

Yeast are quite impermeable to hydronium ions as noted by Eddy (1958) and therefore tend to maintain their intracellular pH constant in spite of extracellular variations in pH. The over-all intracellular pH of resting baker's yeast is 5.8 ± 0.02, while after prolonged oxygenation (8 to 48 hours) the pH is about 6.0 (Conway and Downey, 1950).

Microbial inhibition by alteration of the permeability barrier. Numerous compounds have been implicated as inhibitors of microbial growth through disorganization of the cell permeability barrier and a resulting loss of vital intracellular components. Agents of this type usually exhibit surface active properties. Examples are polymyxin (Newton, 1956), hexachlorophene (Joswick and Gerhardt, 1960) and Nystatin (Sutton et al., 1961). Salton (1951) believes that the effects of such compounds are probably not due to the inhibition of an enzyme since a greater time lag might be expected before the observation of secondary effects, such as the release of cell constituents. However, Newton (1958) states that while Salton's remarks would almost certainly be true for the inhibition of most enzymes involved in metabolism they might not hold for the inhibition of enzymes involved in the maintenance of the protoplast membrane.

Mechanism of inhibition by organic acids. That the effective inhibitory form of organic acids is the undissociated acid is well documented and is adequately reviewed by such workers as Bell et al. (1959) and Weiner and Draskóczy (1961). This phenomenon is explained by the charge neutralization and the resulting increase in lipid solubility so that the acid may penetrate more rapidly to the interior of the cell.

Stoppani et al. (1960) have presented evidence that the permeation of cells by organic acids may also be an active process. The evidence consists of the finding that in the presence of 2,4-dinitrophenol, which uncouples oxidative phosphorylation in yeast (Utter et al., 1958) the following was prevented: (1) the incorporation of C^{14} into yeast, (2) evolution of $C^{14}O_2$, and (3) O_2 uptake. C^{14}-labeled pyruvate, succinate, glutamate, fumarate and acetate were used as substrates for intact cells of yeast.

Weiner and Drasköczy (1961) made the interesting observation that the ionization constant, K, determined for dilute solutions is not applicable in solutions of high ionic strength because of the following relationship:

$pk = pK - \frac{0.5\mu}{1 + 0.33\ a\mu}$ where μ is the ionic strength and "a" is the collision diameter for interionic attractions; this phenomenon had not been accounted for by previous workers in calculating the concentration of undissociated acid required for a given degree of inhibition of intact cells.

Weiner and Drasköczy (1961), employing the above relationship of pk and ionic strength, were able to show a correlation between inhibition of oxidative metabolism of Escherichia coli and the concentration of unionized molecules of several organic acids. The concentration of unionized

acid required to inhibit metabolism was shown to be approximately the same as that required for antiseptic action. In agreement with other workers, they were also able to show that cell-free preparations of the organisms demonstrated inhibition of oxidative metabolism which was proportional to the concentration of ionized (or total) acid. The degree of this dissociation is dependent on the pK of the acid, and the intracellular pH. Therefore, in the intact organism, the inhibitory effect is related to the concentration of unionized acid in the medium, the pK of the acid, and the intracellular pH. These workers suggest that organic acids penetrate the membrane in the unionized form and exert their toxic effect(s) intracellularly in proportion to the intracellular concentration of ionized or total acid.

If the intracellular pH is higher than the extracellular pH, which is usually the case with organic acids acting as inhibitors, and the cell membrane is permeable only to the unionized molecule, the cell will tend to concentrate acid intracellularly. Thus, since it ionizes more completely at the higher pH, growth may be inhibited in the presence of relatively low extracellular concentrations (Weiner and Draskóczy, 1961). This phenomenon was discussed by Jacobs (1940) in describing the selective concentration of weak acids and bases across a membrane permeable only to

the unionized form of the substance, where the pH values of the fluids on each side of the membrane differ considerably. Jacobs points out that the factor of accumulation may be surprisingly large. For example, assuming the maintenance by metabolism of the cell of a constant internal pH of 6.8 while the cell is suspended in a medium at pH 4.8 containing acetic acid, the theoretical accumulation of acetic acid would be 50 fold. The driving force necessary for accumulation in this manner is the pH difference between the cell and its surroundings maintained by metabolic processes within the cell.

Samson et al. (1955) observed that short-chain fatty acids in relatively high concentrations affect yeast metabolism. They concluded that the acids affect a large variety of systems and the mechanism is at a molecular rather than a cellular or tissue level. They speculated that the ultimate mechanism of inhibition is the binding of enzyme protein. If the fatty acid were bound to an enzyme at the active site, noncompetitive inhibition would result; in a few cases, the acid might bind to the substrate site itself and would result in competitive inhibition. These workers were able to show inhibition of glycolysis in cell-free preparations; this was interpreted as disproving the theories that fatty acids affect the cell membrane and also that the inhibition

is due to acidification of the cytoplasm.

Recently, however, Lampen and Weinstock (1962) have reported that in 0.2 M acetate at pH 4.0 the intracellular pH of yeast rapidly drops and a marked decrease in the content or activity of several enzymes results. They suggest that "the unionized fatty acid enters the cell, and the resultant acidification initiates autolytic processes destroying critical glycolytic and oxidative enzymes." Thus, there are indications that acetate, at least, might function by lowering the intracellular pH.

Fencl (1961) has data supporting the theory that, in yeast, inhibitory levels of acetate react with basic components of the membrane which serve as receptors of anions and mediate their entrance into cells and thereby inhibit premeation of these anions.

The suggestion was made by Weiner and Draskóczy (1961) that organic acids (mandelic, hippuric, lactic and acetic) inhibit dehydrogenases. This conclusion was based on an inhibition of indophenol blue reduction, but no data were presented on this point so that the conclusion is difficult to evaluate. Bosund (1960) proposed that benzoic and salicylic acids might inhibit terminal respiration as the primary mechanism of growth inhibition.

Interference of coenzyme A metabolism is a possible mechanism of inhibition. Avigan et al. (1955) theorized that various reactions of acetyl-coenzyme A are specifically inhibited by acyl-coenzyme A compounds. McMurray and Lardy (1958) noted that reduced coenzyme A addition to sonic extracts of rat liver mitochondria greatly increased the P/O ratio and concluded, therefore, that coenzyme A might play a role in oxidative phosphorylation. Thus, alterations of coenzyme A metabolism could effect oxidative phosphorylation.

Penniall et al. (1956), Penniall (1958) and Jeffrey and Smith (1959) showed uncoupling of oxidative phosphorylation by salicylic acid. Mitochrome, a naturally occurring uncoupling agent, has been analyzed by Hülsmann et al. (1960) and Wojtczak and Lehninger (1961) and shown to consist of C_{12}-C_{18} saturated fatty acids and C_{14}-C_{22} unsaturated fatty acids. Pressman and Lardy (1956) in a very interesting study found that latent ATPase (LAS) activity of saturated fatty acids plotted as a function of chain length exhibited a definite optimum around myristic acid. They also noted that the introduction of cis-unsaturation into the C_{18} chain greatly enhanced LAS activity. Two singly cis-unsaturated isomers, Δ^9-oleic and Δ^{11}-cis-vaccinic acids are of equal activity while their geometric isomers, elaidic

and trans-vaccinic acids respectively, show no more activity than their saturated analogue, stearic acid.

Little is yet known about the mechanisms by which high-energy phosphate bonds are generated during the functioning of the electron transport system. Of the possible theories mentioned by Hunter (1951) two are of interest; viz., addition of phosphate to C=C with oxidation to yield an enol phosphate, and addition of phosphate to C=C then dehydration as in 2-phosphoglyceric acid to phosphoenol-pyruvate. The unsaturated bonds of fatty acids could conceivably competitively accept phosphate groups and thereby lower the efficiency of oxidative phosphorylation.

Instead of uncoupling oxidative phosphorylation, there is the possibility that organic acids could inhibit oxidative phosphorylation as does the antibiotic oligomycin. The inhibition of pyridine-linked substrate oxidations by this antibiotic is completely reversible by 2,4-dinitrophenol, the uncoupling agent. Lardy et al. (1958) concluded that oligomycin acts on an enzyme involved in phosphate fixation or in phosphate transfer rather than on enzymes involved in electron transport.

Mechanism of inhibition by sorbic acid. An important characteristic of a food preservative is that it must not

stimulate growth of food poisoning bacteria. Emard and Vaughn (1952) reported that sorbic acid inhibited Salmonella, some strains of Streptococcus faecalis, and Staphylococcus aureus. This was confirmed by Doell (1962) who states that potassium sorbate is bacteriostatic and bacteriocidal against Staphylococcus, Salmonella and Pseudomonas in a concentration of 0.1% at pH 5. However, Doell noted that at the usual pH of foods, potassium sorbate could not be considered to have significant bacteriostatic activity against pathogenic microorganisms. The data are not extensive enough to state conclusively that sorbic acid is effective against food spoilage organisms.

The chief value of sorbic acid lies in its fungistatic activity, probably because it is active only at low pH values and bacteria generally do not multiply well at such high acidities. It has been used in a variety of menstrua as a preservative, mostly foods.

One of the major observations made thus far is that of Emard and Vaughn (1952) who observed that sorbic acid selectively inhibits catalase positive microorganisms with little or no effect on catalase negative organisms. The study included 299 cultures encompassing actinomycetes, bacteria, yeast and molds. Thus, it appeared possible that in the basic differences of metabolism between catalase

positive and catalase negative organisms lay the explanation of the selective inhibition.

The investigation of the effect on catalase *per se* would seem a logical first step in the search for sorbate-sensitive enzymes. Lück (1958, 1960) reported a 70% inhibition of catalase by 1×10^{-2} M sorbic acid at pH 4.5 while less than 5% inhibition was noted at pH 6.8. A negative correlation was noted, however, between catalase inhibition and growth inhibition by sorbic and 22 other organic acids.

Melnick et al. (1954) theorized that sorbic acid might inhibit growth of molds by an inhibition of fatty acid oxidation. No data were given in support of this theory, however, and it is difficult to visualize how this mechanism could be responsible for the inhibition of glycolysis and respiration with such substrates as glucose and pyruvic acid in resting yeast (Hsu, 1957).

York and Vaughn (1955) reported evidence for sorbate inhibition of fumarase. The studies were carried out utilizing growing cultures, intact cells and crude enzyme systems. The report was published as an abstract and therefore it is difficult to evaluate the conclusions.

Whitaker (1959) presented data which were interpreted to show that sorbic acid might be inhibiting the many

sulfhydryl-containing enzymes of a cell by the formation of thiohexenoic acid derivatives.

Wakil and Hübscher (1960) developed a direct spectrophotometric assay for the fatty acid activating enzyme by reacting sorbic acid, adenosine triphosphate, reduced coenzyme A and Mg^{++} in the presence of the enzyme; sorbyl-coenzyme A was formed. It is conceivable that sorbate inhibition is the result of the formation of sorbyl-coenzyme A which is not further metabolized and thus immobilizes the catalytic amounts of coenzyme A available to the cell. Alternatively, it is possible that sorbyl-coenzyme A itself is an inhibitor. Palleroni and De Pritz (1960) speculate that sorbic acid inhibits the formation of citrate from acetyl-coenzyme A, probably by the formation of sorbyl-coenzyme A. They support this possibility by stating that sorbic acid inhibits the formation of acetyl sulfanilamide from sulfanilamide and acetyl-coenzyme A. Palleroni and De Pritz (1960) also reported an inhibition of acetate assimilation by sorbic acid and speculated that this may be due to the inhibition of higher fatty acid synthesis.

Azukas et al. (1961) studied the sorbate inhibition of yeast alcoholic fermentation and concluded that enolase is the primary site of inhibition. However, this does not explain the inhibition of respiration noted by Hsu (1957).

Hsu found that the relationship between the degree of inhibition of glucose and pyruvic acid oxidations and sorbic acid concentrations was similar. Also, he noted that both oxidations were inhibited non-competitively; from these observations he concluded that an identical mechanism was responsible for the inhibition of both glucose and pyruvic acid oxidation.

Sorbic acid is no exception to the rule that organic acids are more effective inhibitors at low pH values. That the mechanism involved is due to the neutralization of the charge of the carboxyl group which in turn allows better cell permeation is indicated by such work as reported by Nomoto et al. (1955). Sorbic acid showed maximal inhibition below pH 4.0 at 0.002% while sorbate esters demonstrated maximal inhibition at 0.002% at any pH level. Oka (1960) reported that in a low pH medium, salicylic, benzoic, dehydroacetic and sorbic acids transfer very rapidly from the medium into the cells and that these acids accumulate in the cells; i.e., much higher concentrations of the acids exist within the cells than in the external medium. He further suggests that the acid in the cell is in equilibrium with the undissociated acid in the medium and that the ratio of the acid in the cell to the total concentration of acid in the medium is limited by the pH of the medium. These conclusions are

in agreement with those of Weiner and Draskóczy (1961) and Jacobs (1940).

The metabolism of sorbic acid. It has been shown that sorbic acid is metabolized in vivo in an identical manner to that of the normally occurring caproic acid, the saturated analogue of sorbic acid. The β-oxidation pathway is employed. With starved rats a ketonuria occurs when sorbic acid is fed, but if glucose is administered along with sorbic acid, a decrease in the ketonuria occurs and it was concluded that under normal feeding conditions sorbic acid is completely oxidized to CO_2 and H_2O (Deuel et al., 1954). β-oxidation is also employed by molds to metabolize sorbic acid (Melnick et al. 1954).

Kennedy and Lehninger (1950), employing liver mitochondria with added succinate or malate, demonstrated that the short-chain fatty acids (hexanoic, octanoic) give rise to more acetoacetate than long-chain acids (palmitic, oleic) which are more extensively oxidized to CO_2 and H_2O. Witter et al. (1950) reported that sorbic, 2-hexenoic and caproic acids are quantitatively metabolized to acetoacetate using rat liver homogenates or washed liver particulate matter.

EXPERIMENTAL METHODS

The following abbreviations will be used: ATP for adenosine triphosphate; DPNH for reduced and DPN for oxidized diphosphopyridine nucleotide; TPNH for reduced and TPN for oxidized triphosphopyridine nucleotide; EDTA for ethylenediamine-tetraacetic acid; MB for methylene blue; DNP for 2,4-dinitrophenol; and ETS for electron transport system.

The yeast used in these experiments was from two sources. The first was baker's yeast purchased locally; in most experiments it was air dried, and maintained in the freezer. Just prior to use, the dried yeast was washed three or four times in distilled water, resuspended in distilled water and incubated on a rotary shaker for 8 to 12 hr to lower the endogenous metabolic rate. In other studies, undried commercial yeast was washed three times in distilled water and used to prepare crude mitochondrial suspensions by the method of Gottlieb and Ramachandran (1961). In preparing crude mitochondrial suspensions by a modification of the method by Gottlieb and Ramachandran (1961), a stock strain of baker's yeast from our laboratories

was used to produce fresh cells in 3-liter fermentors (New Brunswick Scientific Co., New Brunswick, New Jersey) in the following manner: three 230 ml volumes of dextrose broth (Difco) were adjusted to pH 5.0 with tartaric acid, inoculated with stock yeast and allowed to grow in shake flasks at 30 C for 24 hr; three 3-liter fermentors containing the same medium were then inoculated from the flasks and incubated with aeration for 11 hr at 30 C. The cells were harvested with a Sharples Super Centrifuge (The Sharples Specialty Co., Philadelphia, Pa.), washed twice with distilled water, and stored at 0 C until use.

Yeast were treated with dry ice to remove the permeability barrier by a modification of the method reported by Krebs et al. (1952). Two grams of dried, frozen yeast were placed in contact with dry ice for 20 min, the dry ice was allowed to evaporate in the freezer and the cells were washed three times and then resuspended in 0.4 M lactose-0.02 M potassium phosphate, pH 7.0, containing 40 μmoles of DPN, to a total volume of 20 ml.

Acetone dried yeast were prepared by adding an aqueous cell suspension slowly with stirring to ten volumes of acetone previously cooled to -20 C. After brief stirring, the cells were allowed to settle, the solvent removed by filtration and the cells washed with two to five volumes of

-20 C acetone. The cells were then transferred to a desiccator at -20 C which contained paraffin to absorb the acetone.

The method of Gottlieb and Ramachandran (1961) for preparing crude suspensions of yeast mitochondria was modified slightly. A VirTis "45" Homogenizer (The VirTis Company, Inc., Gardiner, New York) was employed to break 4 g (wet weight) of yeast in the presence of 4 g of pavement marking beads (average size, 0.2 mm., manufactured by the Minnesota Mining and Manufacturing Co., Minneapolis, Minn.), 10 µmoles of DPN, 600 µmoles of lactose, and 30 µmoles of potassium phosphate at pH 7.0 in a total volume (excluding beads) of 5 ml. The container was cooled to 0 C in an ice bath and then placed in an alcohol bath at -19 C at the beginning of the 5 min breakage period. The mixture was first centrifuged at 300 X G for 5 min, and the resulting supernate was centrifuged at 1200 X G for 30 min. The final supernate was used in enzymatic studies which were carried out within 10 days. Triple (glass) distilled water was used throughout. All operations were carried out at 0 to 4 C.

Rat liver mitochondria were prepared as described by Carter et al. (1959), and were used the same day. The

method entails isolation of mitochondria by differential centrifugation in 0.25 M sucrose-0.001 M ethylenediamine-tetraacetic acid (EDTA). Triple (glass) distilled water was used throughout. Slight modifications of the method were employed and are described as follows. Rat liver was homogenized 1 min, cooled 1 min, and the process repeated. The entire operation took place in an ice water bath. The isolated mitochondrial precipitate was made up to a total volume of 10 ml in sucrose-EDTA and then rehomogenized for 1 min in an ice bath before use.

Dry weights were determined by placing 1 ml of the cell suspension at 110 C for at least 2 hr. Cell weights given are all as dry weight. Protein levels were determined by the turbidimetric trichloroacetic acid method of Stadtman et al. (1951). Crystalline egg albumin was used as the standard. These assays were carried out with a Spectronic 20 colorimeter (Bausch & Lomb Optical Co., Rochester, N.Y.).

Standard Warburg techniques as described by Umbreit et al. (1957) were employed. CO_2 evolution was determined by the direct method. Unless otherwise noted, all components of the reaction mixture were added to the main compartment of the Warburg flask except substrates which were tipped

in from a side arm after thermal equilibrium was attained. Helium was used as the gas phase in anaerobic experiments; air was the gas phase in aerobic experiments, and 0.2 ml of 20% KOH along with a strip of filter paper was placed in the center well. All components of the reaction mixture were adjusted to the pH of the experiment with dilute KOH or HCl and, usually, the pH was determined at the termination of the experiment (final pH). In manometric work utilizing KCN, 0.2 ml of 1 M KCN was added to the center well in place of 20% KOH. All Q_{O_2} values were derived from manometric techniques. In each case the Q_{O_2} was defined as µl O_2 uptake/mg dry weight of cells/hr with intact, acetone-dried, or dry ice-treated yeast, or µl O_2 uptake/mg protein/hr with cell-free preparations. Aerobic experiments utilized air as the gas phase while anaerobic studies utilized helium.

Oxidative phosphorylation experiments were conducted as described by Carter et al. (1959). In these studies substrates were added to the main compartment of the cups.

The cytochrome c oxidase assay was carried out as described by Umbreit et al. (1957). The method is based on the manometric measurement of O_2 uptake resulting from the reduction of cytochrome c by ascorbate. The mitochondrial suspension was diluted 10 X in cold, triple (glass) distilled

water which allows a maximal reaction rate.

Diaphorase activity was determined by the reduction of methylene blue in the presence of KCN and a measurement of O_2 uptake from the auto-oxidation of methylene blue.

Spectrophotometric assays of DPNH oxidation by yeast mitochondria were conducted by following the decrease in OD at 340 mu; the reaction was initiated by the addition of DPNH. Cytochrome c reductase activity of yeast mitochondria was also determined spectrophotometrically based on the absorbency of reduced cytochrome c at 550 mu. These assays are modifications of methods described by Gottlieb and Ramachandran (1961). With rat liver mitochondria, the DPNH-cytochrome c reductase assay was conducted as follows. Each cuvette contained 2 mg cytochrome c, 1 µmole KCN, 0.1 ml of 0.1% DPNH (in 0.25 M sucrose-0.02 M potassium phosphate, pH 7.4), 0.3 mg protein and, where indicated, 0.2 µg antimycin A or potassium sorbate. The total volume was made up to 3.0 ml with 0.25 M sucrose-0.02 M potassium phosphate, pH 7.4. This is a modification of the method described by Mackler and Green (1956). In one experiment, the mitochondrial preparation was diluted 1:10 in cold, triple (glass) distilled water; while in the other experiment, the mitochondria were undiluted. The total protein per cuvette was the same in each experiment.

Sorbic acid disappearance was measured as follows. Samples (0.3 ml) were removed from the Warburg cups and added to about 90 ml of distilled water in a volumetric flask which had been previously acidified with 1 ml of 1 N HCl, and made up to 100 ml with distilled water. The flasks were shaken, and the pH compared with that of 0.01 N HCl; both had a pH of 2.1. A standard curve was prepared by adding various amounts of sorbic acid to the appropriate amount of mitochondrial suspension. The blank was prepared from a sample from an endogenous Warburg cup. The OD was recorded at 263 mμ on the spectrophotometer and the sorbate concentration determined from the standard curve.

Sorbic acid was obtained from Union Carbide Chemicals Co., New York; the acid was refined sorbic acid (water content 5.5%) which was recrystallized three times from distilled water. The pH of solutions of the acid was adjusted with a KOH solution to that of the particular experiment. The acid was stored in dark brown bottles.

Trans-hexenoic acid was generously supplied by A. F. Mabrouk, Northern Regional Research Laboratory, Peoria, Illinois. The other organic acid inhibitors were of commercial origin and were prepared as sorbic acid was. The cofactors, intermediates, etc. were also obtained commercially and were either prepared fresh at the proper pH for each experiment

or were maintained in the frozen state with the exception of DPN, DPNH, TPN and TPNH which were stored at 0 C since they are more stable at this temperature. Antimycin A was obtained from the Wisconsin Alumni Research Foundation. A stock solution of 1 mg per 10 ml in 95% ethanol was prepared and then dilutions in 50% ethanol (v/v) were made so that a concentration of 4 μg/ml resulted. Appropriate controls of 50% ethanol were always run in inhibition studies.

RESULTS

Studies with Intact Yeast Cells

Inhibition of respiration. Sorbate (5.3×10^{-3}M) did not inhibit the oxidation of pyruvate by intact yeast at pH 7; but it did result in a high level of inhibition of aerobic CO_2 evolution and O_2 uptake from pyruvate oxidation at pH 4.2 (Fig. 1). This is in agreement with Hsu (1957). Ethanol, acetaldehyde, acetate, TPNH and DPNH oxidations were similarly inhibited (Table 1). These results suggested that the electron transport system (ETS) was being inhibited by sorbic acid, especially since TPNH and DPNH appeared to be oxidized by intact cells via the cytochrome system; i.e., the oxidations were markedly inhibited by both KCN and azide.

Attempts were made to reduce cytochrome c directly and observe the effect of sorbic acid on the resulting O_2 uptake. Ascorbic acid, p-phenylenediamine and lactic acid were used as substrates and a strong inhibition of each substrate oxidation by sorbic acid was observed. This suggested that the inhibitory site was between cytochrome c and O_2 (Table 2). However, these observations could also

Figure 1. Effect of sorbic acid on aerobic CO_2 evolution and O_2 uptake by intact yeast. Values were determined manometrically at 30 C. The Warburg vessels contained: 200 µmoles potassium phthalate, 26 mg cells and, where indicated, 30 µmoles potassium pyruvate or potassium sorbate. The cup contents were identical except that in experiment "b", 0.2 ml of 20% KOH was added to the center well. The gas phase was air. The total volume of each cup was 3.0 ml and the final pH was 4.0 - 4.1 for each experiment.

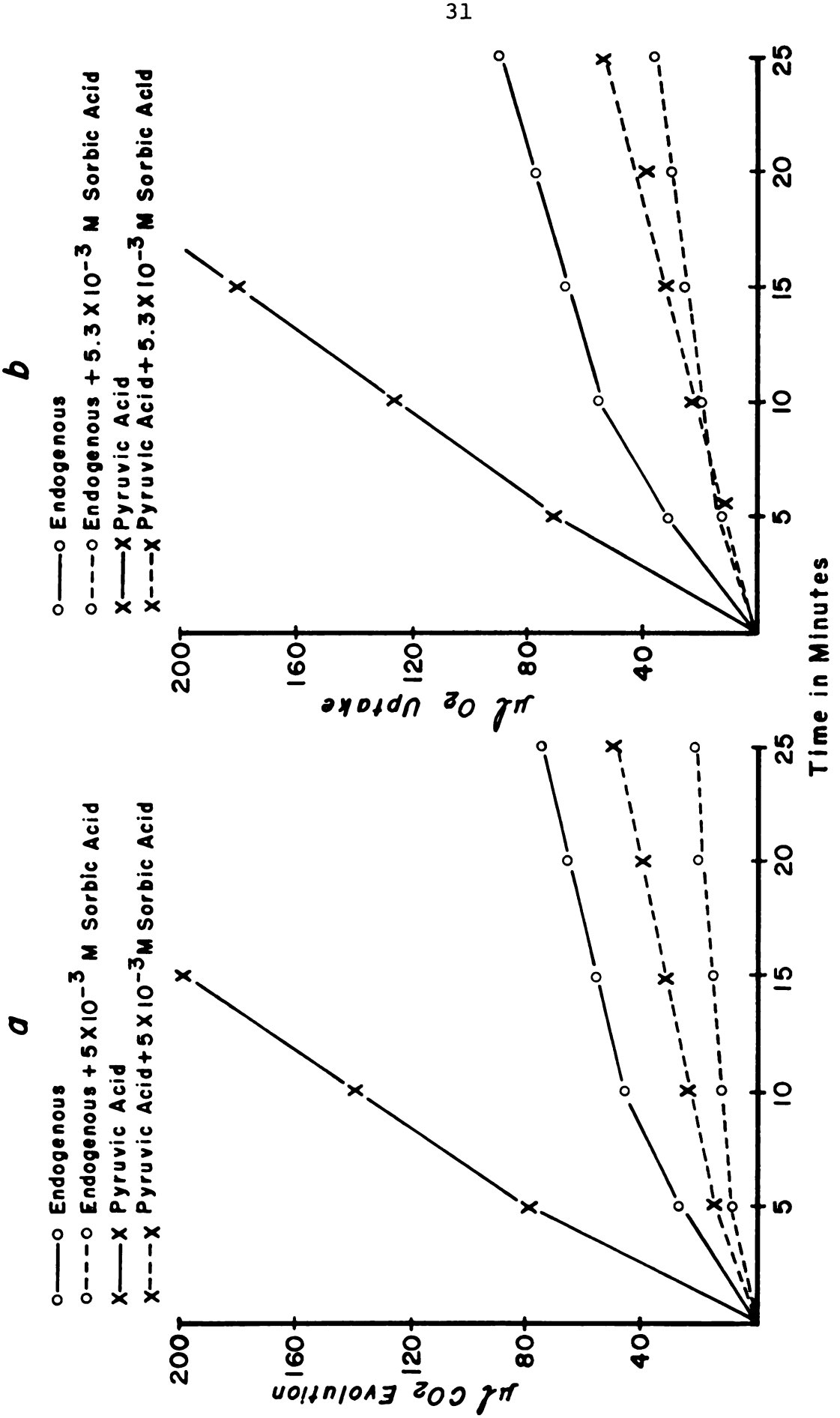

a
b
o——o Endogenous
o- - -o Endogenous + 5 X 10⁻³ M Sorbic Acid
X——X Pyruvic Acid
X- - -X Pyruvic Acid + 5 X 10⁻³ M Sorbic Acid
o——o Endogenous
o- - -o Endogenous + 5.3 X 10⁻³ M Sorbic Acid
X——X Pyruvic Acid
X- - -X Pyruvic Acid + 5.3 X 10⁻³ M Sorbic Acid
μl CO2 Evolution
μl O2 Uptake
200
160
120
80
40
5
10
15
20
25
Time in Minutes

Table 1. Effect of sorbic acid and other inhibitors on ethanol, acetaldehyde, acetate, TPNH and DPNH oxidation by intact yeast.

Experiment	Substrate	Inhibitor	Q_{O_2}	% Inhibition
A	Ethanol		26	--
	Ethanol	Sorbic Acid	7.6	71
B	Acetaldehyde		1.1	--
	Acetaldehyde	Sorbic Acid	0.32	71
C	Acetate		28.5	--
	Acetate	Sorbic Acid	1.1	96
	Acetate	Atabrin (5.3×10^{-2}M)	19.5	32
	TPNH		11	--
	TPNH	Sorbic Acid	1.3	88
	TPNH	Atabrin (5.3×10^{-2}M)	6.7	39
	TPNH	Azide (7.2×10^{-3}M)	0	100
D	DPNH		13	--
	DPNH	Sorbic Acid	4.1	68
	DPNH	KCN (6.7×10^{-1}M)	0	100

O_2 uptake was determined manometrically at 30 C. The Warburg vessels contained: experiment A, 19.7 µmoles sodium citrate, 60.6 µmoles Na_2HPO_4, 20 mg cells and, where indicated, 300 µmoles ethanol; experiment B, 250 µmoles potassium phthalate, 19 mg cells, and where indicated, 73 µmoles acetaldehyde; experiment C, 100 µmoles potassium phthalate, 13 mg cells, and, where indicated, 50 µmoles sodium acetate or 2.5 µmoles TPNH; experiment D, 400 µmoles potassium phthalate, 24 mg cells and, where indicated, 33 µmoles DPNH. Sorbic acid concentration was 8.2×10^{-2}M in experiment A and 5.3×10^{-3}M in the other experiments. Q_{O_2} was corrected for endogenous activity with or without the appropriate inhibitor. The total volume of each cup was 3.0 ml, and the pH was 5.7 - 5.8 for experiment A and 4 for the other experiments.

Table 2. Effect of sorbic acid and azide on the oxidation of ascorbic acid, lactic acid and p-phenylenediamine by intact yeast.

Experiment	Substrate	Inhibitor	Q_{O_2}	% Inhibition
A	Ascorbic Acid		1.3	--
	Ascorbic Acid	Sorbic Acid (5.3×10^{-3}M)	0	100
	Ascorbic Acid	Azide (1.1×10^{-2}M)	0	100
B	p-phenylenediamine		2.5	--
	p-phenylenediamine	Sorbic Acid (5.3×10^{-3}M)	0.76	70
C	Lactic Acid		26.5	--
	Lactic Acid	Sorbic Acid (2.15×10^{-2}M)	5.3	80
	Lactic Acid	Azide (1.1×10^{-2}M)	0	100

O_2 uptake was determined manometrically at 30 C. The Warburg vessels contained: experiment A, 1000 µmoles potassium phthalate, 42 µmoles ascorbic acid and 23 mg cells; experiment B, 400 µmoles potassium phthalate, 10 µmoles lactic acid and 24 mg cells; experiment C, 100 µmoles potassium phthalate, 10 µmoles p-phenylenediamine and 8 mg cells. The Q_{O_2} was corrected for endogenous activity. The total volume of each cup was 3.0 ml, and the pH was 4.0 for experiments A and B and 5.0 for experiment C.

have been the indirect result of other mechanisms of inhibition; e.g., a general inhibition of substrate permeation.

It was of interest at this point to investigate the effect of sorbic acid on the ETS preceding cytochrome c, and the DPNH-methylene blue reductase system proved to be a suitable tool. The mechanism involved is the inhibition of cytochrome c oxidase with KCN so that the electron flow is from DPNH to flavoprotein, possibly to cytochrome b and thence to methylene blue (Singer and Kearney, 1954; Dolin, 1955; Giuditta and Kearney, 1958; Keilin and King, 1958) which is reoxidized by O_2 with the formation of H_2O_2. Results indicated that sorbic acid did not inhibit this pathway since no inhibition was observed (Fig. 2) in the presence of methylene blue and an inhibitor of terminal oxidase (KCN). The 50% inhibition observed with methylene blue and sorbic acid without KCN was probably the result of inhibition by sorbic acid at some point past cytochrome b. No such inhibition was noted with KCN and methylene blue, but these are not comparable since KCN also inhibits catalase which would return one-half of the O_2 taken up in this system to the atmosphere. Sorbic acid does not inhibit catalase under these conditions. It might be suggested that the addition of methylene blue and KCN should have doubled the O_2 uptake rate since catalase was inactivated. Since the

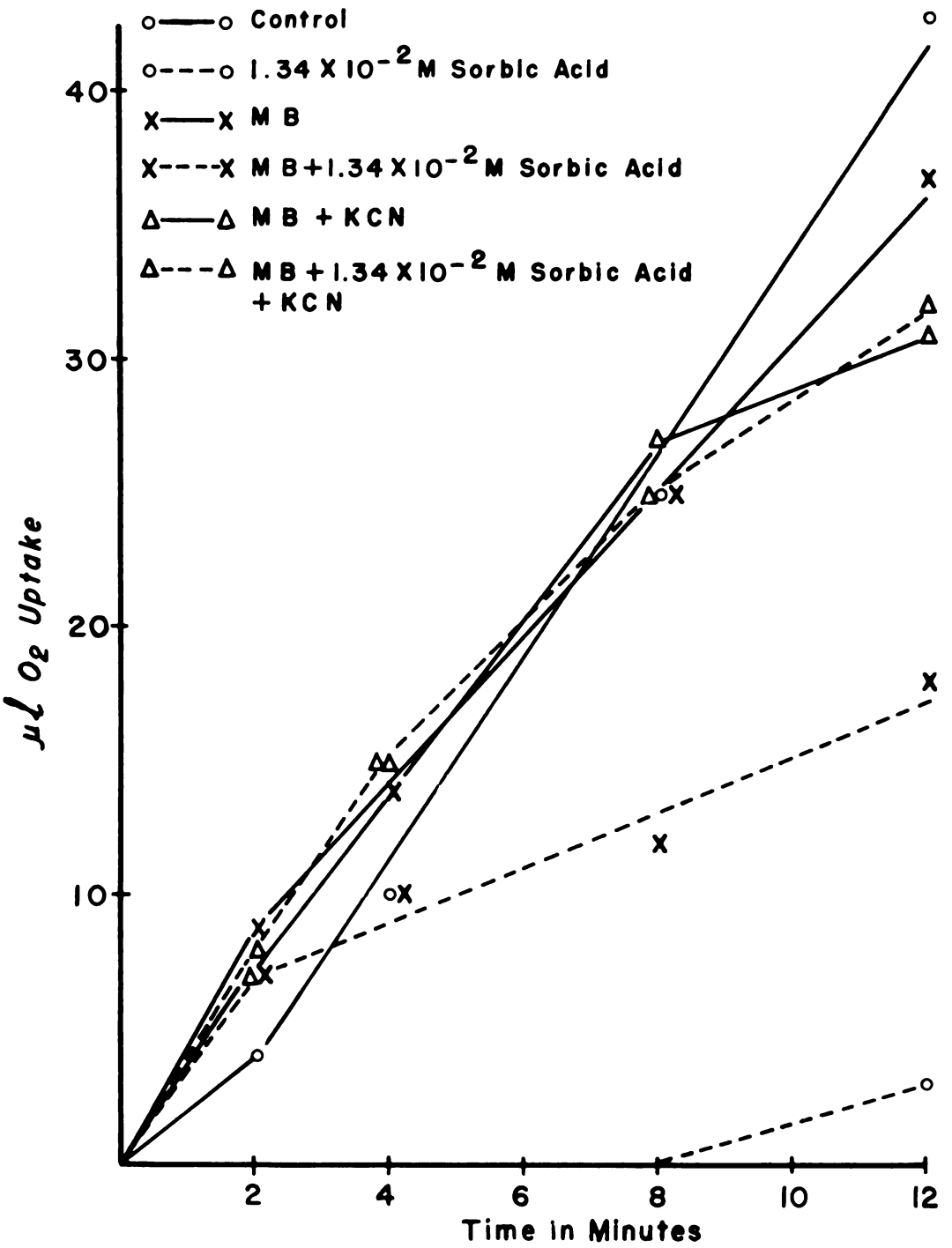


Figure 2. Effect of sorbic acid on the DPNH-methylene blue reductase and the DPNH oxidase systems of intact yeast. O_2 uptake was determined manometrically at 30 C. The Warburg vessels contained: 250 μmoles potassium phthalate, 13 mg cells and, where indicated, 20 μmoles methylene blue (MB) or 9.9 μmoles DPNH. Endogenous rates were zero. The total volume of each cup was 3.0 ml, and the final pH was 5.15 - 5.30.

rate was not altered, it appears that methylene blue reduction occurred at one-half the rate of normal ETS function; i.e., methylene blue reduction was rate-limiting. However, since sorbate almost completely inhibited normal ETS activity, sorbate inhibition in the DPNH-methylene blue reductase system would have been evident had it occurred.

Permeability Studies. As mentioned above, the possibility existed that sorbic acid was inhibiting substrate permeation of yeast cells. Therefore, the effects of sorbic acid and specific inhibitors on endogenous yeast metabolism were studied. Concentrations of sorbic acid were employed which resulted in about the same concentration of exogenous undissociated acid at two pH levels, 5 and 6, so that the pH variable could be minimized. It was believed that if sorbic acid were increasing cell permeability and thereby acidifying the cytoplasm, the inhibition at pH 6 should have been minimized since the intracellular pH of yeast is 5.8 to 6.0 (Conway and Downey, 1950). The fact that equal concentrations of undissociated sorbic acid inhibited endogenous respiration to the same extent at the two pH levels (Table 3) was a further indication that a general inhibition of cell permeation by substrates was not the primary mechanism of action of sorbic acid. Also, a direct effect from lowering

Table 3. Effect of several inhibitors on endogenous metabolism by intact yeast.

Experiment	pH	Additions	Q_{O_2}	% Inhibition
A	6	None	10.3	--
	6	Sorbic Acid (8.2×10^{-2}M)	5.7	45
B	5	None	9.3	--
	5	Sorbic Acid (2×10^{-2}M)	5.0	46
	5	Iodoacetate (6.6×10^{-1}M)	0	100
	5	Arsenite (3×10^{-1}M)	2.6	73
	5	Malonate (8×10^{-1}M)	6.4	31
	5	Azide (6.6×10^{-3}M)	1.0	89

O_2 uptake was determined manometrically at 30 C. The Warburg vessels contained: experiment A, 19.7 µmoles sodium citrate, 60.6 µmoles Na_2HPO_4 and 26 mg cells; sorbic acid was tipped in from the side arm; final pH was 5.9 - 6.1. Experiment B, 100 µmoles potassium phthalate, 27.4 mg cells and inhibitors as indicated. The total volume in each cup was 3.0 ml and the final pH was 5.0 - 5.3.

of intracellular pH from increased permeability was made doubtful. The other inhibitors used implicated glycolysis, the citric acid cycle and cytochrome a_3 as functioning in endogenous metabolism.

Another approach was utilized to investigate the possibility that sorbic acid was inhibiting the permeation of substrates into cells. This consisted of an investigation of the influence of sorbate on the anaerobic decarboxylation of pyruvate. At pH 4, sorbic acid actually increased the rate of anaerobic pyruvate decarboxylation and endogenous CO_2 production (Fig. 3), while O_2 uptake by the same cell suspension was markedly inhibited in a manner similar to that noted in Fig. 1b. Since it is an established fact that glycolysis occurs intracellularly, it would appear that sorbic acid was inhibiting O_2 uptake only after pyruvate permeated the cell. Of course, this is assuming that the same mechanism of permeation functions both aerobically and anaerobically.

It is well known that oxidative phosphorylation (ATP synthesis during ETS function) is "uncoupled" (ATP is no longer generated, but ETS activity continues at a normal or faster rate) by "uncoupling" agents such as 2,4-dinitrophenol (DNP). Conversely, substrate phosphorylation (ATP synthesis

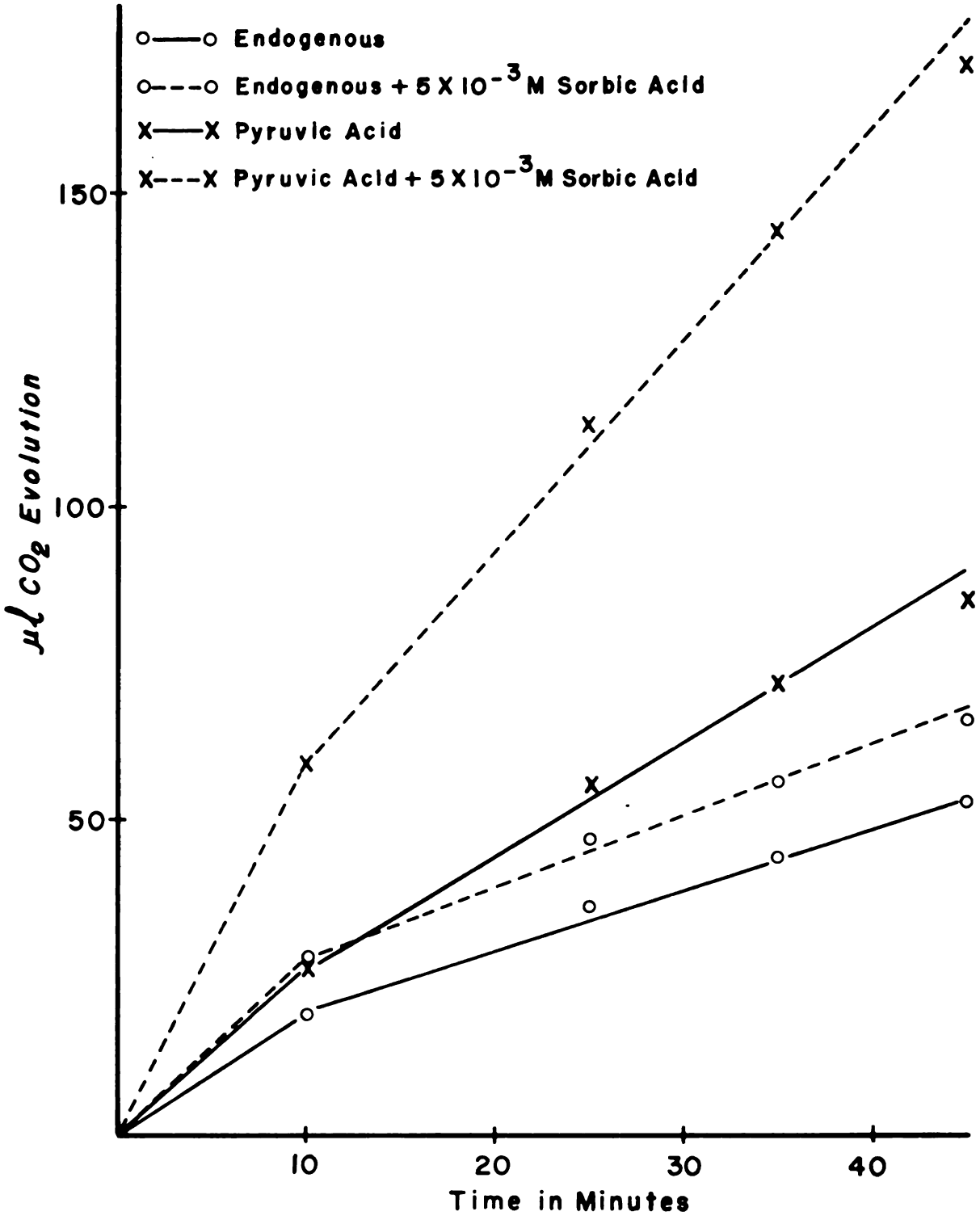


Figure 3. Effect of sorbic acid on the anaerobic decarboxylation of pyruvate. CO_2 evolution was determined manometrically at 30 C. The Warburg vessels contained: 250 μmoles potassium phthalate, 39 mg cells and, where indicated, 300 μmoles potassium pyruvate or potassium sorbate. The gas phase was helium. The total volume of each cup was 3.0 ml, and the final pH was 4.05 - 4.15.

resulting from metabolism along pathways other than the ETS) is not sensitive to uncoupling agents (Dolin, 1961a). Since active transport requires ATP production, uncoupling agents stop active substrate permeation when ATP is produced solely via the ETS and, therefore, oxidation of these substrates. Studies with DNP indicated that pyruvate, ethanol, lactate, acetaldehyde and DPNH required active transport for oxidation by intact yeast since DNP lowered the resulting Q_{O_2} (Table 4), while it failed to affect the rates of endogenous respiration or glucose oxidation. This was as expected since substrate phosphorylation occurs during glucose metabolism. Since sorbate, like DNP, inhibited oxidation of these substrates, it was considered possible that sorbic acid was functioning by uncoupling oxidative phosphorylation and in that way reducing O_2 uptake. Another possibility was that sorbate was actually inhibiting oxidative phosphorylation as oligomycin has been shown to do; and, thereby, stopping active transport and O_2 uptake from exogenous substrates. However, when the active transport factor was circumvented by utilizing cells with a high rate of endogenous activity and oxidative phosphorylation uncoupled with DNP, it was noted that sorbic acid still inhibited O_2 uptake (Table 5). Thus, this would not appear to be the mode of action of sorbic acid.

Table 4. Effect of 2,4-dinitrophenol on respiration of intact yeast.

Experiment	Substrate	Q_{O_2} Control	Q_{O_2} With DNP*	% Inhibition
A	Endogenous	7.4	7.8	0
	Glucose	50	53	0
	Pyruvate	43	13	70
B	Ethanol	43	14	68
	Lactate	44	11	75
C	Endogenous	2.4	11	0
	Acetaldehyde	36	17	53
	DPNH	40	11	73

O_2 uptake was determined manometrically at 30 C. The Warburg vessels contained: experiment **A**, 400 µmoles potassium phthalate, 20 mg cells and, where indicated, freshly prepared 2,4-dinitrophenol to give a final concentration of 5 X 10^{-5}M, 30 µmoles glucose, or 30 µmoles pyruvic acid; experiment B, cup contents are the same as in experiment **A** except that 500 µmoles potassium phthalate, 1,100 µmoles ethanol and, where indicated, 250 µmoles lithium lactate; experiment C, cup contents are the same as in experiment **A** except 250 µmoles potassium phthalate and, where indicated, 364 µmoles acetaldehyde and 66 µmoles DPNH. The total volume in each cup was 3.0 ml, and the final pH was 4.1 - 4.2 in each experiment.

*DNP = 2,4-dinitrophenol.

Table 5. Effect of sorbic acid on endogenous respiration of intact yeast in the presence of 2,4-dinitrophenol.

Additions	Q_{O_2}	% Inhibition
None	96	--
Sorbic Acid	46	52
DNP	101	0
DNP + Sorbic Acid	34	66

O_2 uptake was determined manometrically at 30 C. The Warburg vessels contained: 19.7 μmoles sodium citrate, 60.6 μmoles Na_2HPO_4, 26 mg cells and, where indicated, 6 X 10^{-5}M 2,4-dinitrophenol and 8.2 X 10^{-2}M potassium sorbate. The total volume in each cup was 3.0 ml and the final pH was 6.0.

Some evidence has been presented that sorbic acid was not inhibiting cell membrane permeation per se. More direct proof of this might have been gained by measuring extracellular substrate uptake in the presence and absence of sorbic acid. However, since there were indications that active transport was required for substrate uptake and that sorbic acid was inhibiting the ETS, it was possible that sorbic acid could be blocking substrate uptake indirectly via an inhibition of the ETS. Therefore, proof that sorbic acid was inhibiting substrate uptake might not constitute proof of the primary mechanism of inhibition. For these

reasons, a more direct line of investigation was undertaken; i.e., a study of the effect of sorbic acid on the ETS using preparations having the cell permeability barrier altered or removed by treatment with dry ice or acetone.

Effect of respiration by dry ice-treated and acetone-dried yeast cells. Dry ice-treatment of yeast greatly increases the permeability of the cells. Treated cells oxidized succinate while untreated yeast did not. Substrate oxidation appeared to be mediated via the cytochrome oxidase system since it was KCN-sensitive. However, 5×10^{-2}M potassium sorbate did not decrease the Q_{O_2} resulting from DPNH, succinate or pyruvate oxidation (final pH, 7.4 - 7.8).

Similar results were obtained with acetone-dried cells with which the oxidation of DPNH was azide-sensitive. No inhibition of DPNH oxidation was observed by 8×10^{-3}M sorbic acid at pH 6.3 (Table 6). Similarly, lactate oxidation was not inhibited at pH 5.7 and 6.1 except for an initial lag even by 1.25×10^{-2}M sorbate at pH 5.7. Attempts were made to show inhibition at pH 5.0, but the activity was too low for metabolic studies.

It might be argued that no substantial proof is presented that the membrane was rendered permeable to sorbate by the dry ice and acetone treatments so that one could not

Table 6. Effect of sorbic acid on DPNH oxidation by acetone dried yeast.

Substrate	Inhibitor	Q_{O_2}	% Inhibition
Endogenous		1.4	--
Endogenous	Sorbic Acid	1.2	14
Endogenous	Azide	1.1	21
DPNH		3.6	--
DPNH	Sorbic Acid	3.6	0
DPNH	Azide	1.1	69

O_2 uptake was determined manometrically at 30 C. The Warburg vessels contained: 400 µmoles potassium phosphate, 52 mg acetone dried cells and, where indicated, 16.5 µmoles DPNH, 20 µmoles sodium azide or 8 X 10^{-3}M sorbic acid. The total volume in each cup was 3.0 ml, and the final pH was 6.3.

be certain that sorbate was penetrating the cells at these pH values. However, 1.25 X 10^{-2}M sorbic acid was employed at pH 5.7 and no lactate inhibition was evident (Fig. 4) while approximately this same level was shown earlier (Fig. 2) to almost completely inhibit O_2 uptake from DPNH oxidation with intact cells at pH 5.1 - 5.3. This would indicate that enough undissociated sorbic acid was present to penetrate the membrane. On the other hand, if the treatments were altering the membrane to the point where it was completely

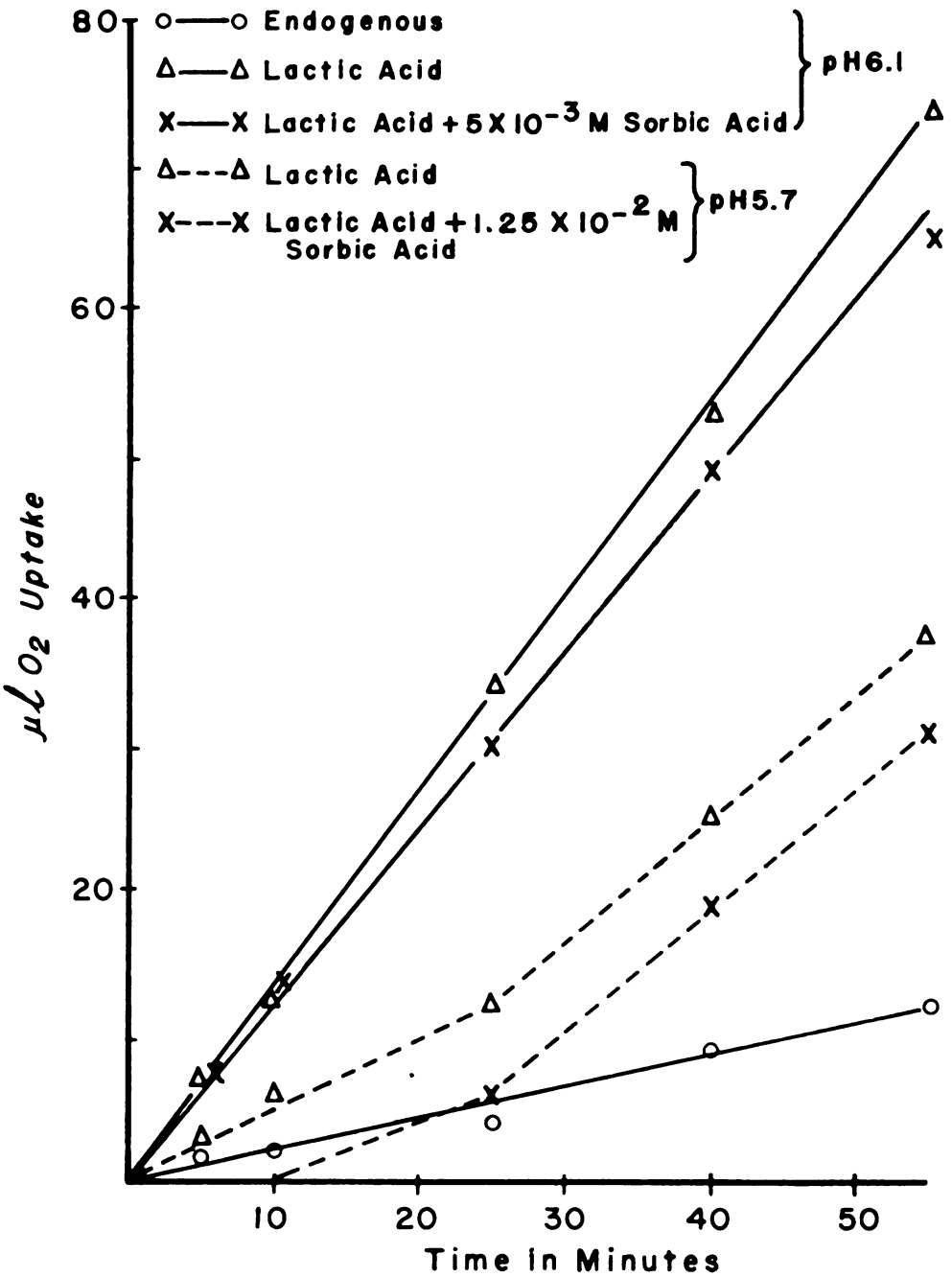


Figure 4. Effect of sorbic acid on lactate oxidation by acetone-dried yeast. O_2 uptake was determined manometrically at 30 C. The Warburg vessels contained: 52 mg cells and, where indicated, 50 μmoles potassium lactate or potassium sorbate; the buffer at pH 6.1 was 900 μmoles potassium phosphate and the buffer at pH 5.7 was 450 μmoles potassium phthalate. Endogenous activity at pH 5.7 was zero and at pH 6.1 the values were corrected for endogenous activity. The total volume of each cup was 3.0 ml.

permeable to the inhibitor, then one would have to add enough sorbate to equal the level attained by accumulation by intact cells since no accumulation should occur if the membrane lost its characteristics of semipermeability. The sorbate level used with dry ice-exposed cells (5×10^{-2}M) would approximate this concentration; and, as will be shown later, this concentration of sorbate was sufficient to strongly inhibit isolated liver mitochondria. Thus, such treatments must alter the system in ways other than simply membrane permeability, or the permability factor must play at least an indirect role in sorbate inhibition.

Studies with Crude Extracts of Yeast Cells

It appeared that the non-inhibition by sorbate with dry ice- and acetone-treated yeast was due to an alteration of the ETS or associated factors during the treatment. Therefore, additional experiments were conducted with cell-free, crude mitochondrial preparations which were prepared by the method of Gottlieb and Ramachandran (1961) except for the cytochrome c studies where the modified method was employed. The results obtained were similar to the above in that DPNH, β-hydroxybutyrate, ethanol, succinate and cytochrome c oxidations were not inhibited by 5×10^{-2}M potassium sorbate. It appeared that the normal ETS was being utilized since

azide and antimycin A exerted strong inhibitions (89 and 61% respectively). High concentrations (1×10^{-1}M) of potassium sorbate inhibited the cytochrome c oxidase system, but it appeared to be a non-specific inhibition since similar levels of KCl and potassium acetate also inhibited. It was thought that the reason for the non-inhibition of O_2 uptake by levels of sorbate below 1×10^{-1}M was the high level of miscellaneous organic material in the Warburg cups which could react with sorbate and lower its inhibitory capacity as was noted by Azukas et al. (1961). In an attempt to overcome this difficulty, spectophotometric investigations were initiated using mitochondria prepared by the modified method of Gottlieb and Ramachandran (1961).

One spectrophotometric assay consisted of measuring the rate of cytochrome c reduction at 550 mu with DPNH as the substrate (the DPNH-cytochrome c reductase system). Sorbate (2.5×10^{-2}M) did not appreciably affect this system when DPNH itself was used as the reductant or when DPNH was generated from ethanol and DPN. Inhibition by antimycin A inferred that the normal ETS was being utilized (Figs. 5 and 6).

However, when the rate of disappearance of DPNH by oxidation was followed spectrophotometrically at 340 mμ (the DPNH oxidase system), sorbate induced a marked stimulation

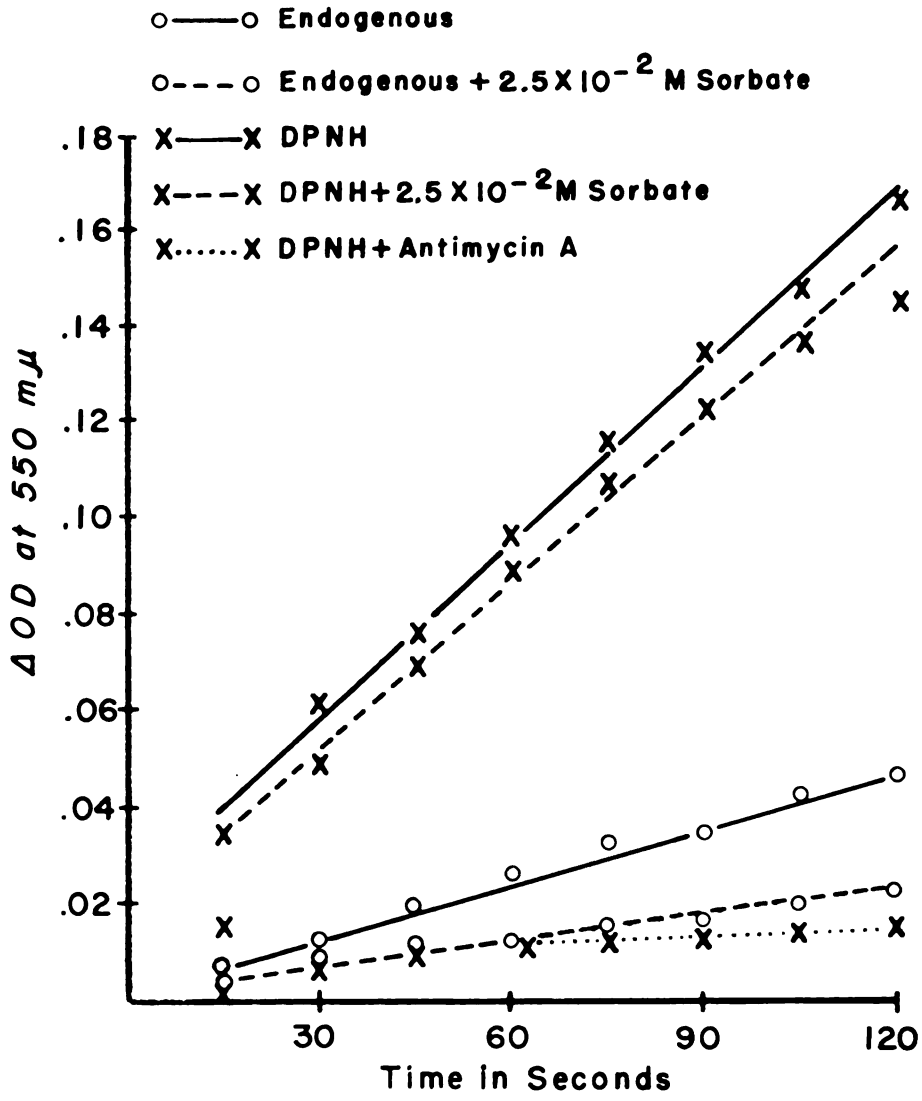


Figure 5. Effect of sorbate on the DPNH-cytochrome c reductase system of crude yeast mitochondria. OD values were determined spectrophotometrically at 25 C. The cuvettes contained: 53 μmoles potassium phosphate, 3.5 mg protein, 1.06 mmoles lactose, 2 mg cytochrome c, 10 μmoles KCN and, where indicated, 1 μmole DPNH, potassium sorbate, or 0.1 μg antimycin A. Endogenous activity was zero. The total volume of each cuvette was 3.0 ml and the pH was 7.0.

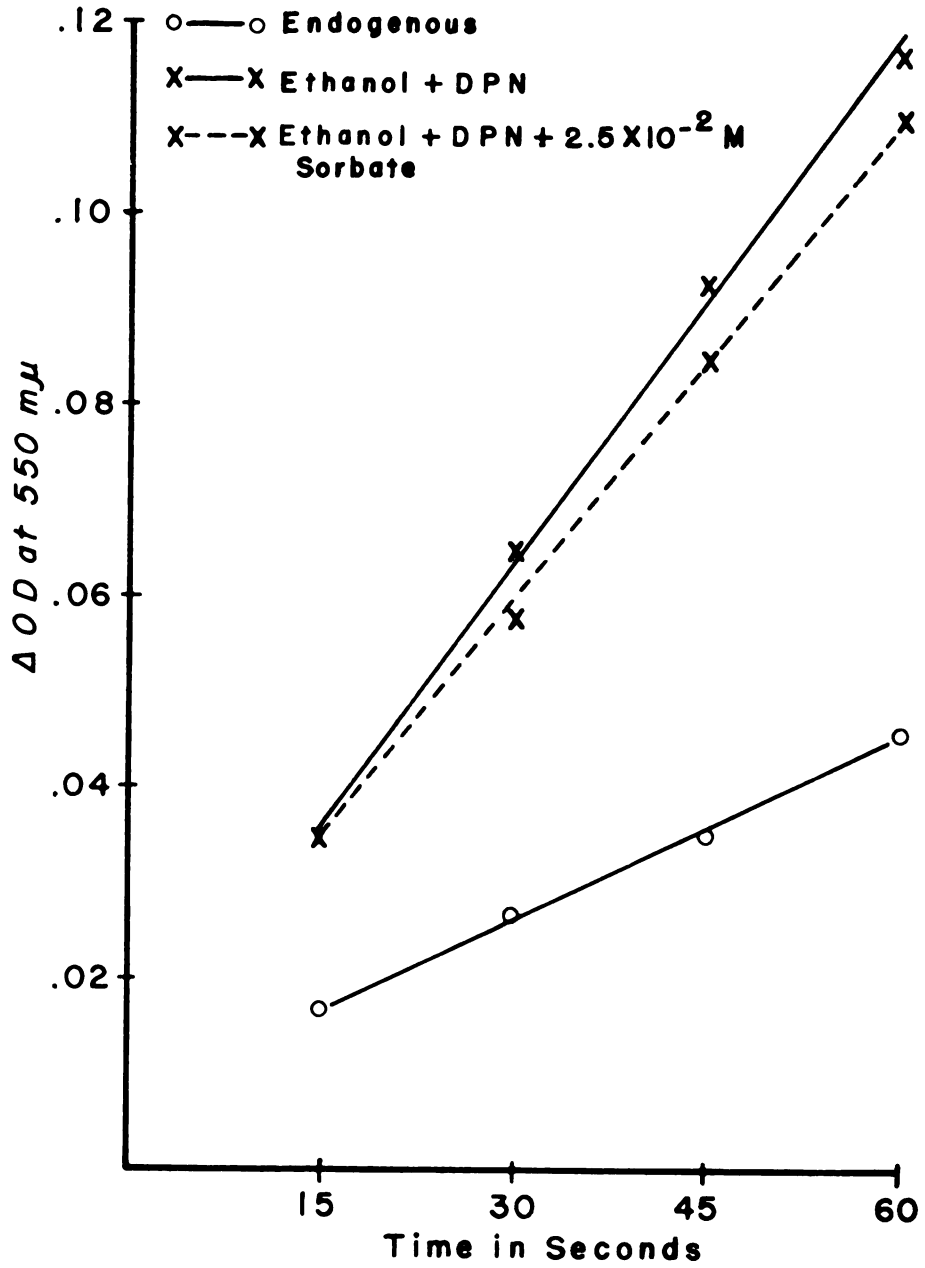


Figure 6. Effect of sorbate on the ethanol-cytochrome c reductase system of crude yeast mitochondria. OD values were determined spectrophotometrically at 25 C. The cuvettes contained: 53 μmoles potassium phosphate, 2.0 mg protein, 1.06 mmoles lactose, 2 mg cytochrome c, 10 μmoles KCN and, where indicated, 0.33 μmoles DPN, 22 μmoles ethanol or potassium sorbate. Endogenous activity was zero. The total volume of each cuvette was 3.0 ml and the pH was 7.0.

(Fig. 7). The system was KCN- and antimycin A-sensitive; but there appeared to be initial stimulation by sorbate even in the presence of these inhibitors, particularly with KCN (Fig. 8). Higher DPNH concentrations were also used so that the substrate would not be so quickly exhausted; and with 0.5 μmole DPNH the stimulation was constant (Fig. 9). Crotonate exerted about the same extent of DPNH stimulation as sorbate while 2-hexenoate and caproate generated approximately 50 and 25% as much stimulation, respectively (Fig. 10). The significance of the effect indicated for caproate is doubtful.

Unlike the stimulation of DPNH oxidation observed spectrophotometrically, it was noted in manometric studies that sorbate did not stimulate or inhibit DPNH oxidation (Table 7). The respiration occurring in the presence of DPNH and sorbate was partially sensitive to antimycin A, KCN, and azide.

Studies with Rat Liver Mitochondria

At this point the data appeared very conflicting. Thus, the intact cell studies indicated an inhibition of the ETS by sorbate, while no inhibition was demonstrable with cells treated to alter membrane permeability or with crude yeast extracts. One possible explanation of this was that

Figure 7. Effect of sorbate on DPNH oxidation by crude yeast mitochondrial preparations. OD values were determined spectrophotometrically at 30 C. The cuvettes contained: 58 µmoles potassium phosphate, 1.5 mg protein, 1.16 mmoles lactose and 0.1 µmole DPNH and, where indicated, potassium sorbate. Endogenous activity was zero. The total volume of each cuvette was 3.0 ml and the pH was 7.0.

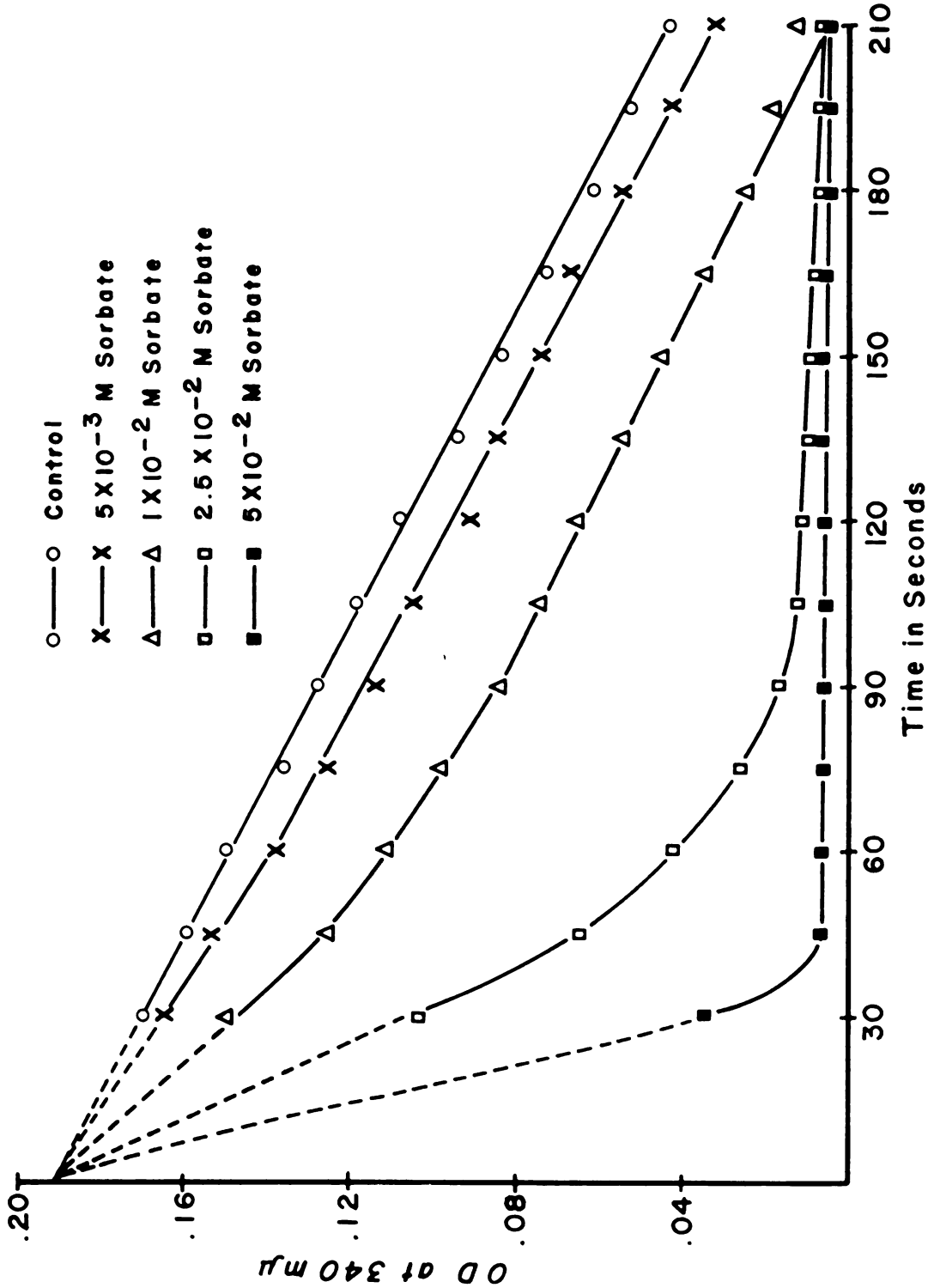
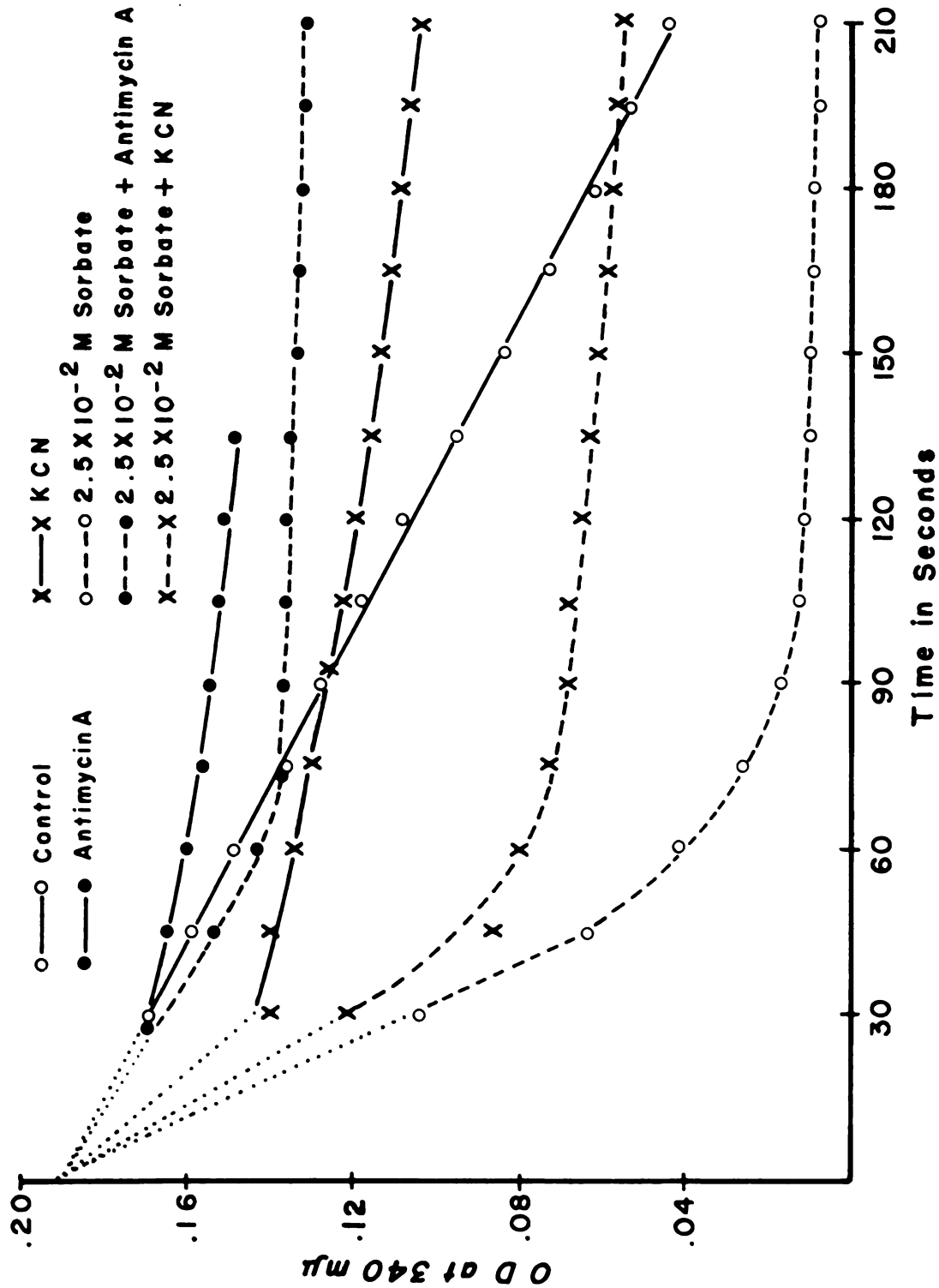

Control
5X10⁻³ M Sorbate
1X10⁻² M Sorbate
2.5X10⁻² M Sorbate
5X10⁻² M Sorbate
OD at 340 mμ
.20
.16
.12
.08
.04
30
60
90
120
150
180
210
Time in Seconds

Figure 8. Effect of KCN and antimycin A on DPNH and sorbate-stimulated DPNH oxidation by crude yeast mitochondrial preparations. OD values were determined spectrophotometrically at 30 C. The reaction mixture was identical to that in Fig. 7 except that, where indicated, 5 μmoles KCN or 0.2 μg antimycin A were added. Endogenous activity was zero. The total volume of each cuvette was 3.0 ml and the pH was 7.0.


O—O Control
●—● Antimycin A
X—X K C N
O---O 2.5X10⁻² M Sorbate
●---● 2.5X10⁻² M Sorbate + Antimycin A
X---X 2.5X10⁻² M Sorbate + K C N
O D at 340 mμ
.20
.16
.12
.08
.04
30
60
90
120
150
180
210
Time in Seconds

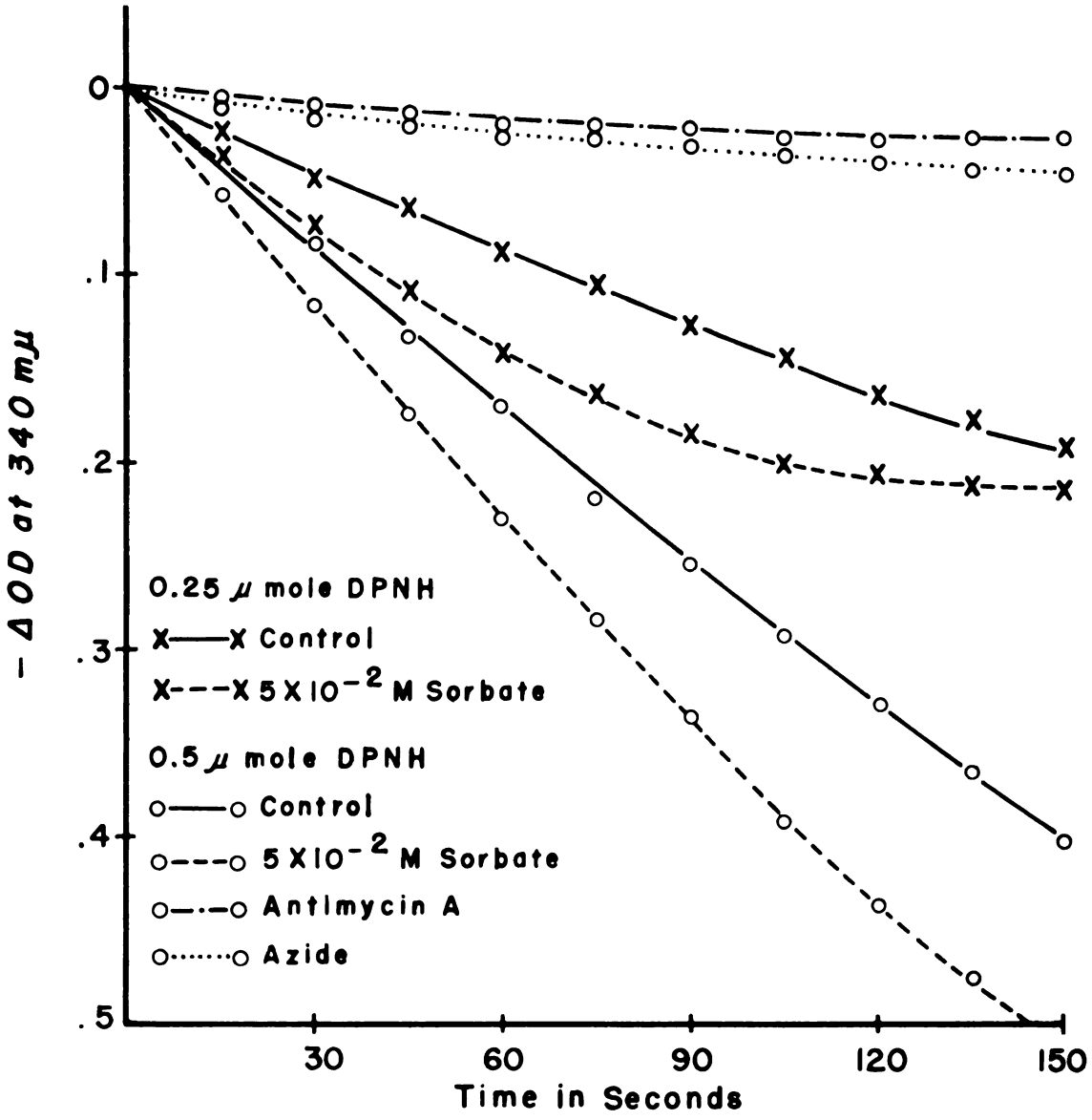


Figure 9. Effect of sorbate on DPNH oxidation by crude yeast mitochondrial preparations. OD values were determined spectrophotometrically at 31 C. The cuvettes contained: 58 μmoles potassium, phosphate, 1.8 mg protein, 1.16 mmoles lactose, DPNH as indicated and, where indicated, 0.2 μg antimycin A, 10 μmoles sodium azide or potassium sorbate. Endogenous activity was zero. The total volume of each cuvette was 3.0 ml and the pH was 7.0.

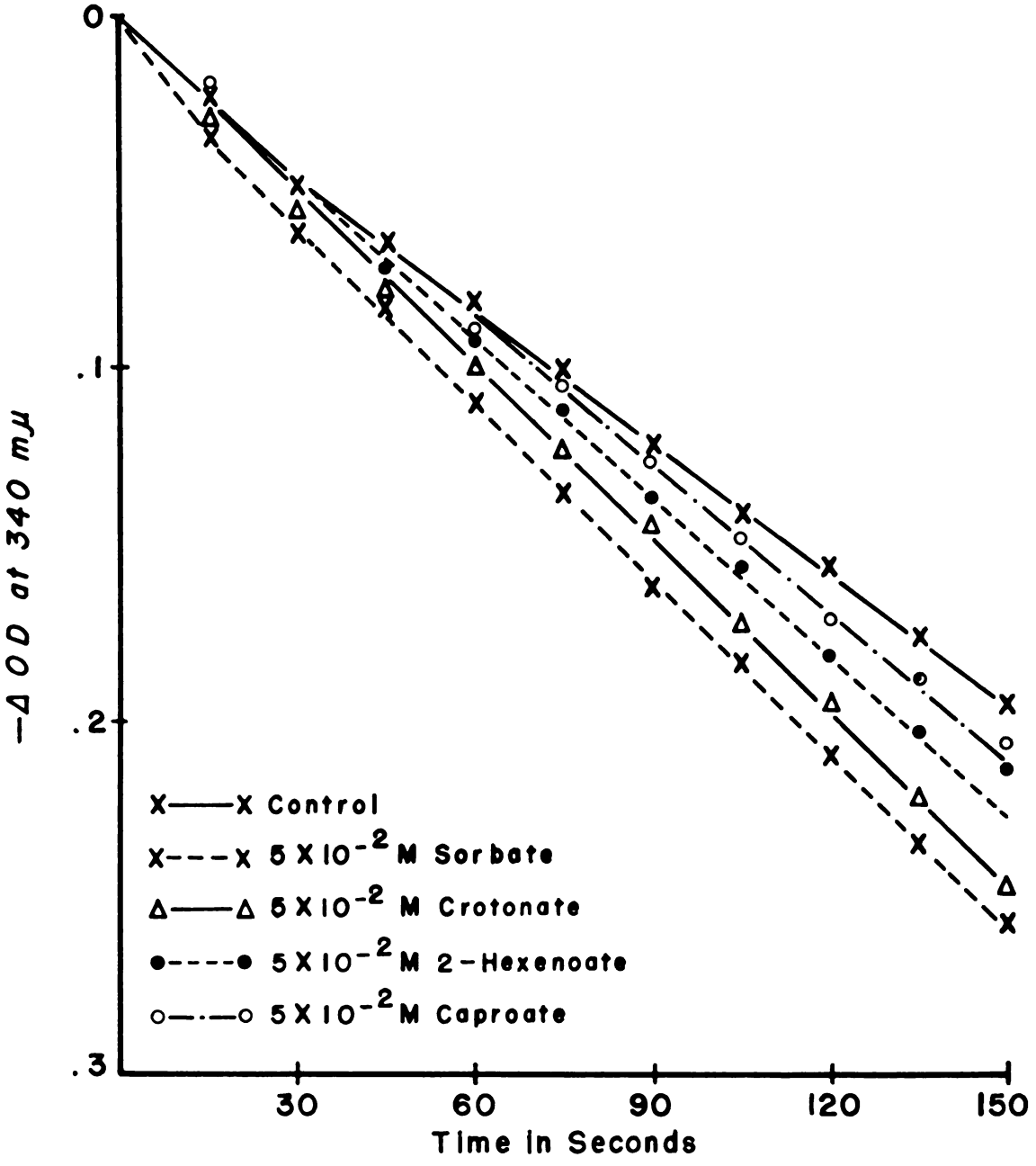


Figure 10. Effect of several α, β-unsaturated acids on DPNH oxidation by crude yeast mitochondrial preparations. OD values were determined spectrophotometrically at 31 C. The reaction mixture was identical to that in Fig. 9. DPNH (0.5 μmole) and the potassium salts of the organic acids were added as indicated. Endogenous activity was zero. The total volume of each cuvette was 3.0 ml and the pH was 7.0.

Table 7. Effect of electron transport inhibitors on the oxidation of DPNH by crude yeast mitochondria in the presence of sorbate.

Substrate	Inhibitor	Q_{O_2}	% Inhibition
Endogenous	None	7.8	--
Endogenous	Azide	5.8	26
Endogenous	Antimycin A	9.9	0
DPNH	None	23.5	--
DPNH	Azide	5.2	78
DPNH	Antimycin A	15.3	35
DPNH	Sorbate	25.2	0
DPNH	Sorbate + Azide	5.9	75
DPNH	Sorbate + Antimycin A	16.0	32
DPNH	Sorbate + KCN	4.2	83

O_2 uptake was determined manometrically at 30 C. The Warburg vessels contained: 23 mg protein (0.25 ml of 90 mg protein per ml of 0.4 M lactose-0.02 M potassium phosphate, pH 7.0) and, where indicated, the following were added to the main cup compartment, 33 μmoles DPNH (in 1.0 ml of 0.4 M lactose-0.02 M potassium phosphate, pH 7.0), 0.147 ml of 0.95 M potassium sorbate, 0.05 ml of 4 μg per ml antimycin A, 0.1 ml of 0.1 M sodium azide. The volume was made up to 3.0 ml total with 0.4 M lactose-0.02 M potassium phosphate, pH 7.0. KCN (0.2 ml of 1 M KCN) was added to the center well as indicated. The final pH was 6.6 - 6.9.

such treatments of cells brought about changes which resulted in protection of the system(s) involved from sorbate inhibition, since similar protection of enolase in extracts of yeast cells and of cells of lactic acid bacteria was observed by Azukas (1962). Also, there are alternate paths of electron transport in yeast cells and it appeared possible that the presence of sorbate resulted in a shift to an alternate route. Therefore, the next step indicated was to study the effect of sorbate on an ETS relatively free of cell debris and alternate systems. Since active yeast mitochondria are difficult to isolate in an active state, mitochondria were isolated from rat liver for these studies; this appeared to be a satisfactory procedure especially since the cytochromes of yeast and mammalian tissue appear to be very similar (Smith, 1961).

Effect on respiration and oxidative phosphorylation. Since previous results with intact yeast had indicated that ATP synthesis was required for the oxidation of several exogenous substrates, it was decided to observe P/O ratios (the number of atoms of inorganic phosphorus incorporated into organic phosphates, primarily ATP, per atom of oxygen consumed) as well as O_2 uptake with isolated mitochondrial preparations in the presence and absence of sorbate. Sorbate exerted an inhibitory effect on pyruvate oxidation as measured

by O_2 uptake, while exhibiting a minimal effect on oxidative phosphorylation (Table 8). None of the controls showed a significant effect on O_2 uptake or oxidative phosphorylation except caproate which seemed to have an uncoupling action. Thus, it would seem that sorbate was acting as an inhibitor of the ETS, but was not lowering the P/O ratio.

Since sorbate was inhibiting O_2 uptake in a system coupled to oxidative phosphorylation, it was possible that sorbic acid was inhibiting ATP synthesis instead of the ETS per se as is the case with oligomycin. To test this possibility directly, the inhibitory effect of sorbate was investigated with rat liver mitochondria which had "uncoupled" spontaneously during isolation; i.e., showed no phosphate uptake. Since sorbate markedly inhibited O_2 uptake in this system in the presence and absence of 2,4-dinitrophenol (Table 9), sorbate inhibition of O_2 uptake must be involved with the ETS itself and not with the coupled phosphorylation concomitant with electron transport. This agrees with previous results obtained with intact yeast.

Effect on DPNH-cytochrome c reductase and cytochrome c oxidase systems. The assay for the cytochrome c oxidase system was conducted by adding a 1:10 water dilution of the mitochondrial suspension, cytochrome c and excess sodium

Table 8. Effect of sorbic acid on pyruvate oxidation by rat liver mitochondria.

Inhibitor	Concentration M	Uptake: Inorganic Phosphate µmoles	Uptake: Inorganic Phosphate % inhibition	Uptake: Oxygen µatoms	Uptake: Oxygen % inhibition	P/O Value	P/O % inhibition
None*		40	--	19.3	--	2.1	--
Sorbate	(1 X 10^{-2}M)	30	25	19.2	1	1.6	24
	(2 X 10^{-2}M)	21	48	12.1	37	1.7	19
	(4 X 10^{-2}M)	--	--	8.3	57	--	--
	(5 X 10^{-2}M)	14	65	7.4	62	1.9	9
Caproate	(1 X 10^{-2}M)	30	25	21.0	0	1.4	33
	(2 X 10^{-2}M)	30	25	19.6	0	1.5	28
	(4 X 10^{-2}M)	--	--	16.4	15	--	--
	(5 X 10^{-2}M)	17	57	17.0	12	1.0	52
Acetate	(1 X 10^{-2}M)	35	12	18.6	4	1.9	9
	(4 X 10^{-2}M)	35	12	20.4	0	1.7	19
	(5 X 10^{-2}M)	31	22	17.8	8	1.7	19
KCl	(2 X 10^{-2}M)	35	12	18.2	6	1.9	9
	(4 X 10^{-2}M)	39	2	19.2	1	2.0	5
DNP	(2 X 10^{-4}M)	0.03	99.9	23.2	0	0	100

O_2 uptake was determined manometrically at 30 C. The Warburg vessels contained: 18 mg protein, 3.3 µmoles ATP, 16.4 µmoles $MgCl_2$, 164 µmoles glucose, 0.33 µmoles DPN, 4.2 µmoles EDTA, 55 µmoles potassium phosphate, 55 µmoles sodium pyruvate, 22 µmoles sodium glutamate, 2.2 mg (67 KM units) hexokinase, and 109 µmoles sucrose. Phosphate and O_2 values were corrected for endogenous activity. The total volume of each cup was 3.0 ml, and the pH was 7.4.

*Average of 3 cups.

Table 9. Effect of sorbic acid on β-hydroxybutyrate oxidation via an uncoupled electron transport system of rat liver mitochondria.

Inhibitor	Q_{O_2}	% Inhibition
None	7.4	--
Sorbate (5×10^{-3}M)	5.4	27
Sorbate (1.25×10^{-2}M)	1.7	77
Sorbate (5×10^{-2}M)	0	100
DNP* (2×10^{-4}M)	4.6	38
Sorbate (5×10^{-2}M) + DNP* (2×10^{-4}M)	0.42	95
Caproate (5×10^{-2}M)	3.8	49

O_2 uptake was determined manometrically at 30 C. The Warburg vessels contained: 4.8 mg protein and 40 μmoles D,L-β-potassium hydroxybutyrate; the cup contents were otherwise identical to those of Table 8 except that pyruvate and glutamate were omitted. The potassium salts of the inhibitors were used. Q_{O_2} values were corrected for endogenous activity. The total volume of each cup was 3.0 ml and the final pH was 7.4 - 7.7.

*2,4-dinitrophenol.

ascorbate to reduce the cytochrome c to Warburg cups and then measuring the rate of O_2 uptake. The water suspension permits maximum rates, probably because of an increase in mitochondrial permeability.

Sorbate exerted an inhibitory effect on the cytochrome c oxidase system at 1 X 10^{-2}M and higher concentrations; while similar concentrations of caproate showed minimal inhibition (Table 10). The fact that the system was inhibited completely by KCN indicated that a true cytochrome c oxidase system was involved with negligible auto-oxidation of reduced cytochrome c.

DPNH-cytochrome c reductase studies were run with water treated as well as control suspensions of mitochondria so that the mitochondria would be as permeable to sorbate as in the cytochrome c oxidase system. Sorbate exerted no inhibition of the DPNH-cytochrome c reductase system either in water-treated or untreated mitochondria (Fig. 11). The reductase system was not inhibited by antimycin A, but this was to be expected on the basis of reports by Maley (1957) and McMurray et al. (1958). These results strongly suggest that inhibition of the ETS was due primarily to inhibition of the cytochrome c oxidase system.

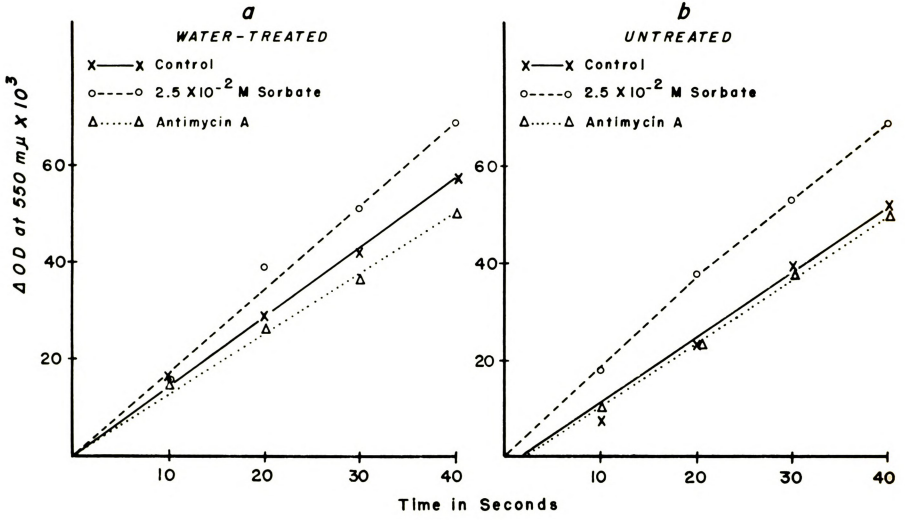
Effect on α-ketoglutarate and succinate oxidations. During studies of the effect of sorbic acid on the ETS

Table 10. Inhibition of the cytochrome c oxidase system of rat liver mitochondria by sorbate.

Inhibitor	Q_{O_2}	% Inhibition
None	590	--
Sorbate (5×10^{-3}M)	480	19
Sorbate (1×10^{-2}M)	440	26
Sorbate (2.5×10^{-2}M)	300	49
Caproate (1×10^{-2}M)	580	2
Caproate (2.5×10^{-2}M)	530	10
Antimycin A	580	2
KCN	10	98

O_2 uptake was determined manometrically at 30 C. The Warburg vessels contained: 0.6 mg protein, 100 µmoles potassium phosphate, 0.24 µmoles cytochrome c, 1.2 µmoles $AlCl_3$, 34.2 µmoles potassium ascorbate and, where indicated, sorbate and caproate as the potassium salts and 0.2 µg antimycin A. KCN (0.2 ml of 1 M KCN) was added to the center well as indicated. Endogenous activity was negligible. The total volume in each cup was 3.0 ml and the final pH was 7.6 - 7.7.

Figure 11. Effect of sorbate on the DPNH-cytochrome c reductase system of water-treated and untreated rat liver mitochondria. OD values were determined spectrophotometrically at 31 C. The cuvettes contained: 0.3 mg protein, 2 mg cytochrome c, 1 μmole KCN, 100 μg DPNH and, where indicated, 0.2 μg antimycin A or potassium sorbate. The total volume was made up to 3.0 ml with 0.25 M sucrose-0.02 M potassium phosphate, pH 7.4. In experiment "a" the mitochondria were diluted 1:10 in cold, triple (glass) distilled water, and in experiment "b" the mitochondria were undiluted.


a
WATER-TREATED
X——X Control
o----o 2.5 X10⁻² M Sorbate
Δ·····Δ Antimycin A
b
UNTREATED
X——X Control
o----o 2.5 X10⁻² M Sorbate
Δ·····Δ Antimycin A
Δ OD at 550 mμ X 10³
60
40
20
10
20
30
40
Time in Seconds

during oxidations of other substrates, a marked stimulation instead of an inhibition of O_2 uptake was observed in the presence of α-ketoglutarate and succinate (Figs. 12 and 13). Potassium caproate was seen to result in an even greater stimulation than sorbate suggesting that these acids were being metabolized in the presence of tricarboxylic acid cycle intermediates as reported by Knox et al. (1948) for fatty acids in general.

To test this hypothesis, high levels of potassium iodide were added to inhibit fumarase so that the effect of sorbate on the succinoxidase system itself could be observed. Preliminary studies showed that 1×10^{-2}M was about the highest level of KI which could be used without completely inhibiting O_2 uptake. At 2.5×10^{-2}M sorbate, succinate oxidation was inhibited in the presence of KI (Fig. 14). Thus, levels of sorbate which inhibited DPN-linked ETS activity did inhibit the succinoxidase system itself. The stimulation by lower levels of sorbate probably resulted due to the fact that 1×10^{-1}M KI does not completely inhibit fumarase so some sorbate oxidation could occur. At higher sorbate levels presumably enough unoxidized sorbate was present to inhibit.

Since the results appeared to agree with the literature which reported that TCA intermediates catalyzed fatty acid

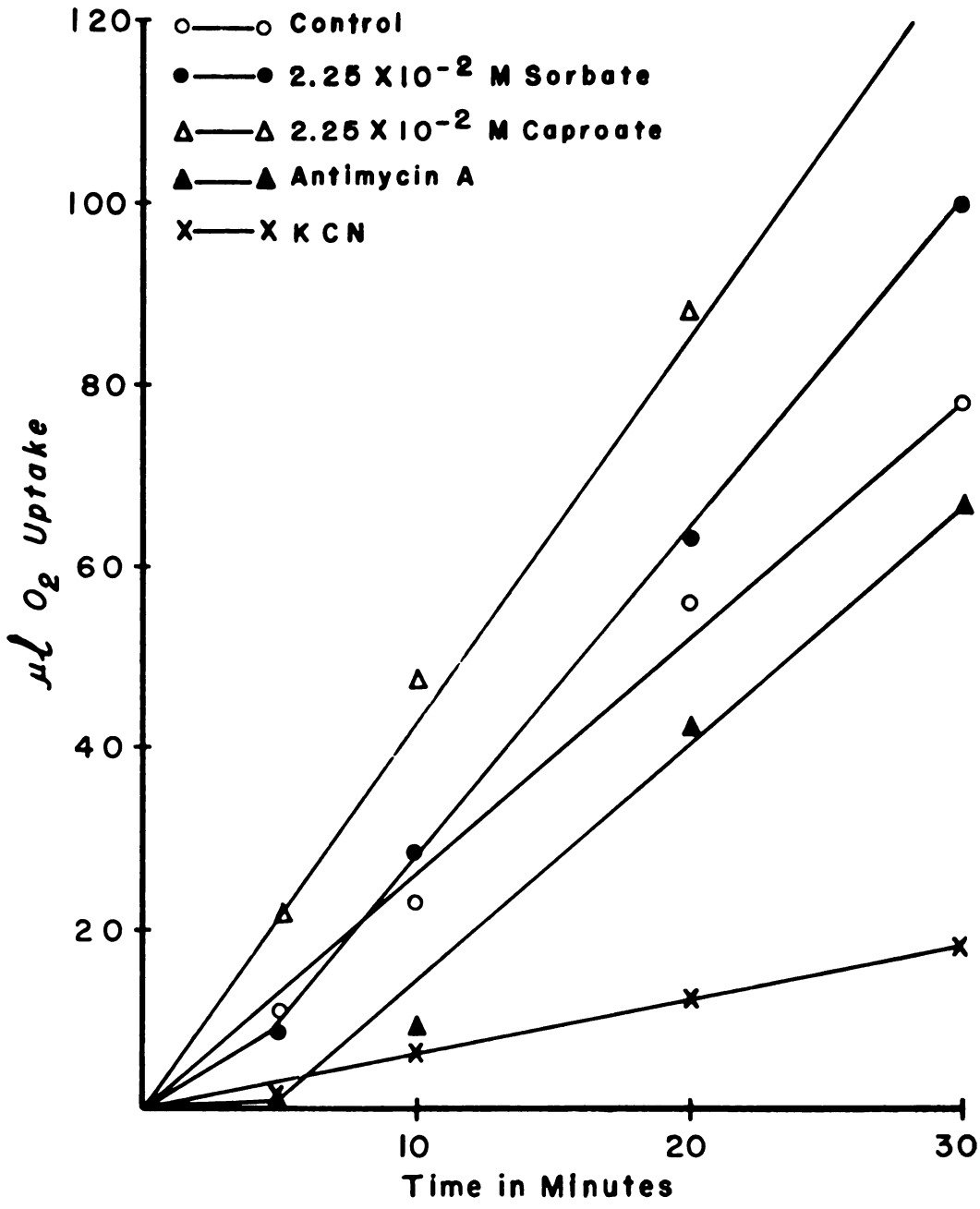


Figure 12. Effect of sorbate on the oxidation of α-ketoglutarate by rat liver mitochondria. O_2 uptake was determined manometrically at 30 C. The Warburg vessels contained: 60 μmoles potassium phosphate, 18 mg protein, 150 μmoles sucrose, 3 μmoles ATP, 15 μmoles $MgCl_2$, 0.3 μmoles DPN, 3.6 μmoles EDTA, 150 μmoles glucose, 40 μmoles potassium α-ketoglutarate and, where indicated, 0.2 μg antimycin A, potassium sorbate, potassium caproate, or 0.2 ml of 1 M KCN in the center well. The total volume of each cup was 3.0 ml, and the final pH was 7.9 - 8.3.

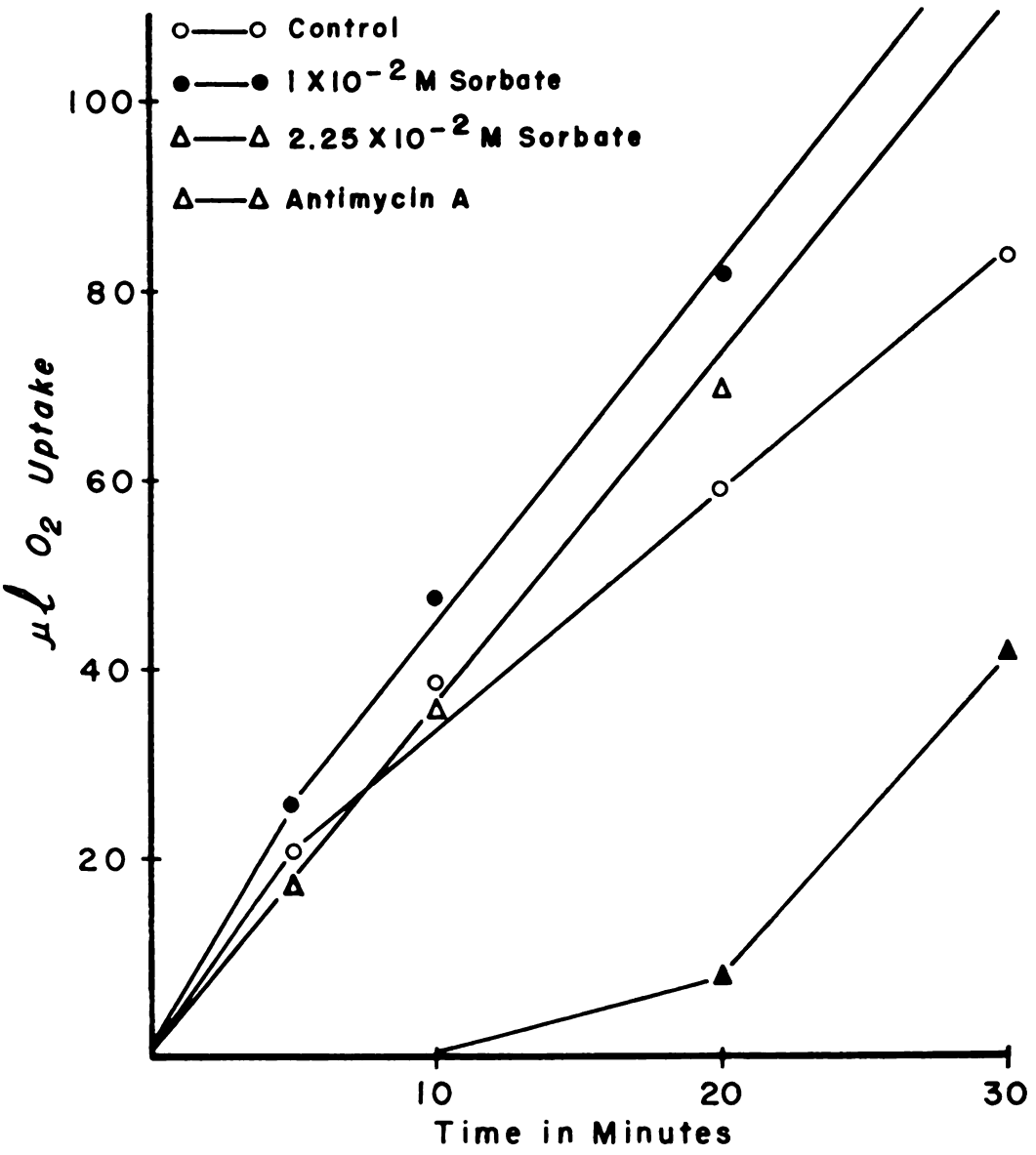


Figure 13. Effect of sorbate on succinate oxidation by rat liver mitochondria. O_2 uptake was determined manometrically at 30 C. The Warburg vessels contained: 60 μmoles potassium phosphate, 15 mg protein, 125 μmoles sucrose, 3.6 μmoles ATP, 18 μmoles $MgCl_2$, 0.36 μmoles DPN, 4.1 μmoles EDTA, 180 μmoles glucose, 40 μmoles sodium succinate and, where indicated, 0.2 μg antimycin A or potassium sorbate. KCN addition resulted in complete inhibition Values were corrected for endogenous activity. The total volume of each cup was 3.0 ml, and the final pH was 7.4 - 7.6.

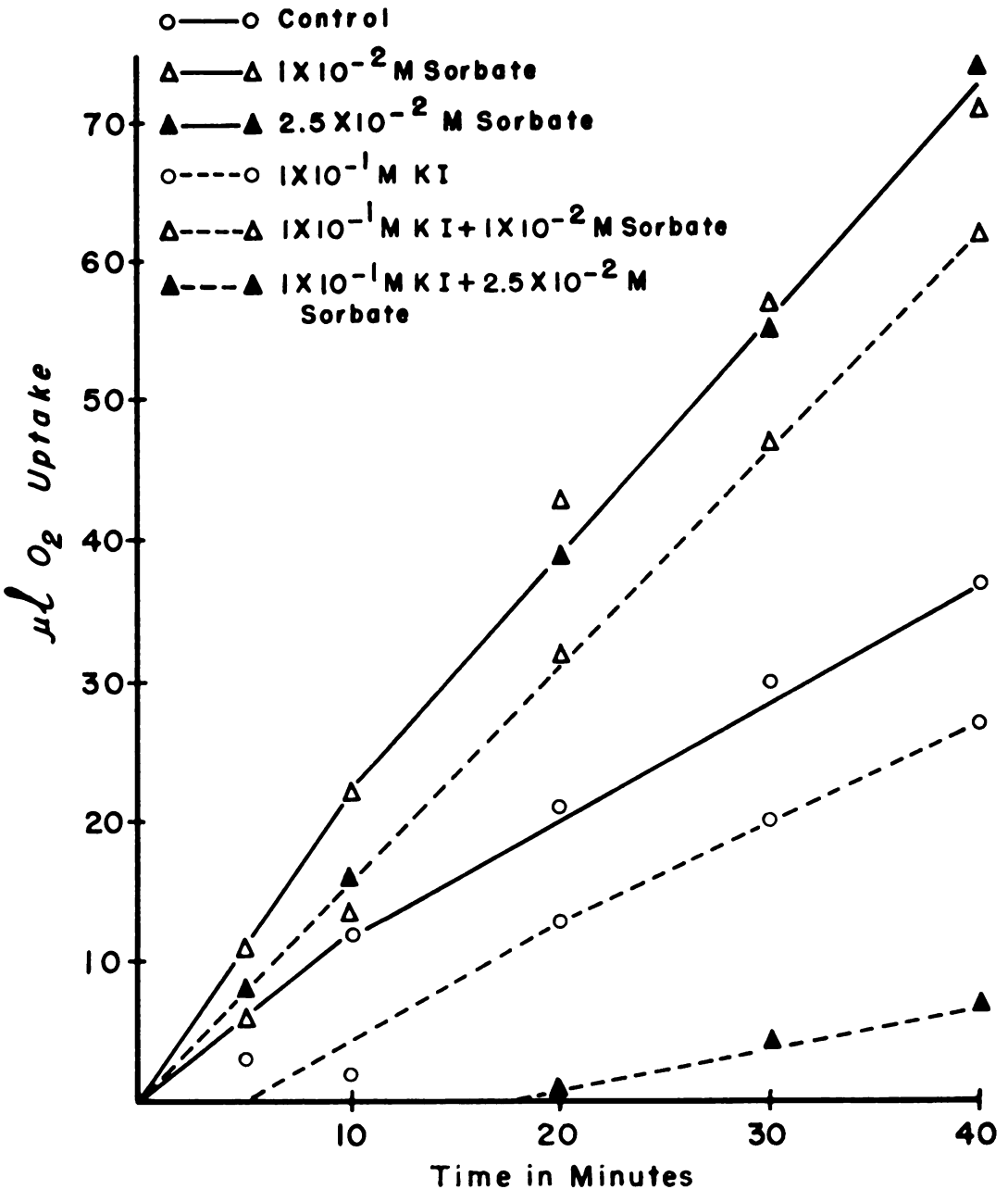


Figure 14. Effect of sorbate on the succinoxidase system of rat liver mitochondria. O_2 uptake was determined manometrically at 30 C. The Warburg vessels contained: 50 μmoles potassium phosphate, 6 mg protein, 150 μmoles sucrose, 3 μmoles ATP, 15 μmoles $MgCl_2$, 3.6 μmoles EDTA, 40 μmoles sodium succinate and, where indicated, potassium iodide and/or potassium sorbate. The total volume of each cup was 3.0 ml, and the final pH was 7.1 - 7.4.

oxidation, low levels of succinate should have exerted a catalytic effect on sorbate oxidation. This was experimentally verified by adding increasing levels of succinate to 2.5 X 10^{-2}M sorbate and observing the increase in respiratory rate (Fig. 15).

The theory of sorbate oxidation was established when the simultaneous disappearance of sorbate and stimulation of respiration occurred in the presence of succinate alone or succinate plus pyruvate (Table 11). The stimulation was not as great at high levels of sorbate (5 X 10^{-2}M), which may indicate some inhibitory action of the sorbate.

The comparative effect of sorbate on O_2 uptake and CO_2 evolution (aerobic) from succinate was investigated (Table 12). A stimulation of O_2 uptake but not of CO_2 evolution indicated that the tricarboxylic acid cycle was not involved in the stimulation. This conclusion was verified when the stimulation was shown not to be inhibited by the aconitase inhibitor, trans-aconitate (Table 12). It was concluded that succinate and α-ketoglutarate were probably stimulating the oxidation of the small quantities of sorbate which penetrated the mitochondria to acetoacetate, and that sorbate could not act as an inhibitor under these conditions.

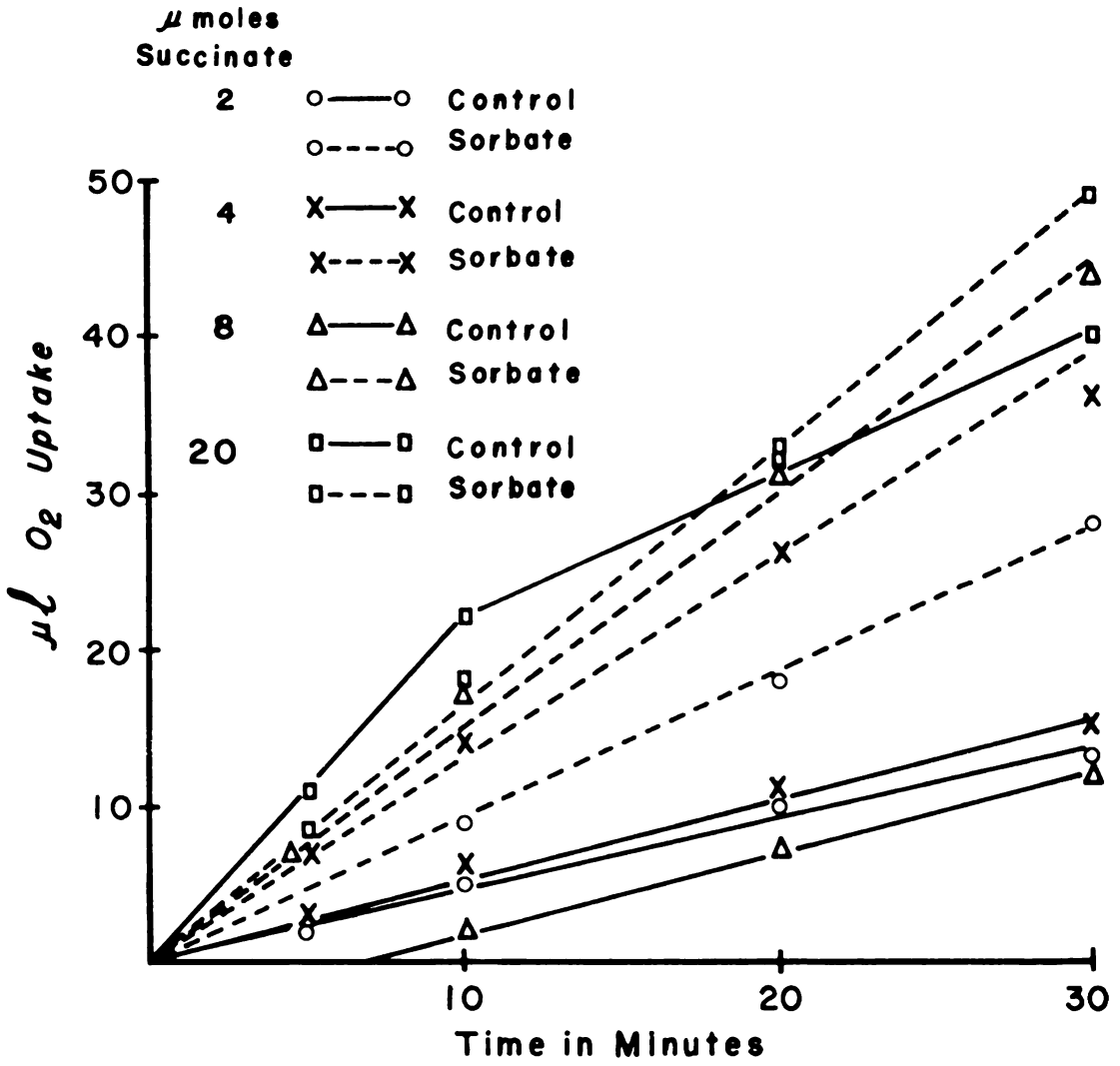


Figure 15. Sorbate-stimulated succinate oxidation by rat liver mitochondria. O_2 uptake was determined manometrically at 30 C. The Warburg vessels contained: 50 μmoles potassium phosphate, 6 mg protein, sodium succinate, 150 μmoles sucrose, 3 μmoles ATP, 15 μmoles $MgCl_2$, 3.6 μmoles EDTA and, where indicated, 2.5 X 10^{-2}M potassium sorbate. The total volume of each cup was 3.0 ml, and the pH was 7.4.

Table 11. Disappearance of sorbate during succinate and pyruvate oxidation by rat liver mitochondria.

Experiment	Reaction time (min)	Additions	Q_{O_2}	$Q_{sorbate}$*
A	50	Succinate	1.1	--
	50	Succinate + 5 X 10^{-3}M Sorbate	10.8	0.50
	190	Succinate	5.0	--
	190	Succinate + 5 X 10^{-3}M Sorbate	11.2	0.20
B	195	Pyruvate	4.0	--
	195	Pyruvate + Succinate	12.9	--
	195	Pyruvate + Succinate + 1 X 10^{-2}M Sorbate	34.0	0.28
	195	Pyruvate + Succinate + 5 X 10^{-2}M Sorbate	14.3	0

O_2 uptake was determined manometrically at 30 C. The Warburg vessels contained: experiment A, 60 µmoles potassium phosphate, 3.6 µmoles ATP, 18 µmoles $MgCl_2$, 4 µmoles EDTA, 150 µmoles sucrose, 8 µmoles succinate, 4.2 mg protein and potassium sorbate as indicated; experiment B, 50 µmoles potassium phosphate, 3 µmoles ATP, 14 µmoles $MgCl_2$, 3 µmoles EDTA, 75 µmoles sucrose, 2.8 mg protein and, where indicated, 60 µmoles sodium pyruvate, 8 µmoles sodium succinate and potassium sorbate in the concentrations stated. The total volume for each cup was 3.0 ml and the final pH for each experiment was 7.25 - 7.35.

*µmoles of sorbate uptake per mg protein per hour at 30 C.

Table 12. Effects of sorbate and trans-aconitate on succinate and citrate metabolism by rat liver mitochondria.

Additions	Q_{CO_2}	Q_{O_2}
Endogenous	1.6	
Endogenous + Sorbate	1.6	
Succinate	2.8	
Succinate + Sorbate	2.9	
Endogenous		2.9
Endogenous + Sorbate		3.7
Succinate		8.8
Succinate + Sorbate		12.4
Citrate		3.7
Citrate + trans-Aconitate		2.4
Succinate		4.8
Succinate + Sorbate		8.1
Succinate + trans-Aconitate		3.5
Succinate + Sorbate + trans-Aconitate		8.9

O_2 uptake was determined manometrically at 30 C. The Warburg vessels contained: 50 µmoles potassium phosphate, 3 µmoles ATP, 15 µmoles $MgCl_2$, 3.6 µmoles EDTA, 150 µmoles sucrose, 10.8 mg protein and, where indicated 8 µmoles sodium succinate, 4 µmoles potassium citrate and a final concentration of 1 X 10^{-2}M potassium sorbate and 1 X 10^{-2}M potassium trans-aconitate. The total volume of each cup was 3.0 ml and the final pH was 7.1 - 7.4.

DISCUSSION

Studies made with intact yeast cells and with isolated liver mitochondria indicate that the site(s) of sorbic acid inhibition of respiration is in the cytochrome c oxidase system. The principle inconsistency among the results which prevents the arrival at a more definite conclusion is the repeated failure to show sorbate inhibition of respiration with either crude preparations of yeast mitochondria or with yeast cells which had been treated to alter their permeability. However, three possible explanations for such results are apparent. First, it is conceivable that all methods of altering or removing the permeability barrier resulted in electron transport by an alternate system (e.g., a peroxidase system) which by-passed the sorbate-sensitive site, or made it no longer rate-limiting. Such a pathway may not involve oxidative phosphorylation, and thus would function only when an active transport of substrate is not necessary. Another possible explanation is that sorbate inhibits only an ETS occurring in the cell membrane and not that occurring in the mitochondria because of a difference in enzyme composition. However, one would have to assume that all ATP for active transport was generated

in the cell membrane and, therefore, sorbate inhibition of the membrane ETS would stop all active transport. Finally, it is possible that all the treatments resulted in a release of intracellular materials from compartments of the cell which reacted with sorbate in some manner and neutralized its inhibitory action. This theory is supported by the observation that sorbate inhibition was observed when a purified mitochondrial system (rat liver) was employed. An attempt was made to prove this hypothesis by adding a crude yeast suspension to isolated liver mitochondria; however, the yeast preparation itself inhibited respiration of the mitochondria so that the results were inconclusive. Azukas et al. (1961) observed the protective effect of crude yeast extracts; i.e., 5×10^{-3}M sorbate inhibited enolase in crude extracts 26 to 29% while crystalline yeast enolase was completely inhibited by 2.5×10^{-4}M sorbate.

Not only did sorbate not inhibit respiration by crude yeast extracts, but a definite stimulation of the rate of DPNH oxidation was observed spectrophotometrically. The same preparations, however, did not show an increased rate of O_2 uptake with DPNH as substrate. If an explanation could be found for these results, the inconsistency noted above would probably be explained.

Several theories can be developed to explain sorbate stimulation of DPNH oxidation at 340 mμ using crude yeast mitochondria. It is well known that ETS activity is stimulated in cell-free extracts by uncoupling agents since oxidative phosphorylation is rate-limiting in coupled ETS activity. However, it was shown that sorbate did not uncouple oxidative phosphorylation of liver mitochondria. Also, using intact yeast, sorbic acid was found to inhibit while 2,4-dinitrophenol stimulated endogenous respiration. An increase in mitochondrial permeability by sorbate does not appear to be the explanation either since liver mitochondria evidenced a lowered respiratory rate upon sorbate addition. One reasonable explanation of the sorbate-induced stimulation is that sorbate is functioning as a terminal electron acceptor, since, if one were measuring O_2 uptake instead of DPNH disappearance, the effect would be one of inhibition. Sorbate would have to be functioning at the cytochrome c oxidase level since the stimulation is partially antimycin A- and KCN-sensitive and ETS inhibition occurs in this region.

One might dispute the theory of sorbate function as an electron acceptor in the cytochrome c oxidase system on the grounds that the electron flow tends to be from the system of lower potential (more negative) to the system of higher potential. Since the E'_o of the succinate-fumarate

oxidation-reduction system which is probably similar to that of sorbate is 0.031 while the E'_o of the cytochrome c couple is 0.25 (Dolin, 1961a) it would seem unlikely that sorbate would accept electrons at the cytochrome c oxidase level. However, Neilands and Stumpf (1958) point out that "simply because a certain system has a slightly lower potential than another system does not mean that the former cannot oxidize the latter. The driving force in the reaction will depend on the actual concentrations or activities of the reactants, and these may be sufficient to overcome an unfavorable potential difference." This quotation takes on considerable significance in the present discussion when one considers the fact that relatively high concentrations of sorbate were required to bring about the stimulation. Also, Dolin (1961a) states that "the potential of electron transfer coenzymes may change on binding of these compounds to protein." Therefore, if part of the sorbate which enters the mitochondria were bound by the proper protein and part remained unbound in solution, the E'_o of each form might differ. Conceivably, the E'_o of the unbound sorbate could be such that it would accept electrons from cytochrome a_3 while the bound sorbate would accept electrons before cytochrome a_3 in the ETS. This theory could explain the partial inhibition by KCN of sorbate-stimulated DPNH oxidation. Thus, KCN inhibits

cytochrome a_3 so that only bound sorbate could accept electrons. But since the sorbate is bound it would not equilibrate rapidly with free sorbate and the bound sorbate would be rapidly reduced and complete inhibition would follow. Since the site of antimycin A inhibition occurs much further back in the ETS, this inhibitor would be expected to completely inhibit DPNH oxidation with or without added sorbate as was observed. In the absence of inhibitors and with excess DPNH available, the sorbate-induced stimulation observed would be expected to be constant since the unbound reduced sorbate should be present in excess and also might equilibrate with extra-mitochondrial sorbate. The lack of apparent inhibition or stimulation observed manometrically could be simply caused by the fact that sorbate was inactivated by the relatively large amounts of crude enzyme preparation required.

Based on the above theory, one might expect a decrease in sorbate concentration to occur in media containing yeast and sorbate under inhibitory conditions. Experimentally, however, no sorbic acid decrease is observable (Costilow et al., 1955). This could be due to the fact that the amount of sorbate reduced might be below the precision of the assay. For example, it has been shown (Oka, 1960) that yeast accumulate

sorbate intracellularly and yet this sorbate loss was apparently not detectable in the previous study. Also, it is conceivable that sorbate is not the true inhibitor; once inside the mitochondria sorbate might be metabolized to another compound which could then be reduced by the ETS to the final inhibitory form. A mechanism such as this might require only very small amounts of sorbate so that the change in sorbate concentration might not be detectable.

Smith and Lester (1961) have postulated the oxidation-reduction of benzoquinones in their interaction with the ETS to explain their stimulatory action on the DPNH-cytochrome c reductase and DPNH oxidase systems as measured by absorbency changes at 340 mμ. It may well be of significance that both benzoquinones and the α, β-unsaturated acids have the α, β-ketone structure which is well known as being active in fungi inhibition. Quinones are widely used in organic chemistry as hydrogen acceptors. It is theoretically possible that sorbate could also accept hydrogen, although it would probably not be as effective as the quinones. This may be reflected by the observation that nonquinonoid α, β-unsaturated ketones are not as active as quinones against fungi (Cochrane, 1958).

An alternative explanation of DPNH stimulation by sorbate is that sorbate could be inhibiting the cytochrome c

oxidase system, while stimulating the cytochrome c peroxidase pathway. This stimulation by sorbate could be due to catalytic levels of sorbate peroxides or simply by the sorbate anion; such a stimulating characteristic was ascribed to anions of the lower fatty acids by Dolin (1957). Chance (1954) has shown that in intact respiring yeast, exogenous H_2O_2 is utilized even more rapidly than O_2 for the oxidation of reduced endogenous cytochrome c; therefore, the cytochrome c peroxidase system would appear to have a greater respiratory capacity so that a shift to it from the cytochrome c oxidase activity would result in a faster rate of DPNH oxidation. KCN and azide will inhibit yeast cytochrome c peroxidase so that the H_2O_2 produced might build up and rapidly destroy the ETS so that inhibition rapidly would ensue as was observed spectrophotometrically and manometrically. The stimulation might not occur with intact cells because the DPNH-cytochrome c peroxidase pathway might not result in ATP production so that active substrate transport could not occur. The inhibition of glucose oxidation by intact cells may be explained by the inhibition of enolase alone (Azukas et al., 1961).

There was no inhibition of cytochrome c reductase systems in either liver mitochondria or in yeast extracts. The reductase system of liver mitochondria was not inhibited by antimycin A. Therefore, it could be argued that the

system was an artifact and that it was only for this reason that sorbate did not inhibit. However, antimycin A insensitivity occurs in rat liver mitochondria when a sufficient level of cytochrome c is added to the system (Maley, 1957). She showed that in the presence of at least 10^{-4}M added cytochrome c, antimycin A inhibition is reversed in the oxidation of external DPNH by rat liver mitochondria. Similarly, McMurray et al. (1958) observed that the DPNH-cytochrome c reductase activity of sonic extracts of liver mitochondria was completely insensitive to 1 μg of antimycin A. McMurray concluded that the addition of sufficient cytochrome c results in a non-phosphorylating (uncoupled) system and that antimycin A sensitivity is always associated with phosphorylation in this region of the ETS. Therefore, the absence of antimycin A inhibition in the present study may be due to the fact that the system was uncoupled. Our data indicated, however, that sorbate will inhibit the overall ETS activity of an uncoupled liver preparation so that it seems probable that sorbate simply is not capable of inhibiting DPNH-cytochrome c reductase activity appreciably. This is confirmed by the lack of sorbate inhibition of yeast DPNH-cytochrome c reductase, a system which is antimycin A-sensitive.

The carbonyl function, $\overset{\backslash}{\underset{/}{C}} = O$ (I), is known to have

the resonance structure, $\dot{C}^{+} - \bar{O}$ (II). During a chemical reaction the molecule reacts as though it possessed that contributing structure most suitable to the electronic demands of the attacking reagent and reaction conditions. Structure II is known to contribute to a great extent to the chemical reactivity of carbonyl groups and explains their reactivity towards nucleophilic reagents; i.e., the carbonyl group reacts by virtue of its positive carbon atom rather than the negative nature of the oxygen atom. The α, β-unsaturated systems constitute an interesting special case of conjugation since the conjugated system may be considered an elongated carbonyl group. Such a system should exhibit electrophilic reactivity at carbon atoms 2 and 4:

$$\overset{4}{C} = \overset{3}{C} - \overset{2}{C} = \overset{1}{O}.$$

It is to be expected, then, that carbonyl and α, β-unsaturated systems should react with such nucleophilic reagents as water, alcohols, amino compounds and sulfhydryl groups (Royals, 1954). There are indications in the literature (Barron and Singer, 1945; Gordon and Quastel, 1948) that the cytochrome c oxidase system is not inhibited by reaction of free sulfhydryl groups with specific reagents. However, sorbate might well inhibit an oxidase enzyme(s) by combining with a free alcohol or amino group which is essential for enzymatic activity.

Not all enzymes in metabolism are equally important when considering them as possible sites of inhibition. The potential activity of many enzymes is much in excess of normal physiological requirements so that a considerable inhibition of their activity does not necessarily cause a significant disturbance of cell metabolism; fumarase is an example. There are, however, rate-limiting reactions in metabolism which determine the over-all rate of a series of reactions because they are the slowest reaction in the series. Rate-limiting reactions, then, are those against which inhibitors can make their effects most easily evident. Two general types of rate-limiting reactions are (a) those reactions which initiate the degradation of primary substrates; e.g., of glucose, amino acids, fatty acids, and molecular oxygen; and (b) reactions occurring at intermediary stages where, after a partial degradation, more than one pathway is open. One main class of respiration inhibitor is the type which interferes with the reactions initiating the utilization of O_2 such as HCN, azide and CO (Krebs, 1958).

As pointed out by Dolin (1961b), it is widely believed that the only physiologically important pathway in yeast for flavoprotein reoxidation by O_2 is mediated by KCN- and CO-sensitive iron carriers (Warburg and Christian, 1933). Dolin also points out that "this belief was further

strengthened by the finding that in intact yeast cells the rate of cytochrome reduction is equal to the over-all respiratory rate (Haas, 1934), and by the subsequent isolation of flavoproteins that catalyzed rapid cytochrome c reduction by reduced pyridine nucleotides." The observation by Smith (1954a) that yeast respiration is inhibited by CO which did not inhibit cytochrome c peroxidase emphasizes the fact that cytochrome c oxidase inhibition of yeast will result in the inhibition of yeast respiration.

Costilow et al. (1955) found that about 1 X 10^{-3}M to 4 X 10^{-3}M sorbic acid at pH 4.6 completely inhibited growth of ten different genera of yeasts common to cucumber fermentations. Hsu (1957) noted that at pH 4.2 this same range of sorbic acid concentration greatly inhibited the metabolism of baker's yeast. Thus, it appeared that the sorbic acid inhibition of growth and metabolism of yeast were closely related. The present studies indicate that ETS activity is inhibited about 50% at 3 X 10^{-2}M sorbate with rat liver mitochondria. Hsu (1957) presented data indicating that about 50% inhibition of aerobic pyruvate oxidation occurred with intact yeast at pH 4.2 in the presence of 1 X 10^{-3}M sorbic acid. If there were a 13 X intracellular concentration of sorbate as reported by Oka (1960), the intracellular concentration of

sorbate would be about 1.3 X 10^{-2}M with this external concentration. If one takes into account the difference in pH of yeast cytoplasm and the pH of *in vitro* liver mitochondrial studies, pH 5.8 and 7.4 respectively, it is evident that more sorbic acid is likely to permeate yeast mitochondria *in vivo* than liver mitochondria *in vitro*. This equates even more the sorbate level required for 50% inhibition for the two systems, and supports the theory of the ETS as the primary site of inhibition of yeast respiration and growth.

The intracellular sorbate concentration must, of course, be very important in microbial inhibition. The most striking example of this, perhaps, is that reported by Melnick et al. (1954) where high mold populations on cheese were able to metabolize sorbic acid and growth was not inhibited. At low mold populations, however, mold growth was inhibited. In regard to yeast, our work has revealed no capacity of these organisms to metabolize significant amounts of sorbate so that it is possible that yeast lack the fatty acid activating enzyme necessary to form the co-enzyme-A derivative. In rat liver mitochondria, about 3 X 10^{-2}M sorbate was found to be necessary for 50% inhibition; but even at this level inhibition was antagonized and sorbate was metabolized if a sufficient level of tricarboxylic acid

cycle intermediate was present. A balance between intracellular concentration and the rate at which the cell can metabolize sorbate may well spell the difference between inhibition and non-inhibition.

Since it appears that the respiration of catalase positive organisms is being inhibited at the cytochrome c oxidase level it follows that oxidative phosphorylation would also be stopped. Therefore, the result would be the inhibition of active transport and hence an inhibition of substrate permeation. The penetration of compounds which are metabolized along pathways yielding substrate phosphorylation would also be inhibited since sorbate has been shown to inhibit enolase (Azukas et al., 1961). Sorbate, then, is probably acting by slowing respiration and ATP synthesis until the cell is at a "starvation" state, and endergonic (ATP requiring) reactions required for protein, carbohydrate, lipid and nucleic acid synthesis are inhibited.

The determination of the primary site of inhibition of an antimicrobial agent is not an easy thing to accomplish, as indicated by Jawetz et al. (1960) who stated that with the possible exception of polymyxin the primary mode of action of antibiotics in chemotherapy is unknown in spite of the extensive work which often has revealed very specific modes of action in vitro. Thus forewarned, one hesitates

to state unequivocally that he has located the primary site of inhibition of an antimicrobial compound, but he can at least be optimistic that this may be the case. We are encouraged in this respect because it is the catalase positive organisms which are most susceptible to sorbic acid and most of these organisms utilize a cytochrome c oxidase system. Therefore, the catalase negative organisms may be much more resistant to sorbic acid primarily because they do not depend on this system. Hsu (1957) concluded from his observations that sorbic acid was probably inhibiting respiration from both glucose and pyruvic acid oxidation by the same mechanism since each substrate oxidation was inhibited to about the same extent; and Palleroni and De Pritz (1960) suggested that sorbate inhibited citrate and higher fatty acid synthesis from acetate. The inhibition of the cytochrome c oxidase system by sorbic acid can explain both of the above findings and theories. The inhibition of synthesis from acetate would follow since the resulting lack of oxidative phosphorylation would minimize the ATP available for anabolic metabolism.

SUMMARY

Taking into perspective the results of the present study, the evidence implicates the cytochrome c oxidase system as the primary site of sorbic acid inhibition of catalase positive microorganisms growing aerobically. This is indicated by the fact that sorbic acid markedly inhibits intact yeast respiration with pyruvate, acetate, ethanol, acetaldehyde, DPNH, TPNH, ascorbate, and lactate as substrates at low pH values; and this respiration is both KCN- and azide-sensitive. Also, sorbate inhibited O_2 uptake by liver mitochondria with pyruvate, β-hydroxybutyrate, succinate or reduced cytochrome c as substrates. The DPNH-methylene blue reductase system of intact yeast and the DPNH-cytochrome c reductase activities of liver and yeast mitochrondrial preparations are sorbate-insensitive which tends to eliminate the DPNH-cytochrome c portion of the electron transport system as containing the site of sorbate inhibition. The inhibition appears to be independent of oxidative phosphorylation since sorbate does not lower the P/O ratio with liver mitochondria nor is the inhibition of respiration by liver mitochondria or intact yeast (endogenous) reversed by 2,4-dinitrophenol. Sorbate inhibition of substrate permeation

might explain the findings with intact yeast; but this hypothesis does not appear to be correct since endogenous O_2 uptake was inhibited and anaerobic pyruvate decarboxylation was not. A breakdown of the cell permeability barrier resulting in a loss of cofactors and acidification of cytoplasm is not a suitable hypothesis either, since this action is usually found with surface active compounds, and sorbate is not surface active.

It should be pointed out, however, that the data are not without contradiction in identifying the site of inhibition. Thus, no sorbate inhibition of respiration was observed with either crude preparations of yeast mitochondria or with yeast cells which had been treated to alter their permeability. Possible explanations for these results are that the treatments permitted the activation of an alternate pathway of electron transport, that only cell membrane electron transport is inhibited, or that a material(s) was released during treatment which neutralized the inhibitory action of sorbate.

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