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The Enzymatic Oxidation of Aldehydes by Xanthine Oxidase

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THE ENZYMATIC OXIDATION OF ALDEHYDES BY XANTHINE OXIDASE

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I. INTRODUCTION

As early as sixty years ago, the existance of an enzyme from mammalian tissues which catalyzed the oxidation of xanthine and hypoxanthine was recognized. At about the same time, Schardinger found that a substance in milk catalyzed the oxidation of aldehydes; presumably to their corresponding carboxylic acids(1). The product of the enzymatic oxidation of the aldehydes was not at this time isolated. Though these two observations remained unrelated for many years, it has now been generally accepted that the two enzymes are one and the same(1). The name given the enzyme was derived from its earliest recognized action, the oxidation of xanthine, hence xanthine oxidase, hereinafter referred to as XO. The Enzyme Commission's numbering system assigns to the enzyme the number 1.2.3.2.(2). The first number indicates the main group to which the enzyme belongs, the oxidoreductases; the second number indicates the group in the donor which undergoes oxidation, an aldehyde or keto group. The third figure indicates the primary acceptor involved, in this case, molecular oxygen. The fourth number is merely the serial number of the enzyme in the sub-sub class indicated by the third figure.

Procedures for obtaining the enzyme from chicken, calf and rabbit liver as well as the main source, milk, have been described(1, 3,4). The purifications from milk constitute most of the commercial preparations, and were obtained 80% pure as long ago as 1939(5), while the crystalline material was first obtained in 1954(6).

The molecular weight of the enzyme has been determined by various methods to be in the neighborhood of 290,000 amu(6). The most reliable data indicate that there is a one to one ratio of molybdenum to FAD(flavin-adenine dinucleotide) in the enzyme(1,7). The FAD to iron ratio has been found to be one to four for the milk enzyme and for the calf liver enzyme(1). Heat denaturation usually liberates both metals and the FAD somewhat, with a corresponding loss of activity(8). The molecular weight and the flavin content of the milk enzyme indicate two moles of FAD and therefore two moles of molybdenum and eight moles of iron(6).

The absorption spectrum, Figure 1, shows characteristic peaks at 280 and 450 nm(9). The ratio of absorbances at 280 and 450 nm is used as an indication of purity, with the lowest ratio indicating the most purity. The lowest 280/450 nm ratio found for the crystalline enzyme was 5.0(7), and the value of $\varepsilon \begin{matrix} 1\% \\ 1cm \end{matrix}$ (450 nm) was 2.3. This plus the molecular weight allow the molar absorptivity coefficient to be calculated as $\varepsilon_{450} = 70{,}000$(1).

The enzyme appears to be very versatile, catalyzing the oxidation of many purines such as xanthine and hypoxanthine to uric acid. Somewhat over one hundred different substrates have been found to be affected by the enzyme(2). Almost all the aldehydes may be oxidized, and although a product analysis has not been reported, the assumed products are the corresponding carboxylic acids(1). The Michaelis constants for the aldehyde reactions are usually higher than for the purine substrates, indicating the enzyme has a greater affinity for

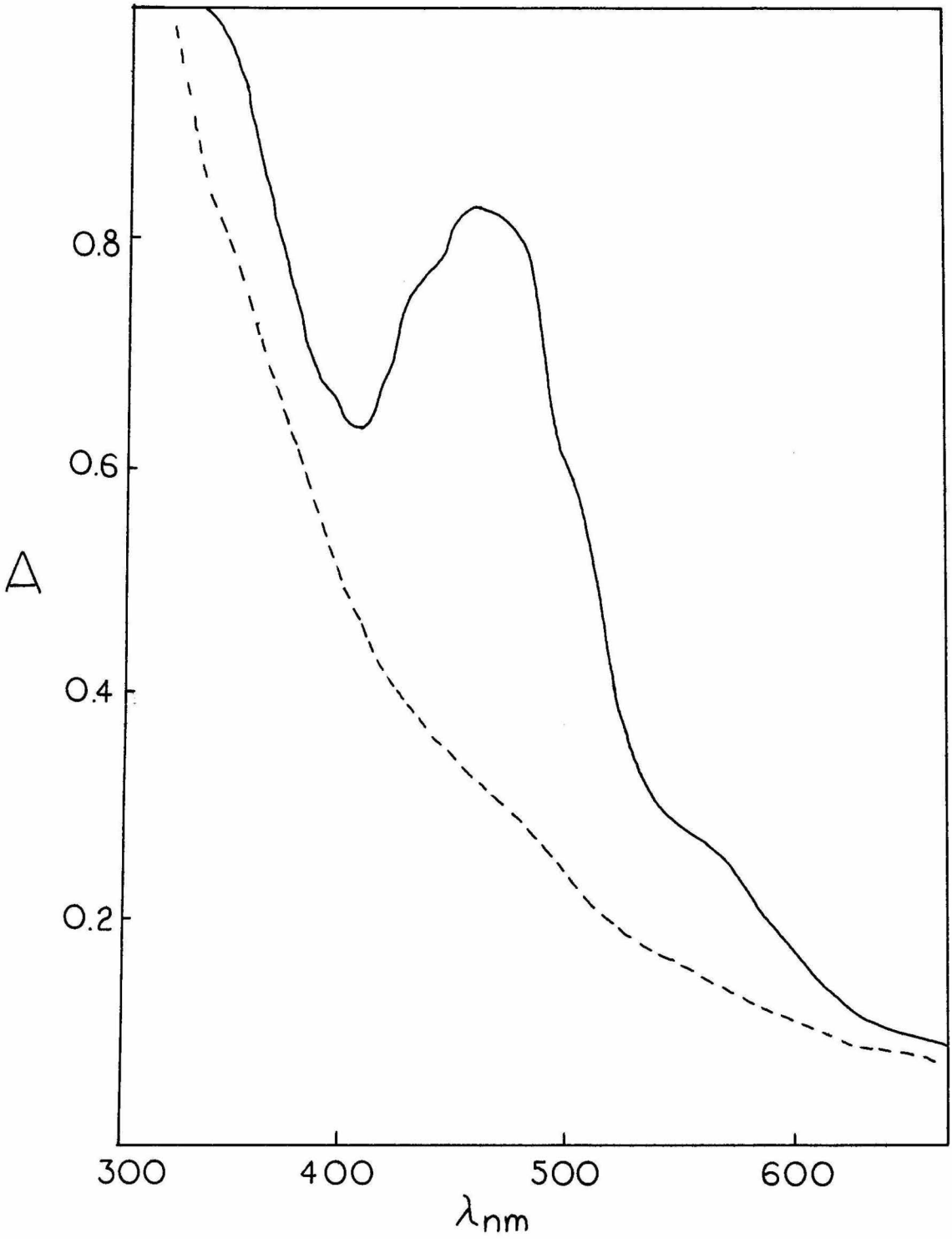


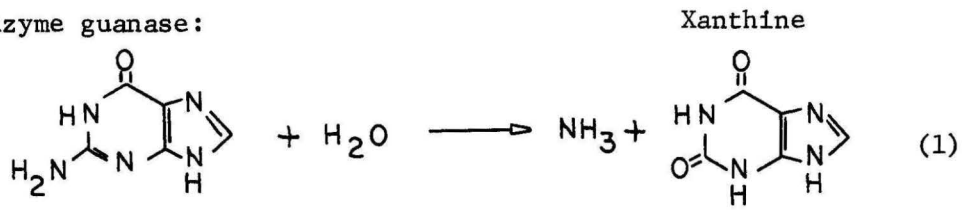
Figure 1. Absorption spectrum of xanthine oxidase, from Massey et al.(9). The solid line represents the oxidized form. The broken line represents the reduced form.

the purines(1). The enzyme shows little specificity for electron acceptors, with oxygen, a number of dyes, certain nitrogen compounds, ferricyanide and cytochrome C all serving in this capacity(5). In the case of cytochrome C, there has been considerable disagreement among investigators, some finding a high degree of affinity, while others find a very low affinity. It is now felt that this disparity in results was caused by the presence of myoglobin, a common contaminant of cytochrome C which has been found to inhibit several of the enzyme reactions(10). Horecker and Heppel(4), have reported that molecular oxygen is essential for the enzymatically catalyzed reduction of cytochrome C by hypoxanthine and acetaldehyde. The present work substantiates this finding. The acceleration of the reduction of cytochrome C with added oxygen concentration has been found to continue up to nearly 100% oxygen(4). While the specificity of xanthine oxidase obtained from sources other than cow's milk has not been widely investigated, available data does not differ appreciably from that of the milk enzyme(11,12).

As might be expected in the case of an enzyme with such a low substrate specificity, there are a great many substances which can act as inhibitors (usually competitive) for XO(9). Certain metals act as powerful inhibitors, as do many purines which themselves are inert to the enzymatic oxidation(7). The enzyme is particularly susceptible to substrate inhibition, as will be shown later in this paper(13,14,15). As previously mentioned, myoglobin and the corresponding globin are strong inhibitors for the reduction of cytochrome C.

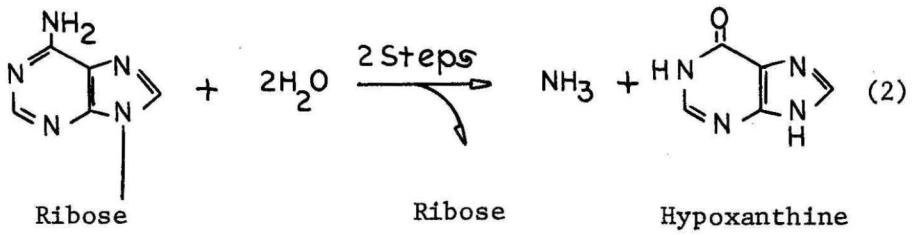
Cyanide has been shown to be an effective, irreversible inhibitor, while arsenite and methanol are reversible inhibitors(10,15).

Although little is known about the biological functions of the enzyme, the high affinity shown for xanthine and hypoxanthine may indicate that these are the most significant substrates biologically (16). Xanthine and hypoxanthine occur naturally in the catabolism or breakdown of the purines in mammals. In man and other primates, the principle excretory product resulting from purine catabolism is uric acid. Xanthine oxidase has been shown to catalyze one of the steps in a pathway by which uric acid may be formed(17). Following cleavage of the N-glycosidic bond, guanine is converted in one step to xanthine by the enzyme guanase:



(1)

The degradation of adenine derivatives in mammals and birds occurs with deamination of adenosine and adenylic acid, followed by conversion to hypoxanthine:

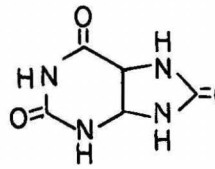


(2)

Hypoxanthine is converted to xanthine and subsequently to uric acid by XO(18):

$$\text{Hypoxanthine} + O_2 + H_2O \rightarrow \text{Xanthine} + H_2O_2 \qquad (3)$$

$$\text{Xanthine} + O_2 + H_2O \rightarrow \text{Uric Acid} + H_2O_2 \qquad (4)$$



A case has been reported of a human who was completely lacking in the enzyme, and who apparently suffered no observable ill effects (19). One investigator has reported that XO production rises markedly during virus multiplication, which may indicate that the enzyme has a controlling effect on the pattern of nucliec acid synthesis(17). Other reports have shown that the enzyme is present in very low concentrations in tumor-bearing animals, and when several of these animals were treated with XO, some retardation of tumor growth was apparant (20). For this reason, cancer research has provided a recent stimulus for work with xanthine oxidase(20).

Although XO is one of the most widely studied enzymes, very little is known regarding the mechanism of its catalytic action. The true importance of the enzyme in areas such as cancer research cannot really be determined until more information is obtained concerning the active site or sites and the mechanism of the enzymatic catalysis. In this light, various studies are being made concerning different substrates under a variety of experimental conditions(21). It has recently been shown, for example, that molybdenum plays a primary role

in the catalytic sequence of internal electron transport(22,23). Using rapid freezing techniques and electron paramagnetic resonance spectroscopy, the investigators obtained results which suggested that molybdenum was the first component of the enzyme to be reduced during the catalytic cycle. Similar studies(15) have shown that characteristic inhibitors such as cyanide, methanol and arsenite all attack and bind the pentavalent molybdenum component of the enzyme. Studies such as these have led to and support the internal transfer scheme of substrate-Mo-FAD-Fe-electron acceptor (O_2, cytochrome C, etc.). Deflavo xanthine oxidase has recently been prepared and studied by Komai and others(9,24). The FAD group on the enzyme was removed by treatment with high concentrations of $CaCl_2$. Rapid reaction studies have shown that the deflavoenzyme is rapidly reduced by xanthine and certain aldehydes, and that the loss of xanthine reductase activity is due to the fact that the reduced deflavoenzyme has a negligable re-oxidation rate with O_2. The deflavoenzyme was catalytically active in the oxidation of xanthine with acceptors such as ferricyanide and cytochrome C. On the basis of these studies, electron transport pathways in the absence of the FAD component have been proposed(24).

It was early recognized that the rate of an enzyme catalyzed reaction increases with increasing substrate concentration until a concentration is reached beyond which further additions give no rise in velocity. Provided the reaction is performed under carefully controlled conditions, the variation of reaction rate with substrate concentration for a large number of enzyme catalyzed reactions is

described by the Michaelis-Menten equation:

$$v_o = \frac{V_m(S)}{(S) + K_m}, \qquad (5)$$

where v_o is the initial velocity, V_m is the maximum velocity and K_m is the Michaelis constant, which will be discussed below. In order for this expression to be valid, the reaction must involve a single substrate, or the concentrations of any other substrates must be held constant. Initial velocities are defined as the rate of disappearance of a substrate or appearance of a product, extrapolated to time zero (17). The substrate concentrations must be much greater than the enzyme concentration, and all other variables such as temperature and pH must be defined and held constant in all measurements. A series of initial velocities at different substrate concentrations may be plotted using the recipricol of equation 5 above as described by Lineweaver and Burke(27). A typical plot is shown in Figure 2:

$$\frac{1}{v_o} = \frac{K_m}{V_m} \cdot \frac{1}{(S)} + \frac{1}{V_m} \qquad (6)$$

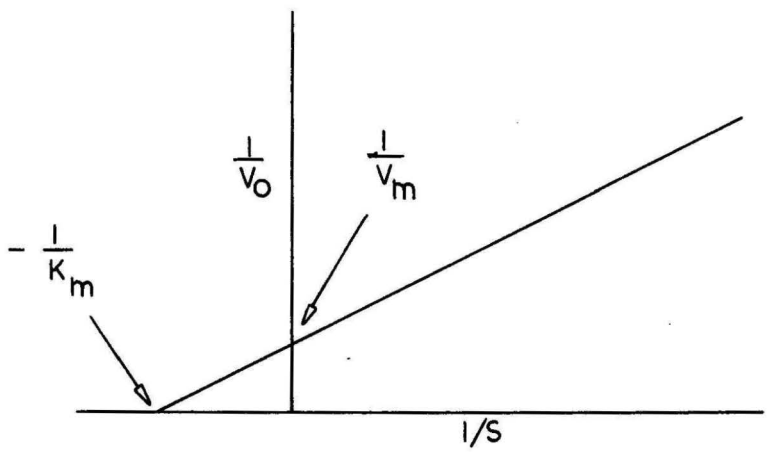


Figure 2. Typical Lineweaver-Burke plot.

As shown, the intercepts of this plot yield V_m and K_m, the latter being numerically equal to the substrate concentration which yields a velocity equal to one-half the maximum velocity. K_m is defined as the rate of breakup of the enzyme-substrate complex into reactants and products divided by the rate of formation of the complex. The value of K_m usually reflects the stability of the enzyme-substrate interaction and is of great practical value. It is not, however, always the true dissociation constant of the enzyme-substrate complex (28). Only when the rate of dissociation of the complex into enzyme and substrate (the reactants) is much greater than the change to products or an enzyme-product complex does K_m actually represent the dissociation constant. A rigorous treatment of these concepts is presented in Dixon and Webb(2). If the same enzyme can attack several substrates, the K_m values give a useful comparison of the affinity of the enzyme for the various substrates. The comparison of K_m values is often used in assessing the specificity and binding groups which are involved in various enzyme-substrate complexes.

In the case of the oxidation of the aldehydes by XO, proposals by various investigators regarding the true nature of the substrate (the aldehyde or its conjugate hydrate) are not in accord(25,26). It is intended that this project, using Michaelis kinetics as described above, will provide compelling evidence as to the true substrate for aldehydic oxidation.

It is now believed that the equilibrated aqueous solutions of aldehydes contain the solvated aldehyde in equilibrium with its

hydrate(29). The belief that the equilibrium:

$$RCHO + H_2O \rightleftharpoons RCH(OH)_2 \qquad (7)$$

is not reached instantaneously when the aldehyde is added to water is founded on the results of several independent investigators. Calorimetric measurements of the solvation and hydration reaction have been cited to indicate and determine the extent of hydration(29). The results of dilatometric studies support the hydration model(31). The ultraviolet spectra of aqueous aldehydes were analyzed by Schou(32) in 1926 and by Herold and Wolf(33) in 1931 and their studies led to the same conclusion. For example, the absorptions characteristic of the aldehydic group in acetaldehyde are greatly diminished in dilute aqueous solutions. The absorbancy of a solution of the aldehyde in a non-aqueous medium (eg. hexane) at 278 nm is three and one-half times that of the same initial concentration in water at 0.0 °C.

The rate of the hydration reaction is measurable and has been the subject of recent studies by Pocker and co-workers(34). NMR and O^{18} studies have supported the hydration theory(30,35). Pocker and Meany have shown that the hydration of acetaldehyde is a general-acid general-base catalyzed reaction(36,37,38).

The hydrations of the pyridine aldehydes were demonstrated spectrophotometrically in 1959 by Sergio Cabani and Piero Cecchi(39), by observing the change in spectra of water-dioxane mixtures in the ultraviolet region at 20.0 °C.

The concepts mentioned above were considered in designing a

study to distinguish the true substrate in the XO-aldehyde reaction. The problem is then to determine whether the oxidation by XO involves attack on the aldehyde:

$$RCHO \xrightarrow{enz} RCO_2H, \tag{8}$$

or on the hydrate:

$$RCH(OH)_2 \xrightarrow{enz} RCO_2H, \tag{9}$$

If the enzymatic oxidation takes place with the free aldehyde, activity should be at a maximum when the equilibrium in Equation 7 above lies to the left, and vice versa. Thus by studying aldehydes for which the position of equilibrium may be monitored, the true substrate may be determined.

Using Michaelis-Menten kinetics and working at substrate concentrations small in comparison to the value of K_m, the reaction rate is first order with respect to substrate. Comparing initial velocities obtained at various stages of hydration equilibrium should give a firm indication of which species is the true substrate. Thus it is necessary to employ conditions under which the rate of hydration is either slower than or comparable to the rate of oxidation. Therefore a detailed kinetic analysis of the reversible hydrations was carried out, which, in addition to being a prerequisite to obtaining definitive conclusions regarding the enzymatic oxidation, has also provided us with important information regarding the mechanism of the acid-base catalyzed hydration.

Fridovich(25) has published work concerning the XO catalyzed

oxidation of acetaldehyde, but restricted his work to the single aldehyde. This restriction, plus the lack of any quantitative data render it impossible to delineate any meaningful conclusions pertaining to the mode of action of xanthine oxidase.

II. EXPERIMENTAL

A. Materials

Substrates The acetaldehyde used in these experiments was obtained from J. T. Baker Chemical Co. and was labeled 99% pure. Since the aldehyde tends to oxidize, it is necessary to distill it immediately prior to use. The distillation was carried out under dry nitrogen gas, using a short Heli-Pak column with a modified finger condenser in a mixture of ethanol and ice as a collection flask. The first and last fractions were discarded, and the middle fraction, bp 21-22 °C, was used for the experiments.

Initially, the aldehyde thus purified was stored under nitrogen in the freezer in Teflon-sealed vials. This however proved unsatisfactory, as an unknown contaminant, perhaps the polymeric form, was in some way introduced. The procedure was therefore abandoned in favor of daily distillation of the acetaldehyde.

Propionaldehyde, n-butyraldehyde and isobutyraldehyde were products of Matheson Scientific, Inc., and were similarly purified daily. The pyridine aldehydes, 2-PA and 4-PA were purchased from Aldrich Chemical Company and twice distilled under nitrogen at 12 mm pressure at the following temperatures: 2-PA 60-61 °C, and 4-PA 76-77 °C. A small vacuum distillation apparatus was employed with a rotating receiver for collecting the desired fraction. The distillations were carried out directly before use to avoid complications arising from oxidation of the pyridine aldehydes.

Buffer Components Phosphate solutions were prepared from commercially available compounds, either analytical or reagent grade or of comparable purity.

Enzyme Preparations Xanthine oxidase was obtained as a product of Worthington Chemical Corporation, prepared and purified from milk using the method of Gilbert and Bergel(40), and offered as a suspension in 0.6 saturated ammonium sulfate. The enzyme was obtained in two different grades, labeled XO and XOP. The XO labeled grade, after further purification, was used in most of the studies. Some xanthine oxidase used in exploratory work was prepared from fresh cream by Mr. R. Tienhaara(41) at this laboratory. Although parallel results were observed from the various preparations, each set of experiments involved only a single preparation.

Cytochrome C Cytochrome C was a product of Sigma Chemical Corporation, Sigma Type VI. Solutions were prepared in small amounts by dissolving in deionized water, and were kept frozen in small vials until needed.

Catalase Beef liver catalase was a product of Worthington Biochemical Corporation, obtained in a purified, powdered form by the method of Tauber and Petit(42). It was dissolved in 0.05 M phosphate buffer, pH 7.0, diluted and kept in the refrigerator until use.

B. Analytical Instruments

Source of Kinetic Data Kinetic data for both hydration and

oxidation studies was obtained on a Beckman Kintrac VII recording spectrophotometer. Beckman glass cells having a pathlength of 10 mm were used throughout the work. The cell chamber of the instrument was attached to a Beckman Thermocirculator Accessory for temperature control within the cells. The Kintrac VII utilizes internal magnetic stirrers within the cells, and has provisions for flushing the cell compartment with nitrogen gas for anerobic studies.

For cooling the cell chamber to less than 10 °C, a low temperature accessory was required. In this case, a Blue M Co. external refrigeration unit was employed to cool a large bath to below zero, and the coolant from this bath was in turn circulated through a coil in the thermocirculator unit. Using this arrangement, temperatures within the cell chamber could be lowered to 0.0 °C with a maximum deviation of ± 0.05 °C. The liquid pump and external refrigeration unit were kindly supplied by Dr. H. S. Habib, of this institution.

pH Measurement The pH values of all buffer solutions were determined using a Beckman Century SS pH meter, the reproducibility of which is ± 0.01 pH units over a range of a few pH units. The electrodes used were a Beckman glass electrode and a Beckman calomel reference electrode.

Weighing Materials A Mettler Model H20T electrobalance was used in all weighing procedures. By weighing previously standardized weights, it was shown that the instrument had a reproducibility of ± 0.01 mg.

Ultraviolet and Visible Scanning Ultraviolet and visible scanning were carried out on a Beckman Model DB recording spectrophotometer.

Colorimetric Determinations Colorimetric protein assays were carried out on a Hitachi Perkin-Elmer Model 139 spectrophotometer due to the ease of handling and the provision for rapid cell changing.

C. Methods

Hydration of Acetaldehyde Acetaldehyde absorbs at a maximum at 278 nm, and the psuedo-first-order rate coefficients, k_{obsd}, were obtained by observing the diminution of this peak(43). A plot of log $(A_t - A_\infty)$ versus time gives a straight line with k_{obsd} equal to -2.303 (Slope). The specific rates obtained were reproducible to about 1%. A first order plot derived from spectrophotometer readings is shown in Figure 3.

Since the reaction is reversible, k_{obsd} is actually a sum of the rate coefficients for the forward and the reverse reactions: $k_{obsd} = k_f + k_r$. These values were obtained from kinetic runs in which the initial absorbancies of the substrates were determined by extrapolation to the beginning of the reaction, and the final absorbancies were those observed from the equilibrated reaction solutions.

Six microliters of acetaldehyde was introduced into exactly 3 ml of the buffer solution by means of a calibrated Hamilton microsyringe. Since the acetaldehyde boils at 21 °C, it was necessary to

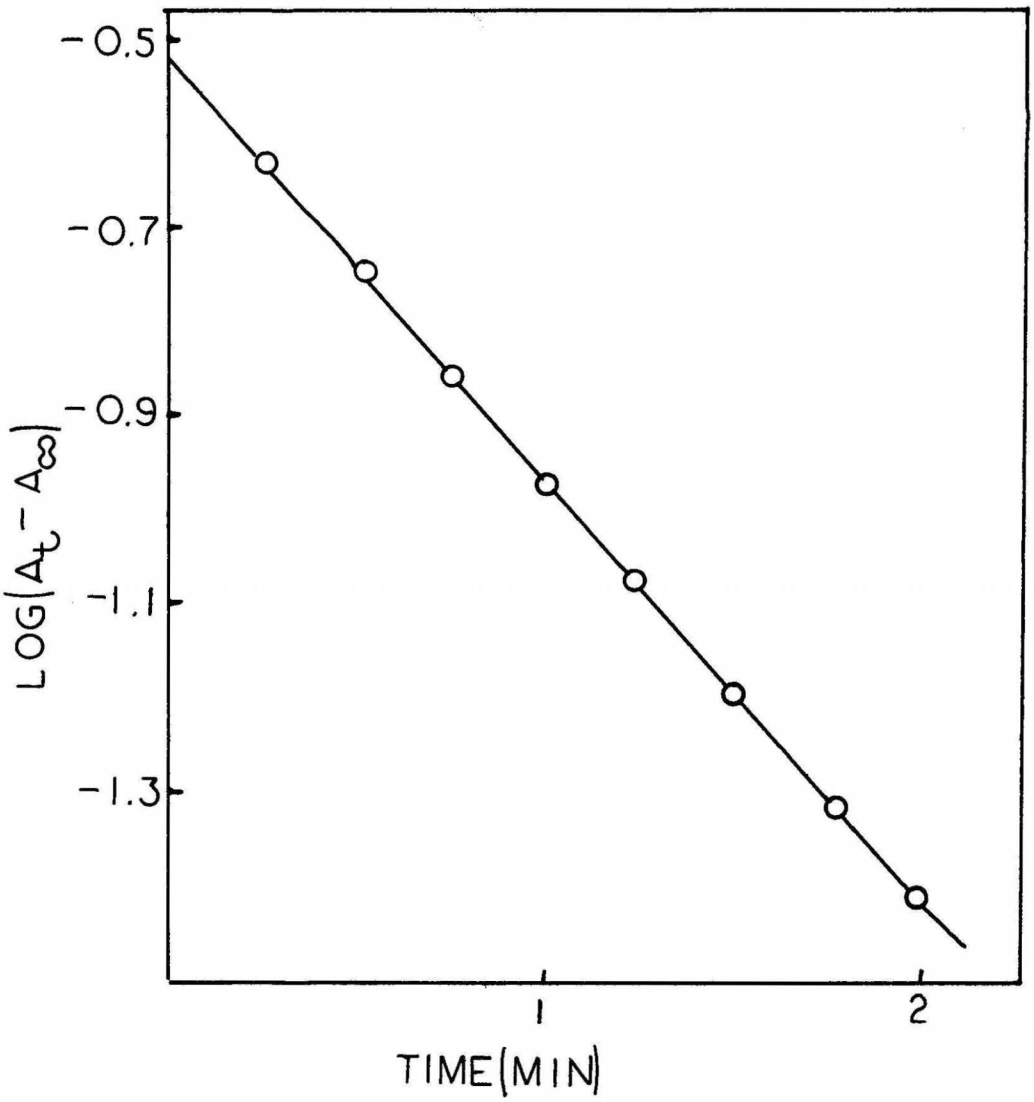


Figure 3. Typical acetaldehyde hydration at 25 °C, 0.002 M phosphate buffer, pH 7.0, $\mu = 0.1$.

chill both the syringe and the aldehyde, because the high vapor pressure of the substance at room temperature otherwise made it impossible to draw the acetaldehyde into the syringe. While the acetaldehyde was being introduced, the magnetic stirrers were running at high speed. This mixing was continued for about 5 seconds after the aldehyde was introduced. The buffer solution was pre-equilibrated to the desired temperature prior to introduction of the acetaldehyde.

It was necessary to accurately determine the substrate concentration for those runs used to determine Michaelis parameters. Calibrated Hamilton syringes were used to inject various volumes of the aldehyde into the reaction mixture. Approximately 30 runs were made of the hydration of acetaldehyde to accurately determine the fraction of hydration, χ. The experimentally determined fraction of hydration was then used to determine the amount of aldehyde and hydrate present in the equilibrated solutions.

Other Hydration Studies The hydration studies on the rest of the aldehydes used were performed in much the same manner as that described for acetaldehyde, except that chilling of the syringe and the aldehydes was not required for these substances(44). The experimentally determined fractions of hydrations for the various aldehydes used are presented in the results section.

Preparation and Storage of Xanthine Oxidase Solutions Enzyme solutions were generally prepared directly prior to use for a given set of kinetic runs. The commercial preparation of the enzyme was

purified by a method which was similar to that used by Rajagopalan and Handler(45). The commercial product was diluted about ten-fold and centrifuged. Enough calcium phosphate gel was used to absorb all the activity (about 2:1, gel:protein). The gel was then washed twice with distilled water, twice with 0.05 M phosphate buffer, pH 7.8, and the washings discarded. Most of the enzyme was then eluted from the gel by washing with small volumes of 0.5 M phosphate buffer, pH 7.8.

Portions of the enzyme preparation not immediately used were frozen and used within a few days. Such frozen preparations when kept for longer periods of time suffered considerable loss of activity. For longer storage, the enzyme was precipitated with ammonium sulfate, redissolved in 0.05 M buffer, pH 7.8, and frozen. Two ml volumes of such preparations were dialyzed overnight against 3 liter volumes of 0.05 M phosphate buffers, pH 7.8.

Assay of Xanthine Oxidase Each batch of enzyme was assayed spectrophotometrically by following the formation of urate from hypoxanthine at 290 nm, as described by the Worthington Biochemical Corp. (46). A unit of activity was defined as that forming one micromole of urate per minute at 25 °C. In this manner, units per ml were obtained. The assay of protein was performed colorimetrically using the method of Lowry(47), with Folin Ciocalteau reagent, purchased from Scientific Products Company. Bovin serum albumin, Fraction V, a product of Sigma Chemical Corporation, was used as a standard. Figures 4 and 5 illustrate typical hypoxanthine assays and protein standard curves. From

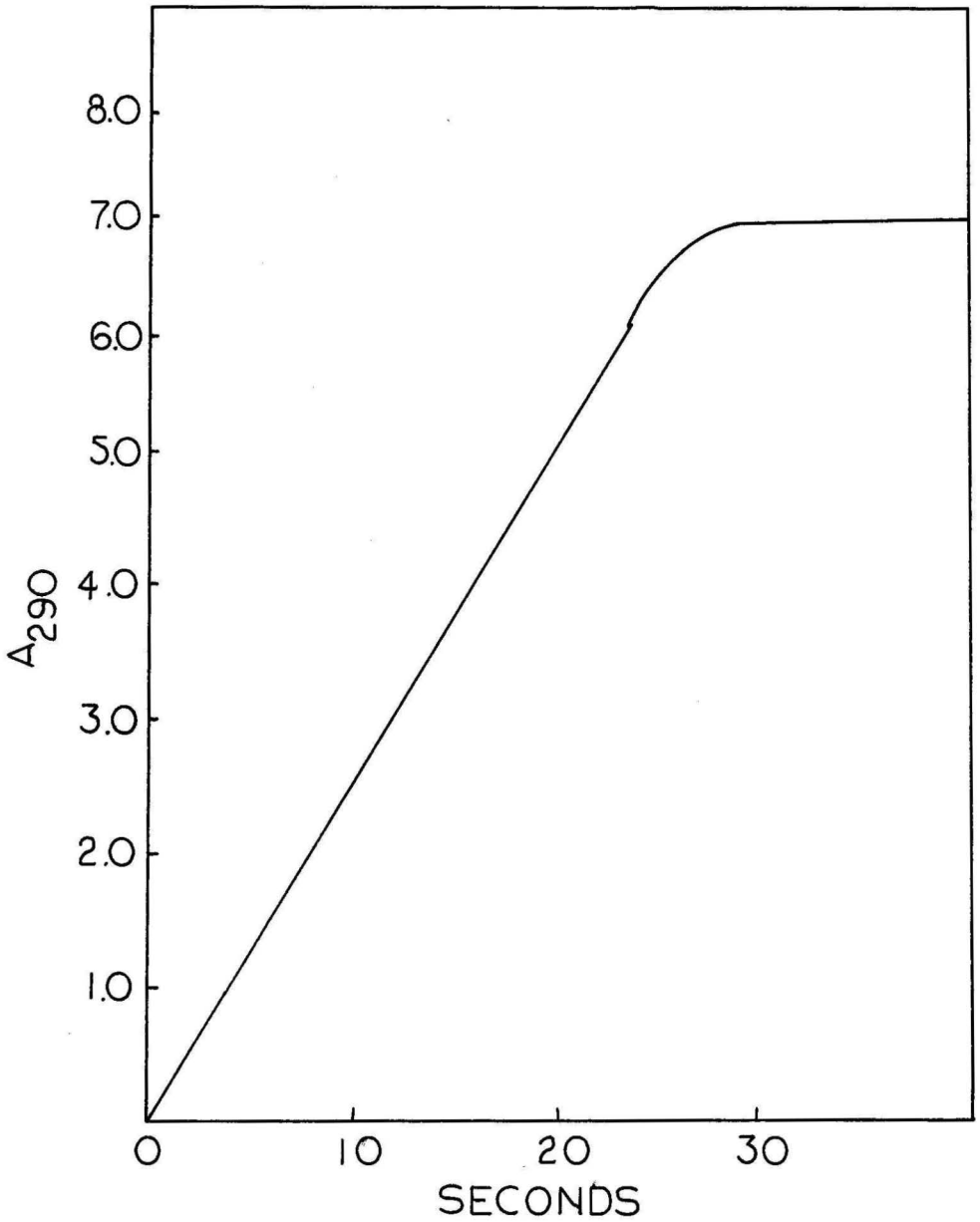


Figure 4. Typical xanthine oxidase assay at 25.0 °C.

these two assays, the specific activity of the enzyme in units per mg was obtained:

$$\frac{\text{Units/ml}}{\text{mg/ml}} = \text{Units/mg.} \qquad (10)$$

Unless otherwise specified, 50 micrograms of XO having a specific activity of 15 was used in all reaction mixtures. When other concentrations of the enzyme were used, the results obtained were normalized to the concentration above by comparing the rates of urate formation of the two concentrations.

Buffer Solutions Phosphate buffers were prepared by diluting accurately weighed KH_2PO_4 and K_2HPO_4 with deionized water to the desired concentrations. Appropriate amounts of NaCl were incorporated to obtain the desired ionic strength.

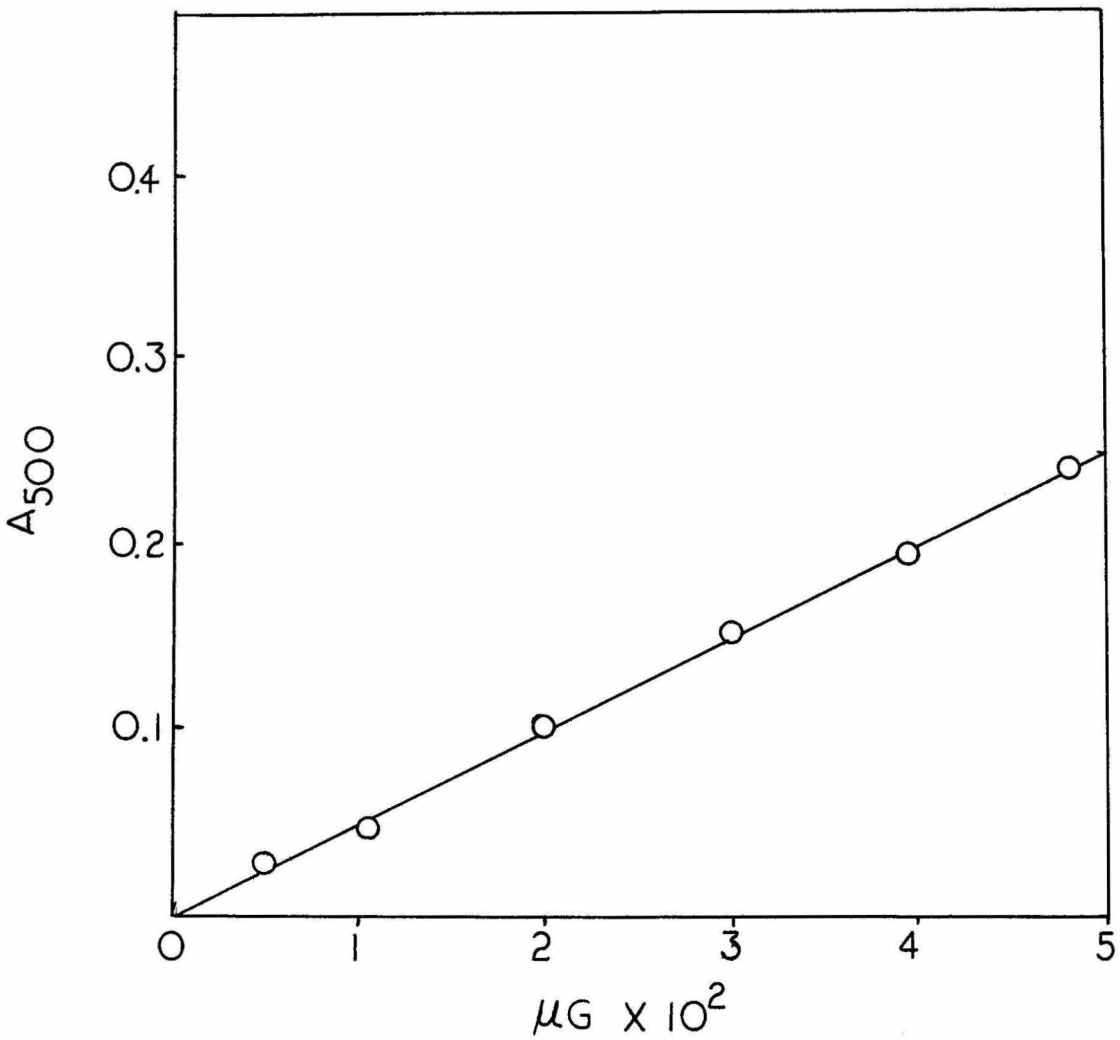


Figure 5. Typical protein standard curve. The absorbancy at 500 nm indicates the number of micrograms of protein in the sample.

III RESULTS

A. Hydration of Acetaldehyde

The reversible hydration of acetaldehyde is a general acid, general base catalyzed reaction in which the acidic and basic components of the reaction mixture independently contribute to the overall reaction rate(43). The psuedo-first order rate coefficients observed from the reaction in phosphate buffers in aqueous medium consist of the sum of catalytic terms for each acidic and basic catalyst present:

$$k_{obsd} = k_o + k_{H_3O^+}[H_3O^+] + k_{OH^-}[OH^-] + k_{HPO_4^=}[HPO_4^=] + k_{H_2PO_4^-}[H_2PO_4^-] \quad (11)$$

where k_o is the solvent catalyzed reaction.

One may deduce the values associated with each of the catalytic species in a buffered solution by proper choice of experiments. Initially a series of runs were carried out varying simultaneously the concentrations of $HPO_4^=$ and $H_2PO_4^-$ while maintaining the same buffer ratio and pH. The buffer ratio, r, is defined throughout as the ratio of the concentration of acid to that of the conjugate base, i.e., $r = [H_2PO_4^-]/[HPO_4^=]$. The kinetic experiments were carried out at the same ionic strength. Rearranging Equation (10) as:

$$k_{obsd} = k_o + k_{H_3O^+}[H_3O^+] + k_{OH^-}[OH^-] + (k_{H_2PO_4^-} + k_{HPO_4^=}/r)[H_2PO_4^-], \quad (12)$$

Table I

Catalysis of Acetaldehyde Hydration as a Function of Phosphate Buffer Concentration at Various Buffer Ratios[a,b]

μ = 0.10, 25.0 °C

		r = 4, pH = 6.30			
$(H_2PO_4^-)$	0.040	0.020	0.008	0.004	0.0020
(HPO_4^{-2})	0.010	0.005	0.002	0.001	0.0005
k_{obsd}	5.86	3.19	1.66	1.05	0.75
		r = 1, pH = 6.85			
$(H_2PO_4^-)$	0.025	0.0125	0.005	0.0025	0.00125
(HPO_4^{-2})	0.025	0.0125	0.005	0.0025	0.00125
k_{obsd}	7.47	4.38	2.07	1.57	0.92
		r = 0.5, pH = 7.30			
$(H_2PO_4^-)$	0.0165	0.00825	0.0033	0.00165	0.000825
(HPO_4^{-2})	0.0335	0.0165	0.0066	0.00335	0.00165
k_{obsd}	8.16	4.44	2.30	1.67	1.15
		r = 0.1, pH = 7.85			
$(H_2PO_4^-)$	0.0045	0.00227	0.0009	0.00045	0.00023
(HPO_4^{-2})	0.0455	0.0227	0.0091	0.00455	0.00227
k_{obsd}	10.42	6.51	3.818	2.79	1.99

a. Concentrations of $H_2PO_4^-$ and HPO_4^{-2} have the units mole l^{-1}.
b. k_{obsd} values have the units min^{-1}.

Then at constant pH, a plot of k_{obsd} against $[H_2PO_4^-]$ would be expected to give a straight line having a slope:

$$S_1 = k_{H_2PO_4^-} + k_{HPO_4^=}/r, \qquad (13)$$

and an intercept:

$$I_1 = k_o + k_{OH^-}[OH^-] + k_{H_3O^+}[H_3O^+]. \qquad (14)$$

If several such series of runs are carried out at different buffer ratios, the values $k_{HPO_4^=}$ and $k_{H_2PO_4^-}$ may be determined by plotting the slopes obtained above against 1/r. In like manner, k_o, $k_{H_3O^+}$ and k_{OH^-} may be obtained from values of I_1, at given $[H_3O^+]$ and $[OH^-]$.

This sequence of experiments was used to determine the specific rate constants associated with the components of the phosphate buffers. Table I gives the observed rate constants resulting from the variation of buffer concentration at the buffer ratios studies. Plots of k_{obsd} versus $[H_2PO_4^-]$ are shown in Figure 6. The slopes of the lines resulting from various values of 1/r are tabulated in table II:

Table II

Catalysis of Acetaldehyde Hydration by Phosphate Buffers

1/r	10	2	1	0.25
Slope[a]	2020	442	311	164

a. In units of 1./mole min.

Figure 7 indicates that S_1 is a linear function of 1/r for the phosphate buffers used. The specific rate constants for the bases and the conjugate acids were obtained from this graph. The slope is equal to $k_{HPO_4^=}$ and was found to be 189 1./mole min. The intercept is equal to $k_{H_2PO_4^-}$, and its value is 105 1/mole min.

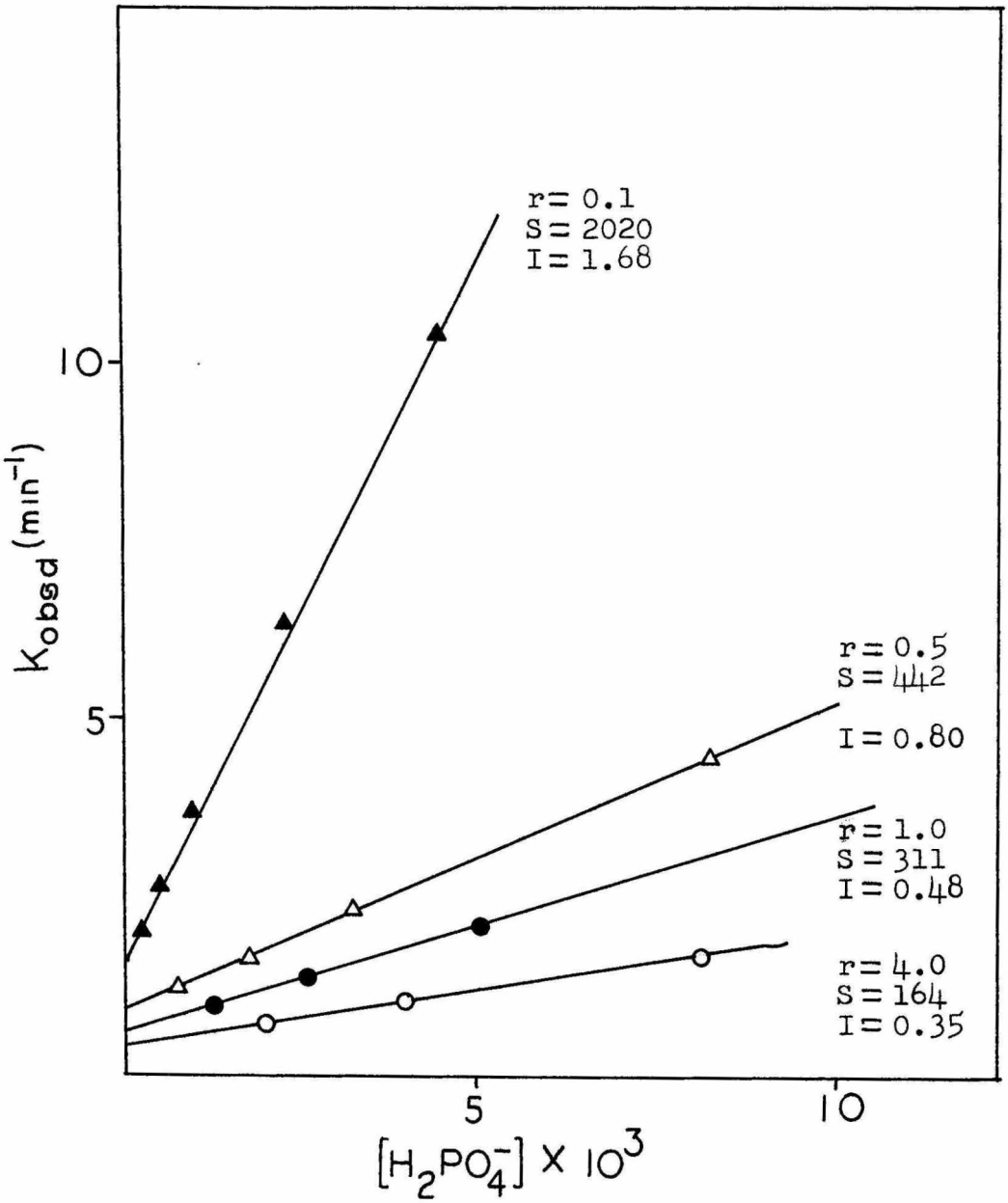


Figure 6. Catalysis of acetaldehyde hydration as a function of phosphate buffer concentrations at various buffer ratios. μ= 0.1, 25.0 °C.

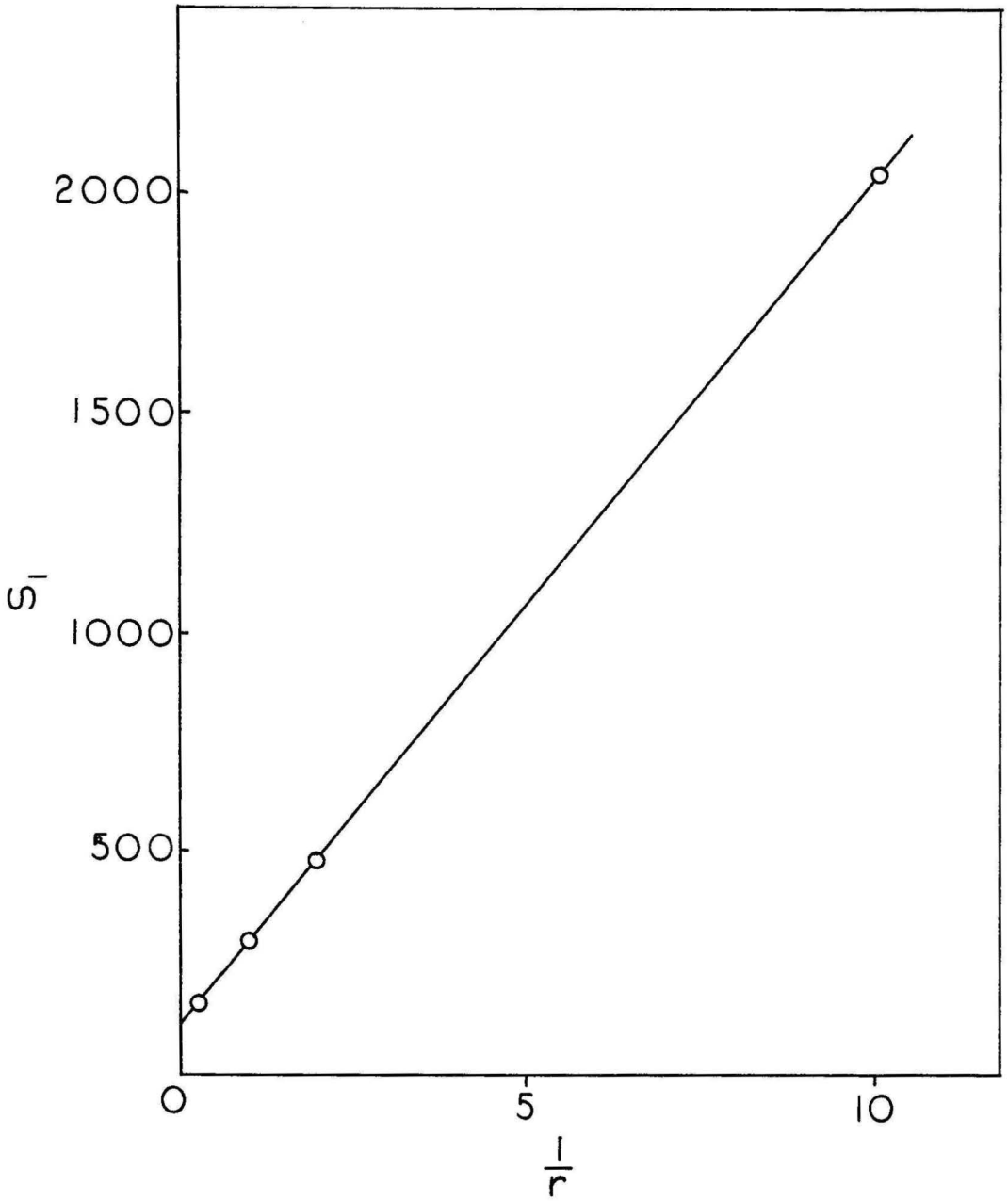


Figure 7. Catalysis of acetaldehyde hydration by phosphate buffers. The slope equals $k_{HPO_4^{-2}}$ = 189 l/mole min. I= $k_{H_2PO_4^-}$ = 105 l/mole min.

The values of I_1 at various hydroxide ion concentrations are tabulated in Table III. Figure 8 shows that I_1 is a linear function of hydroxide ion concentration. The catalytic coefficients for water and hydroxide ions were evaluated from this graph as k_o = 0.305 1/mole min and k_{OH^-} = (Slope)(f_{OH^-}), where f_{OH^-}, the mean ion activity coefficient, was calculated from:

$$\log f_{OH^-} = -0.49\ z^2\ I^{\frac{1}{2}}/(1 + 1.5\ I^{\frac{1}{2}}), \qquad (15)$$

where z and I represent charge and ionic strength, respectively(43). From this, k_{OH^-} becomes 1.88 x 10^6 1/mole min.

Table III

Catalysis of Acetaldehyde Hydration by Water and Hydroxide Ions

I	0.352	0.480	0.800	1.680
$a_{OH^-} \times 10^7$	0.19	0.71	1.99	7.10

The apparent equilibrium constant, K_{eq}, may be determined from the hydration reaction from the following relationship(51):

$$K_{eq} = \frac{\chi}{1 - \chi} = \frac{(H)_t}{(A)_t}, \qquad (16)$$

where $(H)_t$ and $(A)_t$ are the total concentrations of hydrate and aldehyde. Table IV summarizes the experimentally determined data for the various aldehydes at 25.0 °C, pH 7.0.

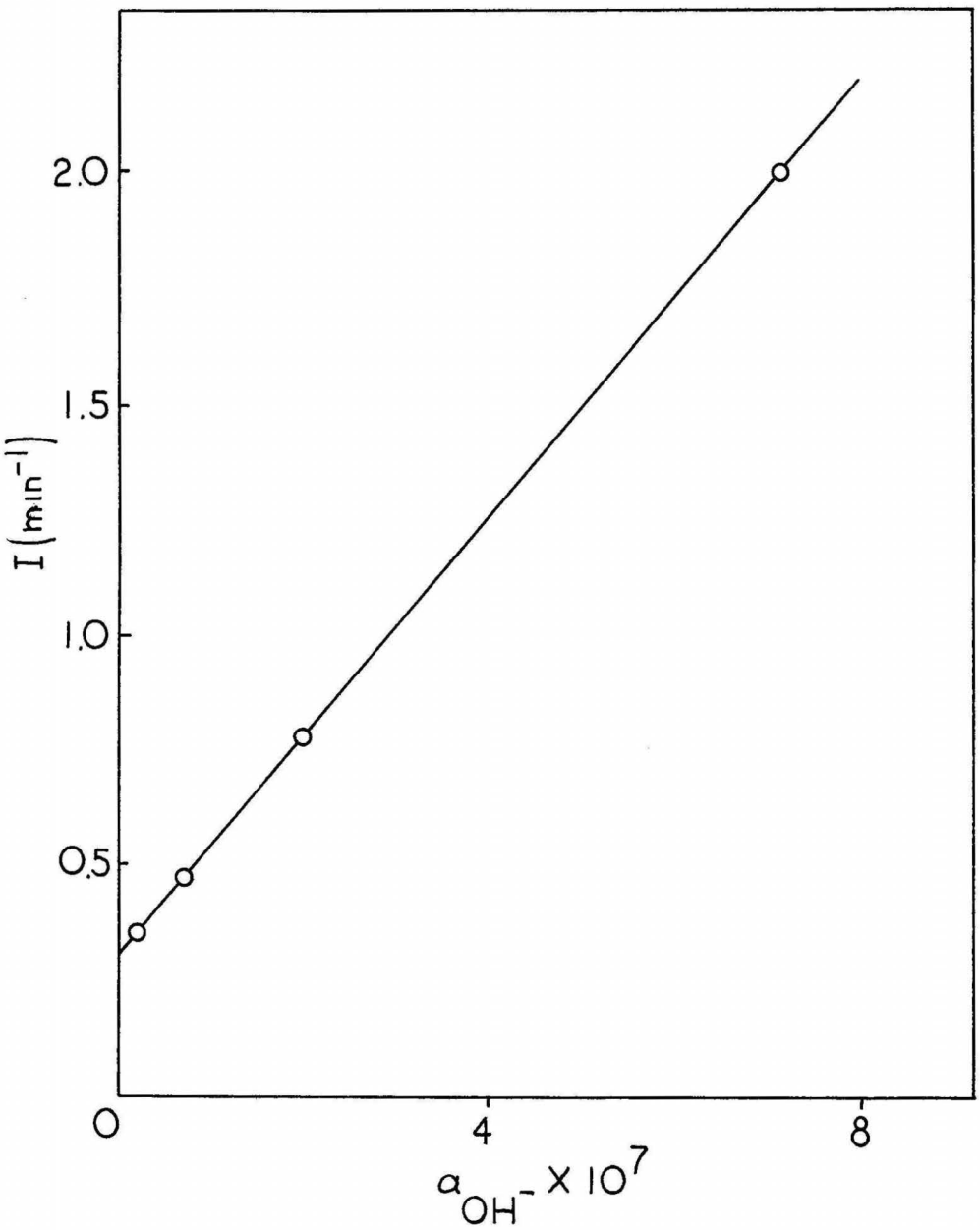


Figure 8. Catalysis of acetaldehyde hydration by water and hydroxide ions. The slope equals $k_{OH^-}/f_{OH^-} = 1.88 \times 10^6$ l/mole min. The intercept equals $k_o = 0.305$ min^{-1}.

Table IV

Hydration Data Obtained at 25.0 °C, pH 7.0

Substrate	χ	K_{eq} [a]
Acetaldehyde	0.503	1.01
Propionaldehyde	0.413	0.71
n-butyraldehyde	0.361	0.57
Isobutyraldehyde	0.345	0.53
2-PA	0.293	0.41
4-PA	0.420	0.73

a. K_{eq} as defined in Equation 15.

B. Preliminary Studies of the XO-Acetaldehyde System

During the initial exploratory work, various enzyme preparations were used, including some prepared in this laboratory. Similar results were generally obtained from all preparations, however the more highly purified solutions seemed to be relatively unstable when stored for a time at lower temperatures. For this reason, enzyme solutions used during kinetic studies were generally prepared immediately prior to use.

Initial runs using the enzyme from the various sources with acetaldehyde as the substrate gave no apparent activity. When following as closely as possible the work of Fridovich(25), a decrease in

absorbance was noted instead of the anticipated increase as the reduced form of cytochrome C appeared. Enzymatic assays with hypoxanthine indicated at least moderate activity, so the acetaldehyde was redistilled and diluted in a freshly distilled sample of dimethoxyethane(48). Using this freshly prepared substrate solution, slight activity was still apparent. Upon doubling the cytochrome C and substrate concenstrations, a sharp drop below zero was encountered followed by a rapid increase in absorbance.

The reaction curve tended to rise after its drop below zero, until apparently all the substrate was consumed, at which time the absorbance again began to decrease as the cytochrome C was re-oxidized. Addition of more substrate at this point increased the rate sharply, following by another decrease.

Some initial runs were made using dialyzed as opposed to non-dialyzed enzyme, to ascertain whether the ammonium sulfate in the solution had any effect on the reaction. Additionally, amounts of ammonium sulfate far in excess of any to be encountered in a dilute stock solution were added to some of the reaction mixtures. These studies indicated that small amounts of the salt had no effect on the reaction.

The reaction of a freshly prepared solution of cytochrome C was compared with that utilizing an older solution. The freshly prepared solution exhibited a much smaller drop during the initial part of the reaction than did the older sample. For this reason, the cytochrome C was prepared in small batches and kept frozen until use. It was also

noted that by using a cytochrome C concentration about three times that employed by Fridovich(25), more consistent results could be obtained.

Anerobic studies were made by bubbling N_2 through the reaction mixtures for several minutes prior to initiation of the reaction. A sintered glass tube was used to deliver fine bubbles of N_2 to the solution. These studies showed that 0_2 is required by the hypoxanthine reaction, and that the cytochrome C reaction will proceed at a slow rate in the absence of 0_2, but is greatly enhanced by the addition of 0_2. When cytochrome C was added to the anerobic hypoxanthine solution, the reaction proceded at a slow rate, but when 0_2 was introduced, the rate of reaction increased sharply. These results parallel those reported by Horecker and Heppel(4). Similar results were obtained in the aldehyde reaction, for when 0_2 was introduced to a previously de-oxygenated solution, an increase in rate was readily apparent. The addition of cytochrome C apparently had no effect on the aerobic oxidation of hypoxanthine, so it was assumed that cytochrome C did not in any way act as an inhibitor for the enzyme.

A series of runs was made with hypoxanthine as the substrate and acetaldehyde added as a possible inhibitor. These runs indicated that acetaldehyde acted as a competitive inhibitor to the hypoxanthine reaction, expecially at higher aldehyde concentrations. This indication of both substrates binding at the same site has previously been reported(7,13).

In many of the preliminary runs, it was noted that after the

absorbance had risen to a maximum, it soon started to decrease again, as the cytochrome C was oxidized. It was presumed that this was caused by the presence of hydrogen peroxide, which is a secondary product of the aldehydic oxidation. To test this hypothesis, catalase was added to a normal acetaldehyde run. Catalase has been shown to be an effective catalyst for the reaction:

$$2H_2O_2 \rightarrow 2H_2O + O_2 \qquad (17)$$

Addition of approximately 10 μg per ml of catalase to the reaction mixture prevented the re-oxidation of cytochrome C by hydrogen peroxide. It was also noted that the pure XO, prepared as previously described, was much less susceptable to the re-oxidation of cytochrome C, indicating that perhaps the stock enzyme is a mixture of more than one enzyme, one of which catalyzes the reaction of peroxide and cytochrome C. In agreement with Fridovich and Handler(49), excess catalase did not inhibit cytochrome C reduction. For these reasons, later kinetic studies were made with either the purified enzyme, or with enough catalase added to destroy the peroxide as it was formed.

If indeed there is a second enzyme present along with XO in the commercial preparations, it apparently has no effect on the oxidation of acetaldehyde. If it catalyzed the reaction, one would expect a lessening of activity from solutions free of the second enzyme. This effect was not noted or could not be identified in the reactions using the purified enzyme. Perhaps, as suggested by Bray(16), certain amounts of an inactive form of XO are present in most enzyme preparations.

A series of runs in which only the ionic strength was varied was made to determine the effect of ionic strength on the reaction. At pH 7.0 and 25.0 °C, the following results were obtained:

Table V

Effect of Ionic Strength upon Reaction Velocity

Ionic Strength	0.01	0.05	0.10	0.15
Initial Velocity[a]	0.86	1.72	1.85	1.22

a. Initial velocities are in μ mole cytochrome C reduced/min.

As may be seen from Table V, the ionic strength has a considerable effect on the reaction rate, the trend being for the reaction rate to increase with increasing ionic strength up to about 0.1 M, then decreasing with increasing ionic strength. This particular aspect of the reaction was not investigated further than to establish the fact that for consistent results the ionic strength must be held constant. Accordingly, subsequent studies were made at a constant ionic strength of 0.1 M.

Effects of cytochrome C, XO, and catalase upon the hydration of acetaldehyde were briefly investigated, and while it was found that a slight change in rate of hydration was apparent in some cases, the fraction of hydration was not changed(37,38). The effects on the rate of hydration by these substances was so small that equilibrium with

the hydrate was reached at nearly the same time as when the aldehyde alone was in solution.

C. Kinetic Studies

Acetaldehyde Previous studies of the xanthine oxidase catalyzed oxidation of acetaldehyde have yielded values of K_m ranging from 6.2×10^{-2} M to 2.0×10^{-2} M(1,25). While these studies were reported to have been carried out at the pH 7.0, the differences exhibited may be attributed to different temperature, buffer concentrations or ionic strengths.

In order to be able to study reaction rates at various degrees of hydration, a relatively slow rate of hydration was desirable. From the hydration studies, a 0.002 M buffer at pH 7.0 and ionic strength of 0.1 was chosen. Under these conditions, equilibrium between the aldehyde and its hydrate was reached in about four minutes, and the fraction of hydration was found to be 0.503.

Using a constant enzyme concentration of 50 μg per cuvet, 3×10^{-5} M cytochrome C and a range of acetaldehyde concentrations, Michaelis-Menten parameters were obtained. The enzyme displayed marked substrate inhibition, in agreement with earlier studies(1,50), and therefore V_m was never reached. Using a Lineweaver-Burke plot, Figure 9, K_m was found to be 2.71×10^{-2} M, and V_m = 21.6 micromoles of cytochrome C reduced per min. Figure 10, which more clearly shows the effect of increased substrate concentration, was drawn from this data. The dotted line represents the curve which would have been expected in

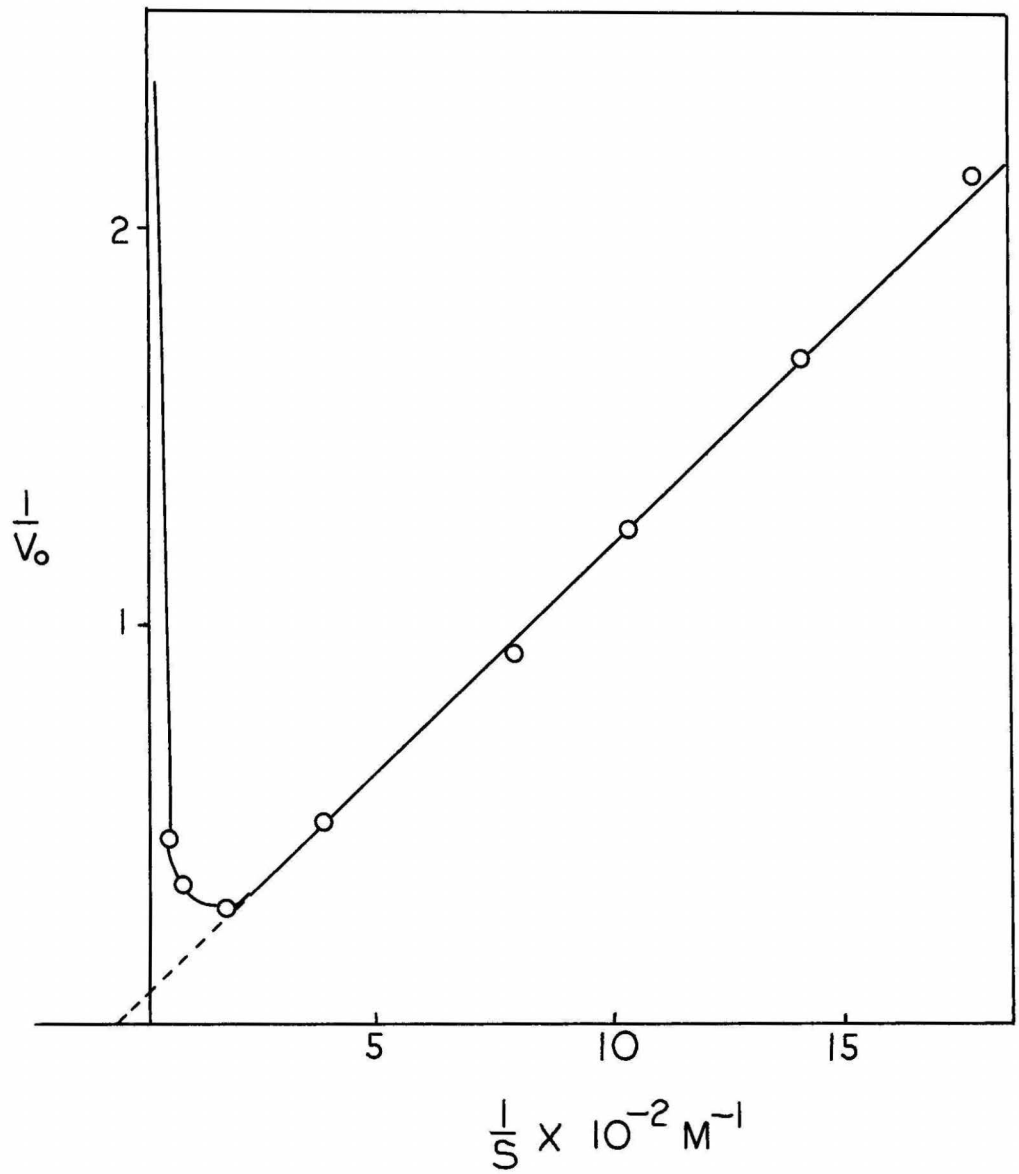


Figure 9. Lineweaver-Burke plot of acetaldehyde oxidation by XO at 25.0 °C, pH 7.0, $\mu = 0.1$. The K_m value is 2.71 x 10^{-2} M. The velocity is in μmoles of cytochrome C reduced per minute.

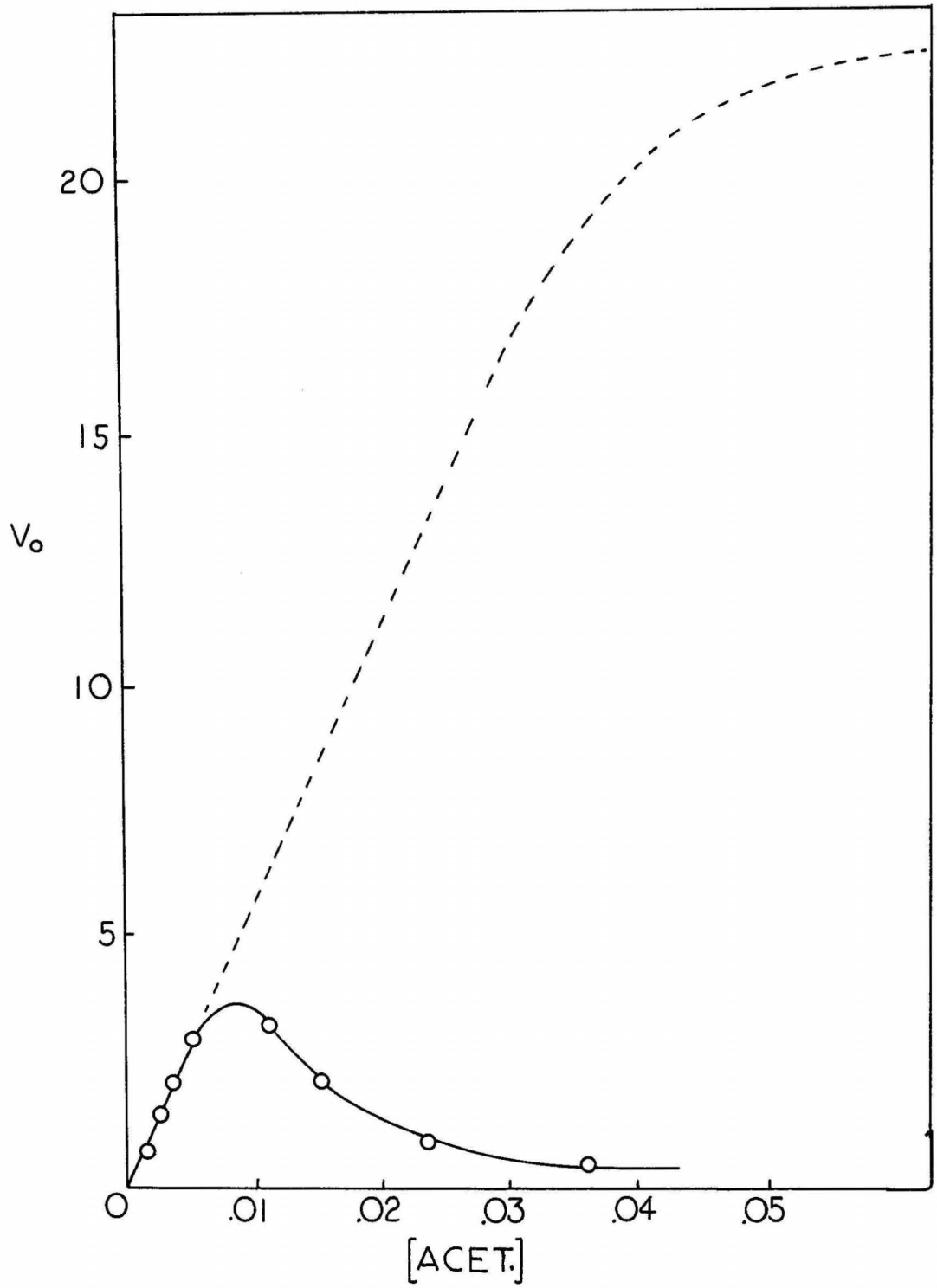


Figure 10. Substrate inhibition by acetaldehyde. The dotted line represents the curve that would have been obtained in the absence of substrate inhibition.

the absence of substrate inhibition, while the solid line is the actual plot obtained from the data. As may be seen from the figure, substrate inhibition is almost 100% above about 0.04 M acetaldehyde.

A pH profile of the acetaldehyde oxidation was obtained in phosphate buffers, ranging from a pH of 6.5 to pH 8.0, at a constant ionic strength of 0.1. An aldehyde concentration of 2.75×10^{-3} M was used, and all other conditions remained as previously stated. The results are illustrated in Figure 11, where the initial velocities, in micromoles of cytochrome C reduced per minute, are plotted against the pH. K_m was also determined at various pH strengths, and very little, if any, deviations were noted over the pH range. These results are presented in Table VI:

Table VI

Dependence of K_m and V_m on pH.

pH	6.5	7.0	7.5
K_m (M)	0.027	0.0271	0.028
V_m (μM/min)	17.0	21.6	15.3

The pH profile is similar to that obtained with hypoxanthine, which exhibits a maximum at about pH 7.5(50).

Propionaldehyde Oxidation Kinetic studies of the propionaldehyde-XO system indicate that the enzyme has less affinity for this substrate than for acetaldehyde, following the pattern set up by

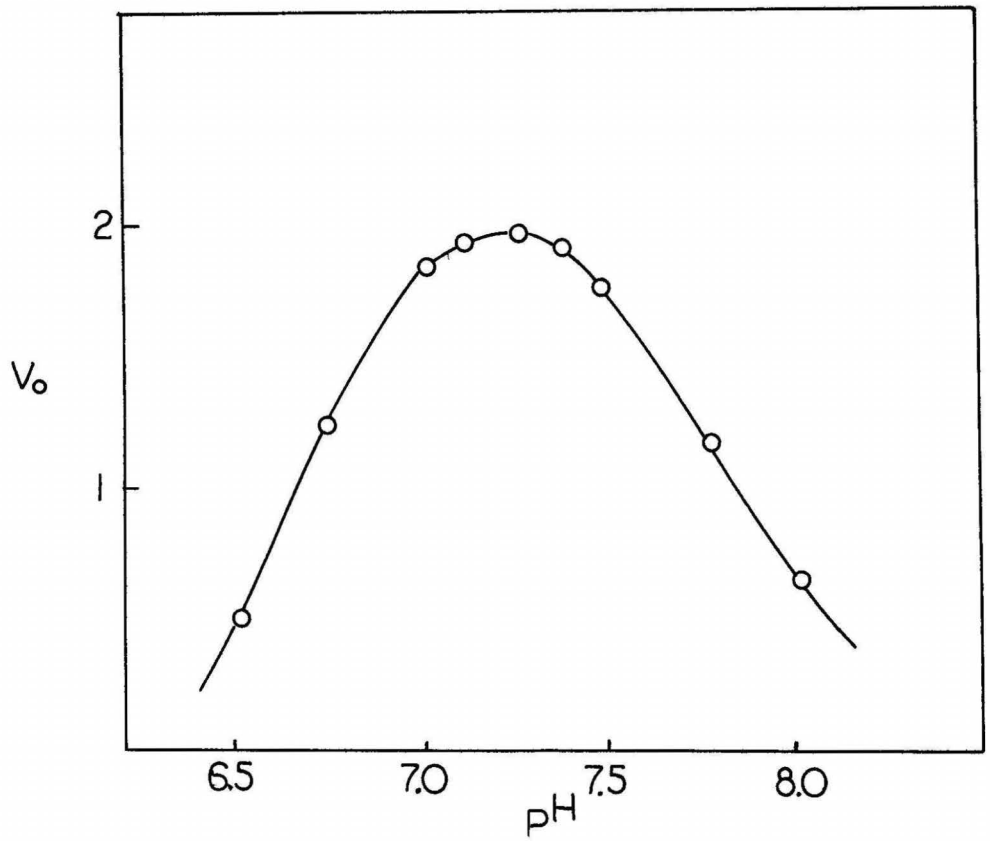


Figure 11. Acetaldehyde oxidation pH rate profile. All solutions were in 0.002 M buffer, $\mu = 0.1$, 2.75×10^{-3} M in acetaldehyde and at 25.0 °C. Initial velocities are in micromoles of cytochrome C reduced per minute.

comparison of formaldehyde-XO: acetaldehyde-XO by Booth(1). As in the case of the acetaldehyde studies presented earlier in this paper, the propionaldehyde studies were run in 0.002 M phosphate buffer, pH 7.0, μ = 0.1 and 25 °C. Results obtained from the Lineweaver-Burke plot, Figure 12, reveal a K_m of 5.3 x 10^{-2} M, and a V_m of 4.5 micromoles of cytochrome reduced per min.

Strong substrate inhibition was again observed, conforming to the results obtained with acetaldehyde. The maximum velocity actually reached with propionaldehyde was 2 μM/min at 4.1 x 10^{-2} M propionaldehyde. The maximum velocity actually reached with acetaldehyde was 3.5 μM/min at 5.5 x 10^{-3} M in acetaldehyde. This observation indicates that substrates with high affinities for the enzyme may also be more subject to substrate inhibition, as indicated by earlier reporters(2).

While no detailed pH study was made with propionaldehyde, a brief study indicated that maximum initial velocities were attained at pH 7.25, paralleling the results from acetaldehyde under the same conditions of temperature and ionic strength.

After K_m was determined, a concentration of 5.5 x 10^{-3} M propionaldehyde was used to investigate the temperature dependence of the reaction over a range from 10° to 40 °C. The results are presented in Table VII: the velocity is reported in micromoles of cytochrome C reduced per min.

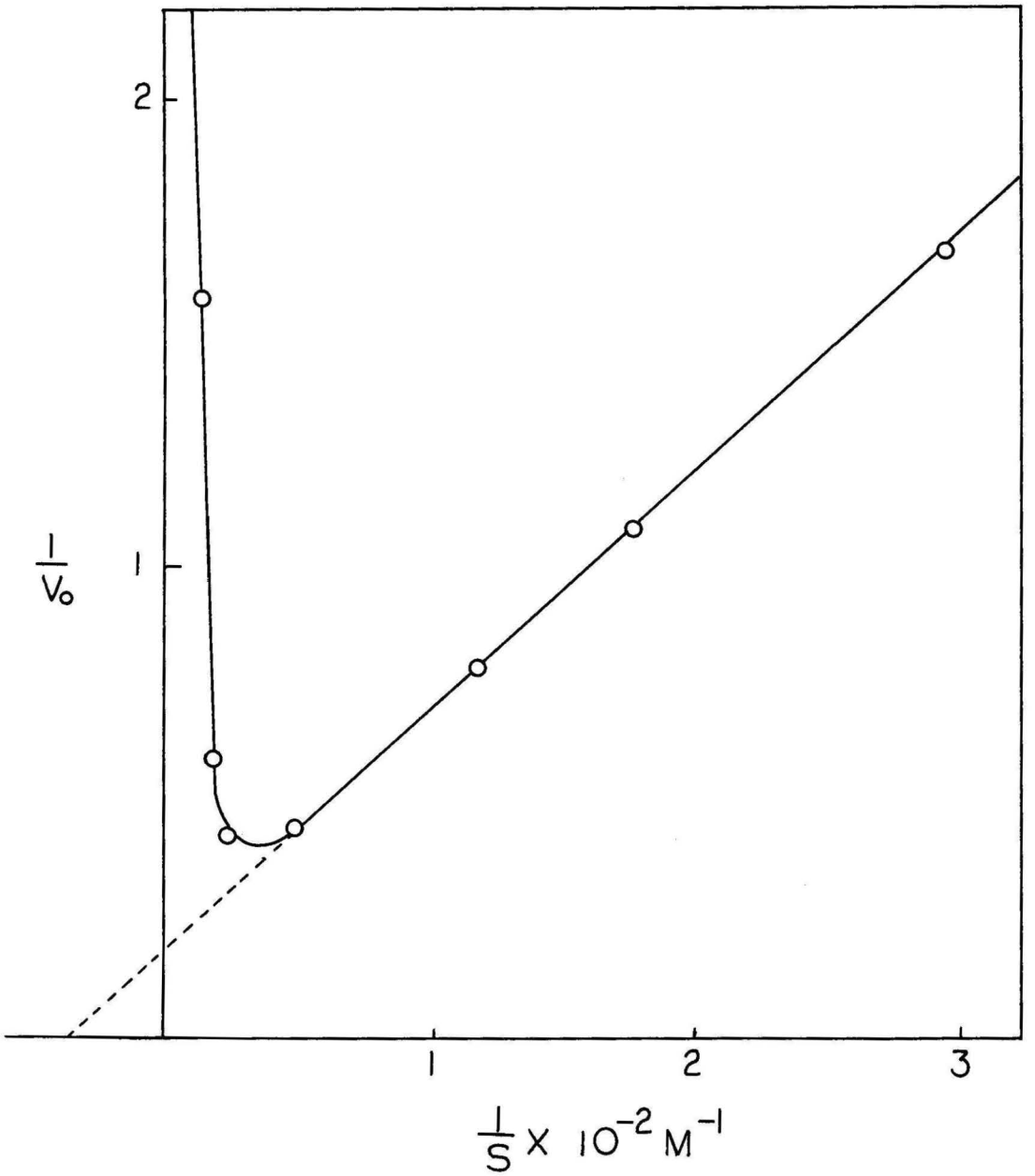


Figure 12. Lineweaver-Burke plot of propionaldehyde oxidation by XO at 25.0 °C, pH 7.0, $\mu = 0.1$. $K_m = 5.3 \times 10^{-2}$ M. The velocity is expressed in μmoles of cytochrome C reduced per minute.

Table VII

Variation of Reaction Rate with Temperature[a]

Temp (°C)	10	20	25	30	40
v_o	0.32	0.70	0.92	1.30	2.70

a. Propionaldehyde oxidation at pH 7.0, μ = 0.1.

This data is plotted in Figure 13. The slope is equal to $-E_a/2.30R$, yielding an activation energy for the reaction of 1.27×10^4 cal/mole. It must be recognized that this is an apparent overall activation energy only, for each individual step in the reaction is characterized by its own particular value of E_a, and these have not been determined here. By way of comparison, Roussos(51) states that the activation energy for the XO catalyzed oxidation of hypoxanthine is 1.21×10^4 cal/mole.

Iso-butyr and n-butyraldehyde Oxidation Hydration studies indicated that the fraction of hydration for iso-butyraldehyde at 25.0 °C was 0.345. Normal distillation procedures were employed and various concentrations of iso-butyraldehyde were pre-equilibrated in the cuvets. In the concentration range studied, from 6.313×10^{-4} M to 2.53×10^{-2} M, no activity was noted. In analogy to the other aliphatic aldehydes, it was assumed that concentrations higher than the above would result in nearly complete substrate inhibition, while the lower concentration was well below the anticipated K_m. Iso-butyralde-

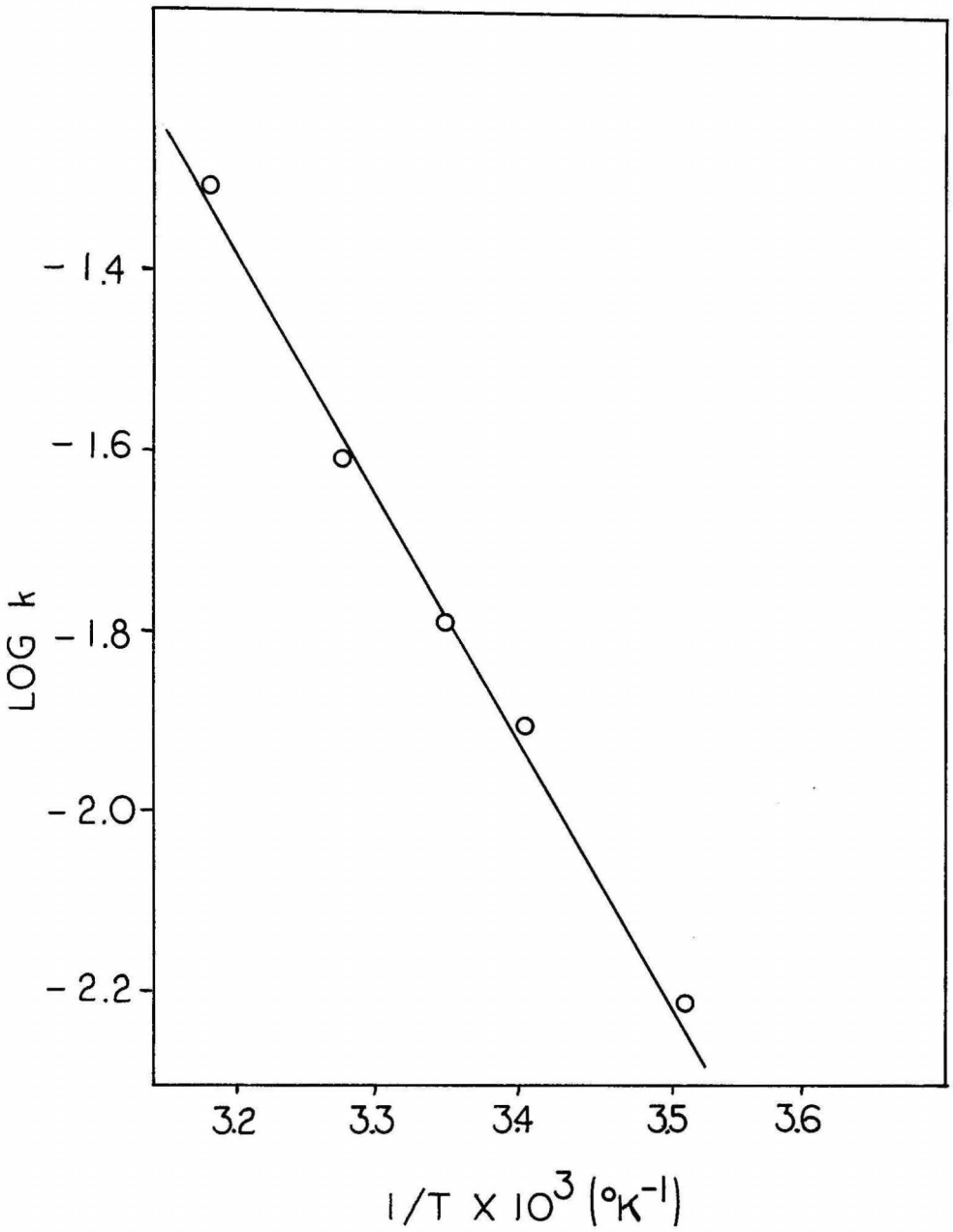


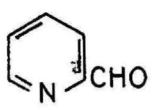
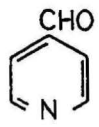
Figure 13. Temperature dependance of propionaldehyde oxidation at pH 7.0. $E_a = 1.27 \times 10^4$ cal/mole.

hyde was therefore assumed to have no affinity for the enzyme.

Isobutyraldehyde does not act as an inhibitor for the other aldehydes. Thus the enzymatic oxidation of acetaldehyde was unaffected by the presence of various concentrations of isobutyraldehyde ranging from 2.53×10^{-3} M to 2.53×10^{-2} M. For example, as shown in Figure 14, the same rate is obtained for the enzymatic oxidation of 7.75×10^{-3} M acetaldehyde alone as is obtained in the presence of 3.79×10^{-3} M isobutyraldehyde. Similar results were obtained when the isobutyraldehyde was injected into an acetaldehyde reaction mixture shortly after the reaction was started. The lack of inhibition by isobutyraldehyde was also demonstrated with the enzymatic oxidation of propionaldehyde.

Studies involving n-butyraldehyde were carried out which showed that the oxidation of this substrate is catalyzed by XO. The value of K_m was found to be 9.2×10^{-2} M. V_m was found to be 0.92 μM/min. The K_m value is appreciably larger than that for propionaldehyde, but still indicative of a moderate affinity for the enzyme.

Figure 15 shows the Lineweaver-Burke obtained for n-butyraldehyde. The usual substrate inhibition was encountered, and it may be seen from Table XII that the Michaelis parameters follow the trend set by the smaller aliphatic aldehydes.

Pyridine Aldehydes The final aldehydes studied were the 2- and 4-pyridine aldehydes: 2-PA  4-PA 

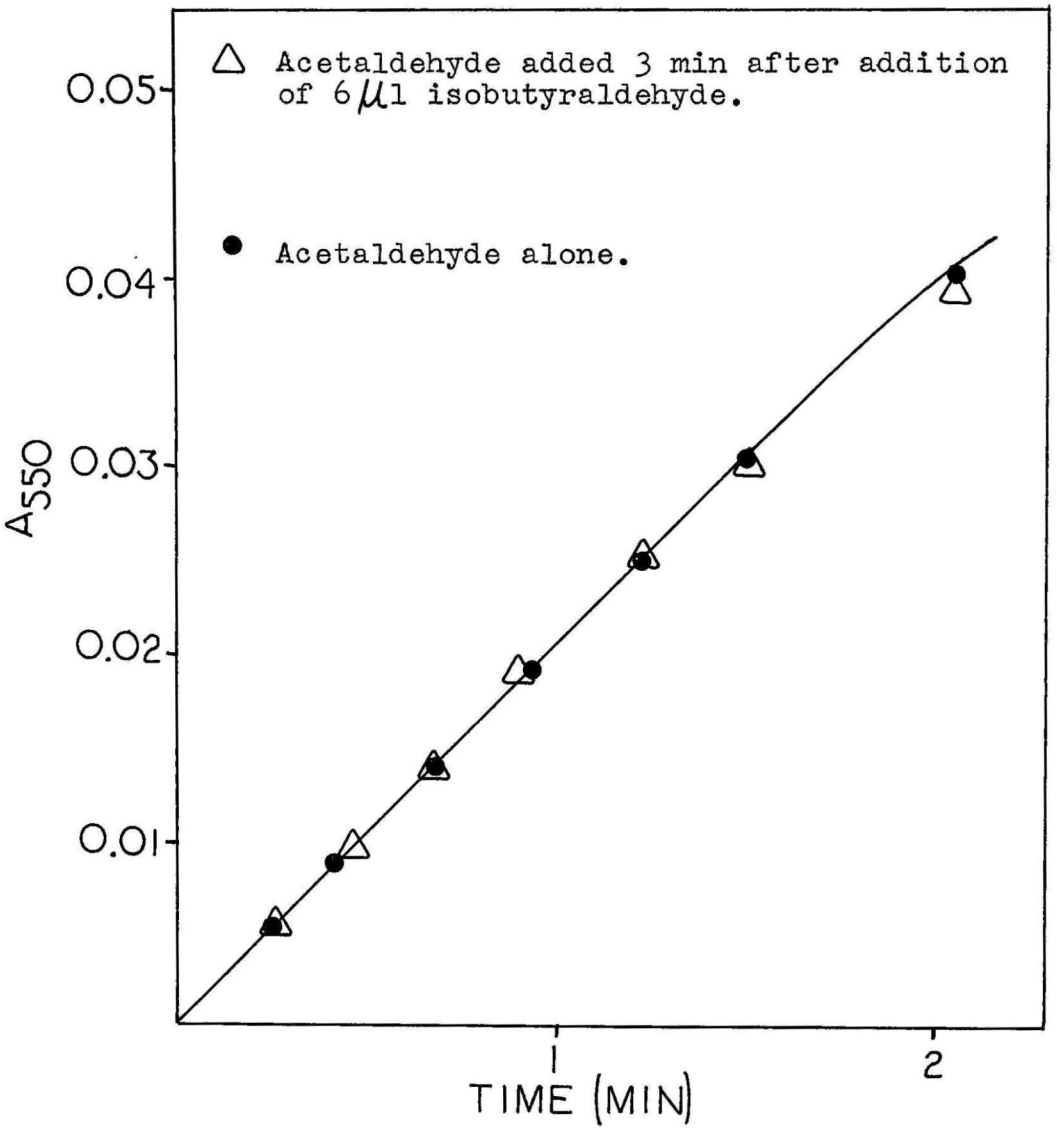


Figure 14. Lack of inhibition of acetaldehyde reaction by previously added iso-butyraldehyde. All reaction conditions were identical, except that in the reaction represented by △, 6 μl of isobutyraldehyde were added three minutes prior to addition of the acetaldehyde.

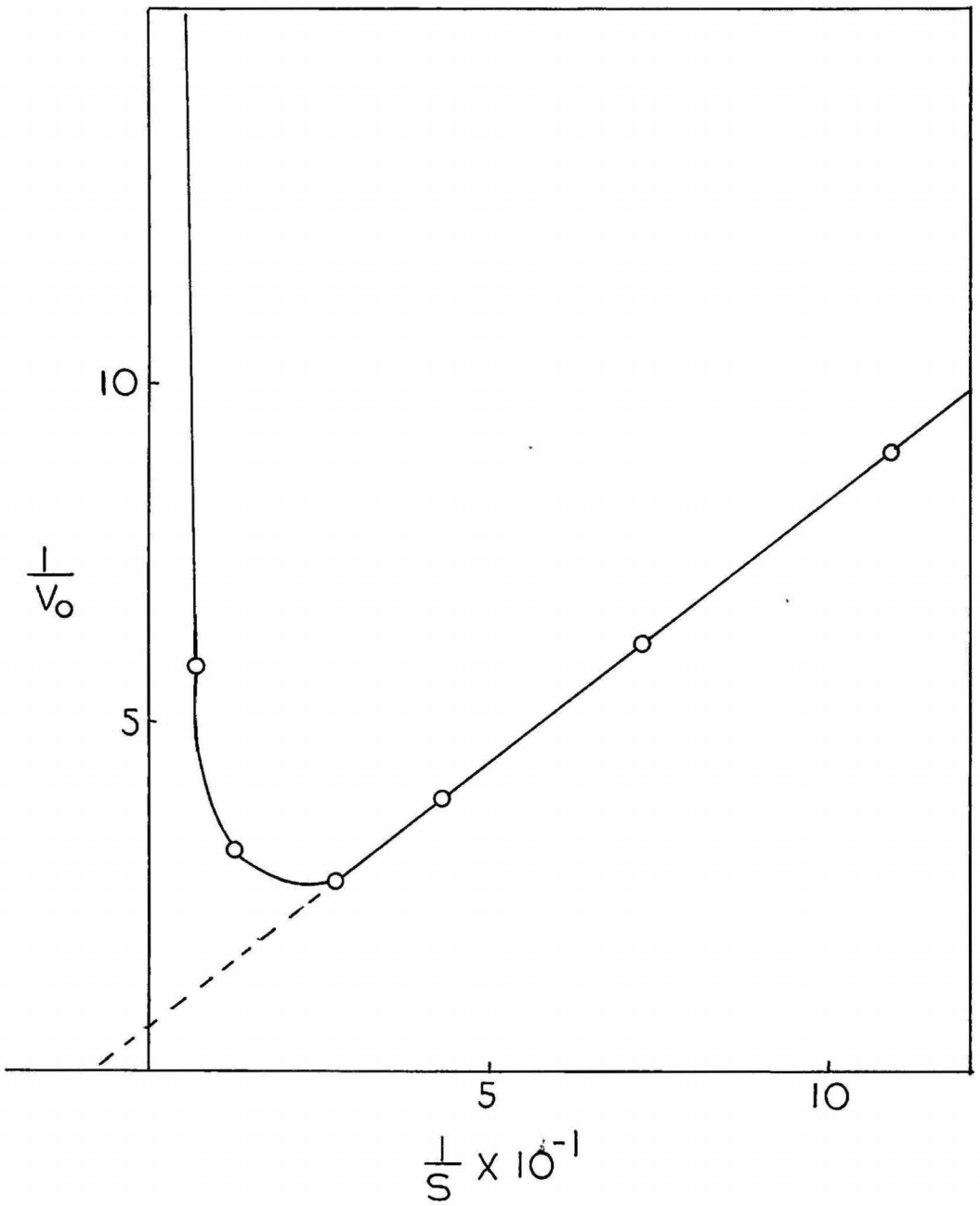


Figure 15. Lineweaver-Burke plot of n-butyraldehyde oxidation by XO, 25.0 °C, pH 7.0, $\mu = 0.1$. $K_m = 9.2 \times 10^{-2}$ M.

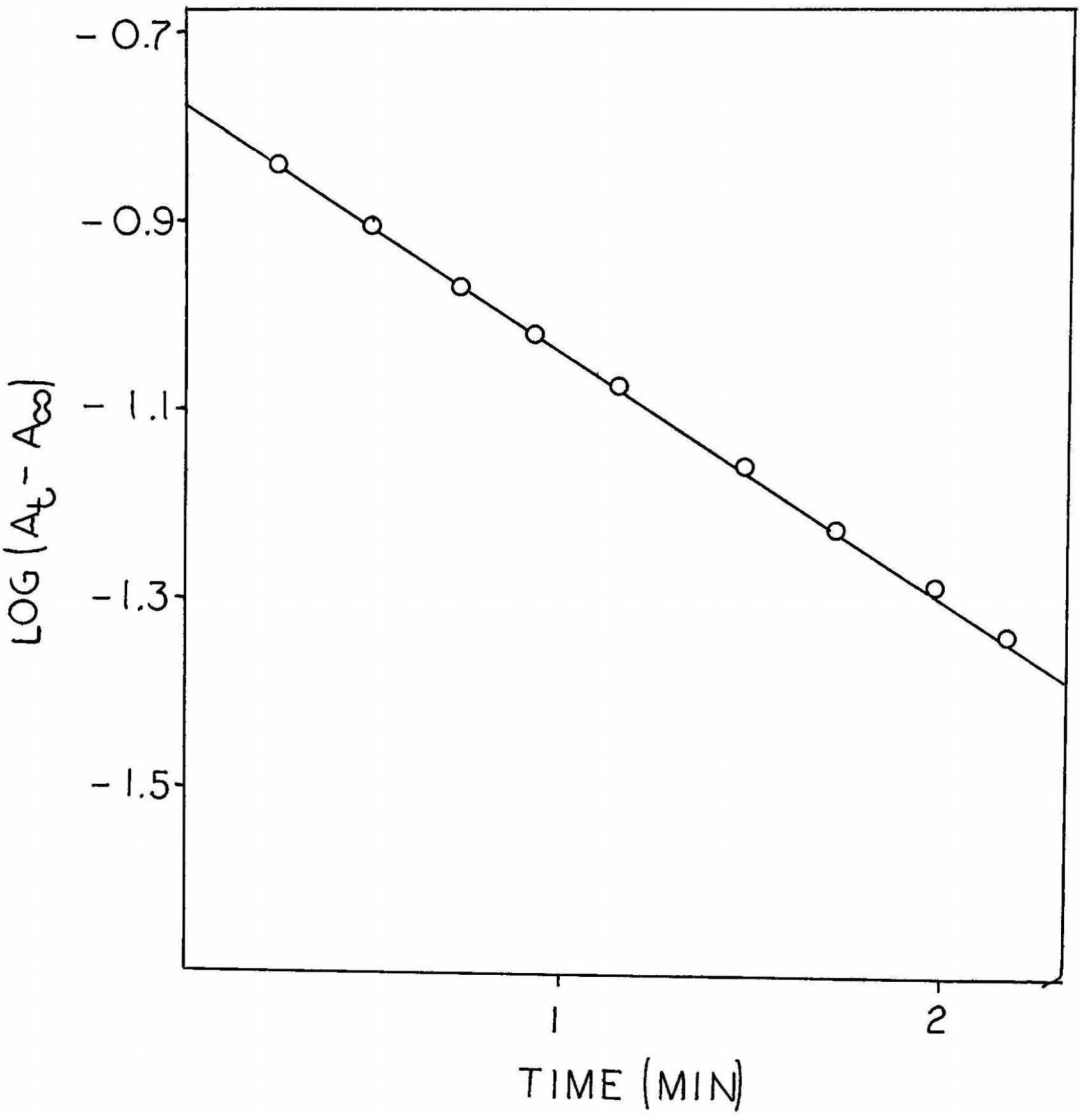


Figure 16. Typical hydration of iso-butyraldehyde at 25.0 °C, pH 7.0, 0.002 M phosphate buffer, μ=0.1.

By inspection, these may be seen to be quite similar to the supposed natural substrates, the purines hypoxanthine and xanthine.

These substrates were distilled under reduced pressure and were used immediately after distillation. A range of from 6.12×10^{-3} M to 2.48×10^{-2} M of 2-PA was initially employed for the enzymatic studies, and little, if any, activity was noted. These initial results seemed to coincide with the possible steric hinderance encountered in the case of isobutyraldehyde.

A concentration of 1.02×10^{-2} M of 4-PA was then tested, and considerably more activity was noted. Lower concentrations gave increased activity, and it was assumed that we were working backward up the substrate inhibition curve (see Fig. 10). Finally, a full range of concentrations from 2.039×10^{-2} M to 2.039×10^{-4} M was employed, with the resultant Lineweaver-Burke plot shown in Figure 17.

A range of concentrations of 2-PA from 2.48×10^{-2} M to 2.48×10^{-4} M was then employed, giving the results shown in Figure 18.

The results obtained with the large, bulky pyridine aldehydes apparently dispel any steric hinderance theory as might have been proposed in the case of isobutyraldehyde, since the larger aldehydes show a marked affinity for the enzyme.

Table XII summarizes the values obtained from the kinetic studies of the various aldehydes.

D. Equilibrated Versus Non-equilibrated Aldehyde Solutions

Using the K_m values obtained in the proceding sections, a series

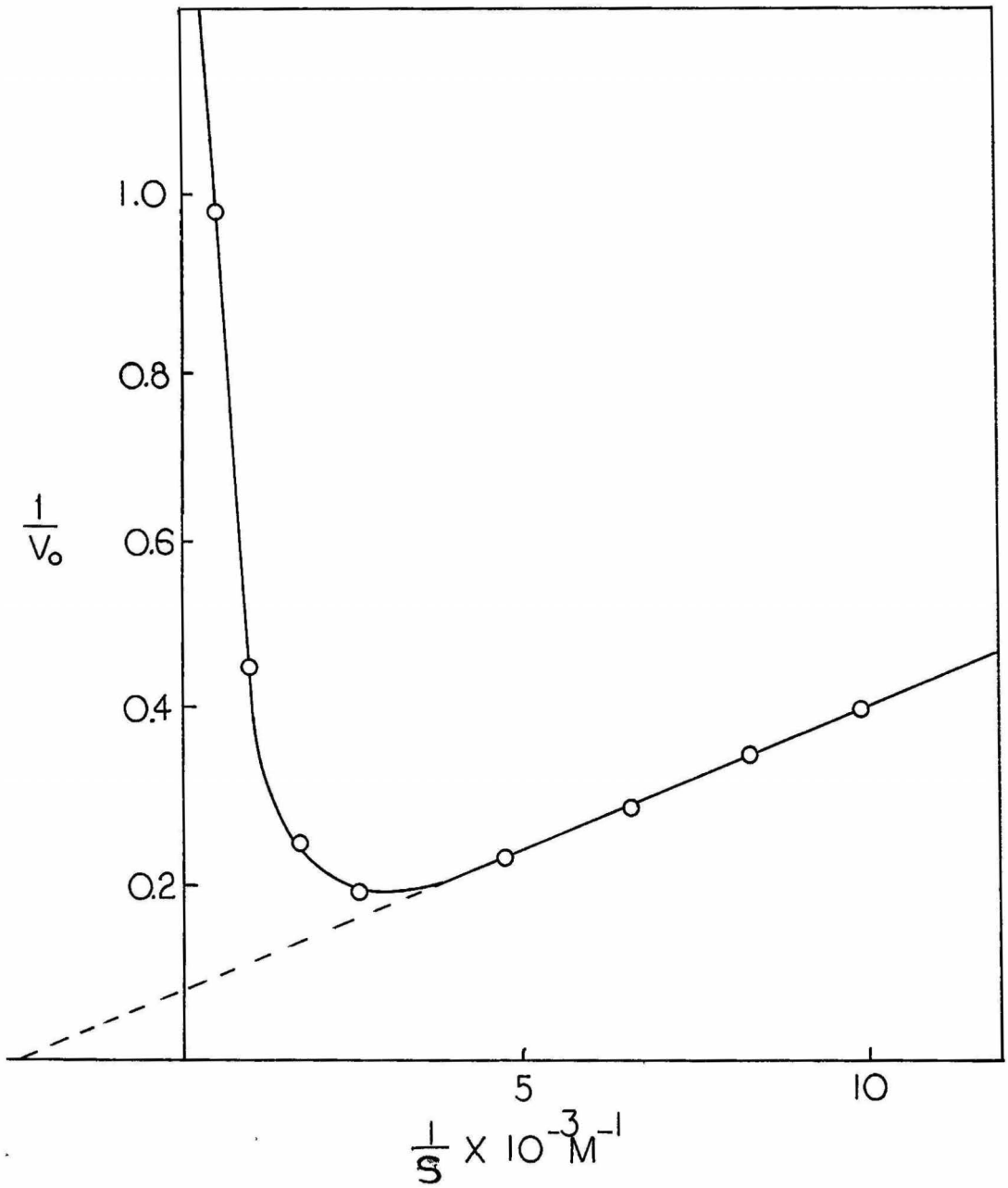


Figure 17. Lineweaver-Burke plot of 4-PA oxidation by XO at 25.0 °C, pH 7.0, $\mu = 0.1$. $K_m = 4.28 \times 10^{-3}$ M. V_m = 13.1 micromoles of cytochrome C reduced per minute.

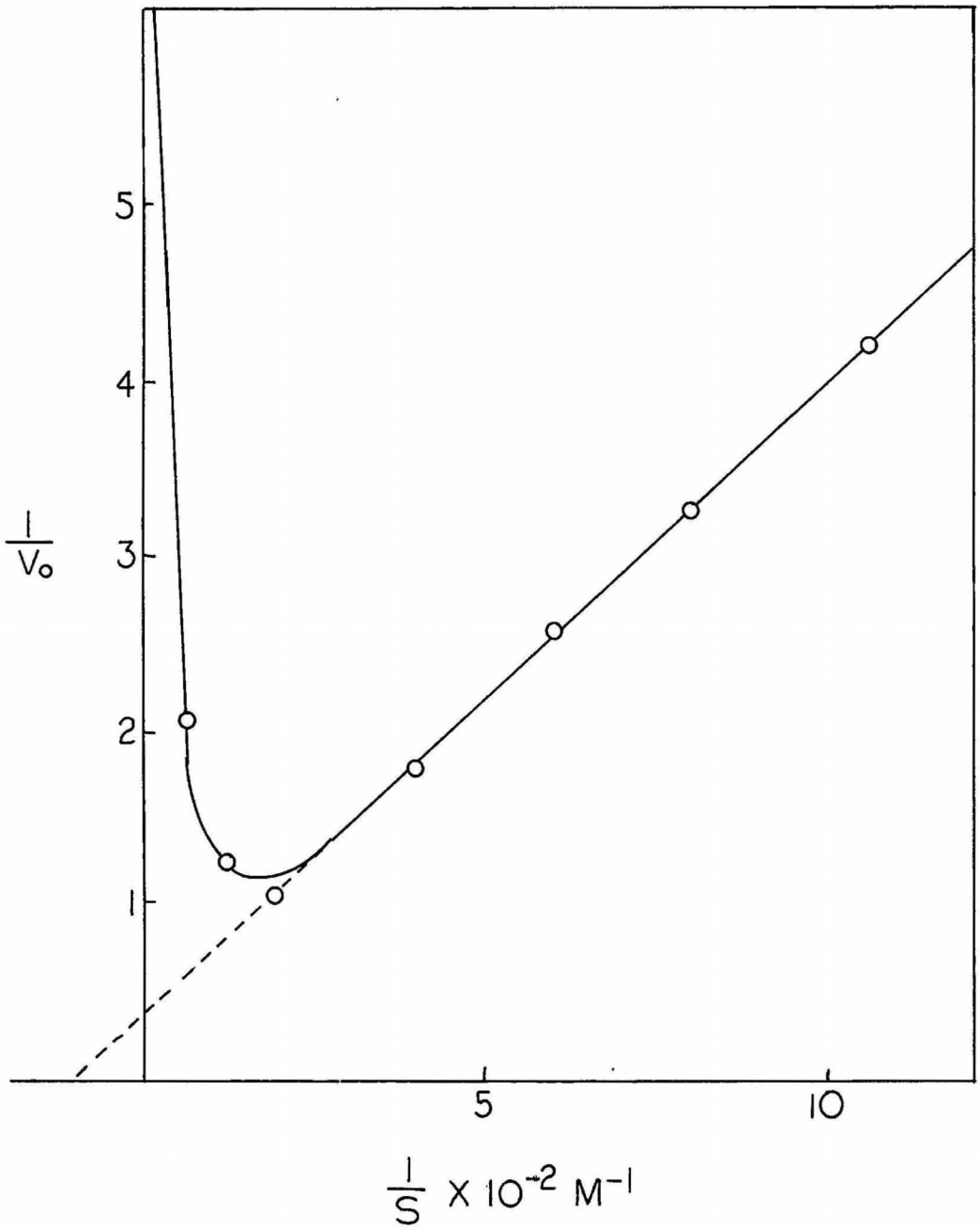


Figure 18. Lineweaver-Burke plot of 2-PA oxidation by XO at 25.0 °C, pH 7.0, $\mu = 0.1$. $K_m = 1.37 \times 10^{-2}$ M. V_m = 3.38 micromoles of cytochrome C reduced per minute.

of studies was made using various concentrations of the aldehydes below the experimentally determined K_m values. These studies entailed injecting the enzyme last into a solution in which the aldehyde had reached equilibrium with the hydrate, and comparing the resultant initial rate of aldehyde oxidation with that obtained from an equivalent solution in which the aldehyde was the last substance added. Several identical runs were made at various concentrations of the aldehydes, and in all cases, the initial rate of cytochrome C reduction from the non-equilibrated aldehyde was greater than that obtained from the equilibrated aldehyde.

The rate for the non-equilibrated aldehyde solutions were observed to decrease at a rate which was comparable to the rate of aldehyde-hydrate equilibration. Thus at a time corresponding to that required for equilibration, the cytochrome C reduction slowed to a constant rate which was identical to that observed from comparable equilibrated aldehyde solutions.

Figure 19 compares the initial rate of cytochrome C reduction observed from a solution of non-equilibrated aldehyde at 2.75×10^{-3} M in acetaldehyde to that obtained from an equilibrated solution of the same amount of aldehyde. Similar results were obtained in all cases of equilibrated versus non-equilibrated solutions. As will be shown in the discussion section, the rate of hydration and the degree of hydration of the aldehyde at any time during the reaction may be deduced from a comparison of the rates obtained from an equilibrated and non-equilibrated solution at that point in the reaction.

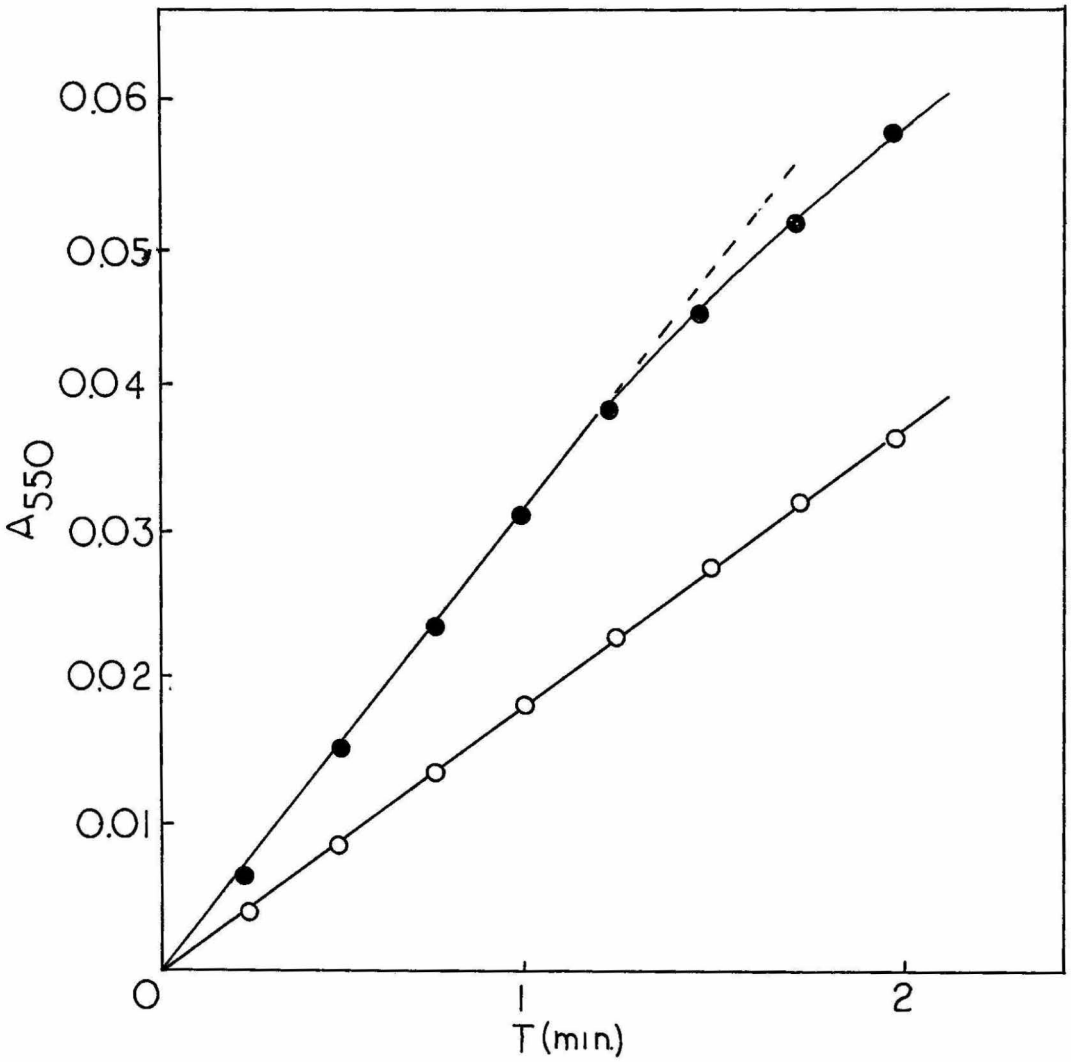


Figure 19. Comparison of initial velocities of equilibrated versus non-equilibrated acetaldehyde. The closed circles represent the non-equilibrated aldehyde. The same concentration of aldehyde, 2.75×10^{-3} M was injected into each solution, but allowed to equilibrate only in the run represented by the open circles.

E. Reaction of Cytochrome C and Acetaldehyde

As has been indicated in previous sections, the addition of acetaldehyde to cytochrome C usually resulted in an initial drop of the absorbance below zero. It was also found that certain conditions minimized this effect.

Several different grades of cytochrome C were tested, and it was found that the higher the grade, the less this effect was noted. Since the highest grade tested, Sigma Type VI, contained the least amount of reduced form of cytochrome C, it was felt that perhaps something in the acetaldehyde was oxidizing the reduced form that was present.

The acetaldehyde was redistilled, and the fresh acetaldehyde tested with the cytochrome C. This was found to minimize the initial absorbance decrease. Therefore, in later kinetic studies, the acetaldehyde used was distilled immediately prior to use. Similarly, it was found that freshly prepared cytochrome C exhibited less of the reduced form than did solutions which had been standing in the freezer.

Comparison of the spectrum of the stock solutions of cytochrome C to the spectrum of the oxidized form, Figure 20, show that there are small amounts of the reduced form present. In the stock solution, small peaks characteristic of the reduced form may be seen at 550, 510 and 415 nm. Upon addition of one ml of acetaldehyde, these peaks were diminished to conform to the spectrum of the oxidized form, which exhibits a single broad peak in the range of 500-560 nm, while the reduced form shows two peaks in this range. A comparison of the spectrum

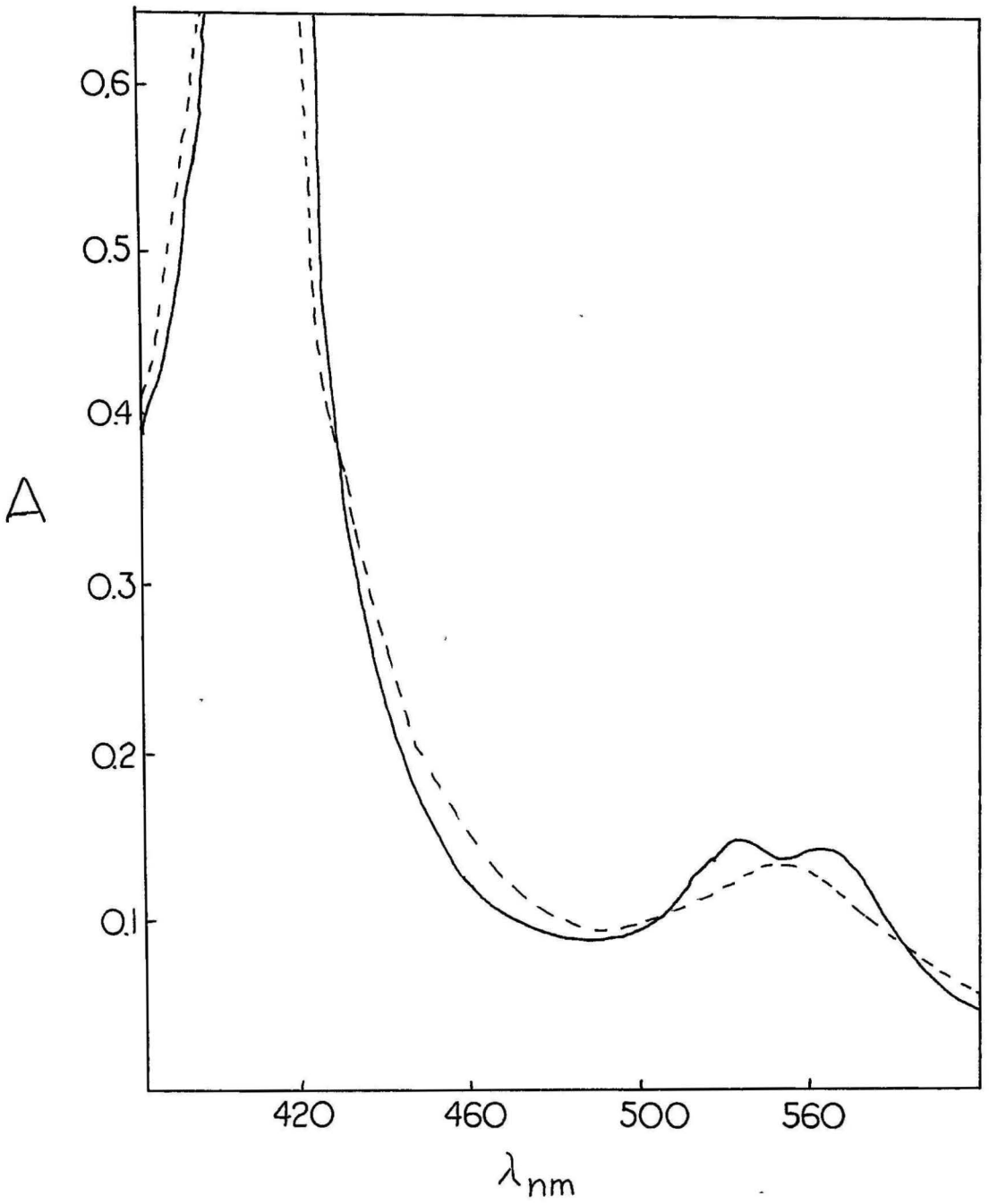


Figure 20. Comparison of the spectrum of stock cytochrome C to that of the oxidized form. The stock solution is represented by the solid line.

of the completely reduced form of cytochrome C and that of the stock solution is shown in Figure 21.

Various other aldehydes were tested with cytochrome C to ascertain whether they had the same effect as acetaldehyde. While the smaller aldehydes gave the same effect, the larger of the aldehydes showed less of the effect. Various related reagents such as ethyl alcohol, acetone, acetic acid and methanol failed to duplicate the effect.

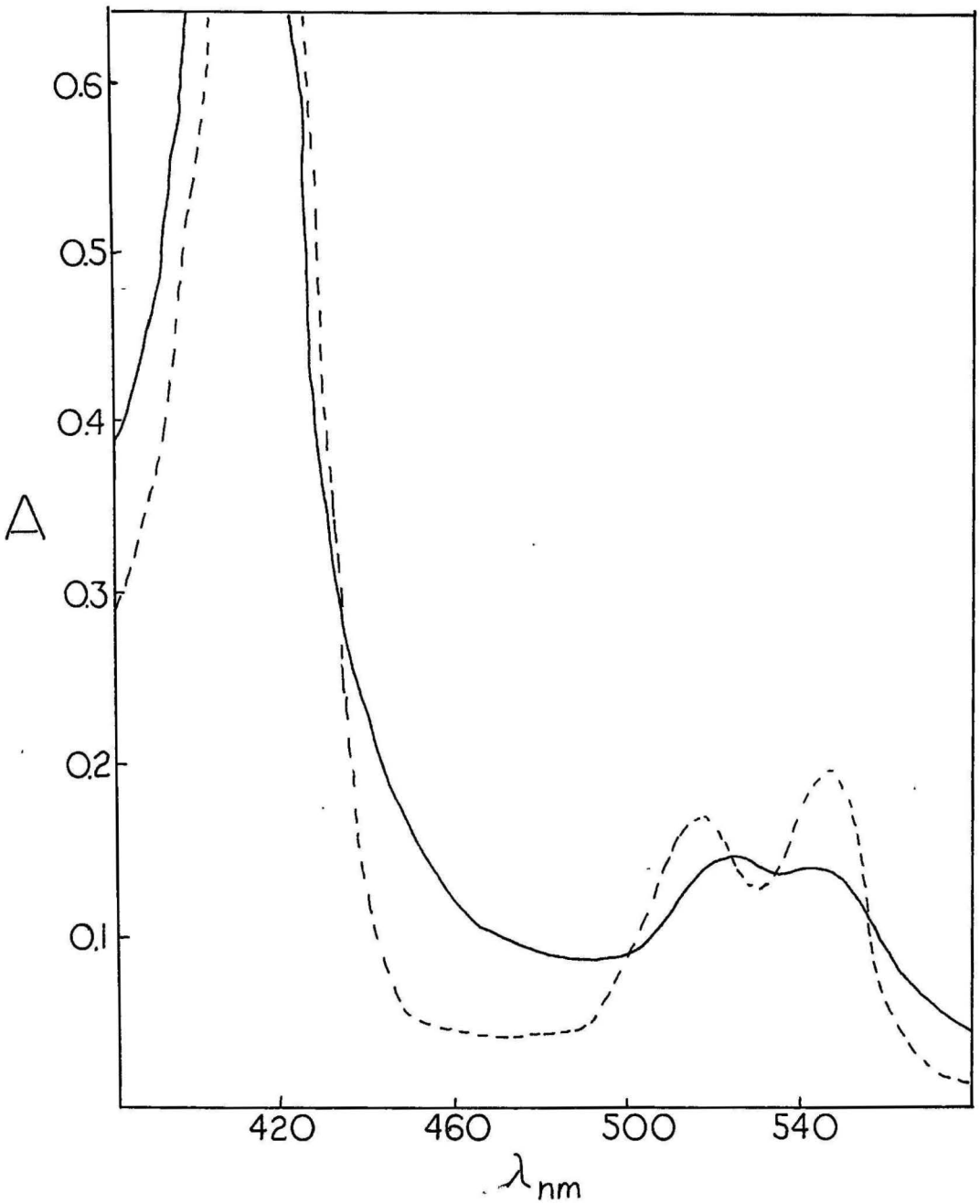


Figure 21. Comparison of the spectrum of stock cytochrome C to that of the reduced form. The stock solution is represented by the solid line.

IV. DISCUSSION

A. Hydration of Acetaldehyde

The capacity of mono- and dihydrogen phosphate to catalyze the hydration of acetaldehyde substantiates earlier findings pertaining to the general acid and general base catalyzed nature of this reaction(31, 32,43). The nature of the catalytic effects noted are consistent with the mechanisms proposed by Bell(31): for general acid catalysis

$$MeCHO + H_2O + HB \underset{slow}{\overset{slow}{\rightleftharpoons}} HOCHMeOH^+_2 + B^-, \qquad (18)$$

$$HOCHMeOH^+_2 + B^- \underset{fast}{\overset{fast}{\rightleftharpoons}} MeCH(OH)_2 + HB, \qquad (19)$$

and for general base catalysis,

$$MeCHO + H_2O + B \underset{slow}{\overset{slow}{\rightleftharpoons}} HOCHMeO^- + BH^+, \qquad (20)$$

$$HOCHMeO^- + BH^+ \underset{fast}{\overset{fast}{\rightleftharpoons}} MeCH(OH)_2 + B. \qquad (21)$$

The experimentally determined fraction of hydration, χ, may be used to determine the apparent equilibrium constant, K_{eq}:

$$K_{eq} = \frac{\chi}{1 - \chi}. \qquad (22)$$

As has been previously mentioned, the forward rate coefficient, k_f, may be evaluated from:

$$k_f = \chi(k_{obsd}). \qquad (23)$$

Table VIII summarizes the values obtained from kinetic runs made at 10 °C and 25 °C. The data at zero degrees is from Pocker and Meany (53).

Table VIII

Dependency of χ and K_{eq} on Absolute Temperature

Temp (°K)	χ	K_{eq}
273	.700	2.33
283	.620	1.64
298	.503	1.01

The enthalpy change, $\Delta H°$, may be determined from a plot of log K_{eq} versus 1/T, as shown in Figure 22,

$$-\Delta H° = 2.303\ R\ (\text{Slope}). \tag{24}$$

Using this enthalpy value and the experimentally determined K_{eq} at 298.0 °K, $\Delta G°$ may be obtained from:

$$-\Delta G° = 2.303\ RT\ \log K_{eq}. \tag{25}$$

The entropy change, $\Delta S°$, may then be determined:

$$\Delta S° = \frac{(\Delta G° - \Delta H°)}{T}. \tag{26}$$

Table IX summarizes the thermodynamic parameters of acetaldehyde hydration at 298.0 °K.

Table IX

Thermodynamic Parameters of Acetaldehyde Hydration

$-\Delta G°$	$-\Delta H°$	$\Delta S°$
0.007 kcal/mole	5.6 kcal/mole	-18.8 eu

As discussed in the results section, the specific rate coefficients for the components of the phosphate buffers may be determined by a series of plots of k_f vs $[H_2PO_4^-]$ at different buffer ratios as shown in Figures 6 to 8. The results of the present study and those of Pocker and Meany at 0.0 °C are summarized in Table X:

Table X

Catalytic Rate Coefficients for Acetaldehyde Hydration

Temp (°K)	k_o (min^{-1})	k_{OH^-} [a]	$k_{H_2PO_4^-}$ [a]	$k_{HPO_4^{-2}}$ [a]
298	0.305	1.88×10^6	105	189
283	0.149	1.14×10^6	51.5	110
273 [b]	0.094	9.60×10^5	16.0	36.0

a. units are 1/mole min.
b. Pocker and Meany(43).

As shown in Figure 6, a plot of k_f versus $[H_2PO_4^-]$ yields a straight line, precluding any significant catalysis arising from the concerted action of the mono- and dihydrogen phosphate. An Arrhenius plot was constructed with respect to the spontaneous hydration by plotting k_o versus 1/T from which the activation energy, Ea, was determined:

$$E_a = -2.303\ R\ (Slope). \qquad (27)$$

From this value, activation parameters for the spontaneous hydration of acetaldehyde may be derived from:

$$\Delta H^{\ddagger} = E_a - RT, \qquad (28)$$

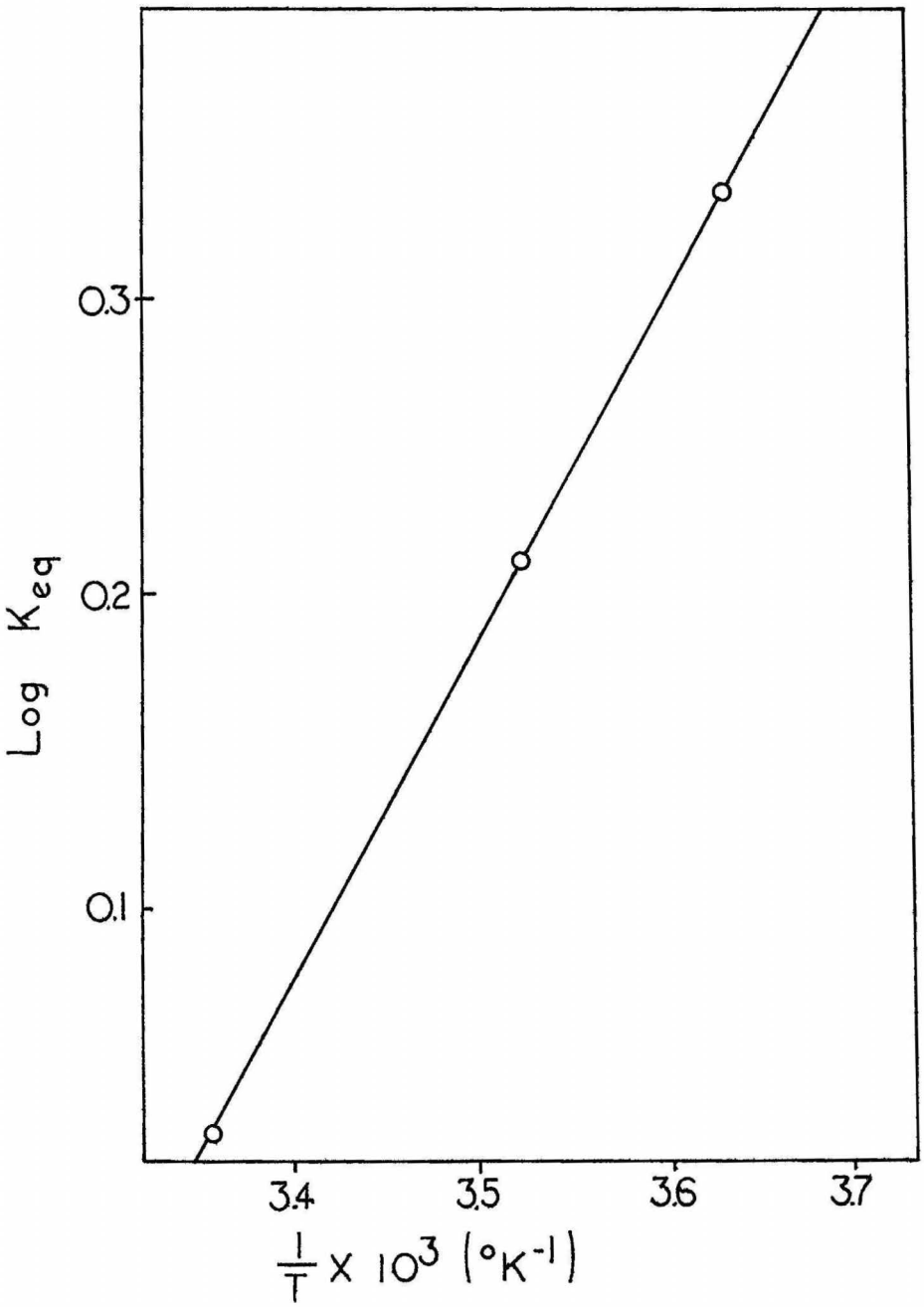


Figure 22. Arrhenius plot for the spontaneous hydration of acetaldehyde.

$$k_f = \frac{kT}{h} e^{\Delta S^{\ddagger}/R_e - \Delta H^{\ddagger}/RT}, \quad (29)$$

and

$$\Delta G^{\ddagger} = \Delta H^{\ddagger} = T\Delta S^{\ddagger}. \quad (30)$$

The activation parameters so obtained are listed in Table XI, and are compared with the activation parameters for the hydration of ethyl and methyl pyruvate, as determined from earlier work(54).

Table XI

Activation Parameters for the Spontaneous Hydrations of Acetaldehyde, Methyl Pyruvate and Ethyl Pyruvate

Substrate	E_a (kcal/mole)	$\Delta H^{\ddagger}$ (kcal/mole	$\Delta G^{\ddagger}$ (kcal/mole)	$\Delta S^{\ddagger}$ (eu)
Acetaldehyde	7.8	7.2	11.9	-16.2
Methyl Pyruvate	8.1	7.5	13.9	-21.3
Ethyl Pyruvate	8.2	7.6	14.0	-21.4

As expected from reactions which involve a decrease in the total number of molecules and in which charge separation begins in the transition state, the entropies of activation for each of the hydration processes is negative. The greater decrease in entropy of activation associated with the hydration of the alkyl pyruvates as compared to the hydration of acetaldehyde may be interpreted as an indication of a greater increase in orientation in the transition state associated with the former. This is consistent with earlier work(34) which showed that

while the stoichiometry of acetaldehyde and its hydrate differ by the elements of a single molecule of water, hydrated alkyl pyruvates and the free pyruvate esters differ by two. Thus, it may be assumed that the transition states in the latter hydration processes also involve more solvation. This would necessitate the involvement of more suitably orientated water molecules and could partially account for the relatively large negative entropy of activation for the hydration of the pyruvate esters as compared to that of acetaldehyde.

Entropies of activation, however, must be treated with reserve and provide no compelling evidence of mechanism especially when determined in polar solvents. Such entropy effects also reflect changes in the randomness of the solvent molecules as new species requiring differing degrees of solvation are formed from the reactants.

B. Catalytic Efficiency

It is of interest to compare the Michaelis parameters obtained for the various substrates studied. Table XII is a compilation of the values obtained in this study and those obtained by Booth and Roussos. The K_m values of Booth were normalized to the current results for comparison by comparing the values obtained for a common substrate, acetaldehyde. Rousses' values for hypoxanthine and xanthine are presented to enable comparison of aldehydic substrates with the assumed "natural" substrates.

Table XII

Michaelis Parameters for the XO Catalyzed Oxidation of Aldehydes and Purines

Substrate	K_m(mole/l)	V_m [c]
Formaldehyde[a]	4.2×10^{-3}	-
Acetaldehyde	2.7×10^{-2}	21.6
Propionaldehyde	5.3×10^{-2}	4.5
n-butyraldehyde	9.2×10^{-2}	0.92
Isobutyraldehyde	-	-
2-PA	1.4×10^{-2}	3.4
4-PA	4.3×10^{-3}	13.1
Salicylaldehyde[a]	1.0×10^{-3}	-
Furfuraldehyde[a]	3.1×10^{-3}	-
Xanthine[b]	2.8×10^{-5}	-
Hypoxanthine[b]	1.5×10^{-5}	-

a. Booth(1). b. Roussos(52). c. V_m is reported in micromoles of cytochrome C reduced per min per 50 µg of XO.

The fact that the pH profiles with respect to K_m and V_m have been shown to be similar for both purines and aldehydes lends support to the argument that both species are catalyzed by the same active site. Both species exhibit bell shaped curves with a rather broad maximum in the area pH 7.25 to pH 7.50 (see Fig. 11), and with points of inflection at pH values of about 6.75 and 8.0. It may be seen from

Table XII that the affinity for the aliphatic aldehydes decreases as the chain length increases. In the case of isobutyraldehyde, the branch in the chain appears to prevent the substrate from binding to the enzyme. It is also noted that isobutyraldehyde does not inhibit other aldehyde reactions when it is present in concentrations from 1×10^{-2} M to 1×10^{-4} M.

In general, while small aliphatic aldehydes and large bifunctional aromatic aldehydes both bind fairly well, a small branched aliphatic aldehyde did not bind at all. The K_m values for the pyridine aldehydes are comparable to, or even smaller than those of the smaller aliphatic aldehydes; for example, from Table XII, K_m for formaldehyde is 4.2×10^{-3} M, while K_m for 4-PA is 4.3×10^{-3} M. As has been previously mentioned, the pyridine aldehydes are structurally more similar to the purines than are the aliphatic aldehydes. This observation leads to the suggestion that the ring nitrogen enhances the enzyme-substrate binding or that there may exist two or more binding sites associated with the active site.

The values cited for the aromatic aldehydes, as reported by Booth, must not be compared too closely with the present results for they were obtained using methylene blue as the electron acceptor, and the pH and temperature were not reported. Booth's data offers a useful comparison of the affinity of XO for aliphatic and aromatic aldehydes, indicating, as does the present study, a higher affinity for the bifunctional aromatic aldehydes. The near proximity of the ring nitrogen and the aldehydic groups in the pyridine aldehydes may account

for the lowered affinity of the 2-PA. Interestingly, the same relative binding capacity of 2- and 4-PA has been observed for another metalloenzyme, carbonic anhydrase from bovine erythrocytes(37). It has also been found that Co^{+3} enhances the oxidation of 4-PA to a larger extent than it does with 2-PA(55).

The general trends of affinity for the various substrates follow the order: purines > aromatic aldehydes > aliphatic aldehydes. It is noted that in all studies of this type reported, the affinities for xanthine and hypoxanthine are on the order of 100 to 1,000 times greater than those shown for the aldehydes. These observations support the theory that the purines are more likely to be the natural substrates than are the aldehydes, since "natural" substrates are normally assumed to be those for which the lowest values of K_m are observed(2).

As has been shown by the kinetic studies, all of the aldehydes which acted as substrates for the enzyme led to severe substrate inhibition. This effect has also been noted by other investigators in the xanthine and hypoxanthine reactions(2,50). Substrate inhibition was encountered regardless of the electron acceptor used. The general trend of substrate inhibition indicated that the higher the affinity of the enzyme for the substrate, the higher was the degree of substrate inhibition. The maximum velocity experimentally reached came closer to the predicted from Lineweaver-Burke plots in the case of substrates with low affinities.

The substrate inhibition may perhaps be explained by assuming

the existence of both an active site and a nearby secondary binding site on the molecule. In the case of the smaller substrates, a molecule attaching to the binding site might block attachment of any molecule to the active site where it can react. In the case of the large aromatic substrates, excess substrate may result in competition for the sites, preventing any single molecule from attaching in the manner required for enzymatic activity. The fact that isobutyraldehyde acts neither as a substrate nor an inhibitor apparently indicates that it is unable to bind at either site.

The active site may then be a small area, capable of binding the small straight chain aldehydes, but unable to bind to a small, but branched molecule. In conjunction with this small restrictive active site may be another binding site which can hold the larger ring substrates in such a manner as to orient the aldehydic group in the direction of the active site for enzymatic activity.

C. pH Dependence

As shown in the results section in Table VI, K_m for the oxidation of acetaldehyde by XO is fairly constant over the pH range 6.5 to 8.0, while V_m varies, falling off 20 to 30% on each side of neutrality. The difference observed between K_m-pH curves and V_m-pH curves indicate that different groups are involved in binding and subsequent turnover. While similar work has been reported with the purines, this study makes the first such comparison for the aldehydes. The constancy of K_m as a function of pH suggests that binding must occur via a

group that does not ionize in the pH range 6-8. The ionizable groups responsible for the inflection points in the pH rate profile, Figure 11, are presumably intimately involved in the oxidation step.

The pH-V_m profile shows inflections at approximately the same pH values as does the pH-rate profile, at about 6.75 and 7.75. These values correspond roughly to imidizole and alpha-amino groups in various amino acids(17). These are approximate indications only, for the pH effects are not only associated with the enzyme, but with the enzyme-substrate complex. Briefly, pH effects can be divided into five groups: (1) effect on denaturation, (2) effect on substrate, (3) effect on buffer, (4) effects on groups binding substrate to enzyme and (5) effects on groups catalyzing the enzymic reaction(2). While the present results seem to indicate that the pH effects noted here are due to effects on the catalytic groups, additional work is needed in order to derive more definitive results.

The current theory is that several acidic and basic amino acid side chains are involved in binding and catalysis(2). However, the assignment of a particular pK_a value to a given amino acid group based solely on pH profiles is treacherous because of the profound effects of neighboring groups and charges. In actuality, for most enzymes, pH maxima and inflection points are found almost continuously over a range of pH 2 to pH 10, indicating many different groups and combinations of groups may be responsible for binding and catalysis(2).

The above considerations show why extensive investigation of pH effects on enzymatic activity must be carried out before definitive

conclusions are drawn. In the present study, the pH effects were investigated mainly to determine a practical pH at which to study the enzymatic oxidation of aldehydes. The pH profiles obtained in the present work are quite similar to those obtained for the purines, which usually have been found to exhibit a maximum between pH 7 and 7.5(50). Other investigators also observed that the K_m values for the purines are unaffected by pH, indicating, as does this work, that the catalytic groups are those effected by pH(50,52). These observations support the theory that the purines and aldehydes utilize the same groups in binding and catalysis.

D. Equilibrated versus Non-equilibrated Aldehydes

The main purpose of this thesis was to determine the true substrate for the aldehydic oxidation catalyzed by xanthine oxidase:

$$[RCHO + H_2O \leftrightharpoons RC(OH)_2] \overset{enz}{\rightarrow} RCOOH. \qquad (31)$$

The initial reaction rates of solutions of non-equilibrated aldehydes, that is, runs in which the neat aldehyde was the last component injected, were compared with initial reaction rates of reactions in which the enzyme was injected into a solution containing the aldehyde already in equilibrium with its hydrate. In every case, the data indicated a more rapid rate from the non-equilibrated solutions, which decreased with time until a constant rate of cytochrome C reduction was reached. This final rate was identical to the initial rate obtained when the aldehyde was allowed to reach equilibrium with its hydrate prior to the addition of the enzyme. This observation suggests

that the aldehyde is the true substrate, since as the concentration of aldehyde decreased due to hydration, a parallel decrease in cytochrome C reduction resulted. Figure 23 is a comparison of the reaction rates obtained from 5.4×10^{-3} M acetaldehyde in a 0.03 M phosphate buffer, pH 7.28. The closed circles represent cytochrome C reduction in the non-equilibrated solution. In Figure 23B, the origin of the equilibrated reaction has been displaced upwards to demonstrate the relationship of the two graphs after equilibration has been reached in both cases. From hydration studies, it was found that equilibration between the hydrate and the aldehyde was reached in about 64 seconds, which corresponds to the point in Figure 23B where the two graphs have identical slopes.

It may be seen that the initial slope of the line associated with the non-equilibrated solution is related to the initial slope of that associated with the equilibrated solution by the fraction of hydration, χ;

$$\frac{S_e}{S_n} = 1 - \chi, \qquad (32)$$

where S_n and S_e are the initial slopes of the non-equilibrated and equilibrated solutions, respectively. From Figure 23, χ is calculated to be 0.508, which is in very good agreement with the value 0.503 as determined by direct measurements using the spectrophotometric method described earlier in this paper. Table XIII summarizes typical values of the fractions of hydration as calculated from Equation 32 and these are compared to those values as deduced through direct hydration studies.

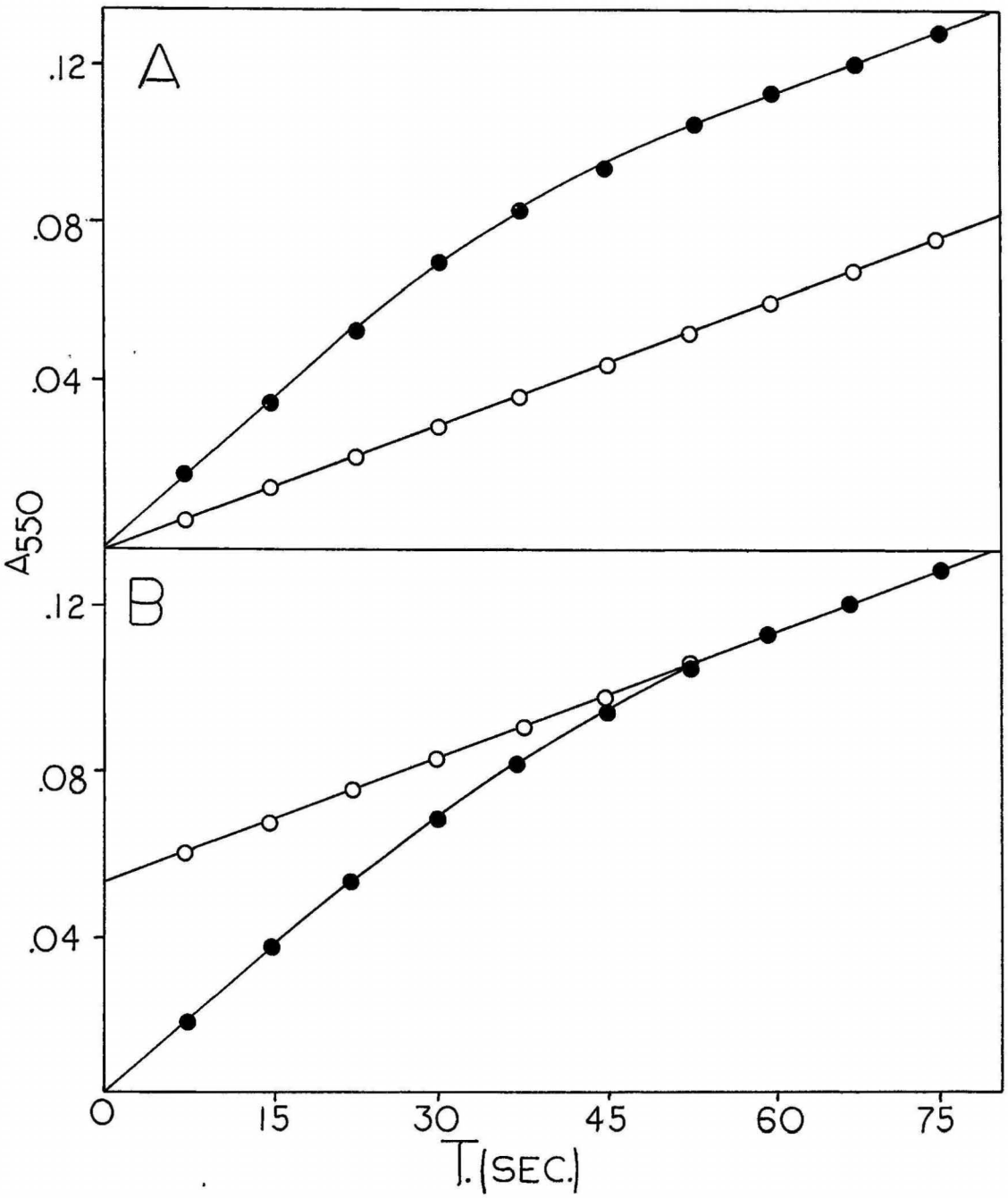


Figure 23. Comparison of initial rates of cytochrome C reduction of equilibrated and non-equilibrated solutions. The open circles represent the equilibrated solutions. Each solution contains 5.4×10^{-3} M acetaldehyde and 3×10^{-5} M cytochrome C.

Table XIII

Comparison of χ Values Obtained from Hydration and Enzyme Oxidation Studies

	Acetaldehyde				Propionaldehyde			2-PA	4-PA
Run	25	59	80	100	22	45	90	14	17
Calc[a]	.504	.512	.501	.500	.410	.415	.411	.290	.418
Obs.		.503				.413		.293	.420

a. Calc. $\chi = (S_n - S_e)/S_n$.

b. Obsd. χ from direct measurements.

The reactions were carried out at aldehyde concentrations much smaller than K_m, and in a region where substrate inhibition was not observed. For example, the range used for acetaldehyde was 2×10^{-3} M to 9×10^{-3} M. Hence the aldehydic oxidations as monitored by cytochrome C reduction were first-order in aldehyde. Furthermore, the concentration of cytochrome C was much smaller than the aldehyde concentration, so that for an equilibrated aldehyde-hydrate solution, psuedo-zero-order kinetics would be expected. When neat acetaldehyde was added directly to the reaction mixture, the rate of cytochrome C reduction, V^r, would be expected to decrease along with hydration equilibration until an equilibrium concentration of hydrate and aldehyde is reached. Thus the reaction rate, under the conditions employed for these experiments is directly proportional to the concentration of

free aldehyde (C),

$$V_t^r = k^r (C_t) \tag{33}$$

which after equilibration has been reached may be expressed as:

$$V_\infty^r = k^r(C_\infty) \quad \text{const.} \tag{34}$$

Using Equations (33) and (34), and by letting A_t' represent the absorbance of the equilibrated solution at time t, and A_t the absorbance of the non-equilibrated solution at that time, the rate expression becomes:

$$V_t^r - V_\infty^r = k^r[C_t - C_\infty] = - \frac{dA_t}{dt} - \frac{dA_t'}{dt} . \tag{35}$$

It is known that for the hydration reaction, the rate of equilibration may be expressed by the relationship

$$- \frac{d\,[C_t - C_\infty]}{dt} = k_{hyd}\,[C_t - C_\infty]. \tag{36}$$

Substituting from Equation (35), we have:

$$C_t - C_\infty \left(\frac{V_t^r - V_\infty^r}{k^r}\right) = - \frac{d\Delta A}{dt} \left(\frac{1}{k^r}\right) , \tag{37}$$

where $\Delta A = A_t' - A_t$. From Equation (36),

$$\frac{-d \frac{d\Delta A}{dt}}{dt} \left(\frac{1}{k^r}\right) = \frac{k_{hyd}}{k^r} \left(\frac{d\Delta A}{dt}\right) , \tag{38}$$

thus,

$$\frac{-d\left(\frac{d\Delta A}{dt}\right)}{\frac{d\Delta A}{dt}} = k_{hyd}\, dt. \tag{39}$$

Then,

$$-\log \frac{d\Delta A}{dt} = k_{hyd}\, t + \text{const.} \qquad (40)$$

If the reaction is first order,

$$\frac{d\Delta A}{dt} = (\text{constant})\Delta A, \qquad (41)$$

and,

$$-\log \frac{d\Delta A}{dt} = -\log \text{constant} - \log\Delta A = k_{hyd}\, t \qquad (42)$$

Thus if the change in rate of cytochrome C reduction is indeed due to the rate of aldehyde-hydrate equilibration, a plot of logΔA against time from Figure 23 should result in a straight line, the slope of which is k_{hyd}. This plot is shown in Figure 24, from which k_{obsd} has been evaluated as 6.53 min^{-1}. The value of k_{hyd} directly observed from the direct hydration was 6.44 min^{-} in the same buffer. Many such analyses showed excellent agreement between the values of k_{hyd} and χ as obtained by the direct hydration of acetaldehyde at 278 mu and those obtained indirectly by the rate of change in cytochrome C reduction as aldehyde-hydrate equilibration was reached. Thus the agreement of the data shown in Table XIII offers unambiguous and quantitative proof that the true substrate for the XO catalyzed oxidation of the aldehydes is the aldehyde itself rather than its conjugate hydrate.

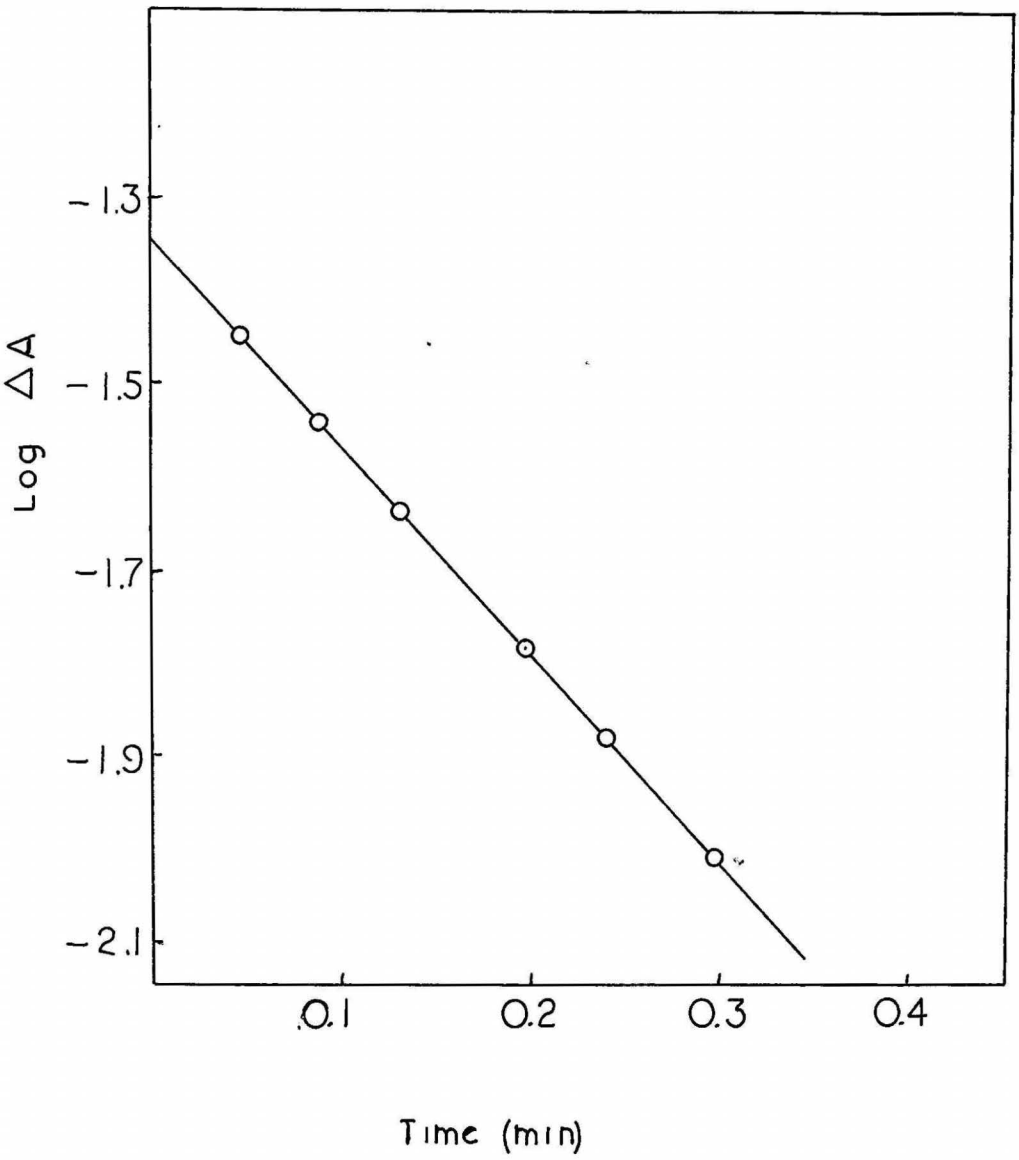


Figure 24. Plot of log ΔA versus time. From the slope, k_{obsd} is calculated as 6.53 min^{-1}.

BIBLIOGRAPHY

1. Booth, V. H., Biochem. J., 1732 (1935).

2. Dixon, M. and E. C. Webb, Enzymes, Academic Press, New York, (1964).

3. Ball, E. G., Science, 89, 51 (1939).

4. Horecker, B. L. and L. A. Heppel, J. Biol. Chem., 178, 683 (1949).

5. Corran, H. S., J. G. Pervan, A. H. Gordon and D. E. Green, Biochem. J., 33, 1694 (1939).

6. Avis, P. G., F. Bergel, R. C. Bray and K. V. Shooter, Nature, 173, 1230 (1954).

7. Avis, P. G., F. Bergel and R. C. Bray, J. Chem. Soc., 1212 (1956).

8. Richert, D. A. and W. W. Westerfield, J. Biol. Chem., 227, 533 (1957).

9. Massey, V., P. E. Brumby, G. Palmer and H. Komai, J. Biol. Chem., 244, 1682 (1969).

10. Westerfield, W. W., D. A. Richert and E. S. Higgens, J. Biol. Chem., 234, 1987 (1959).

11. Bergman, F. and S. Dikstein, J. Biol. Chem., 223, 765 (1956).

12. Gurfreund, H. and J. M. Sturtevant, Biochem. J., 73, 1 (1959).

13. Gilbert, D. A., Biochem. J., 93, 214 (1964).

14. Muroaka, S., Biochem. Biophys. Acta., 73, 27 (1963).

15. Coughlin, M. P., K. V. Rajagopalan and P. Handler. J. Biol. Chem., 244, 2658 (1969).

16. Bray, R. C., in The Enzymes, (edited by P. Boyer, H. Lardy and K. Myrback), Academic Press, New York, Vol 7, (1963), p. 533.

17. White, A., D. Handler and E. L. Smith, Principles of Biochemistry, 3rd ed., McGraw Hill, New York.

18. Mahler, H. R. and E. H. Cordes, Biological Chemistry, Harper and Row, New York, (1966) p. 731.

19. Glassman, E., H. S. Forrest and H. K. Mitchell, Genetics, 42, 372 (1957).

20. Haddow, A., G. Delamirande, F. Bergel, R. C. Bray and D. A. Gilbert, Nature, 182, 1144 (1958).

21. Fridovich, I. and P. Handler, J. Biol. Chem., 231, 899 (1958).

22. Palmer, G., R. C. Bray and H. Beinert, J. Biol. Chem., 239, 2657 (1964).

23. Bray, R. C., G. Palmer and H. Beinert, J. Biol. Chem., 239, 2657 (1964).

24. Komai, H., V. Massey and G. Palmer, J. Biol. Chem., 244, 1682 (1969).

25. Fridovich, I., J. Biol. Chem., 241, 3126 (1966).

26. Deitrich, R. A., L. Hellerman and J. Wein, J. Biol. Chem., 237 560 (1962).

27. Lineweaver, H. and D. Burke, J. Biol. Chem., 56, 658 (1934).

28. Patton, A. R., Biochemical Energetics and Kinetics, W. B. Saunders Co., (1965), p. 81.

29. Ramsey, W. and S. Young, Phil. Trans., 117, 71 (1886).

30. Lombardi, E. and P. B. Sogo, J. Chem. Phys., 32, 635 (1960).

31. Bell, R. P. and J. C. Clunie, Trans. Faraday Soc., 48, 439 (1952).

32. Schou, S. A., C. R. Acad. Sci., Paris, 182, 965 (1926).

33. Herold W. and K. L. Walt, Z. Phys. Chem. B., 12, 165 (1931).

34. Pocker, Y., Proc. Chem. Soc., 17 (1960).

35. Lander, I., Trans, Faraday Soc., 34, 1219 (1938).

36. Pocker, Y. and J. E. Meany, J. Am. Chem. Soc., 87, 1809 (1965).

37. Pocker, Y. and J. E. Meany, Biochem., 6, 239 (1967).

38. Pocker, Y. and J. E. Meany, Biochem., 4, 2535 (1965).

39. Cabani, S. and P. Cecchi, Am. Chim. (Rome), 49, 205 (1959).

40. Gilbert, D. A. and F. Bergel, Biochem. J., 90, 350 (1969).

41. Tienhaara, R., unpublished work at Central Washington State College, Ellensburg, Washington.

42. Tauber, H. and E. L. Petit, J. Biol. Chem., 195, 703 (1952).

43. Pocker, Y. and J. E. Meany, J. Phys. Chem., 71, 3113 (1967).

44. Pocker, Y. and D. G. Dickerson, J. Phys. Chem., 73, 4005 (1969).

45. Rajagopalan, K. V. and P. Handler, J. Biol. Chem., 239, 1509 (1964).

46. Worthington Biochemical Corp., Catalog of Research Enzymes, Xanthine Oxidase, (1967).

47. Lowry, D. H., N. J. Rosebrough, A. L. Farr and R. J. Randall, J. Biol. Chem., 193, 265 (1951).

48. Beste, G. W. and L. P. Hammett, J. Am. Chem. Soc., 62, 3921 (1940).

49. Fridovich, I. and P. Handler, J. Biol. Chem., 233, 1581 (1958).

50. De Renzo E. C., Advances in Enzymology, 17, 293 (1956).

51. Pocker, Y., J. E. Meany and B. J. Nist, J. Phys. Chem., 71, 4509 (1967).

52. Roussou, G. C., in Methods in Enzymology, Vol. 15, Academic Press, New York, (1967), p. 5.

53. Meany, J. E., Unpublished Ph.D. Thesis, University of Washington, 1967.

54. Pocker, Y., J. E. Meany, B. J. Nist and C. Zadorojny, J. Phys. Chem., in press.

55. Pocker, Y., J. E. Meany and C. Zadorojny, J. Phys. Chem., 73, 2879 (1969).

56. Guintoli, R., Unpublished Master's Thesis, Central Washington State College, 1969.