Xanthine Oxidase Catalyzed Oxidation of Aldehydes. Oxidation of Aliphatic Aldehydes and 2- and 4-Pyridinecarboxaldehyde†

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ABSTRACT: The xanthine oxidase catalyzed oxidations of a series of aliphatic aldehydes and 2- and 4-pyridinecarboxaldehydes were studied in phosphate buffers by monitoring the reduction of ferricytochrome *c* at 25.0° In aqueous solutions, these aldehydes exist in equilibrium with their hydrated forms. In order to clearly establish the relationship between the acid–base-catalyzed *hydration* of the aldehydes and the enzymatically catalyzed *oxidation* of the aldehyde–hydrate system, detailed kinetic analyses of both processes were carried out separately. Enzymatic catalysis was studied as a function of pH and of acetaldehyde concentration, and the catalytic components associated with the acid–base-catalyzed hydration of acetaldehyde in phosphate buffers were evaluated. These data, taken together, show not only that the unhydrated aldehyde is the substrate for xanthine oxidase action but also suggest that substantial enzymatic inhibition arises from the formation of the hydrate. The involvement of the unhydrated aldehyde as the preferential substrate is common for the aldehydes studied and in the evaluation of their respective Michaelis constants, K_m: acetaldehyde, 0.0058 M; propionaldehyde, 0.014 M; *n*-butyraldehyde, 0.048 M; 2-pyridinecarboxaldehyde, 0.0061 M; and 4-pyridinecarboxaldehyde, 0.002 M; correction was made to compensate for respective fractions of hydration.

Xanthine oxidase is a very versatile enzyme with the capacity to catalyze the oxidation of many purine derivatives and aldehydes by a variety of electron acceptors (Booth, 1938; Bergmann and Dikstein, 1956; Bergmann *et al.*, 1959, 1960a,b; Bray, 1963; Muraoka *et al.*, 1967; Fridovich, 1970). As might be expected for an enzyme of such low-substrate specificity many inhibitors of this enzyme exist (Coombs, 1927; Doisy *et al.*, 1955; Wyngaarden, 1957; Bergmann *et al.*, 1960a,b; Gilbert, 1964; Massey *et al.*, 1970). Xanthine oxidase is susceptible to substrate inhibition (Hofstee, 1955), the severity of which varies with the nature of the electron acceptor (Dixon and Thurlow, 1924; Fridovich and Handler, 1958; Mazur *et al.*, 1958).

For the enzymatically catalyzed oxidation of aldehydes by xanthine oxidase and related enzymes, the true nature of the substrate (aldehyde *vs.* conjugate hydrate) is in question (Dietrich *et al.*, 1962; Dixon and Webb, 1964; Fridovich, 1966). On the basis of qualitative kinetic studies, Fridovich (1966) has suggested that the unhydrated aldehyde is the preferential substrate. However, because of the complexity of the aldehyde–hydrate–enzyme system, a more detailed kinetic analysis is needed to determine the functions of the aldehyde and the hydrate with respect to the enzymatic process.

In the present work, the enzymatically catalyzed oxidation of acetaldehyde was studied as a function of pH and aldehyde concentration. A kinetic analysis was also carried out on the acid–base-catalyzed hydration of acetaldehyde. Experimental conditions were then chosen so that the influence of the hydration equilibration on the rate of the enzymatically catalyzed oxidation of the aldehyde–hydrate system could clearly be demonstrated. Similar studies studies were carried out on propionaldehyde, *n*-butyraldehyde, and 2- and 4-pyridinecarboxaldehyde.

Experimental Section

The commercially available aldehydes were distilled under dry nitrogen gas directly before use. The aliphatic aldehydes were fractionated through a Heli-Pak column and the pyridinecarboxaldehyes distilled under reduced pressure: bp 60–61° (12 mm) (2-pyridinecarboxaldehyde) and 76–77° (12 mm) (4-pyridinecarboxaldehyde). Phosphate buffer solutions were prepared from the commercially available compounds, in analytical or reagent grade by dissolving accurately weighed KH_2PO_4 and K_2HPO_4 in deionized water to the desired concentrations. Appropriate amounts of sodium chloride were incorporated to adjust the ionic strength to μ = 0.1 M. Cytochrome *c* was purchased from Sigma Chemical Corp., Sigma type VI. Beef liver catalase was a product of Worthington Biochemical Corp.

Xanthine oxidase (prepared and purified from milk) was obtained as a product of Worthington Biochemical Corp. and was further purified in this laboratory using a method similar to that employed by Rajagopalan and Handler (1964). Parallel studies were also carried out with xanthine oxidase samples prepared in this laboratory from fresh cream (J. E. Meany and R. F. Tienhaara, 1970, unpublished results) using a procedure similar to that employed by Horecker and Heppel (1955). Although consistent results were observed from each of the various preparations, each family of experiments involved only a single preparation. Each batch of enzyme was assayed spectrophotometrically by following the formation of urate from hypoxanthine at 290 nm at 25.0° in 0.05 M phosphate buffer (pH 7.5). The assay of protein was performed colorimetrically with Folin Ciocalteau reagent using the method of Lowry *et al.* (1951). The specific activity of the commercial enzyme after purification was 3 units/mg. Unless otherwise specified, 0.25 unit/ml were used in the kinetic runs.

Kinetic studies were carried out on a Beckman Kintrac VII recording spectrophotometer equipped with a Beckman Thermocirculator accessory. Colorimetric protein assays were carried out on a Hitachi Perkin-Elmer Model 139

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TABLE I: Catalysis of the Hydration of Acetaldehyde by the Acidic and Basic Components of Phosphate Buffers, μ = 0.1 M.[a]

Catalyst	$k_{cat}^{25.0°}$ [b]	$k_{cat}^{0.0°}$ [c]
$H_2PO_4^-$	95	16.0
HPO_4^{2-}	183	35.9
OH^-	22×10^5	6.8×10^5
H_2O[d]	0.38/55.5	0.094/55.5

[a] Rate coefficients, having the units M^{-1} min^{-1}, are actually a sum of rate coefficients for the forward, k_c^f, and reverse, k_c^r, processes: $k_c = k_c^f + k_c^r$. [b] Present work. [c] Pocker and Meany (1967). [d] It is assumed that $k_{H_2O} = k_0/55.5$.

spectrophotometer. The pH values of all buffer solutions were determined before and after the kinetic runs using a Beckman Century SS expanded-scale pH meter.

The *hydrations* of the various aldehydes were monitored and kinetically analyzed as described in earlier work (Pocker and Meany, 1967; Pocker *et al.*, 1967; Pocker and Dickerson, 1969). The rate of the enzymatically catalyzed *oxidations* of the aldehydes was monitored at 550 nm by the increase in optical density caused by production of the reduced form of cytochrome *c*. After complete mixing of all reacting species, the initial slopes of the traces produced were used to evaluate reaction velocities which are reported in terms of micromoles of cytochrome *c* per liter reduced per minute:[1] v_0 = slope/1.96×10^{-2}. Because such low aldehyde concentrations were required in these experiments, it was found convenient to employ stock solutions of the substrates prepared in previously distilled anhydrous dimethoxyethane. Approximately 10 μg/ml of catalase was incorporated into the reaction mixtures to destroy any hydrogen peroxide formed as the aldehydic oxidations progressed. It was found that neither the presence of the small quantities of dimethoxyethane nor the catalase altered the rates of enzymatic oxidations. Furthermore, the presence of cytochrome *c*, xanthine oxidase or catalase did not effect the rate of hydration of acetaldehyde.

Results and Discussion

Acid–Base-Catalyzed Hydration of Acetaldehyde. Although the acid–base-catalyzed hydration of acetaldehyde has been rather extensively investigated at 0.0° (Bell, 1966; Pocker and Meany, 1967), a quantitative kinetic analysis at higher temperatures has not been reported. The observed pseudo-first-order rate coefficients for the acid–base-catalyzed hydrations of aldehydes in phosphate buffers in aqueous medium may be expressed as a sum of catalytic terms

$$k_{hyd} = k_0 + k_{H_3O^+}(H_3O^+) + k_{OH^-}(OH^-) + k_{H_2PO_4^-}(H_2PO_4^-) + k_{HPO_4^{2-}}(HPO_4^{2-}) \quad (1)$$

Systematic kinetic analysis involving determination of k_{hyd} as a function of the concentration of the buffer components at several different buffer ratios, $r = (H_2PO_4^-)/(HPO_4^{2-})$ was carried out as in earlier work (Pocker and Meany, 1967). The kinetic parameters as evaluated by this method are listed in Table I. These results enabled us to regulate the rate of the hydration of acetaldehyde at 25.0° so that the variation of the enzymatic oxidation throughout the course of the aldehyde–hydration equilibration can be clearly illustrated.

TABLE II: Results for Lineweaver–Burk Plots for the Enzymatic Oxidation for Acetaldehyde in 0.001 M Phosphate Buffers, Ionic Strength 0.1 M at 25.0°.

pH	6.75	7.00	7.75
V_m	8.6	11	7.6
$K_m^{exp} \times 10^2$ (M)	1.3	1.3	1.4

Enzyme-Catalyzed Oxidation of Aldehydes. Determinations were carried out using acetaldehyde to determine the Michaelis–Menten parameters associated with its enzymatic oxidation. Figure 1A shows the effect of increased aldehyde concentration upon the initial velocities at pH 7.0 and Figure 1B illustrates the corresponding Lineweaver–Burk plot constructed from these data. The dotted line in Figure 1A represents the curve calculated from the extrapolated values of v_0 which would be expected at higher concentrations of aldehyde if "normal" Michaelis–Menten kinetics had been obeyed. Corresponding data were obtained at pH's 6.75 and 7.75 and the resulting Michaelis–Menten parameters appear in Table II.[2]

The rate of the enzymatic oxidation was determined at several values of pH (Figure 2) where the aldehyde concentration (2.75×10^{-3} M) was much lower than the experimental Michaelis constant. Consequently, the values of v_0 obtained are actually a function of the ratio of V_m/K_m. The pH–rate profile shows points of inflection at pH 6.7 and 7.8, and a maximum at 7.25. The data in Table II indicate that variations in V_m are more influential on the profile than those involving K_m. The accuracy of such determinations, however, is limited by the existence of substrate inhibition, since reciprocals of v_0 and aldehyde concentration close to the intercepts cannot be reached experimentally. Bergmann and Dikstein (1956) report the pH maximum (8.3) for xanthine oxidase action on purine derivatives but did not separate the components, K_m and V_m. Fridovich and Handler (1958) indicate that K_m for the enzyme–xanthine–oxygen system is insensitive to pH changes in phosphate buffers, but increases with increasing pH in Tris buffers. For propionaldehyde, a brief study indicated that maximum initial velocities were attained at *ca.* pH 7.2, paralleling the results which were observed for the acetaldehyde.

The variation of initial velocities as a function of substrate concentration for propionaldehyde, *n*-butyraldehyde, 2-pyridinecarboxaldehyde, and 4-pyridinecarboxaldehyde ($[O_2] \simeq 0.001$ M) are illustrated in Figure 3. "Substrate"

[1] The difference in extinction coefficients between ferricytochrome *c* and ferrocytochrome *c* at 550 nm is taken to be 1.96×10^{-2} l. μmole^{-1} cm^{-1} (Horecker and Heppel, 1955).

[2] Differences in the reported values of K_m associated with the aerobic oxidation of acetaldehyde by xanthine oxidase in the presence of cytochrome *c* may be attributed to variations in enzyme purity (Bray, 1959), the presence of myoglobin, a common contaminant of cytochrome *c* (Fridovich, 1962), difference in the oxygen content in the reaction medium arising from variations in atmospheric conditions as well as other differences in experimental conditions, for example, temperature, buffer concentration, ionic strength, etc.

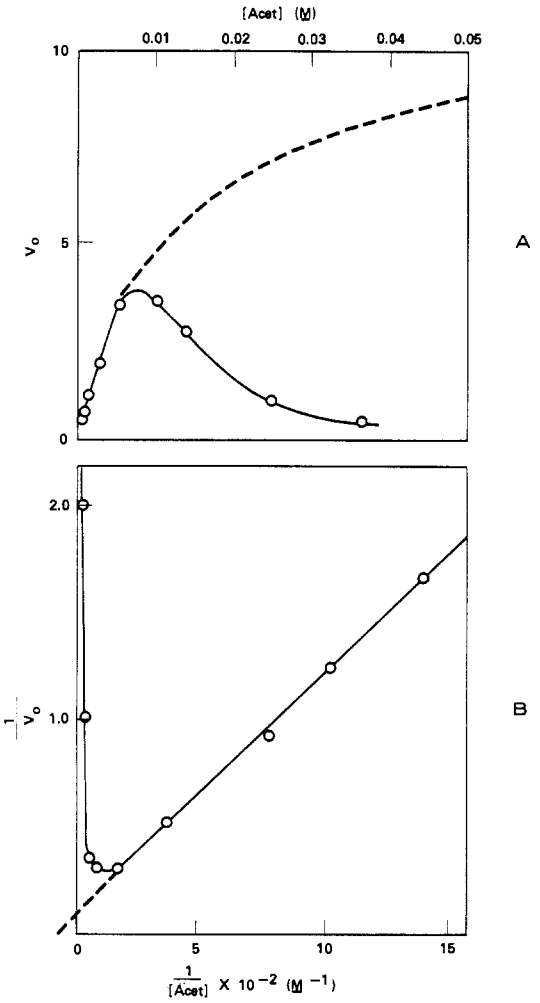
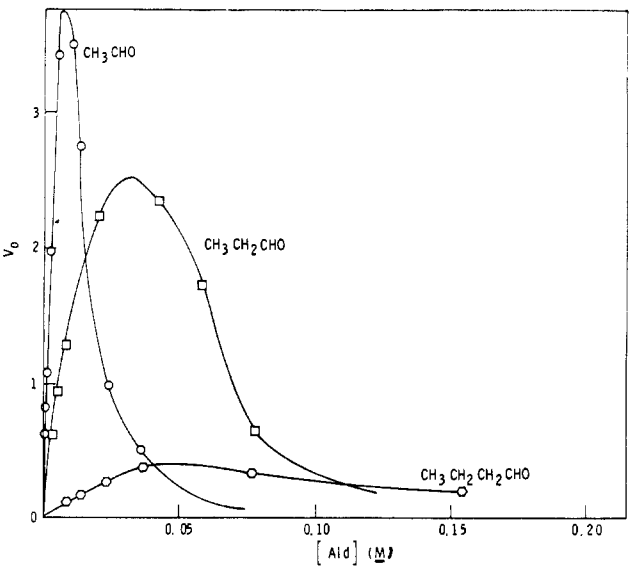


FIGURE 1: (A) Initial velocity of acetaldehyde oxidation as a function of aldehyde concentration. Dotted line represents the curve which would be obtained for a process involving a "normal" Michaelis–Menten mechanism. Data obtained at 25°, pH 7.00 using 0.002 M phosphate buffer, μ = 0.01 M. (B) Lineweaver–Burk plot of the data used in part A.



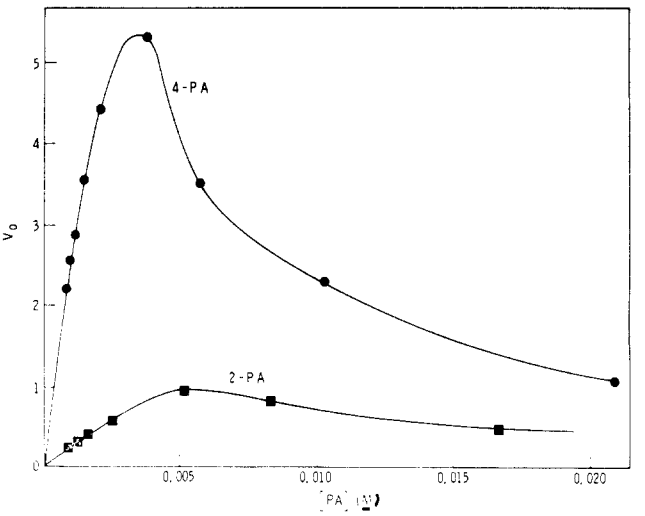


FIGURE 3: Initial velocities of aldehydic oxidations as a function of aldehyde concentration. Data obtained at pH 7.00 in 0.002 M phosphate buffer, μ = 0.1 M.

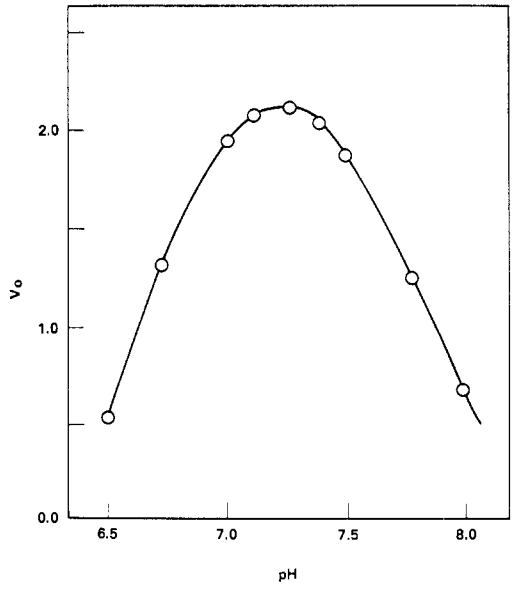


FIGURE 2: The rate of the enzymatic oxidation of acetaldehyde is a function of pH in 0.002 M phosphate buffers at μ = 0.1 M.

inhibition was noted in the case of all xanthine oxidase catalyzed aldehydic oxidations. Thus, it was never possible to experimentally reach the calculated values of V_m (Figures 1 and 3) obtained through extrapolations of the Lineweaver–Burk plots. For acetaldehyde, propionaldehyde, *n*-butyraldehyde, and 4-pyridinecarboxaldehyde, V_m is 2.5 ± 0.3 times as large as that experimentally obtained before "substrate" inhibition becomes dominant. In the case of 2-pyridinecarboxaldehyde, however, the ratio $V_m^{calcd}:V_m^{exp}$ = *ca.* 3.6 is considerably larger. The relatively high degree of inhibition by 2-pyridinecarboxaldehyde may be related to the ability of this aldehyde to chelate to certain metal ions (Pocker and Meany, 1968).

No enzymatic activity was observed for the oxidation of isobutyraldehyde in the concentration range 6.31×10^{-3} to 2.5×10^{-1} M. It was also found that the rate of enzymatic oxidation of acetaldehyde was unaffected by the presence of isobutyraldehyde (2.5×10^{-2} to 2.5×10^{-1} M).

The Michaelis parameters were deduced for the various aldehydes from Lineweaver–Burk plots using the data in Figure 3. It should be noted that the values of both parameters

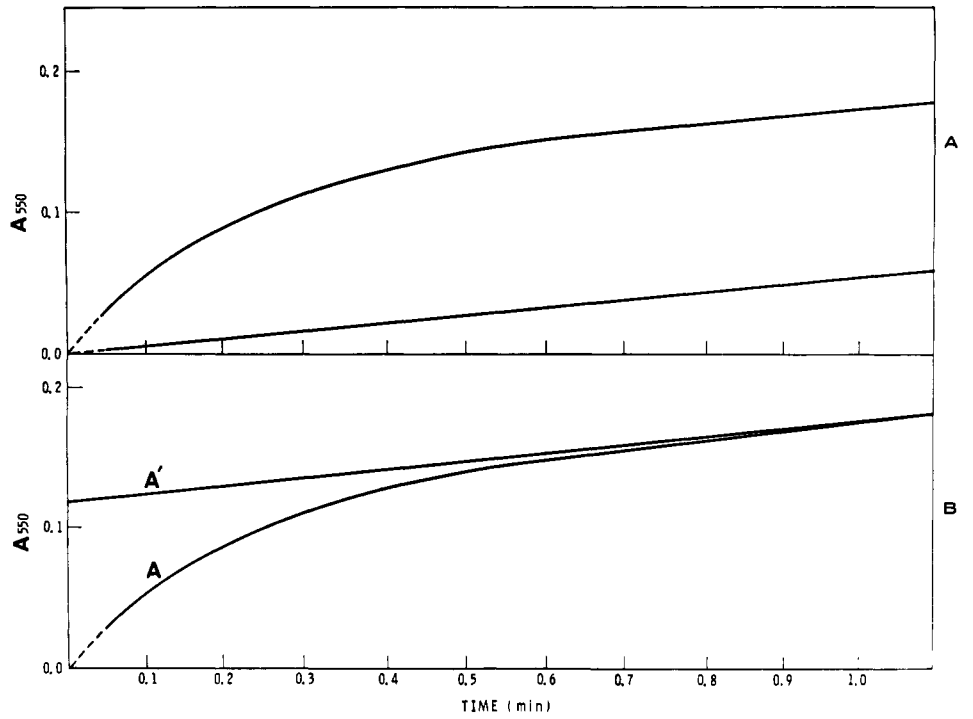


FIGURE 4: Comparison of rate cytochrome *c* reduction of equilibrated and nonequilibrated aldehydic solution. Each solution contains 5.4×10^{-3} M (total concentration) acetaldehyde, 3×10^{-5} M cytochrome *c*, and 0.25 unit of xanthine oxidase/ml. (A) Upper curve: cytochrome *c* reduction initiated by addition of anhydrous acetaldehyde. Lower curve: cytochrome *c* reduction initiated by addition of xanthine oxidase to the preequilibrated aldehyde–hydrate system. (B) A trace of the same run as represented in Figure 4A but where the linear portion reached upon hydration equilibration has been extrapolated back to the kinetic zero.

are dependent upon the oxygen concentration[3] and furthermore, those associated with the various aldehydes may be influenced to different extents by alterations in the oxygen concentration.

Rate of Cytochrome c Reduction at Various Stages of Hydration. In order to meaningfully investigate the relationship between the acid–base-catalyzed *hydration* and the enzymatically catalyzed *oxidation* of the aldehyde–hydrate system, it was necessary to: (i) regulate the pH, phosphate concentration, and the enzyme concentration so the rates of hydration and oxidation were in the region where the effect of the change in aldehyde concentration (due to hydrate formation) on the enzymatic process could be clearly reflected; (ii) operate at an aldehyde concentration which is in the first-order region with respect to the enzymatic process; (iii) to maintain the pH at a value where maximum enzymatic activity was observed so that slight pH changes would show minimum effect on the enzymatic rate. The experimental conditions were chosen on the basis of the data presented earlier (Table I and Figure 1).

The rate of reduction of cytochrome *c* was observed under two sets of experimental conditions. The rate was first monitored in runs which were initiated by adding anhydrous dimethoxyethane solutions of acetaldehyde as the last component to the reaction mixture. Under these conditions, two processes commenced at the time of mixing: (i) the *hydration* of acetaldehyde leading to a first-order decrease in its concentration with the concomitant formation of its hydrate, (ii) the *oxidation* of the acetaldehyde system. A typical example of such a run carried out in 0.03 M phosphate buffer is illustrated by the upper curve in Figure 4A. The lower linear trace in Figure 4A represents a run using the same aldehyde and buffer concentrations as before, but where the aldehyde had first been allowed to fully equilibrate with its hydrate prior to the addition of the enzyme. The initial rates of cytochrome *c* reduction associated with equilibrated aldehyde–hydrate systems were always appreciably lower than those obtained when anhydrous acetaldehyde was added as the last component.

It should be noted, that unless experimental conditions are selected prudently, the interpretation of data such as shown in Figure 4A is not without ambiguity. If the initial anhydrous aldehyde concentration is not held below the velocity optimum (0.01 M for acetaldehyde, see Figure 1) then an observed decrease in rate might be incorrectly interpreted as arising from a resultant decrease in free aldehyde concentration. Thus, the range of aldehyde concentrations used in experiments such as illustrated in Figure 4 must be well below 0.01 M.

Figure 4B represents the same kinetic run as does the upper curve in Figure 4A. Here the linear portion of the curve (reflecting a constant rate of cytochrome *c* reduction once the hydration equilibrium is established) has been extrapolated back to zero time. The rate of cytochrome *c* reduction calculated from the slope of the line obtained ($v = 1.83$ μmole $l.^{-1}$ min^{-1}) is an excellent agreement with that obtained from the run in Figure 4A from a solution containing the preequilibrated aldehyde and hydrate ($v = 1.86$ μmoles $l.^{-1}$ min^{-1}). The data depicted in Figure 4B indicate that the elapsed time necessary for constant rate of cytochrome *c* reduction is the same as that necessary for the hydration reaction to

[3] Horecker and Hepple (1949), noted that the rate of oxidation of acetaldehyde in the presence of cytochrome *c* increased by more than a factor or two when pure oxygen was bubbled into the reaction solutions instead of air, indicating that for this process as carried out under normal atmospheric conditions, the active enzyme complex is not saturated with oxygen.

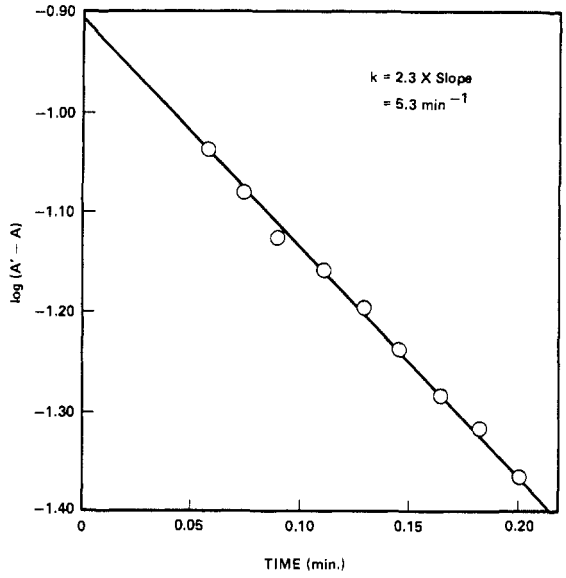


FIGURE 5: First-order rate plot of log $(A' - A)$ (from Figure 4B) *vs.* time.

reach equilibrium (0.03 M phosphate buffer, pH 7.18, and ionic strength 0.1 M). The hydration process is *ca.* 99% complete (7 half-lives, see Table I) after about 1 min under these conditions.

It will further be noted in Figure 4B that the converging of the lower curve with the upper straight line has the appearance of a first-order approach to equilibrium. This is to be expected if the variation of the slope of the lower line is the direct result of the hydration process. Accordingly a plot of log $(A' - A)$ *vs.* time, where A' and A are absorbancies associated with the upper (from the equilibrated system) and lower (from the nonequilibrated system) curves, gives a good fit to a first-order plot up to *ca.* 2 half-lives (Figure 5). By treating the data in Figure 4B in this manner, the slope of the line resulting from the first-order plot × 2.3 gives the constant, $k = 5.3$ min^{-1} which is in good agreement with the first-order rate constant for the hydration process as calculated from eq 1 using our experimental data in Table I, from which $k_{hyd} = 5.43$ min^{-1}.

This same analysis was applied to other runs which were carried out using different buffer concentrations in which the hydration equilibration was allowed to compete with the enzymatic oxidation. Typical results are given in Table IV. Although the reproducibility of such analyses is somewhat limited due to relatively small differences in A' and A, the general agreement of the data clearly shows the *direct* correspondence between the hydration and enzymatic oxidation processes. We believe that the data demonstrate unequivocally that the unhydrated aldehyde is the preferential substrate in the enzymatic process. Similar, although less extensive investigations with respect to the other aliphatic aldehydes studied resulted in the same conclusion. Since the Michaelis constants experimentally obtained for these processes, K_m^{exp}, are based on the total concentration of the aldehyde–hydrate system, they have been corrected by multiplying by the fraction of the unhydrated aldehyde under the reaction concentrations: $K_m^{cor} = K_m^{exp}(1 - \chi)$ (Table III).

One would anticipate that if the aldehyde concentration is within the range where the reduction of cytochrome *c* is first order in aldehyde (Figure 1), that a comparison of the oxidative rates associated with the equilibrated and nonequilibrated aldehyde–hydrate systems should allow a quantitative measure of the fraction of hydration (provided that such rate differences are related *directly and singularily* to the differences in free aldehyde concentration). If so, the slopes of the two curves in Figure 4A associated with the equilibrated, S_∞, and the nonequilibrated, S_0, aldehyde–hydrate systems would allow the calculation of the fraction of hydration, $\chi_{app} = 1 - (S_\infty/S_0) = [(S_0 - S_\infty)/S_0]$. When this approach was applied to Figure 4A, the apparent fraction of hydration calculated has the value, $\chi_{app} = 0.92$, which is much *greater* than the actual fraction of hydration, $\chi = 0.55$, as *directly* determined at 25.0°. This observation was general for all such comparative runs (Table IV). The large value of χ_{app} results because of the relatively slow rate of cytochrome *c* reduction in the equilibrated aldehyde–hydrate system suggesting that the well-known "substrate" inhibition normally ascribed to this enzymatic process may be largely due to the *hydrated* form of the aldehyde.

TABLE IV: Determination of k_{hyd} from the Variation in the Enzymatic Oxidation of Acetaldehyde with Hydration Equilibration.

pH	7.18	7.20	7.24	7.25
$[H_2PO_4^-]$ (M)	0.010	0.0031	0.0015	0.0029
$[HPO_4^{2-}]$ (M)	0.020	0.0069	0.0035	0.0071
k_{hyd} (min^{-1}) (calculated from Table I)	5.43	2.36	1.67	2.47
k (min^{-1}) (from converging curves such as in Figure 4B)	5.3	2.7[a]	1.6	2.4[b]
$\frac{S_0 - S_\infty}{S_0}$	0.92	0.91[a]	0.92	0.93[b]

[a] Values based on the average of two runs. [b] Values based on the average of four runs.

TABLE III: Michaelis Parameters for Xanthine Oxidase Catalyzed Oxidations.

Substrate	K_m^{exp} (M)	χ	K_m^{cor} (M)	V_m[a]
Acetaldehyde	1.3×10^{-2}	0.55	5.8×10^{-3}	11
Propionaldehyde	2.7×10^{-2}	0.47[b]	14×10^{-3}	5.4
n-Butyraldehyde	8.1×10^{-2}	0.41	48×10^{-3}	1.1
2-Pyridinecarboxaldehyde	9.6×10^{-3}	0.36[c]	6.1×10^{-3}	3.4
4-Pyridinecarboxaldehyde	4.2×10^{-3}	0.53[c]	2.0×10^{-3}	13

[a] In units of μmoles of cytochrome *c* reduced per min per 50 μg of xanthine oxidase. [b] Pocker and Dickerson (1969). [c] Pocker *et al.* (1967).

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Kinetic Studies on Solid-Supported β-Galactosidase†

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ABSTRACT: A kinetic investigation was made of the action of β-galactosidase trapped in a polyacrylamide gel, the substrate being *o*-nitrophenyl β-D-galactopyranoside (NphGal). Rate measurements were made over a range of substrate concentrations, and k_c (app) and K_m (app) values were determined. The work was done with disks having thicknesses ranging from 25 to 1000 μ, and at various enzyme concentrations within the gel; under some of the conditions there was diffusion control, under others (thin slices and low enzyme concentrations) there was negligible diffusion control. The results are analyzed with reference to a theoretical treatment of Sundaram, Tweedale, and Laidler [(1970), *Can. J. Chem. 48*, 1498] which makes use of a function F which is related to the extent of diffusion control. It was found that for the thicker slices the rate at low substrate concentrations is proportional to the square root of the enzyme concentration, while for the thinnest slices, where there is little diffusion control, the rate is proportional to the first power of the enzyme concentration. These results are predicted by the theory. The values of *F* calculated from the experimental results were found to be in excellent agreement with the theoretical values.

During the past few years there has been considerable interest in the preparation and properties of solid-supported enzymes (for reviews, see Silman and Katchalski, 1966, Kay, 1968, Barker and Epton, 1970, Mosbach *et al.*, 1971, Volfin, 1971, Sundaram and Laidler, 1971, and Goldman 1971a). This work has application to a number of fields, such as automated analysis (Hornby *et al.*, 1970; Sundaram and Hornby, 1970) and clinical investigations (Chang, 1971). More recently, attention has been focussed on the use of solid-supported enzymes as model systems for the behavior of enzymes *in vivo* when they are attached to membrane surfaces or are embedded in membranes. It has been noted in particular that many enzymes have an increased apparent activity when separated from their natural membrane supports (de Duve, 1959). Experiments to investigate such aspects have included some qualitative studies on the rate of consecutive enzyme reactions when the enzymes are supported (Mosbach and Mattiasson, 1970; Mattiasson and Mosbach, 1971) and more quantitative studies on the effect of diffusion on enzyme systems (van Duijn *et al.*, 1967; Goldman *et al.*, 1968, 1971b).

In addition, a number of theoretical treatments of the kinetics of solid-supported enzymes have been given (Blum and Jenden, 1957; O'Sullivan, 1962; van Duijn *et al.*, 1967; Goldman *et al.*, 1968, 1971b; Sundaram *et al.*, 1970; Goldman and Katachalski, 1971; Kasche *et al.*, 1971; Kobayashi and Moo-Young, 1972). The present work was undertaken as a test of the treatment of Sundaram *et al.* (1970), who gave explicit solutions for the rate equations under the limiting conditions of low and high substrate concentrations. In addition they gave an approximate solution for the general case, and confirmed its reliability by computer calculations. This treatment led to predictions about the conditions under which the

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