A Novel Method for Determining Rate Constants for Dehydration of Aldehyde Hydrates†

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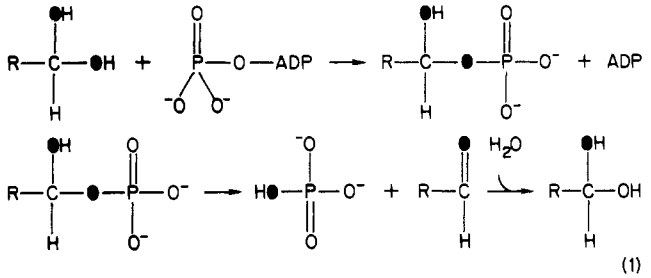
ABSTRACT: A method has been developed for calculating rate constants for dehydration of aldehydes that induce ATPase reactions by kinases and where ^{18}O is transferred from the aldehyde or its hydrate to inorganic phosphate during the reaction. The method involves measurement of the fraction of ^{18}O in phosphate by ^{31}P NMR after the ATPase reaction has proceeded for several minutes with zero-order kinetics. The reaction is started by addition of the aldehyde in a small volume of $H_2{}^{18}O$, and the speed of washout of ^{18}O by reversible dehydration relative to the rate of the ATPase reaction allows calculation of the rate constants if the hydration equilibrium constant is known from the proton NMR spectrum of the aldehyde. Dehydration rate constants (s^{-1} at pH 8–8.5, 0.1 M buffer, 25 °C) for the following aldehydes (all over 95% hydrated) and kinases used are as follows: D-glyceraldehyde with glycerokinase, 0.03; 2,5-anhydro-D-mannose 6-phosphate with fructose-6-phosphate kinase, 0.025; 2,5-anhydro-D-mannose or 2,5-anhydro-D-talose with fructokinase, 0.029 and 0.017, respectively; D-*gluco*-hexodialdose with hexokinase, 0.068. With betaine aldehyde and choline kinase or glyoxylate and pyruvate kinase, no ^{18}O was transferred to phosphate during the ATPase reactions. However, the dehydration rate constant for glyoxylate (0.007 s^{-1} at pH 7 extrapolated to zero buffer concentration and up to 0.11 s^{-1} at pH 9.0 with 0.3 M buffer) was determined by extrapolating the initial rate of reduction of the free aldehyde catalyzed by lactate dehydrogenase to infinite enzyme levels. The lack of a significant ^{18}O isotope effect on the dehydration of glyoxylate hydrate at either pH 6 or pH 8 suggests that C–O bond cleavage is not rate limiting for the reaction.

In the course of our studies on the mechanism of aldehyde-induced ATPase activities of kinases (Rendina & Cleland, 1983, 1984), we wished to know whether the aldehydes or their hydrates were directly phosphorylated to give unstable adducts that decomposed nonenzymatically to release inorganic phosphate. To demonstrate direct phosphorylation, we looked for ^{18}O incorporation from the aldehydes into phosphate during the ATPase reaction. The case where the hydrate is phosphorylated is shown in mechanism 1, where ● represents ^{18}O.



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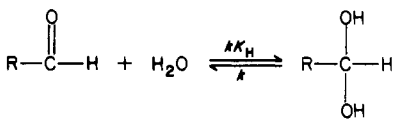
The ^{18}O content of the inorganic phosphate can then be determined by integration of the ^{31}P NMR spectrum of the $P^{18}O^{16}O_3$ and $P^{16}O_4$ species (Cohn & Hu, 1978). In addition to the obvious mechanistic information, which is dealt with in the following paper (Redina & Cleland, 1984), the ^{18}O transfer experiments also provide a novel means for determining rate constants for the hydration and dehydration of aldehydes, which is a relatively fast process (Bell, 1966):



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In mechanism 2, k is the dehydration rate constant and K_H the hydration equilibrium constant. In mechanism 1, the enzyme-catalyzed transfer of ^{18}O into phosphate competes with washout of the label by the reverse of reaction 2. From a model describing the washout of label from the hydrate by both processes and the appropriate experimental parameters (fraction of ^{18}O in phosphate at the end of the experiment, K_H, initial aldehyde level, and elapsed time until ATP utilization stopped), the dehydration rate constants can be calculated for aldehydes that induce ATPase activities by kinases and where ^{18}O is transferred to phosphate during the reaction. In this paper, we report data for several such aldehydes for which data have not been previously available. We also report the rate constant for dehydration of glyoxylate, determined by reduction by NADH in the presence of infinite lactate dehydrogenase, as well as the pH and temperature dependence and the ^{18}O isotope effect on this dehydration reaction.

Experimental Procedures

Materials. $H_2{}^{18}O$ (95.7 and 97.5 atom % ^{18}O) was from the Mound Facility of Monsanto Research Corp. D_2O was from Bio-Rad (99.8 atom % D) or Aldrich (100.00 atom % D). NAD, NADH, phosphoenolpyruvate, Mes,[1] Hepes, Taps, Ches, D-glyceraldehyde, betaine aldehyde, glyoxylate, rabbit muscle lactate dehydrogenase, pyruvate kinase, fructose-6-phosphate kinase (fructose-6-P kinase), yeast hexokinase, choline kinase, *Alcaligenes* choline oxidase, horseradish peroxidase, and *Escherichia coli* glycerokinase were from Sigma. Fructokinase was prepared from beef liver by the method of Raushel & Cleland (1977). All enzymes used were tested for ATPase activity in the absence of aldehydes; in no case was such an activity as much as 5% of the rate in the presence of aldehyde. For kinetic measurements, these background rates

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[1] Abbreviations: Mes, 2-(*N*-morpholino)ethanesulfonate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonate; Taps, 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonate; Ches, 2-(*N*-cyclohexylamino)ethanesulfonate; EDTA, ethylenediaminetetraacetic acid; TLC, thin-layer chromatography; HPLC, high-pressure liquid chromatography.

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were subtracted from the observed ones in the presence of the aldehyde. 2,5-Anhydro-D-mannose (chitose) and its 6-phosphate were prepared as described by Viola & Cleland (1980) and purified on Dowex 50–ethylenediamine by the method of Rendina & Cleland (1981). 2,5-Anhydro-D-talose was prepared by the method of Defaye (1964) and purified in the same fashion as 2,5-anhydromannose.[2] D-*gluco*-Hexodialdose was prepared as described by Fischer & Schmidt (1960). The purity of the aldehydes was checked by TLC, proton NMR, and HPLC by using the system of Hendrix et al. (1981). Stock solutions of ^{18}O-labeled aldehydes (1–2 M) were prepared by dissolving the lyophilized aldehydes in 0.25 mL of $H_2{}^{18}O$ and equilibrating the solutions for at least 12 h at 25 °C prior to use.

Instrumentation. UV spectra were obtained with a Cary 118 spectrophotometer. Kinetic studies were performed by monitoring absorbance changes with either the Cary 118 or a Beckman DU monochromator equipped with a Gilford OD converter and a 10-mV recorder. Assay temperatures were kept at ±0.1 °C of their stated values with thermospacers and a circulating water bath. pH values were measured with a Radiometer 26 pH meter equipped with a combined microelectrode standardized to ±0.01 pH unit. Ultrafiltration to remove proteins was done with a Series 80 Amicon ultrafiltration cell and type PM-10 filter. ^{13}C (50.1 MHz) and ^{31}P (80.99 MHz) NMR spectra were obtained with a Nicolet NT-200 Fourier-transform spectrometer with a 20-mm or 12-mm broad band or a 5-mm fixed-tune probe and a Nicolet 1280 data aquisition system. Proton NMR spectra were obtained with the same instrument or with a Bruker WH-270 spectrometer. Integrations of the ^{31}P spectra were performed with the Nicolet NMCCAP curve-deconvolution program, which permits an analysis of a spectrum of overlapping peaks and provides position, intensities, widths, and relative areas of individual lines.

Determination of Aldehyde Concentrations. For D-glyceraldehyde and 2,5-anhydromannose and its 6-phosphate, the enzymatic end-point assays described by Rendina & Cleland (1981) were used. Since glyoxylate is a substrate for lactate dehydrogenase (Nakada & Weinhouse, 1953), the net change at 340 nm caused by complete reduction of glyoxylate in the presence of excess NADH was used to calibrate this aldehyde (0.1 M Hepes, pH 8.0 25 °C, 0.25 mM NADH, and 140 units of lactate dehydrogenase). Betaine aldehyde solutions were calibrated with choline oxidase by measuring the formation of quinoneinine dye (ϵ_{480} = 4.6 mM^{-1} cm^{-1}) with the peroxidase–phenol–4-aminoantipyrene system as the H_2O_2 acceptor (Ikuta et al., 1977). The assay contained 0.1 M Hepes, pH 8.0, 25 °C, 1.5 mM 4-aminoantipyrene, 2.1 mM phenol, 100 units of peroxidase, and 1.3 units of choline oxidase. The concentrations of these aldehydes and also of D-*gluco*-hexodialdose, 2,5-anhydrotalose, and 2,5-anhydromannose 6-phosphate were also estimated by monitoring the formation of their respective semicarbazones at 230 nm in solutions containing 1% semicarbazide hydrochloride and 1.5% sodium acetate at 25 °C (MacGee & Doudoroff, 1954). Concentrations were calculated by using ϵ_{230} = 11 mM^{-1} cm^{-1} (Brand & Scott, 1963) for the semicarbazones. Solutions of 2,5-anhydrotalose, which contains *cis*-hydroxyl groups, were also calibrated by following cleavage with periodate at 223 nm (Khym, 1972). Unless otherwise state, the units of enzyme are in micromoles of normal substrate per minute at 25 °C.

Determination of Hydration Equilibria for Aldehydes. K_H, expressed as [hydrate]/[aldehyde], was determined by integrating proton NMR signals for the hydrate (ca. 5 ppm) relative to the free aldehyde (ca. 9.5 ppm) in 0.1 M solutions in D_2O at 21–22 °C. The recovery time between pulses (60 s) was sufficient to allow complete return to equilibrium and therefore to allow accurate integrations.[3] Since these aldehydes were heavily hydrated in aqueous solution, a standardized solution containing formate and pyruvate (relative proton integration $^1/_{100}$) was added to each aldehyde solution, so that the aldehyde signal could be measured relative to that of formate at ca. 8 ppm and the hydrate signal could be integrated relative to the pyruvate signal at ca. 2 ppm. Values for D-glyceraldehyde (Angyal & Wheen, 1980) and other aldehydes and ketones (Lewis & Wolfenden, 1977) have been obtained by similar NMR techniques. To obtain the equilibrium constant in H_2O, the [hydrate]/[aldehyde] ratio in D_2O was multiplied by 0.84 to correct for the solvent deuterium isotope effect on the hydration equilibrium (Gruen & McTigue, 1963); the results are reported in Table I.

^{18}O Transfer from Hydrate to Phosphate. In these experiments, the enzymatic ATPase activity competes with the nonenzymatic washout of ^{18}O from the aldehyde and hydrate. In order to observe significant ^{18}O transfer to inorganic phosphate, from 0.3 to 1.0 unit/mL enzyme was required [units expressed as micromoles of P_i released per minute at the reaction temperature; Table I of Rendina & Cleland (1984) gives the ratios of ATPase and kinase activities for the various kinases used]. The ^{18}O-labeled aldehyde added to the reaction mixture was dissolved in a small amount of $H_2{}^{18}O$ (1–3% of the final volume); in controls to test for ^{18}O incorporation into P_i from the solvent, we added unlabeled aldehyde to a reaction mixture containing the same aliquot of $H_2{}^{18}O$ that was added during sample runs. To monitor the time course of P_i formation, 5-μL aliquots were quenched with 45 μL of 4 M HCl or 0.1 M acetic acid at 1-min intervals following initiation of the reaction with aldehyde and then assayed for P_i by the method of Lanzetta et al. (1979). Except where noted, the reactions were stopped by complete consumption of ATP, 10 mM Na_2EDTA was added, and the solution was filtered through a 0.45-μm Millipore or Amicon PM-10 filter and adjusted to 25% D_2O prior to analysis for ^{18}O in P_i by integration of the ^{31}P NMR peaks. The final values for the fraction of ^{18}O in P_i were corrected for the atom % ^{18}O of the water used to make the aldehyde solution, for dilution of ^{18}O during equilibration with the concentrated aldehyde, and for any initial unlabeled phosphate in the samples prior to initiation of the ATPase reaction.

Reaction volumes were 2–8 mL, containing 50–100 mM K-Hepes, pH 8 [NH_4^+ with phosphofructokinase to minimize allosteric inhibition of the enzyme by ATP (Sols & Salas, 1966)], or 60 mM Ches, pH 8.6, with pyruvate kinase and glyoxylate. ATP levels were 2–3 mM, with Mg^{2+} in excess. Pyruvate kinase and phosphoenolpyruvate were used to recycle ADP and prevent product inhibition so that the production of P_i was zero order when fructokinase was incubated with 2,5-anhydrotalose or 2,5-anhydromannose [14 mM phosphophoenolpyruvate in this case, since this substrate is phosphorylated as well as inducing an ATPase reaction with relative

[2] Unlike 2,5-anhydro-D-mannose, 2,5-anhydro-D-talose was a poor substrate for hexokinase (V relative to glucose = 0.07%; K_m = 0.14 M), so we did not try to phosphorylate this aldehyde and test it with fructose-6-P kinase. 2,5-Anhydrotalose did not induce an ATPase activity by hexokinase.

[3] The T_1 relaxation times of the slowly relaxing aldehyde protons of acetaldehyde and glyceraldehyde were determined by the inversion–recovery method (180°–τ–90°) to be 10.4 and 3.3 s, respectively. Similar values are expected for the other aldehydes.

V/K values of ATPase to kinase of 0.15 (Viola & Cleland, 1980). 2,5-Anhydrotalose is not phosphorylated by fructokinase (Rendina & Cleland, 1984)]. With pyruvate kinase and glyoxylate, myokinase was added to recycle ADP and prevent product inhibition, and the reaction was quenched after 4.5 min with hexokinase and glucose to remove remaining ATP (this was also done with fructokinase and 2,5-anhydrotalose).

Dehydration Rate Constants for Glyoxylate. Since lactate dehydrogenase catalyzes reduction of only the free-aldehyde form of glyoxylate (Everse & Kaplan, 1973), the rate of dehydration of the hydrate (the predominant form in solution) will be rate limiting at sufficiently high levels of enzyme according to mechanism 3. Experimentally, the dehydration

$$\text{hydrate} \underset{kK_H}{\overset{k}{\rightleftharpoons}} \text{aldehyde} \xrightarrow[\text{NADH, LDH}]{\alpha E_t} \text{glycolate} \quad (3)$$

rate constant for glyoxylate was determined from the ordinate of a plot of reciprocal initial velocity vs. (volume of lactate dehydrogenase)$^{-1}$ for a fixed level of glyoxylate, that is, at infinite enzyme concentration. A typical experiment was initiated by addition of 10 μL of 10 mM glyoxylate via added mixer to a total volume of 2 mL containing 0.1 M buffer, 0.25 mM NADH, and 100–1000 units of lactate dehydrogenase (micromoles of pyruvate reduced per minute at 25 °C). Mixing times were less than 10 s, chart speed was 12 in./min, and full-scale sensitivity was $0.05A_{340}$ in order to determine the initial velocity.

The temperature dependence of the dehydration rate was determined at pH 8.0. The pH dependence was determined at 25 °C in Mes, pH 5.5–6.5, Hepes, pH 6.5–8.5, and Taps, pH 8.0–9.0. Buffers were adjusted to pH with KOH. The ^{18}O kinetic isotope effect on the dehydration rate was measured by direct comparison of the initial velocities with carefully calibrated solutions of unlabeled and ^{18}O-labeled glyoxylate (added in $H_2{}^{18}O$) to initiate the reaction. Since only the conversion of hydrate to aldehyde should show an ^{18}O isotope effect (the back-reaction is between ^{18}O-labeled aldehyde and $H_2{}^{16}O$ and would show at most a small secondary isotope effect) and an appreciable ^{18}O isotope effect is not expected on the enzymatic reduction of the aldehyde, both the slope and intercept of the reciprocal plot vs. lactate dehydrogenase level should have the same isotope effect (namely, that on the dehydration rate constant k).

Data Processing. Reciprocal initial velocities were plotted vs. reciprocal enzyme concentrations for determination of glyoxylate dehydration rates, and all plots were linear. Data were fitted to the appropriate equations with the Fortran programs of Cleland (1979). Data for individual reciprocal plots were fitted to eq 4, where A is enzyme level, V is the

$$v = VA/(K + A) \quad (4)$$

dehydration rate (k times the glyoxylate concentration), and K is an apparent Michaelis constant (kK_H/α in terms of mechanism 3). The values of k as a function of buffer concentration were fitted to eq 5, where k_0 is the value of k in

$$k = m[\text{buffer}] + k_0 \quad (5)$$

s^{-1} in the absence of buffer and m is the apparent second-order rate constant for buffer catalysis in $M^{-1}\ s^{-1}$. The data for the direct comparison of rates with unlabeled and ^{18}O-labeled glyoxylate were fitted to eq 6, which assumes different isotope

$$v = \frac{VA}{K(1 + F_iE_{V/K}) + A(1 + F_iE_V)} \quad (6)$$

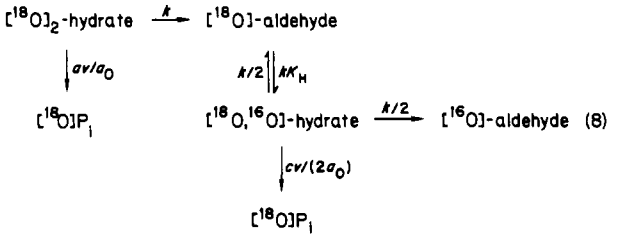
effects on V and V/K, or to similar equations that assume isotope effects on V/K or V only. The data were also fitted to eq 7, which assumes equal isotope effects on V and V/K.

$$v = \frac{VA}{(K + A)(1 + F_iE_V)} \quad (7)$$

In these equations, F_i is the fraction of ^{18}O in glyoxylate at the start of the experiment, and E_V and $E_{V/K}$ are the isotope effects minus 1 on V and V/K, respectively. Data were also fitted to the forms of these equations where the logarithm was taken of both sides (this changes the assumed error distribution from one with constant errors in initial velocities to one with proportional errors).

Theory

^{18}O Transfer from Hydrate to Phosphate. In these studies, the total concentration of aldehyde, as well as the ratio of concentrations of hydrate and free aldehyde, remains constant with time, but ^{18}O is lost during hydration–dehydration cycles as soon as the ^{18}O-labeled aldehyde (preincubated in a small volume of $H_2{}^{18}O$) is added to the reaction mixture. The ^{18}O is also lost via the ATPase reaction itself, but if the aldehyde is present at over 10 times the level of ATP (true in the present experiments), the transfer of ^{18}O to phosphate does not contribute to washout for heavily hydrated aldehydes (this can be allowed for in more precise calculations and is necessary when the free aldehyde is the active species; see below). If the ATPase reaction shows zero-order kinetics until ATP is used up (true in the present experiment),[4] the system can be modeled as



(8)

where K_H is the hydration equilibrium constant, k is the dehydration rate constant, and thus kK_H is the hydration rate constant. v is the zero-order rate of phosphorylation of the hydrate. We assume there are no kinetic or equilibrium ^{18}O isotope effects (see below for a calculation when this assumption is not valid). If a, b, and c are the concentrations of $^{18}O_2$-labeled hydrate, ^{18}O-labeled aldehyde, and $^{18}O,{}^{16}O$-labeled hydrate, respectively, then a_0 and b_0 are initial concentrations of these species ($a_0 = b_0K_H$), and $c_0 = 0$. Since a decreases in first-order fashion

$$a = a_0e^{-kt} \quad (9)$$

The differential equations for the time dependence of b and c are

$$db/dt = kb_0K_He^{-kt} + (k/2)c - kK_Hb \quad (10)$$

$$dc/dt = kK_Hb - kc \quad (11)$$

These equations can be solved simultaneously to give

$$b = \frac{b_0r_2e^{r_1t}}{r_2 - r_1} + \frac{b_0r_1e^{r_2t}}{r_1 - r_2} \quad (12)$$

$$c = \frac{a_0(k + 2r_2)e^{r_1t}}{r_2 - r_1} + \frac{a_0(k + 2r_1)e^{r_2t}}{r_1 - r_2} - 2a_0e^{-kt} \quad (13)$$

[4] ATP should be present at a saturating level so the rate of the ATPase reaction does not slow down as ATP is used up. The equations can also be solved for first-order kinetics of the ATPase, but this is more difficult to achieve experimentally in view of the amount of phosphate needed for the NMR analysis.

where r_1 and r_2 are the two roots given by

$$r = -(k/2)[1 + K_H \pm (1 + K_H^2)^{1/2}] \quad (14)$$

The fraction of ^{18}O-labeled phosphate at the end of the experiment is then

$$\frac{\int_0^t v(a + c/2)/a_0 \, dt}{vt} = \frac{(k + 2r_2)(e^{r_1 t} - 1)}{2(r_2 - r_1)r_1 t} + \frac{(k + 2r_1)(e^{r_2 t} - 1)}{2(r_1 - r_2)r_2 t} \quad (15)$$

where t is the time the ATPase reaction stopped. Since r_1 and r_2 are complex functions of k, eq 15 is solved for k by successive approximations or by tabulating the proportion of phosphate containing ^{18}O as a function of k with the other parameters those of the individual experiment (we have written computer programs to do both of these).

^{18}O Transfer from Free Aldehyde to Product. This model can also be extended to cases where the free aldehyde is the active species leading to incorporation of ^{18}O into an enzymatic product, in which case the loss of ^{18}O via the enzymatic reaction itself could become important. This is especially true when the aldehyde is heavily hydrated and only a small pool of ^{18}O-labeled aldehyde is present.[5] The model now becomes

$$\begin{array}{ccccc} [^{18}O]_2\text{-hydrate} & \xrightarrow{k} & [^{18}O]\text{-aldehyde} & \underset{k/2}{\overset{kK_H}{\rightleftharpoons}} & [^{18}O,^{16}O]\text{-hydrate} \\ & & \downarrow{\scriptstyle v/b_0} & & \downarrow{\scriptstyle k/2} \\ & & [^{18}O]\text{-enzymatic product} & & [^{16}O]\text{-aldehyde} \end{array} \quad (16)$$

Equations 9 and 11 describe the time dependence of a and c, and in addition

$$db/dt = kb_0K_He^{-kt} + (k/2)c - kK_Hb - vb/b_0 \quad (17)$$

In eq 17, the first two terms represent formation from a or c, and the third reflects removal by hydration, while the fourth term accounts for the enzymatic reaction itself (v is the rate of the enzymatic reaction in mM/s, and thus this term is of major importance only when b_0 is small, as with heavily hydrated aldehydes like glyceraldehyde or glyoxylate). Equations 11 and 17 can be solved simultaneously to give

$$b = \frac{b_0(r_2 + v/b_0)e^{r_1 t}}{r_2 - r_1} + \frac{b_0(r_1 + v/b_0)e^{r_2 t}}{r_1 - r_2} \quad (18)$$

where r_1 and r_2 are the two roots given by

$$r = -(k/2)(1 + K_H + v/(kb_0) \pm [1 - 2v/(kb_0) + [K_H + v/(kb_0)]^2]^{1/2}) \quad (19)$$

The proportion of ^{18}O-labeled product at the end of the reaction is then

$$\frac{\int_0^t vb/b_0 \, dt}{vt} = \frac{(r_2 + v/b_0)(e^{r_1 t} - 1)}{(r_2 - r_1)r_1 t} + \frac{(r_1 + v/b_0)(e^{r_2 t} - 1)}{(r_1 - r_2)r_2 t} \quad (20)$$

where t is the time that the enzymatic reaction was stopped. Since r_1 and r_2 are complex functions of k, eq 20 is solved for k by successive approximation, or by tabulating the proportion of ^{18}O in the enzymatic product as a function of k, with the other parameters those of the individual experiment.

^{18}O Transfer in Fructokinase Reaction with 2,5-Anhydromannose. Fructokinase acts on this molecule in two ways, phosphorylating the hydroxymethyl group at C-6 or giving ATPase activity when C-1 of the hydrate is adjacent to MgATP (Viola & Cleland, 1980). Therefore, during an experiment in which ^{18}O transfer from aldehyde to phosphate is followed, the total concentration of 2,5-anhydromannose changes with time until ATP is used up. This alters the differential equations for the system as follows.

First, the time dependence of the concentration of $[^{18}O]_2$-hydrate is

$$da/dt = \frac{-a}{a_0(1 - t/g)}(a_0/g) - ka \quad (21)$$

where g is the time at which all of the 2,5-anhydromannose would be phosphorylated at the initial zero-order rate of phosphorylation. The expression $a_0(1 - t/g)$ is the concentration of total hydrate in the system, while a_0/g is the rate of removal of total hydrate by phosphorylation. The first term of this equation thus represents removal of $^{18}O_2$-labeled hydrate by phosphorylation, while the second term represents dehydration to ^{18}O-labeled aldehyde. Solution of eq 21 gives

$$a = a_0(1 - t/g)e^{-kt} \quad (22)$$

The expression for the change in ^{18}O-labeled aldehyde concentration with time now becomes

$$db/dt = ka_0(1 - t/g)e^{-kt} + (k/2)c - kK_Hb - b/(g - t) \quad (23)$$

The first two terms represent formation by dehydration of a or c, and the third reflects disappearance by hydration. The fourth term represents removal of b by phosphorylation; it is the ratio of b to $b_0(1 - t/g)$, the total concentration of free aldehyde, multiplied by b_0/g, the rate of phosphorylation of total free aldehyde. The equation for the level of c is

$$dc/dt = kK_Hb - kc - c/(g - t) \quad (24)$$

where the third term is for removal by phosphorylation [it is the ratio of c to $a_0(1 - t/g)$ multiplied by a_0/g]. In view of the relative lack of specificity for the substituents in the 6-position of fructokinase substrates (Raushel & Cleland, 1977), we have assumed that V/K for the kinase activity is the same for the hydrate and free aldehyde.

Equations 23 and 24 do not have an analytical solution, and thus we have solved them simultaneously by numerical methods with a computer program based on a differential equation solution package available at our local computer center. Once c is known at closely spaced time intervals ($g/1000$, which was about 1 s in the present case), the ^{18}O content of phosphate formed during the reaction is given by

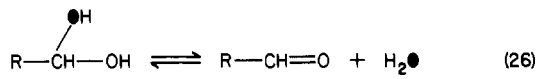
$$\frac{\int_0^t \frac{v(a + c/2)}{a_0(1 - t/g)} \, dt}{vt} \quad (25)$$

This integration was also performed numerically, and ^{18}O proportions in phosphate calculated at 0.005 intervals for k values from 0.005 to 0.100 (these values ranged from 0.894 to 0.142 over this range of k values with the other parameters of the fructokinase experiment).

^{18}O Isotope Effects on ^{18}O Transfer from Hydrate to Phosphate. The derivations above have assumed no ^{18}O isotope effects on the washout of ^{18}O from the aldehyde. However, an equilibrium isotope effect of over 4% is almost certain to

[5] This situation would apply where one determined the ^{18}O content of a reduction product such as glycolate produced by the action of NADH and lactate dehydrogenase on glyoxylate; in this case, the reaction must be stopped before an appreciable amount of the total aldehyde reacts.

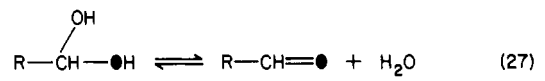
be present, and sizable kinetic effects are possible. The ^{18}O equilibrium isotope effect for dehydration of a mixed $^{18}O,^{16}O$-labeled hydrate to give $H_2{}^{18}O$



(26)

is expected to be 1.033, in analogy with the measured value in the fumarase reaction for the transfer of ^{18}O from a secondary alcohol to water (Blanchard & Cleland, 1980). (We assume that the second OH group has a similar effect on the vibrational frequencies of the first OH group as a saturated carbon atom; if anything, the value will be higher than 1.033, since oxygen substitution for carbon would raise the fractionation factor of a hydrogen in the same position as the OH group by a factor of 1.08.) If C–O bond breaking were rate limiting for this reaction, the kinetic ^{18}O isotope effect might be as high as 1.08 in the forward direction (corresponding to 1.045 in the reverse reaction); a value of 1.073 was seen in the fumarase reaction at low pH (Blanchard & Cleland, 1980).

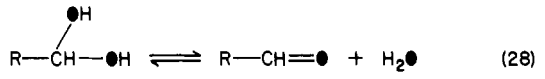
There will probably be a small secondary ^{18}O isotope effect on the elimination of $H_2{}^{16}O$ from the mixed $^{18}O,^{16}O$-labeled hydrate:



(27)

The fractionation factors relative to water of the OH group of malate (1.033) and of the keto group of α-ketoglutarate (1.031) are nearly the same (Blanchard & Cleland, 1980), but the fractionation factor of a ketone will be higher than that of an aldehyde as the result of replacement of hydrogen by carbon. This change causes a discrimination of about 1.008 (Cleland, 1980), and thus we estimate $^{18}K_{eq}$ for eq 27 to be 1.010. The secondary kinetic isotope effect will depend on how early or late the transition state is for the dehydration reaction, but if it were reasonably symmetrical, one would expect a normal kinetic isotope effect of about 1.005 in the dehydration direction and an inverse value of 0.995 in the reverse reaction.

Since the primary and secondary ^{18}O isotope effects should be independent of each other, the overall isotope effects on the dehydration of a fully ^{18}O-labeled hydrate



(28)

will be the product of those on eq 26 and 27, and thus we estimate that $^{18}K_{eq} = 1.033 \times 1.010 = 1.043$, with maximum possible kinetic isotope effects of 1.085 and 1.040 in forward and reverse directions.

With these values, eq 10 and 11 become

$$db/dt = (k/1.085)(1.043b_0K_H)e^{-kt/1.085} + (k/1.005)(c/2) - (k/0.995)K_Hb \quad (29)$$

$$dc/dt = (k/0.995)K_Hb - [(k/1.08) + (k/1.005)](c/2) \quad (30)$$

Note that the original ratio of hydrate to free aldehyde in $H_2{}^{18}O$ is higher than that at equilibrium in $H_2{}^{16}O$, so the total concentrations of hydrate or of free aldehyde do not remain constant during the experiment. Equations 29 and 30 have an analytical solution (although a very complex one), but we have found it more convenient to solve the equations simultaneously by numerical methods and then to carry out the subsequent integration as indicated by eq 15 numerically also to obtain the expected proportion of ^{18}O in phosphate as a function of k. The result of allowing for the isotope effects by using the values above was that the true k value was as much as 7% higher than the value obtained by assuming no isotope effects, although the difference was less for very high proportions of ^{18}O in phosphate. Since we have found no measurable isotope effect on the dehydration of glyoxylate (see below), we have analyzed the data shown in Table I by assuming there were none.

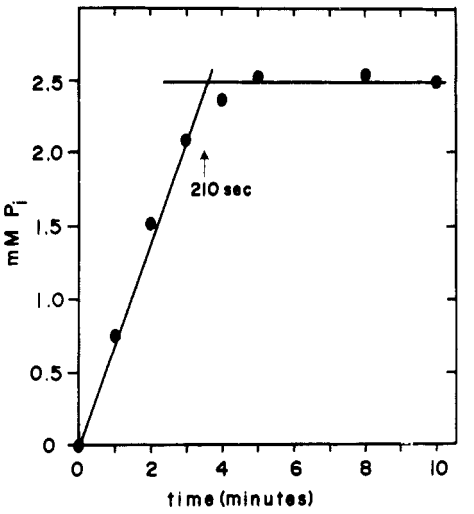


FIGURE 1: Time course for the release of inorganic phosphate during the ATPase reaction catalyzed by glycerokinase in the presence of D-[^{18}O]glyceraldehyde at 25 °C (glycerokinase sample 2; see Experimental Procedures).

Results

Dehydration Rate Constants from ^{18}O Transfer Experiments. In order to calculate rate constants readily, the model for the washout of ^{18}O from the aldehydes or their hydrates requires zero-order kinetics for the release of inorganic phosphate during the ATPase reactions.[4] Figure 1 shows a representative time course for the release of phosphate during the ATPase reaction catalyzed by glycerokinase in the presence of 33 mM D-[^{18}O]glyceraldehyde at 25 °C. This reaction proceeded linearly until all the ATP was consumed, but with other kinases product inhibition by ADP gave curved time courses so that either myokinase or pyruvate kinase with phosphoenolpyruvate was used to recycle the ADP. In those cases, the reactions were linear until quenched after 2–5 min by adding excess glucose and hexokinase to remove the remaining ATP and phosphoenolpyruvate rapidly by formation of glucose 6-phosphate. The elapsed time for the reaction was obtained from the intersection of the zero-order portion of the curve with the average level of phosphate released at the end of the reaction. In order to obtain 1.5–2.5 mM phosphate containing at least 25 atm % ^{18}O, enough enzyme had to be added that the reaction was over in 4–5 min at 25 °C.

Since the ATPase reactions are initiated by the addition of a small aliquot of concentrated aldehyde in $H_2{}^{18}O$, the reaction mixture contains a small amount (<5%) of $H_2{}^{18}O$. To ensure that this ^{18}O was not incorporated in the inorganic phosphate, the ^{31}P NMR spectra of controls similar to that shown in Figure 2 were run, where unlabeled aldehyde was added to a reaction mixture that already contained the same portion of $H_2{}^{18}O$ (2–5%) that would be added during the sample run in the presence of ^{18}O-labeled aldehyde. The spectrum in Figure 2 shows no upfield shoulder corresponding to ^{18}O perturbation of the chemical shift of inorganic phosphate, suggesting either that no ^{18}O is incorporated into phosphate from the solvent during the glycerokinase ATPase reaction or else that ^{31}P NMR cannot detect less than 5% [^{18}O]-

Table I: Rate Constants for Dehydration of Aldehyde Hydrates[a]

aldehyde	temp (°C)	$[aldehyde]_0$ (mM)	reaction time (s)	$[P_i]_\infty$ (mM)	fraction ^{18}O in P_i	K_H	k (s^{-1})
D-glyceraldehyde	2	32.6	96	1.6	0.91	37	0.004
	25	32.6	210	2.5	0.31	15	0.030
2,5-anhydro-D-mannose 6-phosphate	25	27.8	300	3.2	0.26	190[b]	0.025
2,5-anhydro-D-mannose	25	58.7	240	1.7	0.28	190[b]	0.029
2,5-anhydro-D-talose	25	28.8	270	2.4	0.40	110	0.017
D-*gluco*-hexodialdose	25	12.5[c]	102	2.1	0.28	200[c]	0.068
	25	12.5[c]	880	1.9	0	200[c]	
betaine aldehyde	25	28.8	135	1.7	0	150	
glyoxylate	25	47.5	270	1.6	0	260	(0.029)[d]
	25	47.5	3630	1.4	0	260	(0.029)[d]
	17					350[e]	(0.013)[d]
	9					490[e]	(0.007)[d]

[a] $[Aldehyde]_0$ is the total concentration of aldehyde and hydrate. Reaction time is that required to exhaust the ATP. $[P_i]_\infty$ is the net final phosphate concentration. The fraction of ^{18}O in phosphate was calculated from the ^{31}P NMR spectrum. K_H is the hydration equilibrium constant measured from the proton NMR spectrum and corrected for the solvent isotope effect (see Experimental Procedures). k is the rate constant for dehydration calculated as described under Theory. [b] Due to misinterpretation of the proton NMR spectrum as the result of the presence of an impurity, the hydration equilibrium constant for 2,5-anhydro-D-mannose was incorrectly reported by Viola & Cleland (1980). [c] The proton NMR spectrum of D-*gluco*-hexodialdose shows at least 10 different anomeric proton signals in the vicinity of the DHO peak (the ^{13}C NMR spectrum has over 60 resonances as well), so we do not know the actual concentration of the hydrate (that is, of 6-*aldehydo*-D-glucopyranose). Glucopyranose, gulopyranose, and fused-ring furanose–furanose and furanose–pyranose structures are possible. The concentration given is that of total sugar, and the K_H value is from a comparison of total free aldehyde with total other species. The true value is likely to be smaller than 200 and closer to that found for other open-chain sugars. [d] Calculated from the observed rates of reduction by NADH in the presence of infinite lactate dehydrogenase (see Experimental Procedures). [e] Value calculated from that at 25 °C by using $\Delta H = -6.7$ kcal/mol [the value reported for D-glyceraldehyde by Angyal & Wheen (1980)].

phosphate. The expected shift should be 0.021 ppm (Cohn & Hu, 1978), which can easily be observed under the spectrometer conditions in Figure 2, since the line widths at half-height were 0.02 ppm. Further, the resolution of the instrument was checked with a standard preparation of [^{18}O]phosphate (made from $POCl_3$ and $H_2{}^{18}O$) prior to each run. When only one peak for phosphate was observed, as with betaine aldehyde and choline kinase or with glyoxylate and pyruvate kinase, [^{16}O]phosphate was added and the ^{31}P NMR spectrum reexamined to make sure that the singlet was not due to the $P^{18}O^{16}O_3$ species alone.

A typical ^{31}P NMR spectrum for a sample run with ^{18}O-labeled aldehyde at 2 °C (Figure 3) clearly shows the presence of an upfield peak. Figure 3b demonstrates that this peak is due to $P^{18}O^{16}O_3$, since dilution of the sample with unlabeled phosphate clearly enhances only the downfield resonance. These results suggest that glycerokinase ATPase proceeds by direct phosphorylation of the aldehyde or hydrate, and in the following paper we will show that the hydrates are the activators of the ATPase reactions studied here. When corrected for dilution of the ^{18}O label in the aldehyde solution, the initial atom % ^{18}O in the $H_2{}^{18}O$, and the background level of unlabeled phosphate in the reaction mixture, integration of the upfield peak showed that 91% of the ^{18}O was incorporated into phosphate during the ATPase reaction induced by D-glyceraldehyde with glycerokinase at 2 °C (Figure 3a). Initially, all of the ^{18}O transfer experiments were planned at 2 °C because we felt that the rate of the nonenzymatic washout of ^{18}O would exceed the enzyme-catalyzed rate of ^{18}O incorporation into phosphate at 25 °C (limited by the amount of enzyme available and/or its solubility). However, the results in Figure 3 showed that enough enzyme could be addded to give significant levels of ^{18}O in phosphate at 25 °C, and as a result, the remaining experiments were conducted at this temperature in order to compare the calculated rate constants to others determined by different techniques at ambient temperatures. Table I includes the results of these experiments, as well as the other parameters needed to calculate the dehydration rate constant, namely, the time the reaction was stopped, the initial concentration of ^{18}O-labeled aldehyde, the net concentration of phosphate released, and the hydration

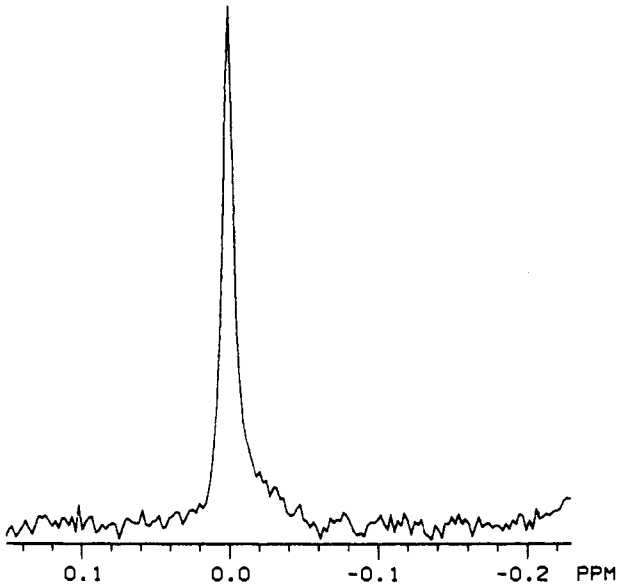


FIGURE 2: Fourier-transform ^{31}P NMR spectrum (80.989 MHz) of the phosphate from the control run for the glycerokinase ATPase initiated with unlabeled glyceraldehyde at 2 °C: 156 scans, acquisition time 5.2 s, sweep width ±750 Hz, delay between pulses 3 s, and 90° pulse angle.

equilibrium constant, K_H, in H_2O determined from integration of the proton NMR spectra of the aldehydes in D_2O and corrected for the solvent isotope effect. Also included are the dehydration rate constants for glyoxylate determinated from reduction of the free aldehyde catalyzed by lactate dehydrogenase (initial velocities were extrapolated to infinite enzyme levels where the reaction is limited by the dehydration of the hydrate).

In order to test the model for washout of ^{18}O from the hydrated aldehydes, the ^{18}O transfer experiment with D-*gluco*-hexodialdose was tried at much lower levels of hexokinase (sample 2). As predicted by the model, when the reaction was quenched after proceeding linearly for 15 min instead of 1.7 min, no ^{18}O could be detected by ^{31}P NMR, since the enzymatic reaction was much slower than the nonenzymatic washout. A similar experiment was tried with glyoxylate

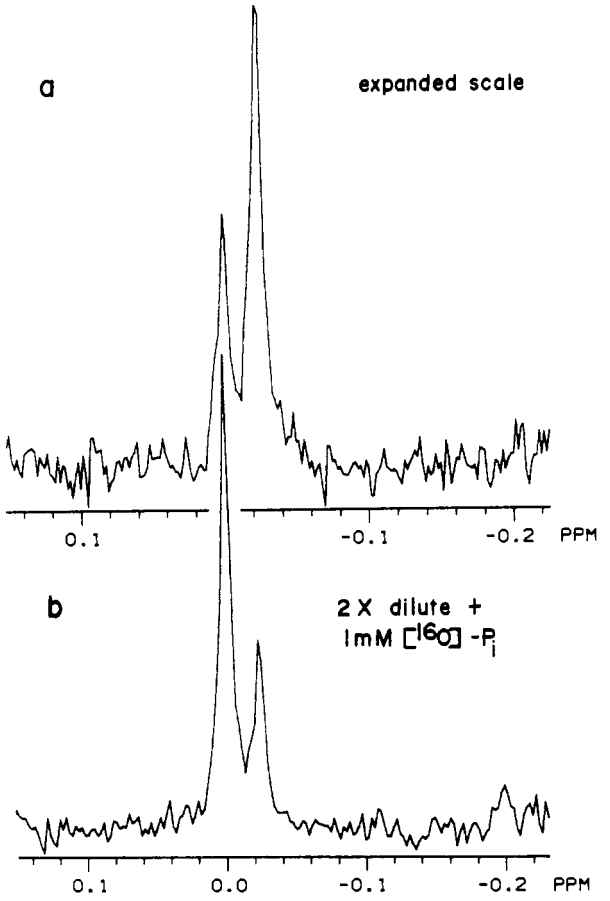


FIGURE 3: ^{31}P NMR spectrum of the phosphate from the sample run for the glycerokinase ATPase reaction initiated with [^{18}O]glyceraldehyde at 2 °C: (a) same conditions as Figure 2 except 300 scans, acquisition time 6.9 s, and sweep width ±296 Hz; (B) after 2-fold dilution with unlabeled phosphate.

and lower levels of pyruvate kinase, and the reaction was quenched after 1 h (sample 2). In this case, no ^{18}O incorporation had been observed at short times (sample 1), raising the possibility that the dehydration rate might limit the incorporation of label if the free aldehyde were the activator and its level were as low as indicated by the large hydration equilibrium constant for glyoxylate.[6] Subsequent experiments have shown that the hydrates are the activators for choline and pyruvate kinases and that the ATPase reactions proceed via induced transfer to water, rather than direct phosphorylation (Rendina & Cleland, 1984), thus explaining why no ^{18}O is observed in the phosphate released by these enzymes from MgATP. Furthermore, the dehydration rate constant for glyoxylate measured by the lactate dehydrogenase experiments agrees well with those calculated from ^{18}O transfer data for other aldehydes, so that ^{18}O should have been incorporated if the mechanisms of the choline- and pyruvate-catalyzed ATPase reactions proceeded by direct phosphorylation.

For the remaining aldehydes tested, we typically found about 30% ^{18}O incorporation into phosphate when 2 mM phosphate was released in 4–5 min at 25 °C (Table I). At pH 8–8.5, ~0.2 M ionic strength, the dehydration rate constants ranged from 0.02 to 0.07 s^{-1}. Although the hydration equilibrium constants vary somewhat for these aldehydes, all were highly hydrated. Whenever K_H is listed in Table I as greater than

[6] In this case, the ATPase reaction would rapidly extract the ^{18}O present initially in the small pool of free aldehyde, and all subsequent oxygen transfers to phosphate would be ^{16}O derived from the breakdown of the phosphorylated hydrate in solution.

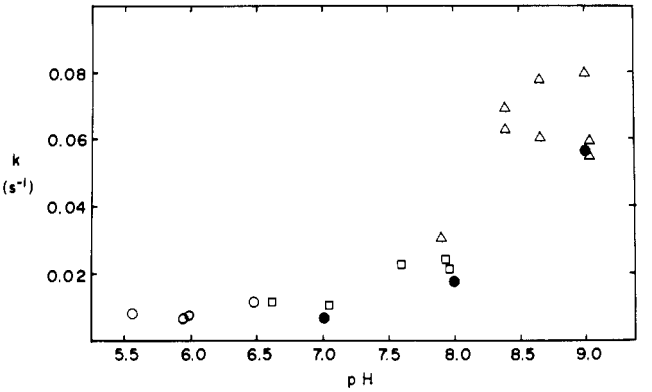


FIGURE 4: Dehydration rate constants for glyoxylate as a function of pH and buffer: (○) 0.1 M Mes, (□) 0.1 M Hepes, and (△) 0.1 M Taps. (●) Rate constants extrapolated to zero concentration of buffer.

Table II: ^{18}O Kinetic Isotope Effect on Glyoxylate Dehydration[a]

pH	equation fitted: eq 7	equation fitted: log form of eq 7
5.95	1.014 ± 0.017	1.017 ± 0.017
5.97	1.010 ● 0.017	1.010 ± 0.018
7.8	1.001 ± 0.026	1.016 ● 0.031
7.94	0.994 ± 0.019	0.995 ± 0.020

[a] The experiments were carried out by varying the concentration of lactate dehydrogenase in the presence of 40 μM glyoxylate, which contained either ^{16}O or ^{18}O in the hydrated aldehyde group. Equation 7 assumes equal isotope effects on V and V/K; the isotope effect is shown for the fit both to eq 7 and to this equation where the logarithm is taken of both sides.

100, the value is a lower limit, since it was difficult to detect and determine accurately the level of the free aldehyde by proton NMR. However, when the hydrate is the ATPase activator and K_H is greater than 40, varying K_H by a factor of 10 gives only a 5% change in the calculated dehydration rate constant. An activation energy of 14.3 kcal/mol was calculated from an Arrhenius plot of the data for the dehydration rate constant for D-glyceraldehyde, and a similar value of 14.2 kcal/mol was calculated for the dehydration of glyoxylate.

pH Dependence of Rate Constant for Dehydration of Glyoxylate. The pH profile for the dehydration rate constant for glyoxylate at 25 °C is shown in Figure 4 for three buffers at 0.1 M concentration. Also shown at pH 7, 8, and 9 are the extrapolated rate constants at zero buffer concentration (0.007 ± 0.001, 0.018 ± 0.001, and 0.056 ± 0.008 at the three pH values). As was the case with formaldehyde (Funderburk et al., 1978), specific base catalysis is evident at pH 8 and above. With Hepes at pH 7 or 8 and with Taps at pH 9, the rate constant was determined as a function of buffer concentration, giving 0.025 ± 0.004, 0.027 ± 0.006, and 0.17 ± 0.05 M^{-1} s^{-1} at the three pH values. Note that the protonated and unprotonated forms of Hepes are equally effective at catalysis, while Mes is clearly less effective and the ionized form of Taps somewhat more so, in line with expectations (Funderburk et al., 1978).

^{18}O Kinetic Isotope Effect on Glyoxylate Dehydration. The ^{18}O isotope effects on the dehydration of glyoxylate hydrate were obtained by direct comparison of reciprocal plots of initial rates with unlabeled and [^{18}O]glyoxylate as a function of lactate dehydrogenase concentration at pH 6 or 8, 25 °C. The data were fitted to eq 6 and 7 and ones that assumed isotope effects only on V/K or V, as well as the logarithmic forms of these equations. No significant ^{18}O kinetic isotope effects were observed on the rate constant for dehydration of glyoxylate

Table III: Comparison of Dehydration Rate Constants and K_H Values for Various Aldehydes and Ketones

aldehyde	temp (°C)	conditions	K_H	k (s^{-1})	ref
acetaldehyde	0	10 mM diethyl malonate, pH 7.2	2.33	0.001	*a*
2-pyridine aldehyde	0	10 mM diethyl malonate, pH 7.2	0.92	0.004	*a*
4-pyridine aldehyde	0	10 mM diethyl malonate, pH 7.2	1.9	0.005	*a*
propionaldehyde	0	10 mM diethyl malonate, pH 7.2	2.04	0.001	*b*
methyl pyruvate	0	10 mM diethyl malonate, pH 7.2	6.52	0.004	*c*
ethyl pyruvate	0	10 mM diethyl malonate, pH 7.2	5.33	0.004	*c*
pyruvate	0	10 mM diethyl malonate, pH 7.2	0.21[k]	0.0007	*c*
formaldehyde	25	unbuffered water, pH 8, 1 M KCl	2273	0.006	*d, e*
acetaldehyde	25	unbuffered water	1.49	0.003	*e*
galactose	26	unbuffered water	~20	0.0005	*f*
formaldehyde	25	0.1 M acetate	2273	0.007	*d, e*
		0.1 M phosphate		0.044	*e*
acetaldehyde	25	0.1 M acetate	1.49	0.015	*e*
glucose	25	barbiturate, pH 8.15	~20	0.016	*g*
D-glyceraldehyde 3-phosphate	20	0.2 M triethanolamine, pH 8.6	29	0.087	*h*
	23	same, but no enzyme, pH 7.3–8.6		0.078	*h*
dihydroxyacetone phosphate	20	0.1 M triethanolamine, pH 7.5	0.8	0.44	*i*
erythrose 4-phosphate	27	0.24 M aldehyde in D_2O, pD 6.4	~9	1	*j*

[a] Pocker & Meany (1967). [b] Pocker & Dickerson (1968). [c] Pocker et al. (1974). [d] Funderburk et al. (1978). [e] Bell & Evans (1966). [f] Wertz et al. (1981). [g] Calculated by extrapolating the ^{18}O exchange rate for glucose to 25 °C (Rittenberg & Graff, 1958) and comparing it with the fraction of free carbonyl group at 25 °C (Hayward & Angyal, 1977). [h] Trentham et al. (1969). [i] Reynolds et al. (1971). [j] Duke et al. (1981). [k] The value is 0.06 at 25 °C (Pocker et al., 1969).

hydrate or on the slopes of the reciprocal plots at either pH 6 or pH 8; the fits of the data to eq 7 are shown in Table II.

Discussion

To facilitate comparison of dehydration rate constants and K_H values determined for the aldehydes in this study with those for other aldehydes, Table III summarizes published values of k and K_H values obtained under conditions similar to those used in this study: pH 8–8.5, 0.1 M buffer (~0.2 M ionic strength), 2 or 25 °C. The dehydration rate constant for D-glyceraldehyde at 2 °C calculated in this study (0.004 s^{-1}) compares favorably with rate constants obtained in 10 mM diethyl malonate buffers at pH 7.2, 0 °C, for aldehydes and ketones whose hydration is catalyzed by carbonic anhydrase (the hydration of the free carbonyl group was followed spectrophotometrically and extrapolated to zero enzyme level). At room temperature, several techniques have been used to measure dehydration rates (see the references cited in Table III), including trapping the free aldehyde with carbonyl reagents and following the product spectrophotometrically (for formaldehyde and acetaldehyde), stopped-flow spectrophotometry of the carbonyl absorbance or of the disappearance of absorbance at 340 nm due to enzymatic oxidation of NADH and reduction of the carbonyl compound (for D-glyceraldehyde and dihydroxyacetone phosphate), exchange rates of ^{18}O at C-1 with water for glucose and galactose, and NMR spin saturation and broadening of the H-1 proton signal for D-erythrose 4-phosphate. The dehydration rate constants of the aldehydes in our study range from 0.017 to 0.068 s^{-1} at 25 °C, in good agreement with the values for formaldehyde, acetaldehyde, glucose, and D-glyceraldehyde 3-phosphate in buffered solutions, which range from 0.007 to 0.087 s^{-1}. The values for dihydroxyacetone phosphate and erythrose 4-phosphate are 1 order of magnitude higher, possibly because of the combined effects of catalysis by the phosphate group (compare glyceraldehyde and its 3-phosphate) and of a lower K_H value.

Since the dehydration rate constants for aldehydes in this study agree closely with values for other aldehydes under similar conditions, it would appear that the kinases and lactate dehydrogenase do not catalyze the dehydration of aldehydes. This has also been found true for D-glyceraldehyde 3-phosphate and α-glycerophosphate dehydrogenases, which do not catalyze dehydration of glyceraldehyde 3-phosphate or dihydroxyacetone phosphate (Trentham et al., 1969; Reynolds et al., 1971).

The dehydration rate constants for formaldehyde and acetaldehyde in unbuffered aqueous solutions are slower than the rates in buffered solutions, since the reaction is both general acid and general base catalyzed (Bell & Evans, 1966; Funderburk et al., 1978). Buffer catalysis of glyoxylate hydrate breakdown was observed in the present work, but for Hepes where general acid catalysis by the protonated form and general base catalysis by the ionized form have equal rate constants, the former is 7-fold faster and the latter 3-fold slower than would be predicted for formaldehyde on the basis of the data of Funderburk et al. (1978) and the Bronsted β and α values determined by these authors (possibly because of the negative charge on glyoxylate). The uncatalyzed rate constant at pH 7 is 1.7-fold faster than that for formaldehyde.

For the heavily hydrated aldehydes examined in the present study, and others shown in Table III, the dehydration rate constants in weakly buffered solutions vary only from 0.01 to 0.09 s^{-1}, despite the fact that the K_H values vary from 1.5 for acetaldehyde to over 2000 for formaldehyde. Thus, the hydration rate constants must vary as much as the equilibrium constants, that is, over 3 orders of magnitude. These differences presumably reflect the much greater effect of substituents α to the aldehyde on the electrophilicity of the carbonyl carbon of the free aldehyde than on the pK of the gem-diol of the hydrated form.

Since dehydration involves breaking a C–O bond, we anticipated that a large primary kinetic ^{18}O isotope effect might be observed on the rate constant for dehydration. However, with [^{18}O]glyoxylate there was little or no ^{18}O isotope effect, with at most a 1% effect at pH 6. We conclude that with glyoxylate C–O bond cleavage is not rate limiting for cleavage of the hydrate and that proton transfer or solvent reorganization may be the slow step.

In summary, dehydration rate constants can be determined for aldehydes that induce ATPase activities by kinases, as long as ^{18}O is transfered from aldehyde to phosphate during the reaction. We have developed the theory for calculating these rate constants when either the hydrate or the free aldehyde is the activator of the ATPase reaction and for correcting these calculations for loss of ^{18}O by the enzymatic ^{18}O transfer process itself, for changing substrate levels during the reaction, and for ^{18}O kinetic and equilibrium isotope effects. The

technique is limited to conditions where kinases are active, that is, pH values between 6 and 9, moderate ionic strength, temperatures between 0 and 40 °C, and, obviously, the presence of MgATP. These are, however, the conditions of greatest interest to biochemists.

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References

Angyal, S. J., & Wheen, R. G. (1980) *Aust. J. Chem. 33*, 1001–1011.

Bell, R. P. (1966) *Adv. Phys. Org. Chem. 4*, 1–29.

Bell, R. P., & Evans, P. G. (1966) *J. Chem. Soc. A 291*, 297–323.

Blanchard, J. S., & Cleland, W. W. (1980) *Biochemistry 19*, 4506–4513.

Brand, J. C. D., & Scott, A. J. (1963) *Tech. Org. Chem. 11*, 61–131.

Cleland, W. W. (1979) *Methods Enzymol. 63*, 103–138.

Cleland, W. W. (1980) *Methods Enzymol. 64*, 104–125.

Cohn, M., & Hu, A. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 200–203.

Defaye, J. (1964) *Bull. Soc. Chim. Fr.*, 999–1002.

Duke, C. C., MacLeod, J. K., & Williams, J. F. (1981) *Carbohydr. Res. 95*, 1–26.

Everse, J., & Kaplan, N. O. (1973) *Adv. Enzymol. Relat. Areas Mol. Biol. 37*, 61–133.

Fischer, F. G., & Schmidt, H. (1960) *Chem. Ber. 93*, 658–662.

Funderburk, L. H., Aldwin, L., & Jencks, W. P. (1978) *J. Am. Chem. Soc. 100*, 5444–5459.

Gruen, L. C., & McTigue, P. T. (1963) *J. Chem. Soc.*, 5217–5223.

Hayward, L. D., & Angyal, S. J. (1977) *Carbohydr. Res. 53*, 13–20.

Hendrix, D. L., Lee, R. E., Baust, J. G., & James, H. (1981) *J. Chromatogr. 210*, 45–53.

Ikuta, S., Matuura, K., Imamura, S., Misaki, H., & Horiuti, Y. (1977) *J. Biochem. (Tokyo) 82*, 157–163.

Khym, J. X. (1972) *Methods Carbohydr. Chem. 6*, 87–93.

Lanzetta, P. A., Alvarez, L. J., Reinach, P. S., & Candic, O. A. (1979) *Anal. Biochem. 100*, 95–97.

Lewis, C. A., & Wolfenden, R. (1977) *Biochemistry 16*, 4886–4890.

MacGee, J., & Doudoroff, M. (1954) *J. Biol. Chem. 210*, 617–626.

Nakada, H. I., & Weinhouse, S. (1953) *Arch. Biochem. Biophys. 42*, 257.

Pocker, Y., & Meany, J. E. (1967) *Biochemistry 6*, 239–246.

Pocker, Y., & Dickerson, D. G. (1968) *Biochemistry 7*, 1995–2004.

Pocker, Y., Meany, J. E., Nist, B. J., & Zadorojny, C. (1969) *J. Phys. Chem. 73*, 2879–2882.

Pocker, Y., Meany, J. E., & Davis, B. C. (1974) *Biochemistry 13*, 1411–1416.

Raushel, F. M., & Cleland, W. W. (1977) *Biochemistry 16*, 2169–2175.

Rendina, A. R., & Cleland, W. W. (1981) *Anal. Biochem. 117*, 213–218.

Rendina, A. R., & Cleland, W. W. (1983) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 42*, 1921.

Rendina, A. R., & Cleland, W. W. (1984) *Biochemistry* (following paper in this issue).

Reynolds, S. J., Yates, D. W., & Pogson, C. I. (1971) *Biochem. J. 122*, 285–297.

Rittenberg, D., & Graff, C. (1958) *J. Am. Chem. Soc. 80*, 3370–3372.

Sols, A., & Salas, M. L. (1966) *Methods Enzymol. 9*, 436–442.

Trentham, D. R., McMurray, C. H., & Pogson, C. I. (1969) *Biochem. J. 114*, 19–24.

Viola, R. E., & Cleland, W. W. (1980) *Biochemistry 19*, 1861–1866.

Wertz, P. W., Garver, J. C., & Anderson, L. (1981) *J. Am. Chem. Soc. 103*, 3916–3922.