Fructose 1,6-Bisphosphate: Isomeric Composition, Kinetics, and Substrate Specificity for the Aldolases†

Christian F. Midelfort,‡ Raj K. Gupta, and Irwin A. Rose*

ABSTRACT: ^{13}C NMR shows fructose 6-phosphate and fructose 1,6-bisphosphate to contain respectively 4.1 and 2.0% keto isomer at room temperature. The lower value for fructose 1,6-bisphosphate can be attributed to the electron-withdrawing effect of the C-1 phosphate. Measurements of the ring-opening rates of the α and β anomers of fructose 1,6-bisphosphate by an NMR line-broadening technique show them to be about 8 and 35 s^{-1}, respectively, at pH 7.2, and 25 °C. The value for the predominant β anomer is threefold greater than the turnover rate of muscle aldolase so that, if the kinetic properties of the keto form were favorable, the reaction could proceed entirely through the keto form in solution. The kinetic properties of a fructose 1,6-bisphosphate(keto) analogue, 5-deoxyfructose 1,6-bisphosphate, in the muscle aldolase reaction are more favorable (V_{max} = 2.6, K_m = 0.11 × 10^{-6} M) than those of fructose 1,6-bisphosphate total (V_{max} = 1, K_m = 2.3 × 10^{-6} M), giving a value of V_{max}/K_m that is 56 times greater for the 5-deoxy analogue. At the 2.0% concentration of the keto form this is sufficient to account for the steady-state rate and requires that the β form, present at 40 times greater concentration, contributes little to the cleavage rate. With yeast aldolase the cleavage rate can also be explained by the rapid spontaneous ring opening and reaction of the keto form with the enzyme. In view of the high rate of ring opening and the excellent properties of the keto form, previous rapid kinetic studies favoring action of cyclic forms may require reevaluation.

The isomeric form(s) of fructose 1,6-bisphosphate (Fru-1,6-P_2)[1] used by the Fru-1,6-P_2 aldolases has been a subject of much recent interest. In the case of the muscle enzyme, it is established that the open chain carbonyl form can be active since xylulose 1,5-bisphosphate is a good substrate (Mehler and Cusic, 1967), although not as good as Fru-1,6-$P_{2(total)}$. However, suggestions that Fru-1,6-$P_{2(keto)}$ is the form to which activity can be attributed (Rutter, 1961), have been considered impractical in view of the low K_m for Fru-1,6-$P_{2(total)}$, 2 × 10^{-6} M, and the failure to detect the keto form by ir or ^1H or ^{31}P NMR (Gray and Barker, 1970; Gray, 1971; Swenson and Barker, 1971) and by natural-abundance ^{13}C NMR (Benkovic et al., 1972; Koerner et al., 1973). Recently, the question of whether one or both of the closed-ring conformers is a substrate along with the acyclic form is thought to have been resolved by rapid kinetic methods (Wurster and Hess, 1973; Grazi, 1974; Schray et al., 1976). These authors found the kinetics to be biphasic, with the first 70–80% of the reaction proceeding at a much faster rate than the final 20–30%. It was concluded that the predominant β anomer was an active substrate along with the keto form. The slow second phase (k_1 = 0.5 s^{-1}) would then represent the spontaneous α-Fru-1,6-P_2 → β-Fru-1,6-P_2 anomerization. In attributing substrate activity to the β anomer it was assumed that the spontaneous β → keto ring-opening rate ($k_{\beta 0}$) was slower than the turnover number for the enzyme (10 s^{-1}), although independent evidence on the value of $k_{\beta 0}$ has not been published.

In this paper, we present the results of ^{13}C NMR measurements on ^{13}C-enriched Fru-1,6-P_2 and Fru-6-P which show that the equilibrium concentration of the carbonyl form in these sugars is higher than previously assumed. The rates of spontaneous ring opening and mutarotation are calculated from measurements of the effect of temperature on the line broadening of the anomeric resonances of Fru-1,6-P_2, a technique previously used to calculate the rates of the chair–chair interconversion of cyclohexane derivatives (Franklin and Feltkamp, 1965), and the unidirectional hydration–dehydration rates of acetaldehyde (Evans et al., 1965), where the absolute values agree well with those obtained by other methods. The kinetic properties of the open-chain analogue, 5-d-Fru-1,6-P_2, are also presented which show this compound to be a far better substrate for the muscle aldolase than Fru-1,6-P_2 itself. Calculations based on the properties of 5-d-Fru-1,6-P_2 show that 100% of the Fru-1,6-P_2 cleavage rate can be accounted for by the cleavage of Fru-1,6-$P_{2(keto)}$ alone.

Materials and Methods

3-Hydroxypropionaldehyde 3-Phosphate (PAP). Although several methods were available for the preparation of the phosphate ester of 3-hydroxypropionaldehyde (Orsi and Cleland, 1972; Putman et al., 1972), we chose to use the following procedure because it gave a stable crystalline product. Fifty grams of 2-deoxyglucose (Sigma) were reduced with 12 g of $NaBH_4$ (Ventron) in a solution of 500 ml for 24 h. After acidification with acetic acid to pH 4.5 the boric acid was removed under reduced pressure by three successive additions of methanol as the volatile trimethoxyborate. The reduced product was crystallized from water–ethanol. Two hundred grams of $NaIO_4$ (Fisher) was then added to a 500-ml solution in an ice bath and left in the cold overnight. The excess IO_4^- was reduced with 10 ml of ethylene glycol. Insoluble $NaIO_3$

† From the Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111. *Received October 22, 1975.* Supported by NIH Grants GM-20940, CA-06927, AM-13351, and RR-05539, and also by an appropriation from the Commonwealth of Pennsylvania.

‡ Supported by NIH Fellowship GM-57317.

[1] Abbreviations used are: Fru, fructose; DHAP, 1,3-dihydroxyacetone 1-phosphate; DHA, 1,3-dihydroxyacetone; Glc, glucose; PAP, 3-hydroxypropionaldehyde 3-phosphate; Fru-1,6-P_2, fructose 1,6-bisphosphate; 5-d-Fru-1,6-P_2, 5-deoxyfructose 1,6-bisphosphate; DPN, diphosphopyridine nucleotide; OD, optical density; EDTA, ethylenediaminetetraacetic acid.

was removed by filtration before and after concentrating to 50 ml. Further desalting was achieved by the addition of methanol to 50%. The mixture was then fractionally distilled twice at 4 mm Hg with the 3-hydroxypropionaldehyde distilling at about 50 °C. This material was converted to the dimethyl acetal, phosphorylated, and crystallized as the dicyclohexylammonium salt by following in detail the methods described by Ballou (1960). Four grams of the crystalline product was obtained. For the hydrolysis to the free aldehyde, the dicyclohexylamine was removed with an excess of Dowex-50 (H^+) and the acetal was hydrolyzed by incubation at 37 °C for 30 min. After adjustment to pH 4 with dilute NaOH, the free aldehyde was stable for several days at 0 °C. It was assayed with glyceraldehyde-3-phosphate dehydrogenase (Boehringer), DPN^+, and arsenate (Orsi and Cleland, 1972). It also could be assayed as inorganic phosphate after incubation at pH 11 by virtue of its extreme alkaline lability ($t_{1/2}$ = 20 min at 37 °C at pH 10.5).

5-Deoxyfructose 1,6-Bisphosphate (5-d-Fru-1,6-P_2). Muscle aldolase (Sigma) was freed of possible contaminating triosephosphate isomerase activity by treatment with D,L-glycidol phosphate (Rose and O'Connell, 1969) followed by dialysis. An excess of the treated enzyme (0.5 mg/ml) was then incubated with 50 mM PAP and 10 mM DHAP in 50 mM triethanolamine-HCl, pH 7.0 until equilibrium was achieved ($K_{eq} \sim 5$ mM). The 10-ml mixture was then applied directly to a 1 × 10 cm Dowex-1 (Cl^-) column and was eluted first with 0.02 N HCl and then 0.05 N HCl. The 5-d-Fru-1,6-P_2 was neutralized to pH 5.5 and was precipitated by the addition of an excess of barium acetate and an equal volume of ethanol. The precipitate was dissolved by swirling with Dowex-50 (H^+) and the solution was stored frozen at pH 4.

Competitive Inhibitors of Muscle and Yeast Aldolase. 2,5-Anhydroglucitol 1,6-bisphosphate and 2,5-anhydromannitol 1,6-bisphosphate (Hartman and Barker, 1965) were the generous gifts of Dr. S. J. Benkovic of Pennsylvania State University. Hexitol 1,6-bisphosphate was made by the $NaBH_4$ reduction of Fru 1,6-P_2 as described by Ginsburg and Mehler (1966).

[UL-^{13}C]Fru-6-P and Fru-1,6-P_2. One-hundred milligrams of uniformly labeled [^{13}C]glucose (either 60% or 13.3% ^{13}C, Merck) was incubated with 50 μmol of ATP, 1200 μmol of creatine phosphate, 300 μmol of $MgCl_2$, and an excess of hexokinase (P-L Biochemicals), phosphoglucose isomerase (Sigma), Fru-6-P kinase (Sigma), and creatine kinase (Boehringer) in 50 ml at pH 7.5. The Fru-1,6-P_2 was isolated by Dowex-1 (Cl^-) chromatography and precipitated as the barium salt. It was converted to the sodium salt with Dowex-50 (H^+) and NaOH; Fru-1,6-P_2 0.5 mmol was obtained. Fru-6-P was made from the Fru-1,6-P_2 by prolonged incubation with potato acid phosphatase (Boehringer) at pH 5 followed by passage through Dowex-1 (acetate) and rephosphorylation of the fructose by ATP and hexokinase with added creatine phosphate and creatine kinase. Care was taken to limit the isomerization of Fru-6-P to Glc-6-P due to the small contaminant of phosphoglucose isomerase present in the hexokinase by stopping the reaction as soon as 95% completion had been achieved. The Fru-6-P was eluted from Dowex-1 with 7 mM HCl and was adjusted to pH 4.5 with NaOH. After concentration the sample contained a twofold excess of NaCl over NaFru-6-P and <10% Glc-6-P.

Fru-1,6-P_2-Aldolase Assays. Fru-1,6-P_2 and 5-deoxy-Fru-1,6-P_2 cleavage rates were followed spectrophotometrically (Racker, 1947) in a coupled enzymatic assay using an excess of α-glycerophosphate dehydrogenase (Sigma) and triosephosphate isomerase (Sigma) in 0.05 M triethanolamine-HCl, pH 7.8, and approximately 0.01 M ammonium sulfate; a kinetically insignificant amount was introduced with the enzymes. Muscle aldolase (Sigma) was 10–12 units/mg and yeast aldolase was 60–70 units/mg (Rutter et al., 1966). Spectrophotometric K_m determinations for Fru-1,6-P_2 were done with a 0.1 OD scale, so that the cleavage of 1–10 μM Fru-1,6-P_2 could be followed. Those for -dFru-1,6-P_2 were done in the presence of several fixed concentrations (0.5, 1, and 2 mM) of the competitive inhibitor butanediol 1,4-bisphosphate ($K_I = 4.5 \times 10^{-5}$ M, determined with Fru 1,6-P_2 as substrate; this is a similar value to the one reported by Hartman and Barker, 1965) in order to raise its apparent K_m into a measurable range. Butanediol 1,4-bisphosphate was prepared by the method of Hartman and Barker (1965).

^{13}C NMR. Nuclear magnetic resonance spectra were recorded at 25.1 MHz on a Varian XL-100-15-FT system with proton noise decoupling. D_2O (5–10%) was present in all samples to permit heteronuclear field-frequency locking on deuterium. Sample volumes of approximately 1.5 ml in 12-mm sample tubes were used and the concentration of the samples was in the range of 0.1–0.5 M. A Teflon plug was used as a vortex suppressor. Transients (4 000–100 000) were time averaged to obtain adequate signal-to-noise ratio in the resulting spectra. In order to obtain maximum signal to noise ratio in a given amount of time in many cases, the rapid pulse FT technique (Ernst, 1966) with pulse repetition rates of up to 5/s and a spin flip angle (θ_{opt}) of 8° calculated according to the expression

$$\cos|\theta_{opt}| = e^{-\tau/T_1} \quad (1)$$

were used, where τ is the time interval between pulses and T_1 the estimated spin–lattice relaxation time of the resonances of interest (~45 s for the carbonyl carbon; Fung et al., 1973). In the quantitation of relative amounts of various forms of Fru-6-P, slow pulsing (90° flip angles and $\tau > 3T_1$) and fast pulsing gave identical results within experimental error. Hence, for similar quantitation with Fru-1,6-P_2 only fast pulsing was used which resulted in significant saving of time. The temperature was controlled within ±1 °C by equilibration with gaseous nitrogen. Internal dioxane was used as reference (67.4 ppm downfield from Me_4Si) in assigning the shifts of the ^{13}C resonances relative to Me_4Si.

^1H-NMR spectra of dihydroxyacetone (Sigma) and dihydroxyacetone phosphate (Calbiochem) were obtained at 100 MHz using the Varian XL-100 spectrometer in the slow sweep continuous wave mode (sweep ratio = 0.2 Hz/s). In some cases noise decoupling was used in working with dihydroxyacetone phosphate to decouple phosphorus. ^1H chemical shifts were measured relative to external Me_4Si.

Results

Detection and Quantitation of the Carbonyl Form of Fru-6-P and Fru-1,6-P_2 by ^{13}C NMR. Several published attempts to detect the open-chain carbonyl resonance of the two fructose phosphate derivatives by ^{31}P NMR (Gray and Barker, 1970) and natural-abundance ^{13}C NMR (Koerner et al., 1973; Benkovic et al., 1972) failed to find any of this form present even in solutions of very high concentration. A lower detection limit of 1.5% was mentioned in several cases. In one study using ir spectroscopy, 2.4% Fru-6-$P_{(keto)}$ was reported, but no Fru-1,6-$P_{2(keto)}$ was seen (Swenson and Barker, 1971). Several considerations led us to make a renewed attempt to detect and quantitate this form by NMR methods: (1) very rapid chemical interconversion of the open and cyclic forms under the conditions of the experiments would have produced a very

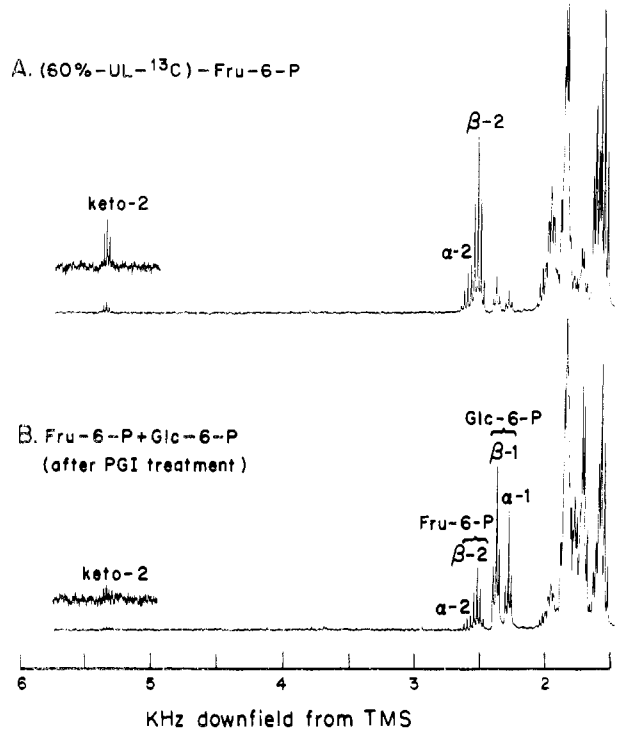


FIGURE 1: Proton-decoupled Fourier transform ^{13}C-NMR spectra at 25.1 MHz of 60% enriched [UL-^{13}C]Fru-6-P at pH 4.5, 17 °C before (A) and after (B) incubation with an excess of phosphoglucose isomerase (PGI). The sample contained initially (A) 0.32 M Fru-6-P, 0.05 M Glc-6-P, 0.005 M EDTA, 0.6 M NaCl in 10% deuterated water. After incubation with PGI (20 units) the same sample (B) contained: 0.12 M Fru-6-P, 0.25 M Glc-6-P, 0.005 M EDTA, 0.7 M NaCl, and 13 units/ml PGI. Each spectrum is the result of time-averaging 93 000 transients of free induction decay signal with a 0.4-s acquisition time and pulse length of 4 μs corresponding to a spin-flip angle of 8°. An exponential filter with a time constant of 0.2 s was used to improve S/N ratio of the spectra and the total time required for data acquisition was ca. 11 h. The inserts show the C-2 keto multiplets at a fourfold higher vertical expansion.

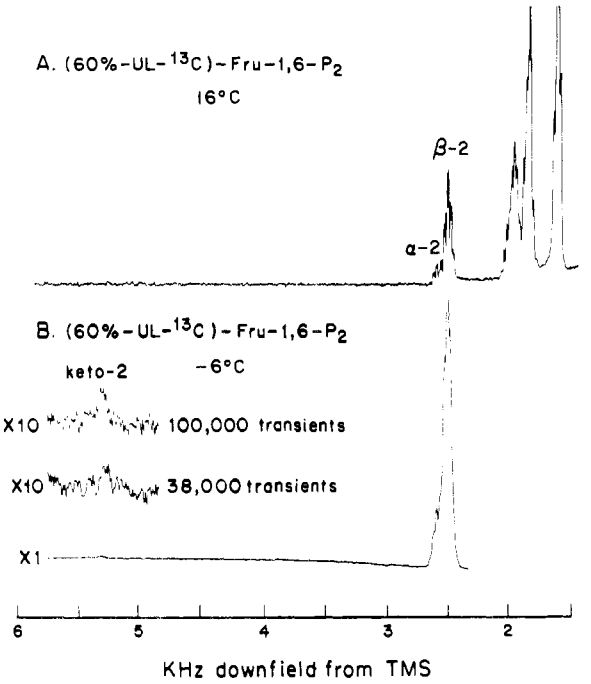


FIGURE 2: Proton-decoupled Fourier transform ^{13}C NMR spectra at 25.1 MHz of 60% enriched [UL-^{13}C]Fru-1,6-P_2. The sample contained 0.5 M Fru-1,6-P_2 (disodium salt at pH 4.5), 0.005 M EDTA in 5% D_2O. The spectrum in (A) was obtained at 16 °C after 43 000 transients. Other NMR instrumental settings were the same as in Figure 1. The spectra in (B) were obtained at −6 °C upon adding 20% Me_2SO to the above sample. The relative vertical expansions and the number of transients used are as indicated. An exponential filter with a time constant of 0.04 s was used to optimize S/N ratio for the keto resonance. Other instrumental conditions were the same as in Figure 1. No keto resonance was observable under these conditions at +16 °C.

appreciable line broadening in the earlier NMR studies and loss of amplitude of the carbonyl resonance (Gupta et al., 1974); (2) the lower detection limit in the published natural abundance spectra might have been as high as 7–10% for Fru-6-P. Fru-6-P was expected to produce a sharper ^{13}C carbonyl resonance than Fru-1,6-P_2 because the presence of the additional phosphate in Fru-1,6-P_2 would accelerate ring closing and produce further line broadening. Also, the phosphate monoanion would anomerize more slowly than the dianion (Bailey et al., 1970). Therefore, we chose pH 4.5 for most of the experiments.

The ^{13}C-NMR spectra of 60% enriched [UL-^{13}C]Fru-6-P are shown in Figure 1. Each C-2 resonance signal in these spectra is analyzed by scale expansion to be split into a quintet (relative areas of 9:24:34:24:9) as expected for intramolecular vicinal ^{13}C–^{13}C coupling (J = 24 Hz) through one bond. A very pronounced carbonyl resonance quintet centered at 5390 Hz or 215 ppm downfield from tetramethylsilane reference is observable (Figure 1A). Treatment of the 60% [^{13}C]Fru-6-P with an excess of yeast phosphoglucose isomerase at pH 6.4 converted two-thirds of the Fru-6-P to Glc-6-P and reduced the size of the keto carbon signal to about one-third, thus showing that the resonance was derived from Fru-6-P. Integration of the keto and the anomeric carbon signals of Fru-6-P showed the former to be 4 ± 0.4% of the total. Since the keto and anomeric resonances may have somewhat different relaxation times (Fung et al., 1973), experiments were done using both fast and slow pulsing with pulse delays of 0.4 and 150 s. These gave integrated areas within the error of the 4% value, showing that no distortion in relative intensities was introduced by pulsing more rapidly than the relaxation time for the excited ^{13}C nuclei.

Attempts to detect any carbonyl in Fru-1,6-P_2 under conditions similar to those of Figure 1 failed. Examination of line broadening in the C-2 resonance of the α and β anomers (see below; also seen in spectra of Koerner et al., 1973) indicated that rapid mutarotation was probably occurring. This would have caused the carbonyl peak to disappear into the noise background. We, therefore, obtained several spectra at temperatures below 0 °C using 20% Me_2SO in H_2O to prevent freezing. The spectrum in aqueous Me_2SO (Figure 2) showed a very definite, broadened carbonyl resonance at the same chemical shift as the C-2 of Fru-6-$P_{(keto)}$. The peak grew steadily out of the noise with time. An average value of 1.3 ± 0.4% for the keto form was calculated from two separate spectra with 10^5 pulses at this temperature.

A summary of the data obtained at several temperatures and two pH's is given in Table I. The error values are a reflection of the signal:noise ratio of the keto resonance in each spectrum. The percentage of Fru-6-$P_{(keto)}$ can be seen to increase slightly with temperature. When the Fru-6-$P_{(keto)}$ and Fru-1,6-$P_{2(keto)}$ are compared under the same conditions (−6 °C, 20% Me_2SO, pH 4.5), the former is about 2.1-fold greater than the latter. As the data in the next section will show, this difference can be assigned to the electron-withdrawing effect of the C_1 phosphate present in Fru-1,6-P_2.

The Hydration Equilibria in Dihydroxyacetone (DHA) and Dihydroxyacetone Phosphate (DHAP). An assessment of the

TABLE I: The Fraction of Ketose Present in Fru-6-P and Fru-1,6-P_2 at pH 4.5 and 6.3 at Various Temperatures Using Fourier Transform ^{13}C NMR.

Sugar	Temp (°C)	Solvent	pH	Relative NMR Intensities (keto/hemiketal)	% Keto
Fru-6-P	25	H_2O	4.5	1:23.4	4.1 ± 0.4
	16	H_2O	4.5	1:24	4.0 ± 0.4
	16	H_2O	6.3	1:24	4.0 ± 0.5
	−6	20% Me_2SO_2 in H_2O	4.5	1:36	2.7 ± 0.6
Fru-1,6-P_2	−6	20% Me_2SO in H_2O	4.5	1:76	1.3 ± 0.4

effect of the C_1-phosphate group present in Fru-1,6-P_2 on the keto ⇌ hemiketal equilibrium can be made by comparing the >C=O ⇌ >C(OH)$_2$ equilibria in DHAP and DHA. Gray and Barker (1970) and Reynolds et al. (1971) have reported a value of 55% keto:45% diol for DHAP in H_2O at 20 °C, pH 7.2, which was obtained by several methods including proton NMR. Figure 3 shows the 100-MHz proton NMR spectra of DHA and DHAP in D_2O under comparable conditions of ionic strength and pH. Integration of the two peaks in DHA and the four in DHAP yields values of 78 (±2) keto/22 (±2) diol for DHA and 56 (±2)/44 (±2) for DHAP, showing that the C_1 phosphate acts as an electron-withdrawing group (Bell, 1966), decreasing the >C=O/>C(OH)$_2$ ratio by about 2.8-fold.

The C_1 phosphate in Fru-1,6-P_2 appears to have a similar effect. Under comparable conditions (−6 °C, pH 4.5, 20% Me_2SO) Fru-6-P contained about 2.7% keto while Fru-1,6-P_2 contained about 1.3% keto, a 2.1-fold difference.

The Ring-Opening Rates of α- and β-Fru-1,6-P_2. It is well known (Gutowsky and Saika, 1953) that when a magnetic nucleus exchanges between different magnetic environments, the absolute rates of exchange may, in suitable cases, be obtained from the line widths in the NMR spectra of such systems. In the case of exchange between two environments, if the exchange times τ_A ($\equiv 1/k_{A\rightarrow B}$) in state A and τ_B ($\equiv 1/k_{B\rightarrow A}$) in state B are sufficiently long compared to the inverse of the frequency separation $[2\pi(\upsilon_A - \upsilon_B)]^{-1}$ where υ_A and υ_B are characteristic resonance frequencies of the nucleus in environments A and B, the resulting spectrum will consist of separate but broadened signals in the vicinity of frequencies υ_A and υ_B. The full width at half-height, $\Delta\upsilon_A$, of the broadened signal centered at υ_A is given by eq 2:

$$\pi\Delta\upsilon_A = \pi\Delta\upsilon^\circ_A + \tau_A^{-1} \quad (2)$$

$$\pi\Delta\upsilon_B = \pi\Delta\upsilon^\circ_B + \tau_B^{-1} \quad (3)$$

The $\Delta\upsilon^\circ$'s are the widths at half-height of the two signals in the absence of exchange. The exchange therefore leads to an additional broadening of the individual signals by $(\pi\tau_A)^{-1}$ and $(\pi\tau_B)^{-1}$. If $\Delta\upsilon^\circ_A$ and $\Delta\upsilon^\circ_B$ are known, a measurement of the widths of these broadened signals in the presence of exchange provides a means of estimating τ_A and τ_B. This procedure is valid, provided the broadening is not large enough to cause appreciable overlap of the signals.

In the case of Fru-6-P and Fru-1,6-P_2, the exchange results from spontaneous chemical conversion of α and β forms to open chain form and vice versa. Thus the equilibrium is:

$$\alpha \underset{k_{0\alpha}}{\overset{k_{\alpha 0}}{\rightleftharpoons}} 0 \underset{k_{\beta 0}}{\overset{k_{0\beta}}{\rightleftharpoons}} \beta \quad (4)$$

The C_2 carbon atom of Fru-1,6-P_2 (or Fru-6-P) sees very different chemical and magnetic environments in the three forms of the sugar. Its resonance frequencies in the α, β, and open-chain forms are 105, 101, and 212 ppm downfield from Me_4Si, respectively.

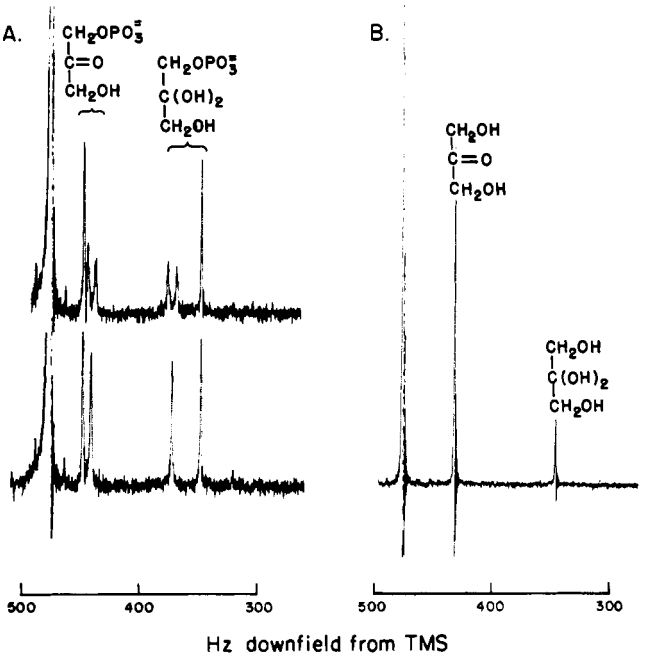


FIGURE 3: Continuous-wave proton NMR spectra at 100 MHz of (A) dihydroxyacetone phosphate (DHAP) and (B) dihydroxyacetone (DHA) in D_2O at pH 7.2, 20 °C. The DHA sample was 0.12 M and contained 0.07 M sodium phosphate buffer. The DHAP sample was 0.07 M of the sodium salt and its spectra are shown with and without ^{31}P noise decoupling to eliminate ^{31}P splitting of the signals of the C-1 protons. The scan speed was 0.2 Hz/s.

With interconversion between closed-ring forms and the single open-chain form, the slow exchange limit applies, and the extension of the same reasoning shows that the line broadening is given by the equations:

$$\begin{aligned} \pi\Delta\upsilon_\alpha &= \pi\Delta\upsilon^\circ_\alpha + k_{\alpha 0} \\ \pi\Delta\upsilon_\beta &= \pi\Delta\upsilon^\circ_\beta + k_{\beta 0} \\ \pi\Delta\upsilon_0 &= \pi\Delta\upsilon^\circ_0 + (k_{0\alpha} + k_{0\beta}) \end{aligned} \quad (5)$$

Since natural ^{13}C line widths are expected to be very small (<0.1 Hz) $\Delta\upsilon^\circ_\alpha$, $\Delta\upsilon^\circ_\beta$, and $\Delta\upsilon^\circ_0$ will essentially be equal, arising from the effects of field inhomogeneity and exponential filtering on the line broadening. These effects on line broadening were determined by measuring the line width of the proton-decoupled ^{13}C resonance of dioxane. $\Delta\upsilon^\circ_\alpha$, $\Delta\upsilon^\circ_\beta$, and $\Delta\upsilon^\circ_0$ were thus estimated to be 1.3 Hz. Since a line broadening ≥0.5 Hz is easily measurable, mutarotation rate constants of ≥2 s^{-1} will produce enough line broadening to be accurately measured. In our experiments with Fru-1,6-P_2, the temperature was varied between 13 and 38.5 °C at pH 7.2. The width

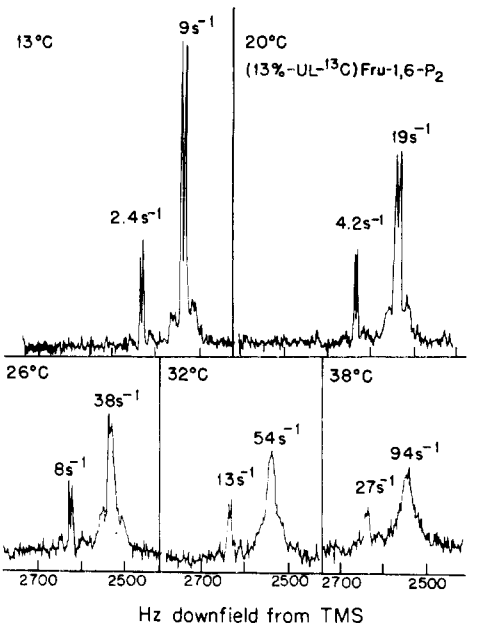


FIGURE 4: Proton-decoupled Fourier transform ^{13}C-NMR spectra of 13% enriched [UL-^{13}C]Fru-1,6-P_2 (pH 7.2) at several temperatures. The sample contained 0.30 M Fru-1,6-P_2 with 3.5 equiv of Na^+, 0.005 M EDTA in 10% D_2O. The ring-opening rates for the α and β anomers obtained from the observed line widths are as indicated. The errors in the rate values are ≤10%. Each spectrum is a time average of 4000 transients with an acquisition time of 0.8 s and an exponential filtering time constant of 0.4 s.

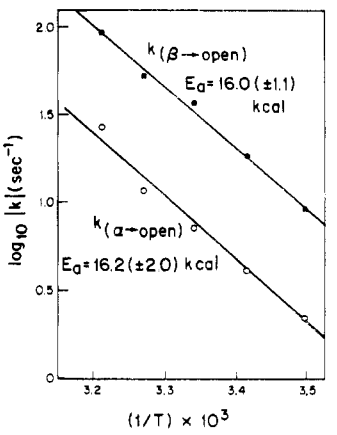


FIGURE 5: Arrhenius plots of the α- and β-ring-opening rates of 0.3 M $Na_{3.5}$Fru-1,6-P_2 at pH 7.2 (Figure 4).

TABLE II: Kinetic Constants of 5-Deoxyfructose 1,6-Bisphosphate.

	Fru-1,6-P_2		5-d-Fru-1,6-P_2	
Enzyme	K_m	V_{max}[b]	K_m	V_{max}[b]
Muscle aldolase	2.3 (±0.4) × 10^{-6} M	1	1.1 (±0.1) × 10^{-7} M[a]	2.7
Yeast aldolase	3.3 (±1.0) × 10^{-4} M	1	1.1 (±0.3) × 10^{-5} M	0.45

[a] Determined with and corrected for the use of a fixed concentration of butanediol 1,4-bisphosphate (K_I = 4.5 × 10^{-5} M) to raise the K_m to a measurable level. [b] V_{max}'s are normalized separately for each enzyme.

at half-height of the dioxane ^{13}C signal was found to equal 1.3 Hz at each temperature.

The effect of temperature on the line broadening of the anomeric C-2 resonances of 0.30 M (13% [UL-^{13}C]Fru-1,6-P_2) is shown in Figure 4. In each case, 4000 transients were accumulated. The line widths of the resonances of both the α and β anomers can be seen to increase dramatically with increasing temperature. For the β anomer the resonances were broadened more than for the α anomer, showing that the rate of $\beta \rightarrow$ open chain was appreciably greater than the rate of $\alpha \rightarrow 0$. The calculated rate constant is shown above each curve.[2]

The rate constants for ring opening shown in Figure 4 can be plotted as a function of temperature to obtain the activation energy (E_a) for each ring-opening step. Both k's have an E_a of about 16 kcal (Figure 5).

The spectra shown in Figure 4 were obtained on a concentrated sample (0.3 M) of $Na_{3.5}$Fru-1,6-P_2, raising the possibility that both intramolecular and intermolecular phosphate catalysis (Bailey et al., 1970) contributed to the observed rates. Since intermolecular effects would be proportional to concentration, spectra were accumulated on several more dilute samples of Fru-1,6-P_2. The calculated ring-opening rates [44 s^{-1} ($k_{\beta 0}$) and 14 s^{-1} ($k_{\alpha 0}$) at 0.2 M, 28 °C, and 44 s^{-1} and 13 s^{-1} at 0.07 M] are within 10% of the values for 0.3 M Fru-1,6-P_2 at 28 °C calculated from the Arrhenius plot in Figure 5. Also the spectrum of 0.7 M Na_4 Fru-1,6-P_2 (Koerner et al., 1973) shows no greater line broadening than reported here. Thus, it is concluded that intramolecular catalysis predominates over whatever intermolecular effects, if any, are present.

5-d-Fru-1,6-P_2. The kinetic properties of Fru-1,6-P_2 and 5-d-Fru-1,6-P_2 toward muscle and yeast aldolases are shown in Table II. These values represent the averages of four different preparations of the analogue. It can be seen that the analogue is an excellent substrate for both enzymes. It is, by far, the best substrate found for the muscle enzyme where the V_{max}/K_m is 50-fold greater than for Fru-1,6-P_2 and the only alternate substrate known for yeast aldolase.

The muscle enzyme has been widely studied with respect to the K_I's of various inactive substrate analogues (Hartman and Barker, 1965; Castellino and Barker, 1965; Ginsburg and Mehler, 1966). Table III gives the K_I's of $NaBH_4$-reduced Fru-1,6-P_2 (hexitol 1,6-P_2, HDP), 2,5-anhydroglucitol 1,6-P_2 (analogue of α-Fru-1,6-P_2) and 2,5-anhydromannitol 1,6-P_2 (analogue of β-Fru-1,6-P_2) under the conditions of pH and temperature used in the 5-deoxy-Fru-1,6-P_2 studies in order to compare the affinities of the cyclic and acyclic hexitol phosphates. With regard to muscle aldolase, two aspects of these data are notable: (1) the enzyme binds the α-Fru-1,6-P_2 and β-Fru-1,6-P_2 analogues with equal affinity and both of

[2] In the calculation of the rate constants, shown above each curve in Figure 3, a complication due to ^{31}P splitting had to be corrected for. When the components of the doublet overlap, the apparent height of each contains a contribution from the side of the other. The line shapes are Lorentzian and the height at any position x Hz away from the center of the doublet is: $h = a^2h_0/(a^2 + (s - x)^2) + a^2h_0/(a^2 + (x + s)^2)$ where s = one-half of the ^{31}P splitting (J = 6.6 Hz for α-Fru-1,6-P_2; J = 8.6 Hz for β-Fru-1,6-P_2); h_0 = the true height of each peak; and a = one-half of the true line width at $h_0/2$. It can be shown that the true line width at $h_0/2$ ($\Delta\nu = 2a$) of the resulting NMR signal is given by: $\Delta\nu = 2a = 16s^2Ah/(16s^2h^2 - A^2)$, when the broadening is small and two separate maxima are seen; and $\Delta\nu = 2a = (A + \sqrt{A^2 - 4h^2s^2})/h$, when the components of the doublet overlap so that only one maximum is seen. Here, h is the measured peak height (assumed to be equal for both peaks) and A is the area of each peak ($A = 2ah_0$). A is obtained as the area under one of the peaks in the absence of overlap (e.g., at 13 °C in Figure 4).

TABLE III: K_I's for Various Substrate Analogues of the Fru-1,6-P_2 Aldolases

Inhibitor	K_I's	
	Muscle Aldolase	Yeast Aldolase
2,5-Anhydroglucitol 1,6-bisphosphate (2-d-α-Fru-1,6-P_2)	14 (±1) × 10^{-6} M	3.1 (±0.3) × 10^{-4} M
2,5-Anhydromannitol 1,6-bisphosphate (d-β-Fru-1,6-P_2)	11 (±1) × 10^{-6} M	23 (±6) × 10^{-4} M
Hexitol 1,6-bisphosphate	0.45 (±0.1) × 10^{-6} M	4.6 (±0.6) × 10^{-4} M
5-Deoxyhexitol 1,6-bisphosphate	1.4 (±0.2) × 10^{-6} M	5.2 (±0.5) × 10^{-4} M

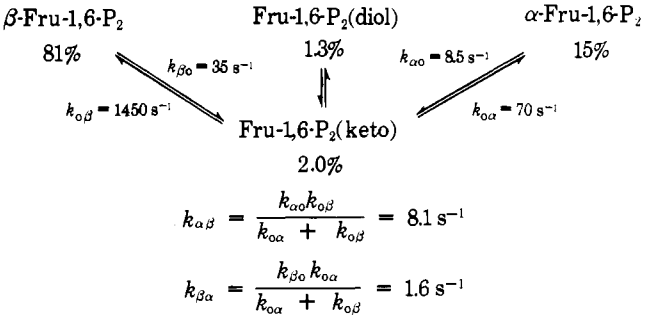
them little better than a bisphosphate compound of comparable interphosphate distance (Hartman and Barker, 1965); and (2) there is a 30-fold preference in binding of the acyclic hexitol bisphosphates and a three-fold tightening of binding by the 5-OH. The yeast enzyme shows no great preference for the open-chain conformation, but does discriminate between analogues of the α and β anomers.

Discussion

The kinetics of Fru-1,6-P_2 mutarotation at 0.3 M, μ = 1.5, 25 °C, and pH 7.2 are summarized in Scheme I. The absence of ionic-strength effects on the observed rates was demonstrated by carrying out the experiments at 0.7 (Koerner et al., 1973), 0.3, 0.2, and 0.07 M Fru-1,6-P_2 which showed no detectable variation in line broadening of the anomeric resonances. Apparently anionic repulsion prevents intermolecular phosphate catalysis by other Fru-1,6-$P_2$$^{-4}$ and Fru-1,6-$P_2$$^{-3}$ molecules. The equilibrium ratio of the α and β anomers (5.4:1) is taken from a number of NMR spectra like those in Figure 4. This ratio differs from the values reported in the literature (4:1, Benkovic et al., 1972; 3.5:1, Koerner et al., 1973; >10:1, Gray, 1971), but it is considered accurate to (±5%) because of the improved signal:noise ratio. The α:β ratio is temperature-independent between 10 and 32 °C. The percentage of Fru-1,6-$P_{2(keto)}$ (2.0%) is an estimate based on the relative amounts of Fru-1,6-$P_{2(keto)}$ and Fru-6-$P_{(keto)}$ at −6 °C in 20% aqueous Me_2SO (1.3% vs. 2.7%) and the amount of Fru-6-$P_{(keto)}$ at 25 °C (4.1%). The assumption is made that the keto/hemiketal equilibria of these two sugar phosphates have the same temperature dependence. The relative hydration equilibria of DHA and DHAP at pH 7.2, 25 °C (Figure 3) support the idea that the keto form is less favored thermodynamically when a phosphate replaces a hydroxyl on the carbon α to the carbonyl. The fourth Fru-1,6-P_2 isomer shown in Scheme I, the gem diol or ketohydrate, has not been detected directly but is assumed to be present at 1.3%, based on the experiments with DHAP (keto/diol = 1.5 at 25 °C, Reynolds et al., 1971).

An interesting aspect of the Fru-1,6-P_2 mutarotation kinetics in Scheme I is that the ring-opening rates are in inverse relation to the thermodynamic stability of the two anomers. The less stable anomer thermodynamically (α) is the more stable kinetically. As a result, the calculated ring-closing rates differ by a factor of more than 20 ($k_{0\beta}/k_{0\alpha}$ = 21). In the only other sugar (glucose) where the unidirectional rates have been

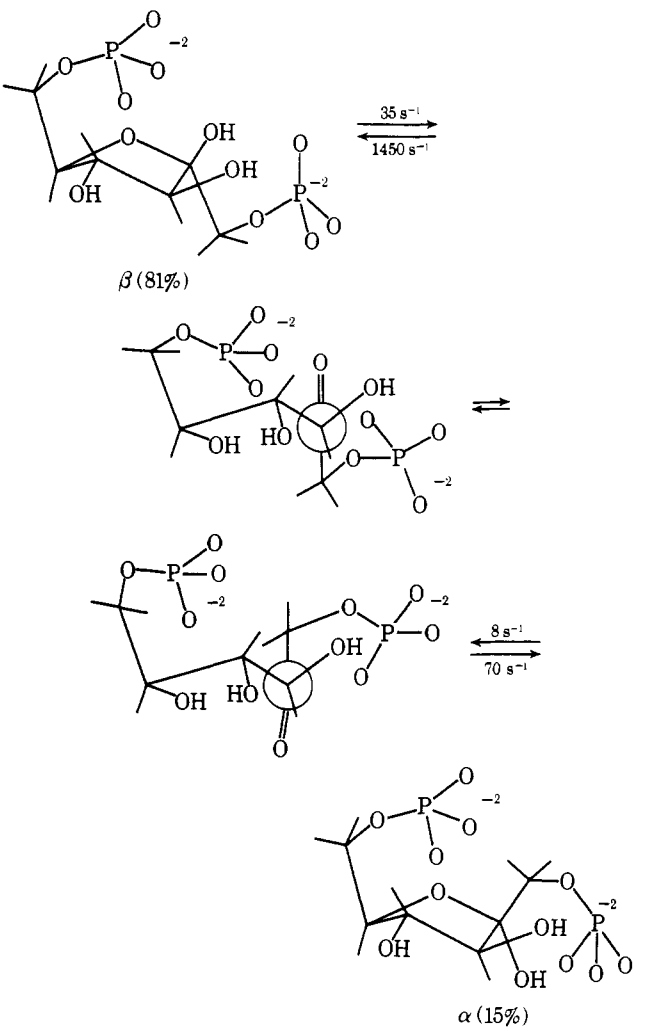
Scheme I



$$k_{\alpha\beta} = \frac{k_{\alpha o}k_{o\beta}}{k_{o\alpha} + k_{o\beta}} = 8.1\ s^{-1}$$

$$k_{\beta\alpha} = \frac{k_{\beta o}k_{o\alpha}}{k_{o\alpha} + k_{o\beta}} = 1.6\ s^{-1}$$

Scheme II



calculated, the ring-opening rates are thought to be proportional to the thermodynamic equilibrium of the anomers ($k_{\beta o}/k_{\alpha 0} \sim k_{\beta\alpha}/k_{\alpha\beta}$) with ring closing to the two anomers occurring at about equal rates (Los et al., 1956). Perhaps the large $k_{0\beta}/k_{0\alpha}$ in Fru-1,6-P_2 is related to the intramolecular catalysis by phosphate at the ends of the sugar. Scheme II shows a model of phosphate-catalyzed α–β conversion of Fru-1,6-P_2. This scheme suggests that the bulky C-1 and C-6 phosphates would have to come closer together in catalyzing $\alpha \rightleftharpoons 0$ than $\beta \rightleftharpoons 0$ and electrostatic repulsion interferes in the former case.

On the other hand, Scheme II also shows that the C-2 carbonyl and C-3 hydroxyl oxygens are gauche in $0 \rightleftharpoons \beta$ and trans in $0 \rightleftharpoons \alpha$ and the enhanced rate of the former could represent

an example of the "gauche effect", the influence of positive interactions between electron pairs of the two oxygens (Wolf, 1972). A determination of the ratio of $k_{0\beta}/k_{0\beta}$ in Fru-6-P would provide a distinction between the two alternatives since possible phosphate–phosphate repulsive effects would be absent. Unfortunately, the presence of very little line broadening in the anomeric resonances of Fru-6-P complicates measurements of $k_{\beta 0}$ and $k_{\alpha 0}$ by the NMR technique, and we have not attempted these experiments. But it is clear that $k_{\beta 0}$ and ($k_{0\beta}$ + $k_{0\alpha}$) are more than an order of magnitude slower than for Fru-1,6-P_2, showing the importance of intramolecular phosphate catalysis.

An important question to be asked is whether Fru-1,6-$P_{2(keto)}$ has kinetic properties in the muscle aldolase system that would allow it to be considered the only active substrate form of Fru-1,6-P_2. First, the NMR line-broadening data indicate that the $k_{\beta 0}$ (35 s^{-1} at 25 °C) greatly exceeds the turnover rate for the enzyme (10 s^{-1}) so that under conditions of excess substrate the interconversion of β-anomeric and keto forms will always be in a preequilibrium. Second, assuming that the parameters for 5-d-Fru-1,6-P_2 reflect those for [Fru-1,6-$P_{2(keto)}$ + Fru-1,6-$P_{2(diol)}$] (a reasonable assumption in view of the broad specificity of the enzyme (Rutter, 1961) and the similarity of the compounds), the data in Table I show that V_{max}/K_m ratio is 50-fold higher for (keto + diol) than for (keto + diol + α and β anomers). The improvement in V_{max}/K_m expected if the anomeric forms were totally inactive would be [Fru-1,6-$P_{2(total)}$]/(Fru-1,6-$P_{2(keto)}$ + Fru-1,6-$P_{2(diol)}$] = (100%)/(2.0% + 1.3%) = 30-fold. An analysis of V_{max}/K_m for a substrate containing inactive forms compared with pure substrate shows them to be related in this way (Rose, 1966). Therefore, the kinetic properties of the 5-d-Fru-1,6-P_2 are well in accord with the hypothesis that the keto isomer of Fru-1,6-P_2 functions as the true substrate form.

A number of rapid kinetic studies have suggested that the β anomer of Fru-1,6-P_2 is an active substrate for muscle aldolase. The maximal rate for the rapid phase involving aldol cleavage of the first 70–80% of the substrate pool has been found to be 7 s^{-1} (Wurster and Hess, 1973), 8 s^{-1} (Grazi, 1974), and ~20 s^{-1} (Schray et al., 1976). The data presented here show that the unhindered $k_{\beta 0}$ is higher than these rates. Therefore, as has been considered above, the uncatalyzed ring-opening step is able to renew the keto form rapidly enough to keep up with aldolase cleavage. In the experiments cited it is necessary to assume that the excess of enzyme over substrate made the rate of ring opening from free β anomer insufficient to explain the observed rates in order to require that the β anomer is also a substrate. In the absence of direct information on the binding constants of the anomers it is difficult to assess the percentages of enzyme-bound and free anomers under conditions of excess enzyme. The observation of a biphasic curve in the product vs. time curves is not inconsistent with reaction proceeding from the free β and α forms by spontaneous ring opening. Therefore, the question of the substrate activity of β-Fru-1,6-P_2 remains unanswered.

A reasonable hypothesis to explain the data obtained at high enzyme/substrate ratios is to assume that ring opening of the β anomer can occur on the enzyme. For example, the anomer could be bound in a conformation which allows intramolecular phosphate catalysis of ring opening. The low value (0.5 s^{-1}) for the second phase of the rapid kinetic data could represent ring opening of the α anomer on the enzyme which in this case would be more than an order of magnitude slower than the spontaneous rate (8.1 s^{-1}).

With respect to the yeast aldolase, 5-d-Fru-1,6-P_2 is the only alternative substrate that has been found to date, with a 30-fold lower K_m than Fru-1,6-$P_{2(total)}$ but a lower V_{max} than the natural substrate. Since one expects the aldol cleavage of Fru-1,6-P_2 to pass through an enzyme-keto intermediate, dFru-1,6-P_2 as an analogue of Fru-1,6-$P_{2(keto)}$ should have no lower V_{max} than Fru-1,6-$P_{2(total)}$ but instead would have a higher V_{max} by the factor of the equilibrium constant for the (enzyme-anomer) $\rightleftharpoons$ (enzyme-keto) reaction. The low V_{max} must signify that the 5-OH is important in determining the cleavage rate of Fru-1,6-P_2 by the yeast enzyme. Schray et al. (1976) interpreted their rapid kinetic studies to exclude a major substrate role of the acyclic form. However, the rate of spontaneous anomerization that was used in their calculations is now known to be much too low, and the cleavage rates they observed were close to those expected for the keto form with a K_m of 10 μM and a concentration of 2% of the total Fru-1,6-P_2.

The suggestion that a minor isomeric form present only in ~2% in equilibrium with the other forms is exclusively used by an enzyme is not without precedent. Aldolase, glyceraldehyde-P-dehydrogenase, and triose-P-isomerase use the aldehyde form of glyceraldehyde-3-P exclusively, although it makes up only 3% of the total and the rate of its production from the diol is quite slow, 0.08 s^{-1} (Trentham et al., 1969).

It should be pointed out that another ketohexosephosphate aldolase, 2-keto-3-deoxy-6-phosphogluconate aldolase, has been found to be specific for the keto form of the substrate. In this case, the turnover number of the enzyme (150 s^{-1}) is far greater than the spontaneous ring-opening rate (3–5 s^{-1}), which sets the upper limit to the steady-state rate at high enzyme concentrations where the keto form is trapped by the enzyme as soon as it is formed nonenzymatically from the anomers (C. F. Midelfort, H. P. Meloche, and R. K. Gupta, unpublished results).

Acknowledgments

The authors acknowledge the generous cooperation of Dr. A. S. Mildvan in the use of the NMR equipment and of Dr. S. J. Benkovic in providing the inactive Fru-1,6-P_2 analogues.

References

Bailey, J. M., Fishman, P. H., and Pentchev, P. G. (1970), *Biochemistry 9,* 1189.

Ballou, C. (1960), *Biochem. Prep. 7,* 45.

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

Benkovic, P. A., Bullard, W. P., deMain, M., Fishbein, R., Schray, K. J., Steffens, J. J., and Benkovic, S. J. (1974), *J. Biol. Chem. 249,* 930.

Benkovic, S. J., Engle, J. L., and Mildvan, A. S. (1972), *Biochem. Biophys. Res. Commun. 47,* 852.

Castellino, F. J., and Barker, R. (1965), *Biochem. Biophys. Res. Commun. 23,* 182.

Ernst, R. E. (1966), *Adv. Magn. Reson. 2,* 1.

Evans, P. G., Miller, G. R., and Kreevoy, M. M. (1965), *J. Phys. Chem. 69,* 4325.

Franklin, N. C., and Feltkamp, H. (1965), *Angew. Chem., Int. Ed. Engl. 4,* 774.

Fung, C. H., Mildvan, A. S., Allerhand, A., Kaworoski, R., and Scrutton, M. C. (1973), *Biochemistry 12,* 620.

Ginsburg, A., and Mehler, A. H. (1966), *Biochemistry 5,* 2623.

Gray, G. R. (1971), *Biochemistry 10,* 4705.

Gray, G. R., and Barker, R. (1970), *Biochemistry 9,* 2454.

Grazi, E. (1974), *Biochem. Biophys. Res. Commun. 59,* 450.

Gupta, R. K., Pitner, T. P., and Wasylishen, R. (1974), *J. Magn. Reson. 13,* 383.
Gutowsky, M. S., and Saika, A. (1953), *J. Chem. Phys. 21,* 1688.
Hartman, F. C., and Barker, R. (1965), *Biochemistry 4,* 1068.
Koerner, T. A. W., Cary, L. W., Bhacca, N. S., and Younganathan, E. S. (1973), *Biochem. Biophys. Res. Commun. 51,* 543.
Los, J. M., Simpson, L. B., and Wiesner, K. (1956), *J. Am. Chem. Soc. 78,* 1564.
Mehler, A. H., and Cusic, M. E. (1967), *Science 155,* 110.
Orsi, B. A., and Cleland, W. W. (1972), *Biochemistry 11,* 102.
Putman, S. J., Coulson, A. F. W., Farley, I. R. T., Riddleston, T., and Knowles, J. R. (1972), *Biochem. J. 129,* 301.
Racker, E. (1947), *J. Biol. Chem. 167,* 843.
Reynolds, S. J., Yates, C. W., and Pogson, C. I. (1971), *Biochem. J. 122,* 285.
Rose, I. A. (1966), *Annu. Rev. Biochem. 35,* 23.
Rose, I. A., and O'Connell, E. L. (1969), *J. Biol. Chem. 244,* 6548.
Rutter, W. J. (1961), *Enzymes, 2nd Ed., 5,* 341.
Rutter, W. J., Hunsley, J. R., Groves, W. E., Calder, J., Rajkumar, T. Y., and Woodfin, B. M. (1966), *Methods Enzymol. 9,* 479.
Schray, K. J., Benkovic, S. J., Benkovic, P. A., and Rose, I. A. (1973), *J. Biol. Chem. 248,* 2219.
Schray, K. J., Fishbein, R., and Benkovic, S. J. (1976), *J. Biol. Chem.* (in press).
Stoddart, J. F. (1971), Stereochemistry of Carbohydrates, New York, N.Y., Wiley, Chapter 5.
Swenson, C. A., and Barker, R. (1971), *Biochemistry 10,* 3151.
Trentham, D. R., McMurray, C. H., and Pogson, C. I. (1969), *Biochem. J. 114,* 19.
Wolf, S. (1972), *Acc. Chem. Res. 5,* 102.
Wurster, B., and Hess, B. (1973), *Biochem. Biophys. Res. Commun. 55,* 985.

A Novel Phosphodiesterase from Cultured Tobacco Cells†

Hideaki Shinshi,* Masanao Miwa, Kunio Kato, Masao Noguchi, Taijiro Matsushima, and Takashi Sugimura

ABSTRACT: A novel phosphodiesterase was purified from cultured tobacco cells to a state which appeared homogeneous on polyacrylamide gel electrophoresis. The enzyme hydrolyzed various phosphodiester and pyrophosphate bonds, including *p*-nitrophenyl thymidine 5′-phosphate, *p*-nitrophenyl thymidine 3′-phosphate, cyclic nucleotides, ATP, NAD^+, inorganic pyrophosphate, dinucleotides, and poly(adenosine diphosphate ribose), which is a polymer synthesized from NAD^+. However, it did not hydrolyze highly polymerized polynucleotides. The molecular weight of the native enzyme was estimated as 270 000 to 280 000 by gel filtration on Sephadex G-200 and Bio-Gel A-5m. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis indicated that the enzyme was composed of subunits with molecular weights calculated to be 75 000. The enzyme did not require divalent cations for activity being fully active in the presence of ethylenediaminetetraacetic acid. The pH optimum for the enzyme was approximately 6 with *p*-nitrophenyl thymidine 5′-phosphate or adenosine cyclic 3′,5′-monophosphate, and 5.3 with NAD^+. Double reciprocal plots of the initial velocity against the concentration of *p*-nitrophenyl thymidine 5′-phosphate gave two apparent K_m values of 0.17 and 1.3 mM, suggesting the presence of at least two active sites.

Although phosphodiesterases that hydrolyze nitrophenyl-pT[1] at acid pH are present in various plant tissues (Razzell, 1966), the enzyme has not so far been purified to determine substrate specificities and other properties.

An enzyme which hydrolyzes nitrophenyl-pT at acid pH is purified to homogeneity on gel electrophoresis from cultured tobacco cells. It is found that many phosphodiesters and pyrophosphate bonds, including cyclic nucleotides, ATP, NAD^+, sodium pyrophosphate, and dinucleotides, are hydrolyzed by the enzyme. Its substrate specificity and enzymological properties are entirely different from those of all other plant and animal phosphodiesterases and pyrophosphatases previously reported. This paper describes the purification and properties of this enzyme.

Experimental Procedure

Plant Material. Cells of tobacco (*Nicotiana tabacum,* cultivar, Bright Yellow 2) were cultured in suspension in the basal medium of Linsmaier and Skoog (1965) supplemented with 1 mg of thiamin-HCl, 0.2 mg of 2,4-dichlorophenoxyacetic acid, and 30 g of sucrose per liter. Cultures were incubated in the dark at 28 °C on a rotary shaker operating at 90

† From the Department of Molecular Oncology, Institute of Medical Science, University of Tokyo, Minato-ku, Tokyo-108 (H.S., M.M., T.M., and T.S.), and The Central Research Institute, Japan Tobacco and Salt Public Corporation, 6-2 Umegaoka, Midori-ku, Yokohama, Kanagawa-227 (H.S., K.K., and M.N.), Japan. *Received December 12, 1975.* This work was supported in part by grants from the Ministry of Education, Science and Culture and Waksman Foundation, Japan.

* Present address: The Central Research Institute, Japan Tobacco and Salt Public Corporation.

[1] Abbreviations used are: nitrophenyl-pT, *p*-nitrophenyl thymidine 5′-phosphate; Tp-nitrophenyl, *p*-nitrophenyl thymidine 3′-phosphate; ADP-Rib, adenosine diphosphate ribose; poly(ADP-Rib), polymer of ADP-Rib synthesized from NAD^+; ppGpp, guanosine 3′-diphosphate-5′-diphosphate; NAD, nicotinamide adenine dinucleotide; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl; ATP, adenosine 5′-triphosphate; AMP, adenosine 5′-monophosphate; TTP, thymidine 5′-triphosphate; UTP, uridine 5′-triphosphate; CTP, cytidine 5′-triphosphate; GTP, guanosine 5′-triphosphate; NMN, nicotinamide mononucleotide; NADH, reduced nicotinamide adenine dinucleotide; NADPH, reduced nicotinamide adenine dinucleotide phosphate.