Catalysis of an Oxygen-Exchange Reaction of Fructose 1,6-Diphosphate and Fructose 1-Phosphate with Water by Rabbit Muscle Aldolase*

Peter Model,† Laura Ponticorvo, and David Rittenberg

ABSTRACT: Rabbit muscle aldolase catalyzes an exchange of the carbonyl oxygens of fructose 1,6-diphosphate (FDP) and fructose 1-phosphate (F-1-P) with the oxygens of H_2O. The rates of these exchanges, measured under conditions such that the substrates are in equilibrium with the triose phosphates which are the products of the cleavage reaction, exceed those of the initial rate of the cleavage reaction in the absence of triose or triose phosphates. The aldolase-catalyzed exchange of [2-^{18}O]F-1-P proceeds at 3% of the rate of [2-^{18}O]FDP. The rate of exchange of [2-^{18}O]FDP is not appreciably affected when carboxypeptidase-treated aldolase was used in lieu of native aldolase, although the initial rate of the cleavage reaction is diminished by a factor of 20.

Treatment of the native enzyme with dihydroxyacetone phosphate and borohydride causes a parallel loss of FDP exchange and cleavage activities. Xylitol 1,5-disphosphate, an inhibitor of the cleavage reaction, also inhibits the exchange reactions of [2-^{18}O]FDP and [2-^{18}O]F-1-P. These results are consistent with the formation of a Schiff base between the substrates and the enzyme. In view of the much slower rate of exchange of [2-^{18}O]F-1-P than of [2-^{18}O]FDP, it is suggested that the rate of cleavage of F-1-P is limited by the rate of formation of a Schiff base. The rates of the nonenzymic exchanges of the ketonic oxygens of the following compounds were measured at 25° and pH 7. The pseudo-first-order rate constants for the exchanges, $k(H_2O)$, are: dihydroxyacetone phosphate, $> 700 \times 10^{-3}$ min^{-1}; dihydroxyacetone, 200×10^{-3} min^{-1}; FDP, 24×10^{-3} min^{-1}; F-1-P, 4.2×10^{-3} min^{-1}; fructose, $<0.2 \times 10^{-3}$ min^{-1}. We propose that a phosphate monoanion α to a carbonyl function can act as a bifunctional acidic and basic catalyst for the hydration–dehydration of a free keto group, and also for the conversion of a hemiketal into the free keto form. The chief effect of phosphorylation at C-6 of fructose (or F-1-P) appears to be to increase the concentration of free ketone. Thus phosphorylation of fructose seems to affect the reactivity of the molecule in addition to strengthening its binding to aldolase.

Aldolase, as well as a number of other enzymes which catalyze carbonyl condensation and cleavage reactions, is inactivated by borohydride in the presence of substrate (Horecker *et al.*, 1963; Rose, 1966). The nature of the products of this reduction suggests that a Schiff base is reduced by the borohydride; the specificity of the inactivation suggests that such a Schiff base is an integral part of the reaction mechanism. This hypothesis predicts that these enzymes catalyze a transfer of the carbonyl oxygen of the substrate to water. Such a transfer has been demonstrated for acetoacetate decarboxylase (Hamilton and Westheimer, 1959) and, recently, for 2-keto-3-deoxy-6-phosphogluconate aldolase (Rose and O'Connell, 1967). We have studied the aldolase-catalyzed loss of ^{18}O from [2-^{18}O] fructose 1,6-diphosphate (FDP)[1] and [2-^{18}O]fructose 1-phosphate (F-1-P) with native and modified rabbit muscle aldolase, both to test the hypothesis of an intermediate Schiff base and to find further evidence on the mechanism of action of the enzyme.

Some experiments designed to determine whether the loss of ^{18}O from [2-^{18}O]FDP is an *obligatory* accompaniment of the cleavage reaction failed because of the rapid exchange with water of the carbonyl oxygen of dihydroxyacetone phosphate (DHAP). The result of a similar experiment has recently been reported by Rose and O'Connell (1967) with 2-keto-3-deoxy-6-phosphogluconate aldolase. The authors report that essentially all ^{18}O at the carbonyl is lost when the hexose phosphate is cleaved to triose.

Materials and Methods

Rabbit muscle aldolase was obtained from Boehringer & Sohne, or was prepared according to the

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† Present address: The Rockefeller University, New York, N. Y.

[1] Abbreviations used in this paper that are not defined in *Biochemistry 5*, 1445 (1966), are: FDP, fructose 1,6-diphosphate; F-1-P, fructose 1-phosphate; DHA, dihydroxyacetone; DHAP, dihydroxyacetone phosphate; BSA, bovine serum albumin.

method of Taylor *et al.* (1948), with three recrystallizations. These preparations contained traces of triosephosphate isomerase. Carboxypeptidase-treated aldolase was prepared by the method of Rose *et al.* (1965). The α-glycerophosphate derivative of aldolase was prepared by a small-scale adaptation of the method of Lai *et al.* (1965). Aldolase (10.8 mg) together with 28 μmoles of DHAP in 1 ml of water was adjusted to pH 6 by the addition of 0.1 M NaOH or 4×10^{-3} M acetic acid, as required. $NaBH_4$ (0.3 ml of 0.1 M) was added alternately with 4×10^{-3} M acetic acid so that the pH was maintained at 6 ± 0.2. Ten minutes after the last addition of $NaBH_4$, a further 28 μmoles of DHAP was added and the cycle was repeated. The solution was stirred with a magnetic stirrer throughout. After the reduction, the foam was removed and the solution was dialyzed overnight against 2 l. of 0.02 M sodium phosphate buffer–10^{-3} M EDTA (pH 7). After dialysis the solution was clarified by centrifugation at 5000 rpm. For the control preparation, no DHAP was added before reduction. Aldolase and its derivative were estimated spectrophotometrically at 280 mμ (Baranowski and Niederland, 1949).

α-Glycerophosphate dehydrogenase and F-1-P were purchased from Boehringer & Sohne. One lot of F-1-P was purified further by conversion of the barium into the dicyclohexylammonium salt, recrystallized twice from acetone–water, and then converted into the sodium salt. The rates of the exchange and cleavage reactions were not affected by such further purification of the substrate. Triosephosphate isomerase, FDP, DPN^+, DPNH, and DHAP were obtained from Sigma. DHA (C grade) was purchased from Calbiochem, and was further purified by the method of Reeves and Renbom (1931). Xylitol 1,5-diphosphate was the gift of Dr. Robert Barker. Carboxypeptidase treated with diisopropyl fluorophosphate was obtained from Worthington.

[2-^{18}O]FDP and [2-^{18}O]F-1-P were prepared by exchange with [O^{18}]H_2O (obtained from Yeda) at neutral pH. The [^{18}O]H_2O was recovered by lyophilization in a closed system. The residue was dissolved in a large volume of cold [^{16}O]H_2O, the pH was quickly adjusted to 7, the solution was frozen, and the water was again removed *in vacuo*. This lowered the ^{18}O concentration of any adhering water to negligible levels. These substrates were analyzed for the concentration of ^{18}O by oxidation to CO_2 by a mixture of $HgCl_2$–$Hg(CN)_2$ (5:1) at 500° (Rittenberg and Ponticorvo, 1956; Anbar and Guttman, 1959) and subsequent analysis of the CO_2 in a mass spectrometer.

The concentrations of FDP and DHAP in solution were measured by enzymatic assay with aldolase, triosephosphate isomerase (for FDP), α-glycerophosphate dehydrogenase, and DPNH. F-1-P was determined by the colorimetric assay of Roe *et al.* (1949) as modified by Hers *et al.* (1953), as well as by release of phosphate in 20 min at 100° in 1 N HCl. Phosphate was determined by the method of Ames and Dubin (1960).

The assay for aldolase was based on the estimation of triose phosphates formed from FDP or F-l-P in the presence of limiting quantities of aldolase by conversion into α-glycerophosphate in the presence of excess triosephosphate isomerase and α-glycerophosphate dehydrogenase. The rate of oxidation of DPNH was determined at 340 mμ in either a Cary Model 14 or a Gilford Model 2000 spectrophotometer, both with thermostated cell compartments. All measurements were made at 25°. All substrates, assay enzymes, etc., were made up in 0.02 M sodium phosphate buffer (pH 7) containing 0.1% bovine serum albumin and 10^{-3} M EDTA. Phosphate, while a competitive inhibitor of the aldolase reaction, was used to avoid the necessity of studying the effects of other buffers on the nonenzymatic exchange of the various ^{18}O-labeled substrates. The assay mixtures contained, other than the buffer described above, 50 or 100 μg per ml of α-glycerophosphate dehydrogenase, 5 or 10 μg per ml of triosephosphate isomerase, $1–2 \times 10^{-4}$ M DPNH, FDP at 10^{-3} M unless otherwise specified, and an appropriate amount of aldolase. The aldolase concentration was routinely varied over a fivefold range to ensure that the assay enzymes were not limiting. Rates are expressed as micromoles of hexose cleaved per minute per milligram of enzyme protein. Each mole of FDP cleaved was assumed to cause the oxidation of 2 moles of DPNH, each mole of F-1-P, 1 mole of DPNH. The molar extinction of DPNH at 340 mμ was taken to be 6220 (Kaplan, 1960).

Exchange of ^{18}O Substrates with H_2O. FDP, F-1-P, Fructose. To 100 μl of the buffer described above at 0°, containing when appropriate a suitable amount of aldolase, $1–3 \times 10^{-5}$ mole of the solid ^{18}O-labeled substrate was added. After mixing, 10 μl was removed, and the remainder was placed in a 25° water bath. Four to five 10-μl samples were taken at various times thereafter, the last at a time calculated to allow complete exchange. The samples were placed in small tubes fitted with $^{10}/_{30}$ joints, capped at once with greased, ground-glass stoppers or parafilm, and quickly frozen in a solid CO_2–ethanol bath. When all of the samples had been collected, each was evacuated while still frozen, and then sublimed, in a very short path length, small closed system, onto 300 mg of a 5:1 mixture of $HgCl_2$–$Hg(CN)_2$, cooled in solid CO_2–acetone. The mixture of water and $HgCl_2$–$Hg(CN)_2$ was contained in 8-mm Pyrex tubes with breakseals at one end. Sublimation took 3–5 min and the water remained frozen throughout. The tubes containing the water and the $HgCl_2$–$Hg(CN)_2$ mixture were sealed while still *in vacuo* and then heated to 450° for 2 hr in order to generate CO_2. The CO_2 in the tubes was transferred to the gas line of a mass spectrometer as previously described (Rittenberg and Ponticorvo, 1956) and the ^{18}O concentration in the CO_2 was measured. When the ^{18}O concentration of the remaining substrate, either FDP or fructose, was to be determined, the solvent used was H_2O or 10^{-3} M phosphate buffer. The reaction volume was increased to 1 ml and the substrate amount was adjusted accordingly (100–300 μmoles). The samples (0.1 ml) were placed into tubes with breakseals. After lyophilization the samples were dried *in vacuo* over P_2O_5 at 61° for 4 hr. The $HgCl_2$–$Hg(CN)_2$ mixture (300 mg) was then added, the tubes were evacuated, sealed, and heated to 500° for 3 hr, and the CO_2 was analyzed.

All glassware that was to be in contact with the samples was heated to 180° for at least 1 hr, cooled, and stored in a desiccator. The parafilm and $HgCl_2$–$Hg(CN)_2$ were dried *in vacuo* at room temperature over P_2O_5. The combustion tubes were heated to 500° for 0.5 hr and stored over P_2O_5 thereafter.

Spectrophotometric Determination of the Exchange Reaction of DHAP and DHA. A solution containing about 10 mg of DHAP was adjusted to pH 7 with 0.01 N NaOH, placed in a conical test tube, and reduced in volume at 30° *in vacuo* to about 0.1 ml, D_2O was added, and the volume was reduced again. This was repeated, and then the mixture was reduced to a syrup, but not to dryness. [^{18}O]D_2O (50 μl) (approximately 60 atom % ^{18}O excess), prepared by exchanging [^{18}O]H_2O with D_2 gas in the presence of platinum oxide, was added and the mixture was quickly transferred to a CaF_2 cell, path length 0.05 mm, and scanned repeatedly in a Perkin-Elmer Model 21 spectrophotometer in the range from 5.5 to 6.5 μ against a D_2O blank. At the conclusion of the exchange a difference spectrum was run between the sample and another sample, treated in the same fashion, to which D_2O had been added rather than [^{18}O]D_2O.

For the DHA-exchange measurement, 50 μl of 0.05 M sodium phosphate (pH 7.0) buffer, previously made up in D_2O, was dried in a small tube. Crystalline DHA (7 mg) was added, and, at $t = 0$, 50 μl of [^{18}O]D_2O (approximately 50 atom % ^{18}O excess). All further procedures were as for DHAP. Since solid DHA may exist as the dimer (Bell and Baughan, 1937) both DHAP and DHA were examined in the ultraviolet and infrared immediately after mixing with phosphate buffer. No differences were found between the spectra of DHAP or of DHA immediately after mixing and some hours thereafter. Early and late spectra of DHA in distilled water, however, do exhibit differences in the intensities of the carbonyl bands.

Enzymatic Formation of Glycerophosphate from FDP or DHAP. Aldolase (10 mg) (Boehringer), 4 mg of α-glycerophosphate dehydrogenase, and 200 μmoles of DPNH were made to 100 ml in 10^{-3} M EDTA (pH 7) and adjusted to pH 7. The solution at room temperature (24°) was stirred with a magnetic stirrer, and the pH was monitored with a Beckman Model G pH meter. The reaction was started by the addition of 165 μmoles of [2-^{18}O]FDP (0.5 atom % excess), and the pH was held to 7 by the addition of 0.1 N HCl. After 10 min the solution was made 1 N with respect to perchloric acid, chilled in an ice bath, and after 15 min filtered through a fluted filter and neutralized with 10 N KOH. The $KClO_4$ was removed by filtration, and the supernatant was reduced to 10 ml on a rotary evaporator at 30°. Newly crystallized $KClO_4$ was filtered, and the filtrate was applied to a Dowex 1 (formate) column of 13-ml bed volume. The column was eluted with an exponential gradient of formic acid, made by allowing 500 ml of 6 N formic acid to flow into 300 ml of water with stirring. Fractions of 5 ml were taken and sampled for organic phosphate. Fractions containing organic phosphate at about the eluent volume corresponding to that at which authentic α-glycerophosphate comes off the column were pooled and reduced in volume on a rotary evaporator at low temperature. Water was added and the volume reduced again. This was repeated until the odor of formic acid could no longer be detected. The final volume was about 1 ml. $Ba(OH)_2$ was added to pH 8–10. The excess barium was precipitated with CO_2, the solution was filtered, and the volume of the filtrate was further reduced to about 0.1 ml. The barium salt of α-glycerophosphate was precipitated with four volumes of ethanol and dried over P_2O_5 *in vacuo*. An aliquot was converted into the sodium salt with an excess of Na_2SO_4 and the *l*-α-glycerophosphate concentration of the resulting solution determined enzymatically with α-glycerophosphate dehydrogenase at high pH in the presence of hydrazine (Hohorst, 1963). The ^{18}O concentration of the remaining barium α-glycerophosphate was determined.

For the reduction of [2-^{18}O]DHAP in [^{16}O]H_2O the same procedure was followed, except that 120 μmoles of lyophilized DHAP was allowed to stand for 4 hr in 1 ml of 1.5 atom % excess [^{18}O]H_2O, and aldolase was omitted from the reaction mixture. The reaction was started by adding the DHAP in the [^{18}O]H_2O to the 99 ml of [^{16}O]H_2O reaction mixture.

For the reduction of [^{16}O]DHAP in [^{18}O]H_2O, the components of the reaction mixture were dissolved in 1.5 ml of [^{18}O]H_2O, and the reaction was started by the addition of 120 μmoles of DHAP. After precipitation with $HClO_4$, filtration, and neutralization, the supernatant was applied directly to the Dowex 1 (formate) column.

Calculation of Rate Constants. THE NONENZYMATIC EXCHANGE. The exchange reaction is formulated below as a reversible bimolecular reaction.

$$S^{18} + W^{16} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} S^{16} + W^{18} \qquad (1)$$

where S^{18} = moles per liter of ^{18}O-labeled substrate, S^{16} = moles per liter of ^{16}O-labeled substrate, W^{18} = moles per liter of [^{18}O]water, and W^{16} = moles per liter of [^{16}O] water. We define $S = S^{16} + S^{18}$, $W = W^{16} + W^{18}$, $f = W^{18}/W$ = atom fraction ^{18}O excess in the water at any time (t), $g = S^{18}/S$ = atom fraction ^{18}O in the exchangeable oxygen of the keto group at any time (t), and f^∞ and g^∞ the respective values of f and g at equilibrium. We assume that $k_1 = k_{-1} = k$, *i.e.*, that there is a negligible isotope effect in the exchange reaction. This is formally equivalent to the requirement that

$$f^\infty = g^\infty \qquad (2)$$

Conservation of ^{18}O in the reaction requires that

$$fW + gS = f^\infty W + g^\infty S = \text{constant} \qquad (3)$$

It is now possible to formulate in differential form the rate of the reaction as

$$\frac{1}{W}\frac{df}{dt} = k(f^\infty - f)\left(1 + \frac{S}{W}\right) \qquad (4)$$

In these experiments S/W varies between 0.002 and 0.005. Neglecting S/W with respect to 1 yields

$$\frac{1}{W}\frac{df}{dt} = k(f^\infty - f) \tag{5}$$

Integration for the case in which $f = 0$ at $t = 0$ yields

$$\ln\left(1 - \frac{f}{f^\infty}\right) = -kWt \tag{6}$$

This equation is suitable for evaluating k in experiments starting with an ^{18}O-labeled substrate and $[^{16}O]H_2O$. In a system starting with $[^{18}O]H_2O$ and unlabeled substrate the appropriate equation may easily be derived in similar fashion and leads to

$$\ln\left(1 - \frac{g}{g^\infty}\right) = -kWt \tag{7}$$

THE ENZYMATIC EXCHANGE. Consider an enzymatic reaction leading to the exchange of S^{18} with W^{16}. Let the rate of reaction, at some given substrate concentration, be v_e, and assume that this exchange involves the participation of only one molecule of substrate and one of water. Then the rate of appearance of ^{18}O in the water will be

$$W\frac{df}{dt} = Ev_e[g(1 - f) - (1 - g)f] \tag{8}$$

which reduces to

$$W\frac{df}{dt} = Ev_e(g - f) \tag{9}$$

where E is the total enzyme concentration in milligrams per milliliter and v_e is the rate of the enzymatic reaction in moles per minute per milligram of enzyme.

Substituting for g from eq 3

$$W\frac{df}{dt} = Ev_e\left[\left(\frac{W}{S} + 1\right)(f^\infty - f)\right] \tag{10}$$

In these experiments W/S varies between 200 and 500, and we neglect 1 in comparison with W/S. Then dividing both sides by W yields

$$\frac{df}{dt} = \frac{Ev_e}{S}(f^\infty - f) \tag{11}$$

Adding the contribution of the nonenzymatic exchange gives

$$\frac{df}{dt} = \left(\frac{Ev_e}{S} + kW\right)(f^\infty - f) \tag{12}$$

Integrating, and noting that at $t = 0, f = 0$

$$\ln\left(1 - \frac{f}{f^\infty}\right) = -t\left(\frac{Ev_e}{S} + kW\right) \tag{13}$$

$$v_e = -\left[\frac{1}{t}\ln\left(1 - \frac{f}{f^\infty}\right) + kW\right]\frac{S}{E} \tag{14}$$

Results

The Rates of the Nonenzymatic Exchange of ^{18}O-Labeled Substrates. The data in Table I show that the carbonyl oxygen of fructose is much more stable to exchange with water than that of F-1-P, which in turn is more stable than that of FDP. Of five experiments measuring the rate of exchange of $[2\text{-}^{18}O]$FDP with the medium, four were concerned with the appearance of ^{18}O in the medium. The values of kW were calculated from eq 6. In a fifth experiment kW was calculated using eq 7 from the measurements of the ^{18}O remaining in the substrate. This last rate agreed well with the results of the other four. After 280 min only 3% of the ^{18}O in $[2\text{-}^{18}O]$fructose had appeared in the water, and

TABLE I: Rates of Nonenzymic Exchange of ^{18}O-Labeled Compounds with $H_2{}^{16}O$.[a]

Compound	$t_{1/2}$ (min)	kW (min^{-1}) $\times 10^3$
$[2\text{-}^{18}O]$FDP[b]	29.5 ± 1.95 (std dev n = 5)	23.5
$[2\text{-}^{18}O]$F-1-P[c]	166	4.2
$[2\text{-}^{18}O]$Fructose[d]	>3600	<0.2
$[2\text{-}^{18}O]$DHAP[e]	<1	>693
$[2\text{-}^{18}O]$DHA[f]	3.2	220

[a] All exchange measurements at pH 7, experimental details in Materials and Methods. [b] At 25°. Four measurements for the appearance of ^{18}O in H_2O, one for the disappearance of ^{18}O from the $[2\text{-}^{18}O]$FDP. Media were water (two measurements) or 0.02 M phosphate buffer containing 0.1% BSA and 10^{-3} M EDTA. ^{18}O concentration in the starting FDP ranged from 0.5 to 4.9 atom % ^{18}O excess (for the whole molecule). [c] At 25°, in 0.02 M phosphate buffer containing 0.1% BSA and 10^{-3} M EDTA. Measurement was for the appearance of ^{18}O in H_2O. Initial ^{18}O concentration in the F-1-P was 3.6 atom % ^{18}O excess. [d] At 25° in 10^{-3} M phosphate buffer (pH 7). Simultaneous measurement of appearance of ^{18}O in water and disappearance from $[2\text{-}^{18}O]$fructose. [e] At 23°, in $[^{18}O]D_2O$, about 60 atom % ^{18}O excess. Measured by infrared spectroscopy. [f] At 23° in $[^{18}O]D_2O$–0.05 M phosphate buffer, about 50 atom % ^{18}O excess. Measured by infrared spectroscopy.

TABLE II: Aldolase-Catalyzed Exchange of [2-^{18}O]FDP and [2-^{18}O]F-1-P.[a]

Preparation	k_{app}[b] ($\times 10^2$)	($k_{app} - kW$)[c] ($\times 10^2$)	S (moles/l.)	E (g/l.)	Rate of Exchange,[d] v_e (μmoles/min per mg of enzyme)	Rate of Cleavage[e] (μmoles/min per mg of enzyme)	Rate of Exchange / Rate of Cleavage
				FDP			
Native aldolase[g]	7.2	4.8	0.23	0.40	28	7.5 ± 0.4 std dev	3.7
Native aldolase[f]	9.7	7.3	0.10	0.17	43		
	4.9	2.6	0.14	0.093	38		
	10.1	7.8	0.21	0.34	47		
	7.7	5.3	0.24	0.36	35		
	6.4	4.1	0.33	0.34	39		
					Mean 40.4	12.2 ± 0.3 std dev	3.3
CP aldolase[g]	6.4	4.1	0.23	0.40	24	0.36	67
CP aldolase[h]	5.7	3.4	0.26	0.32	27	0.68	40
Native aldolase[f] + xylitol 1,5-diP							
0.093 M	2.1	−0.24	0.13	0.093	0		
0.120 M	3.0	0.6	0.16	0.17	5.6		
DHAP–$NaBH_4$	2.3	−0.1	0.15	0.18	0	0.15	
aldolase[i]	3.3	0.9	0.22	0.34	5.9	1.7	3.5
$NaBH_4$ aldolase[j]	8.3	5.9	0.22	0.34	38	11.4	3.4
				F-1-P			
Native aldolase[f]	4.6	4.2	0.25	10	1.02		
	2.5	2.0	0.25	5.1	0.99		
	0.6	0.18	0.26	0.39	1.15		
	2.4	2.0	0.26	6.0	0.88		
					Mean 1.01	0.53[k]	1.9
Native aldolase[f] + xylitol 1,5-diP							
0.027 M	0.95	0.53	0.25	5.1	0.26		
0.016 M	1.1	0.65	0.23	10	0.15		

[a] See Materials and Methods for experimental details. ^{18}O concentrations of substrates used: FDP 4.9 and 3.6 atom % ^{18}O excess, F-1-P 5.2 and 6.0 atom % ^{18}O excess. [b] $k_{app} = 0.693/t_{1/2}$ for the reaction. [c] kW is defined by eq 6. The value for FDP is 23.5×10^{-3}, for F-1-P 4.2×10^{-3} (see Table I). [d] Explanation of v_e is given in Materials and Methods. v_e is calculated from eq 14. [e] Commercial enzyme. [f] Enzyme freshly prepared from rabbit muscle. [g] Commercial enzyme treated with carboxypeptidase (see Materials and Methods). [h] Freshly prepared enzyme treated with carboxypeptidase (see Materials and Methods). [i] Freshly prepared enzyme treated with DHAP and borohydride (see Materials and Methods). [j] Freshly prepared enzyme treated with borohydride alone (see Materials and Methods). [k] Calculated rate at 0.254 M F-1-P using average values of V_m and K_m obtained from three plots of $1/V$ *vs.* $1/S$. [l] Concentration of FDP in the standard assay 10^{-3} M; rates at this concentration were not different from those at 0.234–0.319 M for both native and carboxypeptidase-treated aldolase. For F-1-P, see note *k*.

after about 60 hr only 35% had been exchanged. Analysis of the residual fructose confirmed that it still retained its ^{18}O, and that these results were not due to adventitious dilution of the medium.

Attempts to measure the rate of exchange of the carbonyl oxygen of dihydroxyacetone phosphate by the technique used for the three compounds described above led to inconsistent results. We therefore turned to the use of infrared spectroscopy to measure this exchange. Halmann and Pinchas (1958) have shown that the carbonyl absorption in the infrared is shifted toward lower frequencies when ^{16}O is replaced with ^{18}O, and Byrn and Calvin (1966) have used this technique in a study of the exchange rates of a large number of ketones and aldehydes.

When [2-^{16}O]DHAP was quickly mixed with [^{18}O]-D_2O (H_2O is not clear in the required region) and examined about 100 sec later in the region between 5.5

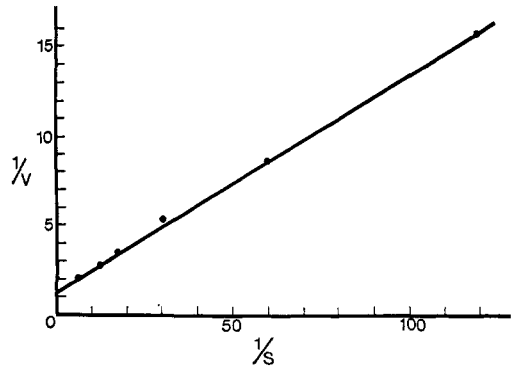

FIGURE 1: Reciprocal of the initial rates of cleavage of F-1-P as a function of the reciprocal of the substrate concentration. Rates were measured in 0.02 M phosphate buffer, 0.1% BSA, and 10^{-3} M EDTA as described in Materials and Methods. V is in moles per minute per milligram, S in moles per liter.

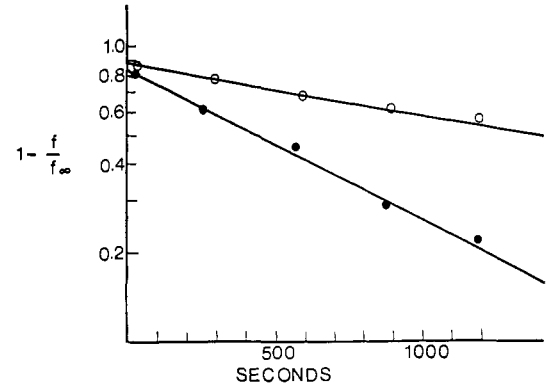

FIGURE 2: Rate of the nonenzymic (O—O—O) and aldolase-catalyzed (●—●—●) exchange of [2-^{18} O]FDP. f is the concentration of ^{18}O in the H_2O of the medium at time t, f^{∞} the concentration at equilibrium. For each experiment 10.7 mg of [2-^{18}O]FDP (4.9 atom % excess) was added to 100 μl of 0.02 M phosphate–0.1% BSA–10^{-3} M EDTA buffer (pH 7) and incubated at 25°. One of the tubes received, in addition, 44 μg of aldolase (commercial product, specific activity 7.5 units/mg). Samples (10 μl) were taken at the times indicated, and the water was analyzed for ^{18}O as described in Materials and Methods.

and 6 μ, the ordinarily sharp band at approximately 5.77 μ had been split into a doublet with a shoulder remaining at 5.77 μ but the most prominent absorption at approximately 5.87 μ. No change was noted over the next 30 min or so. A difference spectrum with DHAP in [^{16}O]D_2O showed a sharp spike at 5.75 μ and a valley at 5.87 μ, confirming that there had indeed been a decrease in absorption at the lower wavelength and an increase at the higher.

As a control, the same procedure was repeated with DHA in 0.05 M phosphate buffer (pH 7). Here the first scan at 110 sec still showed a sharp peak at the usual wavelength (5.77 μ) together with a small shoulder at 5.87 μ. The shoulder grew and the main peak diminished with time, the two having reached equilibrium about 10 min after the start of the reaction. Since the optical densities of the ^{16}O and ^{18}O peaks stood in about the same ratio as did the mole fraction of ^{16}O and ^{18}O in the water recovered from this reaction (as analyzed by mass spectrometry), it was assumed that at equilibrium the $^{18}O/^{16}O$ ratio of the oxygen of the carbonyl of DHA is about the same as that in water. This is in accord with the observations of Byrn and Calvin (1966) on most ketones and aldehydes. The half-life of the carbonyl oxygen was calculated to be 3.2 min (see Table I). We conclude, moreover, that the exchange of DHAP with the medium is too fast to be measured by this technique, which indicates that the carbonyl oxygen has a half-life, under these conditions, of less than 1 min.

Rates of the Enzymatic Cleavage Reactions. The initial rates of the cleavage of FDP by native and altered aldolase are shown in Table II. These measurements were made over the entire time course of the experiments, and it will be noted that the rates are quite reproducible. For the Boehringer enzyme the initial rates were also measured at higher FDP concentrations (0.28–0.32 M). Rates measured under these conditions were not different from those obtained with 10^{-3} M FDP for either the native or the carboxypeptidase-treated enzyme.

The initial rates of cleavage of F-1-P were studied over a range of substrate concentrations from 8.4×10^{-4} to 2.1×10^{-1} M. One such experiment is shown in Figure 1. The average apparent K_m from three experiments was $8.2 \pm 0.8 \times 10^{-2}$ M (std dev), and the average V_m, 0.70 ± 0.09 (std dev) μmole/min mg. The large value for the apparent K_m is due to the high phosphate concentrations used in the assay system. From these average K_m and V_m values, the initial rate at the average F-1-P concentration (0.254 M, range 0.25–0.26 M) used in the exchange experiments was calculated and used in Table II.

Aldolase-Catalyzed Exchange of [2-^{18}O]FDP and [2-^{18}O]F-1-P. Aldolase catalyzes a rapid exchange of the carbonyl oxygen of FDP with those of water (see Figure 2 and Table II). From the rate of appearance of ^{18}O in the water of the incubation medium, together with the rate of the uncatalyzed exchange of substrate, a rate for the enzymatically catalyzed exchange can be calculated, as described in Materials and Methods. This rate, for FDP, was found, within the limits of the experimental error, to be independent of the FDP concentration over the range from 0.103 to 0.326 M.

The rates of exchange of the carbonyl oxygens of both FDP and F-1-P are appreciably greater than the initial rates of the respective cleavage reactions under similar circumstances (see Table II). It should be noted that the initial rates of the cleavage reactions must be faster than the forward rate at equilibrium, since the products of the cleavage are not activators of the enzyme. Equilibrium in this system is reached very quickly, with the consumption of less than 5% of the substrate in the case of FDP, and less than that for F-1-P, as may be calculated from the value of the equilibrium constants found by Lehninger *et al.* (1955) and Rose *et al.* (1965). (Rose *et al.* have, in effect, measured the rates at equilibrium, but at concentrations different from those used here. Because of uncertainty

regarding the values of the relevant kinetic and equilibrium constants, their data cannot shed quantitative light on the rates that would obtain under these conditions.)

When carboxypeptidase-treated aldolase (Drechsler *et al.*, 1959) is used as the catalyst for the exchange of [2-^{18}O]FDP, the rate of ^{18}O exchange from FDP is not appreciably diminished even though the initial rate of the cleavage reaction is reduced to about 5% of its original value (Table II). This is consistent with the lack of effect of carboxypeptidase on glyceraldehyde 3-phosphate–FDP exchange (which includes the steps of ^{18}O exchange) shown by Rose *et al.* (1965).

When the potent competitive inhibitor of the cleavage reaction, xylitol 1,5-diphosphate (Hartman and Barker, 1965), is added to the system, the rate of exchange of both FDP and F-1-P are depressed, though the degree of inhibition cannot be simply related to the values of K_i and K_m for FDP and inhibitor determined in Barker's laboratory. With approximately equal concentrations of FDP and xylitol 1,5-diphosphate the rate of the enzymic component of the exchange fell, in one instance to zero, and in another to 13% of the rate of the uninhibited enzymic component. When xylitol 1,5-diphosphate was added to the system at about one-tenth the concentration of substrate F-1-P, the enzymic component of the rate of exchange decreased to 15–25% of the uninhibited rate. This is a smaller decrease than might have been expected from the reported K_m for F-1-P, 7×10^{-3} (Spolter *et al.*, 1965), and the K_i determined by Barker for inhibition of FDP cleavage (2.8×10^{-6}). Since phosphate, however, affects the apparent K_m of the enzyme toward F-1-P less than toward FDP (Spolter *et al.*, 1965) it may well be that it also inhibits the binding of F-1-P less than that of xylitol 1,5-diphosphate.

Treatment of aldolase with DHAP and $NaBH_4$ reduces the exchange and FDP-cleavage activities to the same extent (see Table II). Treatment with $NaBH_4$ alone has little or no effect on either of these activities. Aldolase at concentrations comparable to those used in the other exchange reactions has no effect on the exchange of [2-^{18}O]fructose.

Obligatory Loss of ^{18}O in the Cleavage Reaction. An attempt was made to determine whether ^{18}O is lost or conserved when [2-^{18}O]FDP is converted to α-glycerophosphate in the presence of aldolase, α-glycerophosphate dehydrogenase, and DPNH. This reaction was carried out at 10^{-3} M FDP so as to favor the formation of product. The α-glycerophosphate was isolated as the barium salt and assayed for its ^{18}O concentration. No ^{18}O was found in the product. When, however, a control incubation was performed in which [2-^{18}O]DHAP contained in a small volume of [^{18}O]H_2O was added to a large volume of normal water containing α-glycerophosphate dehydrogenase and DPHN, no ^{18}O was found in the α-glycerophosphate formed. The reciprocal experiment, the reduction of DHAP in [^{18}O]H_2O, resulted in the incorporation of approximately 1 g-atom of ^{18}O/mole of α-glycerophosphate. This experiment is consistent with our other experiments which suggest that the oxygen of DHAP is too labile to permit a determination of whether ^{18}O loss from [2-^{18}O]FDP is an obligatory accompaniment to the cleavage reaction.

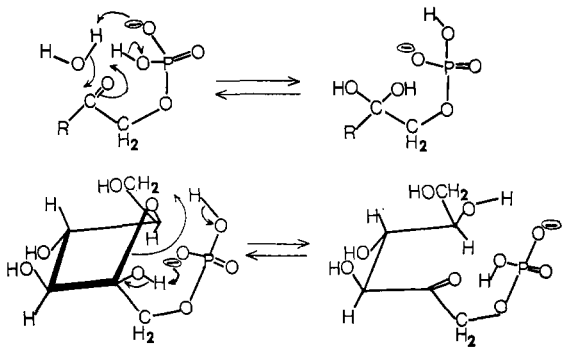

FIGURE 3: Facilitation of hydration–dehydration (top, A) or ring opening (bottom, B) by neighboring phosphate monoanion. R = CH_2OH in DHAP; R = CHOHCHOHCHOH-CH_2OH in F-1-P; and R = CHOHCHOHCHOHCH_2-OPO_3H in FDP.

Discussion

Nonenzymatic Exchange. The studies of the nonenzymatic exchange reactions, while undertaken primarily as controls, have some independent interest. The half-time for the exchange of acetone at pH 6.5 and 25° can be estimated from the data of Hamilton and Westheimer (1959) to be 10 min; that for DHA at 23° and pH 7 is 3.2 min (see Table I). The rate of reaction of DHAP is so rapid that we can give only a maximum value for the half-time (<1 min); the actual half-time is likely to be appreciably less than this. Phosphorylation at the C-1 of fructose appears also to greatly facilitate the exchange of oxygen at neutral pH, the rate of exchange of F-1-P being at least 22 times as great as that of fructose.

Oxygen exchanges at carbonyl functions are believed to occur by two similar mechanisms (Samuel and Silver, 1965). In acid solution, water is thought to add to the protonated carbonyl. In basic solution OH^- may first add to the unprotonated carbonyl. In either case an adduct is formed, the dissolution of which, by reversal, can lead to oxygen exchange. The exchange of the carbonyl oxygen of fructose probably proceeds by the same mechanism. Jencks (1964) points out that substitution reactions at saturated carbonyl derivatives almost invariably go through the free ketone, and the well-known stability of acetals and ketals to hydroxide ion are also indicative of this.

At pH 7, the phosphate radical α to the carbonyl group in DHAP, F-1-P, and FDP exists as a mixture of the mono- and dianion. Consideration of a three-dimensional model (see Figure 3A) of these compounds shows that for the free ketone forms, the acidic hydrogen of the monoanion may approach the carbonyl oxygen, while the O^- of the phosphoryl group can hydrogen bond to a hydrogen atom of a water molecule whose oxygen atom is close to the carbonyl carbon. Electron shifts can give the hydrated form of DHAP (or F-1-P or FDP) which is the intermediate in the exchange reaction.

A model of cyclic F-1-P (see Figure 3B) shows that the phosphate can catalyze the opening of the ring form.

In models of both the α and β isomers of the fructofuranoside and fructopyranoside the monoanion can exist in a conformation in which the O^- of the phosphoryl group is close to the anomeric hydroxyl at C-2 and the phosphoryl OH is close to the ring oxygen. The potential efficiency of bifunctional catalysis of mutarotation has been amply demonstrated by Swain and Brown's (1952) experiments with α-pyridone, while Cunningham and Schmir (1966) have shown that phosphate monoanion can act as a bifunctional catalyst in the hydrolysis of the iminolactone, *N*-phenyliminotetrahydrofuran.

The experiments of Gottshalk (1943) suggest that 80% of fructose is in the pyranose configuration at 22°. While both F-1-P and fructose can exist in the pyranose form, FDP cannot. If we assume that phosphorylation at C-1 does not affect the equilibrium between pyranose and other forms of fructose, and that the effect of phosphorylation at C-6 is to prevent the formation of the pyranose form, the concentrations of the other forms should increase by a factor of five. The rate of exchange of FDP should then be five times that of F-1-P. Experimentally we find a ratio of 5.6 for the two rates (see Table I).

Phosphorylation at C-6 of fructose appears to facilitate exchange by increasing the concentration of the free ketone form, while phosphorylation at C-1 appears to facilitate exchange and, presumably, addition reactions by intramolecular catalysis.

Phosphorylation of fructose at C-1 and C-6 may affect the course of the enzymatic reaction in ways other than by increasing electrostatic binding of the substrate to aldolase. The phosphate on the C-6 of fructose increases the concentration of the free keto form by a factor of 5 by preventing the existence of fructose in the pyranose form. Secondly the phosphoryl group at C-1 can act as a bifunctional catalyst in the conversion of the cyclic to the keto form and subsequently to facilitate the formation of a Schiff base with the amino group of the enzyme.

Enzymatic Exchange. Horecker and his collaborators (Grazi *et al.*, 1962a,b; Horecker *et al.*, 1963; Lai *et al.*, 1965) have shown that DHAP forms a Schiff base with the ϵ-amino group of a lysine residue in the active site of aldolase (see also Cash and Wilson, 1966). The occurrence of an ^{18}O exchange with two substrates of aldolase at rates faster than the catalytic rate suggests strongly that for this enzyme, as for acetoacetate decarboxylase (Hamilton and Westheimer, 1959; Fridovich and Westheimer, 1962) and 2-keto-3-deoxy-6-phosphogluconate aldolase (Rose and O'Connell, 1967), the formation of a Schiff base is an obligatory part of the reaction. This inference is substantiated by the observation that the potent inhibitor of the cleavage reaction, xylitol 1,5-diphosphate, also inhibits the exchange. Further there is a parallel loss of exchange and cleavage activity following treatment of aldolase plus DHAP with $NaBH_4$. The latter treatment has been shown to result in the covalent attachment of no more than three molecules of DHAP (as the glyceryllysine derivative) to aldolase (Lai *et al.*, 1965; Ginsburg and Mehler, 1966; Kobashi *et al.*, 1966). Measurement by a variety of techniques has shown that there appear to be three binding sites for DHAP per aldolase molecule (Ginsburg, 1966; Kobashi *et al.*, 1966; Castellino and Barker, 1966) and it would thus appear that the only effect of the DHAP–$NaBH_4$ procedure is to modify the active sites needed for enzymatic function. We conclude, therefore, that the participation of these active sites of the enzyme is required for the catalysis of the exchange reaction.

The exchange, under equilibrium conditions, proceeds faster than the initial rate of the forward reaction (see Table II). Further, aldolase partially degraded with carboxypeptidase retains its catalytic effect on the oxygen exchange of FDP even though this treatment reduces the rate of cleavage of FDP to about 5% of that found with the native enzyme. These data exclude the possibility that the exchange results from cleavage of the hexose diphosphate, rapid nonenzymatic equilibration of the [2-^{18}O]DHAP with the normal water of the medium, and resynthesis of a [2-^{16}O]hexose diphosphate.

The rate of the aldolase-catalyzed ^{18}O exchange of [2-^{18}O]F-1-P is about 3% that of [2-^{18}O]FDP, and is about twice as fast as the initial rate of F-1-P cleavage at the same concentration. Carboxypeptidase treatment of aldolase markedly reduces the rate of cleavage of FDP without, however, affecting F-1-P cleavage (Drechsler *et al.*, 1959). In the exchange reaction the difference in the catalytic activity of native aldolase acting on F-1-P and of carboxypeptidase-treated aldolase on FDP is consistent with the finding of Rose *et al.* (1965) that the principal effect of the carboxypeptidase treatment is to slow the rate of the last step in the cleavage of FDP, the protonation and release of DHAP from the DHAP–enzyme anion.

The work of Hartman and Barker (1965) and of Ginsburg and Mehler (1966) suggests that Schiff base formation *per se* does not contribute to the binding of FDP. Rutter (1964) has indicated that aldolase can be inactivated in the presence of FDP by borohydride, but no experimental details were given, nor was the product of the reaction identified, so that it is not certain that this inactivation resulted from the reduction of an FDP–enzyme complex. Our observation that the rate of exchange of the carbonyl oxygen of FDP and F-1-P is faster than the rate of cleavage strongly supports the hypothesis that Schiff base formation occurs subsequent to binding but before cleavage and release, and that the formation of the Schiff base is readily reversible. The slow rates of exchange and cleavage of F-1-P relative to those of FDP would reflect, therefore, a slow rate of Schiff base formation. This may be due either to a lower concentration or reactivity of the carbonyl of F-1-P (see above) or, as Rose (1966) has suggested, to the formation of aldolase–F-1-P complexes which are incapable of further reaction.

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References

Ames, B. N., and Dubin, D. T. (1960), *J. Biol. Chem. 235*, 769.

Anbar, M., and Guttman, S. (1959), *Intern. J. Appl. Radiation Isotopes 4*, 233.

Baranowski, T., and Niederland, T. R. (1949), *J. Biol. Chem. 180*, 543.

Bell, R. P., and Baughan, E. C. (1937), *J. Chem. Soc.*, 1947.

Byrn, M., and Calvin, M. (1966), *J. Am. Chem. Soc. 88*, 1916.

Cash, D. J., and Wilson, I. B. (1966), *J. Biol. Chem. 241*, 4290.

Castellino, F. J., and Barker, R. (1966), *Biochem. Biophys. Res. Commun. 23*, 182.

Cunningham, B. A., and Schmir, G. L. (1966), *J. Am. Chem. Soc. 88*, 551.

Drechsler, E. R., Boyer, P. D., and Kowalsky, A. G. (1959), *J. Biol. Chem. 234*, 2627.

Fridovich, I., and Westheimer, F. H. (1962), *J. Am. Chem. Soc. 84*, 3208.

Ginsburg, A. (1966), *Arch. Biochem. Biophys. 117*, 445.

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

Gottshalk, A. (1943), *Australian J. Exptl. Biol. Med. Sci. 21*, 133.

Grazi, E., Cheng, T., and Horecker, B. L. (1962a), *Biochem. Biophys. Res. Commun. 7*, 250.

Grazi, E., Rowley, P. T., Cheng, T., Tchola, O., and Horecker, B. L. (1962b), *Biochem. Biophys. Res. Commun. 9*, 38.

Halmann, M., and Pinchas, S. (1958), *J. Chem. Soc.* 1703.

Hamilton, G. A., and Westheimer, F. H. (1959), *J. Am. Chem. Soc. 81*, 6332.

Hartman, F. C., and Barker, R. (1965), *Biochemistry 4*, 1068.

Hers, H. G., Beaufays, H., and DeDuve, C. (1953), *Biochim. Biophys. Acta 11*, 416.

Hohorst, H. J. (1963), *in* Methods of Enzymatic Analysis, Bergmeyer, H. U., Ed., translated by Williamson, D. H., New York, N. Y., Academic, p 215.

Horecker, B. L., Rowley, P. T., Grazi, E., Cheng, T., and Tchola, O. (1963), *Biochem. Z. 338*, 36.

Jencks, W. P. (1964), *Progr. Phys. Org. Chem. 2*, 63.

Kaplan, N. O. (1960), *Enzymes 3*, 105.

Kobashi, K., Lai, C. Y., and Horecker, B. L. (1966), *Arch. Biochem. Biophys. 117*, 437.

Lai, C. Y., Tchola, O., Cheng, T., and Horecker, B. L. (1965), *J. Biol. Chem. 240*, 1347.

Lehninger, A. L., Sice, J., and Jensen, E. V. (1955), *Biochim. Biophys. Acta 17*, 285.

Reeves, H., and Renbom, E. (1931), *Biochem. J. 25*, 412.

Rittenberg, D., and Ponticorvo, L. (1956), *Intern. J. Appl. Radiation Isotopes 1*, 208.

Roe, J., Epstein, J. H., and Goldstein, N. P. (1949), *J. Biol. Chem. 178*, 839.

Rose, I. A. (1966), *Ann. Rev. Biochem. 35*, 23.

Rose, I. A., and O'Connell, E. L. (1967), *Arch. Biochem. Biophys. 118*, 758.

Rose, I. A., O'Connell, E. L., and Mehler, A. H. (1965), *J. Biol. Chem. 240*, 1758.

Rutter, W. J. (1964), *Federation Proc. 23*, 1248.

Samuel, D., and Silver, B. L. (1965), *Advan. Phys. Org. Chem. 3*, 123.

Spolter, P. D., Adelman, R. C., and Weinhouse, S. (1965), *J. Biol. Chem. 240*, 1327.

Swain, C. B., and Brown, J. (1952), *J. Am. Chem. Soc. 74*, 2534.

Taylor, J. F., Green, A. A., and Cori, G. T. (1948), *J. Biol. Chem. 173*, 591.