Divalent Cation and pH-Dependent Primary Isotope Effects in the Enolase Reaction†

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ABSTRACT: 2-Phosphoglyceric acid has been prepared with deuterium in place of hydrogen on carbon atom 2. The initial rates of dehydration of the normal and deuterated substrates have been compared using rabbit muscle enolase and yeast enolase activated by Mg^{2+}, Mn^{2+}, and Co^{2+}. No difference in Michaelis constant was found between the two substrates. With both Mg^{2+}-activated enzymes, however, a primary isotope effect on the maximum velocity of greater than 2 was found at and below the pH optima. On the alkaline side of the pH optima, the isotope effects decrease and the rates for the two substrates approach the same value at higher pH's. The isotope effect for Mn^{2+} yeast enolase also decreases at high pH's, but the isotope effect is smaller than for the Mg^{2+} enzyme throughout the pH range. The Co^{2+} enzyme, in contrast, shows the same rate with both substrates throughout the accessible pH region. It appears that an unprotonated base is required to catalyze removal of the hydrogen from C_2 of the substrate and that at least one other protonated group assists in another step of the reaction. The lower efficiency of the Mn^{2+} and Co^{2+} enzymes appears to result chiefly from a reduction in rate of some step other than proton removal.

The use of primary isotope effects to investigate enzyme reaction mechanisms has recently been reviewed (Rose, 1970; Richards, 1970). The rate of formation or breaking of a carbon–hydrogen bond will generally be lowered if a heavier isotope of hydrogen, such as deuterium, is substituted for the proton. The dehydration of 2-phosphoglyceric acid to phosphoenolpyruvate, catalyzed by enolase, requires the breaking of both a carbon–oxygen bond and a carbon–hydrogen bond. Substitution of deuterium for hydrogen on carbon atom 2 of the substrate should lower the rate of hydrogen removal, but the effect on the overall rate of dehydration will depend on the relative rates of the different steps in the catalytic sequence. The change in rate of dehydration with pH together with the change in isotope effect with pH might then provide information on the details of the catalytic pathway.

Enolases require divalent cations for activity (Wold, 1971) and the efficiency of the enzyme is strongly dependent on the nature of the activating cation. At present there is very little understanding of the role played by the metal ion in this catalysis. Since the isotope effect can give information on relative rates of different steps in the reaction path, the effect of metal substitutions on the isotope effect could provide information on the detailed role of the metal ion.

Previous attempts to use hydrogen isotope effects to study enzymic dehydrations were not fruitful. Alberty *et al.* (1957) found no primary isotope effect in the fumarase reaction, although subsequently a small effect was reported at lower pH (Berman *et al.*, 1971). Dinovo and Boyer (1971) studied isotope-exchange rates at equilibrium with rabbit muscle enolase, and also looked for, but did not observe, a primary isotope effect on the dehydration rate. Data to be presented here show a substantial primary isotope effect in the reactions catalyzed by both rabbit muscle and yeast enolase. The effect is markedly dependent on both pH and activating cation. Our results complement the findings of Dinovo and Boyer and make it possible to deduce a more detailed picture of the reaction sequence.

Materials

Yeast enolase was prepared as previously described (Westhead, 1966). Rabbit muscle enolase was purchased from Sigma Chemical Co., St. Louis, Mo., and was used without further purification. Dowex 1-X8 (Cl^-) and Dowex 501 (D) were purchased from Bio-Rad Laboratory, Richmond, Calif. P-enopyruvate,[1] PGA and Tris base were purchased from Sigma Chemical Co. Tes and Pipes buffers were purchased from Calbiochem. Other salts were of analytical reagent grade; 99.5% D_2O from General Dynamics Corp., San Carlos, Calif., was used to prepare [^{2}H]-2-phosphoglyceric acid. Tritiated water used to prepare [^{3}H]-2-phosphoglyceric acid was purchased from New England Nuclear Corp., Boston, Mass.

Scintillation counting liquid was prepared as described by Bray (1960).

Methods

Preparation of [^{2}H]-2-Phosphoglyceric Acid. Tes or Pipes buffer salts were dissolved in 99.5% D_2O to make 0.05 M solutions, then adjusted to pD 6.4 at room temperature. The pD is obtained through the formula, pD = pH + 0.4 (Lumry *et al.*, 1951). In a typical preparation, about 100 mg of P-enolpyruvate–tricyclohexylamine salt was dissolved in 10 ml of the above buffer solutions containing 20 mM $MgSO_4$. Between 1 and 2 mg of pure yeast enolase was added and the mixture was incubated at 30°. The progress of the reaction

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[1] Abbreviations used are: P-enolpyruvate, phosphoenolpyruvate; Tes and Pipes buffers, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid and piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) monosodium monohydrate, respectively. Throughout this paper the isotopically substituted substrate, [^{2}H]-2-phosphoglyceric acid, has deuterium substituted for hydrogen only at carbon atom 2.

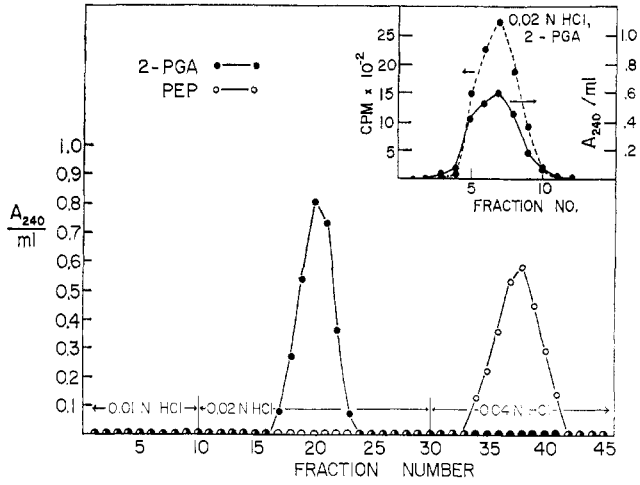

FIGURE 1: Separation of [^{2}H]-2-phosphoglyceric acid or [^{3}H]-2-phosphoglyceric acid from P-enolpyruvate on a Dowex 1-X8 (Cl^-) column. [^{2}H]-2-Phosphoglyceric acid and P-enolpyruvate were assayed as described in Methods. Data for P-enolpyruvate have been divided by four in order to fit them onto the plot. The inset shows a preparation in which tritiated water was added to label the 2-phosphoglyceric acid with tritium.

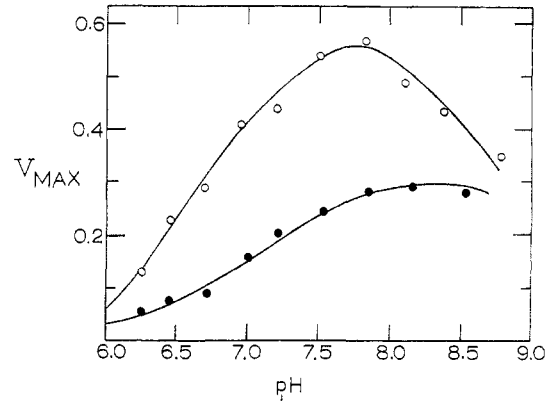

FIGURE 2: pH dependence of V_{max} for Mg^{2+} yeast enolase. Open circles represent extrapolated maximum velocities obtained using normal 2-phosphoglyceric acid. Filled circles represent extrapolated maximum velocities obtained using [^{2}H]-2-phosphoglyceric acid. All points were obtained from Lineweaver–Burk plots of substrate saturation data. Assay conditions are given in Methods.

was observed at 240 nm until there was no further change of absorbance. The reaction was then terminated by the addition of 1 ml of 0.5 N HCl and the mixture was diluted with deionized water to lower the conductivity below that of 0.01 N HCl. A 1 × 14 cm column of Dowex 1-X8 was prepared (Bartlett, 1959) and after the reaction mixture was loaded onto the column, it was eluted stepwise with 100 ml of 0.01 N HCl, 200 ml of 0.02 N HCl, and 200 ml of 0.04 N HCl. The column can be used repeatedly if it is regenerated sufficiently. Control preparations of [^{1}H]-2-phosphoglyceric acid and [^{3}H]-2-phosphoglyceric acid were conducted in the same way but with H_2O or tritiated water instead of D_2O. The fractions were collected at 2 to 3 ml per min, 10 ml/tube.

The presence of 2-phosphoglyceric acid and P-enolpyruvate in the fractions was detected spectrometrically. Aliquots (2 ml) of each fraction were titrated with solid Tris base to pH 7.8 and made 10^{-3} M in $MgCl_2$. The presence of P-enolpyruvate was simply detected by its absorption at 240 nm using the molar extinction coefficient 1400 (Wold and Ballou, 1957). To locate 2-phosphoglyceric acid, 1 ml of each fraction was assayed at 240 nm with yeast enolase at 30°. The concentrations of 2-phosphoglyceric acid were determined when the reactions reached equilibrium, from the known extinction coefficient of P-enolpyruvate, and the known equilibrium constant (Wold and Ballou, 1957). The more concentrated fractions were combined and titrated to pH 7 with NaOH, and then evaporated to about 30 ml on a rotary vacuum evaporator at 47°. The concentrated solution was then lyophilized.

In the [^{3}H]-2-phosphoglyceric acid preparation the radioactivity of each fraction was measured in Bray's solution (Bray, 1960) with a Packard Tri-Carb liquid scintillation spectrometer.

Enzyme Assays. The assay solution for yeast enolase contained 0.05 M Tris-HCl or Tes and 1 mM $MgCl_2$, 5×10^{-5} $MnCl_2$, or 5×10^{-5} $CoCl_2$, at different pH values. At pH values below 7.0, Tes buffer was used in place of Tris. The assay solution used for the muscle enzyme was the same except that it also contained 0.5 M KCl. In a 1-ml assay solution, 0.7–1.5 μg of yeast enolase or 5 μg of rabbit muscle enolase was used. Initial velocities were obtained from recorded curves obtained at 240 or 230 nm. All maximum velocities and Michaelis constants reported were obtained from double-reciprocal (Lineweaver–Burk) plots as exemplified in Figure 4.

Before use in Mn^{2+} and Co^{2+} assays, the purified Mg enzyme was passed through a Dowex 501 (D) column to remove all the metal ions and then $MnCl_2$ or $CoCl_2$ was added to a concentration of 5×10^{-5} M. The buffer solutions for these assays were passed through a large Chelex-100 column to remove divalent metal ions.

Initial nuclear magnetic resonance spectroscopic measurements on the conversion of phosphoglyceric acid to P-enolpyruvate were carried out in D_2O at 40 MHz with substrate at 0.2 M. The results were confirmed and extended using a Bruker X-100 Fourier transform nuclear magnetic resonance (nmr) spectrometer with substrate and $MgSO_4$ each at 20 mM in D_2O. To get quantitative measurements of proton concentrations, small regions of the spectrum were plotted in expanded scale. Tracings were then cut out and weighed. This was necessary because the C_2 proton is split by the phosphorus and other protons to give a cluster of seven lines.

Results

Preparation of [^{2}H]-2-Phosphoglyceric Acid and [^{3}H]-2-Phosphoglyceric Acid. The elution profile of [^{2}H]-2-phosphoglyceric acid is depicted in Figure 1A and the profile of [^{3}H]-2-phosphoglyceric acid in Figure 1B. The [^{3}H]-2-phosphoglyceric acid preparation is only used to confirm the spectrophotometric tracing of [^{2}H]-2-phosphoglyceric acid; only [^{2}H]-2-phosphoglyceric acid was used in all experiments. That there is no absorption at 240 nm in the 0.02 N HCl fractions before enzymatic assay shows the excellent separation of 2-phosphoglyceric acid and P-enolpyruvate on the Dowex 1-X8 (Cl^-) column. All the enolase protein which does not come straight through the column is completely removed by the 0.01 N HCl wash.

Control Experiments. To find out if anything in our preparation of deuterated phosphoglyceric acid could cause low rates in the enzyme assay, we prepared protonated phosphoglyceric acid from P-enolpyruvate by exactly the same procedure used for preparing the isotopically substituted compound. This substrate was dehydrated at pH 7.8 at the same rate as the

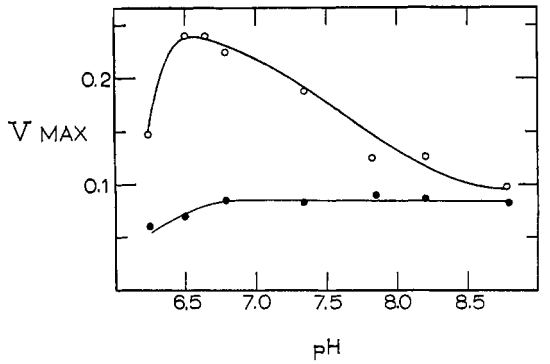

FIGURE 3: pH dependence of V_{max} from Mg^{2+} rabbit muscle enolase. Open circles and filled circles represent data obtained using normal 2-phosphoglyceric acid and [^{2}H]-2-phosphoglyceric acid, respectively, as in Figure 2.

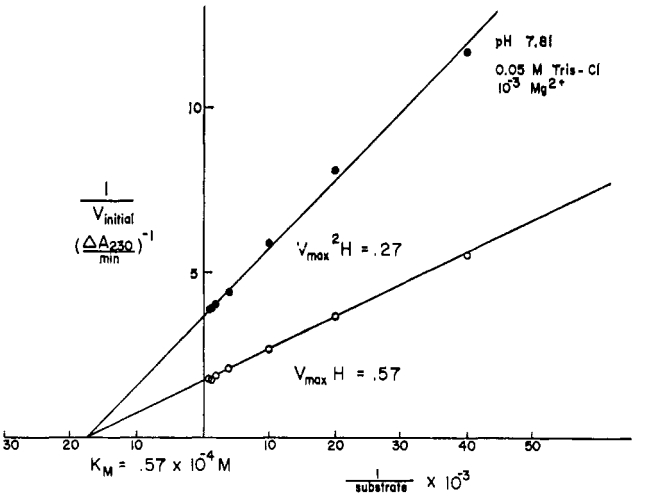

FIGURE 4: Invariance of K_m with isotopic substitution as shown by Lineweaver–Burk plots. Assay conditions were: 0.05 M Tris-Cl, 10^{-3} M $MgCl_2$, 30°, 0.9 μg of yeast enolase/ml at pH 7.81. Data obtained using normal 2-phosphoglyceric acid are shown as open circles and [^{2}H]-2-phosphoglyceric acid as filled circles.

commerical phosphoglyceric acid. The observed effects are therefore truly due to the isotopic substitution.

Because the observed isotope effects decrease at higher pH, it was necessary to know that no base-catalyzed exchange of deuterium could take place. We incubated a sample of the deuterated substrate at pH 8.8 for 10 min and then measured the rate of dehydration of this material at neutral pH. The full isotope effect was observed. Since our rate measurements are normally completed within 2 min, the experiment rules out an important possible artifact. The stability of the hydrogen–carbon bond is in accord with previous and subsequent nmr data showing no exchange over many hours.

Loss of deuterium by an enzyme-catalyzed exchange is to be expected when no isotope effect is observed, since the proton removal step is necessarily fast. This exchange will be discussed later in the paper.

Isotope Effects with Mg^{2+} Enolases. The pH dependence of the maximum velocity (V_{max}) of Mg^{2+} enolase with the normal and deuterated substrates is shown in Figure 2 for the yeast enolase and in Figure 3 for the rabbit muscle enolase. The two enzymes differ appreciably in their pH profiles, a fact previously known from the work of others (Holt and Wold, 1961). Figure 2 and Figure 3 show that in both cases there are very significant differences in V_{max} between normal 2-phosphoglyceric acid and [^{2}H]-2-phosphoglyceric acid at most pH values. To demonstrate that the differences in rates are attributable solely to a change in the catalytic constants, an example of the double-reciprocal plots from which these data are derived is given in Figure 4. These data show that at the pH where the maximum isotope effect is obtained, no change in Michaelis constant (K_m) is seen.

Isotope Effects with Other Divalent Cations. The V_{max} values for the two substrates as a function of pH for Mn^{2+} and Co^{2+} yeast enolase are shown in Figure 5. Figure 6 summarizes the isotope effects for all three yeast metal enolases and the Mg^{2+} rabbit muscle enolase. The ratios of maximum velocities for the normal and deuterated substrates (k_H/k_D) are plotted *vs.* pH. This figure is constructed from the *smooth* curves in Figures 2, 3, and 5, in order not to increase the scatter by dividing one experimental quantity by another at each pH. It will be seen that the ratio (k_H/k_D) for Mg^{2+} yeast enolase and Mg^{2+} rabbit muscle enolase decreases in strikingly parallel fashion with increasing pH. In the case of Mn^{2+} yeast enolase the ratio is significantly lower, but a marked decrease in the isotope effect is apparent in the higher pH regions.

Enolase activity as a function of Mn^{2+} concentration does not reach a plateau as in the case of Mg^{2+}, but shows a sharp maximum due to inhibition by excess Mn^{2+}. The concentration of Mn^{2+} used for the data of Figure 5 (5×10^{-5} M) is near the maximum. pH profiles for the dehydration of both normal phosphoglyceric acid and [^{2}H]phosphoglyceric acid with 1×10^{-4} M Mn^{2+} were also determined and found to be the same as the profiles in Figure 5. Mn^{2+} at 1×10^{-4} M is distinctly inhibitory, so it appears that inhibition does not alter the detailed path of the catalytic reaction.

Finally, we have continued and extended some unpublished experiments begun with Dr. A. Kowalsky in 1961. In these experiments, the proton nuclear magnetic resonance spectra of phosphoglyceric acid and P-enolpyruvate were observed in D_2O during dehydration of phosphoglyceric acid by Mg^{2+} yeast enolase. Since all protons of the substrate and product are easily observable and well separated, it is possible to follow the rate of dehydration of phosphoglyceric acid by the appearance of the vinyl protons of P-enolpyruvate and to measure the rate of exchange of the C_2 proton of phosphoglyceric acid by its disappearance relative to the C_3 protons. At pD 7.8, 30°, with initial substrate and $MgSO_4$ at 20 mM, and 20 mM Tris-chloride buffer, we found that when the reaction had proceeded 50% toward equilibrium, not more than 5% of the remaining phosphoglyceric acid had exchanged its proton for deuterium. At pD 9.2, other conditions the same, we found an approximately linear correlation between net dehydration and degree of C_2 hydrogen exchange. When dehydration reached 50% of its equilibrium value, 50% of the protons of the remaining phosphoglyceric acid had been replaced by deuterium.

Discussion

The information derived from this present study can be summarized as follows. There is no isotope effect on the Michaelis constant under any conditions used (isotope effects in K_m have been seen in other cases (Bush *et al.*, 1971; Page and Vanetten, 1971)).

The pH profiles of Mg^{2+} and Mn^{2+} yeast enolase show modified bell-shaped curves indicating that at least two hydrogen ion equilibria influence the reaction.

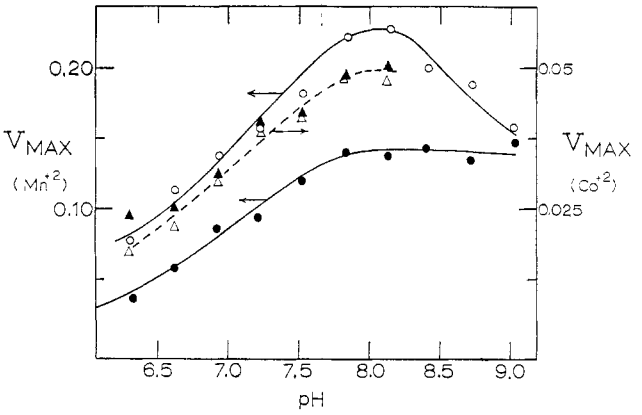

FIGURE 5: pH dependence of V_{max} for Mn^{2+} yeast enolase and Co^{2+} yeast enolase. Solid lines are for Mn^{2+} enolase and the dashed line for Co^{2+} enolase. Open symbols and filled symbols represent normal 2-phosphoglyceric acid and [^{2}H]-2-phosphoglyceric acid, respectively. Insolubility of Co^{2+} at high pH limits cobalt activation data.

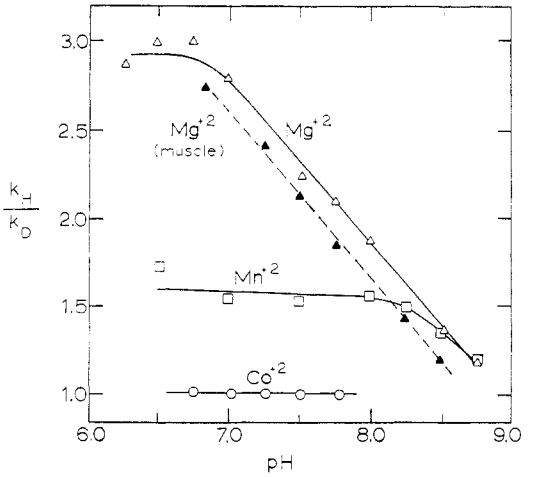

FIGURE 6: Dependence of the isotope effect (k_H/k_D) on pH. Curves are constructed from the data of Figures 2, 3, and 5, using the smooth curves of those figures as described in the text. Solid lines represent data for the yeast enzyme; triangles represent Mg^{2+} enolase, squares for Mn^{2+} enolase and filled circles for Co^{2+} enolase. The dashed line with open triangles represents Mg^{2+} rabbit muscle enolase.

The rate of dehydration is markedly reduced at most pH values for either Mg^{2+} enzyme or for Mn^{2+} yeast enolase when deuterium is substituted for the proton at C_2 of the substrate. The isotope effect diminishes with increasing pH.

Substitution of Mn^{2+} or Co^{2+} leads to considerably lower catalytic efficiency and a marked lowering of the isotope effect. At pH 7.8, the relative rates for Mg^{2+}, Mn^{2+}, and Co^{2+} are: 1.0:0.4:0.1 and the respective isotope effects are 2.1:1.6:1.0.

Nmr data in D_2O, obtained with the Mg^{2+} yeast enzyme show no measurable hydrogen exchange (not over 5%) in remaining phosphoglyceric acid at 50% conversion, while at pH 9.0 there is a moderate exchange rate (50% exchange in remaining phosphoglyceric acid at 50% conversion).

Because the rabbit muscle and yeast enolases differ markedly in their pH optima and because Dinovo and Boyer have published extensive data on equilibrium exchange rates with the Mg^{2+} muscle enzyme, the two enzymes have to be discussed separately although we assume that there is a great similarity in their catalytic mechanisms.

The maximum isotope effect seen with Mg^{2+} yeast enzyme is more than a third of the "theoretical" isotope effect which would be seen if carbon–hydrogen bond scission completely dominated the rate of dehydration. This prominent role of the deprotonation step supports the idea that the need for an unprotonated base, inferred from the pH profile, is to assist in proton abstraction. Dehydration rates of both protonated and deuterated substrate in the acid region (Figure 2) fit a theoretical titration curve with a pK of 6.7 fairly well. The pH profile of Mn^{2+} enzymes with a decreased isotope effect, fits a theoretical titration curve in the acid region less well, and the Co^{2+} enzyme, with no observable isotope effect (Figure 5), fits such a curve very poorly.

The decrease in isotope effect, particularly marked as pH is raised about the pH optima shows that the lowered catalytic activity at higher pH is due to the titration of a group (or groups) which are related to parts of the catalytic sequence other than proton abstraction. The need for a protonated group (or protonation of an intermediate) suggests that the step in question might be hydroxyl ion removal from C_3 of the substrate but product release from the enzyme is another possibility. Either of these steps (or both) could be slowed by the substitution of Mn^{2+} or Co^{2+} for Mg^{2+}, with consequent decrease in isotope effect.

The nmr data which showed very slow exchange at pH 7.2 where the isotope effect is strong agrees with the picture of nearly rate-limiting proton abstraction. At pH 9.2 where there is almost no isotope effect, a moderate rate of exchange is found. If *no* exchange were found, the results would show either sequestration of the removed proton by the enzyme (as shown for fumarase, Rose, 1970) or that the catalysis proceeds by a carbonium ion mechanism in which a slow hydroxyl ion removal precedes rapid proton removal and product release. The rate of exchange found is fast enough to rule out an extreme case of either of these possibilities. The exchange rate measured at pH 9.2 is about double that measured during net conversion by Dinovo and Boyer (1971) at pH 7.8 with the muscle enzyme. These pH values are both about 1.3 pH units above the pH optima of the enzymes. The complexity of the rate equations for a three-step conversion of the ES complex to E + P, including two proton equilibria is too great to allow quantitative assessment of the data without unjustifiable assumptions about relative values of rate constants.

Without further data, we can only assume tentatively that this enzyme uses the same pathway as the rabbit muscle enzyme. For the muscle enzyme (Figure 3) the low pH side of the curve is obscured by a sharp drop in activity which may be due to a conformational change in the protein. (This sharp drop was documented by Holt and Wold, 1961.) An isotope effect which is strikingly similar to that of the Mg^{2+} yeast enzyme is seen, however (Figure 6), and it decreases with pH in a very similar way. The marked difference in pH profiles of the two enzymes might indicate that different ionizable amino acids are used, in a similar catalytic mechanism, by the two enzymes. Indication that a carboxylic acid group is required for activity of the muscle enzyme comes from the work of Rose and O'Connell (1969) who covalently attached the substrate analog glycidol phosphate to a carboxylic acid in the active site of that enzyme. With reagent generously supplied by Rose and O'Connell, we tried to extend their work to the yeast enzyme, but did not get satisfactory results. Perhaps in place of a carboxylic acid the yeast enzyme uses a base with a higher pK (such as a histidine) which may not form a stable product with the reagent.

In work closely related to our present study, Dinovo and Boyer measured the exchange rates of oxygen, carbon, and hydrogen isotopes with substrate and product at equilibrium, using the rabbit muscle enzyme. They looked for, but did not find, the primary isotope effect we discuss here. This is not a serious discrepancy because they looked for the isotope effect at pH 7.8 and we see (Figure 3) that the isotope effect there is relatively small. Their assay at 260 nm would not have permitted them to obtain rate data over as early a portion of the net reaction as is possible at 230 or 240 nm.

Dinovo and Boyer did find the full theoretical isotope effect on the difference of exchange rates between ^3H and ^2H, and calculated that the exchange rate of ^1H in H_2O should be much faster than the exchange of ^{18}O (from C_3), or of ^{14}C in the carbon skeleton. An extremely fast rate of hydrogen exchange would preclude observation of an isotope effect on the overall reaction rate, but the actual rates of ^2H–^1H exchange were equal to the ^{14}C exchange rate at pH 6.5 and only twice the ^{14}C rate at pH 7.8. The rate of C–H bond breaking therefore contributes to an important but not dominating extent to the dehydration rate near the pH optimum. Our maximum observed isotope effect is about a third of the effect on hydrogen exchange rates so the contribution of other steps is significant at all times. From the data of Dinovo and Boyer on the rate of tritium incorporation into phosphoglyceric acid during net dehydration at pH 7.8, we calculate that the loss of deuterium from the substrate would occur at such a rate that at 10% conversion the remaining substrate would have exchanged 10% of its deuterium for a proton. Our rate data are routinely obtained at considerably less than 10% conversion so our rates would not be seriously affected by the exchange.

The previous authors also showed that ^{18}O exchange is always slower than H exchange, demonstrating that the reaction proceeds *via* a carbanion (hydrogen off first), mechanism. Sequestration of the HO^- group removed could conceal a carbonium ion mechanism, but Dinovo and Boyer have presented arguments against the likelihood of that phenomenon.

Our data clearly show that as the pH is raised steps other than hydrogen abstraction increasingly dominate the catalytic rate. Dinovo and Boyer's data show that in going from pH 6.5 to pH 7.8, the rates of *both* H exchange relative to ^{18}O exchange and ^{18}O exchange relative to ^{14}C exchange increase. Apparently an increase in pH slows both of the steps subsequent to proton abstraction and product release appears to be slowed even more than hydroxyl ion removal. The pronounced secondary isotope effect in the reverse reaction at pH 7.8 found by Dinovo and Boyer was interpreted as showing that rehydridization of the Sp^2 carbon to an Sp^3 configuration is an important slow step. We assume that this step must be coordinated with the approach of the HO^- ion. The configurational change would thus be subject to the same pH effects as the hydroxyl-transfer step. It may be noted in passing that although the alkaline side of the muscle enzyme pH profile is observed over two decades of hydrogen ion concentration, the rate does not approach zero at high pH. This appears to show that protonation of some group assists the catalysis but is not critical to it.

In summary, it is clear that no concerted mechanism of dehydration can be utilized by either yeast or muscle enolase. A minimum of three separate steps, proton abstraction, hydroxyl ion removal and product release can be discerned through the use of isotopes, and it appears from the exchange rate data quoted that the steps take place in the order listed. At low pH the first step is slow enough to restrict the dehydration rate but as pH increases, the latter two steps decrease in rate and dominate the dehydration process. The less effective metals are seen to decrease the efficiency of either hydroxyl removal or product release or both, and it seems likely that ^{18}O-exchange rate studies would allow clear cut differentiation between these two possibilities.

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