The Reversible Denaturation Processes of Yeast Enolase*

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The structure of yeast enolase in 0.10 N KCl solution in the absence of divalent metal ions has been investigated by sedimentation-velocity and equilibrium measurements, optical-rotatory-dispersion measurements, ultraviolet-difference spectroscopy, and viscometry. The isoionic point has been determined and the enzyme has been titrated from *p*H 3 to *p*H 11. Variations in the structure as a result of *p*H changes have been investigated over the entire *p*H range. It is shown that at 30° structural changes take place at *p*H 5.2, 4.2, and 9.2. The change at *p*H 5.2 appears to be an opening of the molecule with little loss in helical structure. The change at *p*H 4.2 involves an extensive loss of helical structure, and a subsequent aggregation of the molecules leads to a mixture of higher-order aggregates. At the extremes of *p*H the molecule exists as a very disordered monomer at 30°, but at 0° is still a dimer at *p*H 1. Loss of helical regions does not appear to be complete even at *p*H 1, but appears complete in 8 M urea at *p*H 1 or 8. It is shown that the aggregation of the molecule between *p*H 3 and 4 is largely responsible for the apparent irreversibility of the denaturation at low *p*H. At 2° there is only a single low-*p*H transition which occurs at *p*H 4. This leads to a very stable dimer which has the same loss of helical structure as the 30° monomer. It has also been found that activity may apparently be regained spontaneously, under suitable conditions, no matter how the protein has been denatured.

This study of the conformational states of yeast enolase was carried out with three objectives in mind. The first was to investigate the report by Bücher (1947) that enolase undergoes a major change in structure between *p*H 5.2 and 4 at low salt concentration. The light-scattering data on which this report was based was originally interpreted to show a dissociation of the molecule into subunits. Although that interpretation was later withdrawn (Bücher, 1955), the sharpness of the transition observed was not fully explained.

The second objective was to find a reversible conformational transition which might be used as a sensitive indicator of structural modification. Attempts to discover the composition of the "active site" with chemical reagents (Brake and Wold, 1962; Westhead, 1963) are beset by the difficulty of associating losses in activity with changes specifically at the active site. The study of effects of chemical modification on a conformational transition might provide a more sensitive test for general structural alteration than would physical measurements made on the molecule in a stable state.

Finally, since yeast enolase is devoid of disulfide and sulfhydryl groups (Malmstrom *et al.*, 1959) it is particularly attractive for the study of protein refolding. To date, the only enzymes shown conclusively to regain activity after complete loss of native structure are ribonuclease (White, 1960) and lysozyme (Imai *et al.*, 1963). The refolding of those proteins is complicated by the need to re-form disulfide cross-links. If enolase could be shown to regain activity from a completely denatured state, an interesting study of conditions that affect the speed and efficiency of refolding might be made.

Experimental

Materials.—Enolase was prepared from bakers' yeast as described elsewhere (Westhead and McLain, 1964). Prior to use in any experiments reported here, enolase solutions were made 10^{-4} M in EDTA, then passed slowly through a column of Fisher mixed-bed ion-exchange resin to remove traces of metal ions. After this treatment the enzyme solutions were stored in acid-washed polyethylene containers in the presence of a drop of chloroform or toluene. In all experiments reported, the enolase used had a specific activity at 30° of at least 300 (Westhead and McLain, 1964).

All reagents were analytical reagent grade and were used without further purification except that urea was recrystallized from 10^{-4} M EDTA solution before use.

Methods.—All *p*H values reported were determined on samples of the protein containing salt or buffer solutions at the temperature recorded using a Radiometer Model 22 *p*H-meter equipped with a scale expander.

Enolase was titrated by hand with an Agla micrometer syringe in a thermostated vessel under a stream of water-saturated nitrogen.

Viscosity was measured with a Cannon Ubbelohde dilution viscometer built with a 3-foot coiled capillary and a flow time for water of 6 minutes at 25°. Temperature was controlled to ±0.003° during viscosity measurements. Solutions were passed through a cellulose acetate (Millipore) filter before use.

Difference spectra were recorded with a Cary Model 15 spectrophotometer equipped with a deuterium light source and thermostated cell holders. Unbuffered solutions of enolase in 0.10 N KCl, 1.5 mg protein per ml, were titrated with 0.20 N HCl using a Hamilton microliter syringe and adding the HCl with a plastic plunger to the solutions in the cuvet. Equal amounts of 0.2 N KCl were added to the reference cuvet. The *p*H was read with a small combined electrode set in the cuvet.

Optical rotation data were obtained with a Rudolf polarimeter. Solutions were centrifuged at 20,000 × *g* for 0.5 hour prior to optical rotatory measurements to remove traces of particulate matter. Data from urea solutions were corrected for changes in refractive index (Schellman, 1958).

Ultracentrifugation was done with a Spinco Model E ultracentrifuge, with a platinum wire in place of the phase plate (Von Hippel and Wong, 1963).

Sedimentation equilibria were determined using the multichannelled cell described by Yphantis (1960),

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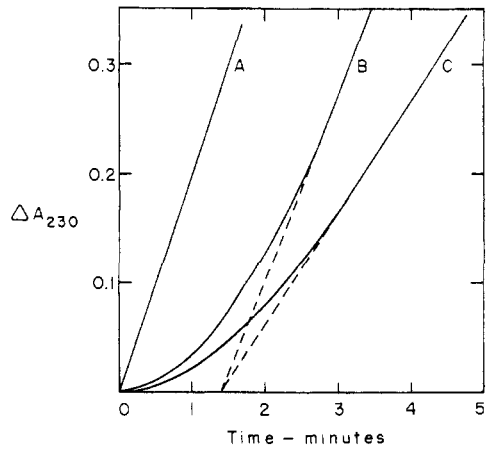

FIG. 1.—Time-dependent recovery of enolase from acid denaturation. Curve A, enolase preincubated at *p*H 7; curve B, enolase preincubated 5 minutes at *p*H 3, 30°; curve C, enolase preincubated 10 minutes at *p*H 3, 30°. Assay temperature, 30°.

and in all cases the weights reported are the extrapolations to infinite dilution of weights calculated at four different protein concentrations between 2 and 10 mg/ml. Corrections for the differences in viscosity and density between salt solutions and water were made only for the data between *p*H 5.6 and 9.0, but corrections for the effect of temperature on viscosity were made for all data. No correction was made for possible changes in the specific volume of the protein. The value used for that quantity was 0.735 ml/g as given by Bergold (1946).

Activity measurements were made as described elsewhere (Westhead and McLain, 1964), except that to follow recovery of activity a wavelength of 230 mμ was used and a salt concentration of 0.5 N KCl was used so that substrate concentrations could be raised to 1.0×10^{-2} M. This combination increases the straight-line portion of the catalytic reaction by a factor of about 7.

RESULTS

When a solution of enolase between *p*H 5.5 and 9.2, uncontaminated by heavy metal ions, is added to a solution of 2-phosphoglyceric acid, an increase in the optical density of the solution in the ultraviolet region takes place as a result of the formation of phosphoenolpyruvic acid. The time course of this reaction is illustrated in Figure 1, curve A. No detectable lag period is observed. When enolase solutions are preincubated at *p*H lower than 5.5 or higher than 9.2, upon addition of the enzyme to the substrate solution at *p*H 7.4, a distinct lag in the enzymic catalysis is observed (Fig. 1, curves B and C). The length of the lag period and slope which the curve approaches as a limit are functions of the temperature and *p*H of preincubation, the length of time of incubation, and the temperature of the substrate solution. Within the normally zero-order region of the reaction there is no effect of substrate concentration on the reaction. Similar curves may be constructed by adding the enzyme first to a substrate-free solution of similar composition and *p*H, and then assaying aliquots of this solution at a series of time intervals, finally plotting initial rates of reaction versus time of incubation at *p*H 7.4.

The limiting slope, if it occurs within the zero-order region of the reaction, or the final activity attained within a few hours after adjustment of the enzyme to *p*H 7.4, is taken as the "recoverable activity" of the enzyme

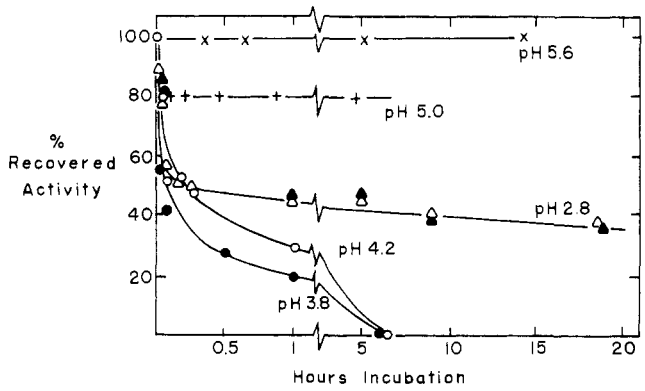

FIG. 2.—"Recoverable activity" as a function of time of preincubation of enolase at acid *p*H as indicated. All incubations and assays were at 30°.

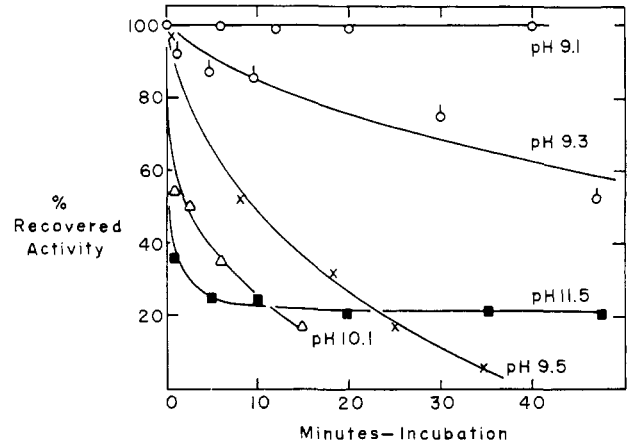

FIG. 3.—"Recoverable activity" as a function of time of preincubation of enolase at alkaline *p*H as indicated. All incubations and assays were at 30°.

under given conditions. Sets of such values of "recoverable activity" are plotted in Figure 2 as a function of the length of time the enzyme has been incubated in solutions of the indicated *p*H. It should be noted that loss of "recoverable activity" is complete at *p*H 4.0–2.8, but that at still lower *p*H values recoverability again reached an almost stable level far from zero. In Figure 3, a similar set of data for the alkaline region is shown.

Sedimentation Velocity.—In order to detect configurational changes that were reflected in the kinetic data, sedimentation-velocity measurements were made at 2° ± 2° and at 29.5° ± 0.5° over the entire *p*H range. (These temperature ranges refer to variations between runs, individual runs had much closer temperature control.) All solutions were 0.10 N in KCl, except that at the lowest *p*H, where HCl was used, Cl^- concentration was approximately 0.1 N. In Figure 4 sedimentation constants are plotted against *p*H for both temperatures. The solid line is drawn through the 30° data; bifurcation of the line in the alkaline region indicates the existence of two separate sedimenting boundaries. The more rapid of these two peaks was always broad and sometimes quite difficult to measure. The relative amounts of protein in each peak were estimated to vary from 4:6 (fast to slow) at *p*H 10.3 to 7:3 at *p*H 11. These were rough estimates only, and of some significance only because of a rough quantitative correlation with recoverability of activity. There was a slow time-dependent shift of material from the slower to the faster peak. For example, in 14 hours, at *p*H 10.3, the area ratio of the slow-to-fast peak changed

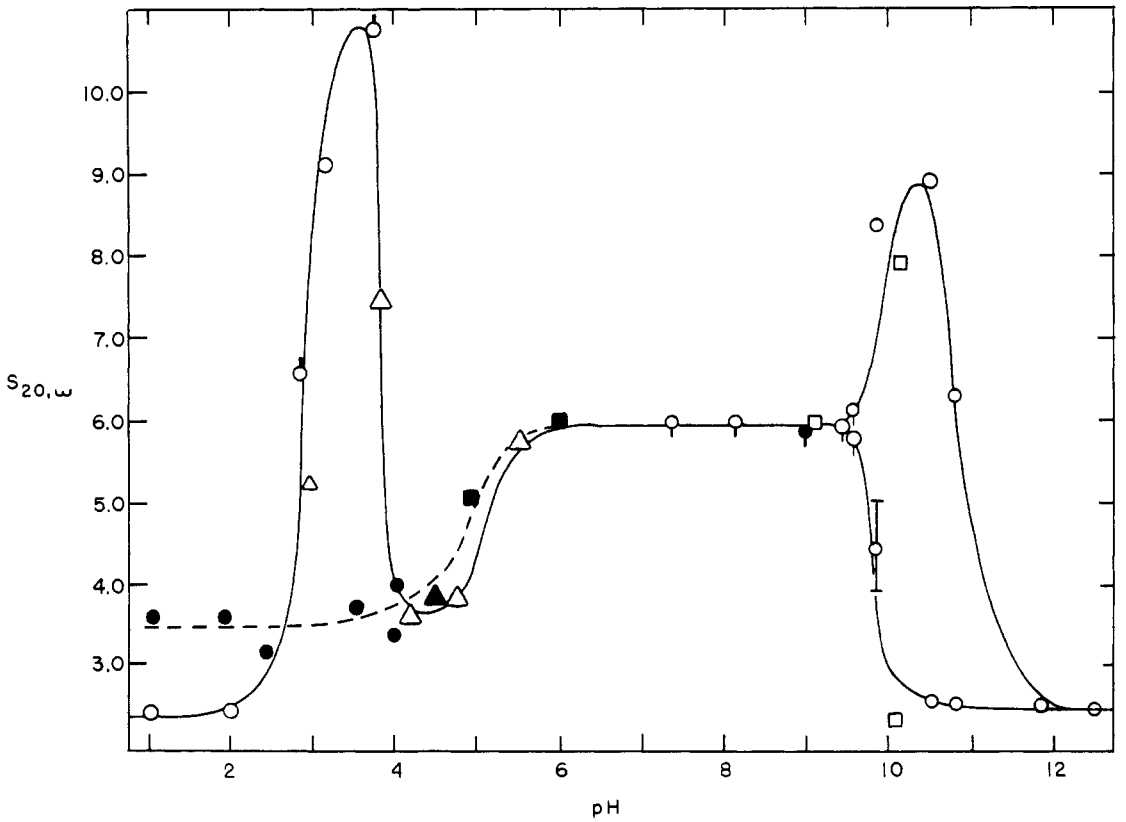

FIG. 4.—Sedimentation velocity of enolase as a function of *p*H at a protein concentration of 2 mg/ml. Open symbols at 30°, closed symbols at 2° ± 2°. Bifurcation of curve in alkaline region indicates presence of two sedimenting boundaries. ○ ●, glycine buffer; ♀ ♀, Tris-HCl buffer; △▲, acetate buffers; ○●, unbuffered solutions, *p*H adjusted with HCl or KOH; □ ammonium chloride buffer; ■ citrate buffer. All buffers were 0.05 M, all solutions were 0.10 N in KCl, except those at *p*H 1.

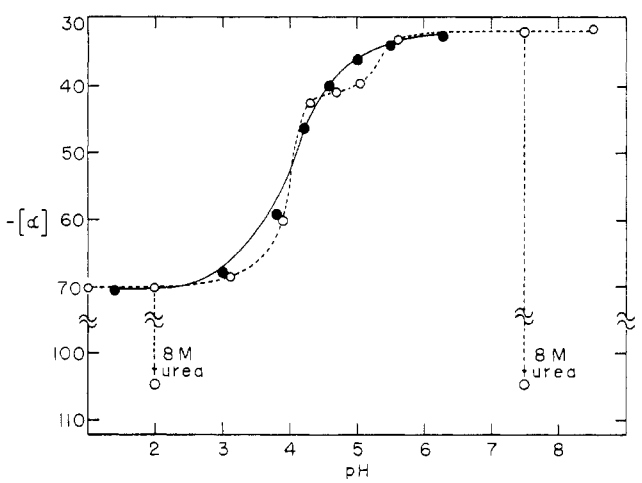

FIG. 5.—Change in specific rotation at 557 mμ as a function of *p*H and urea addition. All data except at *p*H 7.8 and 1.0 obtained in 0.1 N KCl, by adjusting the *p*H with HCl; *p*H 1.0 data were obtained in 0.16 N HCl and *p*H 7.8 data in Tris-HCl buffer 0.05 ionic strength, 0.10 N KCl. Urea solutions were prepared separately. Protein concentration was 5 mg/ml; X, 30.0°; ○, 2.0°; solid line is calculated for first-order hydrogen-ion dependence.

from 4:6 to 6:4. At low *p*H there was no time dependence; several of the sedimentation runs at low *p*H were repeated a day later on the same solutions, and in all these cases the results were identical with the original ones. The data shown are from experiments in which the first pictures were taken between 20 minutes and 40 minutes after the enzyme was mixed with the buffer solution.

Viscosity Measurements.—Viscosity measurements at 25.0° and at 30° were made at five concentrations between 9.5 mg/ml and 3.0 mg/ml at *p*H 6.2 in 0.05 M potassium phosphate buffer, 0.2 N in KCl, and at *p*H 7.4 and 8.6 in 0.05 ionic strength Tris-HCl buffer, 0.50 and 0.10 N KCl. In each case the reduced viscosity was extrapolated to infinite dilution, and showed an almost negligible dependence on concentration. The intrinsic viscosity showed no dependence on *p*H from 6.0 to 8.0 or on temperature, and its average value was 2.9 ± 0.1 cc/g. Efforts to measure the viscosity at *p*H 3.2, 30°, gave estimates of approximately 30 cc/g, but the data were not satisfactorily reproducible, apparently because of a tendency for the protein to aggregate and form surface films under these conditions.

Optical Rotatory Dispersion Measurements.—In order to compare changes in internal organization with changes in the hydrodynamic properties of the protein, optical rotation measurements were made at neutral and low *p*H values. Again all solutions were made 0.10 N in KCl, except that where higher concentrations of HCl were used the KCl concentration was reduced to keep the ionic strength approximately constant. Figure 5 shows the change in optical rotation at one wavelength as a function of *p*H at 30° and 2°. At 2° a single transition occurs, fitting a first-order hydrogen-ion dependence very closely (solid line). At 30° two very distinct transitions occur, one centered at *p*H 5.4 and the other at *p*H 4.0. These transitions show greater than first-order hydrogen-ion dependence and correlate fairly well with the first two transitions of Figure 4, which are centered about *p*H 5.2 and 3.8. Also included in Figure 5 are the effects of 8 M urea on the rotations of enolase solutions at *p*H 1.0 and 8.6 at

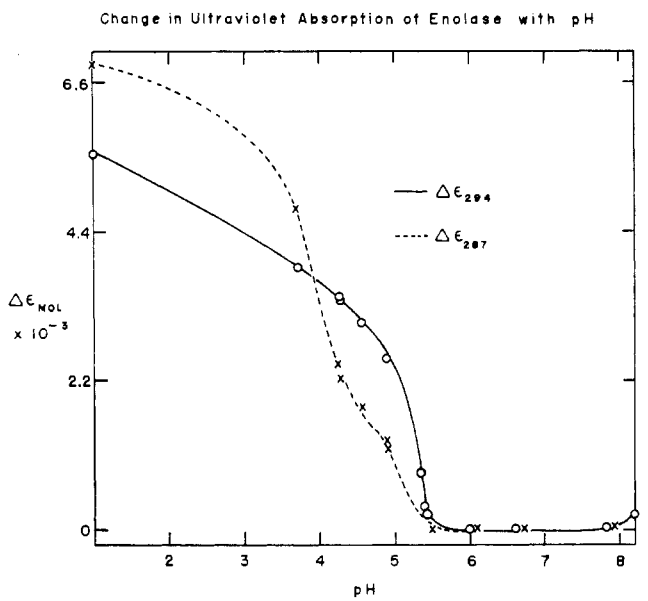

FIG. 6.—Changes in the spectrum of enolase at two wavelengths occurring as a result of *p*H changes. Protein concentration was 1.5 mg/ml, all solutions were 0.10 N in KCl except at *p*H 1.0, which was 0.16 N HCl. Temperature 30°.

TABLE I
ROTATORY DISPERSION PARAMETERS FOR YEAST ENOLASE

		*p*H				8 M Urea[a]
		7.5	4.7	3.0	1.0	
2°	a_0	170	245	410	445	
	b_0	205	200	160	160	
30°	a_0	165	200	420	430	730
	b_0	205	190	130	130	40

[a] Rotatory dispersion at *p*H 1.4 and at *p*H 8.6 was identical.

30°. The loss of structure implied by the increases in $-[\alpha]$ is apparently not complete in the purely aqueous solutions. This impression is substantiated by the data in Table I, where the values of a_0 and b_0 are also recorded. The value of $\lambda_0 = 212$ mμ is used in the calculation of b_0 to make comparison with other data possible, but it might be noted that λ_c (Drude equation) is 223 mμ for enolase in urea and a higher value of λ_0 seems to make a somewhat better fit of the data to a straight line.

Difference Spectra.—The data of Figure 6, except for those at *p*H 1.0, were obtained in unbuffered 0.10 N KCl by adding HCl to the sample cuvet and KCl to the reference cuvet. The data at *p*H 1.0 were obtained by making a separate sample of enzyme 0.16 N in HCl. Experiments were run at both low and high *p*H to see if any rate measurements were possible. It was found that within 3 seconds the absorbancy changes at any wavelength were complete. Since the spectra at high *p*H are complicated by the ionization of tyrosine, only the low-*p*H data are shown. The most striking feature of the data in Figure 6 is the fact that the spectral shifts at 294 mμ (all tryptophane) are out of phase with those at 287 (tryptophane plus tyrosine). This is in sharp contrast to the data obtained by Rosenberg and Lumry (1964) in the companion paper to this one. This difference in results will be considered in the discussion. The approximate mid-points of the curves of Figure 6 are located at about *p*H 4.2 and 5.2, close to the two transitions located by optical rotation and centrifugation.

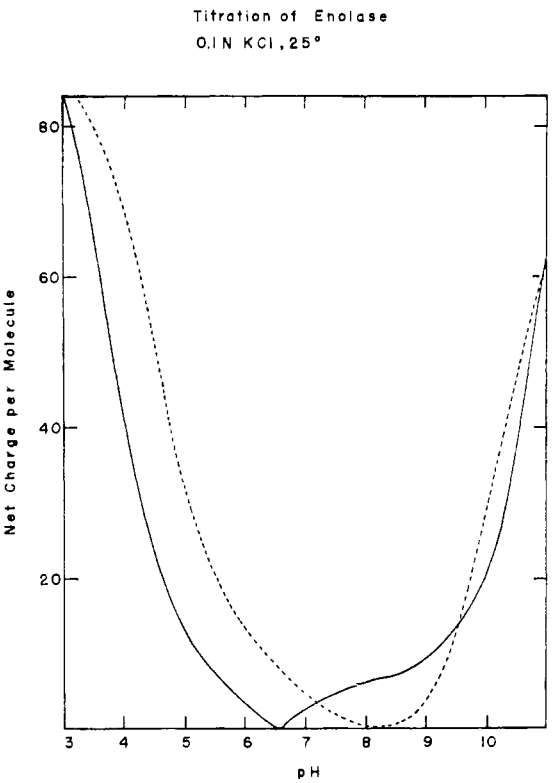

FIG. 7.—Titration curve for enolase at 30°; protein concentration was 7.5 mg/ml. The experimental curve, solid line, was drawn through 80 separate points. The dotted curve was calculated as described in the text.

Titration of Enolase.—Primarily to compare the results of Figure 4 with the charge state of the molecule, solutions of enolase at the isoionic point were made 0.10 N in KCl and then titrated with HCl or KOH. Enolase was made isoionic by passing solutions of about 12 mg/ml slowly through a column of mixed-bed ion-exchange resin at room temperature (23°), and collecting fractions. It was consistently found that the more concentrated protein fractions have a *p*H of 6.58 ± 0.01. No significant change in *p*H takes place upon diluting these solutions with an equal volume of 0.20 N KCl, which indicates that there is no appreciable binding of either K^+ or Cl^- at this *p*H. The curve shown in Figure 7 is one of two such complete curves made on two samples of enolase, and there was no appreciable difference between the two. The first noteworthy feature of the curve is the rather flat region between *p*H 7 and 8.5, where most of the ionization of enolase's 14 histidines would be expected to take place. Only 4 moles of hydrogen ion are titrated in this region which would also be expected to include some contribution from amino and carboxyl groups. The dotted line is a representation of the titration curve that might be expected from a protein of the amino acid composition of enolase. This curve was calculated using average values for *pK* of amino acid side chains found by Tanford (Tanford and Wagner, 1954; Tanford, *et al.*, 1955). The second interesting feature of the titration curve is that at the *p*H of the transitions shown in Figure 4, *p*H 9.2 and 5.2, the net charge on the enolase molecule is the same, i.e., about 12.

Sedimentation Equilibria.—While this work was in progress a report appeared (Deal *et al.*, 1963) which showed that at *p*H 2.9 yeast enolase is a dimer. This phenomenon was investigated more fully with the results shown in Table II. It is clear that the rapidly sedimenting material at 30° in Figure 4 is an aggregate

TABLE II
MOLECULAR WEIGHT OF ENOLASE BY SEDIMENTATION EQUILIBRIUM

	pH		
	7.5	3.8	1.4
2°	65,000	123,000	132,000
30°	68,000	>211,000[a]	70,000

[a] A significant amount of protein had sedimented to the bottom of these cells, so the number is a minimum weight and should not be interpreted as a trimer.

of ill-defined molecular weight, while in strongly acid solution at 30° the molecule is a monomer. At 2°, on the other hand, only a dimer is formed in the transition, and this is stable even at *p*H 1. The 30° monomer at *p*H 1 and the 0° dimer have marked kinetic stability as shown by the following experiments. After lowering the *p*H to 1 at 0°, a sedimentation equilibrium run was made at 2.2° showing a very clear dimer; the cell was then shaken gently to mix the contents and raised to 30°. A new sedimentation equilibrium was then carried out at 30.0°, 45 minutes after the 2.2° run, and again there was only a dimer in solution. A similar experiment involving three equilibrium runs, 30°, 2°, 30°, showed only monomer in each case. Thus over a period of 1 hour the *p*H 1 forms have such kinetic stability that a thermodynamic equilibrium is not approached.

One further experiment bearing on this kinetic stability of the dimer should be described. An enolase solution of 0.1 mg/ml is diluted into ten volumes of 0.10 N HCl and let stand 0.5 hour. The *p*H is then raised to 7.5 with 1.0 N NH_3. If this is carried out at 30°, there is no lag period observed when an aliquot of the enzyme is added to substrate solution. If, however, the cycle is carried out at 0°, addition of an aliquot of the enzyme to substrate results in a very pronounced lag, identical with that obtained if the *p*H 1 enzyme had been diluted with water instead of the NH_3 required to raise the *p*H. (This lag is much greater than the slight lag due to the lowering of the temperature of the substrate solution by cold enzyme.) The 0° *p*H 7.5 enzyme solution has been kept for 2 days without elimination of the lag period. However, if this solution is raised to 30° for even a minute, subsequent addition of the enzyme to substrate gives a linear reaction. This again, is a good demonstration of the strong metastability imposed on the dimer by kinetic factors.

Reversibility of Conformation Changes.—A study of the factors involved in determining the rate and efficiency of refolding is being made in this laboratory and will be reported on at some later time. It is clear already that refolding of the molecule from urea solutions or from either low or high *p*H is very strongly dependent on a variety of conditions, some difficult to control.

In raising the *p*H of a concentrated enzyme solution, or in diluting out a urea solution, the manner of mixing, for example, is very important. Addition of enolase at *p*H 1 to buffer at *p*H 7.5 while stirring by hand yields a much lower recovery of active enzyme than if the addition is made while stirring with an efficient (Vortex) mixer.

So far we have found no conditions of denaturation from which significant recoveries of activity have proved impossible. Enolase has been precipitated with trichloroacetic acid, washed with alcohol and ether, and stored at room temperature in a vacuum desiccator for months. Samples of the enzyme have been boiled at neutral *p*H for 20 minutes to yield a tough insoluble mass. In both cases solution of the enzyme was achieved with 8 M urea, *p*H 1, the urea was dialyzed out against 0.01 M HCl at 0°, and on addition of the enzyme to the assay solution reaction took place with the usual lag period. Recoveries of activity of 20–60% were achieved. Further studies, using hydrogen-exchange techniques, are in progress to document more completely the apparent refolding of enolase from a fully denatured state. Preliminary results show a total absence of slowly exchanging hydrogens in 6 M urea solution.

DISCUSSION

Enolase is a metal ion–activated enzyme, being activated strongly by Mg^{+}, Mn^{2+}, Zn^{2+}, and to a lesser extent by other divalent metals. It is inhibited by low concentrations of Cu^{2+}, Hg^{2+}, and other metals. Since the role of these metal ions is not yet defined, it was considered advisable to study the protein structure under defined conditions of metal-ion binding. The condition chosen was that of no metal binding, and in these studies divalent metals were carefully excluded. As has been shown by Rosenberg and Lumry (1964) in a study complementary to this one, Mg^{2+} exerts very significant effects on the stability of the enzyme.

In its native state enolase is an exceptionally compact and spherical molecule. Bergold's (1946) measurements of sedimentation and diffusion rates indicated a molecule with a frictional ratio of 1.01. The intrinsic viscosity of 2.9 cc/g confirms this high degree of symmetry. By comparison, sperm whale myoglobin, whose X-ray pictures show it to be a compact symmetrical molecule (Kendrew *et al.*, 1960) and whose molecular weight is about one-fourth that of enolase, has an instrinsic viscosity of 3.1 (Wyman and Ingalls, 1943).[1] A change in sedimentation coefficient[2] from 5.9 to 3.5 means a change in frictional ratio to 1.7. Assuming no change in degree of hydration, this would correspond to a change from a nearly spherical molecule to an ellipsoid of revolution with an axial ratio of *13:1* (Oncley, 1941). From the slight change taking place in the optical rotatory parameters, it seems that this transconformation takes place with little net loss in helical structure. The change in absorbance due to tryptophane closely parallels this change in the shape of the molecule, indicating that a significant portion of the seven tryptophanes are exposed to a more polar environment. This point is discussed more fully by Rosenberg and Lumry (1964). If hydrophobic regions of the molecule are in an aqueous environment after the first transition, it is not surprising that the state is one of marginal stability, nor that this state shows increasing stability at lower temperatures (Rosenberg and Lumry, 1964).

At 30°, a further transition in structure takes place near *p*H 4.2. Again, the configurational change observed by optical rotation is paralleled by changes in the sedimentation velocity. The degree of unfolding of helical regions is larger in this transition, but the dimensional changes are masked by the aggregation which takes place. The change which we observe in the spectrum at 287 mμ parallels rather closely this second transition. This change in absorption at 287 mμ is less sharp than the one observed at 294 mμ, but the latter is due almost entirely to tryptophane while

[1] It might avoid confusion to point out that the Einstein limiting viscosity, 2.5, is a volume ratio, so that for a protein of specific volume 0.73, this would become 1.8 cc/g.

[2] It should be noted that the sedimentation coefficients, unlike the sedimentation equilibrium data, are not extrapolations to infinite dilution.

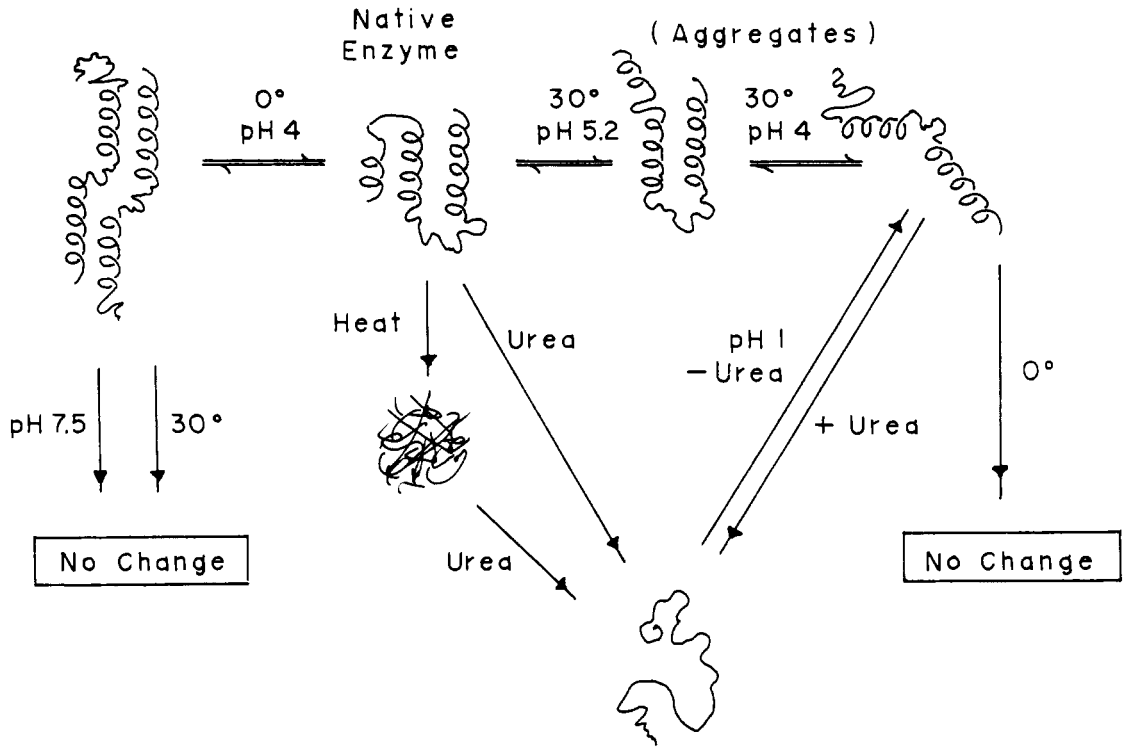

FIG. 8.—Representation of the states of enolase investigated, where double arrows indicate equilibria and single-headed arrows indicate processes which appear not to be reversible.

the former includes contributions from both tryptophane and tyrosine. Since at 287 mμ there is a steep rise in the absorption spectrum of tyrosine while tryptophane absorption is changing much less rapidly, it may be assumed that the difference spectrum at 287 mμ is due to tyrosine with minor modifications due to tryptophane. There is no reason to be surprised that the changes are not parallel, since the tyrosine residues are more polar than tryptophane and may well be in different portions of the molecule. Furthermore it is possible that some of them are involved in hydrogen bonds, which may be disrupted only in the second transition. The fact (not illustrated) that the fifteen tyrosine residue titrate with a common pK of 11.5 (by ultraviolet spectroscopy) may indicate that they are indeed involved in some especially stable organization.

At 0° both optical rotation and sedimentation-velocity and equilibrium studies show only a single transition at pH 4, leading apparently to the same changes in helical structure but to a dimerized form of the molecule. This is reminiscent of the concomitant unfolding and dimerization that have been observed with fumarase, cytochrome c, and other proteins (Reithel, 1963).

The exact interpretation of the optical rotation data is difficult since there is no theoretical justification, with the data available, for one choice of λ_0 over another. While $\lambda_0 = 212$ is used to get the values in Table II, the data appear to fit the Moffitt equation better with a choice of a higher value of λ_0. A higher value, e.g., 218 mμ for λ_0, would make $b_0 = 0$ in urea solution.

The experimental uncertainty in determining a_0 and b_0 from half a dozen points is also great. Nevertheless, it does seem possible to say that the transition from the native state to the low-pH state involves a loss of helical structure which is far less than that which takes place in urea. The small transition which takes place at pH 5.2, 30° in Figure 5 is manifest only by a change in a_0. It is clear that much of the total change in α is due to changes in a_0. If a_0 is a function of the environment of a α-helical regions, it is surprising that the dimer at 2° shows the same a_0 as the monomer at 30° at low pH. Figure 8 is an attempt to show summarily the changes we have observed and the routes by which these changes have been reversed.

A problem remains in explaining the differences between the spectral and rotational data we have presented and those presented by Rosenberg and Lumry (1964). There is very close agreement in both sets of data between the pH of the first transition observed by rotation and that observed by the change in indole spectrum. However, the pH of this transition is higher under our conditions, and it is both larger in proportion to the second transition and farther removed from it than under the conditions used by the others. The major difference in our two conditions was that the work presented here was carried out in 0.1 N KCl while the other data were obtained at much lower ionic strengths. One might expect transitions resulting from charge effects to show marked sensitivity to ionic strength changes. Bücher's early observations (1947) showed that the transition caused by lowering the pH from 5.1 to 4 could be reversed by the addition of $MgSO_4$. Interpreted as an ionic strength effect this would be in the wrong direction to explain our differences, but work being carried out by Dr. David Hanlon in this laboratory has shown that major changes take place in the spectrum of enolase upon addition of activating or inhibiting metal ions to a metal-free solution in KCl. The changes observed with about 10^{-5} M solutions of activating metal ions amount to 20% of the change that takes place in strong acid solution. Inhibitory metal ions cause much greater changes which are in the opposite direction. Our differences, then, may be due either to ionic strength effects or to the presence of divalent ions in one of our preparations.

The striking difference in the spectral changes found in our two laboratories is undoubtedly associated with the differences reflected in the rotational data. One

constructive piece of information resulting from our discrepancies is that we are actually looking at two different things by optical rotation and ultraviolet difference spectra. The tyrosine side chains seem to be so situated that they will become exposed to water with either transition, depending on a delicate balance of factors. It would be interesting to observe the ultracentrifugal behavior of enolase under the conditions used by Rosenberg and Lumry, but charge effects in the lower ionic strength solutions might complicate the interpretation of such data.

It is interesting that no change in rotatory dispersion or ultraviolet spectrum occurs around *p*H 2.7, where the aggregated molecules dissociate. One might expect a change in tryptophyl absorption, for example, but perhaps intermolecular association of hydrophobic groups is replaced quantitatively by intramolecular association.

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Quantitative Study of the Effects of Thyroxine on Components of the Electron-Transfer System*

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The purpose of this work was to examine the effects of thyroxine on several components of the electron-transport system of rat liver. Doses of 0.005, 0.05, 0.1, 0.5, 1.0, and 2.0 mg of L-thyroxine per day were injected into normal white rats for 2 weeks. The highest dose was shown to increase succinoxidase activities of liver homogenates to 165% of the untreated control, succinic-ferricyanide reductase to 177%, succinic–cytochrome c reductase to 230%, cytochrome oxidase to 273%, and coenzyme Q to 279%. NADH–cytochrome c–reductase activity was decreased to 55% of the control value and this decrease is shown to be due to the antimycin A–insensitive microsomal system. These results are in agreement with an effect of thyroxine on protein synthesis which precedes and is responsible for the effect on oxidative metabolism as suggested independently by Tata and by Sokoloff in 1963.

Since the discovery of coenzyme Q (Co Q)[1] by Crane *et al.* (1957), and its involvement in electron transport (Hatefi *et al.*, 1959), there have been several proposals (Hatefi, 1959a, b; Clark and Todd, 1961) suggesting that Co Q might be an intermediate in one of the phosphorylation reactions. Definite proof of a role for Co Q in phosphorylation has thus far been elusive. Recently it has been shown that desiccated thyroid, when fed to rats, could cause dramatic increases in levels of Co Q in the liver (Aiyar and Sreenivasan, 1962; Beyer *et al.*, 1962) as well as smaller increases in other tissues (Beyer *et al.*, 1962). Others have found, however, that at physiological levels of thyroxine no increase could be observed (Pederson *et al.*, 1963).

Our interest in this area stemmed from these observations as well as the report (Leonhauser *et al.*, 1962) that there is a small amount of Co Q in the microsomal fraction of rat liver. The object of this work was to establish a quantitative relationship between the level of thyroxine administered and resulting changes in Co Q level, and to observe any change in the intracellular distribution of Co Q which might result.

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[1] Abbreviations used in this work: Co Q, coenzyme Q; ETP, electron-transfer particle; TSH, thyroid-stimulating hormone.

Experimental

Experimental Material.—Normal white male rats of the Harvard-Wistar strain were placed on experiment