The Reversible Transconformation Processes of Yeast Enolase*

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Studies of the effect of hydrogen-ion concentration and temperature on brewers' yeast enolase (E C 4.2.1.11.) reveal a minimum of five denatured forms. At neutral *p*H heating to 45° produces an irreversible aggregation which can be slowed down by addition of magnesium and phosphate ions. There is a transition centered at *p*H 5 for 25° in which large phenol and indole spectral changes, but very small optical-rotation changes, occur. These changes resemble those occurring during chymotrypsinogen denaturation, but have standard enthalpy and entropy values which are negative, so that the protein denatures on cooling. An explanation for this unusual behavior can be provided on the basis of Brandts' interpretation of chymotrypsinogen behavior (J. F. Brandts, *Federation Proc. 22*, 346 [1963]; *J. Am. Chem. Soc. 86*, in press [1964]). At lower *p*H values there is a *p*H-dependent process, reversible at 25° on base addition, which demonstrates large optical-rotation changes (mainly in a_0 values) and a small additional change in phenol spectrum. It is not reversible at higher temperatures as it is followed by an additional irreversible change characterized by selective reduction in b_0. Thermodynamic quantities for the *p*H 5 transition are $\Delta H^\circ = -20 \pm 5$ kcal/mole; $\Delta S^\circ = -68 \pm 15$ eu/mole. The protein is sensitive to the presence of urea and undergoes a large change, probably unfolding, in 4 M urea, which is known to eliminate catalytic activity.

Yeast enolase is an attractive enzyme for studies of the relationship between protein structure and catalytic function. It has high substrate specificity and low overall free-energy change in its catalytic process, metal ions are involved in the catalytic process, and there are a variety of enolases from different sources which ultimately may be compared. As a protein for studies of reversible transconformation processes, it is attractive because of the absence of cysteine and cystine groups. Furthermore, as will be seen, it demonstrates a variety of "denaturation" reactions, at least one type of which has not been clearly delineated previously. The present study was designed to build on the studies of enolase as an enzyme carried out by Malmström and collaborators (Malmström, 1961) and by Wold and Ballou and collaborators (Brake and Wold, 1962), but with major emphasis on the stability characteristics of the protein, both with an eye to providing further general information about the thermodynamics of protein stability, and to provide the essential base for an investigation of structural involvement of the protein in the catalytic process. Bücher reported a light-scattering change of enolase at low salt in the *p*H region of 5.2 → 4 (Bücher, 1947).

Yeast enolase was prepared from brewers' yeast according to the general procedure of Malmström (1957) with the following modifications. The zone-electrophoretic step of the purification of fraction 1 was replaced by chromatography on DEAE-cellulose.[1] The elution was carried out with Tris-HCl buffer, *p*H 8.2, and an ionic-strength gradient of 10^{-3} −0.25 M in chloride concentration. The elution pattern was similar to that obtained in zone-electrophoresis experiments, but the order of peaks was reversed so that the main enolase fraction was the first major peak. The specific activity was approximately double that obtained with fraction 1. The yield was of the same order as for zone electrophoresis. The next step, selective absorption on the Mg^{2+} form of cation exchanger, was modified to the extent that, instead of sulfocellulose, phosphocellulose was used. The eluting agent was a gradient of citrate buffer of 5×10^{-3} −0.5 M at *p*H = 5.85. The elution pattern was the same as that of Malmström, enolase being the only major component retarded. The solution obtained from this step was dialyzed against pure water and lyophilized. As a rule, the specific activity of the eluate from the last step was within ±10% of the value cited by Malmström (1957). Redissolving the lyophilized product left an insoluble residue. This finding and recent activity determinations (Westhead, 1964) indicate that the pure preparation may contain up to 10% of denatured enolase. Besides enolase purified in the above described manner, enolase purified strictly according to the procedure of Malmström was used for a number of experiments.

METHODS

The ultraviolet-difference spectra were recorded on a Cary Model 11 spectrophotometer equipped with thermostated sample and reference compartments, and a 0–0.10 OD slidewire. The reproducibility of the optical density values was $\pm 1 \times 10^{-3}$ OD units. In order to obtain this degree of reproducibility, the hydrogen arc had to be changed frequently and long (2–3 hour) warm-up time was required. The temperature readings were correct within ±0.05°. The range of protein concentration was 0.2–0.3%. The optical rotation was measured with a Rudolph manual spectropolarimeter 80 Q-200S. The rocking-prism assembly was disconnected and replaced by an a-c line-fed Faraday cell of the type described by J. G. Foss (to be published) constructed to fit into the cell compartment and filled with water. The magnetic fields were around 1000 oersted/cm and gave a symmetrical angle of 2–5° between 800 and 400 mμ. The balance point was determined by minimizing the filtered signal with an oscilloscope as a zero detector. The reproducibility of the measurements was $\pm 1 \times 10^{-3}$ degrees, the accuracy, that of the scale, $\pm 2 \times 10^{-3}$ degrees. The jacketed 2-dm Pyrex cells, used for all the measurements, gave a temperature control of ±0.05°. The range of protein concentration was 0.15–0.20%. The activity measurements were carried out with 2-DL-phosphoglyceric acid prepared according to Kiessling

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[1] DEAE "Selectagel" brand was purchased from Schleicher and Schuell.

TABLE I
CHANGES IN MOLAR ABSORPTION COEFFICIENTS FOR SOME DENATURATION PROCESSES

	No. of Trypto-phans	No. of Tyrosines	$\Delta\epsilon_{294}$	$\Delta\epsilon_{287}$	Ref.
Enolase, *p*H 6 versus 3.8 at 27°	5	15	3200	4400	
Enolase, *p*H 6 versus 3.8 at 20.2°	5	15	3150	4300	
Enolase, *p*H 6 at 20.2° versus enolase, *p*H 3.8 at 50°	5	15	3450	5200	
Enolase at 30° versus 9° (interpolated to cover the whole transition)	5	15	3500		
α-Chymotrypsinogen, *p*H 2.8 at 25° versus 60°	7	4	4800		Brandts and Lumry (1963)
Lysozyme at 25° versus 84° (*p*H 5.4)	6	3	1100		Foss (1961)
Lysozyme at 25° versus 69° (*p*H 2.2)	6	3	1540		Foss (1961)
Aqueous tryptophan versus tryptophan in 75% ethanol			950		Foss (1961)

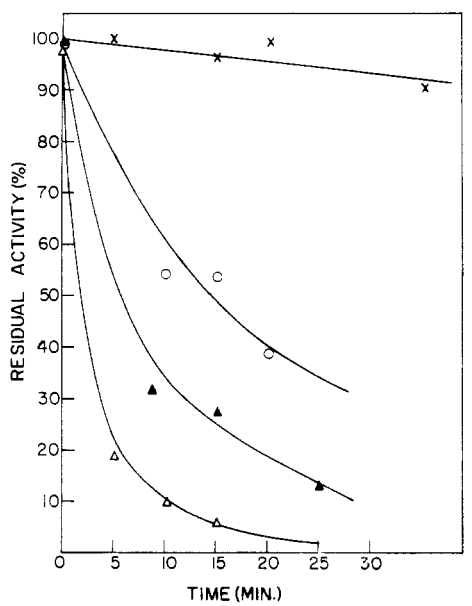

FIG. 1.—The irreversible inactivation of enolase at 44.9° *p*H 7.6. X-X-X, in 0.05 M phosphate buffer in presence of 10^{-4} M Mg^{2+}; O-O O-, in 0.05 M phosphate in presence of 10^{-4} M EDTA; ▲-▲-▲-, in 0.025 M phosphate and 0.5 M KCl; △-△-△, in 0.05 M Tris-HCl in presence of 10^{-4} M EDTA.

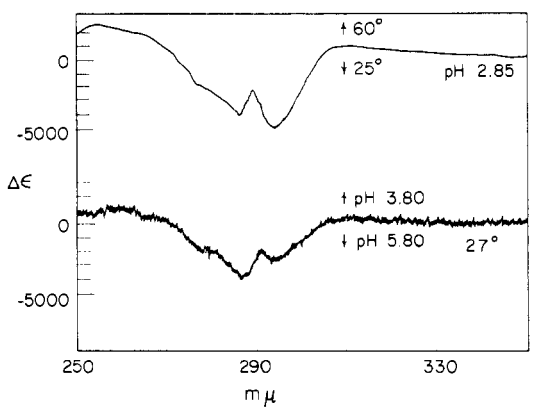

FIG. 2.—The *p*H-difference spectra of enolase compared to the temperature-difference spectra of chymotrypsinogen (Brandts and Lumry, 1963).

(1935) and purified according to Warburg and Christian (1942). The catalytic rate at 25.0° was followed by the change of optical density at 240 mμ and the initial velocity was estimated from the tangent drawn to the curve. The detailed procedure was that given by Malmström (1961).

RESULTS

Thermal Stability.—At neutral *p*H values enolase is unstable at slightly elevated temperatures unless the solution contains anions of phosphate, especially HPO_4^{-2}, and magnesium ions. The data of Figure 1 demonstrate high instability at 44.9° in the absence of either or both of these ions. Solutions of about 2×10^{-6} M protein were incubated at the experimental temperature for the periods of time given on the abscissa of this figure. Aliquots were withdrawn, diluted ten times, and assayed after adding Mg^{2+} to 10^{-3} M under a set of standard conditions for catalytic activity. During incubation of nonstabilized solutions, aggregation resulting in a large increase in light scattering and observable precipitation occurred, even at this concentration of protein. The precipitate could not be dissolved in 1 M urea. Measurements of the indolephenol difference spectrum against a 25° reference solution showed no change prior to the appearance of turbidity. There was also no change in optical-dispersion parameters prior to this condition. It nevertheless cannot be decided whether the aggregates are formed of the native form or that an unfolding occurs prior to aggregation. There is no reason to believe that primary-bond rupture occurred. Irreversibility of this sort we shall term *aggregation metastability* as opposed to *true irreversibility*, which follows the breaking of peptide or other backbone bonds. In cases of aggregation metastability, reversibility can often be obtained by long standing at lower temperature or by addition of weak deaggregation reagents of which urea is a good example. However, no extensive study of the reversibility of enolase aggregation has yet been carried out (but see Westhead, 1964).

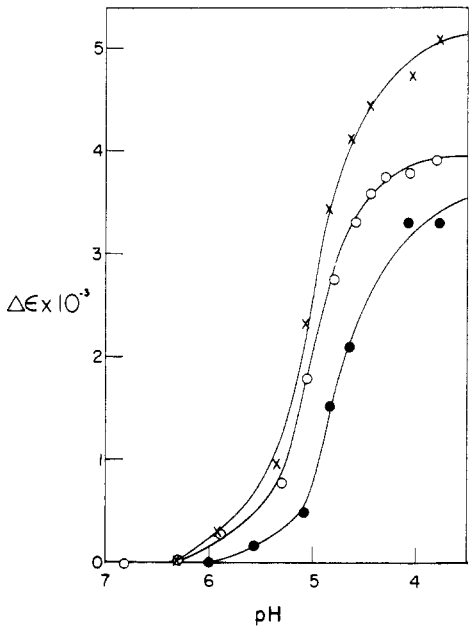

FIG 3.—The difference spectra of enolase at two wavelengths as a function of *p*H, O-O-O, ΔE 294 mμ at 8.6°; X-X-X, ΔE 287 mμ at 8.6°; ●-●-●, ΔE 294 mμ at 27.0°.

Stability as a Function of *p*H.—Stability against hydrogen-ion variations measured by indole-phenol difference spectra was studied in the following way. A dilute solution of enolase (OD at 280 mμ in 1-cm cell = 0.2 to −0.3) in 10^{-3} M sodium acetate buffer (*p*H 6 at 25°) with no added Mg^{2+} was centrifuged for 30 minutes at $10^4 \times g$, then divided into two equal parts. Successive aliquots of 0.01 M HCl were added very slowly to solution A. In a parallel fashion, using the same pipet, aliquots of 0.01 M KCl were added to solution B. After each set of additions the *p*H was recorded. The maximum *p*H shift in solution B was 0.10 unit and was totally unimportant, since there is no change in spectrum of the protein between 6.2 and 5.8. The difference spectra after each pair of additions were recorded immediately and again after 30 minutes. There was no time dependence. Figure 2 gives the *p*H-dependent absorbance changes as changes in molar absorbance coefficients based on an mw of 67,000. In this figure, the difference spectrum between native and thermally denatured chymotrypsinogen is also given (Brandts and Lumry, 1963). The latter spectrum is dominated almost entirely by indole groups and the largest effect is at 294 mμ. The enolase difference spectrum shows the indole difference but also some contribution from changes in phenolic groups which is detectable primarily at about 287 mμ. Several values of the total change in molar absorbance coefficient obtained in denaturation process have been collected in Table I. Data from the thermal denaturation of chymotrypsinogen and lysozyme and the transfer reaction for tryptophan from organic to water solvents are consistent with the interpretation of Yanari and Bovey (1960) that changes in spectrum are predominantly due to changes in the polarizability of the local environment and have been interpreted in this way. This is, of course, a tentative interpretation but suggests conformation changes of either local or more general nature. Enolase demonstrates a dramatic change in indole-phenol spectrum in the *p*H region from 4.5 to 5.5, with a total change in molar absorbance coefficient of 3180 (Fig. 3). The comparisons available in Table I show that this change is of the same order of magnitude as those observed in the reference processes.

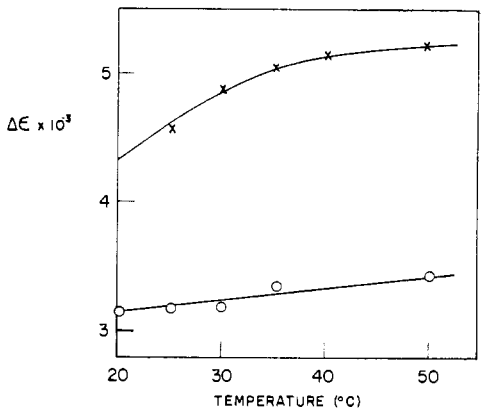

FIG. 4.—The difference spectra of enolase at two wavelengths as a function of temperature. The *p*H was 3.5. O-O-O, ΔE 294 mμ; X-X-X, ΔE 287 mμ.

The enolase spectral changes at 294 and 287 mμ are in phase during the enolase transition caused by *p*H reduction down to *p*H 4. Both give the same mass-law curve with a second-order dependence on hydrogen-ion activity (Fig. 3). At *p*H values below 4, however, phenol-group changes suggested the occurrence of an additional denaturation process at low *p*H values. This possibility was tested in the following experiment. After the *p*H was brought to 3.5 during a standard isothermal experiment, temperature was gradually increased. Figure 4 presents the results of one such experiment. These results show that the transition producing a change in the indole spectrum is over at *p*H 3.5, but that there is a small additional change in the phenol spectrum which is dependent on temperature as well as *p*H. Further evidence supporting the existence of this additional phenolic change appeared in other experiments as discussed in succeeding paragraphs.

The denaturation process following acidification to *p*H 4 and centered at *p*H 5 will be known as *transition I*. The process at lower *p*H values observable only with phenolic spectral changes will be known as *transition II*.

Transition I is totally reversed on neutralization of the protein solutions with dilute base. Base addition must be carried out slowly to avoid turbidity and presumably aggregation. Nevertheless, even when all the base is added quickly and marked turbidity occurs, the turbidity disappears within 1 hour and the protein solution has the usual properties at *p*H 6. The relatively slow dissolution of the catalytically inactive aggregates responsible for the turbidity can be followed by measuring enzymic activity at *p*H 7.6. The lag period observed in this kind of experiment follows closely the loss of turbidity. These aggregates probably consist of protein not yet reversed from transition I and appear to be quite different from those obtained on heating solutions of native enolase at neutral *p*H conditions.

Optical rotatory dispersion studies as a function of *p*H confirm the existence of two distinct *p*H-dependent transitions. The specific rotation at 600 mμ as a function of *p*H is given in Figure 5. Transition I produces relatively small changes in specific rotation even though the largest changes in difference spectrum occurred in this transition. Transition II, on the other hand, produces nearly a 40-degree change in specific rotation. On addition of base a small hysteresis effect is detectable. The hysteresis appears to be an artifact due to a small amount of reversible aggregation. It is obvious that transition II is also reversible.

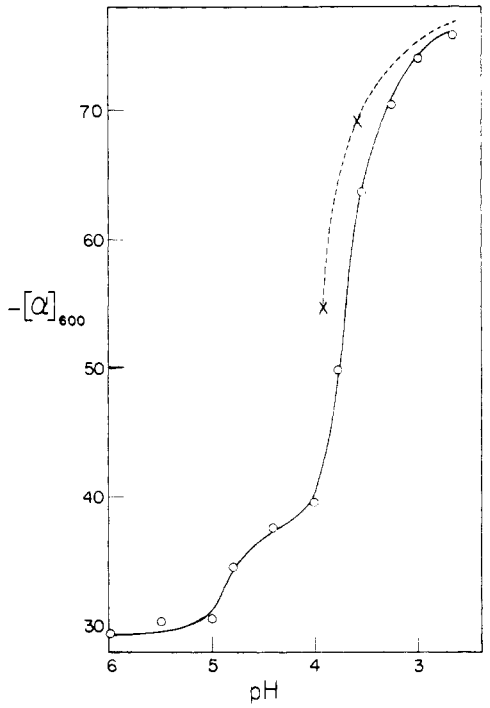

FIG. 5.—The specific rotation at one wavelength as a function of *p*H. Temperature 23.0°; concentration of protein, 0.25%. ○-○-, the *p*H is lowered; X-X-, the *p*H is readjusted with dilute NaOH.

TABLE II
MOFFITT-EQUATION PARAMETERS AT $\lambda° = 212$ mμ, NO BUFFER

*p*H	a_0	b_0	$t°$
6.3	−146	−140	19.6
4.25	−183	−153	19.7
3.05	−303	−125	23.0

The a_0 and b_0 parameters of the Moffitt equation (Urnes and Doty, 1961) are given for selected *p*H points in Table II. Transition I occurs between *p*H 6.3 and 4.05 and is seen to produce no change within the limits of error in determination of either a_0 or b_0. Transition II produces the values measured at *p*H 3.05. Both Moffitt parameters change, but the largest effect is in a_0. The b_0 value changes slightly, indicating only a minor reduction in the low helix content of this protein, which may be estimated from b_0 as 25%.

Temperature Dependence of Transitions I and II.—Although the high precision required for detailed studies of the thermodynamics of reversible protein denaturation was not obtained in this work, good estimates of average enthalpies and entropies of denaturation are possible. These averages are based on the assumptions that there are only two states in each process, the lower-temperature state and the upper-temperature state, that the heat-capacity changes are small, and that the temperature dependencies of the lower and of the upper state are given by thermal behavior below and above the transition, respectively. When possible, linear extrapolation of the thermal behavior of the pure species into the transition range is made in the usual way (Brandts and Lumry, 1963). Although heat-capacity effects are large in the thermal-denaturation reactions which have been studied in detail (Brandts, 1963, 1964) the average values of enthalpy and entropy of denaturation given by the present kind of analysis are of considerable utility.

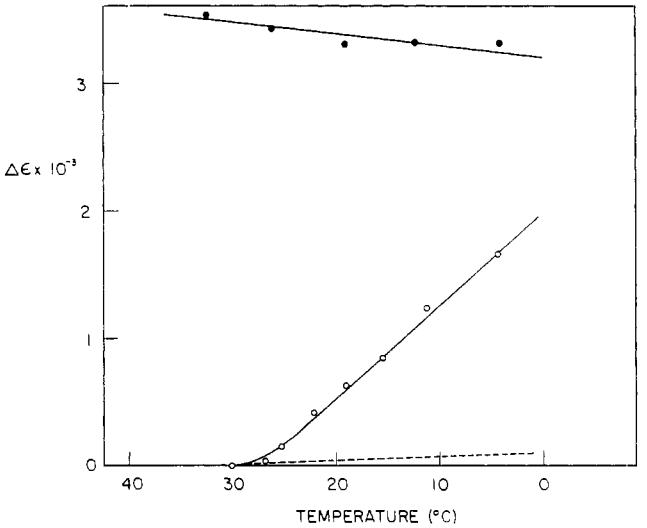

FIG. 6.—The difference spectra of enolase at 294 mμ as a function of temperature, ○-○-○, *p*H 4.98; ●-●-●, *p*H 4.0; - - - -, estimated temperature dependence at *p*H 6.0.

Thermal denaturation was followed by spectral changes at 294 mμ. In Figure 3 data are given for the *p*H dependence of transition I at two temperatures.[2] The most striking conclusion to be drawn from the curves at the two temperatures is that at any *p*H in the transition region the native form of the protein is more stable at 27° than it is at 8.6°. The temperature dependence thus demonstrated is small, but it is in the opposite direction to that usually observed in thermal denaturation studies (Brandts and Lumry, 1963; Brandts, 1963; Kunitz, 1948). If it be assumed that unfolding is the source of changes in local polarizability and thus in indole spectrum, lowering the temperature at a fixed *p*H causes the protein to unfold. A quantitative investigation of this unusual behavior is made difficult by the occurrence of ireversible thermal denaturation at low *p*H values whenever the temperature exceeds about 30°. Studies in the temperature range below 4° are technically impossible so that the temperature range is highly limited. Temperature data could be obtained over only half the transition. The results of a typical experiment are given in Figure 6. The native form, i.e., the stable form at neutral *p*H, was brought to *p*H 4.98 by slow addition of HCl. The solution was then cooled to 4° and in this change approximately one-half transition I takes place. At 4° the *p*H is brought to 4.0, under which conditions the total of transition I has occurred. The protein solution is then slowly reheated to 32°. No reversal of transition I occurs in the last process, so that the observed spectrum measures the temperature dependence of the denatured form at *p*H 4 and this dependence is assumed to be the same as that at *p*H 4.98. Unfortunately, it is not possible to determine the temperature dependence of the native form at *p*H 4.98 with any precision. The dashed line shows the estimated dependence. Acetate buffer was used and no corrections were necessary for the variation of *p*H with temperature. Figure 7 is the van't Hoff plot of the equilibrium constants obtained from Figure 6. The point at 32° is very sensitive to the assumed temperature dependence of the native form, but the other points are not. The value of $\Delta H°$ obtained from Figure 7 is −20 kcal/mole of protein with estimated maximum errors

[2] The usual corrections for changes in optical density due to volume changes on heating or cooling were made. In this case, these changes are in an opposite direction to those produced by denaturation.

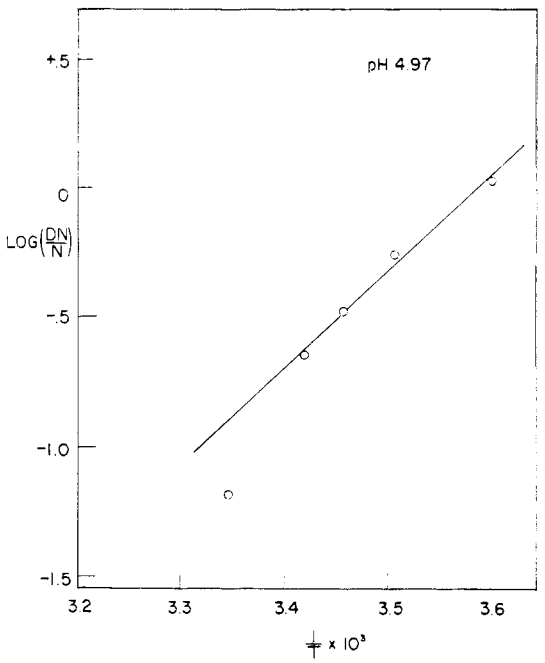

FIG. 7.—The van't Hoff plot of temperature dependence of the first transition at *p*H 4.97. DN/N expresses the concentration ratio of the two forms obtained from ΔE 294 mμ.

of ±5 kcal. The value of $\Delta S°$ is −68 eu/mole of protein with estimated maximum errors of ±15 eu. Additional experiments provided values of the thermodynamic quantities well within the estimated maximum errors. These values are determined at constant *p*H rather than constant degree of protonation.

The temperature dependence of transition II at *p*H 4.2 was studied using optical rotation at 550 mμ. The experiments were not successful. It has previously been shown that this transition is reversible with *p*H change. It was not found to be readily reversible with temperature change. The data given in Figure 8 were obtained by heating for approximately 30 minutes at each temperature, but the reaction did not come to an equilibrium point at any temperature. Hence the reaction is experimentally irreversible and the apparent mass-law appearance of the curve is misleading. Return from higher temperatures did not occur on slow cooling, even after 7 days. The solution remained remarkably clear, with no detectable light scattering and no precipitation. The values of the Moffitt parameters before and after heating are given in Table III. The change in a_0 corresponds to the difference in a_0 obtained on reducing *p*H at 25° (Table II), and suggests that the *p*H-dependent transition II as characterized by a_0 is identical with the thermal transition. However the additional reduction in b_0 values indicates that the thermally denatured form has undergone some loss of helix which is not

TABLE III
MOFFITT-EQUATION PARAMETERS AT $\lambda°$ = 212 mμ

Buffer	*p*H	$t°$	a_0	b_0
Acetate, 0.05 M	4.25	19.4	−151	−143
Acetate, 0.05 M	4.20	47.5	−311	− 87
Tris-HCl, 0.05 M	7.60	19.6	−143	−136
4 M Urea + 0.05 M Tris-HCl	7.60	19.6	−490	−26

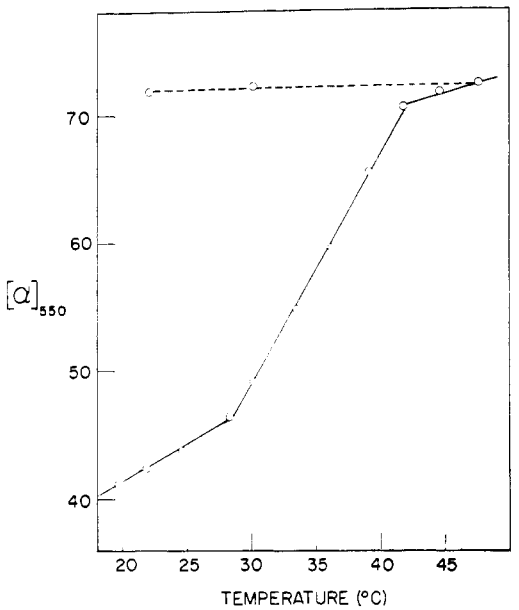

FIG. 8.—The specific rotation of enolase at *p*H 4.25 as a function of temperature. 0.25% protein in 0.05 M acetate buffer. - - - - -, the points obtained during the subsequent cooling of the solution.

reversible. This additional change is labeled *transition III*.

Stability in the Presence of Urea.—Specific rotation was measured in urea solutions. The results in Figure 9, obtained at *p*H 7.60 with 0.2% protein, show that the protein is unstable in urea solutions of relatively low concentration compared with some other proteins which show denaturation only at 7 M urea or higher. It has been shown previously (Malmström *et al.*, 1963) that enolase loses its specific ability to bind the activating metal ion in 4 M urea. The reversibility of the urea effect was not determined in detail. A simple dialysis against water and dilute buffer resulted in precipitation. The Moffitt parameters at *p*H 7.60 for urea and control solutions are given in Table III. The change in specific rotation is slightly greater than occurs in thermal transition II. The Moffitt parameters experience larger changes on urea addition at *p*H 7.60 than they do during transition II, as is shown in this table. The approximate correction for solvent polarizability has been made for the urea experiments. Insofar as this correction adjusts for the difference in solvents, a definite difference in a_0 exists, the value in 4 M urea being 187° more negative than the value after transition II. As compared to transition III, b_0 has changed in both cases so that urea-denaturation product resembles most closely the *p*H 3, 50° form (product of transitions I → II → III).

DISCUSSION

Forms of Enolase.—In the course of this investigation it was possible to recognize six forms of yeast enolase. These are itemized in Table IV and their interconversions are shown in Figure 10. Some forms result from aggregation, others from changes in conformation or character of the conformation. Great care was taken in the preparation to assure that the protein samples contained primarily Malmström's species "a" (Malmström, 1957), since raw enolase consists of several forms. We have no evidence for heterogeneity and may conclude at least tentatively that the forms are unique stages of enolase "a."

Form B is an aggregate and probably a metastable

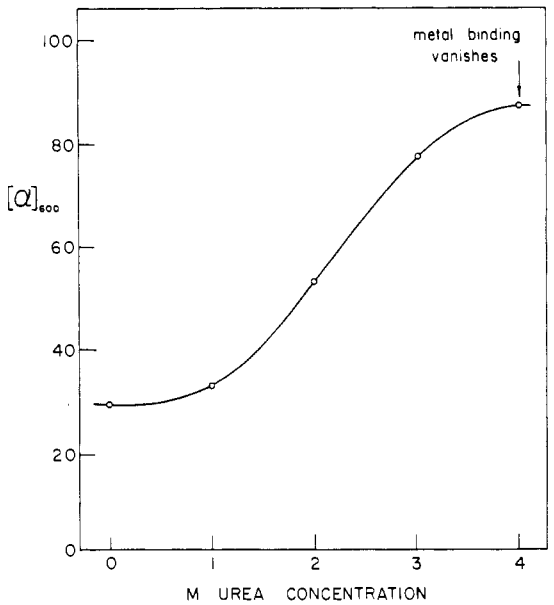

FIG. 9.—The specific rotation of enolase at *p*H 7.6 in Tris-HCl buffer as a function of urea concentration. Temperature 25°.

TABLE IV
FORMS OF YEAST ENOLASE a

(A)	Neutral *p*H, room temperatures, catalytically active
(B)	Neutral *p*H, high temperatures, catalytically inactive, metastable aggregation
(C)	*p*H 4.5, room temperature, catalytically inactive, large indole change, very small rotation change, negative temperature coefficient of stability
(D)	*p*H 3, room temperature, catalytically inactive, tyrosine change, large $a°$ change, little $b°$ change
(E)	*p*H 3, 50°, catalytically inactive, irreversible, no high state of aggregation, $a°$ comparable to (D), $b°$ considerably down
(F)	*p*H 6, room temperature, reversible aggregation of transition I material (form C in metastable state)

aggregate, though we did not succeed in reversing aggregation. It is not surprising that magnesium, which is a cofactor for the catalytic process, stabilizes the native form (A) with respect to the transition to form B. Similarly, substrates of enolase bear phosphate groups which form one valence interaction point with the protein and might be expected also to stabilize form A. If the binding sites for phosphate and magnesium ions disappear on conversion to form B, both substances must stabilize form A. Destabilization against temperature effects has also been reported to be a consequence of specific binding in some other proteins (Caravaca and Grisolia, 1959).

Transition I.—Form C is produced by transition I. Form C resembles the reversible thermally denatured forms of chymotrypsinogen (Brandts and Lumry, 1963) and α-chymotrypsin (Schellman, 1958; Brandts and Lumry, 1961) in that its formation from form A is associated with large difference spectra but small optical-rotation changes. Although it is quite clear that large changes take place in these proteins on heating, unfortunately the description of these changes is not yet clear. An analysis by Brandts (1963) based on a model of extensive unfolding gives remarkable quantitative agreement with experiment and thus suggests gross unfolding. On the other hand, the small changes in viscosity and optical rotation suggest rather limited unfolding. If enolase undergoes the same kind of reversible thermal denaturation as chymotrypsinogen,

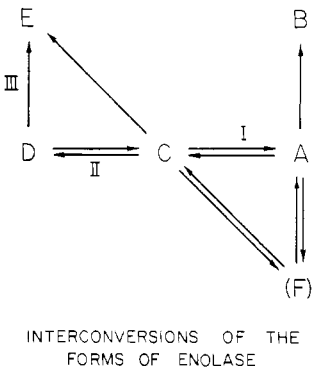

FIG. 10.—A schematic representation of the relations between the different forms of enolase (Table IV).

and measured parameter changes do indicate that this is the case, the following interpretation of the thermodynamics quantities is possible using Brandts' results from chymotrypsinogen studies (Brandts, 1963, 1964).

The right side of Figure 11 is the empirical behavior of the standard free energy of denaturation at *p*H 3 of chymotrypsinogen, which in the temperature range from about 20° up denatures reversibly on heating. Because the conformational changes involved are linked to changes in degree of protonation of a few linked acid groups and because of minor electrostatic repulsion the position of the curve of Figure 11 varies with *p*H, though all such curves have the same shape at the same temperature. The slope of the curve is the standard entropy change and varies rapidly with temperature as the standard enthalpy changes. From this variation the heat capacity changes on denaturation can be determined to allow calculation of the left part of the curve. The curve then demonstrates a minimum point (*a*) which is the point of maximum stability. The standard entropy change is of course zero at this point. To the right of this minimum along *abc* is the path of denaturation of chymotrypsinogen under most conditions of solvent and *p*H, the half-denaturation point lying at *b* where $\Delta F°$ is zero. The heat capacity effects are so large that the curve extended to the left rises along *ade*. The point *d* is the half-denatured point reached by cooling from *a*, and *e* is fully denatured protein essentially the same as *c*. It is path *ade* which appears to be appropriate for transition I, and providing suitable experimental conditions can be found it will be possible to establish this explanation by producing form C via a heating process along *abc*.

The negative values of $\Delta H°$ and $\Delta S°$ for transition I are consistent with predictions based on chymotrypsinogen data. The values of these quantities are not large when compared with $\Delta H°$ = 130 kcal/mole and $\Delta S°$ = 400 eu/mole for chymotrypsinogen denaturation at *p*H 3 (Brandts and Lumry, 1963; Eisenberg and Schwert, 1935) and suggests a much smaller protein change in transition I than occurs in chymotrypsinogen. However, small values may be quite misleading in this respect. It will be noticed in Figure 11 that if point *a* lies close to $\Delta F°$ = 0, $\Delta S°$ and $\Delta H°$ will be small. It is the slope of the $\Delta F°$ curve as it crosses the zero line which determines the magnitude of the average experimental thermodynamic quantities, and not the magnitude of the denaturation process. Thus at *p*H 3 the curve for chymotrypsinogen is very steep at the zero line. For enolase the curve at *d* is not steep yet we cannot say that the transition is of less magnitude than the chymotrypsinogen transition simply because the $\Delta S°$ and $\Delta H°$ are smaller. Only a full determination of these quantities as a function of *p*H, temperature, and solvent composition can establish this fact.

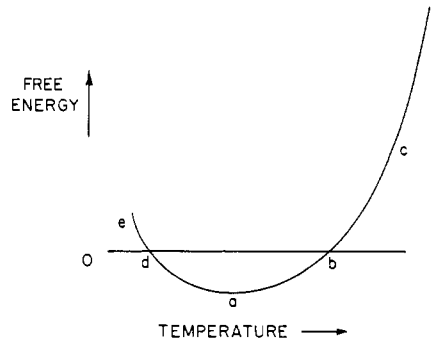

FIG. 11.—A schematic representation of the standard free energy for the conversion of native protein to denatured protein as function of temperature from Brandts (1963).

According to Brandts, the large heat capacities are owing primarily to factors of hydrophobic bonds. The precise description of the curvature of the $\Delta F°$ plot and its position with respect to the zero line must then be determined by the composition of the protein, the nonpolar groups being responsible for the curvature and the net free-energy balance of all stabilizing and unstabilizing factors establishing the vertical and horizontal position of the curve.

Transition II.—The absence of reversibility at low *p*H in transition II following its production by heating is due to the concomitant occurrence of transition III and not an intrinsic characteristic of transition II itself, as shown by the reversibility with *p*H at low temperature. Transition II shows a large a_0 change and only a phenol difference spectrum. Changes in a_0 without concomitant changes in b_0 are currently thought to indicate a change in the polarizability of the environment local to asymmetric carbon atoms, or a change in freedom of motion of groups vicinal to such carbon atoms, or both. The distribution of polar groups near the peptide carbonyl may also influence a_0, though this matter is not yet clear. The direction of the change in a_0 is that also observed when the protein is dissolved in 4 M urea, a solvent additive generally known to cause protein unfolding. Hence, it is probable that there is considerable unfolding during transition II with an increase in freedom of motion of vicinal groups and an increase in exposure of side chains and backbone to water. The interpretation of the transition II is further complicated by aggregation shown to appear during the second transition. The association phenomenon was described by Westhead (1964) in a companion study to this. The results of the two studies where they are from similar experiments are in excellent quantitative agreement, although Westhead used bakers' yeast as his enolase source. Distinct quantitative differences between the papers occur in transitions I and II, and transition III does not appear in Westhead's study. Preliminary experiments here suggest that the differences are largely due to differences in trace amounts of divalent metal ions. For example, on the addition of Zn^{2+} the shape of the difference spectra changed and the ΔE 287 mμ and ΔE 294 mμ changes no longer occurred in phase. The entire transition was shifted toward higher *p*H values (Westhead, 1964). It is also possible that the two enzymes had different degrees of amidation. Further work will have to be carried out to reconcile the differences observed. The preparative treatment we used for enolase should have reduced the concentration of divalent cations to trace levels but a specific study of the dependence of transitions I and II on ion concentrations is called for.

Conclusion.—No other reversible denaturation process of a well-defined protein produced by cooling appears to have been established. It nevertheless seems probable that low-temperature denaturation reactions will prove to be quite common since the thermodynamic basis upon which such transitions rest has already been established experimentally by Brandts and there is no reason to believe that the behavior of $\Delta S°$ and $\Delta H°$ with temperature will vary qualitatively from protein to protein. Enolase, having no disulfide bridges, may indeed be less stable than chymotrypsinogen, for example, which fact could explain the closeness of point *a* to the zero free-energy line and our ability to detect denaturation on cooling.

It is interesting to observe that transition I involves primarily a spectral change whereas transition II produces primarily an optical-rotation change. Similar behavior was suggested by Foss (1961) for lysozyme but was not found following the elimination of experimental artifacts (J. G. Foss, personal communication).

The reversibility of transitions I and II is another example of the well-known importance of free energy as the determinant of protein conformation (Lumry and Eyring, 1954). These reactions are particularly promising for further study because of the absence of disulfide complications. It is to be expected that all the conformation processes reported here will be found to be reversible leading to the fully active enzyme. In some cases reversibility has already been established by Westhead (1964). In any event it is clear that a variety of conformation changes occur and that there is no single all-or-none unfolding process from native protein to random coil.

REFERENCES

Brake, J. M., and Wold, F. (1962), *Biochemistry 1*, 386.
Brandts, J. F. (1963), *Federation Proc. 22*, 346.
Brandts, J. F. (1964), *J. Am. Chem. Soc. 86* (in press).
Brandts, J. F., and Lumry, R. (1961), *J. Am. Chem. Soc. 83*, 4290.
Brandts, J. F., and Lumry, R. (1963), *J. Phys. Chem. 67*, 1484.
Bücher, T. (1947), *Biochim. Biophys. Acta 1*, 467.
Caravaca, J., and Grisolia, S. (1959), *Biochem. Biophys. Res. Commun. 1*, 94.
Eisenberg, M. A., and Schwert, G. W. (1935), *J. Gen. Physiol. 18*, 43.
Foss, J. G. (1961), *Biochim. Biophys. Acta 47*, 561.
Kiessling, W. (1935), *Ber. 68*, 243.
Kunitz, M. (1948), *J. Gen. Physiol. 32*, 241.
Lumry, R., and Eyring, H. (1954), *J. Phys. Chem. 58*, 110.
Malström, B. G. (1957), *Arch. Biochem. Biophys. 70*, 58.
Malström, B. G. (1961), *Enzymes 5*, 471.
Malström, B. G., Rosenberg, A., and Lindskog, S. (1963), *Proc. Intern. Congr. Biochem., 5th, Moscow (1961) 4*, 172.
Schellman, J. A. (1958), *Compt. Rend. Trav. Lab. Carlsberg (Ser. Chim.) 30*, 450.
Urnes, P., and Doty, P. (1961), *Advan. Protein Chem. 16*, 401.
Warburg, J. O., and Christian, W. (1942), *Biochem. Z. 310*, 384.
Westhead, E. (1964), *Biochemistry 3*, 1062 (this issue).
Yanari, S., and Bovey, F. A. (1960), *J. Biol. Chem. 235*, 2818.