# Structural Characteristics of Dehydrogenases*

LUDWIG BRAND,† JOHANNES EVERSE, AND NATHAN O. KAPLAN

*From the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts‡*

*Received September 11, 1961*

Fluorescence and optical rotation methods have been used to investigate the stability of a number of dehydrogenases. Horse liver alcohol dehydrogenase is more stable to heat and urea treatments than the corresponding enzyme from yeast. Beef heart muscle lactic dehydrogenase is more stable than beef skeletal muscle lactic dehydrogenase. There is good agreement between loss of protein structure, as measured by protein fluorescence and optical rotation analyses, and loss of enzyme function. Chicken heart lactic dehydrogenase is also more stable than chicken muscle lactic dehydrogenase. Urea at certain concentrations can bring about loss of enzymatic activity of horse liver alcohol dehydrogenase *without* concomitant changes in protein structure, as indicated by the finding that there is an increase in fluorescence yield of DPNH when it is bound to the horse liver enzyme in the presence of urea. It is suggested that urea may act in this case by complexing with the active site. Denaturation of the horse liver alcohol dehydrogenase results in the loss of capacity to bind DPNH.

It has been possible to show differences in pyridine nucleotide–linked dehydrogenases catalyzing the same activity from different sources, through characterization of the enzymes by catalytic, immunologic, and electrophoretic techniques. Differences can also be shown, in some instances, by determination of molecular weight as well as by determination of the number of moles of zinc found in the crystalline enzymes (Vallee, 1960a). The heterogeneity of the dehydrogenases is further indicated by differences in their capacity to associate with pyridine nucleotides.

Recent investigations employing optical rotation and fluorescence analyses have focused attention on the importance of the secondary and tertiary structures of enzymes for their catalytic activities. In an attempt to show physical differences between the same enzyme from different sources, we have compared the alterations in protein structure with the catalytic changes brought about by the action of various denaturing agents. In this paper we will refer to denaturation as a change in protein structure accompanied by a loss of enzymatic activity.

## EXPERIMENTAL

*Enzyme Preparations.*—Yeast alcohol dehydrogenase was the commercial preparation obtained from Boeringer. It was dialyzed for 24 hours against 0.1 M phosphate buffer, *p*H 7.4. A value of $1.89 \times 10^5$ cm²/mole was assumed as the extinction coefficient of this enzyme. The molecular weight was assumed to be 150,000.

Horse liver alcohol dehydrogenase was the commercial preparation obtained from Boeringer. It was dialyzed for 24 or 48 hours against 0.1 M phosphate buffer, *p*H 7.4. The remaining insoluble precipitate was removed by centrifugation. A value of $0.332 \times 10^5$ cm²/mole was assumed for the extinction coefficient of this enzyme. The molecular weight was assumed to be 84,000.

The beef heart muscle and beef skeletal muscle lactic dehydrogenase as well as the chicken lactic dehydrogenase were isolated in this laboratory by Dr. R. McKay. The isolation procedures will be published in a future communication. Before these enzymes were used, they were dialyzed against 0.1 M phosphate buffer at *p*H 7.4. The extinction coefficient of beef heart lactic dehydrogenase was assumed to be $2 \times 10^5$. The molecular weight was assumed to be 135,000.

*Fluorometric Measurements.*—The Aminco-Bowman spectrophotofluorometer was used for most of the fluorescence measurements. It is equipped with an XBO 150-watt Osram Xenon-Arc and a constant-temperature cell holder. All readings with this instrument were obtained manually. Except where otherwise indicated measurements were taken at 22°. Fluorescence is indicated in arbitrary units for each experiment.

Fluorescence polarization spectra were carried out with a spectrophotofluorometer built according to the general plan of Weber (1960). The light source was an XBO 150-watt Osram Xenon-Arc. The diffraction grating was a 500-mm Bausch and Lomb grating monochromator. The fluorescence emission was observed at right angles to the exciting light. The detector was a 14-stage RCA (7265) photomultiplier with an S-20 response. The fatigue characteristics of this tube limit its use to measurements of very low light intensity. The signal from the detector was amplified by a General Radio DC amplifier type 1230-A and fed into a Minneapolis-Honeywell "Electronik" high-speed strip chart recorder

* Aided in part by grants from the American Cancer Society (P-77) and the National Institutes of Health (CY-3611).

† Fellow of the National Foundation. Present address: Department of Biophysics, Weizmann Institute of Science, Rehovoth, Israel.

‡ Contribution no. 152.

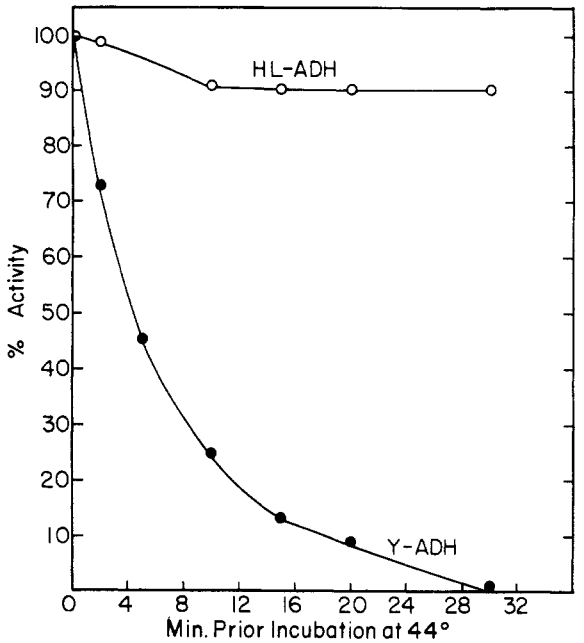


FIG. 1.—The effect of prior incubation at 44° on activity of yeast and horse liver alcohol dehydrogenase. The assays were done as described in the Methods section. The incubation was carried out in 0.05 M glycine buffer (*p*H 9.4).

with a full scale deflection of 1 or 10 mv. The supply voltage for the phototube was obtained with a Northeast Scientific Corporation Regulated High Voltage Power Supply, 0.5 to 3 kv DC. The supply voltage for the light source was obtained from a Sola DC power supply. This unit gave 19.8 v at 7.5 amps. It had to be modified to remove a considerable 120-cycle ripple that interfered with the amplification of the final signal by the recorder.

*Optical Rotation Measurements.*—Optical rotation measurements were carried out with a Rudolph Photoelectric Spectropolarimeter. This instrument was equipped with a General Electric water-cooled mercury arc lamp (A-H6). Good lamp stability was obtained by assuring a high flow rate of cooling water. Only distilled water was used as a coolant, and this was changed frequently to avoid contamination.

All measurements were taken at 22°.

*Denaturing Experiments.*—In general denaturing experiments were carried out at a protein concentration of 1.25 mg per ml. The enzyme was then diluted into the assay mixture, which contained no denaturing agent, for the appropriate assay.

*Assays.*—Horse liver alcohol dehydrogenase was assayed as follows: The sample cuvet contained 0.067 M ethanol, $4.7 \times 10^{-4}$ M DPN, enzyme, and glycine buffer, 0.05 M, *p*H 9.4, in a final volume of 3 ml. The enzyme, treated as described in the text, was added to the reaction mixture at time zero and spectrophotometric readings were taken at 340 mμ every 15 seconds. The assays were carried out at room temperature.

Yeast alcohol dehydrogenase was assayed as follows: The sample cuvet contained 0.335 M ethanol, $4.7 \times 10^{-4}$ M DPN, enzyme, and glycine buffer, 0.05 M, *p*H 9.4, in a final volume of 3 ml. The enzyme, treated as described in the text, was added to the reaction mixture at time zero and spectrophotometric readings were taken every 15 seconds. The assays were carried out at room temperature.

The lactic dehydrogenases were assayed as follows: The sample cuvet contained $2 \times 10^{-4}$ M sodium pyruvate, $1.9 \times 10^{-4}$ M DPNH, and phosphate buffer, 0.10 M, *p*H 7.0, in a total volume of 3.0 ml. The enzymes, treated as described in the text, were added at time zero, and spectrophotometric readings were taken every 15 seconds. The assays were carried out at room temperature.

## RESULTS

*Heat Inactivation of Alcohol Dehydrogenases.*—The effect of temperature of the enzyme activity of yeast and horse liver alcohol dehydrogenase is shown in Figure 1. The enzymes were first incubated at 44° for various lengths of time and then rapidly cooled to 0°. Enzyme assays were then carried out at room temperature. After incubation for 30 minutes the yeast alcohol dehydrogenase was almost completely denatured, whereas the liver enzyme retained most of its activity.

Figure 2*A* shows the effect of increasing temperature on the two alcohol dehydrogenases. The yeast enzyme loses most of its activity by 52°. The liver enzyme, however, retains its activity but begins to precipitate at this temperature.

The decrease in fluorescence intensity (Fig. 2*B*) and the shift in maximum of fluorescence emission (Fig. 2*C*) during the heat inactivation of yeast alcohol dehydrogenase are also shown. The change in emission maximum has been plotted in energy units (kcal/mole) with the relation $E = h\gamma$. It can be seen that the shift occurs quite sharply at about 36°. This is the same temperature at which the enzyme activity falls precipitously. The heat denaturation of liver alcohol dehydrogenase could not be followed by fluorescence measurements because the light scattering caused by precipitation interfered with the measurements.

Heat denaturation of yeast alcohol dehydrogenase as measured by optical rotation is shown in Figure 3. The sharp break in the specific rotation values occurs between 35° and 40°, the same temperature range where the enzymatic activity and fluorescence measurements are also markedly changed.

*Effect of Urea on Alcohol Dehydrogenases.*—The effect of urea on yeast and liver alcohol dehydrogenase is shown in Figure 4. Both activity and protein fluorescence measurements are shown. The enzymes were incubated at a concentration

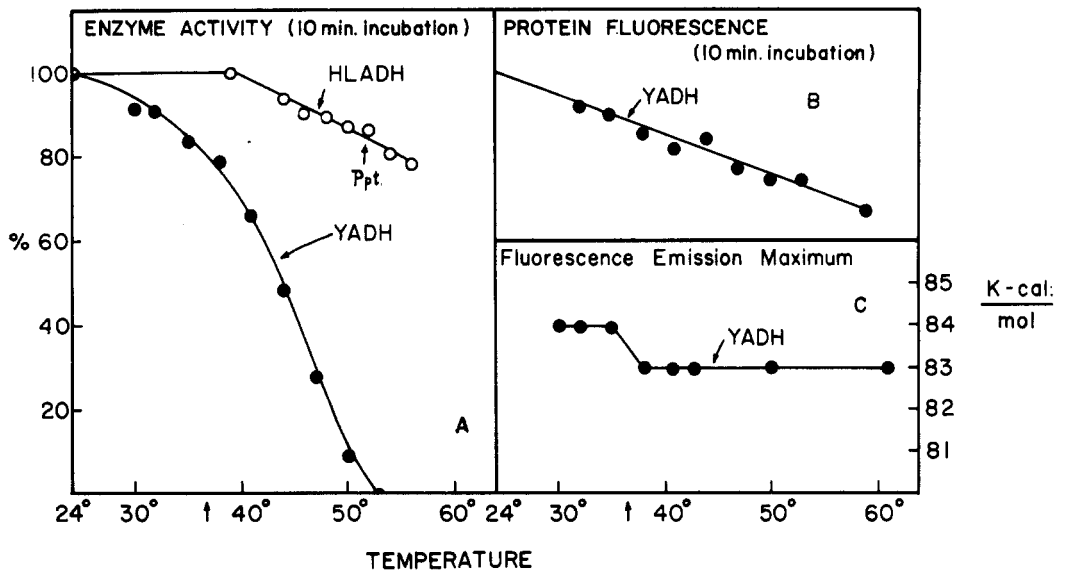


FIG. 2.—The effect of increasing temperature on alcohol dehydrogenase. Experiments were carried out in 0.05 M glycine buffer at *p*H 9.4. The protein was excited at 280 mμ to measure the fluorescence.

of 1.25 mg per ml of 0.1 M phosphate buffer at *p*H 7.0. The incubation time was 15 minutes at 30°. After the denaturation the enzymes were diluted into the assay mixtures that contained no urea. The enzyme assays were carried out in glycine buffer as already described. For the fluorescence measurements the proteins were excited at 280 mμ and the emission measured at 340 mμ.

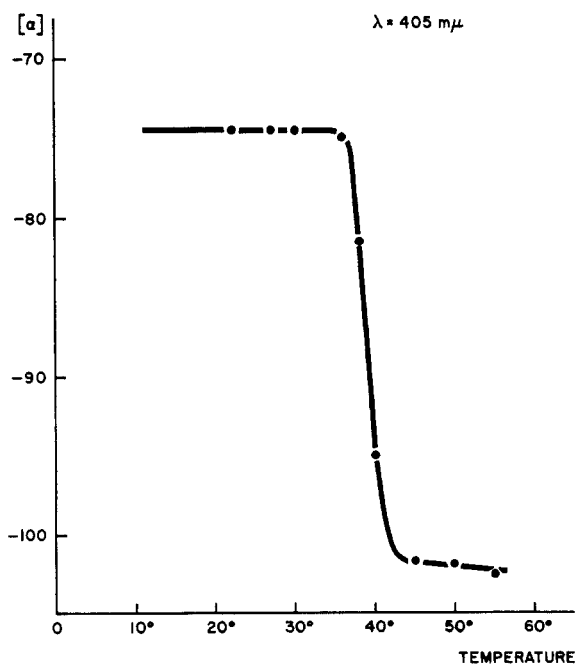


FIG. 3.—Heat denaturation of yeast alcohol dehydrogenase as measured by optical rotation at 404.7 mμ. Conditions were the same as in Figure 2.

As in the temperature studies, the yeast enzyme was shown to be less stable than the liver enzyme, becoming denatured at lower concentrations of urea. The liver protein loses its activity and fluorescence mainly at concentrations of urea greater than 4 M. The total fluorescence change during denaturation is greater for the liver alcohol dehydrogenase than for the yeast alcohol dehydrogenase. The latter enzyme contains more tryptophans, and it is possible that these are affected in different ways by unfolding of the molecule. Quenching of fluorescence of some tryptophans may be compensated for by enhancement of fluorescence of other tryptophan moieties. Cases of enhancement of quantum yield of fluorescence by tryptophan have been observed on denaturation of several proteins (Teale, 1960).

As in the case of the temperature studies it was of interest to study the urea denaturation by more than one technique. An examination of Figure 5 shows the effect of urea on these two enzymes as measured by optical rotation at 435.8 mμ and protein fluorescence. In these experiments the urea was not dialyzed or diluted out before assay. The two measurements show a good correlation.

*Urea and Heat Studies with Lactic Dehydrogenases.*—A considerable amount of work from many laboratories has shown differences between lactic dehydrogenases isolated from a variety of tissues (Gregory and Wroblewski, 1961; Kaplan *et al.*, 1960). It was therefore of interest to determine whether there are any differences in their denaturation properties.

Figure 6 shows the great differences in susceptibilities to heat of two lactic dehydrogenases—one from beef heart muscle and the other from beef skeletal muscle. In these experiments the

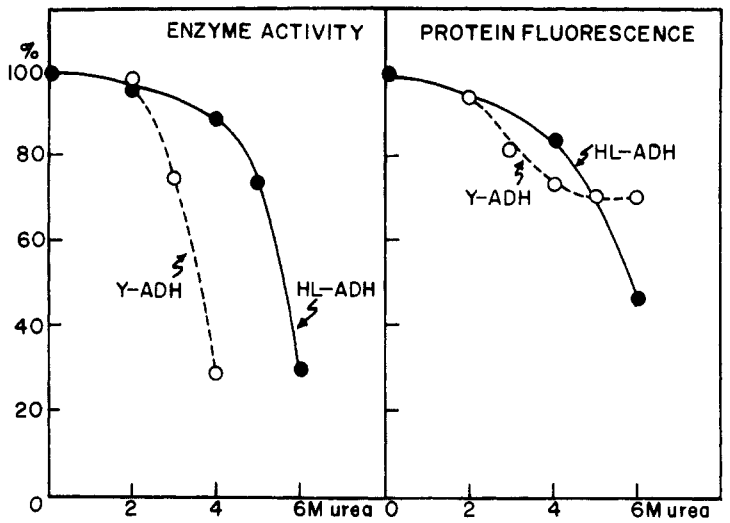


FIG. 4.—The effect of urea on alcohol dehydrogenase. Enzyme activities were as described in the text. Fluorescence excitation was at 280 mμ and emission at 340 mμ. The *p*H of the reaction was 9.2.

enzymes were first incubated at a concentration of 1.25 mg per ml for 15 minutes at the temperature indicated. They were rapidly cooled to 0° and then allowed to come to room temperature. The enzymes were finally diluted into the assay mixture and the catalytic rates observed. The heart enzyme is unusually stable, and loses its activity only between 55° and 65°. Under these conditions it does not precipitate on denaturation. In contrast, the skeletal muscle enzyme loses its

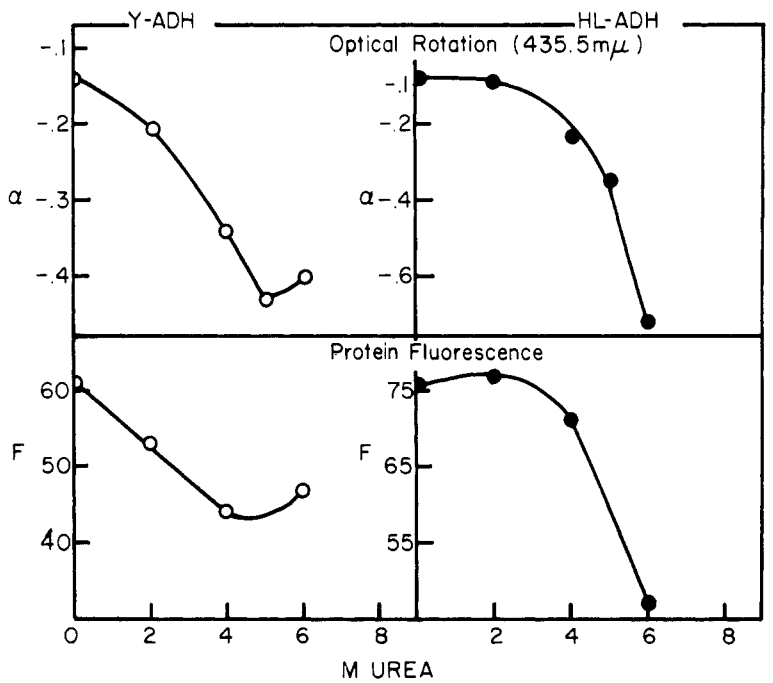


FIG. 5.—Urea denaturation of alcohol dehydrogenase. A comparison of optical rotation and fluorescence measurements. For the optical rotation measurements the concentration of yeast alcohol dehydrogenase was 2.3 mg/ml and that of horse liver alcohol dehydrogenase 2.7 mg/ml. The experiments were done in 0.1 M phosphate buffer at *p*H 7.4. Readings were made with a 1-cm cell. For the fluorescence measurements the concentration of yeast alcohol dehydrogenase was 0.01 mg/ml and that of horse liver alcohol dehydrogenase 0.05 mg/ml

activity at much lower temperatures and precipitates out at 46°. The decrease in activity of beef heart lactic dehydrogenase is paralleled by the decrease of fluorescence emission at 340 mμ when the protein is excited at 280 mμ.

The heat denaturation of beef heart lactic dehydrogenase was also measured by optical rotation at 404.7 mμ. The denaturations were carried out as described above, and the optical rotations of the undiluted enzyme were determined at room temperature in a 10-cm polarimeter tube. The sharp break occurring at about 60° is in very good agreement with the activity and fluorescence measurements. Comparative experiments of this type have given us confidence in the use of protein fluorescence as a tool in the study of enzyme denaturation.

A comparison of the urea denaturation of beef and chicken lactic dehydrogenase from heart and skeletal muscle is given in Figure 7. Both the enzyme activity and protein fluorescence were measured. In these experiments the enzymes were incubated with the urea for 15 minutes at 30°. The urea was then diluted out and the readings made. There is good correlation between the loss of activities of these enzymes and the alteration of structure as measured by fluorescence. The enzymes from heart muscle are more stable than those from skeletal muscle. The beef heart enzyme retains about 70% of its activity in 3 M urea, whereas the beef muscle has lost most of its activity at this concentration. The changes in fluorescence show qualitatively the same difference among the various lactic dehydrogenases as do the changes in enzymatic activities. These results are in accord with the studies of temperature stability described above.

The chick heart lactic dehydrogenase is more stable to urea than the beef heart enzyme. At 4 M urea the chick heart enzyme retains most of its activity, whereas the activity of the beef heart enzyme is less than 50% that of the untreated enzyme. The chick muscle dehydrogenase precipitates during denaturation under these conditions.

We have also been able to show that loss in enzymatic activity of the dehydrogenases is associated with a change in the polarization of fluorescence spectra. This study will be reported elsewhere. Guanidine and stilbesterol produce effects similar to those that urea produces on protein fluorescence and enzyme activity.

*Inhibition by Urea of Alcohol Dehydrogenase Without Denaturation.*—Table I compares the enzymatic activities of the two alcohol dehydrogenases when the assays were carried out in the presence of urea and after removal of the urea by dialysis. Urea of the concentration indicated was present in the reaction mixture. The inhibition is considerably greater when urea is present in the reaction mixture. Thus, at 4 M urea, where the liver enzyme has just begun to denature as measured by the change in protein fluorescence (see Fig. 4), approximately 50% of the activity is lost when the urea is present during the enzymatic assay.

In order to gain more insight into this inhibition the effect of urea on the enzyme-coenzyme com-

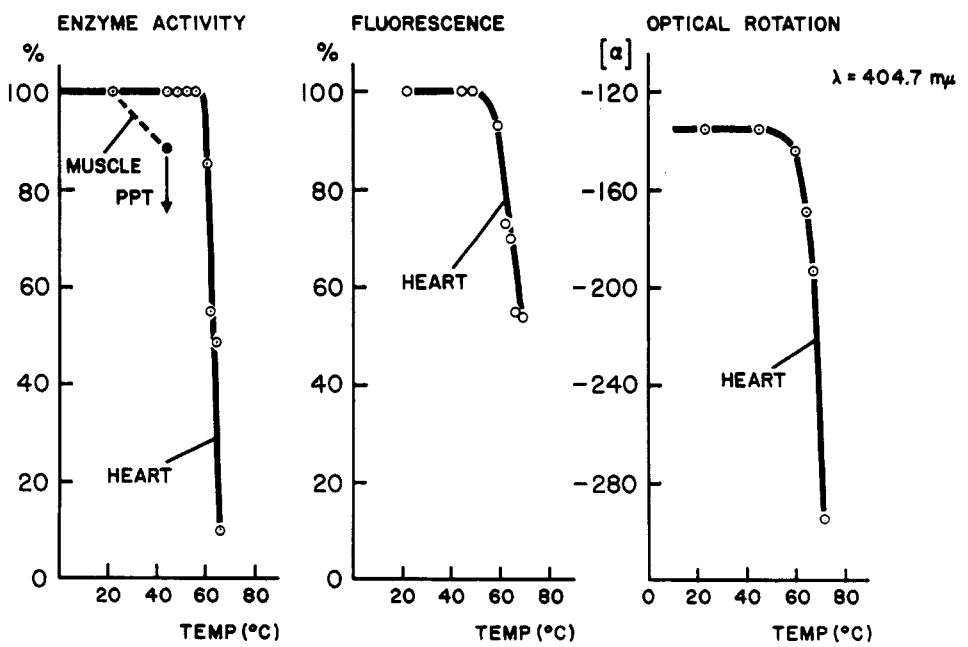


FIG. 6.—The effect of temperature on beef lactic dehydrogenases. Fluorescence was measured by excitation at 280 mμ and emission at 340 mμ. The enzymes were allowed to stand at *p*H 9.4 (0.05 M glycine buffer) for 15 minutes at the various temperatures indicated.

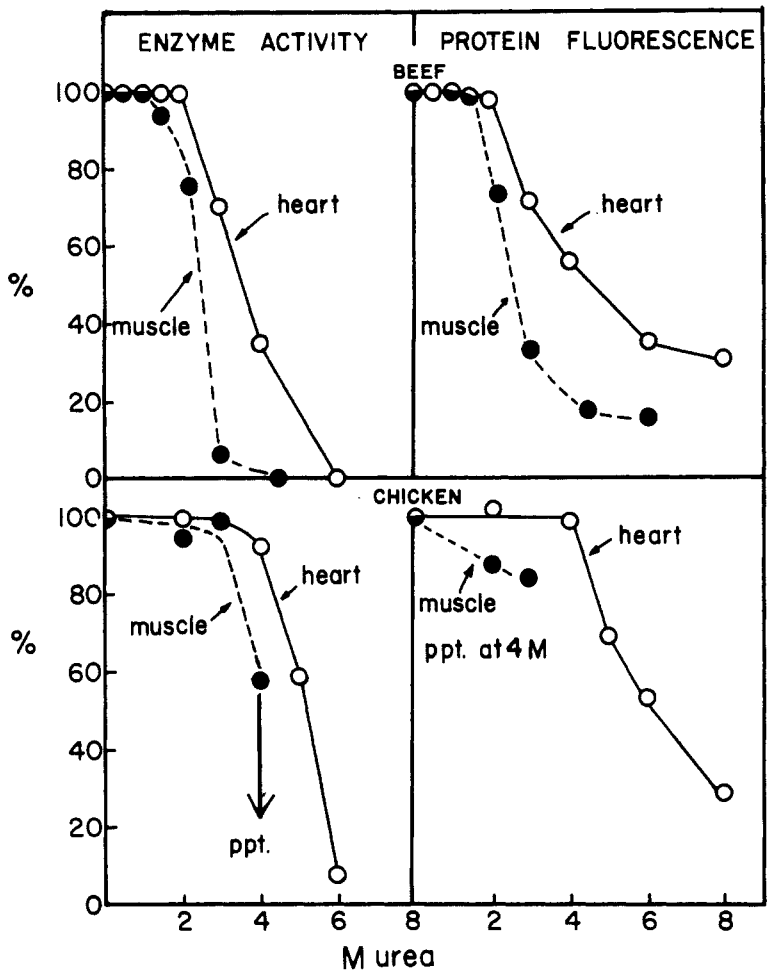


FIG. 7.—The effect of urea on beef and chicken lactic dehydrogenases. Reaction was in 0.1 M phosphate buffer (*p*H 7.4). Enzyme activities were as described in the text. Fluorescence excitation was at 280 mμ and emission at 340 mμ.

TABLE I
UREA INACTIVATION OF YEAST AND HORSE LIVER ALCOHOL DEHYDROGENASE AND THE EFFECT OF UREA REMOVAL

| Urea Concentration (M) | Yeast ADH | | Liver ADH | |
|---|---|---|---|---|
| | % Inhibition | | | |
| | In Presence of Urea | After Dialysis[a] | In Presence of Urea | After Dialysis[a] |
| 0.5 | 36 | 0 | — | — |
| 1 | 65 | 2 | 18 | 0 |
| 2 | 92 | 5 | 28 | 4 |
| 3 | 100 | 15 | 40 | 5 |
| 4 | 100 | 75 | 49 | 8 |
| 6 | — | — | 74 | 70 |

[a] The urea was removed by dialysis against 0.1 M phosphate buffer (*p*H 7.4).

plex of liver alcohol dehydrogenase was investigated in more detail. Boyer and Theorell (1956) have observed that when DPNH binds to this enzyme the maximum of its fluorescence emission is shifted to a shorter wave length and the quantum yield of coenzyme fluorescence is considerably increased. This effect has been used in this laboratory (Shifrin *et al.*, 1959), as well as in other laboratories (Duysens and Kronenberg, 1957; Velick, 1958; Winer *et al.*, 1959), to measure coenzyme binding.

Figure 8 shows the fluorescence of reduced coenzymes bound to liver alcohol dehydrogenase plotted as a function of urea concentration. There is over a fivefold increase in fluorescence of DPNH bound to liver alcohol dehydrogenase at concentrations of urea between 0 and 4 M. The fluorescence of the 3-acetylpyridine analogue of DPNH bound to liver alcohol dehydrogenase showed little enhancement upon addition of urea. Enhancement of this type was not observed with yeast alcohol dehydrogenase and DPNH.

The quantum yield of fluorescence depends on the relative amount of energy lost by a molecule in the excited state, by re-emission as light, by

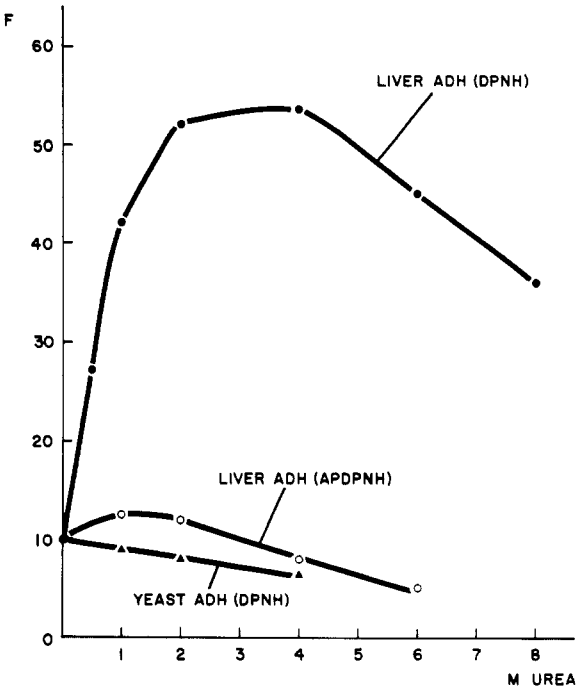


FIG. 8.—The effect of urea on the fluorescence of the DPNH–alcohol dehydrogenase complex. Excitation was at 335 mμ for the DPNH and 345 mμ for acetylpyridine (APDPNH). The fluorescence emission was measured at 440 mμ for DPNH and 450 mμ for acetylpyridine DPNH. The experiments were carried out at room temperature in 0.1 M phosphate buffer, *p*H 7.4. For the enzyme-DPNH complex, the concentration of horse liver alcohol dehydrogenase was $10.8 \times 10^{-6}$ M. The concentration of DPNH was $5.6 \times 10^{-6}$ M. For the enzyme–acetylpyridine DPNH complex, the enzyme concentration was $5.4 \times 10^{-6}$ M. The concentration of acetylpyridine DPNH was $2.8 \times 10^{-6}$ M. For the yeast alcohol dehydrogenase–DPNH experiment the protein concentration was $2 \times 10^{-6}$ M and the DPNH concentration was $5.6 \times 10^{-6}$ M. In each case the fluorescence polarization of the coenzyme indicated that binding to the protein had occurred before the urea was added. Fluorescence is expressed in arbitrary units, and in each case the value obtained with no urea added is called "one."

interaction with other molecules, or by conversion to heat. When DPNH is bound to a number of dehydrogenases it is protected against loss of energy in its excited state by means other than re-emission. It is reasonable that tighter binding of the coenzyme or ternary complex formation would further protect the coenzyme from nonradiative losses of energy and thereby increase the quantum yield of fluorescence. It appears probable that the urea forms a ternary complex with DPNH and liver alcohol dehydrogenase and that this is the reason for the enhanced fluorescence and also for the inhibition of enzymatic activity. The effect may be similar to the increased fluorescence found with acetamide by Winer and Theorell (1959).

The explanation given for the enhanced coenzyme fluorescence is entirely in accord with kinetic experiments (Rajagopalan *et al.*, 1961), which indicate that the inhibition of liver alcohol dehydrogenase by urea is uncompetitive, *i.e.*, the inhibitor combines with the enzyme-coenzyme complex. The lack of enhancement of fluorescence with the 3-acetylpyridine analogue of DPNH may be due to the fact that the amide group of DPNH is involved in the ternary bond formation. Another explanation for the enhancement with liver alcohol dehydrogenase may be that the urea causes slight uncoiling of the molecule, which results in a firmer binding of the DPNH and thereby decreases its dissociation from the protein.

*Binding of DPNH by Liver Alcohol Dehydrogenase.*—Figure 9 indicates the effect of *p*H on the capacity of liver alcohol dehydrogenase to bind DPNH. Polarization fluorescence of the coenzyme was the method used to measure binding. Two fluorescence readings were taken, one with the excitation and emission polarizers parallel and one with the two polarizers perpendicular. The high polarizations are indicative of bound coenzyme, the low values of free coenzyme. The enzyme binds only one mole of DPNH between *p*H 10 and 11 and none at higher *p*H ranges. This is in agreement with the results Theorell and Bonnichsen (1951) obtained by spectrophotometric titrations. The upper curve shows a similar experiment carried out in the presence of 2 M urea. With 2 M urea, the DPNH remains bound at *p*H values where it otherwise becomes free. These findings are in favor of the ternary complex explanation of the fluorescence enhancement in the presence of urea.

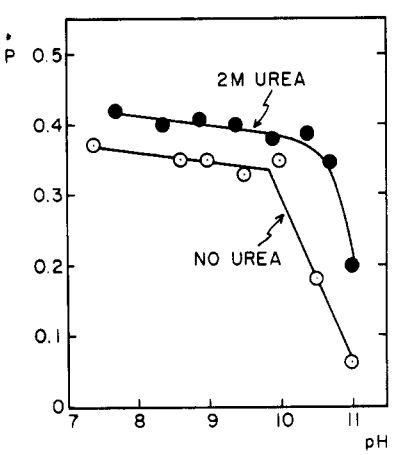


FIG. 9.—The effect of *p*H on the horse liver alcohol dehydrogenase–DPNH complex. The protein concentration was $2 \times 10^{-6}$ M and the coenzyme concentration was $2.6 \times 10^{-6}$ M. The measurements were made in the presence of 0.1 M phosphate at 22°. The instrument used was the Aminco-Bowman spectrophotofluorometer. The polarization of fluorescence was measured by excitation at 340 mμ and emission at 440 mμ.

The dehydrogenase group of enzymes fluoresce at 340 mμ when excited at 280 mμ. The emission of protein fluorescence therefore strongly overlaps the excitation maximum of DPNH. From Förster's theory (1951) one would predict energy transfer from the protein to the bound DPNH. Such transfer has in fact been observed for a number of dehydrogenases (Velick, 1958).

Figure 10 shows the fluorescence excitation spectra for free DPNH and DPNH bound to liver alcohol dehydrogenase under a variety of conditions when the fluorescence emission is measured at 450 mμ. The appearance of the 280 mμ transfer after addition of the enzyme can easily be seen. Upon addition of urea the protein to reduce coenzyme fluorescence is enhanced. As with most fluorescent molecules (Bowen, 1959), the emission of the enzyme-coenzyme complex is enhanced when the temperature is lowered. This is true of the directly excited fluorescence and of the fluorescence excited by energy transfer from the protein. It is of interest that cooling to 1.5° increases the transfer fluorescence to 2.7 times that at 22°, whereas the directly excited fluorescence is increased only 2 times. This may indicate that at the lower temperature the enzyme has folded itself in such a manner as to bring a tryptophan closer to the bound coenzyme. When a similar comparison is made of the fluorescence in the presence of 4 M urea, it is found that lowering the temperature to 1.5° increases both the transfer and direct fluorescence to only 1.3 times that found at 22°. The lesser enhancement of fluorescence, even on direct excitation, may be due to the fact that the factors contributing to the enhancement at lower temperatures are also involved in the enhancement produced by urea—namely, the tighter binding of coenzyme with protein.

In order to obtain information on the relationship of protein structure to the capacity of the enzyme to bind DPNH, coenzyme binding to horse liver alcohol dehydrogenase was studied over a wide range of urea concentrations. Reference to the left half of Figure 11 shows the change in liver alcohol dehydrogenase as measured by protein fluorescence. The enzyme is stable for short periods even in 8 M urea. After incubation for 15 minutes at 38°, the typical denaturation curve for this protein with urea, showing the decrease in fluorescence at concentrations greater than 4 M, is obtained. The curves on the right half of Figure 11 give the binding of DPNH to the enzyme as measured by fluorescence polarization. Without prior incubation the coenzyme remains bound even in 8 M urea. After 15 minutes at 38°, the DPNH remains bound only at urea concentrations of less than 4 M. Experiments such as those summarized in Figure 11 indicate the importance of the folded structure of this dehydrogenase to its coenzyme-binding capacity.

The effect of *p*H on the protein fluorescence of the two alcohol dehydrogenases is shown in Figure 12. There is a decrease in fluorescence at all *p*H values after 5 minutes. This is indicative of unfolding of the proteins at the relatively low

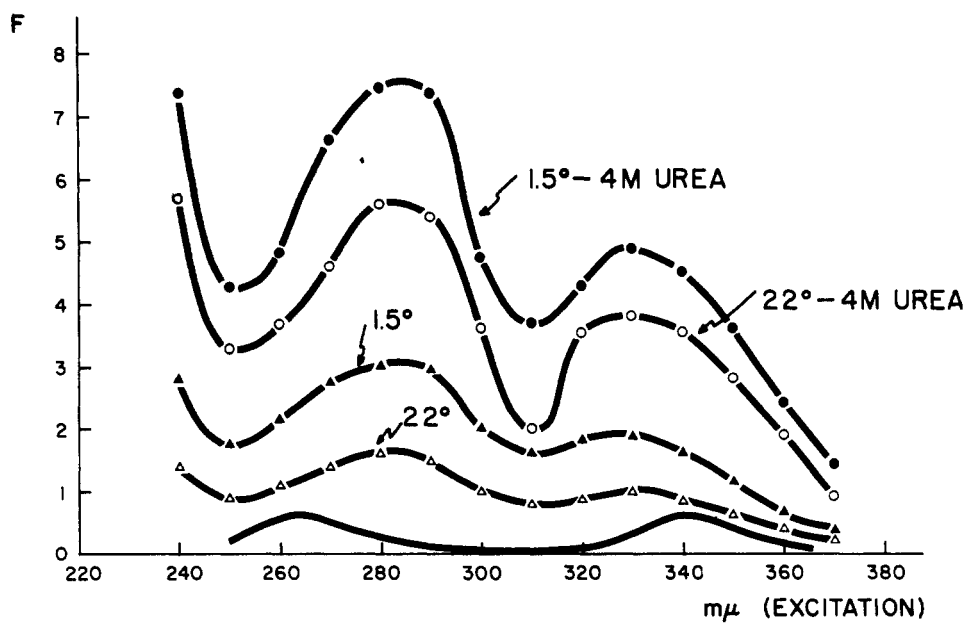


FIG. 10.—The effect of cooling and 4 M urea on the fluorescence excitation spectrum of the liver alcohol dehydrogenase–DPNH complex. The concentration of the enzyme was $4 \times 10^{-6}$ M and the concentration of the coenzyme $6 \times 10^{-6}$ M. The solid points were obtained at 1.5° in the presence of 4 M urea and the open circles at 22° in the presence of 4 M urea. The solid triangles were obtained at 1.5° with no urea and the open triangles at 22° with no urea. The solid line represents the coenzyme alone. The measurements were obtained in the presence of 0.1 M phosphate buffer at *p*H 7.4.

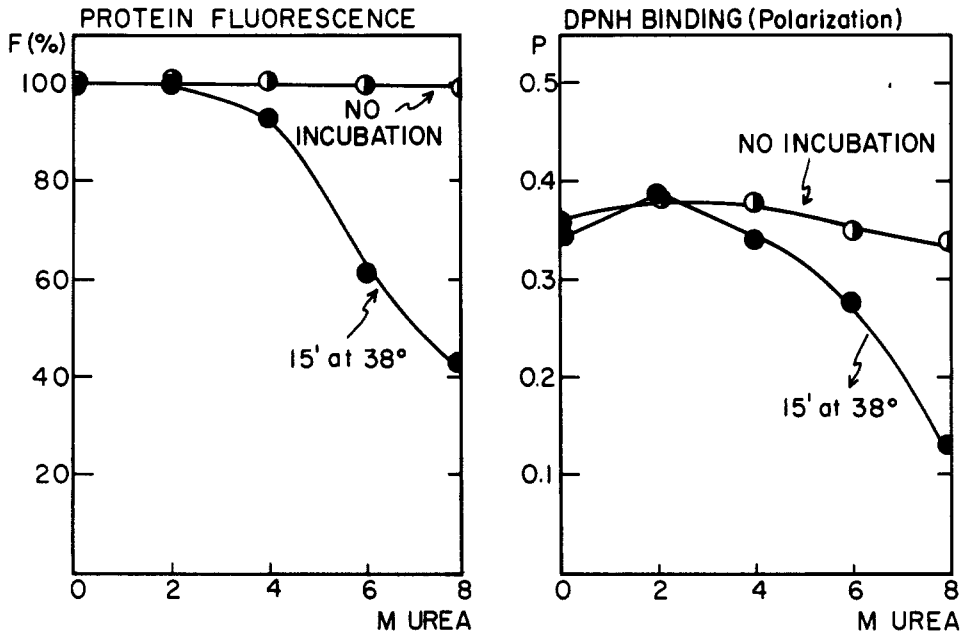


FIG. 11.—The denaturation of horse liver alcohol dehydrogenase by urea and the effect of urea on coenzyme binding capacity. The protein fluorescence measurements were made in 0.05 M glycine at *p*H 9.4 with an enzyme concentration of $0.36 \times 10^{-6}$ M. The binding studies were carried out in 0.1 M phosphate, *p*H 7.7, with an enzyme concentration of $3.7 \times 10^{-6}$ M. The polarization of coenzyme fluorescence was measured by excitation at 340 mμ and emission at 440 mμ. The DPNH concentration was $2.8 \times 10^{-6}$ M.

concentration used for the fluorescence measurements. Both enzymes show a decrease in fluorescence at *p*H values greater than 9. Yeast alcohol dehydrogenase shows a sharp decrease in fluorescence at acid *p*H values. This is not so pronounced with the liver enzyme.

A *p*H profile of liver alcohol dehydrogenase in 8 M urea is presented in Figure 12 (curves *C* and *D*). It has already been shown in Figure 11 that in 8 M urea this protein is denatured in a time-dependent reaction. The fluorescence decreases at all *p*H values after 5 minutes. At acid *p*H in the presence of urea, there is an instantaneous decrease in fluorescence not observed with urea alone. Urea therefore appears to labilize the protein to acid treatment. It is important to note that the fluorescence does not drop to zero. The residual fluorescence remaining after urea treatment is not extensively quenched at any *p*H between 5 and 11 (curve *D*). The fluorescence of tryptophan itself is not quenched in this *p*H range, and the observed quenching of the protein without urea must be related to the environment around the tryptophans. We have tenatatively concluded from these experiments that there are at least two types of tryptophan (as far as environment is concerned) in this enzyme. One is quenched by urea treatment and also at acid or alkaline *p*H values, and the other retains its fluorescence upon urea denaturation or after acid or base treatment. In this connection it should be pointed out that the quantum yield of fluorescence of tryptophan in horse liver alcohol dehydrogenase is roughly the same as that found with free tryptophan.

## DISCUSSION AND CONCLUSIONS

The enzymes studied in this work have been discussed from a number of viewpoints. One of these is the organic model approach (Westheimer, 1959). Other approaches have focused attention on specific functional groups of the enzymes such as Zn (Vallee, 1960b) or sulfhydryl groups (Wallenfels *et al.*, 1959; van Eys *et al.*, 1957). In the present study the emphasis has been on the relationship of over-all protein structure to enzyme function. The specificity and high turnover numbers of enzymes must certainly depend on their folded structure.

In our view a knowledge of the stability of an enzyme under a variety of conditions and the development of a number of methods to study its denaturation should be a prerequisite to the application of specific amino acid reagents. Only in such a manner can the direct effect of these reagents on the active site be differentiated from an indirect effect caused by prior denaturation of the protein. With such precautions the importance of a certain amino acid to the biological function may then be assessed.

The fluorescence of organic molecules is particularly sensitive to the environment. For this reason, and because the measurement of this

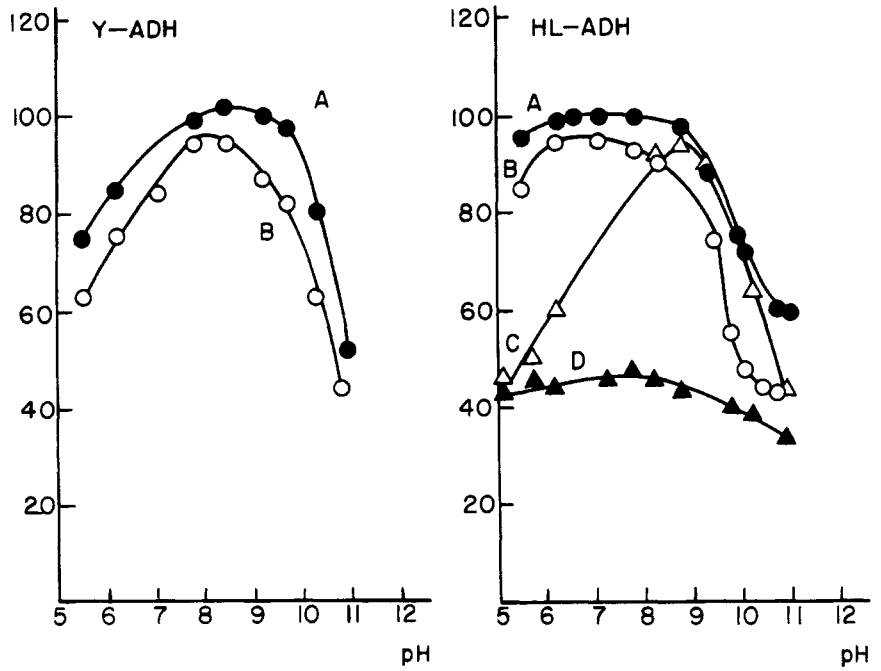


FIG. 12.—The effect of *p*H on fluorescence of alcohol dehydrogenase. Fluorescence is expressed in arbitrary units for each enzyme. Excitation was at 280 mμ. Emission was at 340 mμ. Left, yeast alcohol dehydrogenase. Curve *A* (●) indicates readings taken immediately after dilution of the enzyme at the *p*H indicated. Curve *B* (○) indicates readings taken 5 minutes after dilution. *Right*, the effect of *p*H on the fluorescence of horse liver alcohol dehydrogenase in the presence of 8 M urea. Curve *C* (△) indicates readings taken immediately after dilution of the enzyme. Curve *D* (▲) indicates readings taken 5 minutes after dilution of the enzyme in the 8 M urea. Excitation was at 280 mμ. Emission was at 340 mμ. In all cases the protein concentration was 0.03 mg/ml.

property requires only low concentrations of material, the technique has been used extensively in our studies. The fluorescence intensity depends on the mechanism of energy release as an excited molecule returns to the ground state. This may occur through radiative emission or a variety of nonradiative pathways of energy dissipation (Kauzmann, 1957). Although the literature on fluorescence quenching is extensive (Förster, 1951; Pringsheim, 1949), it is difficult to give more than an empirical interpretation to the quenching that is found in the study of proteins (Weber, 1961). Fluorescence of tryptophan moieties may decrease, increase, or remain unaltered after denaturation, depending on their position in a given protein. In this paper, however, fluorescence quenching has been ascribed to denaturation or uncoiling of the protein that is associated with the loss of enzymatic activity.

Changes in the maximum of fluorescence emission are easier to interpret. The theoretical and experimental work of Lippert (1957) has shown that for most molecules there is a shift to the red in solvents of low dielectric constant. This shift is due to interactions between the solvent and the dipoles of the excited state. The change of protein emission to longer wave lengths as an enzyme unfolds is probably related to the inclusion of water molecules in a previously hydrophobic region of the protein. In contrast, the shift of coenzyme fluorescence to shorter wave lengths when the coenzyme is bound to a dehydrogenase must be indicative of the transfer of this molecule to a hydrophobic region.

We have compared the results obtained by fluorescence methods with similar studies carried out with optical rotation, a more established method of studying protein denaturation. In spite of considerable theoretical work (Moffit and Young, 1961) on optical rotation, this method of measuring protein uncoiling must also be considered empirical. However, in contrast to fluorescence studies, there is a considerable amount of information in the literature comparing the results obtained with proteins to those obtained with a number of polyamino acid models (Blout, 1960). The optical rotation is due to a number of Cotton effects of the peptide bond itself—some in the far ultraviolet region. For this reason the results obtained with the optical rotation method are not dependent only upon the environment around the aromatic amino acids.

The experiments described here show good correlation between the various ways of measuring denaturation. This is apparent from the temperature studies with beef heart lactic dehydro-

genase and yeast alcohol dehydrogenase The beef heart enzyme is stable at temperatures up to 60°. At higher temperatures activity is lost, protein fluorescence decreases, and optical rotations become more negative. The similarity between the results obtained by the various assays is striking. Yeast alcohol dehydrogenase is denatured between 35° and 45°, with the activity falling precipitously in the same temperature range. Similar comparisons with urea denaturation confirm that fluorescence changes may indeed be used to measure denaturation of the dehydrogenases. Since the measurements are rapid and require little material, this may be a preferred method. With none of the enzymes tested did we find evidence for activity after unfolding had occurred.

With all the denaturants and assays for denaturation used, horse liver alcohol dehydrogenase was more stable than yeast alcohol dehydrogenase. The denaturing agents included heat, urea, guanidine, and various detergents. The greater lability of yeast alcohol dehydrogenase is reasonable in view of its higher molecular weight (Hayes and Velick, 1954; Ehrenberg, 1957) and greater substrate and coenzyme specificity (Bliss, 1951; Holzer and Schneider, 1955; Anderson and Kaplan, 1959). This enzyme has a higher turnover number than liver alcohol dehydrogenase under optimal conditions when ethanol is the substrate.

A comparison of enzyme activity and coenzyme binding during urea denaturation of liver alcohol dehydrogenase shows that inhibition of activity is caused by loss of capacity to bind DPNH. The finding that the native configuration of the protein is required for coenzyme binding suggests that it will be difficult to isolate a peptide fragment with bound DPNH. This experiment supports the widely held view (Koshland, 1960) that the active sites of these enzymes are composed of amino acids that may be separated by a great distance in the primary peptide sequence.

The importance of these types of measurements to the intelligent application of specific amino acid reagents is illustrated by the studies of urea on liver alcohol dehydrogenase. The inhibition of activity at low urea concentration is caused by binding of the urea to the enzyme-coenzyme complex. This can readily be distinguished from the inhibition at higher concentrations, which is due to unfolding of the protein with consequent inhibition of coenzyme binding. In the first case, urea causes an enhancement of the fluorescence of the reduced DPN by protecting the coenzyme against nonradiative energy loss. In the second case, the protein fluorescence decreases and the optical rotations become more negative owing to unfolding of the molecule, and the coenzyme becomes unbound. Thus urea can act either as a specific active site reagent or as a general denaturing agent.

At the present time changes in tryptophan fluorescence can be used only as an empirical assay of protein strucural alterations. With proteins where no previous denaturation studies have been done, results must be checked by an independent method such as optical rotation. The danger of equating quenching with denaturation without the appropriate controls is illustrated by the *p*H stability studies with liver alcohol dehydrogenase. These experiments show that this enzyme has at least two "types" of tryptophan. One type has its fluorescence quenched by urea denaturation of the protein or by acid or base treatment; the other is not affected. The fluorescence remaining after unfolding induced by urea is not significantly quenched between *p*H 5 and 10 (see Fig. 12).

The effect of *p*H on the fluorescence of tryptophan, tyrosine, and their derivatives has been studied by White (1959). Tryptophan has an acid $pH_f$ of 2.4. This quenching was interpreted as a migration of $H^+$ ions to the neighborhood of the excited molecule. Removal of the carboxyl group of tryptophan displaced the *p*H of acid quenching to lower values. Since quenching of yeast alcohol dehydrogenase occurs at *p*H values higher than 2.4, the effect observed in the acid region probably is not due to an association of $H^+$ ions with the excited molecules. The native enzyme must be altered and a quenching group brought close to some of the tryptophans.

The alkaline quenching of tryptophan itself has a $pH_f$ of 10.9, and tryptophan methyl ester has a $pH_f$ of 12.8. Both yeast and liver alcohol dehydrogenases are quenched at lower *p*H values. As is the case with acid quenching, the decrease in fluorescence at alkaline *p*H is probably brought about by unfolding of the protein molecule. It is also possible that ionizable groups of the protein in proximity to the aromatic residues in question may be responsible for the quenching. A definitive answer to the cause of the quenching observed here will require a study of the effect of *p*H on the fluorescence of a variety of tryptophan-containing peptides and polyamino acids.

Enzymes catalyzing the same over-all reaction may be found in a variety of organisms and tissues. When isolated and studied many of these enzymes are found to be different from each other. This has been shown with kinetic (Keleti, 1958; Kaplan *et al.*, 1960), physical (Markert and Møller, 1959; Plagemann *et al.*, 1960; Elödi, 1958), and immunologic (Gregory and Wroblewski, 1958; Antoni and Keleti, 1958; Nisselbaum and Bodansky, 1959) techniques. An understanding of the importance of these differences to the chemistry and biology of cells has been of interest to us for some time (Kaplan and Ciotti, 1961). In this paper it has been shown that enzymes that differ by some of the above criteria also have quite different stabilities. These properties may, in fact, be used as another method of distinguishing such proteins. Heart muscle lactic dehydrogenase is more stable to heat and to urea than skeletal muscle lactic dehydrogenase. This holds true

for both the chicken and beef enzymes. It has been our general experience that enzymes catalyzing the same reaction and showing different catalytic characteristics also show differences in their heat stabilities.

## Acknowledgments

We are grateful to Professor Gregorio Weber, who kindly advised us on many aspects of this work. We also appreciate the help of Mr. J. Martin Sandler and Dr. Sonia Bargmann with optical rotation studies. Mr. Sandler's work was supported by the American Cancer Society's Institutional Grant (IN-29C).

## References

Anderson, B. M., and Kaplan, N. O. (1959), *J. Biol. Chem. 234*, 1226.

Antoni, F., and Keleti, T. (1958), *Acta Physiol. Acad. Sci. Hung. 13*, 187.

Bliss, A. F. (1951), *Arch. Biochem. 31*, 197.

Blout, E. R. (1960), *in* Optical Rotatory Dispersion, Djerassi, C., editor, New York, McGraw-Hill Book Co., Chap. 17.

Bowen, E. J. (1959), *in* A General Discussion of the Faraday Society, vol. 27, Aberdeen, Scotland, Aberdeen University Press, Ltd., p. 40.

Boyer, P. D., and Theorell, H. (1956), *Acta Chem. Scand. 10*, 447.

Duysens, L. N. M., and Kronenberg, G. H. M. (1957), *Biochim. et Biophys. Acta 26*, 437.

Ehrenberg, A. (1957), *Acta Chem. Scand. 11*, 1257.

Elödi, P. (1958), *Acta Physiol. Acad. Sci. Hung. 13*, 199.

Förster, T. (1951), *in* Fluorescence organischer Verbindungen, Göttingen, Germany, Vandenhoeck und Ruprecht, Chaps. 10 and 11.

Gregory, K. F., and Wroblewski, F. (1958), *J. Immunol. 81*, 359.

Gregory, K. F., and Wroblewski, F., editors (1961), Conference on Multiple Molecular Forms of Enzymes, *Ann. N.Y. Acad. Sci. 94*.

Hayes, J. E., Jr., and Velick, S. F. (1954), *J. Biol. Chem. 207*, 225.

Holzer, H., and Schneider, S. (1955), *Angew. Chem. 67*, 276.

Kaplan, N. O., and Ciotti, M. M. (1961), *Ann. N.Y. Acad. Sci. 94*, 701.

Kaplan, N. O., Ciotti, M. M., Hamolsky, M., and Bieber, R. E. (1960), *Science 131*, 392.

Kauzmann, W. (1957), *in* Quantum Chemistry, New York, Academic Press, Inc., p. 693.

Keleti, T. (1958), *Acta Physiol. Acad. Sci. Hung. 13*, 103.

Koshland, D. E., Jr. (1960), Advances in Enzymology *22*, 45.

Lippert, E. (1957), *J. Elektrochem. 61*, 962.

Markert, C. L., and Møller, F. (1959), *Proc. Nat. Acad. Sci. U. S. 45*, 753.

Moffit, W., and Young, J. T. (1961), *Proc. Nat. Acad. Sci. U. S. 42*, 596.

Nisselbaum, J. S., and Bodansky, O. (1959), *J. Biol. Chem. 234*, 3276.

Plagemann, P. G. W., Gregory, K. F., and Wroblewski, F. (1960), *J. Biol. Chem. 235*, 2282.

Pringsheim, P. (1949), *in* Fluorescence and Phosphorescence, New York, Interscience Publishers, Inc., p. 313.

Rajagopalan, K. V., Fridovich, I., and Handler, P. (1961), *J. Biol. Chem. 236*, 1059.

Shifrin, S., Kaplan, N. O., and Ciotti, M. M. (1959), *J. Biol. Chem. 234*, 1555.

Teale, F. W. J. (1960), *Biochem. J. 76*, 381.

Theorell, H., and Bonnichsen, R. K. (1951), *Acta Chem. Scand. 5*, 1105.

Vallee, B. L. (1960a), *in* The Enzymes, vol. 3, ed. 2, Boyer, P., Lardy, H., and Myrbäck, K., editors, New York, Academic Press, Inc., chap. 15.

Vallee, B. L. (1960b), *in* Proceedings of the Fourth International Congress of Biochemistry, New York, Pergamon Press, p. 138.

van Eys, J., Ciotti, M. M., and Kaplan, N. O. (1957), *Biochim. et Biophys. Acta 23*, 58.

Velick, S. F. (1958), *J. Biol. Chem. 233*, 1455.

Wallenfels, K., Sund, H., Zarnitz, M. L., Malhotro, O. P., and Fischer, J. (1959), *in* Sulfur in Proteins, New York, Academic Press, Inc., p. 215.

Weber, G. (1960), *Biochem. J. 75*, 335.

Weber, G. (1961), *in* A Symposium on Light and Life, McElroy, W. D., and Glass, B., editors, Baltimore, The Johns Hopkins Press, p. 82.

Westheimer, F. H. (1959), *in* The Enzymes, vol. 1, ed. 2, Boyer, P., Lardy, H., and Myrbäck, K., editors, New York, Academic Press, Inc., p. 259.

White, A. (1959), *Biochem. J. 71*, 217.

Winer, A. D., Schwert, G. W., and Millar, D. B. S. (1959), *J. Biol. Chem. 234*, 1149.

Winer, A. D., and Theorell, H. (1959), *Acta Chem. Scand 13*, 1038.