Kegeles, G., and Gutter, F. J. (1951), *J. Am. Chem. Soc. 73*, 3770.
Kirshner, A. G., and Tanford, C. (1964), *Biochemistry 3*, 291.
Lamm, O., and Polson, A. (1936), *Biochem. J. 30*, 528.
Nozaki, Y., and Tanford, C. (1963), *J. Biol. Chem. 238*, 4074.
Perutz, M. F., Rossman, M. G., Cullis, A. F., Muirhead, H., Will, G., and North, A. T. C. (1960), *Nature 185*, 416.
Rossi-Fanelli, A., Antonini, E., and Caputo, A. (1961), *J. Biol. Chem. 236*, 391.
Scheraga, H. A., and Mandelkern, L. (1953), *J. Am. Chem. Soc. 75*, 179.
Schroeder, W. A. (1963), *Ann. Rev. Biochem. 32*, 301.
Steinhardt, J. (1938), *J. Biol. Chem. 123*, 543.
Svedberg, T., and Pedersen, K. O. (1940), The Ultracentrifuge, London, Oxford.
Tanford, C. (1957), *J. Am. Chem. Soc. 79*, 3931.
Tanford, C. (1961), Physical Chemistry of Macromolecules, New York, Wiley.
Tanford, C. (1964a), *J. Am. Chem. Soc. 86*, 2050.
Tanford, C. (1964b), *Brookhaven Symp. Biol. 17 (BNL869 (C-40))*, 154.
Tanford, C., and Buzzell, J. G. (1956), *J. Phys. Chem. 60*, 1204.
Tanford, C., and De, P. K. (1961), *J. Biol. Chem. 236*, 1711.
Von Hippel, P. H., and Wong, K.-Y. (1964), *Science 145*, 577.
Waugh, D. F., and Yphantis, D. A. (1952), *Rev. Sci. Instr. 23*, 609.
Whitney, P. L., and Tanford, C. (1962), *J. Biol. Chem. 237*, PC1735.
Winterhalter, K. H., and Huehns, E. R. (1964), *J. Biol. Chem. 239*, 3699.

Effect of Side-Chain Deuteration on Protein Stability*

Akihiko Hattori,† Henry L. Crespi, and Joseph J. Katz

ABSTRACT: Thermal denaturation of protio- and deuteriophycocyanin, isolated from the blue-green algae *Plectonema calothricoides*, *Phormidium luridum*, and *Synechococcus lividus*, grown in H_2O and D_2O, was studied in both H_2O and D_2O. The critical temperatures of thermal denaturation of deuteriophycocyanins were always significantly lower than those for protiophycocyanins, in both H_2O and D_2O solutions. The critical denaturation temperature was shown to be higher in D_2O than in H_2O for both protio- and deuteriophycocyanins. These results suggest that substitution of hydrogen atoms by deuterium in exchangeable positions stabilizes the protein conformation, whereas introduction of deuterium in nonexchangeable hydrogen positions appears to decrease nonpolar side-chain interactions.

The mass cultivation of organisms in which all of the hydrogen normally present is essentially completely replaced by deuterium (Crespi *et al.*, 1960; DaBoll *et al.*, 1962) has made possible a new experimental approach to the structure and function of a variety of organic compounds essential to life. Some results obtained with fully deuterated chloroplast pigments, carbohydrates, proteins, and nucleic acids (Strain *et al.*, 1961; Blake *et al.*, 1961; Berns *et al.*, 1963; Crespi and Katz, 1962; Crespi *et al.*, 1962) have already been reported. In our first investigation of deuterioprotein behavior (Berns *et al.*, 1963), deuteriophycocyanin[1] isolated from a deuterated blue-green alga, *Plectonema calothricoides*, was shown to undergo thermal denaturation at a significantly lower temperature than does (ordinary) protiophycocyanin.[1] Since these experiments were carried out in buffer made up with H_2O, it has been inferred that the observed difference in denaturation behavior of deuterio- and protiophycocyanin is probably due to differences in hydrophobic bonding in the protio- and deuterio- forms of phycocyanin. The present investigation was conducted to confirm our preliminary results, to gain further insight into the mechanism of thermal denaturation of proteins, and to explore the applicability of deuterioproteins to the study of the secondary and tertiary structure of proteins.

Pure samples of deuterio- and protiophycocyanin

* From the Chemistry Division, Argonne National Laboratory, Argonne, Ill. *Received March 4, 1965.* This work was performed under the auspices of the U.S. Atomic Energy Commission.

† Resident Research Associate, 1962–1964; on leave from the Institute of Applied Microbiology, University of Tokyo.

[1] The prefix "deuterio-" and the modifiers "fully deuterated" refer to phycocyanin containing 99.6% deuterium at all nonexchangeable positions. The prefix "protio-" refers to ordinary phycocyanin with hydrogen of mass 1 at all nonexchangeable positions.

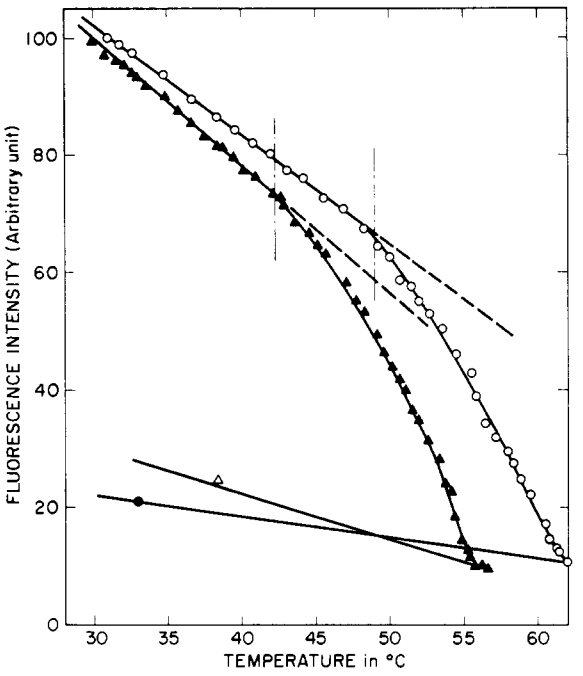

FIGURE 1: The thermal denaturation of protio- and deuteriophycocyanin from *P. calothricoides* in H_2O–phosphate buffer, *p*H 6.22, 0.02 M, as measured by fluorescence quenching. ○, protiophycocyanin, increasing temperature; ●, protiophycocyanin, decreasing temperature; ▲, deuteriophycocyanin, increasing temperature; △, deuteriophycocyanin, decreasing temperature.

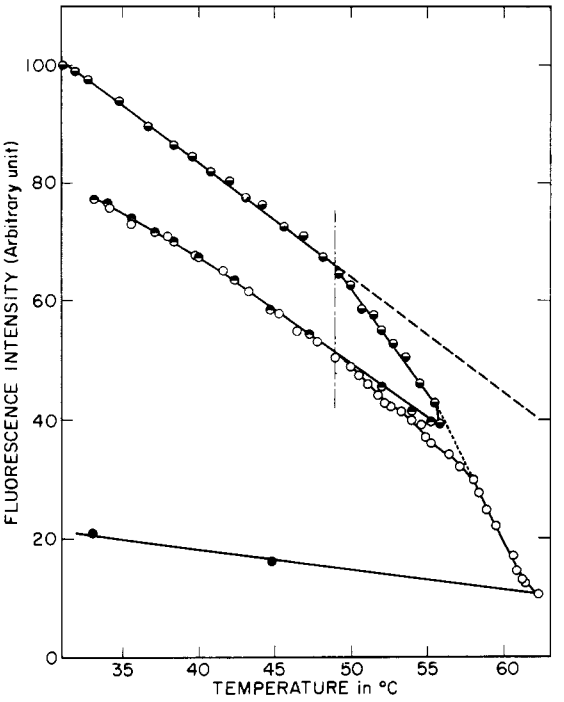

FIGURE 2: Thermal denaturation of protiophycocyanin from *P. calothricoides* in H_2O–phosphate buffer, *p*H 6.22, 0.02 M, as measured by fluorescence quenching. ◒, increasing temperature from 29.0 to 55.8°; ◓, decreasing temperature from 55.8 to 33.0°; ○, increasing temperature from 33.0 to 62.2°; ●, decreasing temperature from 62.2 to 33.0°.

isolated from *P. calothricoides*, *Phormidium luridum*, and *Synechococcus lividus* (DaBoll *et al.*, 1962) were used as test materials. The technique of fluorescence quenching (Steiner and Edelhoch, 1962), which had been used in our earlier study (Berns *et al.*, 1963), was used to obtain the thermal profiles of protein denaturation in H_2O and D_2O. A second procedure was based on changes in absorbance at the peak near 615 mμ as a function of time and temperature (Katz *et al.*, 1964).

The relative susceptibilities of protio- and deuteriophycocyanins to enzymatic proteolysis and γ radiation are also compared. The aim of these experiments was to establish differences in the resistance of the isotopically substituted proteins to a variety of protein denaturants. We believe the experiments described here provide experimental evidence for the importance of hydrophobic bonds in the conformational integrity of proteins. In all cases, fully deuterated phycocyanin is consistently less stable than its hydrogen-containing analog.

Materials and Methods

The deuterio- and protiophycocyanin used in these experiments was isolated by the method of Berns *et al.* (1963) from cells of *P. calothricoides*, *P. luridum*, and *S. lividus* that had been grown in D_2O (99.6%) nutrient medium (DaBoll *et al.*, 1962) or ordinary H_2O nutrient medium. Ordinary water, H_2O, was used in the purification procedures. The proteins were then further purified by adsorption on ECTEOLA- and DEAE-cellulose columns, or by adsorption on tricalcium phosphate gel (Hattori and Fujita, 1959). This further purification is especially important in the case of phycocyanin from *S. lividus;* large amounts of allophycocyanin are generally present in phycocyanin prepared from this organism. Before use, samples were dissolved in sodium phosphate buffer or acetate buffer (prepared with H_2O or D_2O) and dialyzed against the buffer of the same composition for a day or two at low temperature with an occasional change of external solution. The hydrogen ion concentration (*p*H) was determined by a Beckman Model 76 expanded scale *p*H meter. The corresponding values in D_2O buffers (*p*D) were estimated by addition of 0.4 unit to the reading of the *p*H meter (Lumry *et al.*, 1951; Glasoe and Long, 1960).

Thermal denaturation of the deuterio- and protioproteins was investigated by the same procedure used in our previous work (Berns *et al.*, 1963). The concentration of phycocyanin was adjusted to *ca.* 0.8 mg/ml by addition of phosphate buffer on the basis of the optical

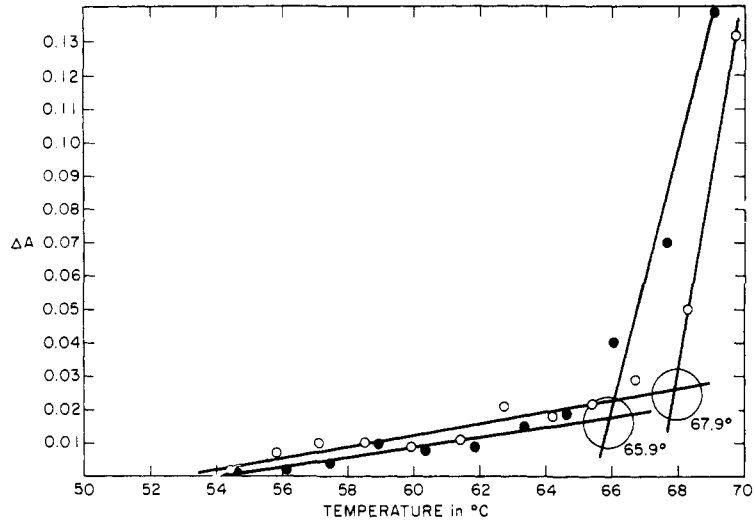

FIGURE 3: Thermal denaturation of protio- and deuteriophycocyanin from *S. lividus* followed by absorbancy change. H_2O–phosphate buffer, *p*H 6.52, 0.02 M. ○, protio; ●, deuterio.

density at the absorption peak near 615 mμ.[2] For thermal denaturation measurements by changes in absorbance the same phycocyanin concentration was used with cells of 0.2 cm optical path.

In the γ-radiation studies dialyzed solutions of phycocyanin (*ca.* 10 ml) were placed in glass tubes (1.5 × 10 cm) equipped with well-fitted glass stoppers. In experiments under anaerobic conditions the glass stoppers were replaced with three-way stopcocks through which the air in the tubes was repeatedly withdrawn and replaced with O_2-free nitrogen gas. Sample tubes were rotated at a distance of 3 cm around a γ source (^{60}Co) of 7.64 × 10^{19} ev/min per liter intensity. At intervals 1-ml aliquots were taken, and, after appropriate dilution, their absorption spectra were measured on a Cary recording spectrophotometer Model 14. From an examination of the difference spectra of irradiated and nonirradiated samples it was found that exposure of phycocyanin to γ rays resulted in decreases in absorptions near 615 and 355 mμ (positions of absorption peaks of native phycocyanin) and an increase in absorption at the wavelengths of 290–300 mμ. In these experiments, therefore, the denaturation was followed by measurement of the optical density at the peak near 615 mμ. Similar results were obtained by following changes in fluorescence intensity. Unless otherwise stated, all radiation experiments were carried out at room temperature.

Trypsin and α-chymotrypsin (Worthington Biochemical Corp.) were dissolved in hydrochloric acid solution, *p*H 3 (Desnuelle, 1960), and kept refrigerated. Enzyme concentrations were estimated from the optical density at 280 mμ (Long, 1961). Rates of proteolytic degradation were determined as follows: An appropriate amount of phycocyanin solution, which had been dialyzed against distilled H_2O, was added to 0.01 M sodium phosphate buffer to give a total volume of 2.9 ml and an absorbance of 0.2–1.0 at the peak near 615 mμ in a 1-cm spectrophotometric cell. At zero time 100 μl of enzyme solution (*ca.* 4.0 mg/ml for trypsin and *ca.* 2.0 mg/ml for α-chymotrypsin) was added. The rate of digestion was followed at 24.5 ± 0.2° by observing the decrease in absorption at the peak near 615 mμ.

Digestions were carried out on thermally denatured phycocyanin as well as native material. The protein was denatured to various extents by heating at fixed temperatures above the critical temperature (*vide infra*). The change in the ratio of the optical density at the peak near 615 mμ to the optical density at 280 mμ was used as a measure of the extent of denaturation. After denaturation the solutions were allowed to equilibrate to room temperature (usually overnight) and then digested as before.

Results

Thermal Denaturation. In our previous report (Berns *et al.*, 1963) it was shown that the fluorescence intensity of phycocyanin decreases with increasing temperature, but the negative increment of the intensity increases abruptly after passing a certain critical temperature. This feature has been reexamined and completely confirmed. A typical experimental result is reproduced in Figure 1. The first process is reversible, and, further, the relationship between fluorescence intensity and the temperature is independent of the rate of temperature change. On the other hand, there is an irreversible

[2] Although phycocyanin does not obey Beer's law, its concentration can be estimated from the optical density at the peak near 615 mμ if the measurements are carried out under the following conditions: *p*H ~7, 0.01 M phosphate buffer, absorbance in 1 cm light path less than 0.8. $E^{1\%}_{1cm}$ = 6.8 is taken as the extinction at the peak near 615 mμ both for protio- and deuteriophycocyanin. The error owing to the deviation from Beer's law is minor.

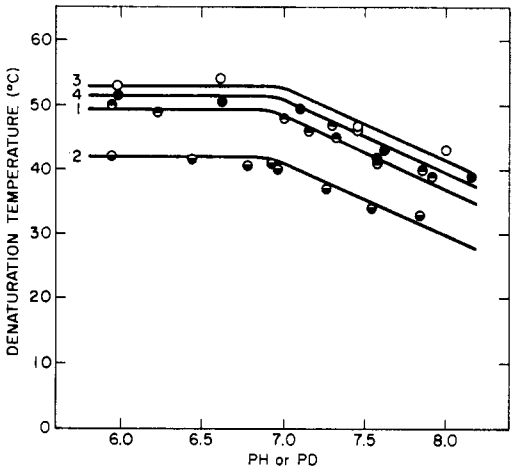

FIGURE 4: The *p*H and *p*D dependence of critical temperature of thermal denaturation. *Plectonema* phycocyanin in 0.02 M phosphate buffer. ◓, H-phycocyanin in H_2O, curve 1; ◒, D-phycocyanin in H_2O, curve 2; ○, H-phycocyanin in D_2O, curve 3; ●, D-phycocyanin in D_2O, curve 4.

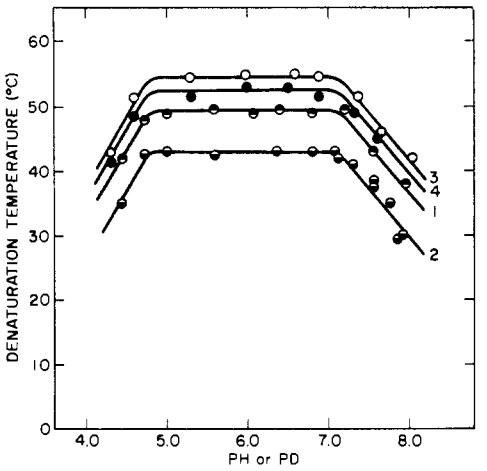

FIGURE 5: The *p*H and *p*D dependence of critical temperature of thermal denaturation. *Phormidium* phycocyanin in 0.02 M phosphate buffer. ◓, H-phycocyanin in H_2O, curve 1; ◒, D-phycocyanin in H_2O, curve 2; ○, H-phycocyanin in D_2O, curve 3; ●, D-phycocyanin in D_2O, curve 4.

decrease in fluorescence intensity as a critical temperature is exceeded.

Figure 2 indicates a coexistence of native and denatured protein in the region of irreversible change, for after partial denaturation followed by cooling the same critical temperature is obtained. However, the shape of the curve in the region of irreversible transition was found to be affected by the rate of temperature change and the duration of heating. The relative proportion of native to denatured form at any particular temperature therefore is a function of both time and temperature. The determination of the temperature at which the velocity of irreversible transition becomes measurable is the more practical from a technical point of view. This temperature will be referred to hereafter as "critical temperature of thermal denaturation" or simply "critical temperature." Since the results of repeated experiments have been shown to be quite reproducible, we have adopted this critical temperature as a measure of the thermal sensitivity of phycocyanin in various environments. Since the phycocyanin from the thermophilic alga *S. lividus* denatures at a relatively high temperature, the fluorescence intensity is quite low at the critical temperature. This makes the critical temperature difficult to obtain by the fluorescence method, but the critical temperature may be readily obtained by measuring absorbance changes. A typical result of such an experiment is shown in Figure 3.

The critical temperature at any particular *p*H (or *p*D) is not affected by ionic strength in the range 0.01–0.2. If buffers of higher ionic strength are used, however, large amounts of protein precipitate during the experiment, so that incremental changes in fluorescence or absorbance become ambiguous. To avoid this complication, 0.02 M phosphate buffers (*p*H 5.6—8.0) or 0.02 M acetate buffers (*p*H 4.3—5.3) were utilized.

In Figure 4 the critical temperatures of thermal denaturation in H_2O of deuterio- and protiophycocyanin obtained from *P. calothricoides* are illustrated as a function of *p*H. The *p*H dependencies of the critical temperature of both deuterio- and protiophycocyanin are similar in the following respects: (1) In the range of *p*H 6.0–7.0 the critical temperatures are on a maximum plateau; and (2) in the range above *p*H 7.0 the critical temperature decreases almost linearly with increasing *p*H. Throughout the *p*H range covered in these experiments, the critical temperature of deuteriophycocyanin is invariably about 7° lower than that of protiophycocyanin. These findings not only agree well with the results of our previous experiments (Berns *et al.*, 1963), which had been carried out with the same phycocyanin preparations in a more limited *p*H range, but also suggest that interactions independent of the ionization of the protein in this *p*H range contribute to the stability of the native protein structure.

Similar experiments were carried out in D_2O-phosphate buffers. In this case, the critical temperature of thermal denaturation is shown as a function of *p*D instead of *p*H (Figure 4). The shape of the curves is almost the same as that obtained in H_2O, since the *p*D-independent region is essentially identical to the *p*H-independent region. However, the critical temperature for both deuterio- and protiophycocyanin is shifted upward, and the difference between the critical temperatures for denaturation of deuteriophycocyanin and protiophycocyanin becomes much smaller, about 2°.

A similar thermal denaturation profile was obtained (Figure 5) when protio- and deuteriophycocyanin from *P. luridum* were used as test proteins. Figure 5 further shows the *p*H (and *p*D) dependence of the critical temperature below *p*H 5 as well as above *p*H 7. It is

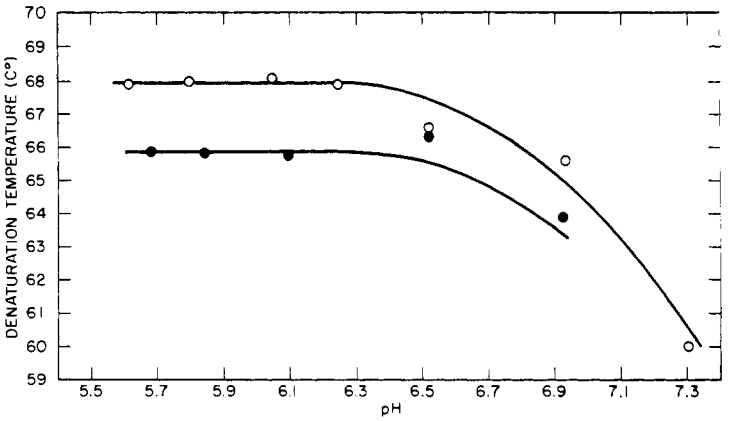

FIGURE 6: The *p*H dependence of critical temperature of thermal denaturation. *Synechococcus* phycocyanin in 0.02 M H_2O–phosphate buffer. O, H-phycocyanin; ●, D-phycocyanin.

noteworthy that the break in the curve in the acidic range of *p*H almost corresponds to the isoelectric point (4.4–4.75 [Lemberg and Legge, 1949; Hattori and Fujita, 1959]) of phycocyanin. The profiles of thermal denaturation in H_2O for *S. lividus* phycocyanin are shown in Figure 6. The higher critical temperatures observed with *S. lividus* phycocyanin are expected from the thermophilic nature of the source organism. A different *p*H dependence of thermal denaturation, and insolubility at low *p*H values, further differentiate the phycocyanin from *S. lividus* from the other phycocyanins used in this study. Preliminary experiments indicate that D_2O also raises the critical denaturation temperature of this protein.

Figure 7 shows the time course of thermal denaturation of phycocyanin followed by measuring the change in fluorescence intensity with time at various fixed temperatures above the critical temperature. The logarithm of the fluorescence intensities was plotted against the time of incubation. A constant temperature was attained within 4 minutes after insertion of the cuvet into the thermostated jacket. Except for these initial few minutes, the observed points fit a straight line on a semilogarithmic plot, an indication that the denaturation is a first-order reaction. The velocity constants for the denaturation in H_2O and D_2O were determined for several temperatures and used to calculate the heat of activation of the denaturation reaction. These data and the values for the free energy and entropy of activation, which were calculated from the values for the rate constant at 53.0°, are given in Table I. The numbers show that the reaction studied here possesses a character typical of protein denaturation.

The upward shift of critical temperature observed when the proteins were dissolved in D_2O indicates that the replacement of exchangeable hydrogen with deuterium stablizes. This stabilization is consistent with the stabilizing action of D_2O on ovalbumin (Maybury and Katz, 1956) and with Hermans and Scheraga's (1959) finding that the substitution of deuterium for

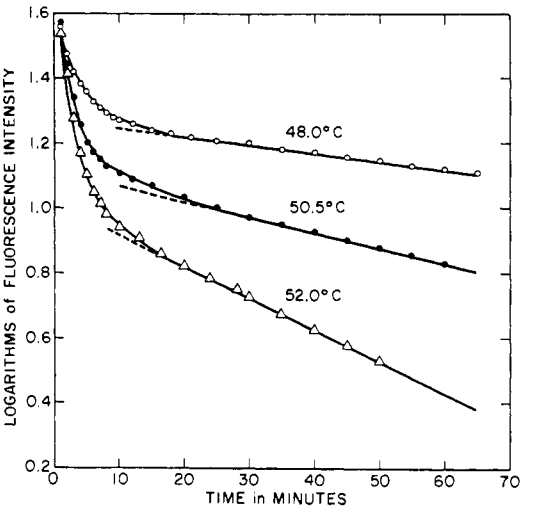

FIGURE 7: Time course of thermal denaturation of *Phormidium* deuteriophycocyanin in H_2O–phosphate buffer, *p*H 7.0, 0.02 M.

exchangeable hydrogen favors the native structure of ribonuclease. In a recent paper, Berns (1963) compared the critical temperatures of protio- and deuteriophycocyanin in H_2O-phosphate and H_2O-acetate with those in D_2O. His conclusion that the critical temperatures in D_2O-phosphate are lower than those in H_2O-phosphate is erroneous because not all his data were derived from the region independent of acidity. The experiments reported here leave no doubt that phycocyanin is more stable in D_2O than H_2O. This view is also supported by the fact that the values of the activation energy in D_2O are higher than those in H_2O (Table I).

The denaturation data of Figures 1 and 2 indicate that two processes are involved in the thermally induced changes. The slope of the first cooling curve of Figure 2 indicates that in the first stages of denaturation the temperature dependence of fluorescence below the

TABLE I: Enthalpy, Entropy, and Free Energy of Activation for Thermal Denaturation of Phycocyanin.[a]

Material	Medium	$\Delta H^{\pm}$ (kcal/mole)	$\Delta S^{\pm}$ (eu)	$\Delta F^{\pm}$ [b] (kcal/mole)
H-Phycocyanin	H_2O	73.9 ± 6.9	161	21.3
H-Phycocyanin	D_2O	138.0 ± 18.3	354	23.0
D-Phycocyanin	H_2O	83.1 ± 12.1	190	20.9
D-Phycocyanin	D_2O	111.2 ± 12.6	274	21.4

[a] *Phormidium* phycocyanin in phosphate buffer, *p*H or *p*D 7.0, 0.02 M. [b] Values at 53.0°.

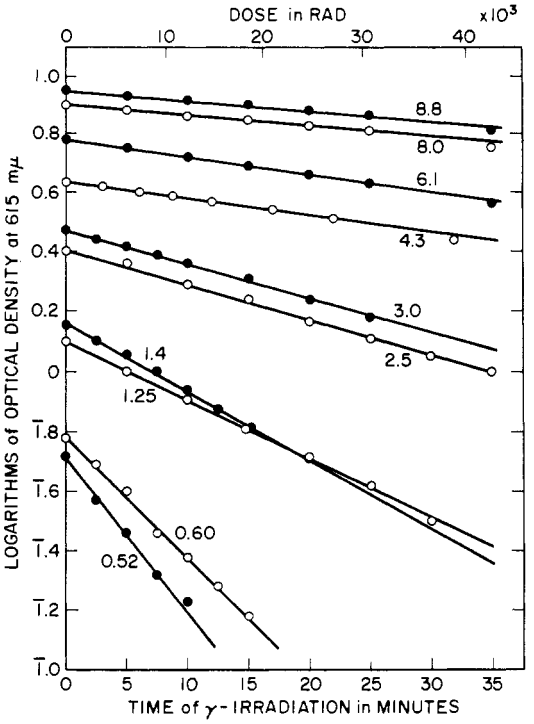

FIGURE 8: γ-Induced denaturation of protio- and deuteriophycocyanin in H_2O–phosphate buffer, *p*H 7.0, 0.02 M. Solutions were exposed to indicated doses of γ-rays from ^{60}Co at a distance of 3 cm. Radiation intensity, 7.64×10^{19} ev/min per liter. O, H-phycocyanin; ●, D-phycocyanin. Figures on individual lines refer to concentrations of phycocyanin in mg/ml.

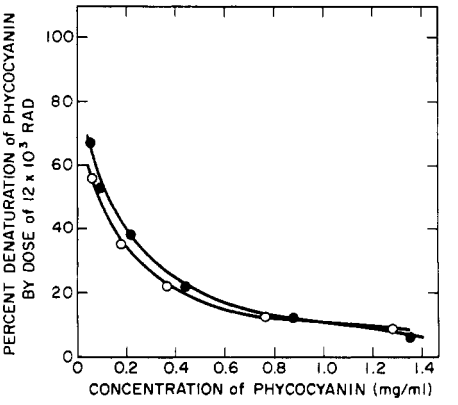

FIGURE 9: Concentration dependence of γ-induced denaturation of phycocyanin. O, H-phycocyanin; ●, D-phycocyanin.

critical temperature is changed only slightly. More extensive denaturation gives the second cooling curve of Figure 2, which shows a marked change in the temperature dependence of fluorescence. Scheraga *et al.* (1962) have suggested that the conformation of proteins is maintained simultaneously by stabilization due to hydrogen bonding (exothermic) and hydrophobic bonding (endothermic). Our data are consistent with this hypothesis, and the critical temperature appears in fact to be determined by these two factors. The endothermic hydrophobic bonding increases in strength with increasing temperature until it reaches a maximal value. We can perhaps interpret the observed thermal denaturation as follows: (1) Below the critical temperature, the deformation of structure due to the breakdown of hydrogen bonding induced by increasing temperature is largely counterbalanced by an increase in the strength of hydrophobic bonding; but (2) above the critical temperature, hydrogen bonding becomes predominant in governing the stability of the protein structure.

γ-Radiation Studies. The dose-response relationship for the denaturation of phycocyanin by γ-ray irradiation is shown in Figure 8. The logarithm of the amount of unchanged phycocyanin decreases linearly with the γ-ray dose with a slope that depends on the initial concentration of the phycocyanin. At a given concentration of phycocyanin, however, denaturation rates were found to be unaffected by changes in acidity between *p*H 5.5 and 8.0, as well as the concentration of the buffer between 0.001 and 0.1 M. All irradiation experiments, therefore, were performed in 0.02 M sodium phosphate at *p*H (or *p*D) 7.0. The effect of γ-radiation was considerably reduced by removal of oxygen from the reaction system, and the rates of anaerobic denaturation of both protio- and deuteriophycocyanin were found to be less than one-fifth of those in the presence of oxygen.

The γ-induced denaturation of phycocyanin is also affected by the protein concentration. Illustrated in Figure 9 is the per cent denaturation at a given dose (12 rad) as a function of the initial concentration of phycocyanin. These results suggest that the denaturation is indirect rather than the result of direct hits (Bacq and Alexander, 1961). An indirect mechanism is also supported by the fact that the effectiveness of γ radiation on frozen samples is much less (about 25%) than at room temperature.

The exponential relationship between the denaturation reaction and the dose is usually ascribed to the protective action of the reaction products. The products of radiation (denatured protein in the present case) continue to interact and thus protect the native (or unreacted) molecules. Adopting this hypothesis, and further assuming that no migration of energy occurs from the activated state of the denatured protein to native protein, the denaturation rate will be expressed: $d \ln P_n/dD = k/P_t$, where P_n represents the concentration of native phycocyanin, P_t that of total (initial) phycocyanin, D is the radiation dose, and k a constant characteristic of the protein.

The values of k calculated from the denaturation rates (Figure 8) and the initial concentrations of phycocyanin are given in Table II. For both protio- and deuteriophycocyanin in H_2O, k-values are almost constant at concentrations above 0.05%. Below this protein concentration there is probably an increase in radical-radical interactions (Bacq and Alexander, 1961). The data indicate that the sensitivity of deuteriophycocyanin to γ radiation, expressed in terms of this k value, is much higher than that of protiophycocyanin.

The results of similar experiments carried out in D_2O are given in Table III. The sensitivity of both protio- and deuteriophycocyanin and deuteriophycocyanin in D_2O is lower than in H_2O. An interesting point is the fact that the difference in sensitivity between protio- and deuteriophycocyanin in D_2O solution is much smaller than that in H_2O. These differences were found, on the average, to be 2.4×10^{-8} mg/ev in H_2O and 1.5×10^{-8} mg/ev in D_2O. This result is in accord with the thermal denaturation behavior of phycocyanin in H_2O and D_2O.

TABLE II: γ Sensitivity of Protio- and Deuteriophycocyanin in H_2O.[a]

H-Phycocyanin		D-Phycocyanin	
Concentration (mg/ml)	$k \times 10^8$ [b] (mg/ev)	Concentration (mg/ml)	$k \times 10^8$ [b] (mg/ev)
0.059	4.3	0.039	4.1
0.15	5.7	0.041	5.6
0.18	6.5	0.051	4.2
0.31	7.6	0.078	7.3
0.37	7.5	0.081	7.1
0.63	7.4	0.090	7.6
0.77	8.1	0.098	6.7
0.96	7.3	0.22	8.8
1.17	8.1	0.44	9.2
1.29	7.3	0.81	11.1
8.8	8.1	0.81	9.9
		0.88	10.0
		1.20	9.6
		1.34	9.7
		2.35	10.5
		9.5	10.0

[a] *Plectonema* phycocyanin. [b] For definition of k, see text.

TABLE III: γ Sensitivity of Protio- and Deuteriophycocyanin in D_2O.[a]

H-Phycocyanin		D-Phycocyanin	
Concentration (mg/ml)	$k \times 10^8$ [b] (mg/ev)	Concentration (mg/ml)	$k \times 10^8$ [b] (mg/ev)
0.34	4.0	0.41	6.3
0.42	5.3	0.45	5.8
0.42	5.9	0.52	7.3
0.53	5.7	0.54	6.8
0.99	5.9	1.08	8.5
1.09	6.4	1.34	7.3
1.61	7.1	1.51	8.3
3.9	6.5	2.14	8.2
4.7	6.9	3.0	8.8
		4.0	7.7
		4.4	8.1

[a] *Plectonema* phycocyanin. [b] For definition of k, see text.

Proteolytic Digestion Studies. When phycocyanin is digested by α-chymotrypsin the absorption peak near 615 mμ is greatly reduced in intensity, while those near 350 and 280 mμ are somewhat increased (Figure 10). Similar changes were observed upon tryptic digestion. It should be noted that most of the apparent initial increase near 280 mμ is due to the presence of the enzyme. In the concentrations used here, trypsin increases the optical density at 280 mμ by 0.19 unit, and α-chymotrypsin causes an increase of 0.11. Any changes in the extinction coefficients of the enzymes (due to self-digestion, for instance) will produce changes with time in the optical density below 300 mμ. Thus changes near 615 mμ are more satisfactory for rate studies than optical density changes near 280 mμ.

The peak near 615 mμ is greatly reduced in a few hours by either trypsin or α-chymotrypsin, but small further changes occur in the height of the peak even after 48 hours. The final optical density is 15–20% of that of the original. The spectral change is similar to that accompanying thermal denaturaton. Initial rates

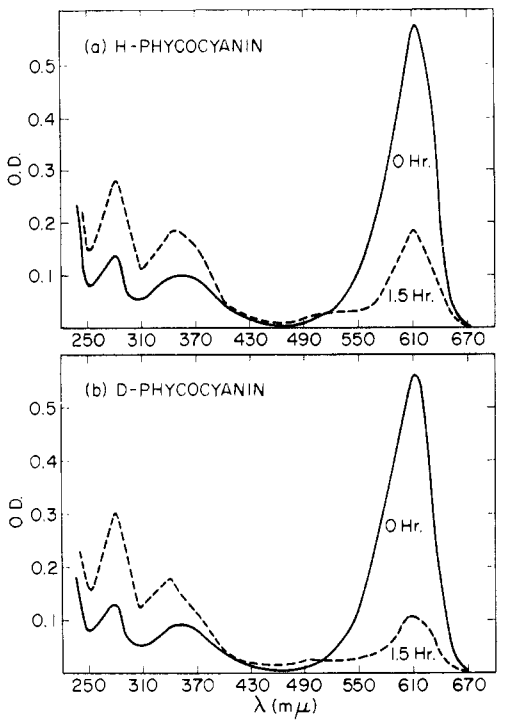

FIGURE 10: Spectral changes on digestion of phycocyanin with α-chymotrypsin. Solid line at zero time; dashed line after 1.5 hours.

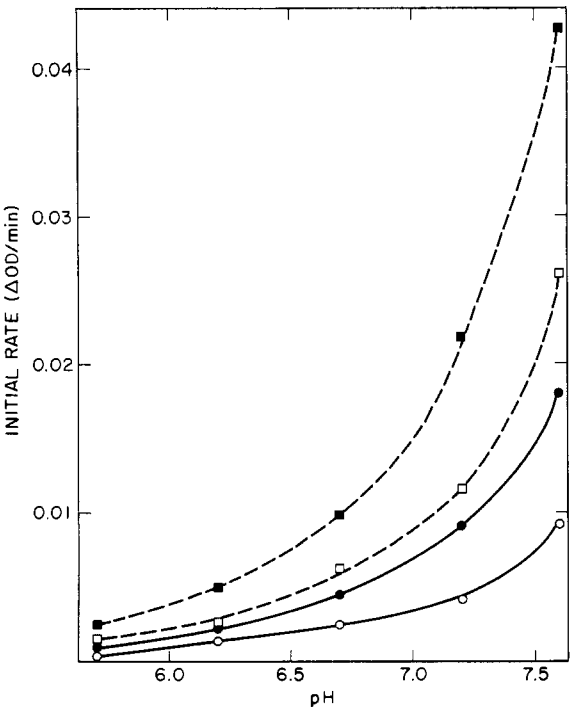

FIGURE 11: Effect of *p*H on rates of digestion of phycocyanin with proteolytic enzymes. With trypsin: ○, protiophycocyanin; ●, deuteriophycocyanin. With chymotrypsin: □, protio; ■, deuterio.

of decrease of the peak near 615 mμ were satisfactorily linear and were used in these comparative rate studies.

Figure 11 shows a plot of the initial rate of decrease of absorbancy near 615 mμ versus *p*H from 5.7 to 7.6. The rate is very low below *p*H 6 but increases rapidly with increasing *p*H. Near *p*H 8 the optical density at the peak near 615 mμ begins to decrease even in the absence of any enzyme because of spontaneous denaturation, so digestions could not be carried out on the native molecule above *p*H 7.6. The *p*H optima of the proteolytic enzymes are near *p*H 7.8 with the synthetic substrates *N*-benzoyl-L-arginine ethyl ester (trypsin) and *N*-acetyl-L-tyrosine ethyl ester (chymotrypsin) (Schwert and Takenaka, 1955). We could not obtain *p*H optima for native phycocyanin because of its unstability above *p*H 8. Figure 11 shows that both enzymes act more actively on deuteriophycocyanin than on protiophycocyanin throughout the *p*H range studied. Figure 11 also shows that α-chymotrypsin is more active than trypsin throughout this *p*H range.

Figure 12 shows the effect of phycocyanin concentration on the rate of digestion by α-chymotrypsin. The variation of the rate with concentration is essentially linear for both protio- and deuteriophycocyanin. The slopes give a comparison of the ease of proteolysis of the two isotopic forms. Deuteriophycocyanin is digested about 50% faster than protiophycocyanin. A similar preference for deuteriophycocyanin was also observed on tryptic digestion. The conditions of these experiments (dialysis of phycocyanin against distilled water before analysis) eliminate the possibility that differences in association equilibria (Hattori *et al.*, 1965) are responsible for the difference in rate of digestion. Digestions were performed on monomer units of the proteins, as the association reactions that follow introduction to buffer are negligible at these dilutions.

Figure 13 shows a plot of the rate of digestion of deuteriophycocyanin by α-chymotrypsin versus the zero-time optical density at 615 mμ. The various lines represent different degrees of denaturation, as measured by the ratio of the optical density at 615 mμ to the optical density at 280 mμ (OD_{615}/OD_{280}). The slopes of the lines in Figure 13 progressively decrease as OD_{615}/OD_{280} decreases. Similar data were obtained for protiophycocyanin, but the rate of decrease of slope is slower, and the lines tend to become parallel as the protiophycocyanin is progressively denatured. Figure 14 shows plots of these rates of digestion versus the ratio OD_{615}/OD_{280}. The curves representing deuterio- and protiophycocyanin are widely separated at an OD_{615}/OD_{280} value of 4.5 (native protein) and come closer together as the ratio decreases, i.e., as the proteins become more denatured. It should be noted that the extinction coefficient at 615 mμ changes as phycocyanin is denatured. Thus the values of "OD_{615} at zero time" plotted in Figure 13 represent concentrations which vary with the extent of denaturation.

Spectrophotometric Titration of Tyrosine. We have also examined the tyrosine titration curves of protio-

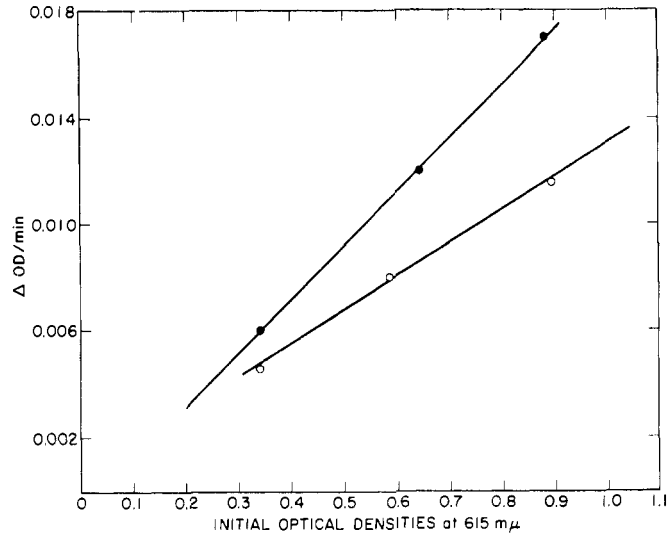

FIGURE 12: Effect of substrate concentration on digestion rate of phycocyanin. The enzyme, α-chymotrypsin; *p*H 7.23. Rate taken as one-fifth the change in A_{615} from 5 to 10 minutes after mixing. ○, protiophycocyanin; ●, deuteriophycocyanin.

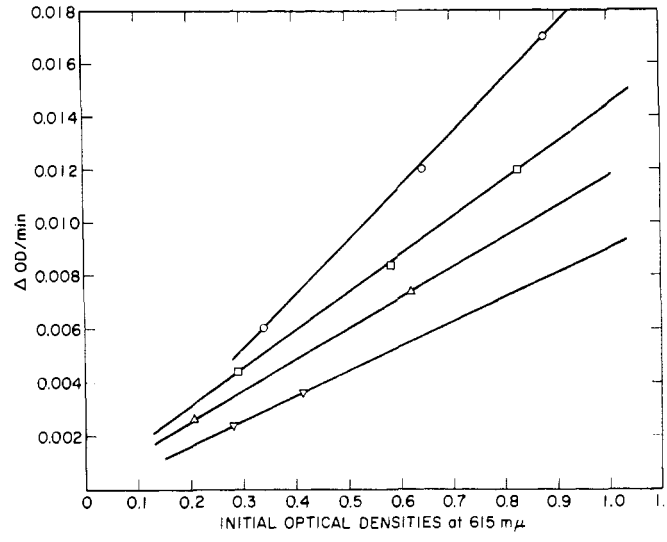

FIGURE 13: Rates of digestion of native and denatured deuteriophycocyanin by α-chymotrypsin in 0.01 M phosphate, *p*H 7.23. The ratios of A_{615}/A_{280}, which indicate the degree of denaturation, are as follows: ○, 4.5; □, 3.0; △, 2.2; ▽, 1.4.

and deuteriophycocyanin, since it is of interest to know whether or not phycocyanin contains any tyrosine residues that titrate abnormally (Beaven, 1961; Wetlaufer, 1962) and whether or not any differences exist in the titration curves of the two isotopic forms of the protein. Unfortunately, phycocyanin denatures above *p*H 8, so that the titration must be done on denatured protein. However, the experiments were carried through by dissolving aliquots of protein solutions in a series of 0.1 M buffers in the *p*H range 8–12.5. Characteristic difference spectra were observed (Hermans, 1962). The difference peak at 245 mμ was used as a measure of tyrosine ionization (Eisenberg and Edsall, 1963) rather than the peak at 295 mμ, since phycocyanin has *p*H-dependent absorption in this region. In both protio- and deuteriophycocyanin, all tyrosines titrated as a single type, with $pK = 10.6 \pm 0.1$.

Since these titration experiments with phycocyanin involve concomitant denaturation, the existence of abnormal tyrosine residues in the native molecule or an isotope effect in the titration of these residues are not precluded. The experiments, however, lend support to the assumption that the two isotopic forms of the protein are identical.

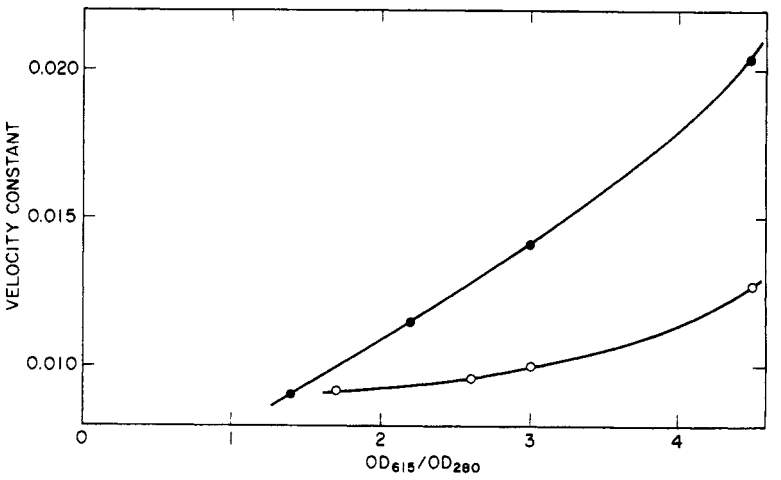

FIGURE 14: Variation of rate of digestion with degree of denaturation. Data taken from Figure 13 and similar data for protiophycocyanin. ○, protiophycocyanin; ●, deuteriophycocyanin.

Discussion

Hydrogen in proteins can be replaced by deuterium in two general ways, and as a result the isotope effects discussed here fall into two distinct categories. The first of these is a solvent isotope effect that results from a change of solvent from H_2O to D_2O. Under such circumstances, hydrogen in exchangeable positions is (reversibly) replaced by deuterium. Mostly it is the hydrogen in hydrogen bonds that is affected, whereas side-chain hydrogen is essentially unaffected (Wishnia and Saunders, 1962). Since hydrogen bonds presumably undergo rupture in thermal denaturation, the solvent effect is a primary deuterium isotope effect. This primary isotope effect will be modified to some degree by an isotope effect on the contribution of structured water (as in hydrophobic bonds and hydrotactoids) to the stability of the protein. The second category of isotope effects involves the hydrogen in the amino acid side chains of the protein. Such hydrogen atoms are nonexchangeable, and proteins containing deuterium in place of hydrogen in nonexchangeable side-chain positions can be obtained only by biosynthesis in fully deuterated media. Replacement of nonexchangeable hydrogen by deuterium primarily involves hydrophobic interactions. Since hydrophobic interactions are nonbonding, isotope effects originating in this fashion may properly be designated as secondary (Halevi, 1963). These secondary isotope effects in proteins can of course be studied in both H_2O and D_2O.

The effects of nonexchangeable deuterium on protein stability have been studied only on biliproteins, and it is hazardous to generalize from so few examples. However, the observations made with phycocyanins isolated from a variety of organisms are similar; these phycocyanins differ considerably in chemical composition, and thus some basis is provided for the presumption that the isotope effects of side-chain deuteration may be general for proteins.

Isotope Effects on the Thermal Denaturation of Proteins. The thermal denaturation of proteins is a kinetic process that may result in a reversible change of conformation, or, as is more often the case, it may lead to irreversible changes in secondary and tertiary structure. The effects of increased temperature on phycocyanin are reversible over a certain temperature range, but at a readily measureable temperature an irreversibly denatured product is produced. The situation may be represented in kinetic terms by

$$N_i \rightleftarrows N_i^* \rightleftarrows [N^{\pm}] \rightarrow D$$

where N_i is the native conformation (or set of conformations); N^* is the configuration (or set of configurations) from which the original conformation(s) can be reversibly restored; $N^{\pm}$ is the activated complex in the irreversible step of the denaturation process; and D is the irreversibly denatured product. The critical temperature for denaturation is that temperature at which the $N^{\pm}$ state is sufficiently populated to give a finite rate of irreversible denaturation. It follows then that a higher critical temperature is indicative of an energetically more demanding activated complex.

Table IV summarizes the observed isotope effects on the critical denaturation temperatures of some phycocyanins. Several conclusions can be drawn from the data: (1) The substitution of deuterium for hydrogen in the side chains consistently lowers the denaturation temperature, but to a greater extent in H_2O than in D_2O. (2) For all the phycocyanins studied, the critical denaturation temperature is higher in D_2O than in H_2O, provided the comparison is made in the *p*H(*p*D)-independent regions (Figures 4 and 5). (3) The *p*D-independent range is essentially the same as the *p*H-independent range (Figures 4 and 5), and the position and magnitude of this plateau region indicates that the thermal denaturation is probably independent of the association state of phycocyanin (*cf.* Hattori *et al.*, 1965).

TABLE IV: Isotope Effects on Critical Denaturation Temperatures for Various Protio- and Deuteriophycocyanins.

Solvent	Protiophycocyanin T_c (°C)	Protiophycocyanin Solvent Effect[a] ΔT_c (°C)	Deuteriophycocyanin T_c (°C)	Deuteriophycocyanin Solvent Effect[a] ΔT_c (°C)	Protein Effect[b] ΔT_c (°C)
H_2O	49.4 ± 0.3[c]		43.0 ± 0.3[c]		6.4[c]
H_2O	49.0 ± 0.8[d]		42.0 ± 0.8[d]		7.0[d]
H_2O	68.0 ± 0.5[e]		65.8 ± 0.5[e]		2.2[e]
D_2O	54.5 ± 0.3[c]	5.1[c]	52.5 ± 0.3[c]	9.5[c]	2.0[c]
D_2O	53.0 ± 0.8[d]	4.0[d]	51.6 ± 0.8[d]	9.6[d]	1.4[d]

[a] Difference in critical denaturation temperature for the indicated protein in D_2O and H_2O. [b] Difference in critical temperature for the indicated solvent between the protio- and deuteriophycocyanins. [c] From *Phormidium luridum*. [d] From *Plectonema calothricoides*. [e] From *Synechococcus lividus*.

Secondary, or Side-Chain Isotope Effect. Protio- and deuteriophycocyanins can be compared in either H_2O or D_2O. The situation here is relatively straightforward, since it is reasonable to assume that the only significant difference between the two proteins will be in the isotopic composition of the amino acid side chains; hydrogen bonding and solvent participation in both the ground state and the activated complexes should be the same for protio- and deuteriophycocyanin in a given solvent. The critical temperature for denaturation is lowered, and, by this token, the rate of denaturation is enhanced by the presence of deuterium in the side chains. In a general way, this secondary isotope effect can be accounted for in terms of a lowered steric requirement of deuterium-substituted nonpolar amino acid side chains. The lowered steric requirements for —C—D versus C—H bonds has been recognized for a long time and has been commented on recently by Brown (1962) and by Bigeleisen (1964); the lower zero-point energy of bonds to the heavier hydrogen isotope reduces the amplitude of its vibrations and results in a lower steric requirement. Where the activated complex is crowded, the lower steric requirement of deuterium relative to hydrogen reduces steric or nonbonding repulsion in the activated complex and so facilitates reaction.

Mislow *et al.* (1964) have described just such a conformational isotope effect in the racemization of 9,10-dihydro-4,5-dimethylphenanthrene. The racemization of this compound is a purely conformational change involving a severely crowded transition state; the smaller steric requirement of deuterium is shown unambiguously to result in a facilitation of racemization when hydrogen in the methyl groups is replaced by deuterium. Similar conclusions were arrived at by Melander and Carter (1964) in a study of the deuterium isotope effect on the racemization of 2,2′-dibromo-4,4′-dicarboxybiphenyl-6-6′-d_2; here also the deuterated compound racemized faster than the hydrogen analog. The increased reaction rate was attributed to a difference in energy of activation occasioned by more favorable nonbonding interactions in the deuterium-containing activated complex (Bartell, 1961). If we assume that the phycocyanin transition state is highly crowded, then the smaller steric requirement of deuterium will promote denaturation. From this view, the smaller steric requirements in deuteriophycocyanin are directly related to the side-chain interactions.

A quantitative assessment of the secondary isotope effect on hydrophobic interactions is not easily made, as it would require a detailed description of the geometry of the transition state relative to the ground state. We consider it unlikely that the phycocyanin denaturation process involves no more than a helix-coil transition.[3]

Nonpolar or apolar interactions are related to the crowding together of nonpolar groups, and since deuterium substitution will lead to less crowding, hydrophobic interactions are reduced. Brandts and Lumry (1963), Zahn (1964), and Schrier *et al.* (1965) have shown that a generalized weakening of hydrophobic interactions tends to lower the temperature of thermal denaturation. The increased susceptibility of deuteriophycocyanin to trypsin is consistent with the idea that the initial step in tryptic digestion involves denaturation of the substrate to overcome steric hindrance to hydrolysis (Putnam, 1953). We have observed that the partial molar volume of deuteriophycocyanin in H_2O is slightly less than that of protiophycocyanin (Hattori *et al.*, 1965). By the criterion of Némethy and Scheraga (1962), this indicates a lessening of hydrophobic interactions. The inverse secondary isotope effect facilitates the denaturation and may thus be construed to be a consequence of relatively more favorable nonbonded interactions in the transition state.

Primary, or Solvent Isotope Effect. The solvent isotope effect is a much more complicated situation.

[3] It has been suggested (Berns, 1963) that rotational isomerism is the important factor in the isotope effect. The importance of hindred rotation cannot be assessed in the absence of any conformational data.

Both the strength of the hydrogen bonds and the water structure are changed in D_2O. All the data directly bearing on this point are in agreement that the introduction of deuterium into hydrogen bonds stabilizes the protein conformation and raises the critical denaturation temperature. Maybury and Katz (1956) found that the urea denaturation rate of ovalbumin in D_2O is only one-third that in H_2O. Calvin *et al.* (1959) observed that thermal transitions in ribonuclease occur at higher temperatures in D_2O, and Von Hippel and Wong (1963a,b) noted similar behavior of collagen in D_2O. The elevation of the denaturation temperature of phycocyanin in D_2O reported here is thus consistent with earlier results and supports the idea that "heavy hydrogen bonds" are more stable than the ordinary ones.

Unfortunately, some troublesome inconsistencies intrude. The experiments of Calvin *et al.* (1959) with poly-γ-benzyl-L-glutamate indicate that deuterated solvents will change the coil-helix transition temperature of this substance, but Doty and Klemperer (cited by Tomita *et al.*, 1962) found no significant difference between the helix-coil transitions in H_2O and D_2O in polyglutamic acid. Further, D_2O has no demonstrable effect on thermal transitions of DNA or RNA (Crespi and Katz, 1962; Mahler *et al.*, 1963). Thus the evidence of the deuterium effect on the strength of hydrogen bonds is contradictory. The magnitude of the isotope effect may be variable for reasons that are now obscure and thus cannot be easily predicted on a priori grounds. In any event, synthetic polypeptides and nucleic acids are equally stable in D_2O and H_2O, but all the natural proteins so far examined are more resistant to thermal denaturation in D_2O.

A puzzling aspect of our data is that side-chain deuteration has a much more pronounced effect in H_2O than in D_2O (Table IV). In both cases where data are available for both protio- and deuteriophycocyanins in both H_2O and D_2O, the difference in denaturation temperature is much smaller in D_2O than in H_2O. In H_2O, the differences between protio- and deuteriophycocyanin are 6.4 and 7.0°, whereas in D_2O the differences are 2.0 and 1.4°. These data are too few from which to generalize. Indeed the small difference in denaturation temperature for protio- and deuteriophycocyanin from *S. lividus* (a thermophilic organism) suggests caution. If water is a component of the transition state, the more structured D_2O (Klotz, 1958; Némethy and Scheraga, 1964; Kavanau, 1964) may impose more severe constraints on the transition state than does H_2O, and the steric aspects of side-chain interaction will thus have less relative importance. Likewise, changes in the geometry of the helix (Tomita *et al.*, 1962) in D_2O could reduce the magnitude of the secondary deuterium effect.

Acknowledgment

We are deeply indebted to Dr. Irving M. Klotz for a critical reading of the manuscript. We acknowledge with thanks the excellent technical assistance of Mr. Robert Stellwagen, Mr. Homer DaBoll, Mrs. Ursula Smith, and Miss Patricia Canton.

References

Bacq, F. M., and Alexander, P. (1961), Fundamentals of Radiobiology, New York, Academic.

Bartell, L. S. (1961), *J. Am. Chem. Soc. 83*, 3567.

Beaven, G. H. (1961), *Advan. Spectry. 2*, 331.

Berns, D. S. (1963), *Biochemistry 2*, 1377.

Berns, D. S., Crespi, H. L., and Katz, J. J. (1963), *J. Am. Chem. Soc. 85*, 8.

Bigeleisen, J. (1964), *J. Chim. Phys. 61*, 87.

Blake, M. I., Crespi, H. L., Mohan, V., and Katz, J. J. (1961), *J. Pharm. Sci. 50*, 425.

Brandts, J., and Lumry, R. (1963), *J. Phys. Chem. 67*, 1484.

Brown, H. C. (1962), The Transition State, A Symposium, London, The Chemical Society, p. 89.

Calvin, M., Hermans, J., Jr., and Scheraga, H. A. (1959), *J. Am. Chem. Soc. 81*, 5048.

Crespi, H. L., Conrad, S. M., Uphaus, R. A., and Katz, J. J. (1960), *Ann. N.Y. Acad. Sci. 84*, 648.

Crespi, H. L., and Katz, J. J. (1962), *J. Mol. Biol. 4*, 65.

Crespi, H. L., Marmur, J., and Katz, J. J. (1962), *J. Am. Chem. Soc. 84*, 3489.

DaBoll, H. F., Crespi, H. L., and Katz, J. J. (1962), *Biotechnol. Bioeng. 4*, 281.

Desnuelle, P. (1960), *Enzymes 4*, 119.

Eisenberg, D. S., and Edsall, J. T. (1963), *Science 142*, 50.

Glasoe, P. K., and Long, F. A. (1960), *J. Phys. Chem. 64*, 188.

Halevi, E. (1963), *Progr. Phys. Org. Chem. 1*, 109.

Hattori, A., Crespi, H. L., and Katz, J. J. (1965), *Biochemistry 4*, 1225 (this issue; following paper).

Hattori, A., and Fujita, J. (1959), *J. Biochem. (Tokyo) 46*, 633.

Hermans, J., Jr. (1962), *Biochemistry 1*, 193.

Hermans, J., Jr., and Scheraga, H. A. (1959), *Biochim. Biophys. Acta 36*, 539.

Katz, J. J., Crespi, H. L., and Finkel, A. J. (1964), *Pure Appl. Chem. 8*, 471.

Kavanau, J. L. (1964), Water and Solute-Water Interactions, San Francisco, Holden-Day.

Klotz, I. M. (1958), *Science 128*, 815.

Lemberg, R., and Legge, J. W. (1949), Hematin Compounds and Bile Pigments, New York, Interscience.

Long, C. (1961), Biochemists' Handbook, Princeton, Van Nostrand.

Lumry, R., Smith, E. L., and Glantz, R. R. (1951), *J. Am. Chem. Soc. 73*, 4330.

Mahler, H. R., Dutton, G., and Mehrota, B. D. (1963), *Biochim. Biophys. Acta 68*, 199.

Maybury, R. H., and Katz, J. J. (1956), *Nature 177*, 629.

Melander, L., and Carter, R. E. (1964), *Acta Chem. Scand. 18*, 1138.

Mislow, K. M., Graeve, R., Gordon, A. J., and Wahl, G. H., Jr. (1964), *J. Am. Chem. Soc. 86*, 1733.

Némethy, G., and Scheraga, H. A. (1962), *J. Phys. Chem. 66*, 1773.
Némethy, G., and Scheraga, H. A. (1964), *J. Chem. Phys. 41*, 680.
Putnam, F. W. (1953), *Proteins 1B*, 893.
Scheraga, H. A., Némethy, G., and Steinberg, I. F. (1962), *J. Biol. Chem. 237*, 2506.
Schrier, E. E., Ingwall, R. T., and Scheraga, H. A. (1965), *J. Phys. Chem. 69*, 298.
Schwert, G. W., and Takenaka, Y. (1955), *Biochim. Biophys. Acta 26*, 570.
Steiner, R. F., and Edelhoch, A. (1962), *Nature 193*, 376.
Strain, H. H., Thomas, M. R., Crespi, H. L., and Katz, J. J. (1961), *Biochim. Biophys. Acta 52*, 517.
Tomita, K., Rich, A., DeLozé, C., and Blout, E. R. (1962), *J. Mol. Biol. 4*, 83.
Von Hippel, P. H., and Wong, K. (1963a), *Biochemistry 2*, 1378.
Von Hippel, P. H., and Wong, K. (1963b), *Biochemistry 2*, 1399.
Wetlaufer, D. B. (1962), *Advan. Protein Chem. 17*, 303.
Wishnia, A., and Saunders, M. (1962), *J. Am. Chem. Soc. 84*, 4235.
Zahn, H. (1964), *Kolloid Z. 197*, 14.

Association and Dissociation of Phycocyanin and the Effects of Deuterium Substitution on the Processes*

Akihiko Hattori,† Henry L. Crespi, and Joseph J. Katz

ABSTRACT: Association-dissociation reactions of deuterio- and ordinary phycocyanin were followed by characteristic changes in the visible absorption spectra under various conditions of *p*H, ionic strength, and protein concentration. Light-absorption peaks of deuterio- and protiophycocyanin are as follows: 617, 625, and 621 mμ for the protio forms, and 610, 617, and 614 mμ for the deuterio forms.

Deuteration of the protein results in a shift of about 7 mμ toward shorter wavelength, irrespective of association state. Complementary information was obtained from sedimentation experiments. Phycocyanin associates or dissociates reversibly to form monomer, trimer, or hexamer, to an extent dependent on conditions. Under identical conditions, the association tendency of deuteriophycocyanin was found to be several times less than that of protiophycocyanin.

Svedberg was the first to show the occurrence of *p*H-dependent association and dissociation of phycocyanin and phycoerythrin (Svedberg and Lewis, 1928; Svedberg and Katsurai, 1929). This observation has been confirmed by subsequent investigations (Svedberg and Eriksson, 1932; Eriksson-Quensel, 1938; Hattori and Fujita, 1959). The most recent of these studies was carried out with samples isolated by improved methods, since some criticism was raised as to the purity of the samples used in earlier work. Unfortunately, most of the earlier studies were mainly concerned with the qualitative aspects of the association phenomena, and the quantitative aspects have not as yet been the subject of active investigation.

The recent success in culturing various algae and microorganisms in pure D_2O has provided a novel way to investigate the structure and function of biologically important substances by the use of fully deuterated compounds. Phycocyanin has been used as a model protein for the investigation of deuterium isotope effects on protein conformation (Berns *et al.*, 1962, 1963; Berns, 1963a,b; Hattori *et al.*, 1965). In one of these studies, we (Berns *et al.*, 1963) observed that phycocyanin does not obey Beer's law. Sedimentation behavior likewise suggested association behavior. Further, the precise positions of the absorption maxima of this protein are variable, depending on the source and even the method of preparation (Haxo and Ó hEocha, 1960; Ó hEocha, 1962). Bergeron (1963) has pointed out the correlation between absorption characteristics and association states, and association-dissociation phenomena appear to be a rather general feature of protein behavior (Reithel, 1963). A detailed knowledge of association-dissociation processes in phycocyanin thus becomes essential if we are to assess the effects of deuterium replacement on the structure and stability of phycocyanin.

In the work reported here, association in phycocyanin was followed by characteristic changes in absorption spectra. These data were correlated with sedimentation

* From the Chemistry Division, Argonne National Laboratory, Argonne, Ill. *Received March 12, 1965.* Based on work performed under the auspices of the U.S. Atomic Energy Commission.

† Resident Research Associate, 1962–1964; on leave from the Institute of Applied Microbiology, University of Tokyo.