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Completely Deuterated Proteins. III. Deuteration Effects on Protein–Protein Interaction in Phycocyanin*

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ABSTRACT: The state of aggregation of fully deuterated phycocyanin is studied as a function of pH, temperature, and ionic strength by sedimentation velocity measurements. Studies of the pH dependence of viscosity and absorption spectra are also presented. The data are compared to those for the normal phycocyanin (Scott, E., and Berns, D. S., *Biochemistry 4*, 2597 (1965)). Under comparable conditions the deuterio protein contains less 11S material and very little 19S material and an enhanced amount of 7S and 3S species. Possible explanations for the results include conventional isotope effects, hydrophobic bonding, and van der Waal's interaction. It is suggested that the 11S aggregate is normally stabilized by dispersion forces in combination with hydrophobic and other forces competing with electrostatic repulsion between the identical subunits, and that in the deuterated protein there is a decrease in polarizability of the groups involved in the protein–protein interaction. More efficient competition of the electrostatic repulsion results in a decreased stability of 11S species with the appearance of more 7S material.

In previous papers in this series (Berns, 1963a,b), we have demonstrated that C-phycocyanins extracted and purified from the blue-green alga *Plectonema calothricoides*, grown in H_2O and in 99.8% D_2O, were antigenically identical but differed in their thermal denaturation characteristics. The aggregation properties of C-phycocyanin have been recently studied in detail, as a function of pH, ionic strength, and temperature (Scott and Berns, 1965), and we now have completed an analogous study on the fully deuterated protein in H_2O. Hattori *et al.* (1965) have recently published a report on the aggregation in the same system and the effects of deuterium substitution. The two studies will be compared where feasible.

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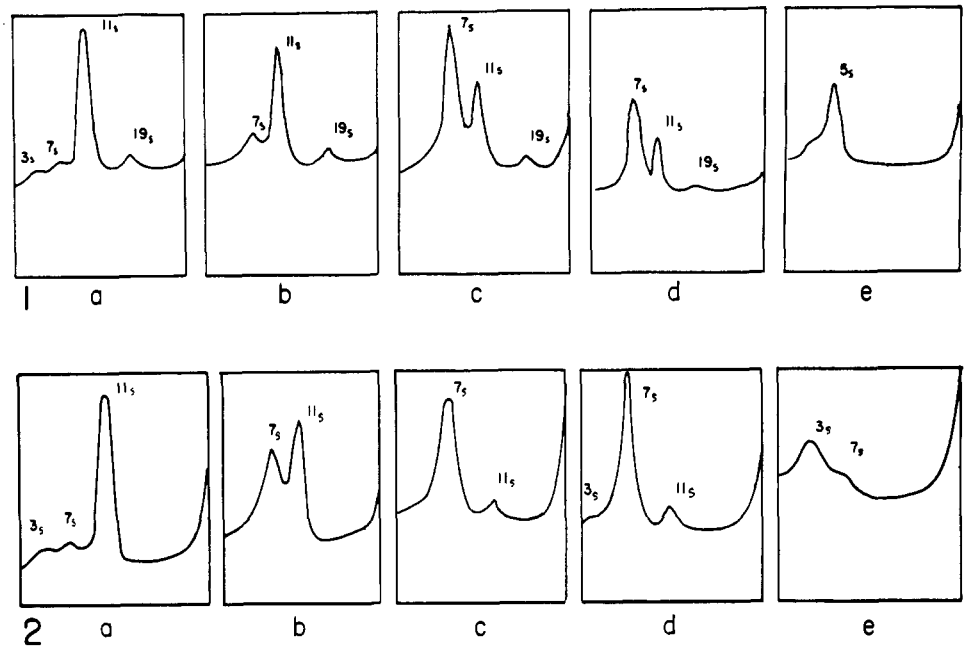
Experimental Section

Materials and Methods. In most of our studies fully deuterated phycocyanin from *P. calothricoides* was used; however, phycocyanin from *Phormidium luridum* was also employed. Previous work (Scott and Berns,



FIGURES 1, 2: Typical sedimentation studies of phycocyanin from protio *P. calothricoides* and deuterio *P. calothricoides*. All sedimentation is from left to right at 59,780 rpm; each picture is at 40 min, ~25°, and H_2O buffers at ionic strength of 0.1 at each pH. Figure 1 is protiophycocyanin. Figure 2 is deuteriophycocyanin. (a) pH 5.0, acetate; (b) pH 6.0, phosphate; (c) pH 7.0, phosphate; (d) pH 8.0, phosphate; and (e) pH 9.0, carbonate.

1965; A. Morgenstern, unpublished data) indicated that these two proteins have essentially identical behavior in the protio form; that is, the sedimentation properties, absorption spectra, diffusion coefficients, and antigenic properties of both are similar. The method of purification of phycocyanin from *P. calothricoides* was identical with that used previously (Scott and Berns, 1965). A step in our ammonium sulfate fractionation procedures, which was of particular utility in separating allophycocyanin from C-phycocyanin, was dialysis overnight at ~4° of a partially purified pH 7.0 buffered sample into an unbuffered 35% saturated ammonium sulfate solution. Phosphate buffers of pH 7.0, μ = 0.1, were generally used in the purification; however, those of pH 6.0, μ = 0.1, were used as controls as a result of recent purification studies (Berns and Morgenstern, 1966). Buffers in this study were μ = 0.1 unless otherwise noted and were similar to the buffers in the protiophycocyanin studies (Scott and Berns, 1965). Protein concentrations were determined by the Kjeldahl method as modified by Markham for micro amounts (Kabat and Mayer, 1961). The per cent nitrogen in the *P. calothricoides* fully deuterated phycocyanin was 14.40 as determined previously (Berns *et al.*, 1962). The purity of protein preparations and the reversibility of all aggregation phenomena were ascertained as in previous studies (Scott and Berns, 1965; Berns and Morgenstern, 1966).

All physical measurements and their analyses were carried out in the same manner as described previously (Scott and Berns, 1965). They included ultracentrifugation, viscosity, density, and immunodiffusion studies. The sedimentation coefficients were investigated as a function of concentration for the deuterio protein at pH 5.0 and 7.0, μ = 0.1, and were analogous to those observed for the protio protein. Sedimentation coefficients at other pH values were the values for a concentration of ~15 mg/ml. The deuterio protein was tested for complete substitution of deuterium by investigation of the infrared spectra of the protein as stated in previous studies (Berns *et al.*, 1962).

Sedimentation. The pH dependence of sedimentation of deuteriophycocyanin is listed in Table I. Comparison of typical sedimentation patterns for deuterio- and protiophycocyanins is seen in Figures 1, 2. At pH 5.0 and 7.0, μ = 0.1, where a detailed concentration dependence of the sedimentation coefficients was investigated, a result analogous to that observed with the protio system was noted. The sedimentation coefficient of the 11S species was far more sensitive to concentration than the 7S in the concentration region investigated, and the relative area under the sedimenting peaks was apparently independent of concentration and time of sedimentation. The extrapolated S values for the deuterio protein were about 15% greater than those for the protio protein and this difference would be predicted from the fact that the molecular weight of the deuterio protein was about 15% greater than that of the protio protein. The effect of pH on sedimentation of the deuterio protein was entirely analogous to the protio system. Increasing

TABLE I: Sedimentation Behavior of Protio- and Deuterio-C-Phycocyanin as a Function of pH.

	Deuterio		Protio[d]	
pH[a] (buffer)	$s_{0,25}$ (× 10^{13})	Rel Area under Peaks (%)	$s_{0,25}$ (× 10^{13})	Rel Area under Peaks (%)
5.0 (acetate)	4.2[b]	5	3.7[b]	6
	7.9[b]	9	6.3	10
	14.6	87	12.1	75
			18.9	9
6.0 (phosphate)	~4[b]	Trace		
	9.4[b]	52	6.2	25
	12.8[b]	48	12.6	68
			19.2	8
7.0 (phosphate)	~4[b]	Trace		
	7.8	89	7.0	62
	14.7	11	12.9	32
			20.8	7
8.0 (phosphate)	4[b]	Trace		
	6.9[b]	83	6.5	65
	12.4[b]	17	12.5	31
			20.1	5
9.0 (carbonate)	3.5[b]	89	5.2[c]	93
	6.5[b]	10	18.9	7
	14.8[b]	Trace		

[a] All buffers are 0.1 ionic strength. Note that all peaks in the protiophycocyanin studies are apparently completely resolved as determined by double-sector cells. In the deuterio studies, the 3S and 7S peaks are usually not resolved even at 59,780 rpm. This finding is indicative of a reaction boundary. [b] Sedimentation coefficients for a single determination, concentration about 15 mg/ml. [c] Trailing edge noted. [d] From Scott and Berns (1965).

TABLE II: Temperature Studies of Sedimentation.

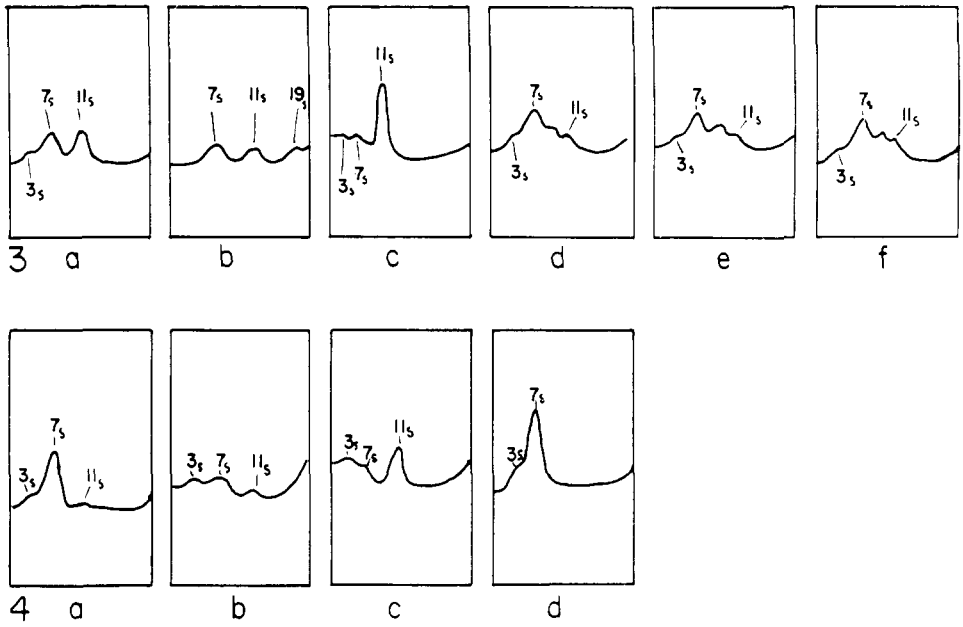
Protein, Buffer, and Temp (°C)	Relative Area under Peaks (%) 3 S	7 S	11 S	19 S
Protio, pH 5.0, μ = 0.1, acetate[a]				
8	17		83	
25	6	10	75	9
Deuterio, pH 5.0, μ = 0.1, acetate				
5	12	18	70	
25	5	9	87	
Deuterio, pH 5.4, μ = 0.02, phosphate				
6		55	45	
22		46	54	
Deuterio pH, 5.4, μ = 0.02, acetate				
8		54	46	
22		38	62	
Protio, pH 6.0, μ = 0.1, phosphate				
3		60	40	
10		45	50	5
25		21	75	4
Deuterio, pH 6.0, μ = 0.1, phosphate				
5		65	35	
15		58	42	
25		52	48	
Protio, pH 9.0, μ = 0.1, carbonate				
8	37	63		
25		93		7
Deuterio, pH 9.0, μ = 0.1, carbonate				
6	51	49		
25	89	10	Trace	

[a] Sedimentation was also carried out with this protein at pH 5.4, μ = 0.02, phosphate at 22°; the behavior was essentially identical with that obtained at pH 5.0, μ = 0.1, acetate.

the pH increased the amount of the slower sedimenting species with an apparently larger amount of smaller aggregates in the comparable deuterio experiment.[1] There was, however, a conspicuous lack of 19S species at all pH values. Careful examination of sedimentation patterns superimposing base lines indicated the presence of trace amounts of 19S material at the lower pH values. When deuterio protein samples purified at pH 6.0 were examined at pH 6.0, μ = 0.1, as much as 10% of the sample was 19 S. This finding is in agreement with the results with the protio protein (Berns and Morgenstern, 1966). Trace amounts of 3S material were evident in all deuterio protein preparations examined at pH 6.0, 7.0, and 8.0.

The temperature effects on sedimentation are indicated in Table II. The trends at pH 6.0 were analogous

[1] In a previous publication (Berns, 1963a) on the immunochemistry of the deuterated proteins, the sedimentation patterns (Figure 4) for the deuterio and protio proteins in saline are incorrectly labeled; the upper pattern is the protio protein and the lower pattern is the deuterio. The deuterio protein in unbuffered saline contained more of the slower sedimenting species, consistent with the present sedimentation experiments.



FIGURES 3, 4: Typical sedimentation studies of phycocyanin from deuterio *P. luridum* purified at pH 7.0. All sedimentation is from left to right at 59,780 rpm; each picture is at 40 min, except 4c which is at 48 min; H_2O buffers at 25° and ionic strength 0.1 unless otherwise noted. (3a) pH 6.0, phosphate; (b) pH 6.0, phosphate purified at pH 6.0; (c) pH 6.0, phosphate +2 M NaCl; (d) pH 6.0, phosphate, μ = 0.01; (e) pH 6.0, phosphate, μ = 0.025; and (f) pH 6.0, phosphate, μ = 0.05. (4a) pH 7.0, phosphate; (b) pH 7.0, cacodylate; (c) pH 7.0, phosphate +2 M NaCl; and (d) pH 7.0, phosphate, μ = 0.01.

to the protio results at pH 6.0 with, at most, trace amounts of 19S material in the deuterio protein. At pH 5.0 at low temperatures in the deuterio protein the 7S species was present, while in the protio system it was absent at low temperatures. It is of interest that the amount of 11 S present at pH 5 at low temperatures was less than the amount present in the protio protein by approximately the same amount as 7S material appearing in the deuterio sample.

The ionic strength dependence of sedimentation was investigated in the deuterio protein at pH 6.0 and 7.0 (Figures 3, 4). In complete analogy to the protio study, in high salt concentration, with buffers at pH 6.0 and 7.0, 2 M in NaCl, a large increase in 11S material was found (Figures 3c, 4c). Small amounts of 3S material were also present. Sedimentation studies at pH 6.0, μ = 0.1 (Figure 3a), indicated the presence of much greater amounts of 7S and 3S material with a small amount of 11S material. The effect of ionic strength on the sedimentation behavior of deuteriophycocyanin from *P. luridum* was investigated in detail. At low ionic strength, μ = 0.01 (Figure 3d), a species appeared to be present intermediate between 7 and 11 S. At μ = 0.025 and 0.05 (Figures 3e, 3f) the species was also evident although its resolution was better at higher ionic strength. It thus appeared that the rate of interconversion of the species might be affected by ionic strength at this pH. At pH 7.0, μ = 0.01 (Figure 4d), *P. luridum* deuteriophycocyanin had no 11S material, while at pH 7.0, μ = 0.1 (Figure 4a), a substantial amount of 11S material was present. The behavior of *P. calothricoides* deuteriophycocyanin in acetate buffer at pH 5.4, μ = 0.02, and also in phosphate buffer showed some variation from the behavior in acetate buffer at pH 5.0, μ = 0.1 (Table II). There is little difference between acetate and phosphate buffers. The deuterio protein does contain a greater amount of 7S species, indicating a greater sensitivity to pH and ionic strength in the deuterio protein.

Viscosity Studies. The intrinsic viscosity of the deuterio protein as a function of pH at 25° is listed in Table III. It appears to go through a maximum in the pH 6.0–7.0 region. The magnitude and trend of

TABLE III: Intrinsic Viscosity as a Function of pH at 25°.

pH[a] (buffer)	[η] (cc/g) Deuterio	Protio[b]
5.0 (acetate)	6.3 ± 0.3	8.3 ± 0.3
6.0 (phosphate)	6.8 ± 0.3	9.3 ± 0.6
7.0 (phosphate)	6.9 ± 0.6	8.4 ± 0.6
8.0 (phosphate)	4.7 ± 0.3	6.1 ± 0.4

[a] Ionic strength is 0.1 for all pH values. [b] From Scott and Berns (1965).

the intrinsic viscosities changes as a function of pH are analogous to those observed for the protio protein and the decrease in intrinsic viscosity from protio to deuterio is in the proper direction predicted from just the decrease in partial specific volume from protio to deuterio. The intrinsic viscosities in this study are consistent with the observed decrease in aggregation in the deuterio protein. These viscosity results with changing pH, and in going from protio to deuterio protein, are not in agreement with those reported by Hattori *et al.* (1965). Their intrinsic viscosities exhibit only a small pH dependence and increase with pH. In addition, they find the intrinsic viscosity of the deuterio protein to be greater than that of the protio protein.

Diffusion Coefficients. The diffusion coefficients for the several species were determined as they were for the protiophycocyanin by the immunodiffusion technique of Allison and Humphrey (1960). The diffusion coefficients for the deuterio protein were within experimental error, the same as those determined for the protio phycocyanin: 4.2 ± 0.4, 7.5 ± 0.6, and 13.2 ± 1.6 × 10^{-7} cm^2/sec at 20° (Scott and Berns, 1965). The buffers used were those employed in all other experiments: acetate, pH 5.0, μ = 0.1; phosphate, pH 6.0, μ= 0.1; phosphate, pH 7.0, μ = 0.1; and phosphate, pH 8.0, μ = 0.1. Analogous to the protio protein, there was a pH dependence of the number of precipitin lines, but within experimental error little pH dependence of diffusion coefficient.

Partial Specific Volume. The apparent partial specific volume of deuteriophycocyanin was determined by the pycnometric method at 25° in the pH range 5.0–9.0. The average partial specific volume was 0.70 ± 0.01 as compared to 0.75 ± 0.01 for the protio protein. This is in good agreement with the value reported by Hattori *et al.* and the value calculated from the known amino acid analysis (Berns *et al.*, 1962) by the method of Cohn and Edsall (1943).

Electrophoresis. Free-solution electrophoresis on a Perkin-Elmer Model 38 electrophoresis apparatus at ~4° was carried out for both deuterio and protio protein in the pH 4.0–9.0 region to characterize the isoelectric point of both proteins. Multiple peaks were present in both systems. A plot of mobility *vs.* pH for both proteins (Figure 5) indicated that in both the isoelectric point was in the pH 4.7 region. The pH dependence of the number and mobility of the peaks in both proteins was quite complex, and the most that could be indicated was a semiquantitative agreement between isoelectric points. Multiple peaks may be due to differences in binding of buffer ions of the several aggregates present. This effect is indicated by the different sedimentation behavior observed in cacodylic acid buffer and phosphate buffer reported for the protio proteins by Scott and Berns (1965). The work of Cann and Goad (1965) is of importance in considering these observed effects.

Spectra. A detailed study of the temperature, pH, and ionic strength dependence of the position and intensity of the absorption maxima indicated similar

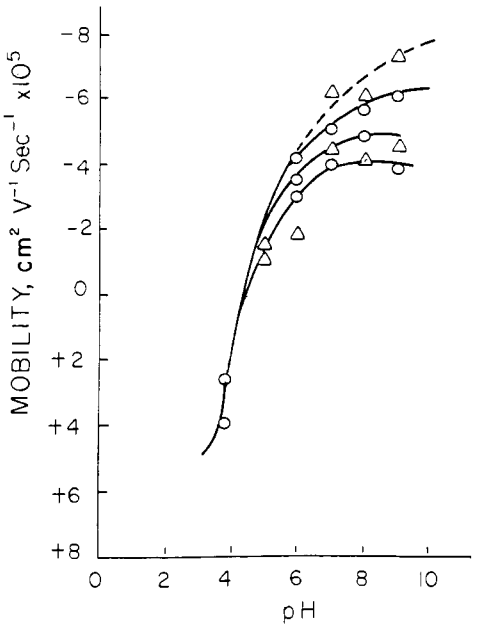


FIGURE 5: A plot of electrophoretic mobility of phycocyanin as a function of pH. All buffers are 0.1 ionic strength; pH 4.0 and pH 5.0, acetate; pH 6.0, 7.0, and 8.0, phosphate; pH 9.0, carbonate. Deuterio- and protiophycocyanin were extracted from *P. calothricoides* and purified at pH 7.0. (open triangle) protiophycocyanin; (open circle) deuteriophycocyanin.

trends to those observed with the protio system (Scott and Berns, 1965) (Table IV). The deuterio protein absorption maximum in the 618-mμ region at 25° shifted to shorter wavelengths with increasing pH and longer wavelengths with increasing ionic strength. At pH 9.0 the maximum is ~603 mμ in the deuterio protein and ~600 mμ in the protio protein. The ratio of 620:278-mμ absorption goes through a maximum in the pH 6.0–7.0 region. There are apparent differences in the absorption maxima in the deuterio and protio system as was observed by Hattori *et al.* (1965). Under comparable conditions, the visible absorption maximum of protio protein is at a wavelength ~4 mμ longer than that of deuterio. The interpretation of these absorption maxima changes is quite complex. Experiments in this laboratory by Dr. O. Kao using sulfhydryl reagents (*p*-mercuribenzoate and methyl mercuric chloride) with subsequent Sephadex gel filtration to separate and characterize this monomeric species have indicated that the 600-mμ absorption maximum is associated with the monomer. Exact assignment of higher wavelength absorption maxima is not possible at present. Samples of *Phormidium* deuteriophycocyanin purified at pH 6.0 that contained 19S material had an absorption maximum at ~620 mμ at pH 6.0, while samples purified at pH 7.0 in which 19S material was not detected when examined at pH 6.0 had an absorption maximum at ~616 mμ.

TABLE IV: Typical Absorption Spectra Experiments.[a]

pH (buffer)[b]	λ_{max}[c] (mμ)	OD Ratio λ_{max}[c]: λ_{278} (mμ)	λ_{max}[d] (mμ)	OD Ratio λ_{max}[d]: λ_{278} (mμ)
5.0 (acetate)	619	4.6	617	4.2
6.0 (phosphate)	617	4.1	615	3.9
6.0 (phosphate + 2 M NaCl)	624	4.9	619	4.3
7.0 (phosphate)	614	3.1	614	3.1
7.0 (phosphate + 2 M NaCl)	622	3.3	618	3.0
7.0 (phosphate, $\mu = 0.01$)	614	3.3	614	3.1
7.0 (cacodylate)	617	4.0	616	3.6
8.0 (phosphate)	614	3.9	612	3.6
9.8 (glycine, $\mu = 0.05$)	605	2.2	603	2.2

[a] These experiments were performed with deuteriophycocyanin from *P. luridum* purified at pH 7.0. Experiments performed with deuteriophycocyanin from *P. calothricoides* yield similar results. Purification of phycocyanin at pH 6.0 results in a red shift of about 4 mμ at pH 5.0, 6.0, 7.0, and 8.0. [b] Ionic strengths of buffers were all $\mu = 0.1$ unless otherwise noted. [c] At 25°. [d] At 4°.

Discussion

In previous studies it was demonstrated by the immunochemical technique that protio- and deuteriophycocyanins from *P. calothricoides* are antigenically identical. The number and character of subunits are antigenically indistinguishable (Berns, 1963a). This result has been supported by the recent study of the optical rotatory dispersion of protio- and deuteriophycocyanin by Boucher *et al.* (1966). In the previous characterizations of thermal denaturation of these proteins (Berns, 1963b) and in the current study, we have been quite cautious in studying and interpreting only effects in identical solvents (H_2O or D_2O). Consequently, interpretations are not complicated by attempts to rationalize hydrogen-bonding differences between the two solvents. In H_2O all hydrogen-bonding sites are populated by protons in both protio and deuterio proteins. Recent studies by Appel and Yang (1965) on synthetic polypeptides have characterized the potential difficulties in interpreting and correlating physical properties of proteins in D_2O and H_2O due to probable changes in the p*K* values of the several amino acids in proteins when exposed to D_2O and H_2O. Since all the present studies were carried out in H_2O buffers, we need concern ourselves only with the secondary isotope effects caused by deuterium substitution at nonexchangeable positions. The potential perturbations due to such isotopic substitution in simple organic molecules have been considered in detail by Halevei (1963), who points out that deuterium bonded to carbon is effectively more electropositive but less polarizable than hydrogen. Streitwieser and Klein (1963) and Paabo *et al.* (1966) have characterized the increased electropositive contribution of deuterium in this situation in several acids. Expected small increases in p*K* values of the several deuterium-substituted acids were found.

The deuterium substitution in simple hydrocarbons and the effect on molar volume and surface tension have been studied recently by Bartell and Roskos (1966). The properties are directly related to steric and cohesive interactions and are of basic importance in a simple discussion of the probable effects of deuterium substitution. The conclusion from the work of Bartell and Roskos is that the lower polarizability of the CD *vs.* that of the CH is the predominant contribution. Another possible effect suggested by Hattori *et al.* (1965) is that deuteration causes a decrease in hydrophobic forces. Solubility studies of model compounds using amino acids, as suggested by the work of Kresheck *et al.* (1965), are not available. Investigation of the solubility of some aromatic compounds by Olsson (1960) and Erlenmeyer *et al.* (1936) gives no clear indication of a general trend.

Under comparable conditions of pH, ionic strengths, and temperatures, the deuterio protein preparations possess a substantially smaller amount of 11S material and a greater amount of 7S material, with the almost complete absence of 19S material. At pH 5.0, close to the isoelectric point, the deuterio and protio proteins behave similarly. There is also a significant amount of 3S material present at all pH values investigated. The 11S species has been previously characterized as a hexameric aggregate and both 11S and 19S aggregates are probably of importance *in vivo* (Scott and Berns, 1965; Berns and Scott, 1966; Berns and Edwards, 1965). In our original characterization of the protiophycocyanin the 7S aggregate was demonstrated to be favored under conditions (high pH, low ionic strength, and low temperature) where the electrostatic repulsion is increased. The 11S and 19S aggregates are stabilized by high temperatures, high ionic strength, and pH values close to the isoelectric point, all of which indicate that the forces stabilizing these aggregates are not electrostatic in nature and may be characterized as being hydrophobic forces, specific side-chain interaction, or dispersion forces. All of these may be contributing to the stabilization of the higher aggregates. Studies with a thermophilic phycocyanin have corroborated this suggestion and have definitely indicated that the 7S aggregate is likely due to electrostatic repulsion (Berns and Scott, 1966). The electrostatic repulsion favoring the 7S aggregate at low temperatures at for instance pH 6.0 is effectively decreased by increasing the temperature. Dispersion forces and hydrophobic interactions are not decreased by higher temperatures, as are electrostatic forces, and therefore they can become the dominant influence in the subunit interaction.

The competition of a specific side-chain interaction with electrostatic repulsion would become more effective at higher temperatures. In the case of the deuterio protein, sedimentation studies at pH 6.0 as a function of temperature indicate less effective competition of the dispersion forces with electrostatic repulsion. This is reflected in a smaller increase in 11S material with increasing temperature in the deuterio protein than in the protio. In addition, less 11S material was observed in the deuterio system temperature studies at pH 6.0 than was the case in the pH studies at pH 5.0. These observations are in direct conflict with any proposed increase in pK's due to deuterium substitution. An increase in pK would result in less charging of the proteins at a particular pH and, therefore, less electrostatic repulsion. These results would appear to eliminate the electropositive nature of CD *vs.* CH as a primary consideration.

The sedimentation results of Hattori *et al.* (1965) are difficult to compare to the present work due to the difference in purification method. In addition, the sedimentation experiments were only at two pH values (5.3 and 7.0, both phosphate buffer) and did not include temperature studies. The sedimentation studies in which the schlieren optics were used are in agreement with results we obtained with hydroxylapatite-treated phycocyanin (Scott and Berns, 1965). There is also some indication from these results that the 7S material is more favored in the deuterio protein. A great deal of Hattori and co-workers' sedimentation data is from studies performed using the absorption optical system at low concentrations, and Berns and Morgenstern (1966) question the validity of these results due to the improper exposure times for the photographic technique.

Hattori *et al.* (1965) indicated in their studies on fully deuterated phycocyanin much smaller amounts of higher aggregates in the deuterio system; however, this does not refer only to the competition between 11S and 7S aggregates but also to the dissociation to the monomeric 3S material. Very little characterization of the 11S and 7S competition is present in their work. The present studies do not indicate a difference in aggregation of the magnitude suggested by Hattori *et al.* (1965), although a definite decrease in aggregation is present. All aggregation phenomena in the work of Hattori *et al.* (1965) are explained in terms of hydrophobic interactions with no implication of electrostatic forces as significant. Results of the present study, in addition to other recent studies (Scott and Berns, 1965; Berns and Scott, 1966; Berns and Morgenstern, 1966), indicate that electrostatic forces are involved as well as, probably, hydrophobic interactions and dispersion forces.

The model compound studies of Kresheck *et al.* (1965) indicate that a greater amount of hydrophobic interaction is expected in D_2O over that in H_2O, and in preliminary D_2O *vs.* H_2O experiments there was a great tendency for increased formation of the 11S aggregate with far less 7S material and little change in 3 S. These studies were not extensive enough to be conclusive and are being continued.[2] The comparison between aggregation in D_2O and in H_2O was striking; however, the difference in aggregation between the protio and deuterio protein in D_2O under comparable conditions was not very significant. The amount of 7S and 3S species, therefore, probably cannot be explained using changes in hydrophobic forces. "Hydrophobic forces" play a role in stimulating the aggregation to 11S and 19S species; however, other factors must contribute. An appealing suggestion is that the dispersion forces are a determining factor in stabilizing the 11S and 19S aggregates once the hydrophobic contribution forces the subunits together. A decrease in the polarizability of a CD bond *vs.* that in a CH bond (Bartell and Roskos, 1966) could result in a sizeable decrease in dispersion forces. This, of course, is a great extrapolation from simple compounds to a summation over the hundreds of CD bonds found in a protein.

van der Waal's forces have been implicated by several investigators as being of possible importance in biological systems (Stockmayer, 1959; Salem, 1962). In simple molecules the dispersion force is the dominant contribution to van der Waal's forces (Wiberg, 1964). Furthermore the interaction energy does not decrease with increasing temperature. With the large number of amino acid side chains and peptide bonds, it is worth considering the contribution of van der Waal's forces to the stabilization of aggregates in proteins. We do not mean to imply that van der Waal's forces, hydrophobic interactions, and specific side-chain interactions are mutually exclusive. Indeed, they are probably cooperative in nature and experimentally it is difficult to discern the contributions of each. In the present study it does appear that the dispersion forces contribute in an important fashion to the stabilization of higher aggregates.

It is tempting to examine a correlation of the thermal denaturation data (Berns *et al.*, 1962; Berns, 1963b) with the change in stability of aggregates; however, at present no simple correlation is evident. For example, near the isoelectric point where aggregation is a maximum no increase in thermal stability of phycocyanin is noted. For the present, this type of correlation appears fruitless.

The equilibria involved in the phycocyanin system are complex and it is difficult to interpret the thermodynamic data calculated from area of sedimentation studies; however, we have tabulated these data for the proposed 7S and 11S equilibrium (Table V). In naively interpreting these data and comparing them to the protio system (Scott and Berns, 1965), it is evident that the change in ΔH going from deuterio to protio is of the same magnitude as that in ΔS. The large decrease in enthalpy of the reaction is of interest in that it brings the value of the enthalpy of this reaction into the range

[2] Several other proteins, in addition to phycocyanin, have been examined in D_2O and H_2O under comparable conditions, and these will be reported shortly. With catalase and trypsin and soybean trypsin inhibitor, where hydrophobic forces have not been implicated in an aggregation phenomenon, no differences were noted. In α-casein, where a competition between hydrophobic and electrostatic forces may be present, there is greater aggregation in D_2O.

TABLE V: Thermodynamics of the Trimer–Hexamer Association.[a]

Temp (°C)	K (l./mole)	ΔF (kcal/mole)	ΔH (kcal/mole)	ΔS (eu)
5.2	1.4×10^3	−4	+7	+40
14.8	2.2×10^3	−4	+7	+40
23.5	2.9×10^3	−5	+7	+40
25[b]	4×10^{4b}	−6[b]	+24[b]	+100[b]

[a] Calculated using sedimentation data from temperature study at pH 6.0, $\mu = 0.1$, phosphate with deuterio protein preparation, unless otherwise noted. Samples for this study were aliquots from a single large sample of concentration ~15 mg/ml. [b] Values from a study on a protiophycocyanin for comparison from Scott and Berns (1965).

of the enthalpy change we observe for the protio phycocyanin at pH 7.0, $\mu = 0.1$ (E. Scott, unpublished observations). This would be explained in terms of a greater electrostatic contribution to the system as we deviate from the isoelectric point and charge up the protein subunits. This observation is in pH 7.0 phosphate buffer, and the phosphate ion is known to bind to proteins; thus, a greater negative charge is probably present. When cacodylic acid buffer is used at pH 7.0, the temperature study on the protio protein exhibits the same temperature dependence and consequently the same ΔH and ΔS as the pH 6.0 protio study. This would seem to be consistent with our suggestion that the electrostatic repulsive forces tend to fragment the hexamer which is held together by van der Waal's forces and other contributing forces and in the deuterio protein a decrease in polarizability and subsequent decrease in the dispersion forces shift the equilibria in favor of the repulsive forces as if it were an increase in pH.

The conclusion drawn from these studies is that van der Waal's forces are to be considered as a significant contribution to protein aggregation. The total protein molecule may not necessarily be the contributing factor in this effect; it may be several side-chain interactions acting cooperatively. The contribution of other effects such as hydrophobic interactions should not be minimized. They may serve to bring the interacting groups close enough so that van der Waal's forces can be most effective in stabilizing the aggregate.

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