The Effect of Ethylene Glycol on the Structure of β-Lactoglobulin*

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ABSTRACT: The effect of ethylene glycol on the structure of β-lactoglobulin A (at pH 3.5, 0.1 M KCl) as observed by optical rotatory dispersion, Cotton effects, sedimentation (at low glycol concentration), and viscometry has been studied. At about 10% glycol the dimeric protein is dissociated into monomeric molecules, and these reassociate at about 20% glycol. We believe that this peculiar transition may be connected with a minimum in the partial specific volume of ethylene glycol which occurs at about 15% glycol. This transition is observed by the polarimetric measurements and verified by sedimentation, but is not seen in viscometric measurements.

At high glycol concentrations our polarimetric data are essentially confirmatory; the Cotton effect data are consistent with Tanford's conclusion that glycol disrupts the native structure and then causes helix formation at very high concentrations. Viscometric measurements made at concentrations of glycol from 60 to 90% show quite clearly that the protein has a minimum of intramolecular structure at about 80% glycol.

The rotatory properties of β-lactoglobulin (β-L[1]) have long been known to be unusual (Christensen, 1952; Tanford *et al.*, 1960). The negative rotation at 589 mμ is unusually low (−24°), which normally indicates the presence of a significant amount of structure; however, the very low negative value of b_0 (−80°) is typical of disordered proteins. It has been known for some time now that certain organic solvents, including dioxane, ethanol, and ethylene glycol (Tanford *et al.*, 1960, 1962), can disrupt the native structure at intermediate concentrations and promote what appears to be α-helical formation at higher concentrations. Tanford *et al.* (1962) reached these conclusions using optical rotations measured at five wavelengths. We thought it would be useful to extend these data by adding Cotton effect measurements and we selected ethylene glycol as the organic solvent of primary interest. While some measurements have also been made with other solvents, only a few of them will be discussed in this paper.

While this work was in progress Timasheff and Townend (1965) reported optical rotatory dispersion data on the three genetic variants β-A, β-B, and β-C. They used methanol, urea, and high pH as denaturants and carried out optical rotatory measurements from 190 to 360 mμ. As will be mentioned below, our data in ethylene glycol are very similar, where comparison is possible, both qualitatively and quantitatively, to the results of Timasheff and Townend in methanol, and they support the earlier conclusions of Tanford *et al.* (1962).

During the course of this work two significant and hitherto unreported phenomena have been discovered. One is an apparent dimer–monomer–dimer transition which occurs in 0.1 M KCl, as glycol is added from 0 to 20%. The other is the demonstration of a maximum in the rate of gelation at about 80% glycol, which we take to be significant new evidence for the conclusion that the protein has more secondary structure at both lower and higher glycol concentrations.

Experimental Procedure

Materials. β-Lactoglobulin (β-L) was isolated from whole fresh milk of Holstein cows from the dairy of the University of Alberta, Edmonton, using the now standard procedure of Aschaffenburg and Drewry (1957). The milk was typed by paper electrophoresis (Timasheff and Townend, 1962). One cow homozygous for β-A and one homozygous for β-B were selected as sources for the two proteins, and several grams of each were prepared though only a few measurements have so far been made with β-B. The concentration of β-L was routinely measured spectrophotometrically using a Beckman DU. $E^{1\%}_{1cm}$ at 278 mμ was taken as 9.6 (Townend *et al.*, 1960b).

Ethylene glycol was Fisher certified grade and was used without further purification. Glass-distilled water was used routinely.

Methods. Optical rotatory dispersion measurements were carried out on a Japan spectroscopic recording instrument at room temperature. The instrument was calibrated with sucrose and was routinely flushed with nitrogen. Protein concentrations of 0.4–1% were used above 300 mμ; 0.04–0.1% from 260 to 300 mμ;

* From the Department of Biochemistry, University of Western Ontario, London, Canada. *Received June 20, 1966.* The work reported here has been supported by the National Research Council and the Medical Research Council of Canada.

[1] Abbreviations used in this paper are: β-L: β-lactoglobulin, β-A, β-B, and β-C: genetic variants of β-L.

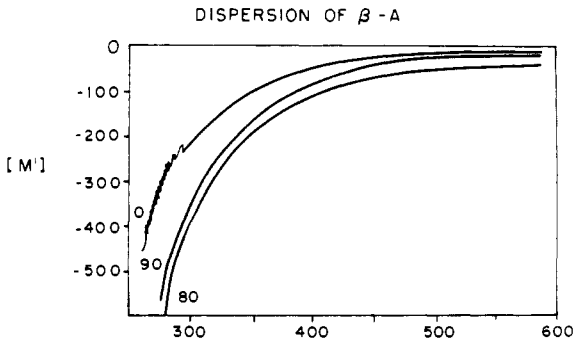

FIGURE 1: The optical rotatory dispersion of β-A in 0, 80, and 90% ethylene glycol. [m'] is plotted against the wavelength. The pH is 3.5, 0.1 M KCl.

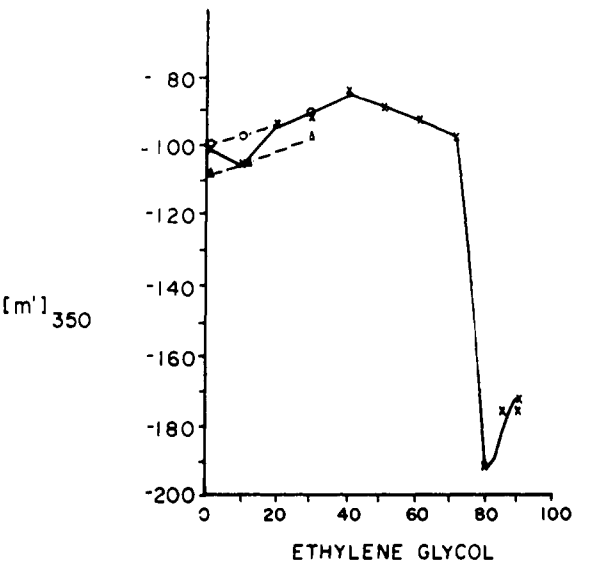

FIGURE 2: The reduced mean residue rotation of β-A at 350 mμ as a function of ethylene glycol concentration at pH 3.5 and various KCl concentration: ×, 0.1 M; ○, 0.06 M; Δ, 0.01 M.

and 0.01–0.03% below 260 mμ. Cells (1 cm) were used above 220 mμ and 1-mm cells below 220 mμ.

Refractive indices of aqueous ethylene glycol were determined with an Abbe refractometer at 589 and 436 mμ; and the Sellmeier approximation (Urnes and Doty, 1961) was used to estimate refractive indices at other wavelengths

$$n_\lambda^2 = 1 + \frac{\lambda^2 a}{\lambda^2 + \lambda_\nu^2} \quad (1)$$

where a and λ_ν are constants. The values of n_λ so determined were used in the determination of the optical rotatory dispersion parameters from eq 2, the well-known Moffitt and Yang (1956) equation

$$[m'] = \frac{3}{n_\lambda^2 + 2}\left(\frac{MRW}{100}\right)[\alpha] = \frac{a_0 \lambda_0^2}{\lambda^2 - \lambda_0^2} + \frac{b_0 \lambda_0^4}{(\lambda^2 - \lambda_0^2)^2} \quad (2)$$

In this equation [α] is the specific rotation, MRW is the mean residue weight (113.3 for β-L), and a_0, b_0, and λ_0 are constant parameters of the dispersion.

Viscometric measurements were made on solutions of β-A (0.5–1.0%) in Cannon–Manning semimicro-viscometers at 25 ± 0.02°. Sedimentation measurements were made on a Spinco Model E analytical ultracentrifuge, kindly made available to us by Dr. P. C. Fitz-James. All runs were made at 25.0°. Protein concentrations of 0.25, 0.50, 0.75, and 1.0% were used and sedimentation coefficients were extrapolated to $c = 0$. The plates were read on a Nikon microcomparator, and $s^0_{20,w}$ values were computed.

The effect of varying concentrations of ethylene glycol on the polarimetric, viscometric, and sedimentation properties of β-A and β-B were studied. Stock solutions of the proteins were made up in 0 or 60% ethylene glycol. The region between 0 and 50% glycol was studied by diluting the 0% stock with the appropriate amount of glycol. Solutions in the region from 60 to 100% glycol were prepared from the 60% stock. The pH of all solutions was maintained at 3.5 as read on a Radiometer pH meter (no corrections were made for the glycol present), and the concentration of KCl was 0.1 M except where otherwise stated.

Results

1. Optical Rotatory Dispersion Measurements. The optical rotatory dispersion of β-A was measured in various concentrations of ethylene glycol between 0 and 100% (v/v). Data are shown in Figure 1 for 0, 80, and 90% glycol, in the region above 250 mμ. From these dispersion curves and other similar curves at other concentrations of glycol we have plotted $[m']_{350}$ (Figure 2) and b_0 (Figure 3) as functions of the solvent concentration. In Figure 4 we show Cotton effect data measured below 260 mμ for 0 and 90% glycol and we have plotted values of $-[\alpha]_{233}$, the trough at about 233 mμ in Figure 3, along with the b_0 data. A scale showing "per cent helix" has been included in Figure 3 to show how the conventional use of b_0 and $-[\alpha]_{233}$ as helix-measuring parameters disagree for this protein, especially seriously at low helix content.

The aromatic Cotton effects (see Figure 1) are observed for β-A at concentrations of glycol up to 60%. At concentrations above 60%, where a major unfolding of the molecule starts, the aromatic Cotton effects disappear. The Cotton effects show troughs at 295 and 286 mμ, peaks at 291 and 283 mμ, and other small peaks and troughs at lower wavelengths. The Cotton trough at 230–240 mμ is actually a split trough in the native protein and this split, or bimodal, trough persists up to 60% glycol, but both minima move to

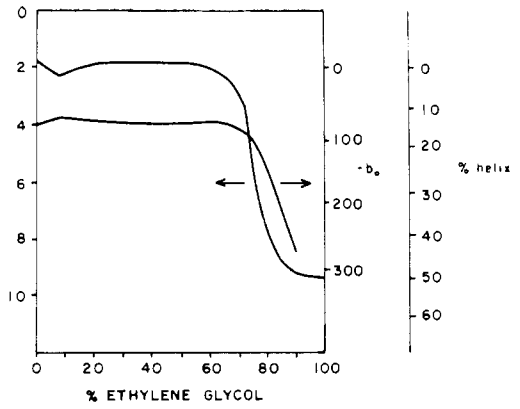

FIGURE 3: The dependence of the value of the specific rotation, $[\alpha]_{233}$ (given in thousands), and b_0 as functions of ethylene glycol concentration at pH 3.5, 0.1 M KCl.

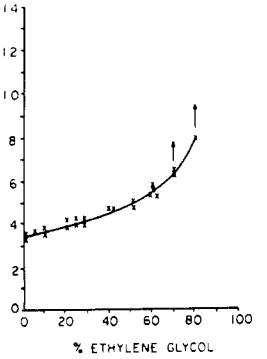

FIGURE 5: Reduced viscosity (milliliters per gram) of 1% solutions of β-A as a function of ethylene glycol concentration at pH 3.5, 0.1 M KCl. The arrows at high glycol concentrations indicate that the results are time dependent.

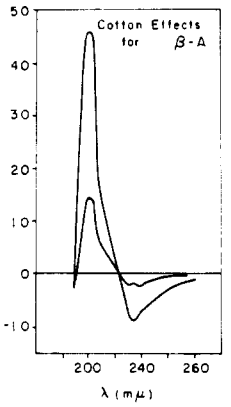

FIGURE 4: Cotton effects for β-A in 0 and 90% ethylene glycol at pH 3.5, 0.1 M KCl. The curve with the large peak and trough is for 90% glycol.

TABLE I: $[\alpha]_{200}$ for β-A in Ethylene Glycol.

Glycol Concn (%)	λ (mμ)	$[\alpha]_{200}$ (deg)
0	204	15,300
10	202	15,900
20	203	15,900
30	203	14,300
40	202	14,300
50	202	15,600
60	203	13,300
70	201	13,300
80	200	47,700
90	201	46,600
100	201	49,300

intermediate wavelengths as the glycol concentration increases. In denaturing concentrations of glycol the splitting of this trough disappears and the new single trough continues to move to lower wavelengths. Similar results, measured in methanol, have recently been reported by Timasheff and Townend (1965).

Values of the Cotton effect peak at 200 mμ are given in Table I for various concentrations of glycol. In general the behavior of this Cotton peak is similar to that of the troughs at 230–240 mμ. Above 60% the magnitude of the peak increases greatly.

2. Viscosity Measurements. The reduced viscosities of 1% solutions of β-A in various concentrations of ethylene glycol (½% in 90% glycol) are shown in Figure 5. Up to about 60% ethylene glycol there is a gradual increase in the reduced viscosity which is independent of time. Above 60% glycol the increase in reduced viscosity is accentuated and the results become time dependent. A study of the time dependence was carried out for reasons explained in the discussion, and the results are shown in Figure 6.

3. Ultracentrifuge Data. Sedimentation coefficients, $s^0_{20,w}$, measured in 0.1 M KCl and various concentrations of glycol between 0 and 30% are shown in Figure 7. Sedimentation rates were determined at four protein concentrations between 0.25 and 1% so that s^0 could be determined. The s^0_{25} values were corrected for the viscosity and density of the medium and for the temperature to give $s^0_{20,w}$ values.

4. Low Glycol Concentrations (0–30%). One of the hitherto unreported phenomena occurring when ethylene glycol is added to aqueous solutions of β-A occurs at about 15% glycol and is evidenced by a small dip or peak in plots of the various rotatory parameters against glycol concentration (Figures 2 and 3). These data are quite reproducible and the size of the dips and peaks, particularly the dip in Figure 2, are considerably larger than the experimental error. Most of the data presented in Figures 2 and all

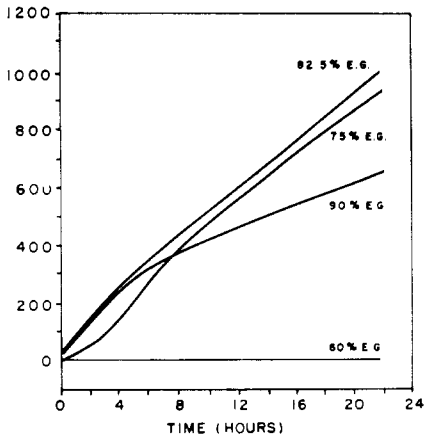

FIGURE 6: Reduced viscosity (milliliters per gram) of 0.5% solutions of β-A as a function of time (hours) in various concentrations of ethylene glycol at pH 3.5, 0.1 M KCl.

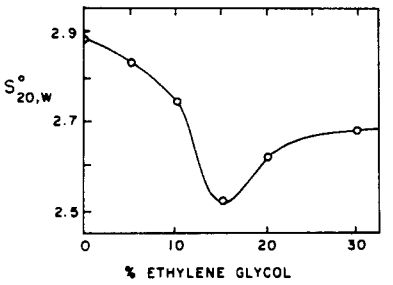

FIGURE 7: Values of $s^0_{20,w}$ for β-A as a function of ethylene glycol concentration at pH 3.5, ionic strength 0.10.

in Figure 3 were measured with an ionic strength of 0.1. For reasons which will be outlined in the discussion it was thought that the salt concentration would affect this transition, and Figure 2 shows this to be true, low values of $-[m']$ being found in 0.6 M KCl and high values in 0.01 M KCl. No transition occurs in either high or low salt. The transition does not cause a significant change in the reduced viscosity (Figure 5) but it does show up when $s^0_{20,w}$ is measured (Figure 7).

5. High Glycol Concentrations (above 30%). A large increase in the negative rotation and in $-b_0$ occurs when the glycol concentration is greater than 60% and below 80% (Tanford *et al.*, 1962). Above 80% glycol we find a decrease in $-[m']_{350}$ (Figure 2). Accompanying this large rotation change are large changes in b_0 and in the magnitude of the Cotton trough at 233 mμ (Figure 3) and the peak at 200 mμ (Table I), though neither of the latter change direction at the highest glycol concentrations. The system is time dependent above 60% glycol and the rotatory and viscometric properties were both studied as functions of time. In general, the rotatory data show small changes lasting for periods of less than 1 hr, while the viscometric data show large changes lasting for long periods and which are due to the onset of gelation. The rotation data in the figures were the values reached after they had stopped changing.

Discussion

1. Low Glycol Concentrations (0–30%). We were surprised to find that as glycol was added to solutions of β-A in 0.1 M KCl a small reversible change in rotatory properties occurred at about 10–15% glycol (Figures 2 and 3). For example, $[m']_{350}$ which equals −100° in water decreased to −106° in 10% glycol and then went back up to −95° in 20% glycol. Similarly b_0 went from −91 in 0% glycol, to −65 in 10%, and back to −83 in 20%. The Cotton trough at 233 mμ went from −1900 to −2195 to 2070°. Interestingly enough, β-B does not exhibit this transition. Townend *et al.* (1960a) showed earlier that at low pH the lactoglobulin dimer–monomer transition favored the monomeric form at low salt concentrations. At pH 2.7 and ionic strength 0.3 the equilibrium favored the dimeric form, but at ionic strength 0.03 it favored the monomeric form. This shift in the equilibrium was later found to be accompanied by small changes in the a_0 parameter of the dispersion equation (Herskovits *et al.*, 1964). We therefore repeated the dispersion measurements in the low glycol region with different salt concentrations, namely, 0.01 and 0.6 M. As can be seen in Figure 2, the value of $[m']_{350}$ found in 0.01 M salt (0% glycol) is −108° (for the monomer) while the value found in 0.1 and 0.6 M salt is −100° (for the dimer). In 0.01 M salt the monomer is apparently favored at least up to 30% glycol, and in 0.6 M salt the dimer is favored. However, in 0.1 M salt, the rotatory properties change from the dimeric values in 0% glycol to the monomeric values in 10% and back to the dimeric values in 20%. Table II shows numerical values for a_0 and $[\alpha]_{233}$ for this transition in both aqueous 0.1 M KCl and in glycol. Values around

TABLE II: a_0 and $[\alpha]_{233}$ Data for the Dimer–Monomer–Dimer Transition.

A. In Aqueous KCl			
KCl	a_0 (deg)	$[\alpha]_{233}$ (deg)	Molecule
0.01	−143	−2140	Monomer
0.10	−126	−1900	Dimer
0.30	−132	−2000	Dimer
0.60	−125	−1940	Dimer
B. In Ethylene Glycol, KCl = 0.1 M			
Glycol	a_0 (deg)	$[\alpha]_{233}$ (deg)	Molecule
0	−126	−1900	Dimer
10	−142	−2195	Monomer
20	−118	−2070	Dimer
30	−118	−1915	Dimer

$a_0 = -143°$ and $[\alpha]_{233} = -2170°$ characterize the monomer, while values around $a_0 = -125°$ and $[\alpha]_{233} = -1925°$ characterize the dimer. Because Timasheff and Townend (1961) had measured sedimentation coefficients for the dimer–monomer transition, we did too, at 0.1 M KCl and at various glycol concentrations. Values of $s^0_{20,w}$ are plotted against the glycol concentration in Figure 7 and a minimum, indicating a shift in the equilibrium to favor monomer, is clearly demonstrated. The values shown are not strictly comparable to those of Timasheff and Townend (1961) since they did not extrapolate their results to zero protein concentration, but the results are in qualitative agreement and fair quantitative agreement with theirs.

The nature of the schlieren pictures is consistent with the interpretation we have given the results. In 15% glycol the schlieren pictures show no evidence of asymmetry, while at higher and lower glycol concentrations some skewing could be detected, as is expected for associating systems (Schachman, 1959). Furthermore, the plots of s against protein concentration are straight lines where we believe the protein is monomeric, but evidence of a downturn at low protein concentrations could be seen in the experiments where we believe both species exist together. These observations, too, are consistent with the interpretation presented above. In spite of the internal consistency of all the data now available, we believe that because of the peculiar nature of the transition, more evidence will be required before the dimer–monomer–dimer transition can be accepted as proved. The transition is now under further study.

Values of $s^0_{20,w}$ have been determined for β-B in 0 and 15% glycol and are 3.02 and 2.91, respectively. These data agree with the polarimetric data that β-B, unlike β-A, favors the dimeric form in 15% glycol.

Ethylene glycol, like ethanol and many other monohydric alcohols, exhibits a minimum value of $\bar{v}$ in aqueous solutions at a concentration around 15% (v/v) (Nakanishi, 1960). This is presumably a consequence of the effect these liquids have on the structure of water.

It is not of course particularly remarkable that the dimer dissociates in 10% glycol, but that it should reassociate in 20% glycol is quite unusual. We are in the process of investigating other alcohols, to see if they too can cause the transition discussed here, and to see if the transition can be more closely related to structural changes in the solvent.

2. High Glycol Concentrations (above 30%). At 60% glycol β-A is denatured as shown by Tanford *et al.* (1962), and our data for $[m']$ and b_0 are in good agreement with their data. In addition we have measured optical rotations in the Cotton effect regions (Figure 3 and Table I). These data serve to verify the conclusion reached by Tanford and De (1961) from $[\alpha]_D$ and b_0 measurements: namely that organic solvents will first cause β-lactoglobulin to unfold, and at very high concentrations will promote the production of α-helical regions in the molecule.

It is also interesting to compare our results with calculations made by Nozaki and Tanford (1965). Working from solubilities of amino acids and related compounds, they made sample calculations of the ethylene glycol concentration required to unfold β-lactoglobulin at pH 3. A glycol concentration of about 75% was found if the fraction of hydrophobic side chains newly exposed in the conformation change was 0.75; of peptide groups 0.50; and of polar side chains 0.25. The calculations also led them to suggest that a conformation containing a large fraction of α-helix would be stable in 90% glycol. Our Cotton effect measurements, and dispersion calculations presented below, show that this is indeed borne out by the experimental results. Both the Cotton trough at 233 mμ and the Cotton peak at 200 mμ are constant and small in magnitude for the native protein, indicating small helical content, but they are both much increased after the denaturation starts, indicating the production of helical content (Simmons *et al.*, 1961; Blout *et al.*, 1962).

It is of interest in this regard to compare our Cotton effect data with those published recently by Timasheff and Townend (1965) for β-A in water and methanol. They found, as we did, that aromatic Cotton effects exist in the 270–290-mμ region when β-A is in water. They were not observed when the protein was in methanol, and we find they disappear when the molecule changes conformation in 60% glycol. These results point to the destruction of an asymmetric environment which exists around the aromatic chromophores in the native molecule. They found a bimodal Cotton trough at about 230 and 240 mμ, in water, as we did. In methanol this trough was unimodal and much larger. Finally, they found that methanol also magnifies the Cotton peak at 200 mμ. Values of our data and theirs for the Cotton troughs and peaks are collected in Table III.

TABLE III: Cotton Effect Data (in degrees). Comparison of Our Results and Those of Timasheff and Townend (1965).

	230 mμ	233 mμ	240 mμ	200 mμ
Water, 0.01 M KCl[a]	−2300	−2100	−2500	10,000
0.1 M KCl, pH 3.5[b]	−1970	−1890	−1930	15,300
Methanol, 0.01 M KCl[a]		−10,400		55,000
100% glycol, pH 3.5[b]		−9050		49,000

[a] Timasheff and Townend (1965). [b] This paper.

In a recent publication, Timasheff *et al.* (1966) analyzed their methanol data on the assumption that the protein contains only α-helix, β-structure, and random structure. They used the equations (Urnes

and Doty, 1961; Tanford and De, 1961)

$$b_0 = f_\beta(b_0)_\beta + f_R(b_0)_R + f_\alpha(b_0)_\alpha \quad (3)$$

$$a_0 = f_\beta(a_0)_\beta + f_R(a_0)_R + f_\alpha(a_0)_\alpha \quad (4)$$

$$f_\beta + f_R + f_\alpha = 1 \quad (5)$$

where f is the fraction of the protein present in a particular fraction and α, β, and R refer to the α-helical, β, or random structures, respectively. Timasheff *et al.* (1966) took the values for the a_0 and b_0 parameters as being: $(a_0)_\alpha = 0$, $(a_0)_\beta = 400$, $(a_0)_R = -650$, $(b_0)_\alpha = -630$, $(b_0)_\beta = 0$, and $(b_0)_R = 0$. In this analysis they found the structural content of native β-A at pH 2 to be 47% random, 13% α-helical, and 40% β, while in 100% methanol it was 10% random, 17% β, and 73% α-helical. A high content of β structure in the native protein has also been indicated by an examination of the infrared spectrum of β-A (Timasheff and Susi, 1966). In this study some α-helix was found in native β-A at pD = 1.0, but a predominance of β structure was indicated.

We have used eq 3–5 and the values listed above for the a_0 and b_0 parameters to analyze our own data for β-A in ethylene glycol. The results are shown in Figure 8. If the assumptions of the analysis are valid, our data for β-A in water of pH 3.5, ionic strength 0.1, are consistent with a structure which is 44% random, 43% β, and 13% α-helical, in good agreement with the results of Timasheff *et al.* Figure 8 shows that in 90% glycol the data are consistent with a structure which is 34% random, 22% β, and 44% α-helical. Comparison with the data of Timasheff and co-workers shows that this structure is very similar to what is found in 50% methanol, namely, 37% random, 16% β, and 46% α-helical. While methanol is a more powerful denaturant than ethylene glycol, all of our data point to the conclusion that both solvents cause the same series of conformation changes, something that is not surprising in view of their similarity.

A high content of β structure was invoked by Timasheff *et al.* (1966) to explain why native β-A has a small value of b_0 and also low values of a_0 and $[m']$. The small negative value of b_0 is a consequence of the presence of only a small amount of α-helix (for 100% helix, $b_0 = -630°$). The large amount of β structure contributes little to the value of b_0 (for 100% β structure, $b_0 = 0°$). Low values of a_0 and $[m']$ occur according to this explanation because the β structure makes a positive contribution to a_0 (400 for 100% β structure) and the random structure contributes negatively (−650 for 100% random structure). The two contributions tend to cancel each other, leaving small values of a_0 and $[m']$. The increase in $-b_0$ at concentrations of glycol greater than 60% is interpreted as being due to the formation of α-helical structure at the expense of β structure; random structure makes no contribution to b_0. The fact that $-a_0$ and $-[m']$ first increase is interpreted as being due to the conversion of β structure to random structure. The subsequent decrease then indicates the conversion of the random structure to α-helical structure.

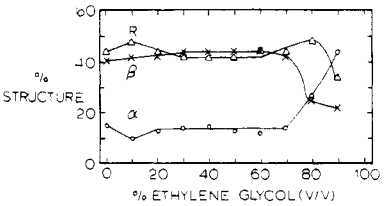

FIGURE 8: Structural content of β-A as a function of ethylene glycol concentration. The assumptions and method of Timasheff *et al.* (1966) have been used (see text).

A phenomenon which can be troublesome at high glycol concentrations is the time dependence of the measurements. We studied this time dependence both polarimetrically and viscometrically. The optical rotation data are time dependent for periods of less than 1 hr, but the viscosity data change for long times, owing to the slow formation of a gel. The viscometric data, which are of considerable interest, are shown in Figures 5 and 6. Figure 5 shows that the reduced viscosity of β-A increases slowly from about 3.4 ml/g in water to about 5.5 ml/g in 60% glycol. None of these data are time dependent, and the dimer–monomer–dimer transition was not observed by this technique. Above 60% glycol the viscosity data are time dependent, and if the solutions are left standing for a long time gelation will occur. It occurred to us that it ought to be possible to use this time dependence to confirm Tanford's conclusion that at very high glycol concentration the molecule folds up in a helical form. If gelation is basically caused by the formation of *inter*molecular hydrogen bonds then the gel should form most easily at some intermediate glycol concentration lower than that which forms the most *intra*molecular hydrogen bonds. We first made up solutions of 0.5% β-A in 75, 82.5, and 90% glycol and allowed them to sit overnight at room temperature. In the morning the solution in 82.5% glycol had set to a turbid gel; the 75% solution, while turbid, had not gelled; and the 90% solution was clear and fluid. This encouraged us to study the time dependence of the reduced viscosity in high glycol concentrations, and some results are shown in Figure 6. Nothing happened to β-A in 60% glycol, while in higher concentrations there are very large increases in the reduced viscosities of 0.5% solutions. (A reduced viscosity of 1000 ml/g in a solution whose concentration is 0.005 g/ml corresponds to a relative viscosity t/t_0 of about 6.) It can be seen from Figure 6 that the rate of viscosity increase is greatest at a concentration between 75 and 90% glycol. We take this to be useful supporting evidence for the conclusion that the molecule passes through what can be called a structural minimum. At this structural minimum the weakest *intra*molecular

hydrogen bonds are formed and hence *inter*molecular hydrogen bonds, leading to gelation, can form. The time dependence of the gelation process and the effect of various solution variables on it is under further study, and we hope to be able to report further results in the near future.

References

Aschaffenburg, R., and Drewry, J. (1957), *J. Dairy Sci. 180*, 376.

Blout, E. R., Schmier, I., and Simmons, N. S. (1962), *J. Am. Chem. Soc. 84*, 3193.

Christensen, L. K. (1952), *Compt. Rend. Trav. Lab. Carlsberg 28*, 37.

Herskovits, T. T., Townend, R., and Timasheff, S. N. (1964), *J. Am. Chem. Soc. 86*, 4445.

Moffitt, W., and Yang, J. T. (1956), *Proc. Natl. Acad. Sci. U. S. 42*, 596.

Nakanishi, K. (1960), *Bull. Chem. Soc. Japan 33*, 793.

Nozaki, Y., and Tanford, C. (1965), *J. Biol. Chem. 240*, 3568.

Schachman, H. (1959), Ultracentrifugation in Biochemistry, New York, N. Y., Academic.

Simmons, N. S., Cohen, C., Szent-Gyorgyi, A. G. Wetlaufer, D. B., and Blout, E. R. (1961), *J. Am. Chem. Soc. 83*, 4766.

Tanford, C., Buckley, C. E., De, P. K., and Lively, E. P. (1962), *J. Biol. Chem. 237*, 1168.

Tanford, C., De, P. K., and Taggart, V. G. (1960), *J. Am. Chem. Soc. 82*, 6028.

Tanford, C., and De, P. K. (1961), *J. Biol. Chem. 236*, 1711.

Timasheff, S. N., and Susi, H. (1966), *J. Biol. Chem. 241*, 249.

Timasheff, S. N., and Townend, R. (1961), *J. Am. Chem. Soc. 83*, 470.

Timasheff, S. N., and Townend, R. (1962), *J. Dairy Sci. 55*, 259.

Timasheff, S. N., and Townend, R. (1965), *Biochem. Biophys. Res. Commun. 20*, 360.

Timasheff, S. N., Townend, R., and Mescanti, L. (1966), *J. Biol. Chem. 241*, 1863.

Townend, R., Weinberger, L., and Timasheff, S. N. (1960a), *J. Am. Chem. Soc. 82*, 3175.

Townend, R., Winterbottom, R. J., and Timasheff, S. N. (1960b), *J. Am. Chem. Soc. 82*, 3161.

Urnes, P., and Doty, P. (1961), *Advan. Protein Chem. 16*, 401.

Dissociation and Association of Phycocyanin*

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ABSTRACT: Phycocyanin from *Anacystis nidulans* dissociates when treated with *p*-mercuriphenylsulfonic acid. Upon removal of the mercurial with glutathione, reassociation occurs. The dissociation is incomplete at pH 5.0 giving rise to various dissociated forms. The dissociation at pH 7.0 is almost complete, yielding two types of subunit which can be separated by gel filtration. The major subunit is characterized by its absorption maximum at 605 mμ and the minor subunit by two maxima at 615 and 652 mμ. The dissociation of phycocyanin results in a decrease of its 620-mμ absorption maximum as well as a shift of it to shorter wavelengths.

Its fluorescence is also decreased. Reassociation of the dissociated subunits brings about a partial regeneration of this decrease in absorption and fluorescence. These reversible spectral changes occurring during dissociation–association are attributed particularly to changes taking place in a highly fluorescent pigment present in the major subunit. Another pigment in the major subunit appears to be less sensitive to conformational changes of the protein.

It has been known ever since the investigations of Svedberg *et al.* (1928, 1929, 1932) and Eriksson-Quensel (1938) that the association–dissociation of phycocyanin, one of the accessory photosynthetic chromoproteins of the red and blue-green algae, is pH dependent. Recent investigations have not only confirmed this (Hattori and Fujita, 1959; Berns *et al.*, 1963), but have also shown a dependence of the reversible aggregation on ionic strength and temperature (Berns and Edwards, 1965; Hattori *et al.*, 1965; Scott and Berns, 1965; Berns and Scott, 1966). Under conditions of varying pH and ionic strength, the association–dissociation of phycocyanin is accompanied by characteristic changes in the absorption and fluorescence spectra (Bergeron, 1963; Goedheer and Birnie, 1965; Hattori *et al.*, 1965; Scott and Berns, 1965). Urea and sodium dodecyl sulfate are capable of causing phycocyanin to dissociate into smaller subunits (Berns *et al.*,

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