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Conformation of Proteins and Polypeptides

I. EXTENSION OF THE SOLVENT PERTURBATION TECHNIQUE OF DIFFERENCE SPECTROSCOPY TO THE STUDY OF PROTEINS AND POLYPEPTIDES IN ORGANIC SOLVENTS

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Previous studies (1-5) related to the present communication have dealt with the development and application of the solvent perturbation technique of difference spectroscopy to studying the location of chromophoric side chains in globular proteins dissolved in aqueous media. The present paper deals with the extension of this new technique to the study of the properties of proteins and polypeptides in helix-forming and random coil-forming organic solvents.

The proteins examined in this study are the milk proteins, α -lactalbumin, β -lactoglobulin, and α_s -casein, as well as some of the proteins previously studied by the author and Laskowski; these are bovine serum albumin (2), ribonuclease (1, 5), insulin,¹ and lysozyme (3, 4).

The accompanying paper (6) reports the optical rotatory properties of these proteins in both nonaqueous and aqueous media.

EXPERIMENTAL PROCEDURE

Materials²—Crystalline bovine insulin (Lot PJ-4086) was obtained through the courtesy of Dr. O. K. Behrens of Eli Lilly and Company; a second sample (Lot K-2459) of essentially identical optical rotatory (6) and difference spectral properties was purchased from Mann Research Laboratories. Crystalline bovine serum albumin (Lot B-2545), pancreatic ribonuclease (five times crystallized, Lot K-1375), egg white lysozyme (three times crystallized, Lot J-2355), sodium salt of poly-L-tyrosine (mol. wt., 80,000; Lot H-1649) and *N*-acetyl ethyl esters of tyrosine, tryptophan, and phenylalanine were also Mann-assayed products. The α_s -casein, β -lactoglobulin, and α -lactalbumin samples used were generously provided by Drs. M. P. Thompson, R. Townend, and L. Weil; the first two proteins were prepared from the milk of single cows according to the procedures of Thompson,³ and Aschaffenburg and Drewry (7), respectively; α -lactalbumin was prepared from the whey of pooled milk by the procedure of Gordon and Ziegler (8).

The organic solvents and reagents employed were analytical grade or of the purest commercially available quality. Dimethylsulfoxide (Fisher-certified reagent) was purified by shak-

ing with sodium bicarbonate followed by vacuum distillation, with the central fraction being retained.

Methods—Nonaqueous solutions of proteins were prepared in stoppered Erlenmeyer flasks. Usually 1 to 12 hours of magnetic stirring was required to dissolve these proteins (either in the cold or at room temperature). Where solution could not be effected by this method, it was found that the addition of small quantities of concentrated HCl (about 1 volume of acid per 1000 of protein solution) would readily effect solution. Where necessary, protein solutions were clarified by centrifugation in a Spinco model L preparative centrifuge. Stainless steel centrifuge tubes and caps were used.

The preparation of aqueous and 8 M urea solutions of proteins, as well as details of the solvent perturbation technique of difference spectroscopy, has been fully described (2). Nonaqueous solutions of proteins were prepared in pairs of matched 5.0-ml volumetric flasks. A given protein stock solution (4 ml) was mixed with pure perturbing solvent (1.0 ml), and a second aliquot of the same stock solution (4 ml, delivered with the same pipette) was mixed with pure solvent (1.0 ml). Where necessary, the volume of the solutions was adjusted with pure solvent. The latter solutions obtained in this manner contained 20% perturbant by volume. Throughout the test, these solutions are simply designated as 20% perturbant solutions.

Protein concentrations were determined spectrophotometrically and gravimetrically. In the latter case the moisture content of the proteins used had to be taken into account. The following extinction coefficients, $E_{1\%}^{1\text{cm}}$, taken from the literature or determined by nitrogen analysis, were employed: α_s -casein equals 10.1 (at 280 m μ);³ β -lactoglobulin equals 9.6 (at 278 m μ (9)); α -lactalbumin equals 21 (at 280 m μ (10)); lysozyme equals 26 (at 280 m μ (10)); ribonuclease equals 7.16 (at 277.5 m μ (10)); insulin equals 10.4 (at 277 m μ); and bovine serum albumin equals 6.67 (at 278 m μ (11)). The extinction coefficient of insulin (Eli Lilly sample) was determined by optical density measurements of acidic solutions and nitrogen analysis. Nitrogen content of 15.9% was assumed (12).

Polytyrosine concentrations were based on the extinction coefficient of 2130 (at 293.5 m μ) per mole of tyrosine in the phenoxide ion form (13). Solutions in ethylene glycol (employed in the difference spectral experiments) were diluted by a factor of 10 with aqueous NaOH, and their optical densities were determined in the presence of 0.2 M NaOH. The effect of

¹ T. T. Herskovits and M. Laskowski, Jr., to be published.

² Mention of specific manufacturers or products does not imply endorsement by the United States Department of Agriculture to the possible exclusion of others not mentioned.

³ M. Thompson, private communication.

residual glycol on the optical density could be neglected since the extinction coefficient of the phenoxide ion in ethylene glycol is only about 2% higher than it is in water (14). The concentrations of *N*-acetyl-L-tyrosine ethyl ester and *N*-acetyl-L-tryptophan ethyl ester were based on the respective molar extinction coefficients of 1340 (at 274.5 m μ) and 5550 (at 280 m μ) in neutral or acidic aqueous solutions (15).

Difference spectral and direct spectral measurements in the 350 to 240 m μ region were made in a Cary model 14 recording spectrophotometer at room temperature ($25 \pm 2^\circ$). Measurements at a single wave length were made in a Zeiss model PMQ II instrument.

The probable error in the difference spectral parameters, $\Delta\epsilon/\epsilon$, ϵ_M , and R_M , defined in the text, are estimated as ± 5 to 7%.

RESULTS AND DISCUSSION

1. Spectra and Difference Spectra of Proteins and Model Compounds in Organic Solvents—The absorption spectra and solvent perturbation difference spectra of proteins in organic solvents are qualitatively similar to those obtained in aqueous media. Fig. 1 compares the direct spectra of one of the milk proteins, β -lactoglobulin, obtained in various solvent media. For most proteins the changes in absorptivity upon denaturation in 8 M urea, acidic methanol, glycol, or chloroethanol are of the order of ± 5 to 10%, while the accompanying shifts in the spectral maxima are from 1 to 2 m μ , toward either shorter or longer wave lengths (blue or red shifts). The shifts in tyrosine and tryptophan difference spectra are of approximately the same order (Figs. 2 and 3). The change from water to ethylene glycol, for example, produces a shift in the two tyrosine peaks of *N*-acetyl-L-tyrosine from 278 to 281 m μ and from 285.5 to 288.5

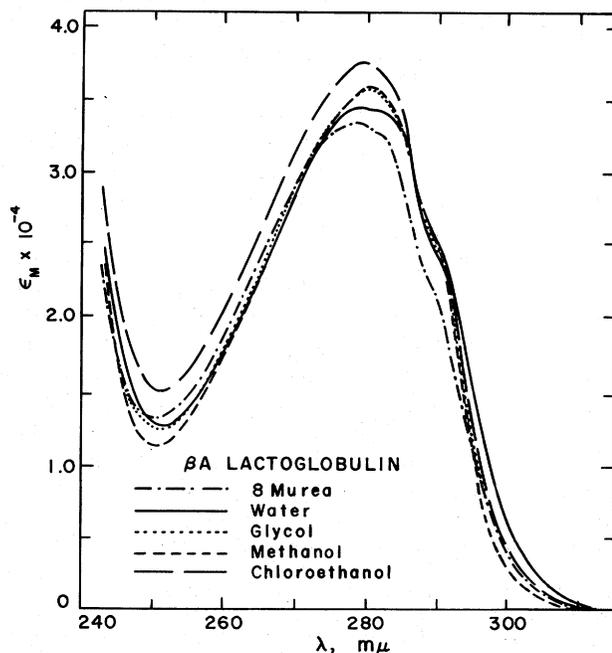


FIG. 1. Ultraviolet absorption spectra of β -lactoglobulin in: water, $\Gamma/2 = 0.03$, pH 5.6; 8 M urea, $\Gamma/2 = 0.03$, pH 3.6; ethylene glycol, 0.01 M HCl; methanol, 0.01 M HCl; and 2-chloroethanol. The molar extinction coefficients, ϵ_M , were based on $E_{1\%}^{1\text{cm}} = 9.6$ at 278 m μ in water (9) and the molecular weight of 36,000.

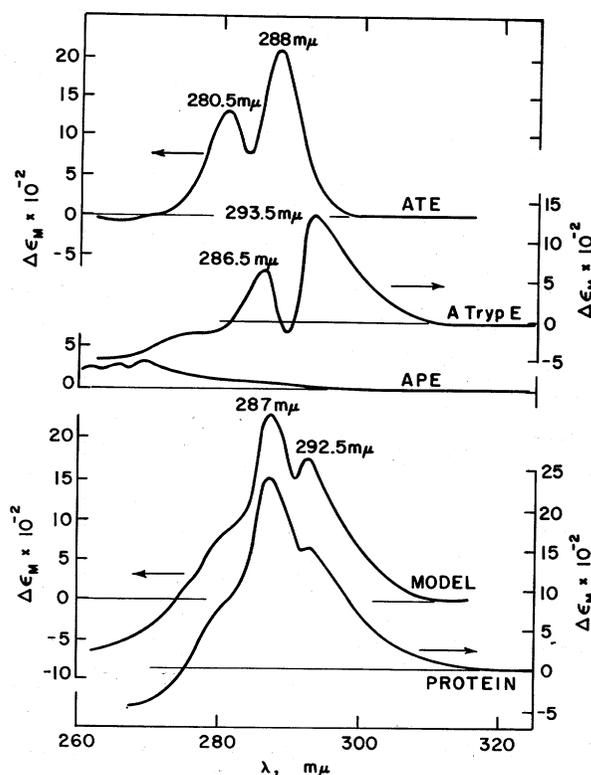


FIG. 2. The solvent perturbation difference spectra of α_s -casein and its constituent chromophoric amino acids in acidic methanol. Perturbant, 20% dimethylsulfoxide. The concentrations of the *N*-acetyl ethyl esters of tyrosine (ATE), tryptophan (A Tryp E), and phenylalanine (APE) used were based on 13 tyrosyl, 3 tryptophyl, and 10 phenylalanyl residues per mole of protein.³ The concentration of model compounds used singly were: 8.7×10^{-4} M *N*-acetyl-L-tyrosine ethyl ester, 2×10^{-4} M *N*-acetyl-L-tryptophan ethyl ester, and 6.7×10^{-4} M *N*-acetyl-L-phenylalanine ethyl ester; the model mixture consisted of 8.7×10^{-4} M *N*-acetyl-L-tyrosine ethyl ester, 2×10^{-4} M *N*-acetyl-L-tryptophan ethyl ester, and 6.7×10^{-4} M *N*-acetyl-L-phenylalanine ethyl ester. The protein concentration used was 0.3%.

m μ (Figs. 3 and 4). In the case of polytyrosine,⁴ the incorporation of tyrosine into the polypeptide matrix produces a slight further red shift in the difference spectral peaks. With 20% dimethylsulfoxide as perturbant and ethylene glycol as solvent, the tyrosine peaks appear at 282 and 289 m μ (Fig. 4). Similar shifts due to the polypeptide chain have been noted by other workers (15, 18, 19).

It should be noted that the changes in the difference spectral parameter, $\Delta\epsilon/\epsilon$, may be as large as 30% in changing from one solvent to another. Moreover, the accompanying changes in $\Delta\epsilon/\epsilon$ for tyrosine and tryptophan are not strictly proportional. In going from water to 8 M urea, for example, there is a slight increase in the $\Delta\epsilon/\epsilon$ values of tyrosine (0.158 as compared with 0.170 with 20% dimethylsulfoxide), whereas for tryptophan there

⁴ The rotatory dispersion properties of the chromophoric biopolymers, poly-L-tyrosine and poly-L-tryptophan are unusual in that both of these polypeptides have positive b_0 values (16). There is evidence in the literature to suggest that poly-L-tyrosine is α -helical in helix-forming solvents (16, 17). While the structure of this polymer has not been established in ethylene glycol, the difference spectral data of Fig. 4 indicates that the tyrosyl side chains in this solvent are very nearly fully accessible to solvent.

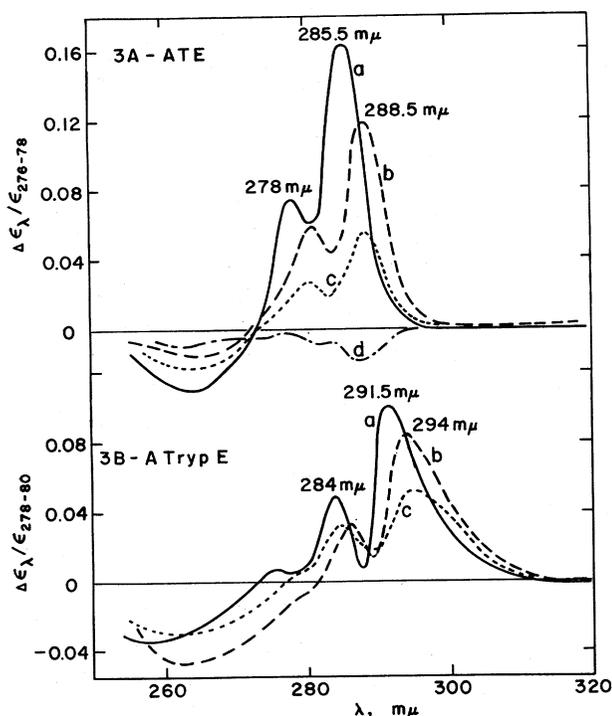


FIG. 3. The effect of perturbants and various solvents on the difference spectra on *N*-acetyl ethyl esters of tyrosine (*ATE*) and tryptophan (*ATrypE*). *A* (*ATE*): Curve *a*, 20% dimethylsulfoxide (*DMSO*) as perturbant in water as solvent; Curve *b*, 20% *DMSO* in ethylene glycol; Curve *c*, 20% polyethylene glycol in ethylene glycol; Curve *d*, 20% glycerol in ethylene glycol. *N*-Acetyl-*L*-tyrosine ethyl ester concentration, 0.8 to 1.4×10^{-3} *M*. *B* (*ATrypE*): Curve *a*, 20% dimethylsulfoxide in water; Curve *b*, 20% dimethylsulfoxide in 2-chloroethanol; Curve *c*, 20% dimethylsulfoxide in ethylene glycol. *N*-Acetyl-*L*-tryptophan ethyl ester concentration 3.8 to 4.0×10^{-4} *M*.

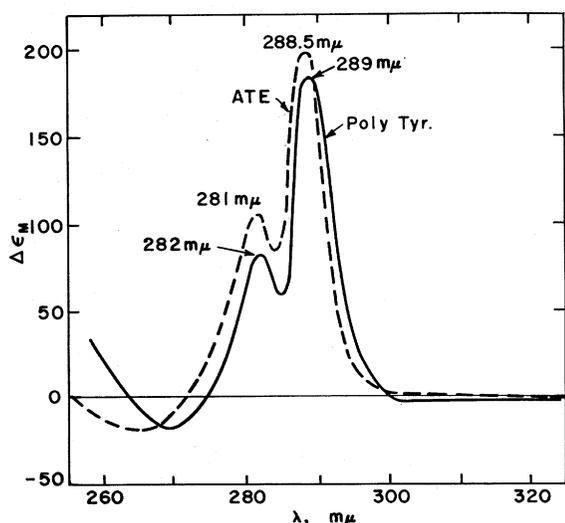


FIG. 4. Comparison of the solvent perturbation difference spectra of poly-*L*-tyrosine and *N*-acetyl-*L*-tyrosine ethyl ester (*ATE*) in ethylene glycol. The perturbant employed was 20% dimethylsulfoxide. The concentration of poly-*L*-tyrosine (solid line) was 1.33×10^{-3} *M* monomer mole per liter, and the concentration of *N*-acetyl-*L*-tyrosine ethyl ester (dashed line) was 1.4×10^{-3} *M*.

TABLE I

Solvent perturbation difference spectral parameters of *N*-acetyl ethyl esters of tyrosine and tryptophan in various solvents due to 20% dimethylsulfoxide

Solvent	Refractive index ^a , n_D	Dielectric constant ^a , D	<i>N</i> -Acetyl- <i>L</i> -tyrosine ethyl ester ($\Delta\epsilon_{286-288}/\epsilon_{276-278}$)	<i>N</i> -Acetyl- <i>L</i> -tryptophan ethyl ester ($\Delta\epsilon_{291-293}/\epsilon_{278-280}$)
Methanol.....	1.3266 ²⁵	32.6 ²⁵	0.128	0.084
Water.....	1.3329 ²⁵	78.5 ²⁵	0.158	0.098
8 <i>M</i> urea.....	1.4003 ²⁰	97 ²⁵	0.170	0.087
Ethylene glycol.....	1.4331 ¹⁵	37.7 ²⁵	0.120	0.054
2-Chloroethanol.....	1.4438 ¹⁵	25.8 ²⁵	0.204	0.085
Dimethylsulfoxide...	1.4787 ²¹	45		

^a Data taken from References 20 and 21. Superscripts refer to the temperature in degrees centigrade.

is an actual decrease in $\Delta\epsilon/\epsilon$ (from 0.098 to 0.087 (Table I)).⁵ In a quantitative study, therefore, it is best to compare the difference spectral constants obtained on a given protein with its model analogue in the same solvent.

The spectral constants used throughout the text are defined in Table II, footnotes *c* and *d*.

2. *Choice of Perturbants and Model Analogues*—The choice of perturbants used in the present study was dictated by the fact that in methanol and in ethylene glycol the difference spectral constants, $\Delta\epsilon/\epsilon$, of proteins and model compounds are appreciably lower than in water (Fig. 3).

From the various perturbants employed in our previous studies (2), dimethylsulfoxide was found to be most suitable because of the relatively large spectral shifts it produces (Fig. 3), and also because of its desirable physical properties (relatively low viscosity and vapor pressure, miscibility with aqueous and many organic solvents, etc.), although other solvents such as tetramethylurea and polyethylene glycol could be employed equally well. The data of Tables II and III on α_s -casein and ribonuclease in 8 *M* urea and ethylene glycol media indicate that these latter perturbants produce essentially the same information as dimethylsulfoxide. Within the experimental uncertainties of the technique the $\Delta\epsilon/\epsilon$ values relative to those obtained with model compounds are very nearly the same. This parameter, designated in Tables II and III as R_M , is a measure of the fraction of chromophoric residues exposed to the perturbing solvent.

Before turning to the discussion of the data obtained on individual proteins, some comments on the choice of model compounds employed as reference are in order. In previous studies on aqueous protein solutions both the proteins in their unfolded state and model compound mixtures corresponding to the tyrosine, tryptophan, and phenylalanine composition of the protein were used for the establishment of the reference values of $\Delta\epsilon/\epsilon$ (1, 2). It is fairly clear, however, that in structure-forming organic solvents the denatured proteins cannot be used

⁵ It should be noted that the data of Table I show no obvious correlation between the magnitude of the spectral shifts and the refractive index or dielectric constant increment produced by the perturbant. Undoubtedly, short range interactions between the solute and solvent contribute to the spectral shifts in these solvents. A discussion of this subject is outside the scope of this paper; the reader is referred to the literature dealing with this subject (18, 22-25).

TABLE II

Effect of polypeptide backbone on perturbation of tyrosine and tryptophan of two disulfide-free proteins in 8 M urea

Perturbant, 20% ^a	Reduced ribonuclease ^b (tyrosine)			α_s -Casein (tryptophan)		
	$(\Delta\epsilon_{287}/\epsilon_{276})^c$		$R_M \times 100\%^d$	$(\Delta\epsilon_{292}/\epsilon_{277})^e$		$R_M \times 100\%$
	Protein	Model mixture ^e		Protein	Model mixture ^f	
Sucrose.....	0.040	0.0455	88 \pm 7%	0.030	0.029	103 \pm 7%
Glycerol.....	0.059	0.068	87	0.032	0.038	84
Ethylene glycol.....	0.064	0.077	83	0.031	0.035	89
Methanol.....	0.059	0.069	86	0.029	0.032	91
Polyethylene glycol ^g	0.098	0.120	82	0.047	0.058	81
Dimethylsulfoxide.....	0.149	0.169	88	0.062	0.069	90
Tetramethylurea.....				0.063	0.065	97

^a With the exception of sucrose, 20 volumes of liquid perturbants were used per 100 volumes of final solution. Sucrose solutions contained 21.6 g of sucrose per 100 ml of solution (such a solution *in water* contains 20% sucrose by weight).

^b Thioglycolic acid-reduced ribonuclease in 8 M urea, 0.02 M thioglycolate, $\Gamma/2 = 0.2$ to 0.25, pH 3 to 6.

^c The difference spectral constant, $\Delta\epsilon/\epsilon_{\lambda, \max}$, is defined as the ratio of the molar difference absorption coefficient, $\Delta\epsilon$, and the molar absorption coefficient, $\epsilon_{\lambda, \max}$ (2); the subscripts refer to the wave lengths at which the difference spectral and direct spectral maxima are observed.

^d R_M is the ratio of the $\Delta\epsilon/\epsilon_{\lambda, \max}$ values of a given protein and its model compound mixture in the same solvent. The model compounds used in this study were mixtures of *N*-acetyl ethyl esters of tyrosine, tryptophan, and phenylalanine.

^e Molar ratio of *N*-acetyl ethyl esters of tyrosine and phenylalanine, 6:3 (26).

^f Molar ratio of *N*-acetyl ethyl esters of tryptophan, tyrosine, and phenylalanine, 3:13:10.³

^g Degree of polymerization, 6.

TABLE III

Effect of perturbant on difference spectral parameters of ribonuclease in acidic ethylene glycol^a

Perturbant, 20% ^b	$(\Delta\epsilon_{287-289}/\epsilon_{276})^c$		R_M^c
	Protein	Model compound	
Dimethylsulfoxide.....	0.062	0.118	0.53
Tetramethylurea.....	0.051	0.086	0.59
Polyethylene glycol ^d	0.031	0.061	0.51

^a HCl, 0.01 M.

^b Ratio of perturbant to solution, 20:100.

^c For the meaning of symbols and abbreviations employed, see Table II, Footnotes *c* and *d*.

^d Degree of polymerization, 6.

for this purpose, since there is no assurance that the chromophoric residues of the protein will remain in free contact with the solvent. Consequently, model compound mixtures consisting of *N*-acetyl ethyl esters of the aromatic amino acids were used for this purpose. Fig. 5 shows the variation of $\Delta\epsilon/\epsilon$ of model mixtures, due to 20% dimethylsulfoxide in water and 2-chloroethanol, as a function of tryptophan content of the mixture. The steepness of the curve for the tyrosine peak at 286 to 288 $m\mu$ suggests that substantial errors may arise in the case of proteins with low tryptophan content. It is gratifying, however, that the variation of $\Delta\epsilon/\epsilon$ for the tryptophan peak at 292 to 294 $m\mu$ as a function of chromophore composition is fairly small; moreover, the tryptophan peak at 292 to 294 $m\mu$ is quite discernible at the tryptophan to tyrosine mole ratio of about 0.2.

From among the seven proteins examined in the present study, only serum albumin belongs to the low tryptophan proteins.

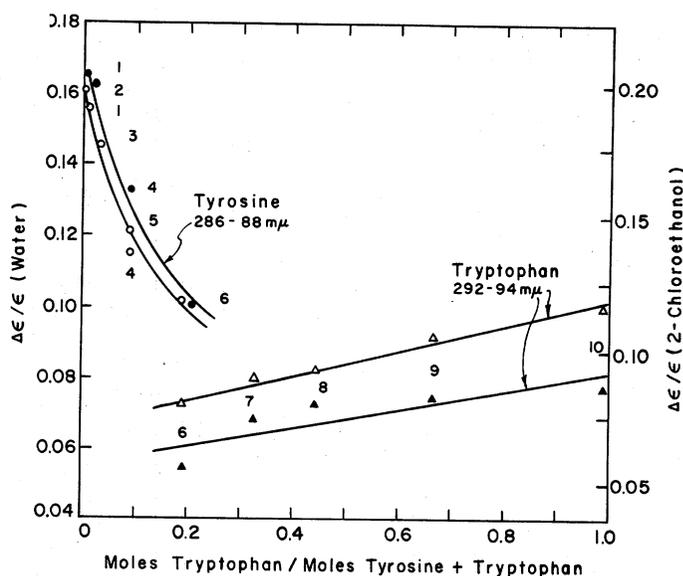


FIG. 5. The variation of the difference spectral parameter, $\Delta\epsilon/\epsilon$ at 286 to 288 $m\mu$ and 292 to 294 $m\mu$ as function of tryptophan content of model mixtures for various proteins. Solvents, water (\circ — \circ and \triangle — \triangle) and 2-chloroethanol (\bullet — \bullet and \blacktriangle — \blacktriangle); perturbant, 20% dimethylsulfoxide. Numbers identify the following model compounds or model compound mixtures for proteins: 1, *N*-acetyl-L-tyrosine ethyl ester; 2, ribonuclease; 3, human serum albumin; 4, bovine serum albumin; 5, ovomucoid; 6, α_s -casein; 7, β -lactoglobulin; 8, α -lactalbumin; 9, lysozyme; 10, *N*-acetyl-L-tryptophan ethyl ester. $\Delta\epsilon/\epsilon$ values were taken from Tables IV to VII and References 2 and 3.

Ribonuclease and insulin contain no tryptophan, while the examination of other proteins, α_s -casein, α -lactalbumin, β -lactoglobulin, and lysozyme, was confined to the 292 to 294 $m\mu$

TABLE IV
Difference spectral parameters of milk proteins in aqueous and organic solvents^a

Protein and solvent	$(\Delta\epsilon_{291-293}/\epsilon_{278-280})^b$		R_M
	Protein	Model mixture ^c	
α-Lactalbumin			
Water, $\Gamma/2 = 0.03-0.1$, pH 6.4-7.0	0.034	0.083	0.45
8 M urea, $\Gamma/2 = 0.03$, pH 3.0-7.4	0.056	0.080	0.70
Ethylene glycol, 0.2 M KCl	0.033	0.050	0.66
Ethylene glycol, 0.01 M HCl	0.038	0.050	0.76
2-Chloroethanol	0.057	0.081	0.70
Methanol, 0.01 M HCl	0.050	0.068	0.74
β-Lactoglobulin			
Water, $\Gamma/2 = 0.03-0.1$, pH 2.2-6.8	0.022	0.087	0.25
8 M urea, $\Gamma/2 = 0.03$, pH 3.2-7.4	0.053	0.083	0.64
Ethylene glycol, 0.01 M HCl	0.032	0.047	0.68
2-Chloroethanol	0.057	0.075	0.76
Methanol, 0.01 M HCl	0.053	0.065	0.82
α_s-Casein			
8 M urea, $\Gamma/2 = 0.03$, pH 3.3-7.4	0.062	0.069	0.90
Ethylene glycol, 0.01 M HCl	0.044	0.045	0.98
2-Chloroethanol	0.062	0.072	0.86
Methanol, 0.01 M HCl	0.046	0.057	0.81

^a For abbreviations and symbols used, see Table II, Footnotes c and d.

^b Perturbant, 20% dimethylsulfoxide.

^c Mixtures of *N*-acetyl ethyl esters of tryptophan, tyrosine, and phenylalanine; respective molar ratios used: α -lactalbumin, 5:5:4 (8); β -lactoglobulin, 4:8:8 (33); α_s -casein, 3:13:10.³ The $\Delta\epsilon/\epsilon$ values are independent of pH and ionic strength.

tryptophan peak. Moreover, it should be noted that even in the case of serum albumin the fairly close agreement between the $\Delta\epsilon/\epsilon$ values of thioglycolic acid-reduced protein (2) and its model mixture in 8 M urea indicates that the possible error in R_M due to the uncertainty in the tyrosine and tryptophan composition of this protein cannot be very large.

3. *Milk Proteins*—The solvent perturbation difference spectra in the 291 to 293 $m\mu$ region of the three principal milk proteins, α -lactalbumin, β -lactoglobulin, and α_s -casein, examined in this study are primarily due to tryptophan residues (Figs. 2, 6, and 7). β -Lactoglobulin and α -lactalbumin are tightly folded globular proteins (27-29), the former being essentially nonhelical (30, 31) while the latter protein is nearly 40% helical (6). α_s -Casein, on the other hand, possesses little, if any, formal structural organization; the best description of its structure in neutral salt solutions is that of a stiff coil or random polypeptide (32).⁶ Kronman, Andreotti, and Holmes (29)⁷ have studied the

⁶ In neutral salt solutions the caseins exist in the form of polymeric aggregates. In addition, since α_s -casein contains no cystine groups its structure seems to be easily deformed. The interpretation of the difference spectra of this casein is complicated by the fact that the addition of perturbant will tend to dissociate or deform these aggregates, producing spectral distortions (2). The difference spectra obtained with 20% sucrose as perturbant appear to be most reliable. Preliminary experiments with this perturbant gave R_M values of 0.6 to 0.8 (pH 6.6 to 7.4, $\Gamma/2$ equals 0.03), suggesting that some of tryptophyl and tyrosyl residues are blocked to perturbant in the casein aggregates.

⁷ M. J. Kronman, L. Cerankowski, and L. Homes, private communication.

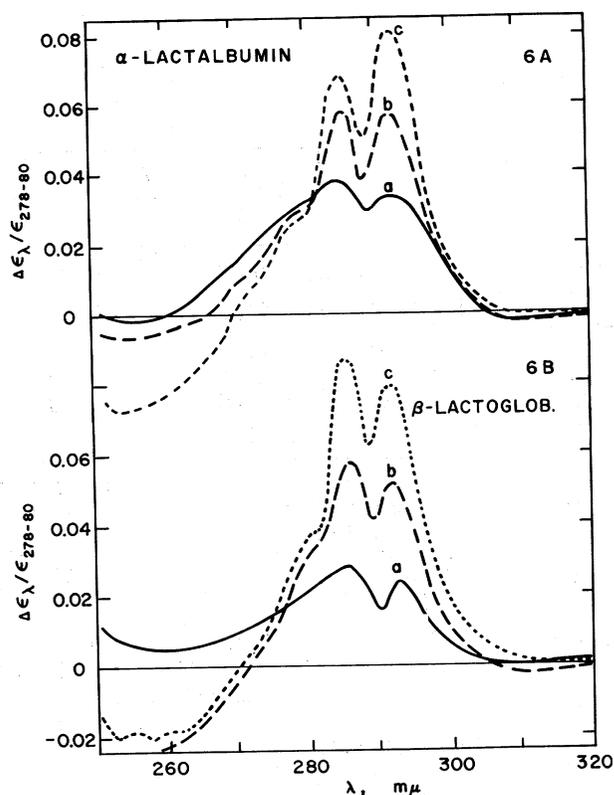


FIG. 6. Comparison of the solvent perturbation difference spectra of α -lactalbumin and β -lactoglobulin and their respective model compound mixtures in aqueous and urea solutions. A, α -lactalbumin: Curve a, 0.11% protein in water, 0.03 M Cl^- , pH 6.7; Curve b, 0.12% protein in 8 M urea, 0.03 M Cl^- , pH 6.2; Curve c, model compound mixture, 3.2×10^{-4} M *N*-acetyl-L-tryptophan ethyl ester, 3.2×10^{-4} M *N*-acetyl-L-tyrosine ethyl ester, 2.6×10^{-4} M *N*-acetyl-L-phenylalanine ethyl ester, 8 M urea, 0.03 M Cl^- , pH 6.8. B, β -lactoglobulin: Curve a, 0.28% protein in water, 0.03 M Cl^- , pH 5.5; Curve b, 0.28% protein in 8 M urea, 0.03 M Cl^- , pH 5.5; Curve c, model compound mixture, 3×10^{-4} M *N*-acetyl-L-tryptophan ethyl ester, 6×10^{-4} M *N*-acetyl-L-tyrosine ethyl ester, 6×10^{-4} M *N*-acetyl-L-phenylalanine ethyl ester, 8 M urea, 0.03 M Cl^- , pH 7. Perturbant, 20% dimethylsulfoxide.

solvent perturbation properties of α -lactalbumin in aqueous media. Their tentative conclusion, based on the sucrose-, ethylene glycol-, and glycerol-induced difference spectra, is that three of the five tryptophyl residues are buried in the interior folds of this protein. The data obtained in this study (Table IV and Fig. 6) indicate that the same fraction of tryptophyl residues is also inaccessible to dimethylsulfoxide. In the case of β -lactoglobulin in neutral salt solutions, it appears that more than half of the tryptophyl residues are buried and inaccessible to perturbant.⁸

In contrast to their state in aqueous media, the unfolding of these two proteins in 8 M urea results in the exposure of a large

⁸ The difference spectral parameters obtained with 20% sucrose, glycol, or dimethylsulfoxide are largely unaffected in the pH region of 2 to 7 at 0.03 ionic strength. In the acid region, below pH 3, β -lactoglobulin is known to dissociate into two nearly spherical subunits (27, 28). Since there are only two tryptophyl residues per subunit, the R_M value of less than 0.5 (e.g. 0.25 to 0.3 with 20% dimethylsulfoxide; Table IV) would mean that either one tryptophyl residue is fully buried and one is partly buried or that both of the tryptophyls are partly buried (T. T. Herskovits, R. Townend, and S. N. Timasheff, to be published).

fraction of the buried tryptophyl and tyrosyl side chains. However, as was noted in previous papers (2, 3) the constraints imposed by disulfide cross-links tend to hinder the access of solvent to chromophores adjoining these cross-links, with the result that the R_M values are lower than the values obtained on disulfide-cleaved or disulfide-free proteins (e.g. α_s -casein).

It should be noted that dissolving these proteins in organic solvents (6, 30) results in the refolding of parts of the polypeptide chains into an altered more helical conformation. While it is reasonable to expect that some of the exposed chromophores could be blocked as a result of these structural changes, the data obtained on α -lactalbumin and β -lactoglobulin, as well as the other proteins of this study (Tables IV to VII), indicate that the fraction of chromophoric side chains exposed in the helix-forming solvents is within about 10% of the fraction of groups exposed in 8 M urea. α -Lactalbumin, for example, has essentially the same R_M value (due to 20% dimethylsulfoxide) in 8 M urea and in 2-chloroethanol, i.e. 0.7 (Table IV), whereas the rotatory dispersion data indicate a randomly-coiled conformation in the former and a nearly 80% helical conformation in the latter solvent (6). It should, however, be noted that the α -helix content of proteins may be largely unaltered, as is the case of α -lactalbumin in ethylene glycol-0.2 M KCl ($b_0 = -240$ as compared with -235 in water, $\Gamma/2 = 0.03$ (6)), while the

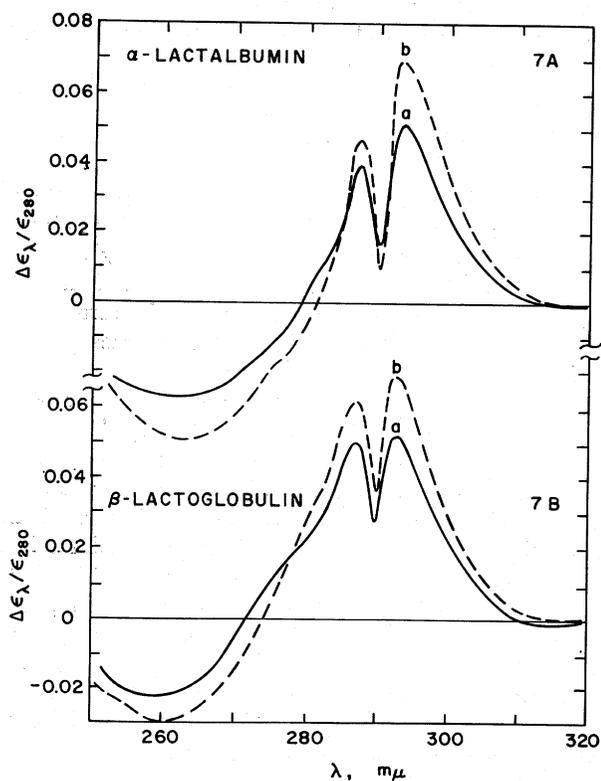


FIG. 7. Comparison of the solvent perturbation difference spectra of α -lactalbumin and β -lactoglobulin and their respective model compound mixtures in acidic methanol. A, α -lactalbumin: Curve a, 0.1% protein, $\Gamma/2 = 0.03$; Curve b, model compound mixture, 3.2×10^{-4} M *N*-acetyl-L-tryptophan ethyl ester, 3.2×10^{-4} M *N*-acetyl-L-tyrosine ethyl ester, 2.6×10^{-4} M *N*-acetyl-L-phenylalanine ethyl ester. B, β -lactoglobulin: Curve a, 0.3% protein, $\Gamma/2 = 0.01$; Curve b, model compound mixture, 3×10^{-4} M *N*-acetyl-L-tryptophan ethyl ester, 6×10^{-4} M *N*-acetyl-L-tyrosine ethyl ester, 6×10^{-4} M *N*-acetyl-L-phenylalanine ethyl ester. Perturbant, 20% dimethylsulfoxide.

TABLE V
Difference spectral parameters of lysozyme in aqueous and organic solvents^a

Protein and solvent	$(\Delta\epsilon_{291-293}/\epsilon_{280})^b$		R_M
	Protein	Model mixture ^c	
Lysozyme			
Water, ^d $\Gamma/2 = 0.025$	0.056	0.088	0.64
8 M urea, ^d $\Gamma/2 = 0.1-0.2$	0.056	0.081	0.69
Thioglycolic acid-reduced lysozyme ^e			
8 M urea, ^d $\Gamma/2 = 0.2$	0.075	0.081	0.93
Lysozyme			
Ethylene glycol, 0.01 M HCl.....	0.039	0.054	0.72
Ethylene glycol, 0.2 M KCl.....	0.029	0.054	0.54
2-Chloroethanol.....	0.051	0.084	0.61

^a For abbreviations and symbols used, see Table II. Footnotes c and d.

^b Perturbant, 20% dimethylsulfoxide.

^c Mixture of *N*-acetyl ethyl esters of tryptophan, tyrosine, and phenylalanine; respective molar ratios, 6:3:3 (37). $\Delta\epsilon/\epsilon$ values are independent of pH.

^d pH 3 to 7. Some of the data were taken from References 4 and 5.

^e Thioglycolic acid-reduced lysozyme in 8 M urea (4).

change in the difference spectral parameter, R_M , indicates a substantial degree of unfolding in the protein structure; in α -lactalbumin the change in R_M is from 0.41, in aqueous salt solution, to 0.66 in ethylene glycol-0.2 M KCl.

4. *Lysozyme*—Among the various globular proteins examined so far by the solvent perturbation technique (1-5, 19),¹ only lysozyme has a substantial fraction of its chromophoric side chains exposed to solvent in the native state. In the native protein about two-thirds of the tryptophyls (4, 34, 35) and tyrosyls seem to be accessible to solvent or reagent. It should be noted that one of the 3 tyrosyl residues is slowly ionizing (36) and unreactive toward cyanuric fluoride (38).

The difference spectral behavior of this enzyme in denaturing solvents is also unusual. As is shown by the data in Table V (see also Fig. 8), neither the helix-forming nor the random coil-forming solvent (2-chloroethanol and 8 M urea, respectively) has a really significant effect on the R_M parameter, despite the fact that the conformation of the native enzyme is substantially altered in these solvents, as is shown by the changes in the rotatory dispersion parameters (e.g. $b_0 = -350$ in 2-chloroethanol as compared with -150 in water (6)).

It is also worth noting that neither the difference spectral parameters nor the rotatory dispersion parameter, b_0 , seem to be affected to an appreciable degree in ethylene glycol (Table VIII). This suggests that the native protein is fairly tightly organized and possesses few, if any, easily deformable hydrophobic regions. The absence of such regions or clusters in the polypeptide folds of lysozyme has been suggested by Wishnia on the basis of hydrocarbon gas adsorption experiments in aqueous media (39).

5. *Ribonuclease and Insulin*—Since the primary structure of these two enzymes is known (26, 40) and some of the abnormal tyrosyl residues have been identified (38, 41-43), their solvent perturbation behavior in both aqueous and organic solvents is of particular interest. Studies on aqueous solutions of these

two proteins (1)¹ suggest that somewhat more than one-half of the tyrosine chromophores are blocked to the various perturbants employed (20% sucrose, ethylene glycol, glycerol, methanol, polyethylene glycol, and dimethylsulfoxide). For reasons of comparison, some of the data obtained with dimethylsulfoxide as perturbant are given in Table VI and Figs. 9 and 10A. The data obtained on ribonuclease led to the conclusion that two of the six tyrosyl residues are buried in the interior of this enzyme, while the remaining four residues are exposed and accessible to solvent to varying degrees. In insulin, two of the four tyrosyl residues seem to be buried also. This has been suggested by both our findings¹ and by the fact that two of the tyrosyl residues do not react with cyanuric fluoride (38).

The denaturation of these two tyrosine-containing proteins is qualitatively similar to denaturation of the other proteins discussed so far, whose ultraviolet spectra are mainly due to tryptophan, in that with both groups of proteins, denaturation is accompanied by a net increase in the fraction of chromophores rendered accessible to solvent.

What is, however, of greater interest here is the marked difference in the physical properties of ribonuclease and insulin in random coil- and helix-forming solvents, and the assignment of some of these differences to the known structural features of

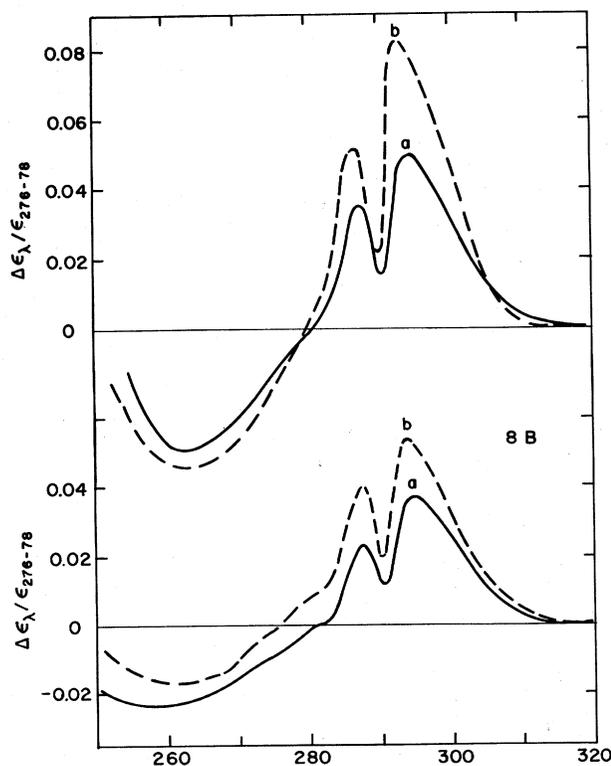


FIG. 8. The solvent perturbation difference spectrum of lysozyme and its model compound mixture in 2-chloroethanol and in acidic ethylene glycol. A: Curve a, 0.1% protein in 2-chloroethanol; Curve b, model compound mixture, 3.2×10^{-4} M *N*-acetyl-L-tryptophan ethyl ester, 1.6×10^{-4} M *N*-acetyl-L-tyrosine ethyl ester, 1.6×10^{-4} M *N*-acetyl-L-phenylalanine ethyl ester in 2-chloroethanol. B: Curve a, 0.1% protein in ethylene glycol, 0.01 M HCl; Curve b, model compound mixture, 3.2×10^{-4} M, *N*-acetyl-L-tryptophan ethyl ester, 1.6×10^{-4} M *N*-acetyl-L-tyrosine ethyl ester, 1.6×10^{-4} M *N*-acetyl-L-phenylalanine ethyl ester, in ethylene glycol. Perturbant, 20% dimethylsulfoxide.

TABLE VI

Difference spectral parameters of ribonuclease and insulin in aqueous and organic solvents^a

Protein and solvent	$(\Delta\epsilon_{296-288}/\epsilon_{276-278})^b$		R_M
	Protein	Model mixture ^c	
Ribonuclease			
Water, $\Gamma/2 = 0.25$, pH 3-7	0.060	0.159	0.38
8 M urea, $\Gamma/2 = 0.03-0.25$, pH 3-5	0.115	0.169	0.68
Thioglycolic acid-reduced RNase ^d			
8 M urea, $\Gamma/2 = 0.25$, pH 3-5	0.149	0.169	0.88
Ribonuclease			
Ethylene glycol, 0.2 M KCl	0.062	0.118	0.53
Ethylene glycol, 0.01 M HCl	0.062	0.118	0.53
2-Chloroethanol	0.137	0.202	0.68
Insulin			
Water, $\Gamma/2 = 0.1$, pH 2-3	0.064	0.159	0.40
8 M urea, $\Gamma/2 = 0.03$, pH 4.6-5.2	0.117	0.169	0.69
Ethylene glycol, 0.01 M HCl	0.098	0.118	0.83
Methanol, 0.01 M HCl	0.107	0.124	0.86
2-Chloroethanol	0.163	0.200	0.82

^a For abbreviations and symbols used, see Table II, Footnotes c and d.

^b Perturbant, 20% dimethylsulfoxide.

^c Mixtures of *N*-acetyl ethyl esters of tyrosine and phenylalanine; respective molar ratios used: ribonuclease, 6:3 (26); insulin, 4:3 (40). $\Delta\epsilon/\epsilon$ values are independent of pH and ionic strength.

^d Thioglycolic acid-reduced ribonuclease in 8 M urea.

these two proteins. The difference spectral parameters of ribonuclease summarized in Table VI indicate that the structure of the molecule is only partly unfolded in ethylene glycol. It is noteworthy that the change in R_M for this protein is from 0.38 in water to 0.53 in ethylene glycol, whereas the corresponding change for insulin is from 0.40 to 0.83. In the case of ribonuclease, this corresponds to the uncovering of approximately 1 buried tyrosyl residue (or perhaps the partial uncovering of two to three such groups¹). Denaturation in 2-chloroethanol results in further uncovering of tyrosyls, with an R_M value of 0.68 being reached. With insulin, however, the maximum degree of unfolding is already reached in ethylene glycol. This is indicated by the fact that the R_M values in acidic glycol, methanol, and 2-chloroethanol are very nearly the same, *i.e.* 0.82 to 0.86. A comparison between the properties of these two proteins could not be made in acidic methanol because of the low solubility of ribonuclease. However, it is fairly clear from the chloroethanol data alone that under maximal conditions of helix formation, the fraction of exposed tyrosyls is greater in insulin than in ribonuclease, despite the fact that the apparent helix content of ribonuclease is greater.

Partly as a consequence of the unusually low b_0 value of insulin in 2-chloroethanol, it has been proposed that this protein possesses both left-handed and right-handed helical regions (16). The contribution of left-handed helical regions to the rotatory dispersion would cancel out the contribution of some of the right-handed helical regions and thus lead to low b_0 values, the implicit assumption being that the left-handed helical region escapes disorganization in 2-chloroethanol. It is difficult to reconcile these findings with ours since (a) the R_M values ob-

tained in acidic glycol, methanol, or 2-chloroethanol indicate extensive structural disorganization or rearrangement, and (b) there is an actual decrease in b_0 in acidic glycol compared with water (6). To explain this latter observation, one would have to postulate that the intrinsically less stable left-handed helical regions are preserved in this solvent while some of the normal right-handed regions are destroyed (44). The confining influence of the three cystine bridges on the conformation and rotatory properties of insulin will be considered in the accompanying paper (6).

Finally, the low perturbation parameters of fully unfolded ribonuclease (e.g. $R_M = 0.68$ in both 2-chloroethanol and 8 M urea; Table VI) and the more normal values obtained on insulin ($R_M = 0.82$ to 0.86 in the three pure organic solvents and 0.69 in 8 M urea) deserve some comments. In ribonuclease, three of the six tyrosyl side chains are in the close vicinity of cystine groups; residues 25 and 73 adjoin disulfide bridges, while in the modified amino acid sequence (26), residue 97 is two amino acids removed from a third disulfide bridge. In insulin, however, there is only one such tyrosine side chain; residue 19 in the A chain adjoins half-cystine residue 20 which connects the two chains. The fact that the cleavage of these cross-links in 8 M urea produces a substantial increase in the R_M value of ribonuclease (from 0.68 to 0.88) to values comparable with those obtained on other disulfide-cleaved proteins (2, 3)¹ indicates that

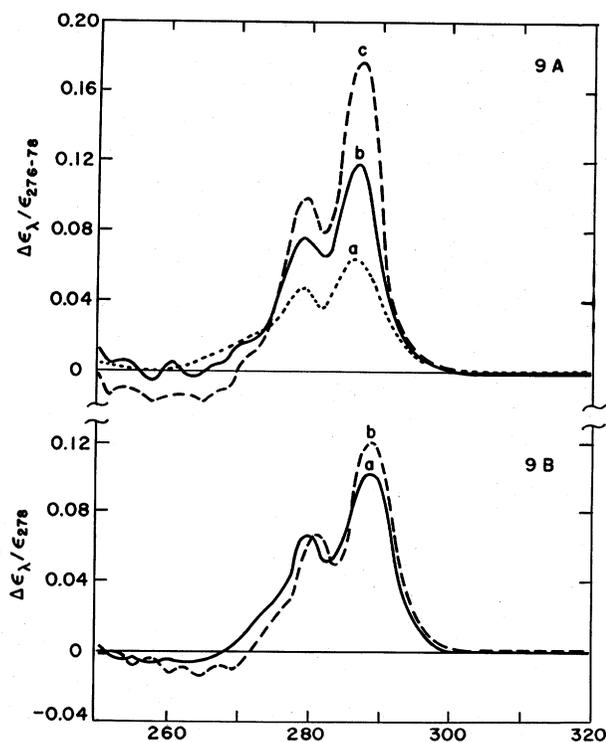


FIG. 9. The solvent perturbation difference spectra of insulin in water, 8 M urea, and acidic methanol. A: Curve a, 0.2% insulin in water, 0.1 M Cl⁻, pH 2.5; Curve b, 0.2% insulin in 8 M urea, 0.03 M Cl⁻, pH 4.6; Curve c, model compound mixture, 1.6×10^{-3} M *N*-acetyl-L-tyrosine ethyl ester, 1.2×10^{-3} M *N*-acetyl-L-phenylalanine ethyl ester in 8 M urea, 0.03 M Cl⁻, pH 2.9. B: Curve a, 0.2% insulin in methanol, 0.01 M HCl; Curve b, model compound mixture, 1.6×10^{-3} M *N*-acetyl-L-tyrosine ethyl ester, 1.2×10^{-3} M *N*-acetyl-L-phenylalanine ethyl ester, in methanol. Perturbant, 20% dimethylsulfoxide.

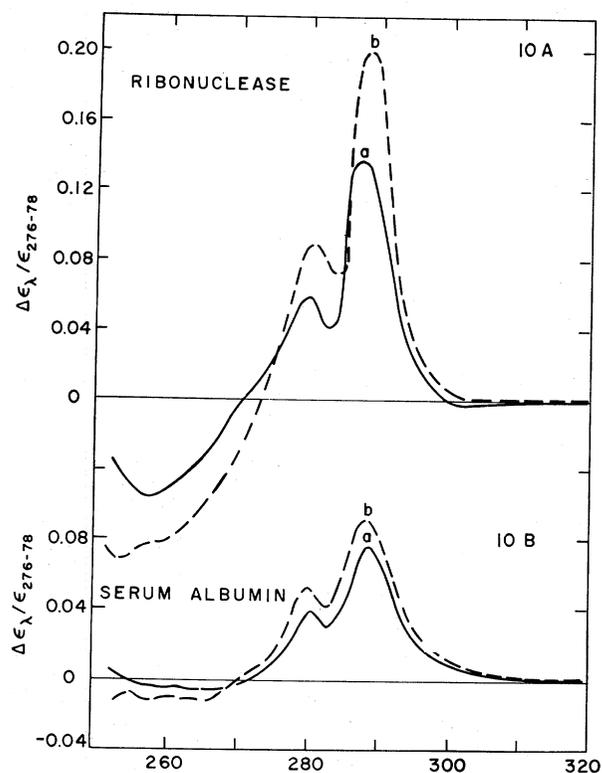


FIG. 10. The solvent perturbation difference spectra of ribonuclease and bovine serum albumin. A, ribonuclease and its model compound mixture in 2-chloroethanol: Curve a, 0.2% ribonuclease; Curve b, 1.6×10^{-3} M *N*-acetyl-L-tyrosine ethyl ester and 8×10^{-4} M *N*-acetyl-L-phenylalanine ethyl ester. B, bovine serum albumin and its model compound mixture in ethylene glycol, 0.01 M HCl: Curve a, 0.3% bovine serum albumin; Curve b, 1.0×10^{-3} M *N*-acetyl-L-tyrosine ethyl ester, 9.5×10^{-5} M *N*-acetyl-L-tryptophan ethyl ester and 1.25×10^{-3} M *N*-acetyl-L-phenylalanine ethyl ester. Perturbant, 20% dimethylsulfoxide.

the low R_M value in this case is due to steric factors imposed by bulky neighboring groups (2).

6. *Bovine Serum Albumin*—The serum albumins are fairly helical proteins (6, 45). Probably due to the steric constraints imposed by the 17 disulfide linkages in bovine serum albumin, further helix formation in organic solvents such as 2-chloroethanol and acidic methanol is fairly limited (6, 20). Since about 70% of the tyrosyl residues are known to be buried in the native molecule (2), the effect of helix-forming solvents on the state of these buried residues is of interest.

The changes in the fraction of exposed tyrosyls in serum albumin, from about 0.3 (2) in aqueous solutions to 0.83 in acidic glycol, suggest that the buried tyrosyl regions of the molecule are more readily disrupted than similar regions in lysozyme or ribonuclease (Table VII and Fig. 10B). Again, as with ribonuclease, the change in b_0 upon denaturation in ethylene glycol is fairly small (6). In fact, even under conditions of maximal helix formation, in acidic methanol or 2-chloroethanol, the increase in helical content of serum albumin is only of the order of 10 to 15% (see Table VIII). These latter observations plus the presence of 17 disulfide bridges in the albumin molecule argue against the possibility of extensive structural rearrangements in these solvents. At the same time, however, it is difficult to imagine why more than half of the 21 tyrosyls in this protein

TABLE VII
Difference spectral parameters of bovine serum albumin in aqueous and organic solvents^a

Protein and solvent	$(\Delta\epsilon_{286-288}/\epsilon_{276-278})^b$		R_M
	Protein	Model mixture ^c	
Serum albumin			
Water, $\Gamma/2 = 0.1-0.25$, pH 4-7.....	0.039 ^d	0.115	0.34
8 M urea, $\Gamma/2 = 0.03-0.25$, pH 3-6.....	0.077-0.088 ^d	0.130	0.60-0.68
Thioglycolic acid serum albumin ^e			
8 M urea, $\Gamma/2 = 0.2$, pH 3-6.....	0.112 ^d	0.130	0.86
Serum albumin			
Ethylene glycol, 0.01 M HCl.....	0.075	0.090	0.83
Methanol, 0.01 M HCl.....	0.078	0.100	0.78
2-Chloroethanol.....	0.116	0.167	0.69

^a For abbreviations and symbols used, see Table II, Footnotes *c* and *d*.

^b Perturbant, 20% dimethylsulfoxide.

^c Mixtures of *N*-acetyl ethyl esters of tyrosine, tryptophan, and phenylalanine; respective molar ratios used, 21:2:26 (46). $\Delta\epsilon/\epsilon$ values are independent of pH and ionic strength (2).

^d The $\Delta\epsilon/\epsilon$ values in water and 8 M urea are slightly dependent on pH and ionic strength (2).

^e Thioglycolic acid-reduced serum albumin in 8 M urea (2).

TABLE VIII
Changes in difference spectral and rotatory dispersion parameters of proteins accompanying denaturation in ethylene glycol and 2-chloroethanol

Protein	Ethylene glycol, 0.01 M HCl		2-Chloroethanol	
	ΔR_M^a	$-\Delta b_0/630^b$	ΔR_M^a	$-\Delta b_0/630^b$
Lysozyme.....	0.08	0.12	-0.03	0.32
Ribonuclease.....	0.15	0.03	0.30	0.46
α -Lactalbumin.....	0.35	0.17	0.29	0.41
Insulin.....	0.43	-0.10	0.42	0.13
β -Lactoglobulin.....	0.43	0.55	0.51	0.60
Bovine serum albumin....	0.49	0.08	0.35	0.17

^a The ΔR_M represents the difference between the R_M values obtained in the denaturing solvent specified and in water (Tables IV to VII).

^b $-\Delta b_0/630$ represents the change in helix content of proteins upon dissolving in the denaturing solvents specified; the b_0 values of the native proteins in water and in the denaturing solvents are taken from Reference 6; the normalizing factor, -630, represents the contribution to b_0 of a fully helical polypeptide chain (16).

should be confined to 10 or 15% of the total polypeptide chain, which is presumably nonhelical. While in ribonuclease, the buried tyrosyl residues seem to be confined to nonhelical regions of the protein fold (14), it appears that in other proteins such as serum albumin the buried chromophoric groups may be accommodated equally well in hydrophobic areas located between neighboring helices or between helical and nonhelical segments of the protein fold.

7. *Concluding Remarks*—The data obtained in the present study indicate that the structural changes accompanying the denaturation of globular proteins in both helix-forming and random coil-forming solvents renders the chromophoric side chains, which are normally buried in the interior of the protein (1-5, 19),^{1, 4} largely exposed and accessible to the approach of solvent. Recent studies on the solubility of amino acids and peptides corresponding to the side chains of these amino acids in

organic solvents (47-50) and gas adsorption studies of proteins (39) have suggested that in aqueous media, the nonpolar side chains will tend to cluster among themselves, and thus form hydrophobic regions in the native protein fold. The enhanced solubility of these model compounds in organic solvents has also offered a plausible mechanism for the unfolding of proteins in these solvents and thus may also serve to explain some of the present findings.

The relatively inert character of ethylene glycol (14, 35, 51) has made it a useful solvent for studies directed toward the elucidation of the sources of conformational stability in proteins (18, 47, 50). In particular, the solubility studies of Nozaki and Tanford (50) on amino acids in glycol media have suggested that hydrophobic regions in proteins will be less stable in glycol solutions than in water. While the effects of glycol on hydrogen bond-stabilized regions in proteins have not been assessed by appropriate studies on model compounds, it has been generally assumed (14, 35, 51, 52) that intramolecular hydrogen bonds, as well as other stabilizing factors in proteins (18, 53), are not affected substantially by this solvent. While the latter assumptions must be kept in mind, it is still significant that a rough correlation is found between the denaturability or extent of unfolding, estimated from the change in R_M upon denaturation, and the relative importance of hydrophobic interactions of at least three of the proteins of this study, *i.e.* lysozyme, serum albumin, and β -lactoglobulin (30, 39). It is hoped that the order of assignment of the importance of these interactions (Table VIII) will be borne out by other experiments more quantitative in nature such as the hydrocarbon gas adsorption studies of Wishnia on lysozyme and serum albumin (39).

Among the various globular proteins which have been studied by the solvent perturbation technique (1-5, 19),^{1, 4} only lysozyme has a large fraction of its chromophoric side chains accessible to perturbant. Evidence has been presented in the literature (54, 55) implicating some of the tryptophyl residues in the catalytic activity of this enzyme, which would require that the key amino acid side chains be confined to the surface of the molecule, coming freely in contact with the substrate. A hydrophobic mechanism of enzyme-substrate complex formation

in this case would offer some clue concerning the unusual location of these tryptophyl side chains.

SUMMARY

The solvent perturbation method of difference spectroscopy is extended to the study of proteins and polypeptides in helix-forming and random coil-forming solvents. The fraction of chromophoric residues exposed in 2-chloroethanol, acidic methanol, ethylene glycol, and 8 M urea is estimated by comparing the solvent perturbation parameter, $\Delta\epsilon/\epsilon$, of a given protein with the same parameter obtained with model compound mixtures (*N*-acetyl ethyl esters of tyrosine, tryptophan, and phenylalanine) corresponding to the chromophoric amino acid composition of the proteins.

The milk proteins, α -lactalbumin, β -lactoglobulin and α -casein, together with some other proteins previously studied, are examined in this way. It is found that approximately 70 to 85% of the chromophoric residues of α -lactalbumin, β -lactoglobulin, bovine serum albumin, and insulin are exposed in 2-chloroethanol and acidic methanol. With the disulfide bonds intact, the fraction of exposed tyrosyls and tryptophyls is found to be about the same in the random coil-forming denaturant, 8 M urea. In contrast, in the native form, these proteins have only 30 to 40% of their chromophores exposed and accessible to solvent. Lysozyme is the only exception; here the fraction of exposed tryptophyl residues is largely unaffected upon denaturation in 2-chloroethanol or 8 M urea. In these solvents, as well as in aqueous salt solutions studied previously, about two-thirds of the tryptophyl residues are found to be exposed.

The difference spectral studies indicate that α -lactalbumin, β -lactoglobulin, bovine serum albumin, and insulin are also nearly fully unfolded in ethylene glycol. Again this denaturant has little or no effect on the fraction of exposed tryptophyls in lysozyme. Ribonuclease is found to be partly unfolded in this solvent.

The tryptophyl and tyrosyl residues in the disulfide-free polypeptides, α -casein and poly-L-tyrosine, are also very nearly fully exposed in ethylene glycol.

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