The Relationship of Structure to the Effectiveness of Denaturing Agents for Proteins

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The effect of a large number of compounds on the optical rotation of bovine serum albumin has been determined. The changes in optical rotation are interpreted as an indication of serum albumin denaturation. The effectiveness of the compounds examined shows no correlation with their expected hydrogen-bonding ability, as either hydrogen donors or acceptors, and thus provides no support for the hypothesis that their mechanism of action involves *simple*, monofunctional hydrogen bond formation with the protein. Bifunctional hydrogen bonding may explain their effectiveness, but it is necessary to postulate more than a single type of such bifunctional hydrogen bonding to explain the action of all active compounds. In the case of serum albumin, the observed effects are not due to an increase in the hydrophobic character of the solution, since there is either no correlation or a negative correlation with the hydrophobic character of the denaturing agents. More limited studies with ovalbumin, based on solubility as well as optical rotation measurements, confirm the conclusions reached with bovine serum albumin with respect to the role of hydrogen bonding in denaturation, but suggest that in the case of ovalbumin there is a significant contribution of the hydrophobic character of the compounds examined to their denaturing effectiveness.

Although it is often assumed that the denaturing action of urea, guanidinium salts, and related compounds toward proteins is due to their hydrogen bond–forming properties, there is little or no direct evidence to support this assumption. Hopkins (1930) carried out studies, dating back to 1899, on the structural requirements for effectiveness of denaturing agents toward ovalbumin, measured by evaporating a solution of protein and denaturing agent to dryness and testing the residue for released sulfhydryl groups with nitroprusside. Hopkins found that a free amide group was required for denaturing effectiveness and noted a negative temperature coefficient and an increase in levorotation of the protein associated with this type of denaturation. Some of his conclusions are uncertain, however, because of the insolubility of many of the compounds tested. Greenstein (1938, 1939) and Greenstein and Edsall (1940) extended this work to several other proteins. Their quantitative measurements of sulfhydryl group release showed that guanidinium chloride is more effective than urea and that methyl substitution decreases the denaturing effectiveness of urea and guanidinium salts, but that substituted ammonium salts and amino acids not only are ineffective but cause a decrease in the titer of free sulfhydryl groups of native myosin. Other early work has been summarized by Neurath *et al.* (1944) and by Bawden and Pirie (1940); surprisingly little work on the structural requirements for protein denaturation by small organic molecules has been carried out since this time.

The present investigation was carried out to examine more closely the relationship between simple hydrogen bonding ability and the effectiveness of protein denaturants of the urea-guanidinium class. If simple hydrogen bond–forming ability were the only property of these reagents required for denaturing effectiveness, then compounds which are better hydrogen-bonding agents than urea and guanidinium salts, because of a greater acidity or basicity, should also be better denaturing agents. Water is itself a very effective hydrogen-bonding agent, and the hydrogen-bonding effectiveness of other compounds in aqueous solution must be greater than that of water itself to exert an effect through this mechanism. Recent reports have strongly suggested that the hydrogen-bonding abilities, in aqueous solution, of the amide groups of urea (Schellman, 1955; Gill *et al.*, 1961), guanidinium ion (Tanford, 1954), and *N*-methylacetamide (Klotz and Franzen, 1960) are either only slightly greater or smaller than the hydrogen-bonding ability of water, which is itself an effective denaturing agent (Altman and Benson, 1960).[1]

The earlier findings that methyl substitution decreases denaturing effectiveness and that ammonium salts, which are much stronger acids than urea, are inactive as denaturing agents constitute strong evidence against a simple, monofunctional hydrogen-bonding mechanism or a hydrophobic mechanism for denaturation by urea and related compounds. Our experiments were carried out in order to examine the effect of a larger number of compounds on a single protein and to obtain more evidence on this question. Measurement of the change in optical rotation of bovine serum albumin at 579 mμ was chosen as the method for determining the denaturing effectiveness of the 104 compounds examined; the optical rotation results were confirmed by measurements of the decrease in solubility on denaturation of ovalbumin with a more limited series of compounds. A number of compounds were examined by observing the change in optical rotation upon addition of a relatively small concentration of reagent to a solution of partially denatured protein in 5.45 M urea; this technique makes possible the examination of compounds which are too insoluble for direct measurement and also permits studies under conditions in which there is a relatively very small change in the optical and solvent properties of the solutions.

"Denaturation" is an ambiguous term and is best defined operationally for a particular series of experiments. Denaturation, as used in this paper, refers to only that type of denaturation which is accompanied by a change in optical rotation, unless otherwise stated.

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[1] Levy and Magoulas (1961, 1962) have reported that aqueous urea does not break the intramolecular hydrogen bond of certain dicarboxylic acid monoanions. This result shows that the intermolecular interaction of the carboxylate monoanion with urea is not stronger than the intramolecular interaction of the internally hydrogen-bonded compound. It does not, however, provide information on the relative hydrogen-bonding strengths of urea and water, but only shows that neither urea nor water breaks the internally hydrogen-bonded structure.

This type of denaturation is characteristic of denaturing agents of the amide class, such as urea and guanidine hydrochloride, and probably reflects a change in the secondary, helical structure of the protein (Cohen, 1955). Our conclusions apply only to this type of denaturation; a number of other types of denaturation are known, but will not be considered here.

A preliminary report of this work has appeared (Gordon and Jencks, 1961).

Materials and Methods

Commercial preparations of crystallized bovine serum albumin (BSA) (Armour Pharmaceutical Co., Kankakee, Ill., lot numbers V68802, T68204, and T68304) were used without further recrystallization. A 10% (g/vol.) solution of native bovine serum albumin was prepared by dissolving crystalline bovine serum albumin in cold 0.05 M tris(hydroxymethyl)-aminomethane hydrochloride (Tris) buffer, *p*H 7.6, with a minimum of agitation. The solution was clear and did not require clarification. Two grams of bovine serum albumin were processed at a time and dialyzed in boiled dialysis tubing overnight against Tris buffer. The protein solution was then divided into small vials and stored at −15°. The concentration of each stock preparation in Tris buffer was determined spectrophotometrically, based on a value of $E^{1\%}_{1cm}$ = 6.7 at 279 mμ (Yang and Foster, 1954).

A commercial preparation of twice-recrystallized ovalbumin was used (Mann Research Laboratories, lot number C2434). A 15% stock solution of ovalbumin was prepared as described above. Prior to dialysis, the protein solution was clarified by low-speed centrifugation. The final concentration of the ovalbumin stock solution was determined spectrophotometrically, based on a value of $E^{1\%}_{1cm}$ = 7.5 at 279 mμ (Maybury and Katz, 1956).

The majority of the reagents tested were reagent-grade compounds, obtained from commercial sources. Practical-grade reagents, reagent-grade compounds with melting points differing by more than a degree from published data, and compounds found to be effective denaturants were recrystallized or redistilled. Compounds which were obtained as the sulfate salt were converted to the hydrochloride by treatment of an aqueous solution with $BaCl_2$ and HCl and evaporation of the filtrate to dryness. 1,1-Dimethylurea, m.p. 180°, was synthesized according to Ingersoll and Armendt (1948). Reagent-grade urea was twice recrystallized from 80–90% methanol which had been saturated with urea at 60°. Ten molar stock solutions of urea in 0.05 M Tris buffer, *p*H 7.6, were refrigerated until used. Distilled water was used throughout.

Measurements of *p*H were made with a radiometer *p*H meter (model PHM4b). Measurements of *p*H in urea solutions are apparent, uncorrected values.

Optical rotation measurements were carried out with a Rudolph photoelectric polarimeter (model 200), utilizing an oscillating polarizer and the method of symmetrical angles. A water-jacketed polarimeter cell (1 decimeter, 0.9 ml), which was maintained at 37° ± 0.1°, was used for all measurements of optical rotation. The glass end plates were routinely checked for strain. The measurements were performed at a fixed symmetrical angle of 3°, and the slit opening was usually less than 0.10 mm, with the 579 mμ line of the low pressure mercury arc as a light source. Solutions with absorbancies requiring slit openings greater than 0.2 mm were not reported. The experimental data comparing the effectiveness of the tested denaturants are expressed in terms of the change in specific optical rotation, $\Delta[\alpha]^{37°}_{579m\mu}$, where

$$[\alpha]^{37°}_{579m\mu} = \frac{100\alpha}{lc}$$

$$\Delta[\alpha]^{37°}_{579m\mu} = [\alpha]^{37°}_{reagent} - [\alpha]^{37°}_{control}$$

and $[\alpha]$ = specific rotation (degrees), l = length in decimeters, c = concentration in g/100 ml, and α = observed rotation. Better reproducibility with the same reagent in different experimental runs was obtained if the results were expressed as the change in rotation, compared to a control run at the same time and under the same conditions, than if expressed as the absolute value of the rotation. The rotation of the control solutions varied from −60° to −64° over a period of several months. The reproducibility of the change in rotation obtained with the same reagent at different times was generally in the range of ± 1°–2°.

Optical rotation measurements at 366 mμ and 435 mμ, which were obtained for approximately 30 randomly chosen reagents, showed no change in the order of effectiveness of the reagents in causing changes in optical rotation compared to the results obtained at 579 mμ.

The change in optical rotation of ovalbumin in the presence of the various denaturants was measured as described above, after incubation for 3 hours at 37°. At the same time that the change in optical rotation was measured, the loss of solubility of ovalbumin was determined. After incubation with denaturant for 3 hours at 37°, an aliquot of the ovalbumin solution was transferred to 30 volumes of 0.1 M acetate buffer, *p*H 4.7, and allowed to stand for 30 minutes at room temperature. The *p*H was readjusted to 4.7 if necessary. The solution was then centrifuged at 18,000 rpm in a Servall centrifuge for 20 minutes at 5°. Any precipitate was washed twice with acetate buffer. The washed precipitate was dissolved in 0.5 ml of 1.0 N NaOH for 30 minutes at room temperature. The amount of insoluble protein was measured by the biuret reaction (Gornall *et al.*, 1949). Under these conditions, ovalbumin incubated in Tris buffer gave no precipitate. With only a few exceptions, which are noted in Table III, the insolubilization of ovalbumin on incubation with various reagents was found to be either all (+) or none (0), compared to a control which was denatured by boiling for 10 minutes.

Results

The results obtained from an examination of the effects of 104 compounds on the optical rotation of bovine serum albumin (BSA) at 579 mμ are summarized in Table I. Unless otherwise noted, all experiments were carried out at 37° in 0.05 M Tris buffer, *p*H 7.6 in water. Solutions were adjusted to an apparent *p*H of 7.6 before addition to the protein; small variations in *p*H would not be expected to have a large effect on the results, since the optical rotations of bovine serum albumin and of ovalbumin are independent of *p*H between *p*H 4.7 and 10 (Almquist and Greenberg, 1934). Imidazole (pK_a 7) was examined and found to be effective at *p*H values of 6.2, 7.0, and 7.9; this indicates that both the free base and its conjugate acid, imidazolium ion, are active denaturing agents.

The low solubility in water of many compounds of interest is a major obstacle to a comprehensive survey of the structural requirements for denaturing effectiveness. In order to avoid this problem, the effect of a number of compounds on the optical rotation of bovine serum albumin in 5.45 M urea solution was examined.

TABLE I
EFFECTIVENESS OF DENATURING AGENTS TOWARD BOVINE SERUM ALBUMIN AT 37°[a]

	Reagent in Tris Buffer[b]		Reagent in 5.45 M Urea	
	M	$\Delta[\alpha]_{579}$[c]	M	$\Delta[\alpha]_{579}$[c]
Urea derivatives				
Urea	2.15	0		
	5.5	−25		
	8.0	−30	0.9[d]	−7
	9.1	−45	1.8[d]	−18
Methylurea	8.0	−9		
Ethylurea	8.0	−9	1.8	−13
Allylurea	8.0	−9		
Formylurea	1.0[e]	−1	0.9	−5
1,1-Dimethylurea			0.9	−6
1,3-Dimethylurea	8.0	+2	0.9	−6
			3.6	−11
Ethyleneurea	3.6	−12	0.9	−8
	5.8	−20		
Semicarbazide[f]	3.8[e]	0	0.9	−3
Carbohydrazide	2.6[e]	+1	1.8	−3
Sodium hydantoate	2.1[e]	+1	0.9	+17
Thiourea derivatives				
Thiourea	2.6	−13	0.5	−14
			0.9	−22
Allylthiourea			0.9	−16
Methylthiourea			0.5	−6
1,3-Diethylthiourea			1.8	−6
Amidine derivatives (as the HCl)				
Guanidine	1.3	+1	0.9	−22
	1.8	−20		
	2.7	−31		
	3.6	−43		
	4.0	−47		
	5.0	−47		
	5.8	−49		
S-Methylisothiourea	3.3	−31	0.9	−24
O-Methylisourea			0.9	−16
Cyanoguanidine[g]			0.9	−20
Biguanide[f]	3.8	−16	0.9	−17
			1.8	−24
Methylguanidine	4.0	−32	0.9	−14
1,1-Dimethylguanidine	3.8	−20	0.9	−9
1,1,3,3-Tetramethylguanidine	3.3	−2	0.9	−6
Aminoguanidine			0.9	−5
Formamidine	8.0	−40	0.9	−11
Acetamidine	8.0	−35	0.9	−14
3-Aminotriazole[g]	2.7	−27	0.9	−13
Imidazole				
*p*H 7.9	6.0	−22		
*p*H 7.0	6.0	−22		
*p*H 6.2	6.0	−23		
Carbamates				
Urethan			0.9	−10
N-Methylurethan			0.9	−13
n-Propyl carbamate			1.8	+5
n-Butyl carbamate			0.9	+8
Simple amides				
Formamide	8.0	−7	0.9	−2
			3.6	−13
N-Methylformamide	8.0	0		
N,N-Dimethylformamide			0.9	0
			3.6	0
N-Ethylformamide	8.0	0		
Acetamide	7.3	0	0.9	−5
			1.8	−7
Glycolamide	3.5[e]	−1	0.9	−5
N-Ethylacetamide	7.3	0	0.9	−7
N,N-Dimethylacetamide	7.3	0		
2-Chloroacetamide			0.9	0
Propionamide	8.0	−1	0.9	−2
			3.6	−5
Thioacetamide	1.0[e]	0	0.9	−7
γ-Butyrolactam (2-pyrrolidone)	3.6	−12	0.8	−6
δ-Valerolactam	2.6[e]	+2	0.9	−2
Succinimide	2.7	−1	0.9	−3
Amino acids				
Glycine	2.0[e]	−1	0.9	+21
Glycylglycine	2.0[e]	−1		
Glycine ethyl ester HCl			0.9	+1
Formiminoglycine	0.9[e]	−2	0.9	+2
N-Acetylglycine[h]	0.77[e]	−1	0.9	+13
N-Acetyl-D,L-alanine[h]			0.9	+21
N-Acetyl-D,L-leucine[h]			0.9	+22
D,L-Aspartate[h]	0.69[e]	+6	0.9	+35
N-Acetyl-D,L-phenylalanine[h]			0.9	+11
Ammonium chloride derivatives				
Ethylamine HCl	8.0	+3	0.9	+3
Propylamine HCl	8.0	+17		
Dimethylamine HCl	8.0	+18		
Triethylamine HCl	8.0	+10		
Ethanolamine HCl			0.9	−2
Hydrazine HCl[f]	2.6[e]	0	0.9	+38
Tetramethylammonium Cl			0.9	+16
Methoxyamine[f]	4.2	+3	0.9	+27
	5.45	+7		
Hydroxylamine[f]	3.60	+18		
Ethylenediamine HCl[f]	2.5	+3		
Other (uncharged)				
Pyridine	4.0	−7	0.9	−10
Dioxane	0.91	+3	1.8	−1
	2.70	+2	2.7	−2
	4.60	+2		
Boric acid	1.80[e]	+1	1.8	+1
Sulfamide	4.10	−4	0.9	−3
Ethanol			1.8	0
Chloroethanol			0.9	+5
Other (charged)				
Ammonium formate	8.0	0	0.9	+15
Sodium formate	8.0	+3	0.9	+20
Potassium acetate			0.9	+19
Sodium perchlorate	8.0	−3	1.8	+4
Sodium sulfamate	3.6	−2	1.8	+25
Ammonium chloride	8.0		1.8	+15
Potassium iodide	3.5	+1	0.9	+3
Sodium chloride	2.7	0	0.9	+12
			1.8	+19
Sodium fluoride			0.5	+1
Potassium fluoride	1.8	0	0.5	+9
			0.9	+16
Lithium chloride	4.3	+5	1.8	+7
Sodium bromide			1.8	+4
Dipotassium acid phosphate	1.8	+6	0.9	+19
Potassium cyanate	4.7	+7		
Potassium thiocyanate	3.6	−7	0.9	−2
Sodium salicylate, *p*H 7.5	1.8	−9	1.8	+3
Sodium thioglycolate			0.9	+6

[a] In 0.05 M Tris buffer, *p*H 7.6. Other reagents tested but not tabulated because of low solubility alone and in aqueous urea and giving no significant optical rotatory change are acetylurea (0.14 M), 1-acetyl-3-methylurea (0.39 M), *t*-butylurea (0.21 M), acetonesemicarbazone (0.32 M), biuret (0.32 M), hydantoin (0.37 M), thiosemicarbazide (0.51 M). Compounds too insoluble to be tested alone or in urea: nitroguanidine (<0.1 M), biurea (0.86 M), guanylurea sulfate (0.33 M), phenylurea (insoluble), dithioxamide (<0.1 M), oxamide (0.1 M), *N,N*-dibutylacetamide (insoluble), dimethylolurea (polymerizes). [b] The following reagents gave an immediate turbidity upon addition of bovine serum albumin to an 8.0 M solution: ethanol, ethanolamine HCl, urethan, *N*-methylurethan, *N,N*-dimethylformamide, *N,N*-diethylformamide, tetramethylammonium chloride, potassium acetate, and pyridine. [c] Change in rotation compared to a control in Tris buffer ($[\alpha] = -62 \pm 2°$) or in 5.45 M urea ($[\alpha] = -87 \pm 2°$). [d] *I.e.*, 6.35 and 7.25 M urea. Included for comparison with other compounds. [e] The relatively low solubility of these reagents alone prevents any conclusion concerning their effectiveness as denaturants. They are sufficiently soluble in urea, however, for conclusions to be drawn. [f] Equimolar in sodium chloride. [g] As the free base. [h] Sodium salt.

TABLE II

ABSENCE OF MAJOR CHANGES IN ROTATION DUE TO "SOLVENT" EFFECTS *per se*

A.	No change in the observed rotation of native serum albumin in:			
	Up to 3 M urea	−62°	7.3 M *N,N*-dimethylacetamide	−62°
	1.25 M Guanidine HCl	−59°	2.0 M glycine	−61°
	2.6 M Carbohydrazide	−61°	4.0 M dioxane	−62°
	2.1 M Sodium hydantoate	−60°	8.0 M ammonium formate	−62°
	8.0 M *N*-Ethylformamide	−61°	3.6 M sodium sulfamate	−64°
	7.3 M Acetamide	−62°		
B.	The final rotation of fully denatured serum albumin does not differ significantly in:			
	a 3.4–5.0 M Guanidine HCl	−109°	9–10 M urea	−108°
	b 5.45 M Urea (−88°) plus:			
	0.9 M Thiourea	−106°	0.9 M biguanide HCl	−104°
	0.9 M Guanidine HCl	−109°	0.9 M cyanoguanidine	−105°
	4.4 M Guanidine HCl	−106°	2.7 M 3-aminotriazole	−103°
	c 1.8 M Guanidine HCl (−77°) plus:			
	1.8 M Thiourea	−108°	3.2 M acetamidine HCl	−105°
	d 2.7 M Guanidine HCl (−99°) plus:			
	1.8 M Thiourea	−108°	3.2 M acetamidine HCl	−105°
	e 3 M Guanidine HCl (−103°) plus:			
	3.6 M Dimethylurea	−101°	3.6 M formamide	−106°

Under these conditions the protein is partially denatured and a relatively small additional concentration of an effective denaturing agent causes a large change in optical rotation. An additional advantage of this technique is that it permits the examination of compounds under conditions in which there is comparatively little change in the optical or solvent properties of the solution. Results were obtained in both aqueous and 5.45 M urea solutions for 48 compounds. There is generally a good agreement between the results obtained in the two solvents for those compounds for which comparison is possible (Table I). An arbitrary classification of effectiveness of the different compounds in changing the optical rotation of bovine serum albumin in the two solvents may be made as follows: For 8 M solutions in water, $> -30°$ = excellent; -20 to $-30°$ = good; -7 to $-20°$ = fair; $< -7°$ = inactive. For 0.9 M solutions in 5.45 M urea, $> -10°$ = excellent; -7 to $-10°$ = good; -5 to $-6°$ = fair; $< -5°$ = inactive. Of the fifteen compounds which were examined at these concentrations in the two solvents, eleven fall into the same class, three are in the neighboring class, and one (*N*-ethylacetamide) differs by two classes in the two solvents (compounds which gave changes in optical rotation of $> -30°$ at concentrations of less than 8 M were classified as "excellent"). It may be concluded that examination of compounds in 5.45 M urea provides a valid indication of large differences in effectiveness between different compounds; small differences should not be considered significant.

Corrections of the optical rotation measurements for refractive index differences of the solutions of denaturing agents were not made for the following reasons: (1) Most of the asymmetric groups in the native protein are exposed to a refractive index close to that of the pure protein, approximately 1.60, which is considerably greater than that of the solvent. While the observed change in rotation on denaturation may be subject to a solvent effect because of exposure of the interior of the protein to the solvent, it is difficult to correct for this effect, particularly in the case of partially denatured proteins. (2) Although there is a sound theoretical basis for the refractive index correction, in practice it is found that other, less predictable effects of the solvent often cause considerably larger changes in rotation in small molecules (Beckmann and Cohen, 1936). (3) The refractive index corrections are small and in most cases may be neglected. The refractive index correction, $(n_w^2 + 2)/(n_s^2 + 2)$, was calculated for 18 representative denaturant solutions and was found to be less than 5%; similar calculations for the experiments carried out in urea solutions gave correction factors of 1–2%.

The results are given in terms of the change in specific rotation at 579 mμ, measured a few minutes after addition of reagent, compared to a control in buffer alone ($-62 \pm 2°$) or in 5.45 M urea solution ($-87 \pm 2°$). The rapid response of bovine serum albumin to denaturing agents made possible the examination of compounds, such as cyanate and *O*-methylisourea, which would react chemically with the protein on prolonged incubation. The observed rotations of native, partially denatured, and fully denatured serum albumin show a satisfactory agreement with values reported in the literature (Kauzmann and Simpson, 1953; Yang and Foster, 1954; Yang and Doty, 1957; Schellman, 1958). No further changes in rotation were observed during the time of measurement, and the rotations in the presence of 20 representative compounds were found to remain constant for at least several hours. A number of compounds caused no change in rotation, but were too insoluble, even in urea solution, to permit meaningful comparison with effective reagents; these are listed in footnote *a* of Table I. Several compounds which could not be examined in aqueous solution because of the development of turbidity after the addition of protein are listed in footnote *b*.

In a series of different organic solvents the optical rotations of organic compounds, including polypeptides, show a greater sensitivity to the nature of the solvent than can be accounted for by refractive index differences, and certain salts, such as lithium bromide, may cause changes in the rotation of proteins which are due to a "solvent" effect rather than to a change in the conformation of the protein (Beckmann and Cohen, 1936; Yang and Doty, 1957; Albinak *et al.*, 1961; Bigelow and Geschwind, 1961; Mandelkern and Roberts, 1961). It is, therefore, necessary to show that in aqueous solution under the experimental conditions described in this work there are no large "solvent" effects on the optical rotation of bovine serum albumin which might bring the correlation of these rotation changes with denaturation into question. Experimental data bearing on this point are given in Table II. A number of different neutral and charged compounds have no effect on the observed rotation of native serum albumin in the range of concentrations examined; some of these compounds cause a rapid increase in levorota-

tion at higher concentrations, due to denaturation. Since it is unlikely that a denaturing effect would be exactly cancelled by an equal and opposite "solvent" effect on the rotation for each of these compounds, it may safely be concluded that these compounds do not affect the rotation of native serum albumin through a solvent effect. This conclusion is in agreement with previous results, notably those of Simpson and Kauzmann (1953), who found that there is no immediate change in rotation which might be attributed to a "solvent" effect on the addition of ovalbumin to 7.3 M urea, although there is a progressive increase in levorotation with time, due to denaturation. Since it might be argued that these molecules exert no "solvent" effect because they are unable to penetrate to the interior of the native protein under these conditions, a more critical test is the effect of denaturing agents on the optical rotation of denatured protein, which is presumably unfolded and exposed to the solvent. It is unlikely that "solvent" effects of the denaturing agents studied here cause large effects on the rotation of denatured albumin, because the final rotations obtained in the presence of a number of different denaturing agents and mixtures of denaturing agents are essentially the same (Table IIB); such a result is unlikely if the observed rotation is a function of the nature of the bound denaturing agent and suggests that the observed rotations reflect primarily the conformation of the denatured protein. Furthermore, a large number of experiments were carried out in 5.45 M urea solution, so that the addition of a relatively small amount of denaturing agent would be expected to have little or no nonspecific "solvent" effect on the rotation, and, as described below for ovalbumin, there is a satisfactory correlation between optical rotation changes and denaturation as determined by measurement of insoluble protein, both in a quantitative study at varying urea concentrations and with a series of different denaturing agents.

Compounds which were obtained as the hydrochlorides and which have pK_a values below 8 were neutralized to *p*H 7.6 with NaOH before use, so that it is necessary to consider the effect of the associated NaCl in comparing their effectiveness with that of other compounds which were examined in the absence of concentrated salt. Although certain salts affect the optical rotation of proteins by "solvent" effects as well as by effects on the extent of denaturation, NaCl is one of the least effective salts in these respects (Burk, 1943; Simpson and Kauzmann, 1953; Bigelow and Geschwind, 1961; Mandelkern and Roberts, 1961). NaCl, 2.7 M, has no effect on the optical rotation of bovine serum albumin in water, but 0.9 and 1.8 M NaCl cause partial reversal of the negative change in rotation caused by 5.45 M urea (Table I). Thus, it is possible that compounds such as semicarbazide have some denaturing effectiveness which is masked by the accompanying NaCl; it appears certain, however, that such compounds are much less effective than amidinium derivatives, which are highly active in the presence of equimolar chloride ion. Hydroxylamine, methyoxyamine, and hydrazine HCl cause positive changes in rotation in the absence of urea or positive changes larger than those caused by NaCl in the presence of urea and, therefore, appear to be ineffective as denaturing agents.

The results with bovine serum albumin may be summarized as follows:

(1) Within a given series of compounds, substitution by alkyl or other relatively nonpolar groups and increases in the chain length of such alkyl substituents abolishes or progressively decreases their effect on the optical rotation of bovine serum albumin. Ethyleneurea and γ-butyrolactam, at least in aqueous solution, are considerably more effective than similar alkyl-substituted open-chain compounds.

(2) Substitution of electron-withdrawing groups, as in semicarbazide, carbohydrazide, aminoguanidinium ion, and chloroacetamide, causes decreased activity, generally to a much larger extent than does alkyl substitution.

(3) Among different series, amidinium cations are more effective than urea and its derivatives, which are in turn more effective than simple amides. Urethan appears to be approximately equal to urea in effectiveness. Compounds containing a sulfur atom are generally more active than their oxygen-containing analogs.

(4) All active compounds are unsaturated, are capable of resonance, and contain at least one —NH— (preferably $—NH_2$) group with a partial positive charge. A number of neutral molecules and cations are active, but anions are invariably inactive.

(5) Ammonium chloride, substituted ammonium salts, and amino acids and their derivatives are inactive, and, in fact, generally cause positive changes in rotation. Simple saturated nitrogen bases, such as methoxyamine, hydroxylamine, and hydrazine, which contain at least one free $—NH_2$ group at neutral *p*H, are inactive.

(6) Under the conditions of these experiments, simple compounds which decrease the polarity or increase the hydrophobic character of the solvent, such as dioxane and ethanol, are inactive.

(7) Under the conditions of these experiments, simple salts, with the exception of thiocyanate, either have no effect or cause positive changes in rotation.

The denaturing effectiveness of a group of compounds toward ovalbumin is summarized in Table III. Denaturation by reagents in aqueous solution was determined by measurement of the solubility of diluted aliquots in acetate buffer, *p*H 4.7, and denaturation by reagents in 5.45 M urea solution was determined by measurements of the optical rotation of the solutions and the solubility of diluted aliquots. Quantitative measurements of the amount of insoluble protein were carried out by the biuret method, but only the presence or absence of insoluble protein is indicated in the Table, since in nearly every case denaturation was either absent or complete. Estimations of the extent of ovalbumin denaturation at various urea concentrations show a satisfactory agreement between the results of the solubility and optical rotation methods (Fig. 1).

Discussion

Methodology.—Changes in the optical rotation of proteins may result from (1) changes in the helical content, (2) other changes in conformation, or (3) effects which do not involve a change in conformation. The first two of these changes will be defined here as "denaturation" and the third will be called a "solvent effect." Our interpretation that the changes (or absence of changes) in optical rotation observed in this study represent denaturation (or absence of denaturation) rests on evidence in the literature and the preceding text and is summarized here:

(1) An increase in levorotation upon protein denaturation was noted by Hopkins in 1930 and has been further described and correlated with other manifestations of protein denaturation in a literature too voluminous to record here. The sigmoid curve for the increase in levorotation of bovine serum albumin with increasing urea concentration was described by Kauzmann and Simpson (1953) and has been confirmed by us (unpub-

TABLE III
DENATURING EFFECTIVENESS TOWARD OVALBUMIN AT 37°

Reagent	Reagent in Tris Buffer[a] M	Insoluble[c] Protein	Reagent in 5.45 M Urea[b] M	$\Delta[\alpha]_{577}$[b]	Insoluble[c] Protein
Urea	4.5	0	4.2[d]	−49	+
	9.0	+	5.0[d]	−48	+
Methylurea	8.0	+			
Ethylurea	4.0	+	0.9	−18	+[e]
			1.8	−36	+
			3.7	−36	+
1,3-Dimethylurea	8.0	0	2.7	−36	+
1,1-Dimethylurea			2.7	−43	+
Ethyleneurea			2.6	−48	+
Semicarbazide[g]			1.8	+ 8	0
Carbohydrazide			2.7	+ 7	0
Thiourea	2.7	+	1.8	−50	+
O-Methylisourea HCl			1.8	−21	+[e]
Guanidine HCl	2.7	+[e]	1.8	−48	+
	7.2	+			
1,1-Dimethylguanidine HCl			1.8	−43	+
Biguanide HCl			2.7	−37	+
Cyanoguanidine			0.9	−38	+
Acetamidine HCl			2.7	−37	+
Imidazole[h]					
*p*H 6.3	6.3	+			
*p*H 7.6	6.3	+[e]	2.7	−44	+
Formamide	7.2	+	2.7	−39	+
Acetamide	7.2	+	2.7	−45	+
Propionamide[f]	8.0	+	2.7	−45	+
γ-Valerolactam			2.7	−35	+
Thioacetamide			1.8	−36	+
Hydrazine HCl[g]			1.4	+15	0
Methoxyamine[g]	5.4	0	1.8	+ 6	0
Tetramethylammonium chloride			2.7	+11	0
Ammonium formate	8.0	0	2.7	+ 5	0
Methylamine HCl	7.2	0	2.7	+ 9	0
Dimethylamine HCl	8.0	0			
Triethylamine HCl	7.2	0			
Glycine			2.7	+ 9	0
Boric acid			1.8	+ 3	0
Pyridine	3.6	+[e]			
Sodium chloride			0.9	0	0
			1.8	+ 1	0

[a] Incubated with ovalbumin for 30 minutes at 37°. The following reagents at the indicated concentrations gave an immediate precipitate upon the addition of ovalbumin so that meaningful conclusions as to their denaturing ability could not be drawn: *N*-2-hydroxyethylacetamide (7.2 M), *N*-2-hydroxyethylformamide (8.0 M), urethan (4.5 M), *N*-ethylformamide (8.0 M), *N*-ethylacetamide (7.2 M), *N,N*-dimethylacetamide (7.2 M), and sodium perchlorate (7.2 M). The following reagents gave no precipitate upon dilution with acetate buffer, but the reagent concentration was below that necessary for urea to give a precipitate under these conditions: nitromethane (1.5 M), glycine (1.8 M), hydroxylamine (2.3 M), tetramethylammonium chloride (4.5 M), acetylurea (0.1 M), acetylmethylurea (0.39 M), semicarbazide (3.8 M), *t*-butylurea (0.2 M), hydantoin (0.4 M), and aminoguanidine HCl (1.8 M). [b] Incubated with ovalbumin for 180 minutes at 37°. The specific optical rotation of ovalbumin was found to be −29° and −39 ± 2° in 0.05 M Tris buffer *p*H 7.6 and in 5.45 M urea in the same buffer, respectively. The results are given as the change in rotation compared to a control in 5.45 M urea. The following reagents caused gel formation at the following concentrations in urea solution and prevented meaningful optical rotation and solubility determinations: urethan (2.7 M), *N*-methylurethan (2.7 M), sodium perchlorate (2.7 M), *N*-ethylformamide (2.7 M), dimethylformamide (2.7 M), butyramide (2.3 M), methanol (2.7 M), pyridine (2.7 M), dioxane (2.7 M), and *t*-butanol (2.7 M). Glycine ethyl ester HCl (2.0 M) gave a white coagulum. [c] Within experimental error, the loss of solubility was all (+) or none (0), compared to a boiled control, except as noted. [d] Total [urea] = 9.65 and 10.45 M. [e] Approximately 75% insoluble protein. [f] Increased viscosity noted. [g] Equimolar in NaCl. [h] Neutralized with HCl.

lished data); a similar curve for ovalbumin denaturation is shown in Figure 1 of this paper.

(2) Although it is known that certain optically active molecules may exhibit changes in rotation due to "solvent effects" without undergoing a helix-coil transition, these effects are observed only with high concentrations of organic solvents or salts. Furthermore, many so-called "solvent effects" may themselves be due to changes in conformation other than a helix-coil transition (Djerassi, 1960). The concentrations of reagents examined here, especially in those experiments in which a compound was added to protein in 5.45 M urea solution, are considerably lower than those generally required to cause nonspecific solvent effects.

(3) The following evidence further suggests that the observed optical rotation changes are, in fact, due to denaturation and not to "solvent effects" which do not involve a change in conformation:

(*a*) A number of compounds have no effect on the rotation of bovine serum albumin. Unless there is a fortuitous exact cancellation of solvent and denaturing effects for all of these compounds, it must be concluded that they do not exert solvent effects on the rotation of the native protein.

(*b*) The same end points of optical rotation change are reached with a number of different reagents and the optical rotation of fully denatured protein is not changed by addition of other compounds. The optical rotation of fully denatured bovine serum albumin is, therefore, not subject to "solvent effects" by the compounds examined.

(*c*) The results obtained by measurement of change in optical rotation agree with those measured by decrease in solubility of ovalbumin, (*i*) as a function of urea concentration, and (*ii*) with a number of different compounds which were examined for their denaturing activity.

(*d*) There is generally good agreement between the results obtained with concentrated reagents in aqueous solutions, in which any solvent effects might be expected to be relatively large, and the results obtained with dilute reagents in 5.45 M urea solution, in which solvent effects would be expected to be much smaller.

(*e*) In several instances, particularly in respect to the effect of alkyl substitution on denaturing effectiveness and the ineffectiveness of ammonium salts (Greenstein, 1938, 1939; Greenstein and Edsall, 1940), the results obtained here are in agreement with previous results obtained by different methods.

We conclude that, with the possible exception of the results obtained with certain salts, the observed optical rotation changes are a semiquantitative measure of protein denaturation. Even if nonspecific "solvent effects" were significant in one or a few cases, each of the conclusions which are developed below rests on data from several series of compounds, and it appears unlikely that solvent effects could be producing identical artifacts in several different series of compounds. Furthermore, the conclusions regarding hydrogen bonding are confirmed by the solubility method for the case of ovalbumin, and those regarding the effect of alkyl substitution and acidity are supported by previous work utilizing other methods, referred to above. In view of the possibility of small solvent effects and the sigmoid relationship of optical rotation change to denaturant concentration, small changes in rotation

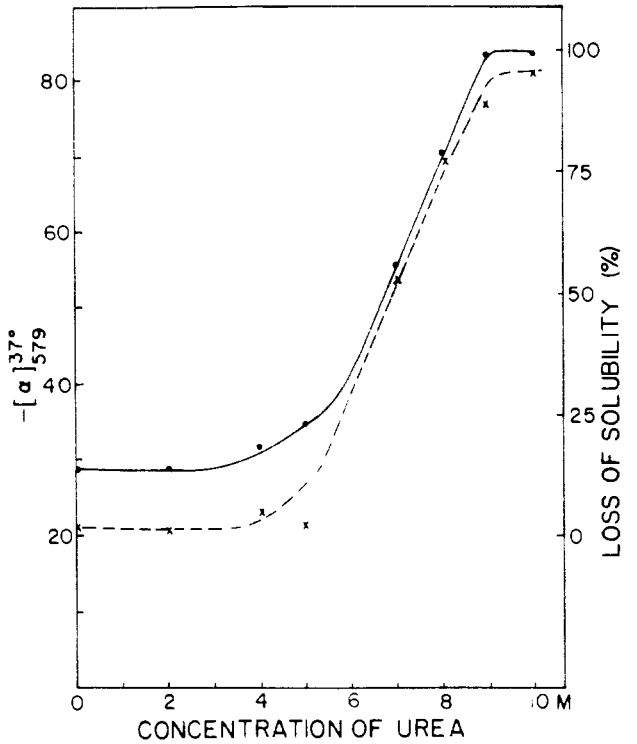

FIG. 1.—Denaturation of ovalbumin by urea after 3 hours at 37°. Specific optical rotation, ●; decrease in solubility, ×.

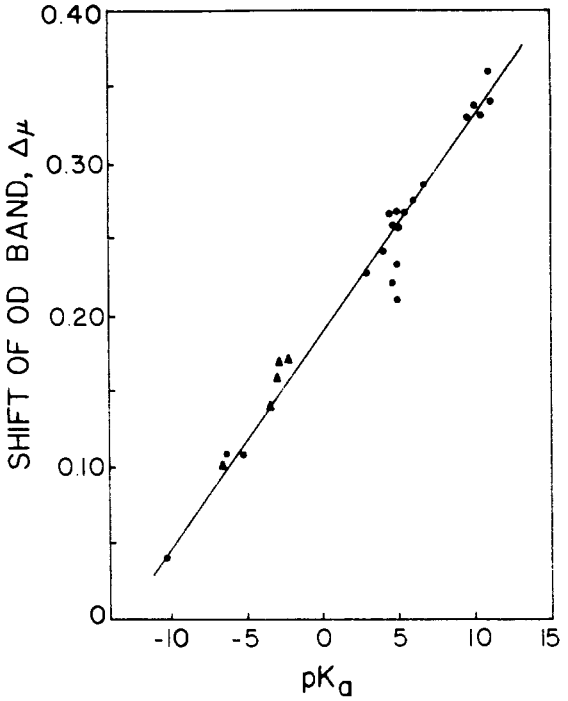

FIG. 2.—The relationship of basicity to hydrogen bonding ability, as measured by the shift in the infrared stretching frequency of the O-D band of CH_3OD (Gordy and Stanford, 1941, ●; Arnett and Wu, 1960, ▲).

should not be regarded as significant and results obtained with different concentrations of different reagents generally cannot be compared. However, the presence or absence of denaturing activity may be inferred from the presence or absence of an effect of a given compound on rotation and the order of denaturing effectiveness may be estimated, at least approximately, from a comparison of the effects of a series of compounds at the same concentration and under the same conditions.

It should be noted that there are different kinds of protein denaturation. The denaturation studied here, which appears to be characteristically caused by amide-type reagents, is accompanied by a change in optical rotation and probably represents a decrease in the α-helix content of the protein (Cohen, 1955). Both bovine serum albumin and ovalbumin fall into Jirgensons' (1961) Class I proteins, which show a decrease in λ_c and an increase in levorotation, presumably accompanying a decrease in helical content, upon denaturation. This exact structural interpretation is not, however, required for the conclusions drawn here. Other types of denaturation may occur without large changes in optical rotation and may represent changes in the tertiary structure of the protein without large changes in the helical content (Bresler, 1958; Weber and Tanford, 1959). The conclusions reached here apply only to the former type of denaturation.

Hydrogen Bonding.—A hydrogen bond involves the partial transfer of a proton from an acid, H-A, to a base, B, and its strength may be expected to be proportional to the tendency for complete proton transfer to occur, *i.e.*, to the acidity of H-A and the basicity of B. This expectation is generally realized experimentally, although exceptions are known. For example, the O-D stretching frequency of deuteromethanol, which is an approximate measure of hydrogen bond strength, is proportional to the basicity of a variety of proton acceptors of different kinds over a range of basicity of 10^{22} (Fig. 2). Different classes of amines exhibit deviations from this line, which appear to be due largely to steric and solvation effects (Tamres *et al.*, 1954). Correlations of hydrogen-bonding ability with the acidity of H-A and the basicity of B have been demonstrated in a number of systems by measurement of equilibrium constants for hydrogen bond formation, as well as by measurement of H-A stretching frequencies (Baker and Shulgin, 1959; Henry, 1959; Gordon, 1961). Probably the most serious exception to this relationship has been reported by Becker (1961), who found that dimethylformamide (which is inactive as a denaturant toward bovine serum albumin in 5.45 M urea) is more active than pyridine as a hydrogen acceptor from alcohols, as estimated from equilibrium constants, although less active in terms of O-H frequency shifts. The limited data available do not suggest that ureas have exceptional hydrogen bonding ability compared to amides, at least as proton acceptors, since the infrared data of Cook (1958) and of Baker and Harris (1960) show that the hydrogen-bonding ability of tetramethylurea is similar to that of dimethylformamide and dimethylacetamide. Mizushima and Shimanouchi (1957) have reported that acetanilide, dimethylacetamide, and methylacetamide fall in approximately the expected order of both hydrogen acceptor and hydrogen donor ability in respect to their basicity and acidity respectively. Thus, although a precise correlation should not be expected, particularly when hydrogen bonding to different classes of compounds is compared, available data generally support the expected proportionality of hydrogen bonding ability to the acidity of the hydrogen donor and the basicity of the hydrogen acceptor.

As shown in Figure 3, there is *no* correlation of acidity or basicity with denaturing effectiveness, as estimated from the effect of 0.9 M solutions of each compound on the optical rotation of bovine serum albumin in 5.45 M urea. The range of acidity and basicity included in the series is sufficiently large to

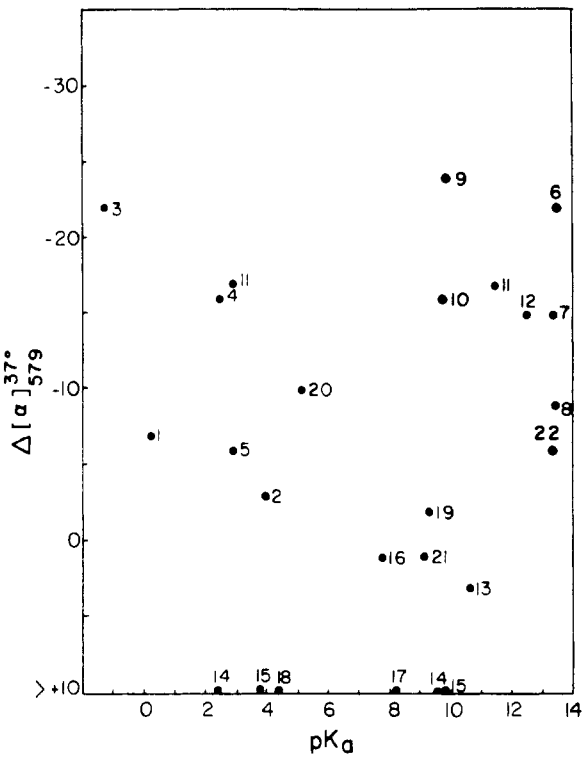

FIG. 3.—The change in the specific optical rotation of a 1% solution of bovine serum albumin in 0.91 M reagent and 5.45 M urea as a function of the pK_a of the reagent or its conjugate acid: 1, urea; 2, semicarbazide; 3, thiourea; 4, allylthiourea; 5, methylthiourea (pK_a's determined in 50% dioxane, Walter *et al.*, 1956); 6, guanidine HCl; 7, methylguanidine HCl; 8, 1,1-dimethylguanidine HCl (Angyal and Warburton, 1951); 9, *S*-methylisothiourea HCl; 10, *O*-methylisourea HCl (Dippy *et al.*, 1959); 11, biguanide HCl (Sarma, 1952); 12, acetamidine HCl; 13, ethylamine; 14, glycine; 15, D,L-aspartic acid; 16, glycine ethyl ester; 17, hydrazine HCl; 18, methoxyamine; 19, ethanolamine HCl; 20, pyridine; 21, boric acid; 22, 1,1,3,3-tetramethylguanidine HCl.

strongly suggest, therefore, that there is no relationship of denaturing effectiveness to simple hydrogen bonding effectiveness.

If the denaturing agents were acting as simple proton acceptors (Brönsted bases), it would be expected that compounds that are stronger bases than urea, for example, would be better denaturing agents. However, as summarized in Table IV, compounds such as semicarbazide, methoxyamine, hydroxylamine, ethylenediamine hydrochloride, and a number of anionic bases are inactive as denaturing agents, although they are 3 to 6 orders of magnitude more basic than urea. Furthermore, it is hardly conceivable that amidinium ions such

TABLE IV
INACTIVITY OF RELATIVELY STRONG BASES

Active	pK_a[a]	Inactive	pK_a[a]
Urea	0.2	Semicarbazide	3.6
Thiourea	−1.3	Methoxyamine	4.6
Guanidine HCl	[b]	Hydroxylamine	6.0
S-Methylisothiourea HCl	[b]	Ethylenediamine HCl	7.0
O-Methylisourea HCl	[b]	Glycine	2.4
		Potassium acetate	4.7
Formamidine HCl	[b]	Potassium fluoride	3.1
Acetamidine HCl	[b]	Dipotassium phosphate	6.8

[a] Of the conjugate acid. [b] No detectable basicity.

TABLE V
INACTIVITY OF RELATIVELY STRONG ACIDS

Active	pK_a	Inactive	pK_a
Urea	>15	Glycine ethyl ester HCl	7.8
Guanidine HCl	13.7		
Thiourea	[a]	Glycine	9.7
Acetamidine HCl	12.5	Ammonium Cl	9.2
		Ethylamine HCl	10.6
Thioacetamide	13.4	Dimethylamine HCl	10.6
γ-Butyrolactam	15	Triethylamine HCl	10.6
		Ethanolamine HCl	9.5
		Hydrazine HC	8.1
		Ethylenediam ne HCl	10.0
		Boric acid	9.2

[a] Not known.

as guanidinium, isourea, and amidinium cations, which have no detectable basic properties, are acting as hydrogen acceptors (all of these compounds are more than 99% in the protonated, isouronium form at *p*H 7.6). Finally, methyl substitution either has no effect or slightly increases basicity, but decreases or abolishes the denaturing activity of ureas and amides.

If the denaturing agents were acting as simple proton donors (Brönsted acids), it would be expected that compounds which are stronger acids than urea and guanidinium ion would be better denaturing agents. However, as summarized in Table V, compounds which are 3 to 6 orders of magnitude stronger acids than guanidinium ion, and even stronger compared to urea, are inactive as denaturing agents and may even cause a reversal of denaturation. The compounds include a number of substituted ammonium ions which have the same charge as guanidinium ion; compounds which carry a negative charge, such as glycine, may be omitted from this and the previous comparison without affecting the conclusion. Furthermore, methyl substitution has no effect on the acidity of guanidinium ions (Angyal and Warburton, 1951), but decreases denaturing activity. The effect of introducing an amino group into urea or guanidinium, as in semicarbazide, carbohydrazide, and aminoguanidine, is to introduce a new basic group and to increase the acidity of the remainder of the molecule by its electron-withdrawing effect. Either or both of these effects might be expected to increase hydrogen-bonding ability, but both result in an elimination or marked decrease of denaturing effectiveness. Urea and simple amides have no detectable acidic properties in aqueous solution.

Although a single comparison would not prove the point because of the possibility of an exception to the correlation of hydrogen bond strength with acidity and basicity, the absence of any relationship between denaturing effectiveness and expected hydrogen-bonding ability in the examples cited provides strong evidence that the denaturing effectiveness of the active denaturants examined is not due to simple, monofunctional hydrogen bonding between the denaturing agent and the protein. It should be emphasized that this conclusion does not imply that hydrogen bonds may not be important in maintaining the structure of native proteins.[2]

[2] The assumption that the mechanism of action of a denaturing agent *necessarily* reflects the nature of the forces which maintain the structure of a native protein is a common fallacy. If one could attach a fishhook to each end of a protein and pull, one could denature the protein, but it does not follow that the protein is held together by fishhooks.

The results do not rule out the hypothesis that denaturing agents of the amide class act by polyfunctional hydrogen bonding to the protein, and, indeed, such a mechanism provides the simplest (but not the only) hypothesis to explain their action. Such polyfunctional combination could occur through either an acid-base or an acid-acid interaction, as shown in Figure 4. There is no direct experimental support for such a mechanism, although it is known that polyfunctional hydrogen-bonded complexes, as in the dimer of δ-valerolactam, are more stable than monofunctional complexes, as in the case of *N*-methylacetamide (Mizushima and Shimanouchi, 1957). Furthermore, it would be necessary to postulate at least two types of such polyfunctional bonding to account for the activity of reagents, such as formamide and urethan, which can act only as acid-base reagents, and reagents of the guanidinium class, which can act only as acid-acid reagents. It is of interest that ethylenediamine HCl, hydrazine HCl, and glycine, which have the potentiality for strong bifunctional hydrogen bonding, are inactive as denaturing agents.

Klotz and Stryker (1960) have reported that the "abnormal" pK_a of a dye bound to polyvinylpyrrolidone returns to the normal pK_a value in the presence of urea, a phenomenon similar to that observed upon urea denaturation of proteins to which this dye is bound. Bifunctional acid-base hydrogen bonding to polyvinylpyrrolidone is not possible because the amide groups in this compound do not have a proton on the nitrogen atom.

Solvent Effects.—The results suggest that the denaturation of bovine serum albumin by reagents of the amide class is better described as the result of a specific interaction of the denaturing agent with the protein, which favors the denatured form (Anson and Mirsky, 1933–34), than as the result of a nonspecific change in the properties of the solvent or solvation shell around the protein molecule. The low effectiveness of hydrophobic compounds supports this view. Furthermore, the addition of a number of salts of widely different structure does not promote denaturation and may even reverse it. Salts have large effects on the properties of aqueous solutions and they may be classified as small "structure-making" ions, such as Li^+ and F^-, which increase the "structure" of aqueous solutions, and large "structure-breaking" ions, such as ClO_4^-, which have the opposite effect (Gurney, 1953; Frank and Wen, 1957). For example, the rate of the "water" hydrolysis of acetylimidazolium salts, which is highly sensitive to activity coefficient effects and to the structure of the solvent, is markedly decreased by sodium perchlorate (500-fold at 8 M), is much less sensitive to other, smaller salts, and is actually increased by F^- and $SO_4^=$ (which may act as nucleophilic reagents), but is almost unchanged by 4 M urea (Marburg and Jencks, 1962). One of the striking properties of urea is the very small effect it has on the structure of water, as evidenced by its small effect on the activity of water (Lewis and Burrows, 1912; Scatchard *et al.*, 1938). It is well known that some proteins are denatured by certain ions, and it is likely that such denaturation may represent a solvation effect, since large anions decrease the activity coefficient of the amide group (Meyer and Klemm, 1940), but this appears to be a quite different phenomenon from that observed in the denaturation of bovine serum albumin by reagents of the urea class.

Hydrophobic Forces.—The effect of hydrophobic solvents on protein structure is not altogether clear at the present time. It is probable that an important force tending to hold a protein in its native conformation arises from the reluctance of the hydrophobic interior of the protein to become exposed to the aqueous solvent (Kauzmann, 1959). Addition of a hydrophobic solute to the solvent will tend to overcome this reluctance and thus may favor an unfolding or denaturation of the protein. On the other hand, it is also likely that internal hydrogen bonds exist which may contribute to the stability of a native protein, and the addition of a hydrophobic solvent will generally strengthen these bonds by decreasing the competing hydrogen-bonding ability of the solvent. There is evidence for the existence of both of these effects. It is well known that hydrophobic solvents, such as alcohols or acetone, may denature proteins. On the other hand, nonpolar solvents cause a positive change in optical rotation and presumably *increase* the hydrogen-bonded structure of polypeptides and some proteins (Yang and Doty, 1957; Weber and Tanford, 1959; Bresler *et al.*, 1959; Doty, 1959). In one instance, both of these opposing effects have been observed with the same protein, depending on the concentration of the organic solvent (Tanford *et al.*, 1960; Tanford and De, 1961).

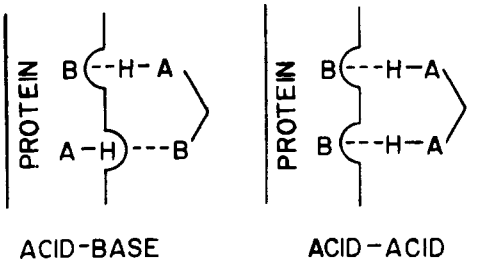

Fig. 4.—Bifunctional hydrogen bonding.

It has been suggested that urea may owe its denaturing effectiveness to its hydrophobic properties (Kauzmann, 1959), and it appears, in spite of the high dielectric constant of urea (Wyman, 1933), that aqueous urea solutions have hydrophobic and poor ion-solvating properties, compared to water alone. Thus, urea prevents the formation of micelles (Alexander and Stacey, 1952; Bruning and Holtzer, 1961), increases the solubility of adenine in water, decreases the solubility of tetrasodium pyrophosphate in water (Levine *et al.*, 1963) and increases the solubility of the nonpolar moiety of certain amino acids in water (Whitney and Tanford, 1962). Although this hydrophobic character of urea solutions appears to be of major importance in the denaturation of deoxyribonucleic acid (Levine *et al.*, 1963) and may be of some importance with certain proteins (see below), it does not appear to be significant in the denaturation of bovine serum albumin by this class of reagent. If denaturation were due to the hydrophobic nature of urea and related compounds, then increasing the hydrophobic nature of the denaturant should increase denaturing effectiveness. An increase in hydrophobic character may be brought about by increasing the number or the size of alkyl substituents on the denaturing agent. Such changes in substituents cause an increase in adenine solubility and a decrease in tetrasodium pyrophosphate solubility in solutions of several different classes of denaturants, as expected from the increase in hydrophobic character (Levine *et al.*, 1963). However, the effect of alkyl substitution or increasing alkyl chain length on urea, thiourea, guanidinium ion, urethan, and formamide is to decrease or abolish denaturing effectiveness. As shown in the examples summarized in Table VI, an increase in the hydrophobic character of the denaturing agent decreases and, in every series but one, causes a complete loss of activity under conditions in which the unsubstituted compound is active. Furthermore, diox-

TABLE VI

EFFECT OF ALKYL SUBSTITUTION AND OF HYDROPHOBIC REAGENTS ON THE OPTICAL ROTATION OF BOVINE SERUM ALBUMIN

Compound	$\Delta[\alpha]_{579}$
8 M Urea	−30
8 M Methylurea	−9
8 M 1,3-Dimethylurea	+2
0.5 M Thiourea in 5.45 M urea	−14
0.5 M Methylthiourea in 5.45 M urea	−6
1.8 M 1,3-Diethylthiourea in 5.45 M urea	−6
2.7 M Guanidine HCl	−31
4.0 M Guanidine HCl	−47
4.0 M Methylguanidine HCl	−32
3.8 M 1,1-Dimethylguanidine	−20
3.3 M 1,1,3,3-Tetramethylguanidine	−2
0.9 M Urethan in 5.45 M urea	−10
1.8 M *n*-Propyl carbamate in 5.45 M urea	+5
0.9 M *n*-Butyl carbamate in 5.45 M urea	+8
8 M Formamide	−7
8 M *N*-Methylformamide	0
8 M Propionamide	−1
1.8 M Dioxane in 5.45 M urea	−1
1.8 M Ethanol in 5.45 M urea	0
0.9 M Chloroethanol in 5.45 M urea	+5

ane or ethanol, known hydrophobic compounds, do not enhance the denaturing effect of urea. These results appear to rule out a hydrophobic mechanism for the denaturation of bovine serum albumin by urea and related compounds.

Structural Requirements for Denaturing Effectiveness.—The observed structural requirements for denaturing effectiveness toward bovine serum albumin are summarized in Figure 5. The active compounds contain an N—H group with a partial positive charge and a partial double bond to a carbon atom, which is in turn bound by a partial double bond to another atom, X. In order of decreasing activity, X may be $H_2N > S > O$ and the other substituent on carbon may be $H_2N\sim CH_3S\sim CH_3O > S > O\sim H\sim CH_3$. Substitution on the nitrogen atom gives decreasing activity in the order H, alkyl, NH_2, CH_2COO^-. Nearly all of the active compounds contain the structure I or II. Pyridine and salicylate do not fit into this classification, and it seems likely that these compounds exert their denaturing effect by a different mechanism; Putnam (1953) includes salicylate in the detergent group of denaturing agents. In open-chain molecules, substituents on the nitrogen atom will force the molecule into a conformation in which there is minimum steric interference with substituents on the carbon atom, and may therefore eliminate denaturing effectiveness if the preferred conformation is inactive as a denaturing agent. It is, therefore, of great interest that cyclic compounds such as ethyleneurea, γ-butyrolactam, imidazolium ion, imidazole, and 3-aminotriazole, which contain substituents that cause inactivation of denaturing effectiveness in open-chain reagents, are all active denaturing agents. It appears that the structural requirements for effective protein denaturation are highly specific, which suggests that denaturation involves a specific and intimate interaction of the denaturing agent with the protein.

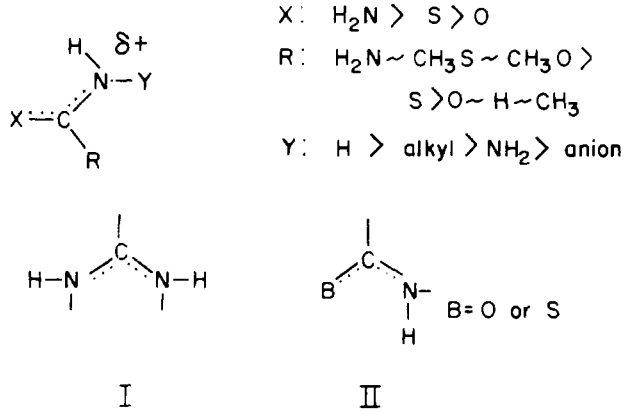

FIG. 5.—Structural requirements for denaturing effectiveness.

Other Proteins.—A series of experiments with ovalbumin was carried out to determine the extent to which the results and conclusions reached with bovine serum albumin apply to the denaturation of a different protein. The denaturation of ovalbumin is slow, so that the results reflect the rate of denaturation. This is in contrast to the situation with bovine serum albumin, which undergoes reversible denaturation very rapidly so that the experimental measurements refer to the extent of denaturation in an equilibrium process (Simpson and Kauzmann, 1953; Kauzmann and Simpson, 1953). The experiments with ovalbumin were generally carried out at high reagent concentration and the results are essentially qualitative, since in most cases denaturation either had not occurred at all or had gone to completion by the end of the incubation period. The results provide a further check on the validity of optical rotation as a measure of denaturation, since the amount of denaturation determined by the change in rotation parallels that found by measurement of insoluble protein, both in a series of different concentrations of urea (Fig. 1) and with different denaturing agents (Table III).

The results are similar to those obtained with bovine serum albumin insofar as they give no support for a mechanism of denaturation based on simple hydrogen bonding. Mono- or diamino substitution eliminates the activity of urea; the relatively strong bases, formate ion and methoxyamine, and the relatively strong acids, hydrazine HCl, substituted ammonium ions, glycine, and boric acid, are all ineffective or give positive changes in rotation.

In contrast to the results obtained with bovine serum albumin, activity is generally retained upon alkyl substitution or increase in alkyl chain length in the denaturing agents. This is found in the urea, guanidinium, and, most prominently, in the amide series. Simple hydrophobic reagents, such as alcohols and dioxane, give an insoluble gel, presumably indicative of denaturation, even in the presence of urea. It may be concluded that ovalbumin has a greater sensitivity to denaturing agents in the hydrophobic class than does bovine serum albumin and that, with this protein, certain compounds may be effective denaturing agents by virtue of their content of both amide and hydrophobic groups. This is supported by Simpson and Kauzmann's finding (1953) that ethanol or acetone increases the rate of ovalbumin denaturation in the presence of urea. Preliminary results with a carotenoid-protein pigment of the lobster shell, which undergoes color changes upon denaturation, indicate that this protein is even more sensitive to the hydrophobic character of the denaturing agent,[3] and the results obtained with deoxyribose nucleic acid suggest that the denaturation of this compound is principally a function of the hydrophobic and poor ion-solvating properties of the reagents (Levine *et al.*, 1963).

[3] B. Buten, unpublished experiments.

It appears, then, that denaturation, as caused by amide derivatives, hydrophobic reagents, certain salts, and detergents, may be the result of a number of different factors, more than one of which may be present in a given molecule, which may upset the delicate balance between the native protein and the solvent (Putnam, 1953). The difference in the relative importance of amide and hydrophobic character in determining the effectiveness of denaturing agents toward different proteins and toward DNA illustrates these differences. In retrospect, it appears fortunate that bovine serum albumin was chosen as a test protein for extensive study, since it is possible to study the properties of the amide class of denaturing agents with this protein with little interference from other factors, such as hydrophobic forces, which are of greater importance in causing denaturation of some other proteins.

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