cent. each of the Mn and of the radioactivity was precipitated; the precipitate contained the appropriate amounts of pyridoxal and amino acid.

To 4 ml. of ice-cold 0.020 *M* CuPyrVal, previously crystallized, was added 0.33 ml. of 0.060 *M* $Cu^{64}Val_2$, both solutions at *p*H 7.2 and the solution concentrated quickly in the cold to about 0.5 ml. After holding 5 min. at 0°, the suspension was centrifuged 4 min. at high speed, and the crystals washed twice with water. In a representative test, 32.2% of the copper and 33.1% of the radioactivity had been precipitated.

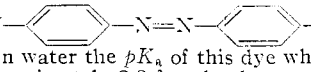
ANN ARBOR, MICHIGAN

[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF NORTHWESTERN UNIVERSITY]

The Hydrogen Ion Equilibria of a Single Group Attached to Serum Albumin: Some Implications as to the Surface Characteristics of Protein Molecules

BY IRVING M. KLOTZ AND JANET AYERS

RECEIVED MARCH 18, 1957

The ionization equilibria of the dimethylamino group of $(CH_3)_2N$—  —Hg— attached to bovine serum albumin have been studied in various aqueous solvents. In water the pK_a of this dye when attached to cysteine is 3.3; when linked to protein, 1.8. In 8 *M* urea, the pK_a is approximately 3.3 for the dye on the protein or amino acid, near 5 in 0.03 *M* dodecyl sulfate in both cases. These results and the more detailed behavior of the ionization equilibria are not in concordance with electrostatic expectations. It is suggested that the pK_a's reflect the structure of the water envelope of the protein. A variety of observations can be described in terms of changes in the order in this aqueous framework.

Introduction

Proton equilibria of acid–base groups on proteins have been the subject of numerous investigations for over 50 years. The early literature has been summarized by Cohn and Edsall[1] and more recent work has been reviewed by Steinhardt.[2] In general, the maximum uptake or release of protons corresponds well with the number of acidic and basic groups presumed to be on the protein from analyses of its content of specific amino acids. The course of the *p*H-titration curve, however, usually differs markedly from what one might calculate for the corresponding number of comparable proton-dissociating groups in an ordinary aqueous environment.

The modified acidity of groups on a protein molecule has been ascribed in part to electrostatic interactions between successively dissociating sites.[3–6] These interactions account reasonably well for the titration curves of some proteins[4,7] but more often there are large deviations, in both directions, from electrostatic predictions. Such discrepancies have been attributed to changes in molecular weight or shape,[8–10] to binding of small ions[6] and to the influence of hydrogen bonding between polar groups on the pK's of proton-dissociating groups.[7,11]

The interpretation of acid–base ionizations of protein molecules is complicated in part by the fact that the measurements reflect the composite behavior of a large number of groups. Even in limited regions of *p*H, for example, 3–5, where only one type of equilibrium is involved, —COOH ⇌ —COO^-, the number of participating carboxyl groups is very large. There are usually fewer imidazole or tyrosine groups in a protein, but even these are commonly present in appreciable number and their proton equilibria overlap those of amino groups, so that a sharp separation cannot be made by electrometric methods.

The proton-donor group usually present in smallest number in proteins is the sulfhydryl. A direct study of its dissociation by electrometric methods is not feasible, however, since the equilibria of amino and tyrosine groups would obscure the contribution of –SH. In principle, it might be possible to adapt the optical method used by Benesch and Benesch[12] for the determination of pK_{SH} of cysteine, a method which takes advantage of the appearance of an absorption peak at 240 mμ when –SH is converted to $-S^-$. In most proteins, however, absorption of light at this wave length is very strong even in dilute solution and even when the mercaptan group is not ionized. It would be difficult, therefore, to measure the small differences in absorption accompanying ionization of the –SH, against the strong background absorption of the relatively concentrated protein solution which would be needed to supply an appreciable concentration of mercaptan groups.

An alternative approach which could still take advantage of the small number of –SH groups on a protein would be to introduce a molecule which reacts specifically with the mercaptan side-chain, and which contains a substituent whose proton uptake, or release, is accompanied by a marked change in visible absorption spectrum. A class of such molecules are the azomercurials, (I), in which

I

(1) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Chap. 20, Reinhold Publ. Corp., New York, N. Y., 1943.

(2) J. Steinhardt, *Advances in Protein Chem.*, **10**, 151 (1955).

(3) K. Linderstrøm-Lang, *Compt. rend. trav. lab. Carlsberg*, **15**, No. 7 (1924).

(4) R. K. Cannan, A. C. Kibrick and A. H. Palmer, *Ann. N. Y. Acad. Sci.*, **41**, 243 (1941).

(5) G. Scatchard, *ibid.*, **51**, 660 (1949).

(6) C. Tanford, THIS JOURNAL, **72**, 441 (1950).

(7) C. Tanford and J. D. Hauenstein, *ibid.*, **78**, 5287 (1956).

(8) G. Scatchard, *Am. Scientist*, **40**, 61 (1952).

(9) C. Tanford and J. Epstein, THIS JOURNAL, **76**, 2163 (1954).

(10) C. Tanford, S. A. Swanson and W. S. Shore, *ibid.*, **77**, 6414 (1955).

(11) M. Laskowski, Jr., and H. A. Scheraga, *ibid.*, **76**, 6305 (1954).

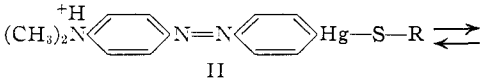
(12) R. E. Benesch and R. Benesch, *ibid.*, **77**, 5877 (1955).

X is the substituent with acidic or basic properties (*e.g.*, $(CH_3)_2N-$ or $-OH$). These substances combine specifically with the mercaptan groups of proteins[13] and undergo marked alterations in absorption over a characteristic *p*H range, depending on the nature of the substituent. From changes in the spectra one can follow the ionic state of these substituents in the protein environment without obscurant effects from other groups of the protein which are simultaneously involved in acid–base equilibria.

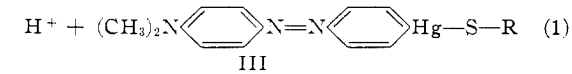
The complex between one such mercurial, 4-(*p*-dimethylaminobenzeneazo)-phenylmercuric acetate [(I) with X = $(CH_3)_2N-$], and a protein with one –SH group, bovine serum albumin, has been previously prepared.[13] Its proton equilibria, described in this paper, provide a basis for examining the surface characteristics of this protein.

Experimental

The acidity constant for the ionization



II



III (1)

was computed from changes in the visible absorption spectrum with *p*H. The acidic species II has an absorption peak near 520 mμ, the conjugate base at about 460 mμ. The fraction of the azomercurial in the acid form, α, was calculated by means of the equation

$$\alpha = \frac{\text{optical density soln. } \alpha - \text{optical density basic soln.}}{\text{optical density very acid soln.} - \text{optical density basic soln.}} \quad (2)$$

the optical densities all being measured at the same wave length, usually 520 mμ. A graph was then made of α *vs.* *p*H. For most comparisons the value of the *p*H at which α = 0.50 was taken as the *pK*, since for reaction 1

$$pK = p\text{H} - \log \frac{(\text{III})}{(\text{II})} = p\text{H}_{\alpha=0.5} \quad (3)$$

Where α = 0.5, (II) = (III). For some purposes, *pK*'s were calculated at several *p*H's by substitution of the appropriate values for II and III in eq. 3.

In eq. 1, R represents either the protein, bovine serum albumin, or a small-molecule mercaptan, such as cysteine. When the protein was studied, a 0.2% solution (3×10^{-5} *M*) was prepared in the desired solvent and to this was added a few crystals of azomercurial. The solution was stirred gently for several hours, until an appreciable amount of dye had dissolved, as judged by the intensity of the color. The protein-linked dye was in the range $(0.8–1.5) \times 10^{-5} M$. A similar procedure was used with cysteine, except that cysteine hydrochloride was at a concentration of 1×10^{-4} *M*; the cysteine–azomercurial complex, nevertheless, was maintained at about 1×10^{-5} *M*.

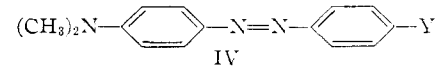
To the solution of bound azomercurial was added a small portion of hydrochloric acid (0.05–0.5 *M*) or sodium hydroxide (0.01 *M*) and the optical density was measured. A second portion of acid or base was then added and the absorption measured again. This was repeated[14] until some 20 points were available for graphing. Suitable corrections were made for dilution of the solution by added acid or base. In most cases a full absorption spectrum was taken over the region 350–600 mμ. From a graph of optical density *versus* *p*H, α was computed, and then it was plotted as a function of *p*H, so that the *pK*'s could be determined readily.

Crystallized bovine serum albumin was purchased from Armour and Company. Previous experiments[13] with the same samples have shown 0.66 mole SH per mole protein. Cysteine hydrochloride was obtained from Nutritional Biochemical Corp., and cysteine ethyl ester and glutathione from Schwarz Laboratories. Thioglycolic acid was given to us by the Toni Research Laboratories and thiomalic acid by Evans Chemetics. The sodium dodecyl sulfate was a specially purified sample supplied by the Fine Chemicals Division of E. I. du Pont de Nemours and Company. The urea and inorganic salts were reagent grade. Sorbitol monohydrate was purchased from Mann Research Laboratories.

The absorption of light was measured with the Beckman spectrophotometer, model DU, at about 25°; *p*H was obtained with a Beckman model G *p*H meter.

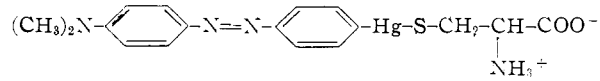
Results

Choice of Reference Compound.—The pK_a of dimethylaminoazobenzene (IV, Y = H)



IV

in aqueous solution is 3.5 and is not very sensitive to the nature of the opposite substituent Y.[15] For example, with Y = OH or Y = SO_3^-, the *pK* is 3.4. Nevertheless, if we wish to examine the effect of protein environment on the acidity of the $(CH_3)_2N-$ group, it seemed desirable to have information on the behavior of the azomercurial attached to a small-molecule mercaptan, so that any effect of the –Hg–S– linkages could be accounted for. Among the small molecules examined were thioglycolic acid, thiomalic acid, glutathione, cysteinyl ethyl ester and cysteine. The last proved most convenient experimentally, for it combined with the azomercurial rapidly and the complex obtained was stable over long periods of time. S-(*p*-Dimethylaminobenzeneazophenylmercuric) - cysteine (V)



V

was used, therefore, as a reference compound to ascertain the behavior of the $(CH_3)_2N-$ group in a non-protein environment.

Effect of Salts on pK_a.—Titrations with the cysteine–azomercurial (V) were carried out in water and in 0.1 *M* NaCl. The optical titration curves (Fig. 1) were normal, that is, similar to those

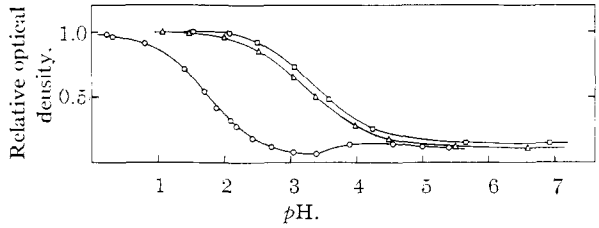
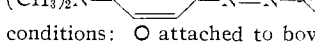


Fig. 1.—Relative optical densities *versus* *p*H for  under various conditions: O attached to bovine serum albumin in 0.1 *M* NaCl; △, attached to cysteine, in aqueous solution, with no salt; □, attached to cysteine, in 0.1 *M* NaCl.

(13) M. G. Horowitz and I. M. Klotz, *Arch. Biochem. Biophys.*, **63**, 77 (1956).

(14) In some cases a separate fresh portion of solution was used for each *p*H measurement. This procedure was followed whenever there was an indication of fading in acid solutions.

(15) I. M. Klotz, H. A. Fiess, J. Y. Chen Ho and M. Mellody, THIS JOURNAL, **76**, 5136 (1954).

for related substituted azobenzenes in aqueous solution, and the pK in solutions of constant ionic strength (3.39) was not appreciably different from that in water (3.26). For an ionization of the type represented by eq. 1, one would not expect much sensitivity to ionic strength.

When the azomercurial is attached to the protein, the titration curve of the $(CH_3)_2N-$ group is markedly different than in the cysteine complex. The course of a typical titration is shown in Fig. 1. Proceeding from pH 6 down, we find first a plateau, followed by a region between pH's 5 to 3.5 with a small hill and then a very steep rise in absorption between pH 3 to about 1, with every indication of a plateau below pH 1. It was not feasible to determine the height of the acid plateau by adding further quantities of strong acid both because of the instability of the protein in such solutions and because the quantities of acid required would make a large contribution to the ionic strength. The acid plateau was estimated, therefore, from an extrapolation of the optical density readings *versus* $1/(H^+)$. Having obtained the absorption of the fully acid form and taking the lowest readings of the optical density, near pH 3, as the absorption of the basic form of the dye, we can read off the pK readily as the pH at which the absorption is midway between the two extremes. For the azomercurial attached to bovine serum albumin in water the pK is 1.9 (Table I), in 0.1 M NaCl, 1.72.

One can also compute pK's from *each* of the optical density readings not too close to the completely acid or basic forms. Such a calculation has been made for the experiments in 0.1 M and in 0.5 M NaCl, and the results have been assembled in Fig. 2. There is some indication of an increase in pK with decreasing pH. We are not certain, however, that this trend is significant, for the points at the lower end of the α coördinate (higher end of pH scale) are sensitive to the choice of optical density for the conjugate base III. Yet it is in this range (pH 3) that the protein undergoes some modification leading to changes in the spectrum of III, as is apparent from the small hill in Fig. 1.

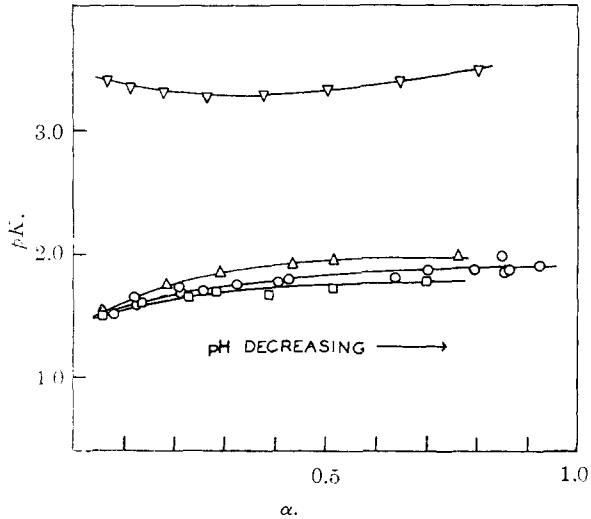
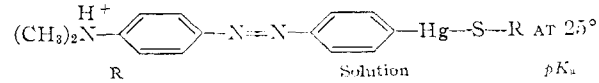


Fig. 2.—Variation of pK with extent of formation (α) of $(CH_3)_2NH$-form of the azomercurial attached to protein: △, in water; □, in 0.1 M NaCl; ○, in 0.5 M NaCl; ▽, in 8 M urea.

In any event, whether the trend in Fig. 2 is real or negligible, it is apparent that the pK does *not decrease* with decrease in pH as one might expect from a consideration of electrostatic factors. As the pH is lowered, one would expect the protein to acquire an increasingly positive charge which ought to lower the pK of the $(CH_3)_2NH^+-$ group. In solutions of moderate ionic strength, bovine albumin becomes almost saturated with hydrogen ions when the pH drops to near[10,16] 2; thus that portion of the ionization of the azomercurial on the protein which takes place below pH 2 occurs at essentially constant protein charge and the pK's in this region should not change appreciably with pH. In the range from pH 2 to 3, however, the protein does change its charge appreciably. In the absence of salt, for example, bovine albumin acquires 25 more protons as the pH is lowered from[16] 2.8 to 2.1; but simultaneously the binding of chloride increases by nearly 17 ions,[16] so the net increase in charge is eight units. For an ionic strength near 0.01, a change in charge of eight units on the protein ought to *decrease* the pK of the $(CH_3)_2NH^+-$ group by about 0.4 unit. Actually an *increase* is observed (Fig. 2) over the pH range 3 to 2. It would seem, therefore, that the ionization of the $(CH_3)_2NH^+-$ group is unaffected by changes in charge on the protein, at least within the pH region open to examination.

TABLE I

ACIDITY CONSTANTS OF



R	Solution	pK_a
$-CH_2-CH(NH_3^+)-COO^-$	Water	3.26
	0.1 M NaCl	3.39
	8 M urea	3.42
	0.03% sodium dodecyl sulfate	(~3)
	1.0% sodium dodecyl sulfate	5.18
Bovine serum albumin[a]	Water	1.9
	0.1 M NaCl	1.72
	0.5 M NaCl	1.80
	1 M urea	2.38
	6 M urea	3.25
	8 M urea	3.32
	0.03% sodium dodecyl sulfate	<1.3
	1% sodium dodecyl sulfate	4.7
	40% sorbitol	1.4
	60% sorbitol	1.3

[a] It should be emphasized that in the presence of protein, the pK_a listed refers only to the midpoint of the titration curve, for titrations of the azomercurial linked to serum albumin cannot be fitted to the simple form of equation (3).

(16) G. Scatchard, J. S. Coleman and A. L. Shen, THIS JOURNAL, **79**, 12 (1957).

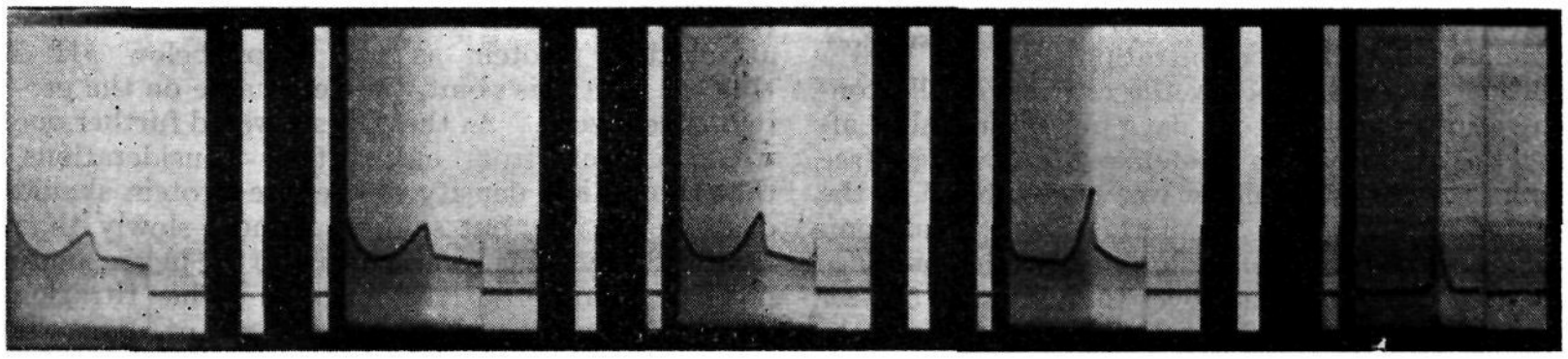


Fig. 3.—Ultracentrifugation of azomercurial–cysteine complex V in presence of 1% sodium dodecyl sulfate in water. The boundary of the dye, shown by the edge of the yellow color (dark area), coincides with the boundary of the dodecyl sulfate micelles, given by the peak in the schlieren pattern. Spinco ultracentrifuge model E was used with synthetic boundary cell rotating at 59,780 r.p.m. Purple filter inserted to increase color contrast. Times (in minutes) after formation of boundary (reading from right to left): 0, 11, 27, 43, 59. In absence of dodecyl sulfate, the color of a corresponding dye (methyl orange) diffused throughout the entire field in about 20 minutes.

A similar conclusion would be reached from the small effect of 0.5 *M* NaCl, as compared to water, on the *pK* of this group (Table I).

Effect of Urea.—The significance of *p*H, as measured with a glass electrode standardized in aqueous buffer, may be somewhat ambiguous in concentrated urea solutions. This ambiguity was minimized by obtaining first the *pK* of the $(CH_3)_2$-NH^+– group of the azomercurial complex of cysteine. This *pK* in 8 *M* urea, 3.42 (Table I), is not significantly different from that in water, and hence the mercurial attached to the small molecule provides a stable reference point.

Turning to the protein complex, we find a marked change in *pK* in 8 *M* urea, 3.32 as compared to 1.9 in water. It is particularly interesting that in aqueous urea the dye attached to protein behaves in its ionization just like the dye in water free from the protein. The urea thus acts like an "unmasking" reagent just as it does in connection with other groups, such as mercaptan, on proteins. An important distinction must be made in the present instance, however, in that naturally masked groups (such as –SH) were built into the protein during its biosynthesis, whereas the $(CH_3)_2N$– group was attached thereafter.

A 6 *M* concentration of urea has about the same influence as the 8 *M* solution (Table I). Substantially lower concentrations, such as 1 *M*, have much smaller effects, but nevertheless in the same direction, *i.e.*, an increase in *pK* in the presence of urea. Our observations with $(CH_3)_2NH^+$– thus parallel those of Foster and Sterman[17] in their electrometric titrations of the carboxyl groups of serum albumin in the presence of urea.

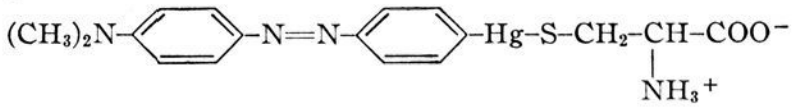
Effect of Sodium Dodecyl Sulfate.—This reagent, in a class with urea as a denaturant, has markedly different effects on the ionization of the $(CH_3)_2NH^+$– group than does urea. In a 1% solution (0.03 *M*), sodium dodecyl sulfate forms micelles[18] which carry a large negative charge. In this solution the cysteine complex of the azomercurial has a *pK* of 5.18 (Table I), very substantially higher than in water alone. This elevation of the *pK* might be due to the preferential absorption of the cationic acid form of the dye by the anionic micelles, or to the existence of a higher concentration of protons within the micelles. The former explanation may be ruled out, however, from ultracentrifugation experiments with the dye and 1% dodecyl sulfate (Fig. 3); the yellow basic form of the dye travels with the anionic micelle. Thus it seems clear that a mixed micelle is formed by the azomercurial and dodecyl sulfate and that the equilibrium of eq. 1 is shifted to the left because of the higher H^+ concentration within the anionic micelle.[19] If the *p*H of the solution external to the micelle is used to compute pK_a within the micelle, the resultant pK_a comes out higher than that in the aqueous phase.

The behavior of the azomercurial attached to the protein in the presence of 1% dodecyl sulfate is similar to the cysteine complex, the *pK*, 4.7, being almost three *p*H units above that in water alone. It seems fair to conclude, therefore, that this detergent forms micelles on the protein surface, since the dye attached to the protein shows acid–base properties very similar to those of the cysteine–dye complex in the mixed micelle in water. The micelle on the protein surface must have a substantial amount of water built into its framework, since protons have no difficulty reaching the $(CH_3)_2N$– group buried within. The strong negative charge of the sulfate groups at the ends of the aliphatic chains produces an increase in the local concentration of hydrogen ions, so that the concentration of $(CH_3)_2\overset{H^+}{N}$– is greatly increased.[20]

(17) J. F. Foster and M. D. Sterman, THIS JOURNAL, **78**, 3656 (1956).

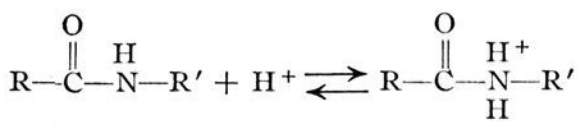
(18) M. L. Corrin and W. D. Harkins, *ibid.*, **69**, 683 (1947).

(19) It is of interest to note in passing that these results give some insight into the constitution of detergent micelles. In a mixed micelle of



with dodecyl sulfate, the dipolar amino acid end would presumably be facing the aqueous phase, whereas the azobenzene framework would presumably be buried inside the aliphatic chains of the C_{12} sulfate. Hence the $(CH_3)_2N$– substituent must also be buried within the lipophilic phase. If this substituent has no trouble picking up an H^+ ion, as is evident from the high *pK*, the proton must have no difficulty penetrating the cluster of aliphatic chains. It seems likely, therefore, that within this cluster is a substantial amount of H_2O acting as a vehicle for the transfer of protons.

(20) Micelle formation on the surface of protein molecules would also shift the equilibrium



The effect of sodium dodecyl sulfate is critically dependent on its concentration. In an 0.03% solution (0.001 M), below the critical micelle concentration, the detergent does not appreciably affect the pK[21] of the cysteine–dye complex (see Table I). Some difficulty was encountered in the determination of the pK of the dye–protein compound because of its insolubility in the presence of 0.03% dodecyl sulfate over the pH range 5.4–2.7 and below pH 0.8. Nevertheless the optical observations showed that the acid form II did not even begin to form until the pH dropped to about 2, and that the pK for the ionization of eq. 1 must be below 1.3. It is immediately evident, therefore, that at 0.001 M concentration the detergent does not form micelles on the surface of the protein; otherwise the pK ought to be much above 1.8–1.9. There can be little doubt, nevertheless, that the detergent ions are bound to the protein since even at higher pH's where the protein carries a negative charge essentially all of the anionic detergent in solution is bound to the protein.[22] In our solutions, acid to the isoelectric point, binding would be further strengthened by the positive charge of the protein.

Despite the binding of some 30 anions by each albumin molecule[23] and the consequent acquisition of some 30 negative charges by the protein, the pK_{H^+} of the $(CH_3)_2N-$ group was not elevated, as one would expect from simple electrostatic considerations. Quite the contrary, its acidity was increased by at least 0.6 pH unit over that in water in the absence of detergent (see Table I). It is of interest in this connection that very concentrated sorbitol solutions have a similar effect on the pK of the protein-linked dye (Table I) as does sodium dodecyl sulfate, the pK being lowered to approximately 1.3.

Discussion

The fact that the pK of the $(CH_3)_2NH^+-$ group is 1.8–1.9 when it is attached to the protein and 3.3 when it is in a small molecule in water shows that the protein environment has a very profound effect on the ionizing abilities of this group. It seems unlikely that electrostatic effects are directly responsible. As has been mentioned above, appreciable changes in the charge of the protein, either by change in pH in the region of 2.7 to 2 or by addition of some 30 dodecyl sulfate anions, do not shift the pK_a in the expected direction. Furthermore a careful comparison of the optical titration curves of the dye in protein and in cysteine complex shows behavior not in accord with electrostatic expectations. In the cysteine complex, a rise in absorption becomes detectable with increasing acidity as soon as one passes below pH 5 (Fig. 1). The same initial rise is shown by the dye attached to protein as one drops below pH 5 (Fig. 1). At this point, the net charge on the protein is near zero. As the pH is lowered further one would expect, from electrostatic considerations, that the optical density of the dye–protein should continue to rise, but somewhat more slowly than that of the cysteine complex, due to the increasingly positive charge on the protein. The titration curve in the protein should start to rise at about the same pH (near 5), should be less steep and spread out more over a wider pH range, but should be of the same general form as the cysteine complex.

Actually the optical density of the azomercurial on the protein reaches a small maximum at about pH 4.2 and then drops rapidly to a minimum at about pH 3.3. Clearly some profound change has occurred in the environment of the dye as the pH drops below about 4.2. This effect is shown even more strikingly if one examines the full spectra (Fig. 4). A marked shift in spectrum occurs at about pH 3, with the maximum of the dye (on the protein) shifting from about 455 to 420 mμ. (Only at much lower pH's does the characteristic spectrum of the acid form of the dye appear with a peak at 520 mμ.)

It is of course well known[24] now from a variety of experiments that serum albumin undergoes marked changes in physical characteristics at pH's below 4, and these have usually been interpreted in terms of swelling of the protein molecule.[8,10,24] Such an expansion is not in itself adequate to explain our observations, however, for the net result should be merely a decrease in the electrostatic broadening effect on the titration curve of the $(CH_3)_2NH^+-$ group.

The actual appearance of the optical titration curve of the dye–protein in the region below pH 3, where the absorption rises smoothly with decreasing pH (Fig. 1), is similar to that of the dye–cysteine complex except that the whole curve is displaced by about 1.5 pH units. The steepness of rise, once it starts, is if anything greater with protein than with cysteine. From electrostatic influences plus molecular expansion, one would expect the titration curve of dye–protein not to be displaced but to start upwards at about the same pH as does the cysteine complex, to rise less steeply at first and to increase in steepness, without exceeding that of the cysteine complex, as the pH is lowered further. The observed displacement of the titration curve of the dye–protein suggests that the protein effect is rather one of "masking" or hindering access to the $(CH_3)_2N-$ group similar to the masking of other, but naturally occurring, functional groups in protein molecules.

It seems inappropriate in the present case to attribute such masking to hydrogen-bonding between $(CH_3)_2\ddot{N}-$ and a donor group (see formula VI) because of our background of spectroscopic information. The dye–cysteine complex has a peak at 458 mμ. In the protein the absorption peak of the dye is much flatter, but the maximum is near 455 mμ at pH 5 and drops to 420 mμ below pH 4. If hy-

toward the right. Perhaps this effect is responsible for the catalysis of amide and peptide hydrolysis in the presence of dodecylsulfonate described by J. Steinhardt and C. H. Fugitt, *J. Research Natl. Bur. Standards*, **29**, 315 (1942). The explanation offered by these authors is slightly different; they attributed the catalysis to the binding of anion directly to the amide or peptide group.

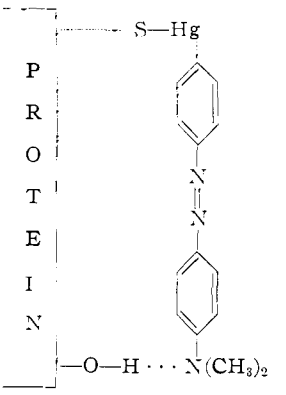
(21) The pK could not be determined precisely in these solutions because of instability of the dye–cysteine complex in the presence of these low concentrations of dodecyl sulfate.

(22) F. Karush and M. Sonenberg, THIS JOURNAL, **71**, 1369 (1949).

(23) The concentration of dodecyl sulfate was 1×10^{-3} M, of serum albumin 3×10^{-5} M.

(24) For rather complete references see M. D. Sterman and J. F. Foster, THIS JOURNAL, **78**, 3652 (1956).

drogen bonding occurred in the protein one would expect the peak to shift to higher wave lengths ($\sim$ 500 mμ) instead of lower, and to higher extinctions, as seems to occur in related dimethylaminobenzeneazo dyes.[25,26] The observed shifts



VI

with the azomercurial albumin to lower wave lengths and reduced extinctions argue against a hydrogen bond from protein to $(CH_3)_2N-$ substituent.

In essence the phenomena observed show that the azo dye attached to serum albumin does not behave as it would in a normal aqueous environment, that attachment to the protein hinders access from the water to the $(CH_3)_2N-$ group. One is led to ask, therefore, whether the water in the vicinity of the protein is not different in structure from that in the bulk of the solution.

The importance of "icebergs" around polar (and non-polar) species in an aqueous environment was perhaps first emphasized by Frank and Evans,[27] who also mentioned that these structures might be of significance biologically. We have previously obtained good indications that "frozen" water molecules play a role in determining the enthalpy and entropy changes accompanying the formation of protein complexes.[28] Likewise Jacobson[29] has shown that dielectric behavior of aqueous protein solutions may be interpreted in terms of oriented water molecules. From a variety of very striking fluorescence experiments in ordinary ice, Szent-Györgyi[30] has suggested that muscular contraction and relaxation (as well as other biological phenomena) may be a response to a rearrangement in structure of the water envelope of proteins.

In the context of the present experiments we would view the drop in pK_a of the dimethylamino group in the environment of the protein as a consequence of the rigid iceberg structure in the neighborhood of the macromolecule. With serum albumin this freezing seems to become extensive as the pH drops below about 4.2. In the neighborhood of pH 5 to 4.2, the dye gives indications of normal ionization behavior and hence, at least in

(25) I. M. Klotz, R. K. Burkhard and J. M. Urquhart, THIS JOURNAL, **74**, 202 (1952).
(26) W. L. Peticolas and I. M. Klotz, *ibid.*, **78**, 5257 (1956).
(27) H. S. Frank and M. W. Evans, *J. Chem. Phys.*, **13**, 507 (1945).
(28) I. M. Klotz and J. M. Urquhart, THIS JOURNAL, **71**, 847 (1949).
(29) B. Jacobson, *ibid.*, **77**, 2919 (1955).
(30) A. Szent-Györgyi, *Science*, **124**, 873 (1956).

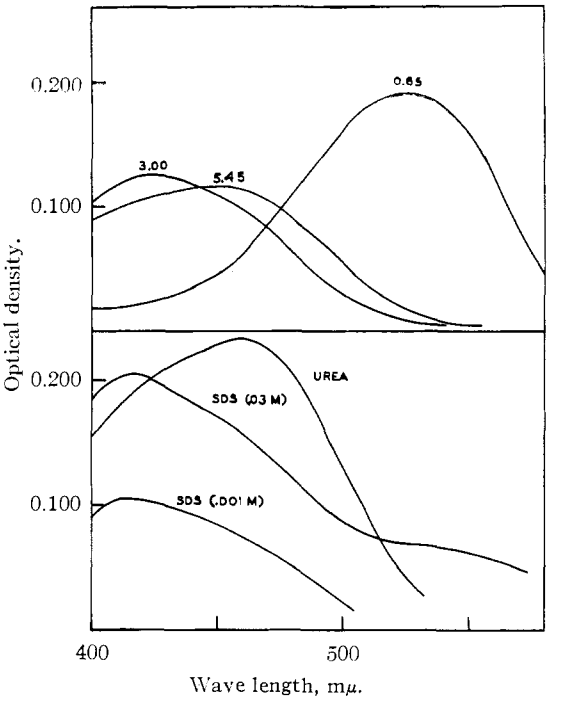


Fig. 4.—Spectra of $(CH_3)_2N-C_6H_4-N=N-C_6H_4-Hg-$ attached to bovine serum albumin: top, in water at pH's 5.45, 3.00 and 0.65; bottom: in 8 M urea at pH 7.22; in 0.001 M sodium dodecyl sulfate at pH 6.50; in 0.03 M sodium dodecyl sulfate at pH 5.45.

the vicinity of the $(CH_3)_2N-$ group of the dye attached to the cysteinyl residue of the protein, there is no indication of "frozen" water. Below pH 4.2, the accumulation of substantial net positive charge may increase strongly the extent and rigidity of the hydration envelope of the protein so as to produce effects on ionization properties as well as perhaps to contribute to the marked changes in hydrodynamic properties and other optical characteristics described in the literature.[24]

In line with this viewpoint, the effect of urea, bringing the pK_a back up to 3.3, is easy to understand. In essence in the presence of urea, the dye attached to protein behaves in its ionization just like the dye in water, free from the protein. An appropriate inference might be, therefore, that urea, because of its strong hydrogen-bonding characteristics, breaks down the "frozen" structure of the water envelope of the protein and transforms it into one more nearly like that of the bulk aqueous environment.[30a] It is of interest in this connection that the absorption spectrum of the protein-linked dye in the presence of 8 M urea shows a peak only at about 455 mμ (Fig. 4) and fails to develop the absorption peak at 420 mμ, characteristic of the masked $(CH_3)_2N-$ group, as the pH is lowered. Only the usual acid spectrum of azo dye, with a

(30a) In terms of the picture of masking as a steric effect, it is difficult to see (1) how the protein can provide side chains to block the $(CH_3)_2N-$ group at the extreme end of the azomercurial substituent attached to the surface of the protein; (2) why urea, a hydrogen-bond breaker, should be able to abolish completely any such lipophilic type of interaction even if it could be established *de novo*.

peak near 520 mμ, is obtained with decreasing *p*H in aqueous 8 *M* urea.

In contrast, 0.001 *M* sodium dodecyl sulfate lowers the *pK* of the protein-linked dye and hence must not interfere with the oriented water envelope, but instead must reinforce it somewhat. Such a reinforcement is quite possible since polarizable organic structures are believed to increase the order of water structures.[27] One cannot be certain of course that the dodecyl sulfate anions are spread uniformly around the serum albumin molecule, but at least in the neighborhood of the dye, which is attached to a cysteinyl residue of the protein, these anions show their influence. It is of interest in this connection that the spectrum of the dye–protein in the presence of 0.001 *M* dodecyl sulfate shows only the 420 mμ peak (Fig. 4) and not the 455 mμ one, even at *p*H's as high as 6.5 (Fig. 4). Furthermore as the *p*H is lowered to *p*H 2.7, there is no change in position of the peak, although it becomes a little sharper. Evidently the environment of the $(CH_3)_2N-$ group on the protein in the presence of dodecyl sulfate is established by these anions even at *p*H 6.5, and there is no large change in its character as the *p*H is lowered, except when protons are finally accepted around *p*H 2.

At a higher concentration of dodecyl sulfate, 0.03 *M*, the nature of the water envelope is dominated by the surface micelles. It is clear that these micelles must have water within their framework since protons have no difficulty reaching the $(CH_3)_2N-$ group attached to the protein. At the surface of the protein, the micellar water seems to be highly ordered, nevertheless, for the spectrum of the protein-linked dye shows a peak at 420 mμ even at *p*H 7.5. As with more dilute detergent, this peak remains unchanged as the *p*H is lowered. With 0.03 *M* dodecyl sulfate, however, the micellar charge more than compensates for the rigidity of the water and protons are bound by the $(CH_3)_2N-$ groups at a much higher *p*H.

It is perhaps worthwhile to extend the scope of concepts of protein structure to include the organization which the macromolecule imposes upon its aqueous envelope. One might speak perhaps of the *meta*-structure of the protein, the framework which it imposes upon its environment beyond its own boundaries. The ordering of water molecules so produced may be limited to local regions of the surface or may be general around the whole protein molecule, and would probably depend on the distribution of polar groups within the protein. In the present studies with serum albumin and, as far as ionization of the specific dye of the class I is used as a probe (over the limited *p*H range of about 5 and below), the freezing of the water seems to be general. For the region of "masking" of the $(CH_3)_2N-$ group parallels that in which hydrodynamic properties indicate an increase in effective volume.

"Masking" of other, *naturally occurring*, functional groups of proteins might also be due to a "freezing" of the water structure in the neighborhood of the group. Lack of reactivity of such hindered groups (*e.g.*, mercaptan) is usually only relative and dependent on the reagent. The effectiveness of a reagent in combining with a masked group might very well be related to its ability to "dissolve" in the iceberg covering the group. Similarly, the effects of denaturing agents, such as urea, in "unmasking" blocked functional groups may be due to the rearrangements which they produce in the structure of the water envelope characteristic of the native protein. In terms of the "melting" of water of the metastructure it is quite reasonable that urea denaturation of proteins should be accompanied by a substantial contraction in volume, as observed in the Carlsberg Laboratory.[30b] Likewise the volume contractions accompanying enzymatic breakdown of proteins,[30b] and particularly their large values at the onset of proteolysis, may be a reflection of disarrangements in the water lattice at the surface of the macromolecule.

The reversibility of some denaturation processes, for example after removal of urea, is also more readily understandable. It is not necessary to postulate a highly specific refolding of the peptide chain into its original configuration but merely the refreezing of water when the disturbing, melting influence has been removed. Other denaturing factors, such as strong acid or alkali, or increased temperature can also be interpreted in terms of their influence on the structure of bound water. For example, the marked decrease in rate of exchange of deuterium in ribonuclease[30c] as the temperature is lowered would seem most plausible since the extent of the rigid iceberg structure should surely increase as the solution is cooled.

Likewise coöperative effects, such as the increased reactivity of the successively reacting sites after the first one on a protein has combined with a substrate,[31] may be transmitted through the water envelope. The original protein would have a *meta*-structure determined in part by its prosthetic groups and in part by its specific amino acid residues. When a molecule, *e.g.*, O_2, combines with the prosthetic group or specific site, the structure of water at that point could be changed significantly. Since the structure at this point is part of a more extensive water framework, it is evident that the effects due to combination at a specific site could be transmitted some distance and result in a disordering of the water structure some distance away. In consequence, a prosthetic group or specific site at a distance from the initially reactive one could have its water mask loosened and hence access to the second site could be significantly eased. Similarly specific ions bound at one site or region of a protein molecule could modify the framework for some distance by changing the water structure at that point and hence produce effects on a reactive site not in its immediate vicinity.

As has been mentioned, the present experiments have been limited in range of *p*H and in protein used. However, it is readily evident that corresponding experiments can be carried out with the present class of dye (I) and other proteins with mercaptan groups.[13] In addition analogous investigations can be made over other *p*H ranges by sub-

(30b) K. Linderstrom-Lang, *Cold Spring Harbor Symposia on Quantitative Biology*, **14**, 117 (1950).

(30c) A. Hvidt, *Biochim. et Biophys. Acta*, **18**, 306 (1955).

(31) The increased affinity of hemoglobin for the second oxygen molecule is a prime example of this phenomenon.

stituting groups in place of the $(CH_3)_2N-$ substituent on the azobenzene nucleus of I. A corresponding dye with a *para* O–H group, for example, would allow one to study ionization behavior in the region near *p*H 7–8, an *ortho* O–H group, higher *p*H ranges. One is also not limited to attachments at mercaptan side chains of proteins. The use of a sulfonyl chloride substituent in place of –HgAc in I, for example, would permit one to make adducts to lysine residues. There is thus a wide range of covalent complexes which can be prepared. These should offer suitable examples for establishing how much emphasis should be put on the role of "frozen" water in determining the chemical and physical behavior of dissolved protein molecules.

Acknowledgments.—This investigation was assisted by a grant from the National Science Foundation. We also are indebted to Dr. Howard K. Schachman for his suggestions in the use of the synthetic boundary cell.

EVANSTON, ILL.

[CONTRIBUTION NO. 2170 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

Further Studies on the Kinetics of the α-Chymotrypsin Catalyzed Hydrolysis of Acetyl-L-tyrosinamide and of Acetyl-L-tryptophanamide in Aqueous Solutions at 25° and *p*H 7.9 ± 0.1[1]

BY RICHARD A. BERNHARD AND CARL NIEMANN[2]

RECEIVED JANUARY 25, 1957

It has been found that the values of K_S, k_3 and K_P for the system α-chymotrypsin-acetyl-L-tyrosinamide, in aqueous solutions at 25° and *p*H 7.9 ± 0.1, obtained by observing the rate of formation of either one of the two reaction products, are identical within the limits of experimental error, that the values of K_S and K_P are independent of the nature and concentration of the buffer components of the four buffer systems investigated and that the values of k_3 are dependent upon the concentration of the buffer components and indirectly upon the nature of the buffer species. More limited observations with the system α-chymotrypsin–acetyl-L-tryptophanamide suggest that the behavior of this system is identical with that observed for the system α-chymotrypsin–acetyl-L-tyrosinamide. An experimental criterion for the recognition of bi- and trifunctionality in anionic competitive inhibitors of the type $RCH_2CO_2^-$ and $R'CONHCHRCO_2^-$ has been noted.

In a previous study Thomas, MacAllister and Niemann[3] examined the kinetics of the α-chymotrypsin catalyzed hydrolysis of acetyl-L-tyrosinamide in aqueous solutions at 25° and *p*H 7.9 ± 0.1 and 0.02 *M* in the THAM[4] component of a THAM–HCl buffer, or 0.02 *M* in the EDA[5] component of an EDA–HCl buffer, by determining the rate of formation of acetyl-L-tyrosinate ion. The results obtained by these investigators were re-evaluated by Foster and Niemann[6] and by Foster, Shine and Niemann[7] and it was concluded that the above reaction systems could be represented throughout their course by equations 1 and 2, where P_{1f} is acetyl-L-tyrosinate ion and P_{2f} either ammonia or ammonium ion, and that their rates could be described, within the limits of experimental error, by eq. 3, where $K_S = (k_2 + k_3)/k_1 = 32 \pm 4 \times 10^{-3}$ *M*[6],

$$E_f + S_1 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E_f + P_{1f} + P_{2f} \quad (1)$$

$$E_f + P_{1f} \underset{k_5}{\overset{k_4}{\rightleftharpoons}} EP_1 \quad (2)$$

$$k_3[E]t = K_S(1 + [S]_0/K_P)\ln([S]_0/[S]_t) + (1 - K_S/K_P)([S]_0 - [S]_t) \quad (3)$$

$K_P = k_5/k_4 = 110 \pm 30 \times 10^{-3}$ *M*[7] and $k_3 = 2.4 \pm 0.3 \times 10^{-3}$ *M*/min./mg. protein nitrogen per ml.[8].

When the above values of K_S and k_3 are compared with those reported earlier,[8-11] and which were obtained by determining the rate of formation of ammonia and ammonium ion in aqueous solutions at 25° and *p*H 7.8 and 0.1 *M* in an unspecified phosphate buffer containing sufficient lithium chloride to bring the initial ionic strength to 0.292, it is seen, *cf.* Table I, that such a comparison leaves unanswered a number of questions whose answers are required for a more complete understanding of the reaction under consideration. It is the purpose of

TABLE I

KINETIC CONSTANTS FOR THE α-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF ACETYL-L-TYROSINAMIDE IN AQUEOUS SOLUTIONS AT 25° AND *p*H 7.9 ± 0.1

Ref.	K_S[a]	k_3[b]	K_P[a]
3, 6, 7	32 ± 4[c,d]	2.44 ± 0.3[c,d]	110 ± 30[c,d]
8	23[e,f]	2.7[e,f]	.....
9	32.6[e,f]	2.7[e,f]	.....
10	27[e,f,g]	3.0[e,f,g]	.....
11	29[e,f]	3.1[e,f]	.....

[a] In units of 10^{-3} *M*. [b] In units of 10^{-3} *M*/min./mg. protein-nitrogen per ml. [c] Reaction system 0.02 *M* in the THAM component of a THAM–HCl buffer or 0.02 *M* in the EDA component of an EDA–HCl buffer. [d] Based upon the determination of the rate of formation of acetyl-L-tyrosinate ion. [e] Reaction system 0.1 *M* in an unspecified phosphate buffer containing sufficient lithium chloride to bring the initial ionic strength to 0.292. [f] Based upon the determination of the rate of formation of ammonia and ammonium ion. [g] Said to be preferred to the two sets of values immediately above.

(1) Supported in part by a grant from the National Institutes of Health, Public Health Service.

(2) To whom inquiries regarding this article should be sent.

(3) D. W. Thomas, R. V. MacAllister and C. Niemann, THIS JOURNAL, **73**, 1548 (1951).

(4) Tris-(hydroxymethyl)-aminomethane.

(5) Ethylenediamine.

(6) R. J. Foster and C. Niemann, THIS JOURNAL, **77**, 1886 (1955).

(7) R. J. Foster, H. J. Shine and C. Niemann, *ibid.*, **77**, 2378 (1955).

(8) S. Kaufman and H. Neurath, *Arch. Biochem.*, **21**, 245 (1949).

(9) S. Kaufman and H. Neurath, *J. Biol. Chem.*, **180**, 181 (1949).

(10) G. W. Schwert and S. Kaufman, *ibid.*, **180**, 517 (1949).

(11) S. Kaufman and H. Neurath, *ibid.*, **181**, 623 (1949).