[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, PURDUE UNIVERSITY]

An Investigation of Bovine Plasma Albumin by Differential Ultraviolet Spectroscopy[1]

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Bovine plasma albumin (BPA) exhibits a *p*H-dependent differential absorption spectrum in the ultraviolet with a peak at 287 mμ and a secondary peak at 280 mμ. The spectrum is thus very similar to that which has been observed with a number of other proteins and attributed to changes in the extent of hydrogen-bonding of the tyrosyl residues. The magnitude of the differential extinction coefficient (ΔE) at 287 mμ has been studied systematically as a function of *p*H in 0.02 and 0.1 *M* chloride in both water and 2 *M* urea, at 0.02 *M* chloride in 4 *M* urea and in 0.02 *M* thiocyanate. The major effect on the spectrum takes place below the isoionic *p*H in the carboxyl titration region. Changes in the magnitude of ΔE do not, however, parallel protonation of carboxylate groups. Thus, an increase in ionic strength or replacement of chloride by thiocyanate, both of which factors effect an increase in hydrogen-ion binding at a given *p*H, results in a decrease in the magnitude of ΔE instead of the expected increase if tyrosyl residues were hydrogen-bonded to carboxylate anions. This fact together with a consideration of the effect of urea leads to the conclusion that the perturbation of the spectrum results from general changes in molecular structure which are known to occur in BPA in the carboxyl titration region. The spectral perturbation can be explained in terms of alterations in the degree of hydrogen bonding of tyrosyl residues to unidentified acceptor groups. Alternatively, it is pointed out that the results could be explained on the basis of changes in the polarity and polorizability of the environment of the tyrosyl groups.

Introduction

Absorption of radiation by proteins at wave lengths between 280 and 300 mμ is attributed primarily to the absorption characteristics of the aromatic amino acid residues, tryptophan and tyrosine.[3] Above *p*H 8 the absorption spectrum of a protein containing tyrosine exhibits a pronounced bathochromic shift as the *p*H is increased due to ionization of the phenolic groups of the tyrosyl residues. Crammer and Neuberger[4] utilized a spectrophotometric procedure to investigate the ionization of the phenolic groups in ovalbumin and insulin. Similar investigations have been concerned with plasma albumin[5] and ribonuclease.[6,7] These investigations suggest that the phenolic hydrogens in some proteins do not dissociate freely but are rendered less labile because of having been incorporated in the protein structure. The formation of intramolecular hydrogen bonds between phenolic groups and side-chain carboxylate groups[4,8,9] has been proposed as a possible explanation of this anomalous dissociation. Recently the technique of differential spectrophotometry[10] has been employed to study the effect of low *p*H on the absorption spectra of insulin[11] and ribonuclease[12] in an attempt to confirm such bonding.

It has been suggested that as the *p*H is decreased below the isoelectric point the native form of BPA is converted into an isomeric form[13–15] which is capable of expansion.[16–18] The present work was undertaken to determine whether there might be changes in the differential absorption spectrum of BPA in the 280–300 mμ region which could be correlated with the isomerization and expansion results. Since the completion of this research a paper by Glazer and co-workers[19] reporting the effect of *p*H and urea on the differential spectra of BPA and ovalbumin has come to our attention.

Experimental

Materials.—Crystalline BPA (Lot No. 67908) was purchased from Armour and Company. Deionized water was used in the preparation of all solutions. It was observed initially that low *p*H solutions (*p*H <3.6) became turbid after standing several hours. These solutions had been prepared from a fresh stock protein solution which had been deionized using a mixed-bed ion-exchange column.[20] The appearance of turbidity in low *p*H BPA solutions had been observed previously[16,21] and had been attributed to the presence in the protein of a fatty acid or ketone[22] impurity. Infrared analysis by the KBr pellet method[23] and determination of the neutralization equivalent has subsequently shown that the impurity contains stearic acid.[24] To eliminate and identify the impurity a procedure was devised whereby BPA was dissolved in 0.1 *N* HCl. After having stood 48 hr. at 5°, the turbid solution was filtered while cold and the filtrate first passed through an ammonium acetate ion-exchange column and then through a mixed-bed column. The precipitate on the filter was recovered for analysis by extraction with acetone. Centrifugation is an alternate method for removing the impurity, the impurity rising to the surface of the solution.[16]

Each set of solutions for each series of spectrophotometric determinations consisted of a common reference solution and several sample solutions at different *p*H values. Isoionic 0.02 *M* sodium chloride solution served as the common reference solution. The concentration of BPA was the same in all solutions of a particular set and all sample solutions of a set were at the same ionic strength and urea concentration. BPA concentrations utilized in the investigation ranged from 0.2 to 0.5%, most of the experiments being conducted at concentrations of 0.3–0.4%. Thus, the total

(1) This investigation was supported in part by the National Cancer Institute, National Institutes of Health, Grant C-2248, and by the National Science Foundation, Grant G-1953.

(2) Fellow of the Visking Corporation, 1957–1958.

(3) G. H. Beaven and E. R. Holiday, *Advances in Protein Chem.*, **7**, 324 (1952).

(4) J. L. Crammer and A. Neuberger, *Biochem. J.*, **37**, 302 (1943).

(5) C. Tanford and G. L. Roberts, THIS JOURNAL, **74**, 2509 (1952).

(6) D. Shugar, *Biochem. J.*, **52**, 142 (1952).

(7) C. Tanford, J. D. Hauenstein and D. G. Rands, THIS JOURNAL, **77**, 6409 (1956).

(8) C. Tanford, *Proc. Iowa Acad. Sci.*, **59**, 206 (1952).

(9) M. Laskowski, Jr., and H. A. Scheraga, THIS JOURNAL, **76**, 6305 (1954).

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(13) K. Aoki and J. F. Foster, THIS JOURNAL, **79**, 3385 (1957).

(14) K. Aoki and J. F. Foster, *ibid.*, **79**, 3393 (1957).

(15) J. F. Foster and K. Aoki, *ibid.*, **80**, 1117 (1958).

(16) J. T. Yang and J. F. Foster, *ibid.*, **76**, 1588 (1954).

(17) C. Tanford, J. G. Buzzell, D. G. Rands and S. A. Swanson, *ibid.*, **77**, 6421 (1955).

(18) M. D. Sterman and J. F. Foster, *ibid.*, **78**, 3652 (1956).

(19) A. N. Glazer, H. A. MacKenzie and R. G. Wake, *Nature*, **180**, 1286 (1957).

(20) H. M. Dintzis, Ph.D. Thesis, Harvard University, 1952.

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

(22) P. Bro and J. M. Sturtevant, *ibid.*, **80**, 1789 (1958).

(23) R. D. Meiklejohn, R. J. Meyer, S. M. Aronovic, H. A. Schuette and V. W. Meloche, *Anal. Chem.*, 329 (1957).

(24) E. J. Williams and J. F. Foster, to be published.

optical density at 287 mμ, the wave length of primary interest, did not exceed 2.7 in most experiments.

Measurements.—The absorbance of each of the sample solutions relative to that of the reference solution was measured at 25° with a Beckman DU spectrophotometer with photomultiplier attachment. A Beckman Model G *p*H meter was employed for *p*H measurements at 25° and infrared analyses were accomplished with a Perkin-Elmer Model 21 spectrophotometer. The neutralization equivalent of the fatty acid impurity was determined by the method of Cohn and co-workers.[25]

Results

Experiments were carried out in 0.02 *M* and 0.1 *M* chloride and in 0.02 *M* thiocyanate to determine the effect of *p*H, ionic strength and intrinsic anion-binding ability on the differential spectra. Additional experiments were carried out in 2 and 4 *M* urea. A few reversibility experiments conducted in 0.02 and 0.1 *M* chloride indicated that the changes in differential spectra were reversible to *p*H changes. Figure 1 depicts typical differential

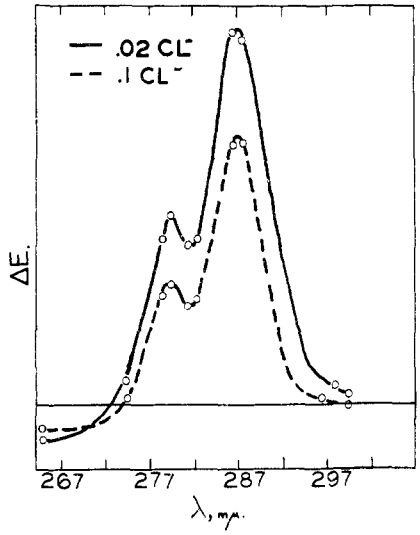

Fig. 1.—Differential spectra of BPA: solid line represents *p*H 2.04, 0.02 Cl^- solution; dashed line *p*H 2.20, 0.1 Cl^- solution. In this and later figures ΔE is defined as E of the reference solution minus E of the sample.

spectra while Figs. 2 and 3 illustrate the influence of the various solution factors on the differential extinction coefficient ($\Delta E^{1\%}_{1\,cm}$)[26] at 287 mμ, the wave length of maximum differential absorption.

The dependence of the differential absorbance on BPA concentration was tested and found to obey Beer's Law. Neither the *p*H nor the differential absorbance of the chloride and thiocyanate solutions appeared to undergo significant changes over a 24 hr. period following preparation of the solutions. The urea solutions, however, exhibited a systematic decrease in differential absorbance and an increase in *p*H during the same interval. This effect was most pronounced in the case of the 4 *M* urea solutions. The differential absorbance of a particular urea solution at a given *p*H (between *p*H 2.8 and *p*H 5), when measured 24 hr. following preparation of the solution, agreed, in most cases,

(25) E. J. Cohn, W. L. Hughes, Jr., and J. H. Weare, THIS JOURNAL, **69**, 1753 (1947).

(26) ΔE (molar) = $7000\Delta E^{1\%}_{1\,cm.}$, assuming a molecular weight of 70,000 for BPA.

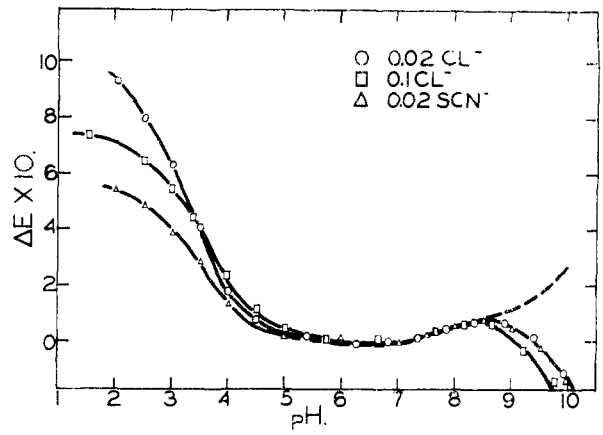

Fig. 2.—Effect of *p*H on the differential extinction coefficient at 287 mμ: ○, 0.02 Cl^-; □, 0.1 Cl^-; △, 0.02 SCN^-. The dashed line represents a crude correction to compensate for tyrosyl ionization.

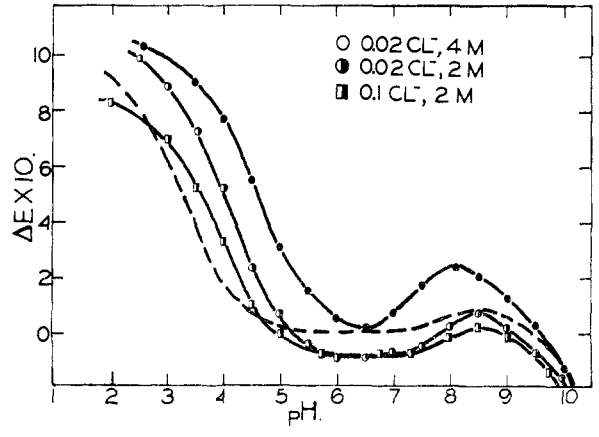

Fig. 3.—Effect of *p*H on the differential extinction coefficient at 287 mμ in urea: ○, 0.02 Cl^-, 2 *M* urea; □■, 0.1 Cl^-, 2 *M* urea; ●, 0.02 Cl^-, 4 *M* urea. The dashed line represents the 0.02 Cl^-, 0 *M* urea curve.

with the differential absorbance of a freshly prepared solution at the same *p*H. It appears that the decrease in differential absorbance with time was a consequence of the increase in the *p*H of the solutions and that the effect of *p*H on ΔE in urea solutions is essentially reversible.

Discussion

At low *p*H the contribution to the first excited state of tyrosine is predominantly by dipolar quinoid structures (Fig. 4a) while at high *p*H the excited state can be represented adequately by ionic structures (Fig. 4b) which are possible because of dissociation of the phenolic hydrogen. These ionic structures should be more stable than the dipolar structures since they do not necessitate a separation of charge. The energy difference between the ground and first excited state should therefore be less for ionized tyrosine than for un-ionized tyrosine. This decrease in the absorption energy (ionized form compared to un-ionized form) accounts for the observed bathochromic shift of the tyrosine spectrum when the *p*H is increased above *p*H 8.[3] If hydrogen-bonding of the phenolic hydrogen stabilizes the first excited state the excitation

energy will be decreased (Fig. 4c), and a small bathochromic shift should be evident. Since this shift will be small, the measurement of differential spectra rather than difference spectra is desirable for optimum precision.[10]

In line with these ideas the differential spectra of BPA might be postulated as being due primarily to changes in the extent of intramolecular tyrosyl hydrogen-bonding as a consequence of changes in the solution environment. For example, if two BPA solutions are of equal concentration but differ in *p*H the absorbances of the solutions will be different because the extent of intramolecular tyrosyl hydrogen-bonding is different in the two solutions and the perturbation of the tyrosyl spectrum is assumed to be proportional to the extent of hydrogen-bonding of the phenolic hydrogen. In accordance with the presentation in the preceding paragraph, a solution in which there is considerable hydrogen-bonding would be expected to absorb more strongly than one in which there was less hydrogen-bonding. Consideration of solute–solute and solute–solvent intermolecular hydrogen-bonding has been neglected. The latter effect is neglected on the assumption that the tyrosyl residues are not accessible to or do not react appreciably with solvent molecules (salt, urea, water). Evidence that this assumption is fallacious will be discussed in a later section. The effect of solute–solute interaction is neglected because of the apparent concentration independence of ΔE for the concentrations of BPA which were used.

Crammer and Neuberger[4] were the first to suggest anionic carboxylate groups as the most likely acceptors in tyrosyl hydrogen bonds. The *p*H dependence of the differential spectra observed with insulin[11] and ribonuclease[12] has in both cases been interpreted in terms of tyrosyl-carboxylate hydrogen bonds. In the former case the pK of the acceptor groups appeared to be about 3.0, in the latter case near 2.0. The present results again show that the major effect takes place precisely in the carboxyl titration range and at first thought would be taken as evidence for tyrosyl-carboxylate hydrogen bonds. However, as has been pointed out earlier by one of us[27] such results must be treated with caution and are at best only presumptive evidence in the identification of the acceptor groups. This is particularly true if one admits the possibility of pronounced coöperative changes in the structure of the protein. In the case of BPA, in view of the overwhelming evidence that drastic reversible structural alterations take place in the *p*H range below 4.5[13–18,27] it is particularly important to examine the results with care. Thus regardless of the nature of the acceptor groups it might be anticipated that the tyrosyl bonds would be broken as a consequence of isomerization and especially molecular expansion. It will now be shown that general structural alterations are in all probability responsible for the low *p*H change in tyrosyl absorption and that carboxylate anions are not necessarily the acceptor groups.

In the first place the effect of *p*H on the differential spectra is not limited entirely to the carboxyl

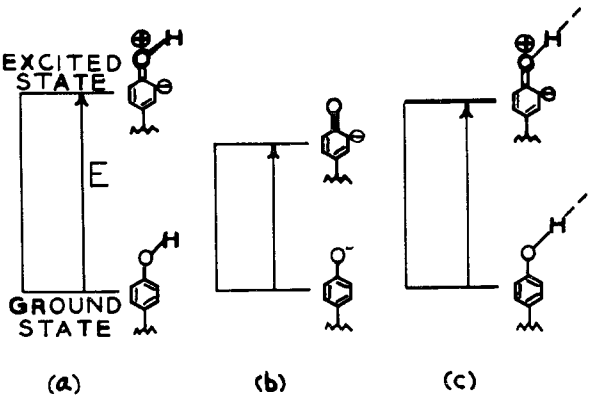

Fig. 4.—Schematic energy-level diagrams of (a) tyrosine, (b) ionized tyrosine, (c) hydrogen-bonded tyrosine. Ground state levels are not necessarily the same in the three cases.

titration range. Examination of Figs. 2 and 3 shows a small but significant increase in ΔE above *p*H 7. This rise is not due to ionization of tyrosine which takes place at still higher *p*H and results in a decrease rather than an increase in ΔE. (This effect is discussed in detail below.) If the effect of *p*H on ΔE is to be attributed directly to protonation of groups directly involved in the hydrogen bonds, it is evident that carboxylate anions cannot be the sole acceptors of tyrosyl hydrogen bonds. Further, however, no acceptor groups should be destroyed by increasing *p*H in any *p*H range. Increasing *p*H would be expected to increase the number of acceptors or to decrease the number of donors. The simplest explanation of the rise in ΔE with increasing *p*H in this range, in terms of hydrogen bonding, is that other hydrogen bonds are being disrupted by deprotonation of donor groups, most probably imidazolium residues, and that tyrosyl hydrogen bonds are disrupted secondarily because of coöperative changes in the protein structure.

Additional evidence tending to minimize the importance of the carboxylate groups as acceptors in tyrosyl hydrogen bonds arises from the behavior of the ΔE–*p*H curves upon variation of the ionic environment. The titration curve of BPA (as for proteins in general) in the carboxyl titration region is shifted to higher *p*H with increasing ionic strength[28] and also when chloride ion is replaced by thiocyanate.[13] In other words, protonation of the carboxylate groups at a given *p*H is enhanced by either an increase in ionic strength or an increase in anion binding.[29,30] It would thus be expected that both the 0.1 M chloride curve and the 0.02 M thiocyanate curve in Fig. 2 would lie well above the 0.02 M chloride curve. The shift is actually in the opposite direction, except for the slight upward shift in the 0.1 M chloride curve between *p*H 3.5 and 5. This latter point will be considered in subsequent paragraphs. Similarly, in 2 M urea (Fig. 3) the effect of ionic strength is just opposite to that expected on the basis of protonation of carboxylate

(27) J. F. Foster and K. Aoki, *J. Phys. Chem.*, **61**, 1369 (1957).

(28) C. Tanford, S. A. Swanson and W. S. Shore, THIS JOURNAL, **77**, 6414 (1955).

(29) G. Scatchard, I. H. Scheinberg and S. H. Armstrong, Jr., *ibid.*, **72**, 535 (1950).

(30) G. Scatchard, I. H. Scheinberg and S. H. Armstrong, Jr., *ibid.*, **72**, 540 (1950).

groups involved in tyrosyl hydrogen bonds. Figure 5 illustrates graphically the fact that ΔE is not a unique function of hydrogen-ion binding. In view of these results it appears that changes in the differential spectra in the carboxylate titration region do not arise by virtue of direct protonation of tyrosyl–carboxylate hydrogen bonds. The carboxylate residues may or may not be the primary acceptors. If they are, indeed, the principal or sole acceptors, it seems clear that their rupture is more a function of general changes in the protein configuration than of direct protonation of the bonded carboxylate groups *per se*.

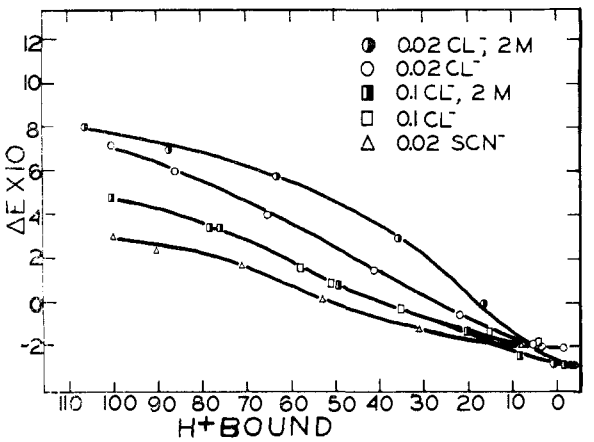

Fig. 5.—Correlation of differential extinction coefficient at 287 mμ with number of moles of hydrogen ion bound per mole of BPA: O, 0.02 Cl^-; □, 0.1 Cl^-; △, 0.02 SCN^-; ◑, 0.02 Cl^-, 2 *M* urea; ◨, 0.1 Cl^-, 2 *M* urea.

On the other hand, the effects of increasing ionic strength, of replacement of chloride by thiocyanate and of urea are all in substantial qualitative accord with their effects on the expansion of the protein.[16–18] If the differential extinction coefficient at 287 mμ (ΔE) is plotted *versus* the radii calculated from viscosity[17] Fig. 6 is obtained. (Similar plots are obtained using radii calculated from sedimentation coefficient[31] and electrophoretic mobilities.)[13,14] It is seen that over much of the expansion range ΔE is proportional to the radius of the molecule. Alternatively, similar results are obtained if ΔE is plotted *versus* the optical rotation, a property which has already been shown to be a convenient measure of the molecular expansion.[16] The upturn in the curves in Fig. 6 at the upper extremities, corresponding to very low *p*H, suggests a continuing rupture of hydrogen bonds after attainment of the limiting degree of expansion. The initial rise in the ΔE–*p*H curve prior to any appreciable increase in radius occurs in the range in which the isomerization (N–F transition) of BPA occurs.[13–15] Indeed, plots of ΔE *versus* percentage of the F form as deduced from electrophoresis measurements in the *p*H range above 3.5 yield roughly linear plots in all cases (Fig. 7). It is suggested that a part of the total attainable ΔE values is associated with the isomerization process. Relating changes in differential absorption to the expansion and isomerization phenomena explains a

(31) M. J. Kronman and J. F. Foster, *Arch. Biochem. Biophys.*, **72**, 205 (1957).

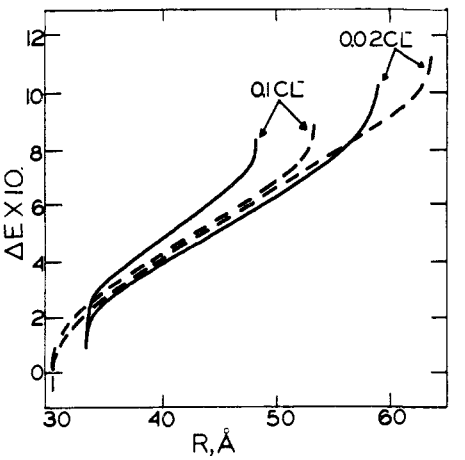

Fig. 6.—Correlation of the differential extinction coefficient at 287 mμ with the molecular radii of BPA. Dashed lines represent 2 *M* urea curves.

fact pointed out above, namely, that increasing ionic strength has inverse effects on the ΔE *versus* *p*H curves above and below *p*H 3.5 whereas thiocyanate shifts the entire curve to lower *p*H. Both increasing ionic strength and increasing anion-binding affinity have been found to depress the expansion which occurs below *p*H 3.5.[16,18,31] On the other hand, increasing ionic strength shifts the isomerization curve slightly to higher *p*H[18] while replacing chloride by the more strongly binding thiocyanate anion shifts it to lower *p*H.[14] Thus thiocyanate would be expected to repress the differential spectra shift in both the isomerization and expansion regions while increasing chloride concentration would be expected to have opposing effects above and below *p*H 3.5, as is observed.

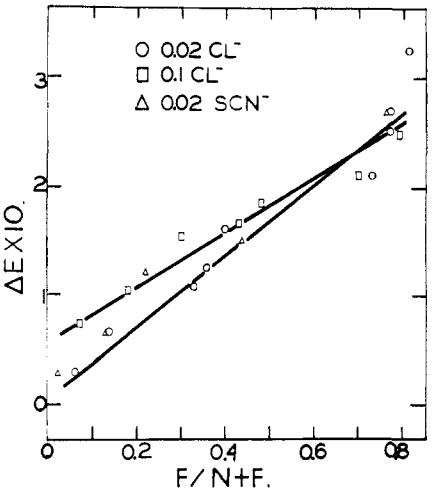

Fig. 7.—Correlation of the differential extinction coefficient at 287 mμ with the fraction of F isomeric form: O, 0.02 Cl^-; □, 0.1 Cl^-; △, 0.02 SCN^-. Lines are drawn through the 0.02 Cl^- and 0.1 Cl^- points.

Further evidence that changes in the absorption spectrum are due to general structural changes can be found in the analysis of the effect produced by urea. Classically, urea is presumed to exert its profound effect on proteins through rupture of

intramolecular hydrogen bonds. This rupture presumably consists of the substitution of urea–protein intermolecular hydrogen bonds for the previously existing intramolecular bonds. From this point of view it is difficult to predict the effect of urea on the differential spectra. There is evidence that weak hydrogen bonds between urea and phenolic compounds exist.[32,33] Actually, a spectral shift in either direction could be rationalized on the basis that the tyrosyl–urea bond was either weaker than or stronger than the original bond. Examination of Fig. 3 shows that urea exerts a strong effect. For all *p*H values below 5 increasing urea concentration increased ΔE indicating a net reduction in hydrogen-bonding. This implies that either urea (a) does not hydrogen bond to the liberated tyrosyl residues or (b) forms much weaker tyrosyl hydrogen bonds than those preëxisting in the native protein.

Above *p*H 5 it is seen that 2 *M* urea depresses ΔE while 4 *M* urea enhances it. It is possible to rationalize this surprising result in terms of the known behavior of BPA in aqueous urea if it is assumed that not all tyrosyl residues in BPA are intramolecularly hydrogen-bonded and that these non-bonded tyrosyl residues are capable of hydrogen-bonding to urea. Kauzmann and co-workers[34,35] observed that at *p*H 7–8, urea in 2 *M* concentration had no observable effect on the viscosity and optical rotation of BPA whereas at 4 *M* there were pronounced increases in both of these properties. On the other hand, below *p*H 3 even 2 *M* urea caused increases in rotation and viscosity. Similarly, Sterman and Foster[18] found 2 *M* urea to elevate the viscosity and rotation below *p*H 5 but not above this *p*H. It is quite plausible, then, that 2 *M* urea is incapable of producing the necessary coöperative alteration in the protein structure above *p*H 5 to promote rupture of tyrosyl hydrogen bonds. Under these conditions, the effect of 2 *M* urea on the differential spectra could result from formation of hydrogen bonds with tyrosyl residues which are unbonded in the native protein structure. From the effect of 2 *M* urea on ΔE between *p*H 5.5 and 7 and comparison with the magnitude of the effect of 2 *M* urea on the differential absorption of acetyltyrosine ethyl ester at 285 mμ,[36] it might be inferred that approximately 12 of the 21 tyrosine residues in BPA are non-hydrogen bonded in the native state.[37]

Glazer, *et al.*,[19] also concluded that changes in differential absorption parallel molecular expansion of BPA. Further, it is of considerable interest that they noted essentially no low *p*H differential spectrum in the case of ovalbumin, a protein which is known not to expand significantly in acid solution.[16] Thus the facts seem to demand an interpretation of the differential spectrum as arising from coöperative breakdown of the native protein structure.

The positions of the maxima in the various ΔE versus *p*H curves in the alkaline region (Figs. 2 and 3) coincide approximately with the region in which ionization of the tyrosyl groups would be expected to commence.[5,38] The maximum in 4 *M* urea is at a distinctly lower *p*H than in either 0 or 2 *M* urea, an observation which is easily reconciled with the hypothesis of tyrosyl hydrogen-bonding which would result in an increase in the intrinsic *pK* of the tyrosyl residues. Destruction of such bonds by urea, even with formation of weaker tyrosyl–urea hydrogen bonds, would result in a decrease in the intrinsic *pK*. The fact that 2 *M* urea does not affect the position of the maximum to any great extent is in agreement with the deduction made above that 2 *M* urea does not disrupt the hydrogen-bonded structure above *p*H 5.

An attempt has been made in Fig. 2 to indicate the expected course of ΔE above *p*H 8.5 if ionization of the tyrosyl residues did not occur. The calculated curve was obtained using *pK* values as reported by Tanford, *et al.*,[28] and spectral data for ionized and un-ionized tyrosine at 287 mμ as reported by Beaven and Holiday[3] and Cramme.r and Neuberger.[4]

Although the differential spectra have been interpreted above as arising predominantly from changes in intramolecular hydrogen-bonding of tyrosyl residues, the possibility that other factors contribute to the spectra must not be ignored. Wetlaufer, *et al.*,[39] have reported that O-methyl tyrosine and glycyl-O-methyltyrosine, in which there are no available phenolic hydrogens, have *p*H-dependent difference spectra. Donovan, *et al.*,[40] have reported similar results for acetyl-l-tryptophan and glycyl-l-tryptophan; however, acetyl-l-tryptophan ethyl ester in which both ionizable groups are blocked did not yield a differential spectrum with change in *p*H. These studies indicate that the proximity of charged groups to tyrosyl residues (as well as to other aromatic residues) in the protein molecule might influence the spectral properties. Wetlaufer, *et al.*,[39] also found that urea and acetate ions are capable of producing difference spectra with O-methyltyrosine[41] and attributed such effects to dipole–dipole and ion–dipole interactions. Ion–dipole and dipole–dipole interactions are quite similar to hydrogen bonding, all three effects being essentially electrostatic in nature.[42] However, hydrogen bonds possess some covalent character and hence are subject to certain covalent-bonding requirements (availability of bonding orbit-

(32) D. B. Wetlaufer, *Compt. Rend. Trav. Lab. Carlsberg. Ser. Chim.*, **30**, 135 (1956).

(33) M. Laskowski, Jr., Abs. 131st Meeting Amer. Chem. Soc., 47C, 1957.

(34) H. K. Frensdorff, M. T. Watson and W. Kauzmann, THIS JOURNAL, **75**, 5167 (1953).

(35) W. Kauzmann and R. B. Simpson, *ibid.*, **75**, 5154 (1953).

(36) M. Laskowski, Jr., private communication.

(37) By comparison of the maximal ΔE obtained in our results at 0.02 ionic strength in chloride with the magnitude of ΔE obtained for ribonuclease,[12] it is estimated that 6 or 7 intramolecularly hydrogen bonded tyrosines exist in native BPA. These together with the 12 unbonded tyrosines would account almost quantitatively for the 21 present. Such calculations must be regarded, however, with grave reservation in view of the many assumptions which are involved and especially the possibility, discussed later, that factors other than tyrosyl hydrogen-bonding are involved.

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

(39) D. B. Wetlaufer, J. T. Edsall and B. R. Hollingworth, *J. Biol. Chem.*, in press. We are indebted to Professor Edsall for sending us a copy of the manuscript in advance of publication.

(40) J. W. Donovan, M. Laskowski, Jr., and H. A. Scheraga, *Biochim. Biophys. Acta*, in press.

(41) It seems significant that the direction of their shift in urea was the same as we find for BPA in 2 *M* urea but opposite to that in 4 *M* urea.

(42) C. A. Coulson, "Valence," Oxford Press, 1952, p. 298.

als and electrons, proper orientation of bonding orbitals, etc.)

In line with the findings of Wetlaufer, *et al.*,[39] it may be significant that our results indicate a systematic dependence of the differential absorption on net molecular charge. This is shown in Fig. 8. It

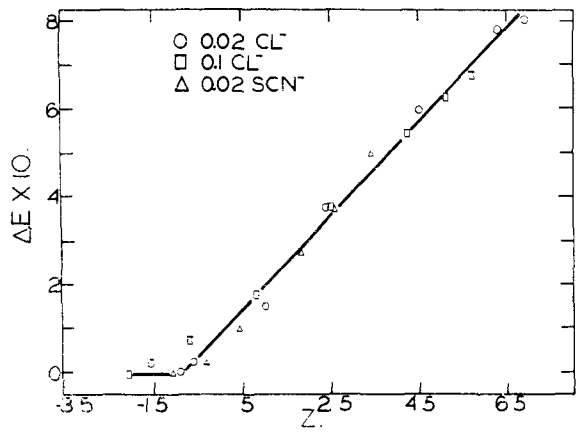

Fig. 8.—Correlation of the differential extinction coefficient at 287 $m\mu$ with net charge: O, 0.02 Cl^-; □, 0.1 Cl^-; Δ, 0.02 SCN^-.

should be pointed out, however, that the effects they observed were monotonic in net charge, as is indeed to be expected for a direct charge effect. The small but definite upturn of the curves in Figs. 2 and 3 with increasing negative charge, which is not shown in Fig. 8 because of the uncertainties of the chloride-binding correction at high *p*H, argue strongly against a simple electrostatic perturbation of the spectrum. This is indeed to be expected since the direct charge effect must be largely inductive in character, operating through the covalent-bonded structure. The charged centers in a protein, in contrast to the case of a simple amino acid, are at least several atoms removed from the aromatic rings. Furthermore, the magnitude of the effects obtained by Wetlaufer, *et al.*, are considerably smaller than ours (about one-fifth as great) when compared on a per tyrosyl residue basis. While general electrostatic effects doubtless make some direct contribution to the spectra, our observed correlation between ΔE and net molecular charge probably arises in an indirect manner through the relation of the latter to the over-all protein configuration.

It is well known that the dielectric constant of the environment produces an effect on the absorption spectrum of aromatic molecules.[43-45] Such effects are complex, indeed, and it appears that one must consider at least two entirely different effects based, respectively, on the dipole moment (referred to for convenience as the D effect) and the electronic polarizability (P effect). A solvent of high polarity will normally tend to stabilize the ground state of phenolic-type molecules more than the excited state, in view of the Franck–Condon principle. The D effect will thus be in such direction that increasing polarity will result in a blue shift of the spectrum. The polarizability, usually approximated as n^2 (the square of the refractive index of the refractive index of the solvent) operates in the opposite direction. That is, increasing polarizability results in a greater stabilization of the excited state than of the ground state, giving a red shift.[46,47] An alternative explanation of the observed spectral perturbations in proteins can be proposed on the basis of changes in the D and P effects without resorting to a hydrogen-bonding argument. It seems probable that the tyrosyl residues are imbedded, in the native protein, in a medium of relatively high polarizability ($n \sim 1.6$).[48] Furthermore, in this state it may be presumed that there is little or no opportunity for orientation of surrounding dipoles about the aromatic centers, so that effectively these centers are in an environment of low polarity. Any change in configuration such as expansion, isomerization or unfolding would be expected to remove the aromatic centers to the aqueous environment or at least permit greater access of water to such centers. The net effect of any such configurational change would thus be to increase the polarity and diminish the polarizability[49] of the environment of the aromatic residues. *Both effects would operate in the same direction as rupture of hydrogen bonds*, namely to give a blue shift and consequently increase ΔE as defined in this paper.

Much further work will clearly be required before it is possible to estimate the relative importance of the various factors in producing the observed differential spectra. The data reported by Baba[47] suggest that the magnitude of the P effect may be as great as that due to hydrogen-bonding. Bayliss[51] has stated that for anisole, dimethylaniline and chlorobenzene, all of which are analogous to phenol and hence to the tyrosyl residues, the P effect appears to be predominant over the D effect. Clearly, extreme caution must be exercised in the interpretation of differential spectra on proteins at the present time.

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