onic point only if it carries a sufficiently large number of solvating groups to outweigh the attraction both due to Coulomb as well as van der Waals forces between the individual macromolecules. This effect is quite strikingly shown by the studies on the solubility of copolymers of 2-diethylaminoethyl methacrylate and methacrylic acid as a function of composition,[24] in which it was found that copolymers containing less than 35 mole % or more than 79.6% basic groups were insoluble at the *p*I. Inasmuch as at the isoionic point the number of positive and negative charges must be equal,[31] the maximum total number of ionized groups in a two component copolymer cannot exceed $2(1 - X)$, where X is the mole fraction of the component present in excess. Thus the total number of charges per polymer molecule,[32] and therefore also the solvation of the polyampholyte, decreases as the composition is varied from the equimolar mark, resulting in precipitation of the copolymers at the extremes of the composition scale.

It is apparent that the determination of the state of ionization is particularly simple in polymethacrylic acid and the copolymers of methacrylic acid and 2-dimethylaminoethyl methacrylate because the carboxylate ions absorb in a region not otherwise obscured by extraneous bands. That this is *not* a prerequisite for the success of this technique, however, is shown by the measurements on the copolymer of acrylic acid and vinylpyridine, in which there is considerable overlapping of the frequencies characteristic of ionized groups. The more difficult problem of the determination of the ionization of proteins, in which bands due to peptide links dominate the spectrum in the region of interest, will be considered in the following communication.

I would like to acknowledge my indebtedness to Dr. G. B. B. M. Sutherland for his generous advice and for many stimulating discussions during the course of this study, which was supported by Contract N6 onr-232249 with the Office of Naval Research and Contract DA-44-109-QM-1037 with the Quartermaster Corps.

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(31) J. T. Edsall, H. Edelhoch, R. Lontie and P. R. Morrison, THIS JOURNAL, **72**, 4641 (1950).

(32) Inasmuch as the molecular weight in this series of copolymers was not held constant, and the dissociation constants of the monomers do not suffice to quantitatively describe the dissociation of the groups on the polymer, calculations of the actual number of charged groups have not been carried out.

[CONTRIBUTION FROM THE H. M. RANDALL LABORATORY OF PHYSICS, UNIVERSITY OF MICHIGAN]

Infrared Studies on Solutions of Polymeric Electrolytes. II. Proteins

BY GERT EHRLICH[1] AND G. B. B. M. SUTHERLAND

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The possibility of using infrared methods to detect carboxylate groups in globular proteins at the isoionic point has been investigated. Using N-methylsuccinamic acid in D_2O solution as a model compound, it has been shown that spectral changes in the region between 5 and 8 μ associated with variations in *p*D can be attributed to changes in the number of COOD and COO^- groups, while the absorptions in this region due to the peptide link are essentially unaltered. Two proteins were investigated, *viz.*, bovine plasma albumin and β lactoglobulin. Both were shown to contain COO^- groups, which in acid solution could be converted into COOD groups, thus providing direct evidence for the existence of these proteins as dipolar ions at the isoionic point. The absorption band which best characterizes the COO^- group lies at 6.4 μ, *i.e.*, very close to a peptide band at 6.45 μ. The latter, however, is shifted to 6.8 μ when the protein is deuterated, thus revealing the carboxylate group band. In undeuterated proteins the two bands overlap and this may affect deductions on protein structure made from observations on the 6.45 μ band in undeuterated proteins. The possibility of extending the infrared method to the detection of other ionic groups in proteins is discussed.

The numerous investigations of the electrochemical behavior of proteins, and of the dependence of their colligative properties on the state of charge,[2,3] provide an impressive body of evidence testifying to the importance of ionized groups in determining the physical chemistry of solutions of globular proteins. The majority of infrared studies on proteins have hitherto been aimed at elucidating the structure of the peptide linkages and the configuration of the individual polypeptide chains forming the protein skeleton, and with a few exceptions[4,5] have ignored the vital part played by ionized groups in determining protein structure and reactions. It has been shown in the preceding paper[6] that information on the state of ionization of certain synthetic polymeric electrolytes in solutions of heavy water can be obtained from infrared bands, characteristic of the carboxyl and carboxylate groups. The most numerous of the ionizable groups which have been identified in globular proteins are the carboxyls of glutamic and aspartic acid residues. If present in sufficiently large numbers, these should contribute to the infrared absorption spectra of proteins and the possibility arises of using infrared analysis for determining the state of ionization of protein molecules in solution. The feasibility of such a method depends mainly on the bands of the ionizable groups not being obscured either by (a) bands due to other parts of the protein molecule or (b) bands due to the solvent.

The infrared spectrum of a typical protein is

(1) Research Laboratory, General Electric Co., Schenectady, New York.

(2) W. Pauli and E. Valko, "Elektrochemie der Kolloide," Julius Springer, Wien, 1929, Chs. 42–52.

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(4) I. M. Klotz, P. Griswold and D. M. Gruen, THIS JOURNAL, **71**, 1615 (1949).

(5) H. Lenormant and J. Chouteau, *J. physiol. et path. gén.*, **41**, 203A (1949).

(6) G. Ehrlich, THIS JOURNAL, **76**, 5263 (1954).

shown diagrammatically in Fig. 1, together with the infrared bands found to be most useful for following the ionization of the carboxyl group in synthetic polymeric electrolytes.[6] It will be observed that the strongest band due to the carboxylate ion, at 6.35 μ, almost coincides with a very intense band in the protein spectrum at 6.45 μ. The latter has always been assumed to be entirely due to a vibration of the peptide link,[7] since it is an equally characteristic feature of the spectra of monosubstituted amides.[8] It would be difficult to detect changes in the intensity of a carboxylate absorption band overlapped by an intense peptide absorption. Furthermore, since water has a wide, intense band between 5.5 and 8.0 μ, the observation of carboxylate groups in ordinary aqueous solution of proteins is quite impracticable. If, however, the protein is dissolved in D_2O, the difficulty due to the solvent is largely eliminated[6]; moreover, the resulting deuteration of the hydrogen in the peptide link shifts the 6.45 μ peptide band to 6.8 μ.[9,10] The region between 6.2 and 6.8 μ is thus cleared of interfering absorption and the COO^- groups in deuterated proteins should be readily detectable. Carboxyl groups should also be apparent through the 5.87 μ band, although the fact that this is near a relatively much more intense protein absorption at 6 μ (due to the CO of the peptide link) may be expected to cause some trouble in detecting small concentrations of COOH groups.

It was considered advisable to study the effect of *p*H on solutions of N-methylsuccinamic acid as a model for a protein with ionizable carboxyl groups, since this compound contains one peptide link and one carboxyl group, and observation in acid and base should thus afford a check on possible changes in the peptide frequencies under these conditions.

Materials and Spectroscopic Techniques

The spectrometer, the absorption cells, and the solvents used in this investigation already have been described.[6] Two proteins were studied: (1) crystallized bovine plasma albumin and (2) β-lactoglobulin. Both were furnished by the Armour Laboratories. In addition, studies were made on N-methylsuccinamic acid, the model compound referred to in the preceding section.

N-Methylsuccinamic acid was synthesized in the Chemistry Department from succinic anhydride and methylamine and recrystallized from ethanol[11]; m.p. 108.5–109.3° (cor.). The dry material was allowed to dissolve in D_2O for ten hours, yielding a stock solution from which, by dilution, were obtained solutions of the following concentrations, on which absorption measurements were performed: (a) 10% N-methylsuccinamic acid in 0.35 *N* DCl, (b) 6% N-methylsuccinamic acid in 0.27 *N* NaOD.

The bovine plasma albumin was obtained in the isoionic form by exhaustive dialysis of a 10% solution against redistilled water. Measurements on bovine plasma albumin solutions were performed in the following sequence: a stock solution of the albumin in D_2O (weight concentration 8%) was prepared, from which, through addition of DCl, was obtained a 6.1% solution in 0.3 *N* DCl; addition of an equivalent amount of NaOD yielded a neutral protein solution in NaCl. The effect of excess alkali on the protein spectrum was determined on an 8% solution, obtained by addition of 2 *N* NaOD to a stock solution of bovine plasma albumin.

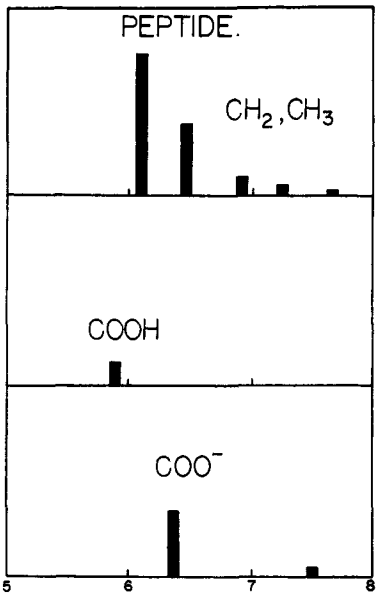

Fig. 1.—Principal infrared absorption bands of globular protein, carboxyl and carboxylate groups from 5–8 μ.

Films of deuterated bovine plasma albumin for absorption measurements were cast from a 1% D_2O solution in an atmosphere of nitrogen and then sealed between silver chloride plates. Undeuterated albumin was regained by exposing the deuterated film and permitting exchange of deuterium with atmospheric water vapor. Films of the acidified albumin were prepared by dissolving an isoionic protein film in 0.01 *N* HCl and evaporating the solvent by exposing to the atmosphere.

Since β-lactoglobulin is insoluble in distilled water, it was prepared as a suspension and dialyzed in this form; the isoionic material was isolated by freeze-drying. Solutions of β-lactoglobulin were prepared by dissolving the isoionic protein in D_2O acidified with DCl. The isoionic deuteroprotein was then isolated as a precipitate by adding NaOD. Through the addition of DCl or NaOD to the acidic stock solutions, 7% solutions of β-lactoglobulin (0.4 *N* in either acid or base) were obtained.

Results

N-Methylsuccinamic Acid.—The spectrum of this model compound was examined carefully between 5 and 8 μ. It was found to be identical in D_2O and in DCl (Fig. 2A) but markedly different in NaOD solution (Fig. 2B). In the former case, the un-ionized carboxyl group gives rise to the band at 5.87 μ (1704 cm.$^{-1}$); but in alkaline solution this band disappears entirely, being replaced by a very intense carboxylate band at 6.39 μ (1565 cm.$^{-1}$). It will also be observed that the intensity of absorption in the 7.1 μ region is enhanced on passing from acidic to alkaline solution and that a third band, at 7.66 μ, is also associated with ionization of the carboxyl group. The two bands at 6.39 μ (1565 cm.$^{-1}$) and 7.09 μ (1410 cm.$^{-1}$) are known to be due, respectively, to the antisymmetric and symmetric stretching vibrations of the COO^- group.[12] The origin of the band at 7.66 μ (1305 cm.$^{-1}$) is not so obvious. In investigations of the Raman

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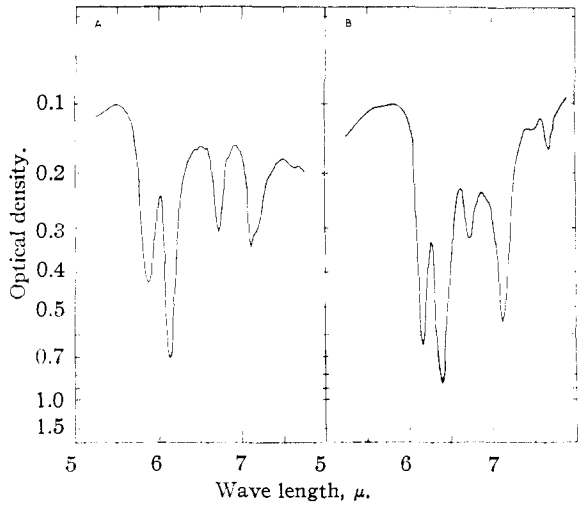

Fig. 2.—Absorption spectrum of N-methylsuccinamic acid: A. in 0.35 *N* DCl solution; B, in 0.27 *N* NaOD solution.

spectra of amino acids and related compounds it has been observed[13] that a frequency at 1320 cm.$^{-1}$ (7.58 μ) in glycylglycine disappears on adding acid, *i.e.*, on removing the COO^- group. Moreover, a Raman frequency of 1300 cm.$^{-1}$ (7.69 μ) appears in the spectrum of propionic acid when it is ionized.[14] However, no such frequency has been observed in the vibration spectrum of sodium acetate,[12] nor in the absorption spectra of the polymeric acids and polyampholytes reported in the preceding paper.[6] N-Methylsuccinamic acid, glycylglycine and propionic acid all have a CH_2 group adjacent to the carboxyl group. Since this is not the case for the other compounds examined it suggests that the 7.66 μ (1305 cm.$^{-1}$) band is due to a deformation vibration of the CH_2 group adjacent to a carboxylate ion.

The ratio of the extinction coefficient of the key band for carboxylate ion (6.39 μ) to that for the carboxyl group (5.87 μ) was determined as 2.5 from spectra of N-methylsuccinamic acid in NaOD and DCl. In order to do this it was assumed that the extinction coefficient of the peptide CO band at 6.12 μ was unaffected by ionization of the carboxyl group. This seems reasonable since no appreciable shift was observed in the position of this band in going from acidic to basic solutions; moreover, the value of 2.5 is in good agreement with the value of 2.3 found from the spectra of synthetic polymeric electrolytes.[6]

Bovine Plasma Albumin.—The spectrum of a film of bovine plasma albumin cast from H_2O solution is shown in Fig. 3B (full line) for the region from 5 to 8 μ. The most prominent bands appear to be the two peptide link frequencies at 6.08 μ (1645 cm.$^{-1}$) and 6.45 μ (1550 cm.$^{-1}$). If, however, the film is cast from HCl it will be noticed that certain changes occur in the spectrum (Fig. 3B, broken line). A new weak absorption appears near 5.9 μ forming a shoulder on the 6 μ peptide band and the absorption on the short wave length side of the 6.45 μ peptide band is significantly reduced. This indicates that in a film of bovine plasma albumin there exist ionized carboxyl groups (giving rise to absorption near 6.4 μ) which have largely been converted into COOH groups (absorbing at 5.9 μ) in a film prepared from HCl solution.

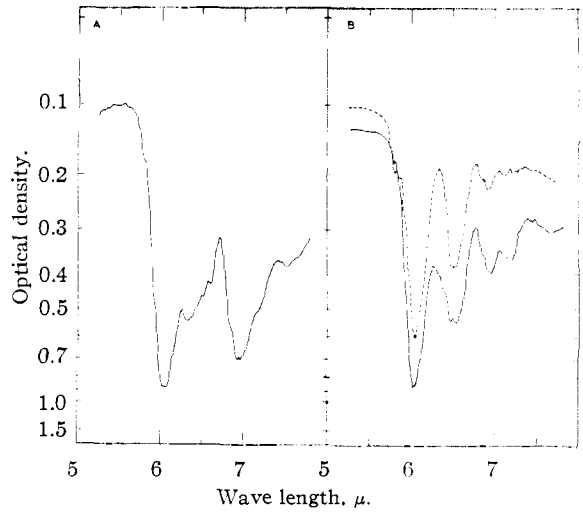

Fig. 3.—Absorption spectrum of bovine plasma albumin: A. cast from D_2O; B. cast from H_2O (—), cast from HCl (- - -).

The corresponding spectrum of a film cast from D_2O solution is shown in Fig. 3A. The effect of partial deuteration has been to reduce very considerably the absorption on the long wave side of the 6.45 μ band. As mentioned earlier, deuterated peptide links absorb strongly near 6.8 μ and a great increase in absorption is noticeable in this region. The fact that the absorption on the short wave side of the 6.45 μ band is not appreciably affected by deuteration confirms the conclusion drawn above that the absorption near 6.4 μ in bovine plasma albumin is mainly due to COO^- ions.

These effects can be seen much more clearly when we carry out ionization experiments on D_2O solutions of the albumin. Thus the spectrum in Fig. 4A indicates that in D_2O solution the deuteration has been much more complete, as the absorption on the long wave side of the 6.4 μ band has almost entirely disappeared. Addition of DCl (to give a 6.1% solution in 0.26 *N* DCl) now causes the elimination of all absorption in the 6.4 μ region as well as the disappearance of the other carboxylate band at 7.1 μ (Fig. 4B). However, the characteristic CO absorption of the carboxyl group becomes evident near 5.9 μ. Although it is not possible to make accurate measurements, the intensity of the absorption near 5.9 μ in acid solution is consistent with the ratio of 2.3 to 2.5 discussed in the preceding section.

It appears, therefore, that in D_2O solution of bovine plasma albumin there are a significant number of carboxylate groups present in the protein and that addition of DCl converts them completely into the un-ionized form. Confirmation comes from the addition of an equivalent quantity of NaOD to the acidic protein solution making it equivalent to an NaCl solution. This causes the bands characteristic of carboxylate groups to reappear at 6.35

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TABLE I

ABSORPTION BANDS OF N-METHYLSUCCINAMIC ACID, BOVINE PLASMA ALBUMIN AND β-LACTOGLOBULIN IN THE REGION 5.5 TO 7.5 μ

N-Methylsuccinamic acid				Bovine plasma albumin				β-Lactoglobulin				
DCl		NaOD		DCl		D_2O		DCl		D_2O		Tentative assignment
μ	cm.$^{-1}$	μ	cm.$^{-1}$	μ	cm.$^{-1}$	μ	cm.$^{-1}$	μ	cm.$^{-1}$	μ	cm.$^{-1}$	
5.87	(1704)			5.85?	(1709)			5.85?	(1709)			ν(C=O) in COOH
6.12	(1634)	6.15	(1626)	6.08	(1645)	6.08	(1645)	6.12	(1634)	6.12	(1634)	ν(C=O) in peptide link
		6.39	(1565)			6.35	(1575)			6.37	(1570)	ν(COO^-) antisymmetric
								6.50	(1538)			(CONHR)
6.70	(1493)	6.70	(1493)	6.91	(1447)	6.93	(1443)	6.93	(1443)	6.93	(1443)	(CONDR) + δCH_2
7.08	(1412)											$\delta(CH_3)$?
		7.09	(1410)			7.13	(1403)			7.13	(1403)	ν(COO^-) symm.
		7.66	(1305)									$\delta(CH_2)$?

and 7.13 μ, while the absorption due to carboxyls near 5.9 μ disappears (Fig. 4C full line). Further addition of NaOD does not significantly affect the spectrum (Fig. 4C, broken line).

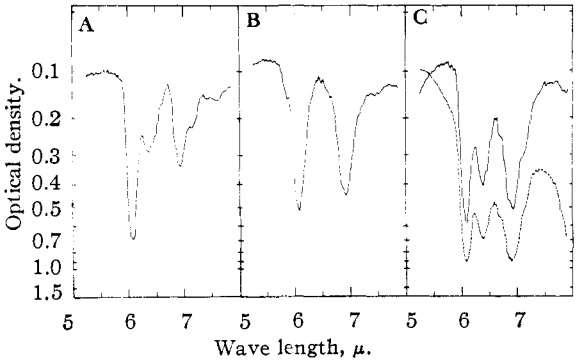

Fig. 4.—Absorption spectrum of bovine plasma albumin: A. in D_2O solution; B. in 0.26 *N* DCl solution; C, in NaCl solution (—), in 0.4 *N* NaOD (- - -).

β-Lactoglobulin.—The spectrum of β-lactoglobulin in D_2O (Fig. 5A) is very similar to that of bovine plasma albumin in D_2O (Fig. 4A), showing characteristic carboxylate bands near 6.4 and 7.1 μ. Addition of DCl eliminates the 7.1 μ band but does not quite remove the absorption in the 6.4 μ region where a weak band remains centered at 6.5 μ (Fig. 5B). The latter is probably due to peptide linkages which have not been fully deuterated; this may be connected with the relative insolubility of this protein in D_2O.

The disappearance of the carboxylate ion bands is again accompanied by the appearance of the weak COOH band near 5.9 μ (Fig. 5B). An alkaline solution of β-lactoglobulin exhibits the carboxylate ion bands near 6.4 and 7.1 μ (Fig. 5C) and no absorption is detectable from COOH groups near 5.9 μ.

Thus the results for β-lactoglobulin are essentially the same as those found for bovine plasma albumin. The wave lengths of the peaks of the principal absorption bands for all three materials (together with their assignments) have been collected in Table I.

Discussion

An isoionic solution of a protein is, by definition, a solution in which the only non-colloidal ions are H^+ and OH^- (or, for solutions in heavy water, D^+ and OD^-). Thus the requirement of electrical neutrality ensures that any positive charges on the isoionic protein molecule are balanced by an appropriate number of negative ones.[15] That such charges do exist on a protein in isoionic solution has been inferred from measurements of the heat of dissociation as a function of *p*H and from titrations in the presence of formaldehyde. The foregoing work, in which carboxylate ions in the protein have been observed through their characteristic infrared absorption gives a much more direct proof of the existence of charged groups in isoionic solutions of proteins. Moreover, the infrared method makes possible the detection of COO^- ions in protein films and crystals which are insoluble in water.

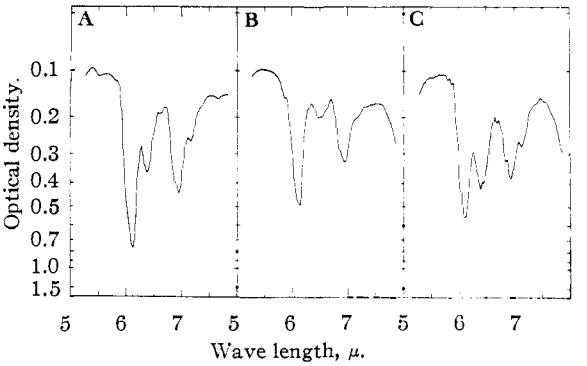

Fig. 5.—Absorption spectrum of β-lactoglobulin: A. in D_2O solution; B. in 0.4 *N* DCl solution; C. in 0.4 *N* NaOD solution.

The question immediately arises whether other ionic groups in proteins can be detected by infrared analysis, *e.g.*, those arising from the ionization of the residues of arginine, lysine, histidine, cysteine and tyrosine. In the case of arginine, the change from the guanidine group to the guanidinium ion should cause the disappearance of the band near 6 μ due to the C=N group. Whether this would be obscured by the intense peptide CO band near 6 μ can only be determined by experiment. However, the stretching frequency of the NH bond in the C=NH group of guanidine may be sufficiently different from the other NH frequencies of the guanidine, guanidinium and peptide groups to allow it to be used.[16] Moreover, there is evidence that the ND_2^+ and ND_3^+ groups have characteristic weak

(15) Negligibly small deviations from electrical balance may occur in dilute solutions, as pointed out by J. T. Edsall, H. Edelhoch, R. Lontie and P. R. Morrison, THIS JOURNAL, **72**, 4641 (1950).

(16) H. Thompson, D. Nicholson and L. Short, *Faraday Soc. Disc.*, **9**, 222 (1950).

absorption bands near 4.5 μ[17]; the possibility of using these for arginine and lysine should be investigated. In the case of cysteine, the SH band has been located near 3.9 μ, and the SD band can be expected near 5.5 μ; these, although perhaps weak, are in regions free from interfering absorption and their disappearance on ionization may be detectable.[13] For histidine, it is necessary to know the effect of ionization on the imidazole ring. Some preliminary experiments we have made indicate that 4-methylimidazole in DCl has a characteristic band at 6.22 μ (1608 cm.$^{-1}$) which is absent in alkaline solution. In the case of tyrosine, the effect of the removal of the phenolic hydrogen on the spectrum has not yet been established. Edsall[18] reports no significant differences between the Raman spectra of aqueous solutions of phenol and sodium phenolate. The removal of the OH frequency on ionization is probably impossible to detect in aqueous solutions, but changes in the fundamental frequencies of the benzene ring accompanying ionization are to be expected and we have obtained evidence of small shifts in these frequencies between acid and alkaline solutions of *p*-cresol in D_2O. Ionization of the tyrosine residues in a protein may therefore also turn out to be detectable.

Although in the two proteins we have investigated only the carboxylate ions have been determined, it would seem from the above discussion that there are possibilities of detecting and estimating other ionic groups in proteins by means of infrared analysis. Even when such groups are not detectable in the native protein they may well be observable in breakdown products, or in model compounds which simulate the behavior of the more complex protein in reactions involving these groups.

The results described here have another bearing on investigations of protein structure. Elliott and his co-workers[19] have associated the α-form of a polypeptide chain with characteristic absorption bands close to 6.03 μ (1660 cm.$^{-1}$) and 6.45 μ (1550 cm.$^{-1}$) and the β-form with bands close to 6.10 μ (1640 cm.$^{-1}$) and 6.55 μ (1525 cm.$^{-1}$). Using this empirical criterion, they have attempted to correlate denaturation in certain proteins[20] with a change from the α- to the β-form by observing the alterations in the shapes of the contours of broad, ill-defined bands in the region between 1500 and 1800 cm.$^{-1}$. The possible contribution of ionization effects has been ignored, but examination of their curves for insulin shows that the COOH absorption near 5.85 μ (1710 cm.$^{-1}$) underwent a marked change on heat denaturation so that at least part of the change observed in the region of 6.45 μ (1550 cm.$^{-1}$) must be due to a decrease in the number of COO^- ions. We do not mean to imply that changes in the state of ionization can account for all of the spectral changes observed on denaturation, but that ionization effects contribute to the absorption significantly and should be more fully investigated.

General confirmation of our conclusions (a preliminary account of which has already appeared)[21] has been given in a recent brief note by Lenormant and Blout[22] who independently carried out experiments similar to ours on bovine plasma albumin. The only difference between their results and ours involves the peptide band near 6.45 μ (1550 cm.$^{-1}$). They find that this band is quite strong in D_2O solution and that it does not diminish on acidification (in contrast to Fig. 4B). However, in alkaline solution it disappears and is *not* restored by reacidification. Since Lenormant and Blout report that the 1550 cm.$^{-1}$ band is completely eliminated in denatured plasma albumin, it is possible that this discrepancy can be explained on the assumption that our starting material was already denatured. Since it had been in storage for some time this is not improbable.[23] Alternatively, it is possible that Lenormant and Blout did not allow the deuteration of the protein to go to completion.

In conclusion it should be pointed out that although only proteins have been considered in this paper, another promising field to which this method is likely to have important applications is the structure of nucleic acids.

The foregoing investigation forms part of a program of research on protein structure supported by Contract N6 onr-23224 with the Office of Naval Research and Contract DA-44-109-QM-1037 with the Quartermaster Corps.

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