determined by Hedström at 25° for the ionic strength of 3, a value of $15(\pm 2) \times 10^2$ being obtained.[22] At this temperature and ionic strength we find k_d to be $16(\pm 5) \times 10^2$. However, we find the dimerization is strongly dependent on ionic strength, becoming much less important at lower ionic strengths.

For the determination of the first hydrolysis constant 10^{-4} M iron(III) solutions of moderately large acidities were used. Under these conditions polynuclear species can be neglected. A comparison between reported values for the first hydrolysis constant at 25° has been given in Fig. 2. The values obtained by Brosset,[12] Bray and Hershey,[13] Olson and Simonson,[6] and Hedström,[3] are in good or reasonable agreement with us. All these workers were dealing with iron(III) solutions under true equilibrium conditions, and with the exception of Hedström, who took polynuclear species into account, all used iron(III) solutions that were sufficiently dilute to avoid serious errors from polynuclear species.

(22) In terms of the constants reported by Hedström, k_d is given by $x_{22}/(x_{11})^2$.

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Infrared Spectroscopy of Human Hemoglobins. I. The Effects of Varying *p*H and *p*D on the Spectra Obtained in the Solid and Liquid Phase

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Infrared absorption spectra have been obtained on normal adult human hemoglobin in the dried and liquid phases. Sixteen peaks have been studied over the range of 3.1 μ (3220) to 9.1 μ (1100), and compared when run under acid, neutral or alkaline conditions. In the *liquid* phase dissolved in *deuterium oxide* the peaks at 3.1, 3.4, 4.7, 6.15, 6.9 and 7.65 μ showed no change; the peaks at 5.8 and 6.5 μ increased in intensity in acid *p*D; and the peaks at 6.35 and 7.15 μ increased in intensity at alkaline *p*D. In the infrared spectrum of human hemoglobin from *dried* films cast from *water* solutions the peaks at 3.1, 3.4, 6.15, 6.5, 6.9, 7.65 and 8.6 μ showed no change; the peaks at 3.3, 5.8 and 8.1 μ increased in intensity at acid *p*H; and the peaks at 7.2 and 9.1 μ increased in intensity at alkaline *p*H. In the infrared spectrum of human hemoglobin from *dried* films cast from *deuterium oxide* solution the peaks at 3.1, 6.15, 7.65 and 8.6 μ showed no change; the peaks at 3.4, 4.15, 5.8, 6.9 and 8.1 μ showed increased intensity at acid *p*D; and the peaks at 6.5, 7.2 and 9.1 μ showed increased intensity at alkaline *p*D.

In the course of the rapid development of applied infrared spectroscopy over the last decade, many substances of biologic interest have been examined. However, relatively little information has appeared in the literature on the infrared characteristics of proteins. Technically, it has been difficult to obtain satisfactory patterns on proteins in solutions except by use of deuterium oxide as the solvent.[1] The patterns obtained on dried protein films cast from watery solutions are usually a mixture of varying degrees of denatured and undenatured proteins. And, finally, the interpretation of infrared patterns of proteins is made difficult due to the large number of infrared-sensitive groupings in the molecules.

Recent observations have established that the position and intensity of absorption peaks in the infrared analysis of proteins may be influenced by the *p*H or *p*D of the material under examination. In the study of the Raman spectra of amino-acids, Edsall, Otvos and Rich have shown[2-4] that the disappearance of the carbon double bond oxygen frequency at 5.8 μ (1745 Kaysers)[5] on ionization, and the replacement by a lower frequency near 7.12 μ (1400), is characteristic of ionized carboxyl groups.

In the examination of dried deuterated protein films, Lenormant has shown[6] that the absorption band at 6.45 μ (1550) shifts to 6.9 μ (1450), but this shift is incomplete.

Ehrlich and Sutherland noted[7] that upon the addition of base, the band at 5.87 μ (1703), characteristic of un-ionized carbonyl groups, is replaced by a band at 6.4–6.45 μ (1560–1550), and by another less prominent band at 7.1 μ (1410). They observed that in the examination of isoionic bovine plasma albumin in deuterium oxide, the band present at 6.4 μ (1560) disappears upon the addition of DCl and is replaced by a shoulder on the low wave length side of the band at 6.08 μ (1655) simultaneous with a disappearance of the shoulder at 7.13 μ (1405). Upon the addition of NaOD, however, the bands at 6.45 μ (1550) and 7.1 μ (1405) were restored. These variations in the spectra were attributed to changes in ionization of the carboxy groups.

Lenormant and Blout observed[8] the presence of the band at 6.35 μ (1575) in alkaline heavy water, and that the band disappeared in acid heavy water; however, the band could be made to reappear on bringing the *p*D back to 9 or 10. Similar, but less marked, changes occurred at the 7.15 μ (1400) region. The band at 6.45 μ (1550) remained upon acidification and disappeared in alkaline solution. This change was said to be irreversibly completed. It was also noted that when deuterium solutions of albumin, gamma globulin or ovalbumin were heated to 100° for 2 minutes, the 6.45 μ (1550) band disappeared. They suggested that this behavior could

(1) R. C. Gore, R. B. Barnes and E. Peterson, *Anal. Chem.*, **21**, 382 (1949).

(2) J. T. Edsall, J. W. Otvos and A. Rich, THIS JOURNAL, **72**, 474 (1950).

(3) J. T. Edsall, *J. Chem. Phys.*, **4**, 1 (1936).

(4) J. T. Edsall, *ibid.*, **5**, 508 (1937).

(5) The term "Kayser" is identical with the term "reciprocal centimeter." See Joint Commission for Spectroscopy, C. J. Bakker, *J. Opt. Soc. Amer.*, **43**, 410 (1953). Throughout the remainder of the paper, the number in parentheses following the wave length refers to Kaysers or reciprocal centimeters.

(6) H. Lenormant, *Ann. chim.*, **383**, 459 (1950).

(7) G. Ehrlich and G. B. B. M. Sutherland, *Nature*, **172**, 671 (1953).

(8) H. Lenormant and E. R. Blout, *ibid.*, **172**, 770 (1953).

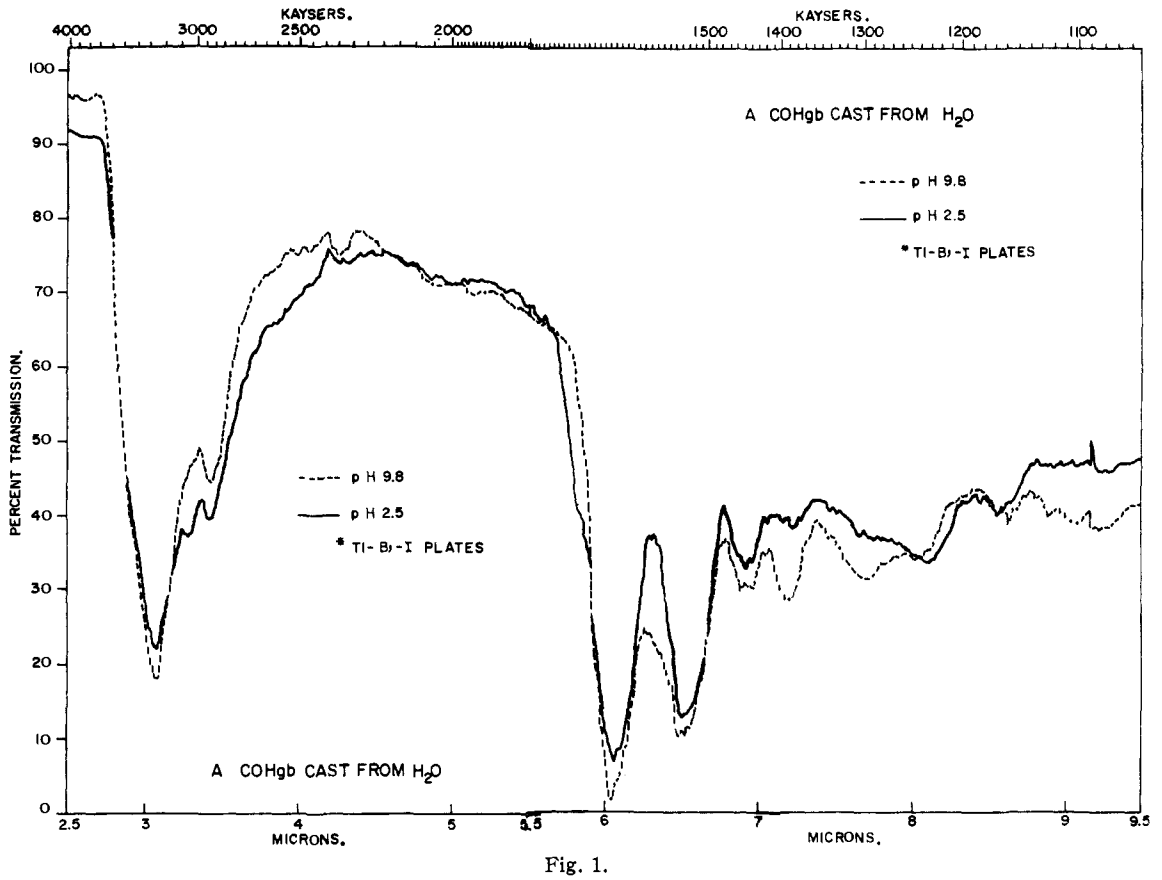

Fig. 1.

be explained on the basis of two types of peptide linkages; one is deuterated at room temperature, the other deuterated only in alkali or by heating to 100° for two minutes.

Human hemoglobin is a readily available protein that is easily prepared in a good state of purity and is relatively stable when converted to carboxyhemoglobin. (The abbreviation for normal adult human carboxyhemoglobin is A COHgb.) This protein has been studied extensively with other chemical and physical techniques, and is well characterized. The purpose of this communication is to report studies on the infrared characteristics of human hemoglobin under varying conditions of hydration, acidity and alkalinity. The procedures described easily can be applied to the examination of other proteins of biologic interest.

Methods

Hemoglobin specimens were prepared according to the method of Drabkin,[9] converted to carboxyhemoglobin, dialyzed against distilled water, dried *in vacuo* from the frozen state, and stored in sealed glass ampoules until used. Hemoglobin samples prepared in this manner retained their original solubility, visible and ultraviolet absorption spectra, and electrophoretic mobility, when redissolved. In order to ensure maximum removal of water from the dried specimens, some of the samples of hemoglobin were subjected to an additional drying period of 18 hours at 25° using an oil diffusion pump. By pumping directly on the specimen, pressures were reached as low as 3.6×10^{-6} millimeter of mercury, as measured by an ionization gage. Under these conditions, a driving force of 23.756 mm. of mercury vapor pressure is exerted by unbound water in the sample. Therefore, the retention of unbound water in the sample after this treatment is unlikely.

Dried films of hemoglobin were cast from a 10% solution of hemoglobin; dried films cast from water were dried in a stream of air or in a vacuum desiccator; dried films cast from deuterium solution were dried in a stream of super-dry nitrogen, or cast in a dry-box and sealed between silver chloride plates with a rubber gasket before removal from the dry-box. In comparing the effects of change in *p*H or in *p*D, aliquots from the same sample of hemoglobin were treated with hydrochloric acid or deuterium chloride for studies in acid solution, and sodium hydroxide or sodium deuteroxide for studies in basic solution. Spectra of hemoglobin in deuterium solutions were obtained by placing the sample between silver chloride plates, using a platinum foil spacer 0.001 inch thick. Deuterium oxide was used as the blank in the reference beam in studies with hemoglobin solutions.

Analyses were carried out on a Perkin–Elmer Model 21 infrared double beam recording spectrophotometer. Window materials used were silver chloride plates and thallium bromide–iodide plates. The plates were matched so as to give nearly 100% transmission and not more than 5% variation away from the baseline. A rock salt prism was used throughout all of the studies. The correct electrical balance of the instrument at 2 μ (5000) was usually established before each set of determinations. Most experiments were designed to scan the wave lengths from 2 μ (5000) through 10 μ (1000).

Results and Discussion

Wave length 3.1 μ (3220) is shown in Figs. 1, 2 and 3. There was a strong peak in solid films cast from water and from deuterium as well as a strong peak in the liquid phase in deuterium. This peak probably represents hydrogen stretching involving O–H and N–H; the O–H and the N–H may be associated or unassociated. In the deuterated

(9) D. L. Drabkin, *J. Biol. Chem.*, **164**, 703 (1946).

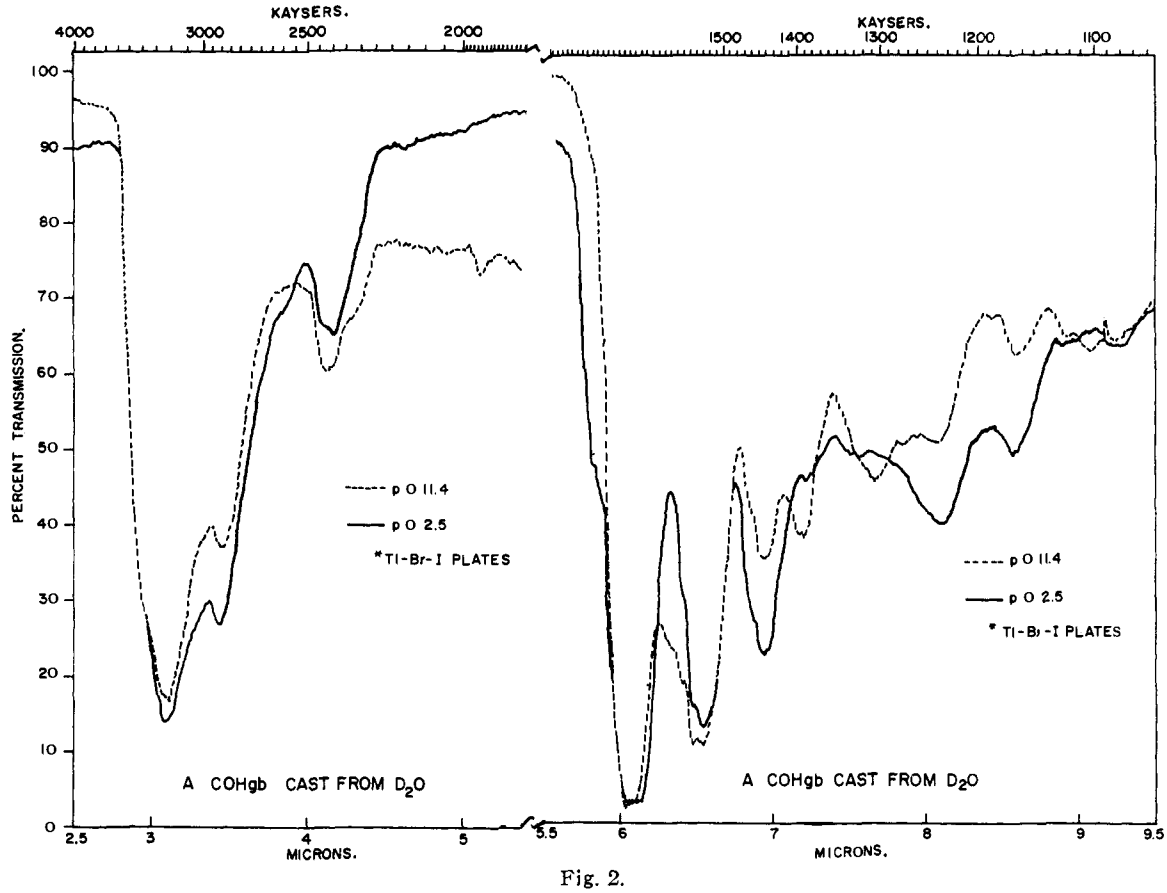

Fig. 2.

specimens, one can be fairly certain that this is not due to deuterium oxide since deuterium oxide run against air does not show heavy absorption except in the regions 3.75 (2665) to 4.74 μ (2120) and from 7.75 (2105) to 9.0 μ (1110). It is possible that this peak is partially a result of some residual water in the hemoglobin molecule; however, it must then be water that is not removed at extremely low drying pressures, *i.e.*, 3.6×10^{-6} mm. of mercury; furthermore, it must be water that does not exchange with deuterium oxide even after prolonged exposure.

Wave length 3.3 μ (3030) is shown in Fig. 1. This was present as a strong peak only in acid solution films cast from water. It was somewhat more apparent using thallium bromide–iodide windows than with silver chloride windows. It is not certain whether this peak is the vibration of N–H stretching or of N–H deformation.

Wave length 3.4 μ (2930) is seen in Figs. 1, 2 and 3. It was present as a strong peak in all solid films and in the liquid phase as well. In films cast from deuterium solution and examined with the thallium bromide–iodide plates, the peak was strongest in acid solutions and decreased noticeably in intensity with increasing alkalinity. This peak is associated with carbon–hydrogen stretching.

Wave length 4.15 μ (2410) see Fig. 2. This peak was seen only in films cast from deuterium solution. Unfortunately, this peak was somewhat more apparent using the silver chloride windows and is not well seen using the thallium bromide–iodide plates. The peak was most prominent at *p*D 1.0, in association with the peak at 4.1 μ; it faded to a shoulder by *p*D 5.0 and disappeared from all samples by *p*D 7.35. It is possible that this double peak represents O–D and N–D bond stretching. It is of interest that this peak did not appear in any spectra obtained in deuterium solutions; however, due to the heavy deuterium oxide absorption in this region the peak may have been masked. The peak at 4.07 μ (2460) remained relatively constant in solid films cast from deuterium, and was not seen in films cast from H_2O.

Wave length 4.7 μ (2120) is seen in Fig. 3. This peak was present only in deuterium solutions and not in any of the solid films. It was a very strong peak over a range of 4.6 (2170) to 4.8 μ (2080). Deuteration of a carbon atom would produce a peak in this region; inadequate compensation of the deuterium oxide background must be eliminated to properly evaluate this peak.

Wave length 5.8 μ (1730) is seen in Figs. 1, 2 and 3. This peak was apparent in all films and in the liquid phase as a shoulder on the 6.1 peak below a *p*H or *p*D of 3.0. It was not present in alkaline solution or in films cast from alkaline solution. The peak is associated with carbonyl groups.[2,7,8]

Wave length 6.0 (1665) to 6.15 μ (1625) is seen in Figs. 1 and 2. This was the strongest peak in the spectrum and stable over the entire *p*H or *p*D range in both the solid and in the liquid phase. This peak is associated with carbon–oxygen double bond stretching.

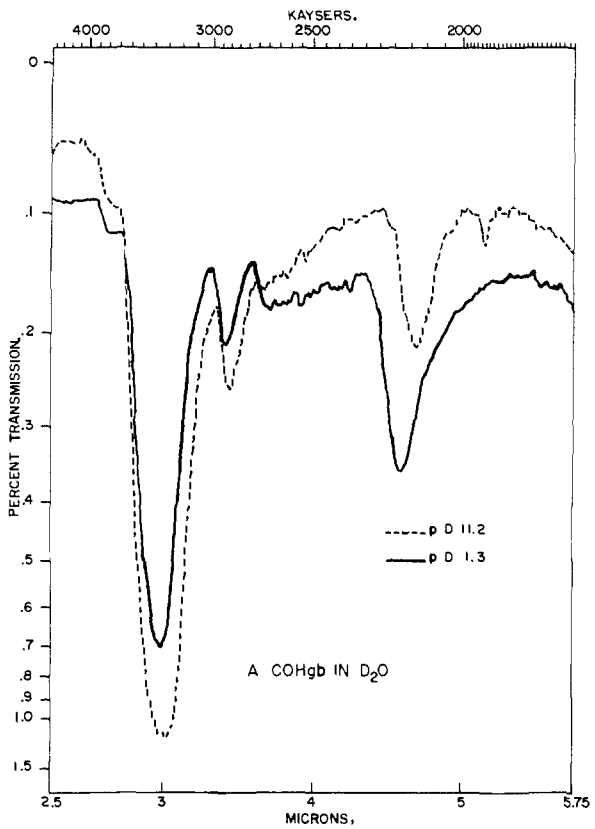

Fig. 3.

Wave length 6.35 μ (1570) was seen only in the liquid phase (see kinetic studies). It first appeared at a pD of 5.0; with increasing the pD, the peak increased in intensity up to pD 9.0; above pD 9.0, the peak widened and became U-shaped. This wave length is associated with carboxyl ions.[2,7,8]

Wave length 6.45 to 6.55 μ (1550 to 1530) is seen in Figs. 1 and 2. It was present in all films cast from water and was of relatively uniform intensity over the entire pH range. It was second only to the 6.1 peak in intensity. In films cast from deuterium solution, the peak was small at pD 1.0 and increased progressively up to a pD of 11.0. Above pD 7.35, this peak is moderately strong immediately after dissolving the hemoglobin but rapidly shifts to the peak at 6.9 μ. This phenomenon will be described in detail in another communication. Bonds under consideration are carbon–nitrogen stretching and nitrogen–hydrogen deformation.

Wave length 6.9 μ (1450) is seen in Figs. 1 and 2. It presented a band of moderate intensity and showed little or no change over the entire pH range in solid films cast from water. In contrast to this, films cast from deuterium solution showed the peak at greatest intensity at pD 1.0 and its intensity decreased progressively up to pD 11.0. In the liquid phase there was an initial rapid increase in intensity of this peak immediately after dissolving the hemoglobin in deuterium oxide solution (see kinetic studies). This peak is associated with carbon–hydrogen deformation as well as the shift of the peak at 6.5 μ following deuteration.

Wave length 7.15 μ (1400) was not seen in the solid films. In the liquid phase, it appeared as a shoulder on the 6.9 μ peak in neutral solution, and with increasing pD up to 11.0, a small peak became apparent (see kinetic studies). This is characteristic of ionized carboxyl groups.[2,7,8]

Wave length 7.25 μ (1390) is seen in Figs. 1 and 2. It was scarcely detectable at pH 2.0 in solid films cast from water, then steadily increased in intensity up to pH 11.0; at pH 11.0, it had a greater intensity than the peak at 6.9 μ. This change was more notable in films cast on silver chloride plates. In films cast from deuterium solution the peak first appeared at pD 5.0 to 7.35 and then increased slightly up to pD 11.0. The peak was not seen in the liquid phase. This wave length is probably related to carbon–hydrogen deformation.

Wave length 7.65 μ (1310) is seen in Figs. 1 and 2. It showed a peak in all films cast from water or deuterium at a neutral or alkaline pD or pH. In the liquid phase, there was a small peak which was stronger immediately after deuteration. This peak was more apparent when examined with the thallium bromide–iodide plates.

Wave length 8.1 μ (1230) is seen in Figs. 1 and 2. It showed a peak in films cast from either water or deuterium. The peak was considerably stronger in acid solution.

Wave length 8.6 μ (1160) is seen in Figs. 1 and 2. It showed a peak in all solid films, of moderate intensity and was reasonably stable over the entire pH and pD range.

Wave length 9.1 μ (1100) are seen in Figs. 1 and 2. It showed a peak in all solid films which was not present in acid pH or pD, but became apparent in films cast from alkaline solution.

Since completion of this manuscript, it has been called to our attention through a personal communication that Drs. Ehrlich and Sutherland have submitted papers to THIS JOURNAL providing a more detailed report on their work on the effects of changes in pD and pH on the infrared spectra of proteins in solution.

It is a pleasure to acknowledge the criticism and advice of Prof. R. M. Badger. We wish to thank the Dept. of Instrumentation, AMSGS, for their construction of a dry-box and silver chloride plate holder. The use of a platinum foil spacer between silver chloride plates was suggested by Dr. R. B. Gochenour. Valuable technical assistance was given by Cpl. Forrest Nutter.

WASHINGTON, D. C.