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Comparison of Protein Structure in the Crystal and in Solution. I. The Tyrosyl Ionization of Crystalline Methemoglobin*

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The tyrosyl ionization of horse methemoglobin was measured spectrophotometrically in solution and in crystal suspensions. No difference was observed for solution and crystal maintained at the same ionic strength, showing that upon crystallization there is no change in structure about the tyrosines. However, the tyrosyl pK of soluble methemoglobin increased with increasing ionic strength, suggesting there may be conformational changes in passing from physiological conditions to those under which crystallization occurs. No electrostatic interaction was observed for the crystalline protein, presumably a result of the high ionic strength of the buffer. Hydrogen-ion equilibria in the crystal were established rapidly and reversibly, and were normal, which indicated that no fundamental impediment exists for comparison of crystal and solution structures through comparisons of their chemical reactivities.

The recent determination of the three-dimensional structure of crystalline myoglobin and hemoglobin (Kendrew, 1962; Cullis *et al.*, 1961–62) has greatly deepened our general understanding of proteins. However it is the structure of a given protein in solution which is necessary for the detailed understanding of its properties, and to this end some assessment must be made of changes in conformation attending crystallization. Richards (1963) has thoroughly reviewed the relevant data, which, although largely indirect, strongly suggest that extensive changes are unlikely. Nevertheless, small rearrangements in structure cannot be excluded, particularly in view of the flexibility of a protein molecule while part of the crystal (Richards, 1963), and in view of the small free-energy changes associated with denaturation and structural fluctuations (Linderstrom-Lang and Schellman, 1959).

One approach to defining the extent of these rearrangements is through quantitative determination of the chemical reactivities of the same groups in the crystal and in solution. This paper reports a study of the ionization of the two titratable tyrosines of crystalline and soluble horse methemoglobin, a comparison which does not detect any conformational change in crystallization. Since the demonstration of structural changes by studies of this sort is analogous to the determination of protein homogeneity, the more experiments there are which quantitatively compare the special reactivities of specific groups, the more closely the limits of putative conformational changes can be set. The data below then represent only one of several necessary approaches, and lead only to conclusions concerning the environment of the tyrosines.

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Experimental

Materials.—Red blood cells were harvested from fresh[1] citrated horse blood, washed five or more times with 0.9% saline, and laked; the cell debris was removed by centrifugation. The resulting solution of oxyhemoglobin was converted to one of methemoglobin by the addition of 1.5 equivalents of potassium ferricyanide, and was then exhaustively dialyzed against cold deionized water. After dialysis the stock solution contained 50–100 mg/ml hemoglobin and was of pH 6.6–6.7, the pH of the isoionic protein (Cohn *et al.*, 1937). It was stored frozen. The extinction coefficient, used for routine concentration determination, was determined by drying at 103° to be $E^{1\%}_{500m\mu} = 5.12$. Inorganic chemicals were reagent grade, and deionized water was used throughout.

Preparation of Crystalline Methemoglobin.—In early experiments crystals were grown by dialysis against 2 M $(NH_4)_2SO_4$, conditions like those used by Perutz and co-workers in preparing material for crystallographic analysis (Cullis *et al.*, 1961–62). The resulting suspensions were exhaustively dialyzed against 2 M Na_2SO_4 to remove ammonium ions which would interfere with the titrations. No disruption of the crystal or change in habit was observed. In later experiments crystals were grown by adding methemoglobin stock solution to

[1] Obtained at the slaughterhouse of the Tucson Dog Food Co., through the courtesy of Mr. Dalrymple.

2 M Na_2SO_4, so the final concentration was 1.8 M. Under the microscope the resulting crystals were like those grown from ammonium sulfate, and they were used in the experiments presented below.

Preliminary studies showed that better spectra could be obtained from suspensions of small rather than large crystals, because suspensions of large crystals, in which all light incident upon a single crystal was absorbed, appeared of constant optical density. Fragmentation was accomplished by five cycles of freezing (in dry ice–acetone) and thawing. The fragmented crystals were harvested by centrifugation, washed with 1.6 M Na_2SO_4 five or more times, until the supernatant remained clear, and then suspended in 2 M Na_2SO_4. The fragments, 1–10 μ diameter, appeared crystalline under the microscope, and when dissolved by dilution in water the crystals gave a clear solution of methemoglobin, indicating that negligible denaturation had occurred.

Buffers.—A stock buffer solution, containing 2.0 M Na_2SO_4, 0.5 M NaH_2PO_4, and 0.2 M $NaHCO_3$, of *p*H 8.3, was adjusted to appropriate *p*H with 10 M NaOH. More dilute buffers were prepared from this set of solutions, excepting buffers of the lowest ionic strength used, which were prepared from a stock solution of 0.15 M NaH_2PO_4, 0.15 M $NaHCO_3$. The various buffer concentrations are distinguished below by their ionic strength, calculated for all components present as the sodium salts. The *p*H was determined at 30.0°, using a Radiometer TTT1 meter with a GK-2021-B, low-sodium-error electrode. The calibration buffers were among those recommended and described by Bates (1954); *p*H 4.01 phthalate, *p*H 6.85 phosphate, and *p*H 11.69 saturated calcium hydroxide. The *p*H of the 6.85 standard was read before and after about each four buffer *p*H determinations. The meter required correction above *p*H 12.1 (e.g., 0.20 *p*H unit at *p*H 12.6), determined using solutions of 0.1–1.0 M NaOH, whose *p*H was calculated according to Bates (1954), using the sodium hydroxide–activity coefficients measured by Akerlof and Kegeles (1940). Corrections for sodium-ion error were determined using the nomogram supplied with the electrode, adjusted to accord with *p*H values measured for 0.1 M NaOH[2] at sodium-ion concentrations to 4 M.[2] At high *p*H and sodium-ion concentration long times (30–45 minutes) were required for attainment of a constant *p*H reading.

Absorbance Measurements.—Absorbance measurements were made with a Zeiss PMQII spectrophotometer, using 1-cm cells, at 30.0°. Wide slits of 1–1.5 mm were necessary, leading to a half-band width of 3–5 mμ. In a typical experiment, 2 ml of buffer of appropriate *p*H was pipetted into a cell, and 50 μl of crystal suspension was added using a polyethylene cup attached to a small rod, which in several up and down passes served to mix. The absorbance was read against a similarly prepared reference of *p*H 8.5–9.0. These mixtures were crystal suspensions or solutions depending upon the buffer concentration, the hemoglobin crystals dissolving instantaneously at concentrations below ionic strength 7.[3] In buffers of ionic strength 7.2, at *p*H above 10.5, the crystals dissolved and denatured rapidly leading to time-dependent changes in optical density. Initial values could be determined from measurements begun within 5–10 seconds after mixing, using a Varicord linear-log recorder coupled to the Zeiss. In these experiments an acetone-water solution was used as a stable reference, and all solutions were read against it, including the one which served as the low *p*H reference.

[2] For example, the correction for 4 M sodium ion was 0.25 units at *p*H 12.0.

[3] The preparation of hemoglobin solutions from the same stock crystals used to prepare the crystal suspensions minimizes the effect of small amounts of impurities which may be present in the protein preparation.

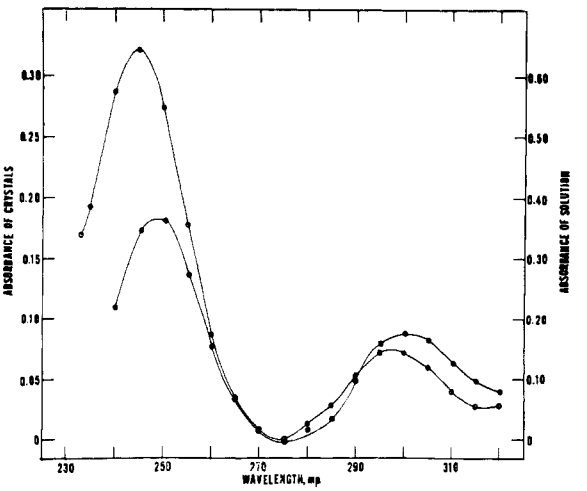

FIG. 1.—Ultraviolet-difference spectrum for a solution (open circles) and crystal suspension (closed circles) of methemoglobin. High versus low *p*H reference.

Absorbance readings were made at 248 and 276.5 mμ for crystal suspensions, and at 245 and 276.5 mμ for hemoglobin solutions. These wavelengths correspond to the greatest difference and the adjacent isosbestic point for the spectrum of the ionized versus the un-ionized phenolic group (see Fig. 1). The difference between these readings was used as a measure of the tyrosyl ionization, to eliminate errors owing to differences in concentration between reference and sample crystal suspensions. A given error in concentration, which would be unimportant for solution measurements, produced a significant error for the crystal suspensions owing to the higher ratio of total to difference-spectrum absorbance, a result of the turbidity. Hermans (1962) first suggested the use of the 245-mμ difference-spectrum peak to follow tyrosine ionization in heme proteins, and in accord with his work interference from changes in heme absorption with *p*H was found negligible in this wavelength region.

RESULTS

The High pH Difference Spectrum of Methemoglobin in the Crystal and in Solution.—The ultraviolet-difference spectrum of hemoglobin at high *p*H against a low *p*H reference is shown in Figure 1 for a crystal suspension and a solution of the same concentration. The difference spectrum for the solution is characteristic of the tyrosyl ionization and in agreement with that of Hermans (1962). The wavelengths of the maxima and minima for the difference spectrum of the crystal suspension were nearly identical to the solution values, but the intensity ratio for the 245- to the 300-mμ peak differed significantly from that for the solution (2.03 for the crystals, 4.47 for the solution). This difference probably arises from scattering behavior of the type considered by Latimer (1963) in connection with spectrophotometric measurements of algal and red-cell suspensions. The scattering by a particle increases in approximate proportion to the intensity of the absorption at a given wavelength, and causes in the observed spectrum an apparent diminution of the intense absorption bands relative to the less intense. Latimer also noted that the scattering maximum fell slightly to the blue of the absorption maximum, which could account for the slight red shift (245 to 248 mμ) found for the crystal suspension.

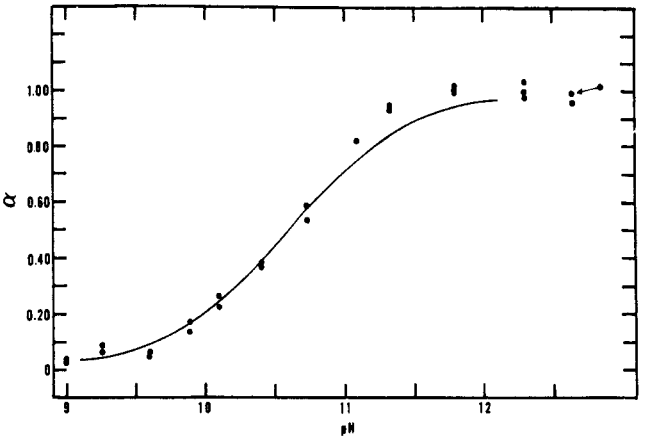

FIG. 2.—Spectrophotometric titration data for crystalline methemoglobin at ionic strength 9.6. α = 1.00. This represents the titration of two tyrosines in the soluble protein, and is assumed to represent two for the crystals. The curve is calculated for two independent ionizations, of pK 10.62.

Spectrophotometric Titration Curves.—Figures 2 and 4 show spectrophotometric titration data obtained for crystalline methemoglobin at ionic strength 9.6, and for solutions at ionic strengths 4.8 and 1.9. The data were obtained from measurements of the 245- 250-mμ band,[4] and are presented as the fractional change in spectrum. Since the difference spectrum of the crystals cannot be interpreted in terms of an extinction coefficient, owing to the importance of light scattering, the changes were assumed to represent ionization of the same two tyrosines which titrate in the soluble protein (Hermans, 1962). This is reasonable in view of, first, the internal consistency of the results, and second, the improbability of the large conformational changes required to uncover or bury a tyrosine. The change in molar extinction per tyrosine for soluble methemoglobin was 9700, an average of five experiments and in agreement with the values found by Hermans (1962) for tyrosine and several hemoglobins and myoglobins.

The interpretation of titration data for proteins and other polyampholytes requires consideration of the electrostatic interactions between the charge units. Classically this is done by introducing a free-energy term dependent upon the macromolecular charge and shape (Tanford, 1962):

$$pH - \log \frac{\alpha}{1-\alpha} = pK - A\bar{Z} \qquad (1)$$

where α is the fraction ionized, $\bar{Z}$ is the net charge on the macromolecule, and A is a constant. A treatment of this sort, using as a model an impermeable sphere with smeared surface charge, has generally been adequate for titration studies of rigid compact proteins; the constant A in this case is $0.869w$, where w is the familiar electrostatic interaction factor. Protein crystals present a different situation, in that they are open and permeable to solvent, with charges distributed throughout the interior. Detailed calculations of the electrostatic free energy have been made for structures similar to protein crystals, i.e., ion-exchange resins (Rice and Nagasawa, 1961) and polyelectrolyte gels (Katchalski, 1954). The electrostatic-interaction correction for the titration behavior of these systems is of the form $A\bar{Z}$, as in equation (1), although the constant A is of course no longer $0.869w$. These developments are restricted to low ionic strength (below 0.01), and unfortunately no theory which covers high ionic strengths has gained general acceptance. Several considerations, however, support the use of equation (1) for the evaluation of electrostatic-interaction effects in protein crystals and solutions at high salt concentrations. (1) Several attempts to extend the Debye-Huckel theory to high ionic strength have led to a free energy which varies as the square of the ionic charge, and therefore an electrostatic interaction term $A\bar{Z}$ (Falkenhagen and Kelbg, 1959). (2) If the titration behavior at high ionic strength is independent of the charge (as will be shown), then as long as no further interpretation is sought, the exact form of the assumed dependence on Z is unimportant.

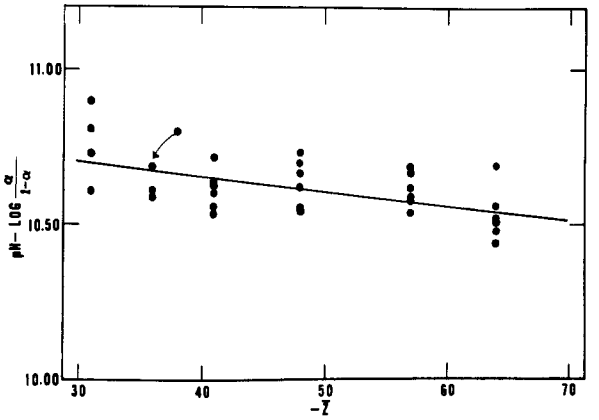

FIG. 3.—Determination of the electrostatic-interaction parameter for the tyrosyl ionization of crystalline methemoglobin. The line was fitted by least-squares analysis of the data.

Figure 3 is a plot of the left-hand side of equation (1) against $-\bar{Z}$ for the titration of crystalline methemoglobin. Values of $\bar{Z}$ were calculated from the crystal titration data of M. Christie and J. A. Rupley (unpublished experiments). The value of -0.0047 for A was obtained by a least-squares treatment of the data of Figure 3; similar small negative values[5] were obtained for hemoglobin solutions at lower buffer concentrations. The absence of appreciable dependence of the titration behavior on the macromolecular charge and on ionic strength shows that for both protein crystals and solutions, at the buffer concentrations studied, electrostatic interaction can be neglected. The lack of interaction is especially interesting for a crystal composed of highly charged molecules ($\bar{Z}$ approximately -70) in close contact with one another, and electrostatic shielding at high salt concentrations must be complete. The Donnan distribution can also affect the hydrogen-ion equilibria of protein crystals, but it is quantitatively of less importance than the electrostatic interaction and may be likewise neglected at high ionic strengths (Katchalski, 1954). In summary, at the high salt concentrations studied, incorporation into the crystal does not *by itself* affect the general ionization behavior of the protein (although it may change the titration of certain specific groups through introducing new intermolecular interactions or altering the conformation). This point is crucial, because if crystallization had an intrinsic effect upon the ionization, it could not be used

[4] The 300-mμ peak gave solution and crystal titration data in agreement with those obtained at the shorter wavelength, but less precise owing to the significantly smaller change. Also, analysis of the supernatant from crystal suspensions in pH 11.33 and 12.65 buffers showed less than 5% of the difference spectrum could be attributed to dissolved hemoglobin.

[5] Compared with a value of A approximately 0.1 for proteins like hemoglobin at low ionic strength (Tanford, 1962).

for comparing structure in the crystal and in solution. These conclusions are not surprising, in view of studies on related systems. Identical ionization at high ionic strengths of the same group in solution and attached to a porous solid has been found for polyelectrolyte gels (Katchalski, 1954); the titration of ion-exchange resins (Kunin, 1958) and protein fibers (Steinhardt and Zaiser, 1955) approaches a limiting behavior as the ionic strength is increased.

In heterogeneous reactions the activity of the solid phase is constant and, by convention, unity, which leads to a question concerning the validity of applying the foregoing thermodynamic treatment to reactions of the crystal. Resolution of this problem follows from observations of Perutz (1946), that the unit cell of a methemoglobin crystal remains constant within certain ranges of *p*H. Since over the same *p*H regions the crystalline protein takes up stoichiometric quantities of hydrogen ion, similar to those taken up by the soluble protein (R. Christie and J. A. Rupley, unpublished data; see Fig. 6), there can be no experimentally significant phase change upon titration of the crystalline protein. This conclusion is supported by failure of the crystals when titrated to rupture, whereas oxy- or methemoglobin crystals are destroyed by conversion to reduced hemoglobin, which exhibits a different crystal structure. The protein crystal is then like inorganic ion-exchange crystals, presumably because of the open, porous nature of the solid. The validity of a standard thermodynamic treatment of the ionization under these conditions may be supported on more theoretical grounds. The continuity of the titration curve of crystal suspensions suggests that a single crystal contains at any instant protein molecules with a number of different distributions of charge, all of which must be isomorphous as a consequence of the overall regular structure. The activity of any one ionization state will then not be constant with *p*H or unity, and the titration curve for the crystal suspension will define the relationship between average ionization states as a function of *p*H, as is true for protein solutions.

Comparison of the shapes of the titration curves of Figure 4 is aided by normalization on their *pK*, through presentation of the fractional change in ionization as a function of the difference, *p*H − *pK*. The *pK* calculated for a given titration was the value best fitting the accurate data in the midrange of the titration, and in all cases was close to the *p*H of 50% ionization. The titration curves for crystals and solution at different ionic strengths have an identical shape, and therefore differ only in *pK*. The curve in Figure 4 is theoretical, and was calculated for ionization without electrostatic interaction, which, following the preceding discussion, can be neglected in these studies. In fact, the small deviation of the experimental curves from the theoretical, at *p*H above the *pK*, is opposite to that explainable by electrostatic interaction and must result from some intrinsic property of the ionizing groups, perhaps a cooperative effect in which ionization of the second tyrosine is rendered more likely by ionization of the first.

Reversibility of the titrations was studied by maintaining crystals or solution for 5–10 seconds at high *p*H, sufficient time for the spectral change to be established, and then adding a second buffer to lower the *p*H. Reversibility obtained under all conditions tested: soluble hemoglobin at ionic strength 4.8, reversed from *p*H 10.90 to 9.36, exhibited $\alpha = 0.113$, compared to 0.107 for a control at *p*H 9.36, not exposed to high *p*H; soluble hemoglobin at ionic strength 1.9, reversed from *p*H 10.89 to 9.52, exhibited $\alpha = 0.149$, compared to 0.121 for the control; crystals at ionic strength 7.2, reversed

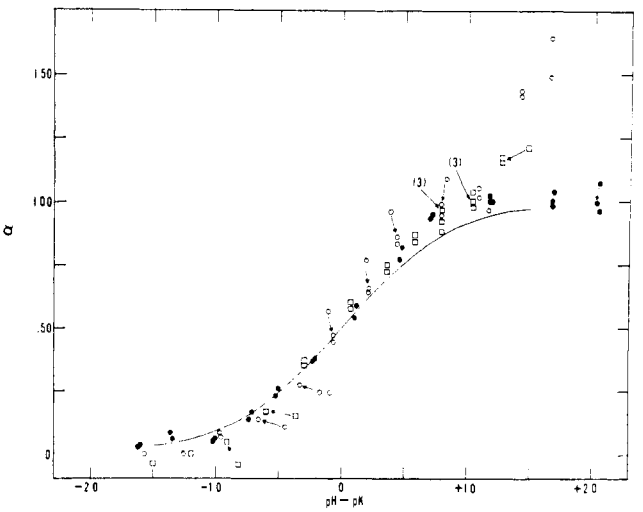

FIG. 4.—Spectrophotometric titration of crystalline methemoglobin at ionic strength 9.6 (closed circles), and soluble methemoglobin at ionic strengths 4.8 (open circles) and 1.9 (open squares). The data are normalized to their respective *pK*. The curve is theoretical for two independent ionizations.

from *p*H 11.09 to 9.59, exhibited $\alpha = 0.026$, compared to −0.026 for the control.

The absorbance of the methemoglobin solutions increased with time above *p*H 10.5, a result of denaturation with concurrent spectral changes, which follow changes in the heme environment and ionization of the one abnormal tyrosine. Under conditions where this occurred, the absorbance values of the native protein were obtained by extrapolation of the spectral data to the time of mixing. In contrast, the absorbance of crystal suspensions, which was established instantaneously, remained constant at all *p*H for considerable periods of time (e.g., at least 10 minutes at *p*H 12.30). Incorporation of hemoglobin into the crystal must therefore result in appreciable stabilization, and similar stabilization at low as well as high *p*H has been noted in other titration studies (R. Christie and J. A. Rupley, unpublished experiments). This reduction in denaturation rate upon crystallization is not unusual, and has long been accepted as an aid in preserving proteins. Also, reactivity differences of this sort do not permit conclusions concerning differences in structure between crystal and solution, since the denaturation rate for the crystalline protein is almost certainly limited by the rate of solubilization. The protein, while part of the crystal, is likely to be sterically restrained from the large conformational changes characteristic of denaturation.

It should be noted that instantaneous attainment of the equilibrium absorbance by the crystal means that both the hydrogen-ion equilibria of the protein in the crystal and the proton transfer through the crystal are rapid. Moreover, since for these measurements the crystals were transferred from 2 M Na_2SO_4 to a buffer of different ionic strength, either the change in solvent composition has little effect on the tyrosyl ionization and spectrum, or equilibration of solvent inside the crystal with the surrounding solvent is immeasurably fast. The half-time for equilibration of salt ions can be crudely estimated at 3 minutes for a crystal of 1–10 μ diameter, about 0.1 the thickness of dialysis membranes which exhibit a half-time of approximately 30 minutes for the passage of salt (Craig, 1960). Alternatively, drawing on the similarity of protein crystals to ion-exchange resins, a half-time for equilibration of less

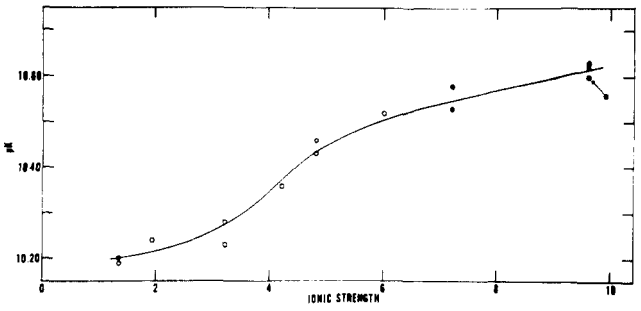

FIG. 5.—pK of the tyrosyl ionization as a function of ionic strength. Closed circles, crystals; open circles, solution.

than 30 seconds may be estimated from the data of Boyd and Soldano (1953) on the self-diffusion of various ions in resins. Hydrogen-ion equilibria would be established more rapidly than equilibria involving salt ions, since proton transfer may occur through a Grotthuss-type mechanism, and since the self-diffusion of water in resins is an order of magnitude greater than that for salts (Boyd and Soldano, 1953). It is not surprising, then, that the equilibrium absorbance of the crystals is established rapidly, in particular since there is only a small effect of ionic strength (see Fig. 5) upon the tyrosyl ionization in the crystal.

The Effect of Transfer into the Crystal and of Ionic Strength upon the Tyrosyl Ionization.—Figure 5 presents the pK of the tyrosyl ionization of methemoglobin as a function of ionic strength. There is no discontinuity in passing from the solution to the crystalline environment, proving that any conformational change accompanying crystallization is not reflected in the tyrosyl ionization. However, there is a continuous increase in pK in passing from dilute to concentrated buffers. This change cannot be ascribed to general electrostatic interactions, which have been shown here to be unimportant. The change in pK must then result either from a salt-induced change in conformation or charge distribution, or from some property of the concentrated salt environment, other than just the high ionic strength. In this connection, first, at high buffer concentrations (approximately 30% by weight salt) the water activity is appreciably reduced. Second, high salt concentrations can produce conformational changes in proteins (Kauzmann, 1959). Since the change in pK with ionic strength continues in the crystal, if it is the result of some conformational change, it must be small or localized. Finally, specific ion binding at these buffer concentrations may influence the titration.

DISCUSSION

The identity of the tyrosine ionization of methemoglobin in the crystal and in solution shows that the environment and interactions of the tyrosines do not change in crystallization. From this it may be inferred that a conformational change does not occur as measured by the tyrosine ionization. The qualification must be stressed, since the chemical reactions of a protein may reflect only a particular element of the structure or the environment of a few groups. Moreover, the two normal tyrosines cannot participate in the crystal in any intermolecular bonds or contacts of measurable strength; this is in agreement with crystallographic data on myoglobin (Kendrew, 1962) and will probably be so for hemoglobin in view of the close structural similarity of these two proteins.

The foregoing statements hold strictly for comparison of the crystal and solution at the same buffer concentration and at pH 9–11, the range of tyrosyl ionization. It is necessary to distinguish the comparison of the crystal structure with the structure in solution under identical conditions from comparison of the crystal structure with that in solution under physiological conditions. Also, the crystallographic data on hemoglobin have been obtained on crystals at pH near 7, in ammonium sulfate–ammonium phosphate mixtures (Cullis *et al.*, 1961–62). Perutz (1946) has considered the crystal parameters of methemeglobin as a function of pH and salt concentration. Changes in the unit cell were observed, similar to changes which have been ascribed to repositioning of molecules of constant conformation (see the discussion in Richards, 1963). Therefore it is likely that the tyrosine environment is the same for crystals under the conditions of this study and under those of the crystallographic work. A consideration of the effect of ionic strength upon the conformation of soluble hemoglobin would make this more certain, as well as permit a conclusion about the conformation at low ionic strength in solution compared to that at high ionic strength in solution and in the crystal.

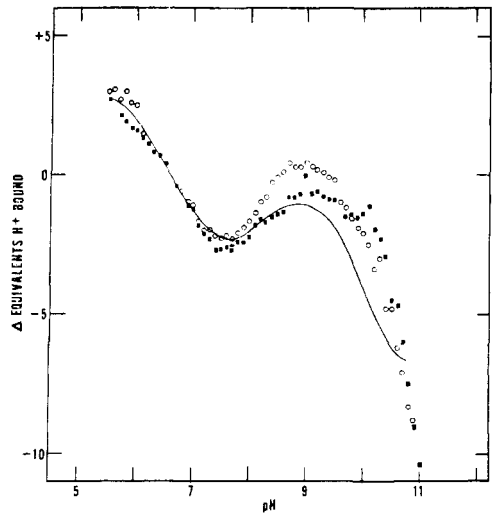

FIG. 6.—Difference titration for crystalline methemoglobin in 2.0 M Na_2SO_4 and soluble methemoglobin in 0.4 (open circles) or 0.7 M Na_2SO_4 (closed squares). The ordinate is the equivalent of H^+ bound per mole methemoglobin in solution less the number bound in the crystal. The curve is theoretical, calculated for changes in pK of 24 groups: 6 of pK 6.0 in solution to pK-5.0 in the crystal, 6 of pK 7.2–8.0, and 12 of pK 10.2–11.3.

Two previous studies of hydrogen-ion equilibria of crystalline proteins have been made. R. Christie and J. A. Rupley (unpublished experiments) have compared the titration behavior of crystalline methemoglobin in 2 M Na_2SO_4, and soluble methemoglobin in 0.4 and 0.7 M Na_2SO_4. The soluble proteins exhibited identical titration curves from pH 4.5 to 11, but differed from the crystals at pH below 9.0 and above 10.5. Figure 6 shows the differences observed. Tanford and Epstein (1954) have titrated zinc-insulin through the region of crystallinity. Differences between the crystalline, amorphous, and soluble proteins were found. Neither of these experiments is clearly interpretable in terms of structural changes or new interactions of the groups titrated. The hemoglobin titrations were performed at ionic strengths sufficiently different to show tyrosyl pK shifts which were unrelated to conformational changes upon crystallization, and other groups may behave similarly; the insulin was titrated at low ionic strength, where a large electrostatic interaction for the crystal may be a dominant factor. Differences in ionization

of certain groups between soluble and crystalline proteins are expected, since a small number of these groups are likely to participate in intermolecular interactions responsible for molecular order in the crystal. Five to ten ionizable groups are involved in interactions of this sort in myoglobin, a sufficient number to explain the titration differences found for the larger hemoglobin molecule. Any structure comparison between crystal and solution which is based upon their reactivities must consider new intermolecular interactions as a source of differences, and only a difference unaccountable in these terms can be used to infer a structural change.

The titration data just quoted and those obtained in this work show that ionizing groups in the crystalline protein are accessible and behave normally. They participate with hydrogen ions in rapidly established equilibria, characterized by constants the same or nearly the same as for the soluble protein. Consequently, it is reasonable to expect that reactions such as those routinely used for protein study will yield data interpretable in terms of a comparison between the crystal and solution. Several such experiments have recently been reported. Banaszak *et al.* (1963) studied the reaction of bromoacetate with the histidines of myoglobin, and found that the same groups reacted in the crystal and solution. Doscher and Richards (1963) have shown that ribonuclease is enzymically active in the crystal, and that only small changes in X-ray diffraction pattern are caused by substrate or inhibitor binding to the crystalline protein. Praissman and Rupley (1964) have studied the tritium-hydrogen-exchange behavior of insulin in solution and in the crystal; differences were observed which require explanation in terms of a structural change. More experiments of this sort are needed, and one hopes they will be forthcoming.

ACKNOWLEDGMENT

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Conformational Aspects of Polypeptide Structure XIII. A Nonionic Helical Polypeptide in Aqueous Solution*

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Poly-δ-hydroxy-*O*-acetyl-L-α-aminovaleric acid was prepared from L-glutamic acid and was shown to be in a fully helical conformation in trifluoroethanol and dimethylformamide. In order to avoid the problem of racemization during deacetylation of poly-δ-hydroxy-*O*-acetyl-L-α-aminovaleric acid, we utilized a method involving amidolysis with 3-aminopropanol. Poly-δ-hydroxy-L-α-aminovaleric acid was isolated and found to be soluble in 0.75–9.8 M aqueous lithium bromide and in hexafluoroacetone trihydrate. The conformational studies are based primarily on optical rotatory dispersion measurements, and reveal a transition from the random coil at lithium bromide concentrations greater than 2.75 M to the helical conformation at about 2.0 M lithium bromide concentration. In 0.75 M lithium bromide and in hexafluoroacetone trihydrate poly-δ-hydroxy-L-α-aminovaleric acid is essentially completely helical. The polypeptide precipitates from solution at concentrations of lithium bromide below 0.75 M.

Since water forms the natural environment of proteins, poly-α-amino acids can serve most effectively as model structures of protein conformations if they are studied in aqueous solution. Fasman and Blout (1960) attempted to prepare a water-soluble, nonionic, high-molecular-weight, synthetic polypeptide, composed of one optical isomer. Their studies were carried out on poly-L-serine since hydroxyl side groups usually facilitate water solubility. However the poly-L-

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