# Horse Heart Cytochrome *c*. Spectrophotometric Titration and Viscosity Changes in Alkaline Solution*

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Horse heart cytochrome *c* contains four tyrosyl residues. Their ionizations were measured spectrophotometrically at 242 mμ, and were found to be reversible and to occur over a wide range of *p*H, from 9 to 14. The data were consistent with the dissociation of four groups of *pK* 10.05, 11.00, 12.35, and 13.10, respectively, suggesting that only one of the ionizations is normal. Appreciable changes in viscosity were observed only above *p*H 13, indicating that ionization of two of the abnormal tyrosines was not associated with gross alterations in structure. This behavior of the abnormal tyrosines, as well as the high stability to alkaline *p*H, is unusual in proteins.

Cytochrome *c* is one of the few proteins whose amino acid sequence is known (Margoliash *et al.*, 1961), and it has consequently become of more than usual interest in studies relating protein structure to side-chain group reactivity. Reported below are spectrophotometric titration and viscosity data which show abnormal reactivities for three of the four tyrosines of horse heart cytochrome *c*, and a remarkable stability of this protein at alkaline *p*H.

Previous titration studies with cytochrome *c* (Theorell and Akesson, 1941; Paleus, 1954) have demonstrated the presence of one or two heme-linked groups, but data were not obtained at sufficiently alkaline *p*H to describe the tyrosyl residues. Spectral changes reflecting alterations in the heme environment occur as a function of *p*H (Theorell and Akesson, 1941; Boeri *et al.*, 1953) and might be expected to preclude spectrophotometric study of the tyrosyl ionization. However, Hermans (1962) has shown recently that the large change in absorbance at 245 mμ can be used to follow this ionization in hemoglobin and myoglobin, and that differences in the heme spectrum in fact do not interfere. This approach was used to obtain the data described.

## Experimental

*Materials.*—Boehringer horse heart cytochrome *c* was purified on Biorex-70 columns according to the block-elution procedure of Margoliash (1957). The purified material was obtained in 55% yield. Other reagents were analytical grade; deionized water was used throughout.

*Buffers.*—A stock buffer solution of 0.15 M $NaH_2PO_4$, 0.15 M $NaHCO_3$ was adjusted to appropriate *p*H values with 10 M NaOH. The *p*H of these solutions was determined at 30.0°, using a Radiometer TTT1 *p*H 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 sodium-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 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 (e.g., 0.25 unit for 4 M sodium ion at *p*H 12.0) were determined using the nomogram supplied with the electrode, adjusted to accord with *p*H values measured for 0.1 M NaOH at sodium-ion concentrations to 4 M. At high *p*H and sodium-ion concentration long periods (30–45 minutes) were required for attainment of a constant *p*H. For several experiments at high *p*H, 0.17, 0.5, and 1.0 M NaOH were used as buffers, and their *p*H values were calculated as before.

*Absorbance Measurements.*—Absorbance measurements were made with a Zeiss PMQII spectrophotometer, using 1-cm cells, at 30.0°. Wide slits of about 1 mm were necessary, leading to a half-band width of 3 mμ. In a typical experiment, 2 ml of buffer of appropriate *p*H was pipetted into a cell and 25 μl of 55 mg/ml cytochrome *c* stock solution was added, using a polyethylene cup attached to a small rod, which in several vertical passes served to mix. The absorbance was read against an appropriately dense acetone-water solution, used as a stable reference. The absorbance of a *p*H 8.80 protein solution, the low *p*H reference, was subtracted from the absorbance of solutions of higher *p*H to obtain the difference curve. Absorbance was recorded on a Varicord linear-log recorder coupled to the Zeiss galvanometer, permitting readings within 5–10 seconds after mixing.

Absorbance readings were made at 242 and 275 mμ (wavelengths, respectively, of the greatest difference and the adjacent isosbestic point for the spectrum of the ionized versus the un-ionized tyrosyl groups of cytochrome *c*; see Fig. 1). The difference between these readings was used as a measure of tyrosyl ionization, to eliminate errors owing to differences in concentration between reference and sample. No systematic drift with *p*H was observed in the 275-mμ absorbance, indicating the only significant contribution to the difference spectrum was the change in tyrosyl absorption.

*Viscosity.*—A standard Cannon-Fenske capillary viscometer with 59-second water-outflow time was used in a bath thermostated at 25.0 ± 0.005°. One % solutions of unchromatographed Boehringer cytochrome *c* in appropriate buffers were centrifuged to remove small amounts of aggregated material, 5-ml aliquots were transferred to the viscometer, and ten or more flow times were averaged for comparison with that of the solvent. Data are reported in terms of the specific viscosity, in units $(g/100\ ml)^{-1}$, where *c* is the protein

$$\eta_{sp} = \left(\frac{t_{solution}}{t_{solvent}} - 1\right) \cdot \frac{1}{c}$$

concentration in g/100 ml, and $t_{solution}$ and $t_{solvent}$ are the outflow times of solution and solvent, respectively.

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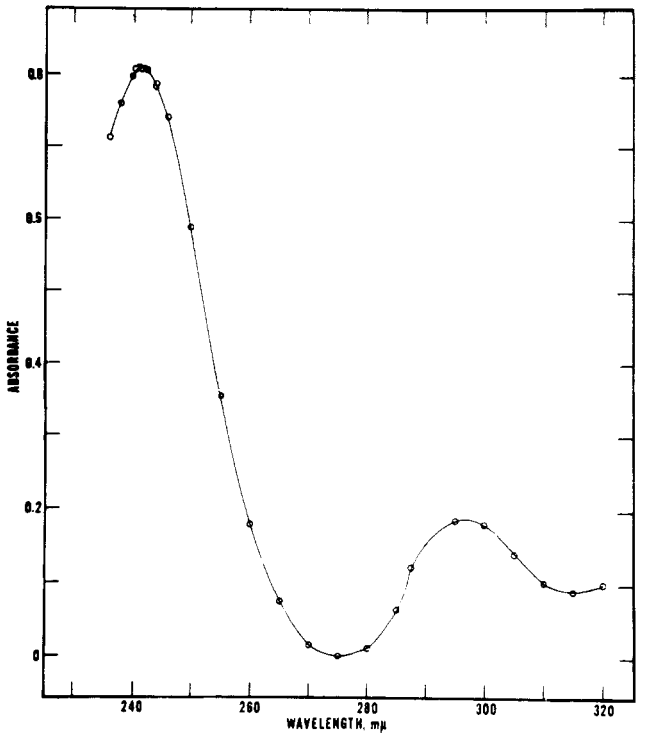


FIG. 1.—Ultraviolet difference spectrum of cytochrome *c*. See text for details.

## RESULTS

*The High-pH Difference Spectrum of Cytochrome c.*—The ultraviolet difference spectrum of cytochrome *c* at *p*H 11.5 against a *p*H 8.9 reference is shown in Figure 1, and is characteristic of the tyrosyl ionization (Hermans, 1962; Wetlaufer, 1962). The maximum at 242 mμ is shifted to the blue of the corresponding 245-mμ maximum in hemoglobin (Hermans, 1962; Rupley, 1964). The intensity ratio of the 242- to the 295-mμ peak is 4.32, similar to the values found for tyrosine (4.25) and methemoglobin (4.47) (Hermans, 1962; Rupley, 1964).

*Spectrophotometric Titration Curve.*—Figure 2 shows the change in ionization of the four tyrosine residues of horse heart cytochrome *c* as a function of *p*H. The number of tyrosines ionized was calculated from the per cent of the total spectral change at 242 mμ. The change in molar extinction coefficient per tyrosine, calculated as one-fourth the total change, was 9700, in agreement with reported values for tyrosine, myoglobin, and hemoglobin (Hermans, 1962; Rupley, 1964).

Electrostatic interactions may be neglected for interpretation of the data. The high buffer concentration, corresponding to ionic strength 1.4 at high *p*H, effectively swamps any interaction, shown in a titration of methemoglobin using these same buffers (Rupley, 1964). Also, cytochrome *c* has an isoelectric *p*H of 10.7 (Theorell and Akesson, 1941), and electrostatic interactions would be minimal in this region. The data were therefore analyzed by assuming the four tyrosyl groups dissociated independently and by selecting four $pK$ values to give the best fit of the data. These were $pK_1 = 10.05$, $pK_2 = 11.00$, $pK_3 = 12.35$, and $pK_4 = 13.10$.[1] The last ionization constant is of doubtful significance, since it characterizes a *p*H region where conformational changes occur (*vide infra*) and where large *p*H corrections are required. The curve in Figure 2 was calculated using the foregoing constants.

Reversibility was tested by exposing the protein to

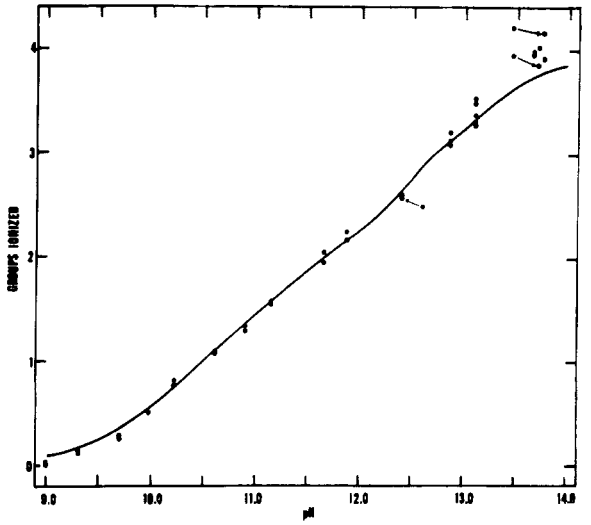


FIG. 2.—Spectrophotometric titration of cytochrome *c*, using the change in absorbance at 242 mμ.

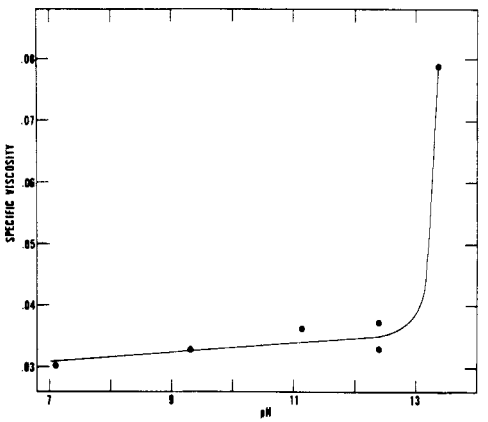


FIG. 3.—Specific viscosity of cytochrome *c* as a function of *p*H.

*p*H 13.1 for approximately 5 seconds, then reducing the *p*H to 11.5. The reversed sample differed from a control not exposed to high *p*H by only 0.05 tyrosine residue ionized (1.2% of the total change, close to the experimental error). At any *p*H, equilibrium was reached within the 5–10 seconds elapsing before measurements were begun, and the absorbance did not change over the approximately 5 minutes of measurement.

*Viscosity.*—In Figure 3 the specific viscosity of 1% solutions of cytochrome *c* is shown as a function of *p*H. At *p*H to 13.1 the low and relatively constant specific viscosity is typical of a compact globular protein. The marked rise in viscosity in alkaline solution suggests a conformational change occurs between *p*H 13 and 13.5.

## DISCUSSION

The wide spread of the four tyrosyl ionizations of cytochrome *c* is unusual, with $pK$ values ranging from

[1] A variation of more than 0.1 *p*H in the assigned $pK$ values results in the calculated titration curve's differing significantly from the experimental data. If electrostatic interaction is allowed (in spite of the above argument), the data can be fitted by assuming two tyrosines ionizing with a normal $pK$ of 10.60, two tyrosines of $pK$ 12.15 and 12.90, respectively, and an electrostatic interaction factor, $w$ (Tanford, 1962), of 0.02. It is not possible to fit the ionization of either the first three or all four tyrosines with one $pK$ and a constant value of $w$.

10 to 13, and with only one residue having a normal value of about 10. The abnormality of these ionizations is certainly a result of their environment; it is reasonable that the residues of altered $pK$ might participate in intramolecular interactions and be responsible in part for stabilizing the native conformation. Although ionization of these groups would be expected to disrupt the folding, the viscosity data show that for the ionization of two and part of the third abnormal tyrosines there is no appreciable change in external shape. However, small changes in *internal* structure may still be associated with the titration of one or more of the tyrosyl groups. This is consistent with data on the iodination of cytochrome *c* (Ishikura *et al.*, 1959). Incorporation of four atoms of iodine led to disruption of certain functional properties of the cytochrome (i.e., it produced inactivity in an oxidase system, autoxidizability, and a capacity to bind carbon monoxide) without appreciable change in heme spectrum, explainable by local conformational changes following iodination (and presumably ionization) of one or more abnormal tyrosine residues.

The stability of the compact molecular structure to *p*H above 13 itself is noteworthy. Proteins generally denature in less alkaline solution, often irreversibly and rapidly. The spectral difference is reversible for cytochrome *c* from *p*H 13.1 (reversibility was not tested at higher *p*H), and at all *p*H values the spectrum did not change over the time of measurement. Previous studies on the guanidine hydrochloride denaturation of cytochrome *c* (W. Kauzmann and J. A. Rupley, unpublished data) have shown that the molecule is unusually stable (8 M urea does not affect the structure), and that conformational changes when they occur are rapid and reverisible. The behavior at alkaline *p*H is in agreement with these properties of the molecule.

Similar experiments on the tyrosyl ionization of cytochrome *c* have been recently reported by Stellwagen (1964), who observed under different experimental conditions (lower salt concentrations) approximately the behavior described. Stellwagen did not analyze the ionizations in terms of dissociation constants, but concluded from other evidence that all four tyrosines were abnormal, in contrast to the single normal ionization inferred from this work.

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# The Macromolecular Organization of Dentine Matrix Collagen. I. Characterization of Dentine Collagen*

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Bovine dentine matrix collagen and corium collagen were isolated and purified. These hard- and soft-tissue collagens were then compared in terms of amino acid composition, hexose and hexosamine content, and reactivity of the $\epsilon$-amino groups of lysine and hydroxylysine. The principal differences were that the dentine matrix collagen was found to contain phosphorus (at least partially in the form of phosphate bound to serine as phosphoserine) and that the $\epsilon$-amino groups of lysine were more readily reactive with fluorodinitrobenzene than were similar groups in the corium collagen. Comparative swelling and solubility studies show that the dentine matrix collagen is a highly cross-linked system. Consideration of the potential cross-linking mechanisms leads to the tentative conclusion that the enhanced stabilization of the dentine matrix collagen stems from the presence of phosphate-mediated di- or triester intermolecular cross-linkages.

The major emphasis in research on the basic structure of collagen has been centered on the collagens of the soft tissues because of the relative ease with which these can be purified and, frequently, rendered into soluble form. The collagens which form the principal organic matrices of bones and teeth have not been studied so extensively, although they play an important, if not crucial, role in calcification and tissue stabilization.

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