*p*H 3 and above *p*H 13 the stability of the hemochromogen is decreased, since one molecule of carbon monoxide then can be added, replacing one of the nitrogen atoms.

Type I gives the absorption spectrum of free hemin c. This proved that both the hemochromogen-forming nitrogen atoms are reversibly split off from the iron in strongly acidic solution.

An attempt to interpret the linkage of the iron atom to nitrogenous groups in cytochrome c on the basis of titration curves, determinations of the amino acid composition, the spectrophotometric data in this paper, and measurements of the magnetic susceptibility at different *p*H values, is described in the last paper of this series.

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[CONTRIBUTION FROM THE BIOCHEMICAL INSTITUTION OF THE MEDICAL NOBEL INSTITUTE, STOCKHOLM]

Studies on Cytochrome c. III. Titration Curves

BY HUGO THEORELL AND Å. ÅKESSON

It was of particular interest to investigate the titration curves of ferro- and ferricytochrome, in order to compare these with the results of the spectrophotometric and magnetic measurements. In 1935 we had already found[1] what we have now confirmed (see part I), that the ionic mobility of cytochrome c remains almost constant from *p*H 6 to 9. The same phenomenon obviously ought to be evident in the titration curves.

Experimental.—A number of titrations were carried out with pure cytochrome c in vessels that were tightly closed with rubber stoppers.

Ferricytochrome was titrated in the following way. By first adding 3 *N* hydrochloric acid the *p*H was brought to a value (*p*H about 1.5) where complete oxidation of Fe^{++} to Fe^{+++} was assured, and carbonic acid was removed quantitatively in a few minutes by means of a slow stream of oxygen. We then titrated with 2–3 N sodium hydroxide from a Linderstrøm-Lang microburet, graduated in 0.2 cmm. per line. The determinations of *p*H were carried out by means of a glass electrode; a $Pt–H_2$-electrode would have reduced the Fe^{+++} to Fe^{++}.

The following procedure was used in the titration of ferrocytochrome. First we reduced the cytochrome in water solution at approximately neutral reaction with 10–20 mg. of platinum black and hydrogen gas. After the reduction was complete we added from a microburet the same amount of hydrochloric acid as that used in the corresponding experiment with ferricytochrome, and then titrated with 2.3 *N* sodium hydroxide. The determinations of *p*H were carried out both with a H_2–Pt and a glass electrode for the sake of control. The temperature was kept constant by means of a water thermostat at 20 or 0°.

From a series of experiments, which all gave practically the same result, we shall select a few for discussion.

1. **Ferri- and Ferrocytochrome at 20°.**—0.0066 millimole of pure cytochrome c from cow hearts was dissolved to the volume 7.0 ml. in water; 0.1000 ml. of 3.00 *N* hydrochloric acid was added in one case directly for the titration of ferricytochrome (glass electrode), in the other case after reduction with $Pt–H_2$ for the titration of ferrocytochrome. 1.82 *N* sodium hydroxide was successively added from a microburet. In addition the curve was determined for 0.1000 ml. 3 *N* hydrochloric acid + 7 ml. water + successively added sodium hydroxide. The difference between this blank curve and the curves with cytochrome, recalculated to equivalents per mole of cytochrome, may be seen in Fig. 1.

In order to check the correctness of the curves in relation to one another, the following three determinations were carried out. Each solution contained 0.0066 millimole of cytochrome in 7 ml. of water, and 0.1000 ml. of 3 *N* hydrochloric acid under oxygen. Sodium hydroxide was then added so that *p*H became 5.59, 8.60 and 10.595, measured by the glass electrode. To each of the solutions 10 mg. of platinum black had been added. Hydrogen was then introduced. Before appreciable reduction could be shown spectroscopically, the potential was established also at the hydrogen electrode, the same *p*H value being obtained as with the glass electrode. After this the bubbling through of hydrogen was continued for several hours, until new potentials had become established at both electrodes and the spectroscopic investigation showed full reduction. In this way we obtained exactly corresponding *p*H values for ferri- and ferrocytochrome.

TABLE I

*p*H, Ferri	*p*H Ferro
5.59	5.03
8.60	7.14
10.595	10.69

(1) H. Theorell, *Biochem. Z.*, **285**, 207 (1936).

The results agree with the curves of Fig. 1, which cross at *p*H 9.6. Below this value the solution becomes more acid on reduction, which is to be expected considering the fact that a hydrogen ion is formed by the reaction $Fe^{+++} + H = Fe^{++} + H^+$.

Between *p*H 3.5 and 8 the difference between the ferri and ferro curves is one equivalent. In other words, throughout this region the change in charge of the iron atom does not affect the dissociation of any groups in the vicinity. Below *p*H 3.5 the difference becomes greater, and seems at *p*H 1.5 to be about 2 equivalents. Although the values at this high degree of acidity are rather uncertain, it nevertheless seems very probable that the observed difference is correct, since ferricytochrome, but not ferrocytochrome, shows a spectrophotometrically determinable dissociation stage with pK 2.5 (see part 2). Above *p*H 8 the curves approach one another and cross at about *p*H 9.6. At *p*H 10.6 the difference between the curves amounts again to an equivalent, though with reversed sign. In the ferri form, then, within the *p*H range 8 to 10.6 one titrates two heme-linked acid groups. It is strange that only one of these seems to be spectrophotometrically operative, for the spectrophotometric measurements of mixtures of the forms III and IV gave a typical dissociation curve with $n = 1.0$ and $pK' = 9.35$. It is difficult, however, to draw definite conclusions about these details, owing to the difficulty of determining the point of intersection of the titration curves with sufficient precision.

The existence of two heme-linked acid groups with pK' between *p*H 9 and 10 in ferricytochrome is of the greatest interest, as this gives us a clue in the investigation of the nature of the hemochromogen-forming groups of cytochrome. As is described in greater detail in part IV, we can expect, according to Russell and Pauling,[2] that the imino-nitrogen in the imidazole rings of two histidine molecules will be titrated with pK' about 9.5. On the other hand, it seems to be out of the question that primary amino groups, for example the NH_2 groups of the cysteine residues that are bound to the porphyrin,[3] should combine with the iron atom in the cytochrome, for this would make it impossible to explain the occurrence of the two heme-linked acid groups with pK' 9.35 and $\sim$ 9.85.

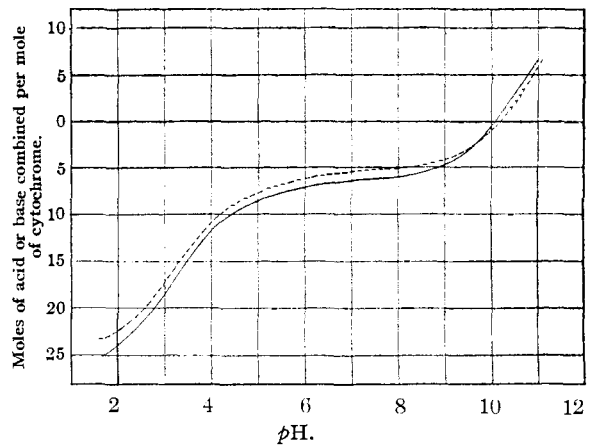

Fig. 1.—The acid and base combining capacity of ferro- and ferricytochrome at 20°: —, oxidized form; - - -, reduced form.

Further information is obtained from the form of the titration curves between *p*H 5.5 and 8.5, where the imidazole groups of histidine are generally titrated. In cytochrome two equivalents are titrated between these *p*H values, while the molecule according to the analyses contains three moles of histidine per mole. However, not even these two equivalents which are titratable between *p*H 5.5 and 8.5 belong entirely to imidazole groups. This is shown by our titration curves at both 20 and 0°. These experiments were carried out, for the sake of exactness, in such a way that the same solution of ferrocytochrome was saturated with hydrogen and the *p*H value determined by means of a hydrogen electrode at both 0 and 20°. The temperature was regulated by holding the titration vessel in the one case in ice water and in the other in water at 20° until the temperature and potential had become constant. In this way 33 pairs of *p*H values for 0 and 20° were determined, after the successive addition of 2 N sodium hydroxide. From the values obtained we calculated the "apparent heat of dissociation," Q' according to Wyman,[4] and plotted them

$$Q' = -4.579\, T_1T_2 \frac{pH_2 - pH_1}{T_2 - T_1}$$

against *p*H (see Fig. 2).

The imidazole groups of histidine should show an "apparent heat of dissociation" of about 6500 cal. per mole. According to Wyman hemoglobin shows a constant plateau where $Q' = 6500$ between *p*H 6.5 and 8.0, while the regions *p*H 5.5–

(2) C. D. Russell and L. Pauling, *Proc. Nat. Acad. Sci.*, **25**, 517 (1939).

(3) K. Zeile and H. Meyer, *Naturwissenschaften*, **27**, 596 (1939); *Z. physiol. Chem.*, **262**, 178 (1939).

(4) J. Wyman, *J. Biol. Chem.*, **127**, 1 (1939).

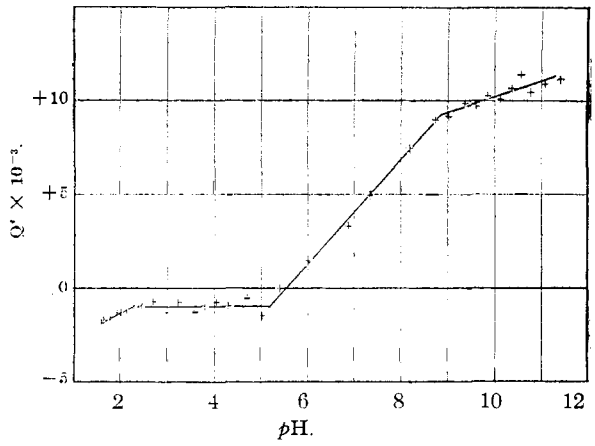

Fig. 2.—Apparent heat of dissociation, Q', in the range 0-20°, plotted against *p*H.

6.5 and 8–8.5 constitute transition zones to the dissociation regions of the carboxyl and amino groups.

Cytochrome shows a different picture. In the region between *p*H 5.5 and 8.5 Q' rises continually from 0 to 9000, which shows that *within this pH region also other groups than imidazole groups are to a certain extent titrated.* Since the total titration value is 2 equivalents from *p*H 5.5 to 8.5, and since only a part of this can be ascribed to imidazole groups, it is at least plausible to assume that only one of the three imidazole groups is titrated within its normal *p*H region, the two abnormal ones being the two which are bound as hemochromogen-forming groups to the iron atom.

From *p*H 1.5 to 7 about 18 equivalents are titrated. The distribution of these among free dicarboxylic acid carboxyls is discussed in part I.

It is difficult to determine the isoelectric point accurately by titration, on account of the difficulty of obtaining the cytochrome absolutely free of electrolyte. It would, however, be possible to get good values by determining quantitatively the electrolytes in the investigated material, *e. g.*, chloride and sodium ions, and correcting the titration curve accordingly. The curve reproduced in Fig. 1 gave the value I. P. = 10.09 for oxidized, and 10.17 for reduced cytochrome. Other determinations gave values close to these. Electrophoretic determinations at 0° and ionic strength 0.1 gave I. P. = 10.65, which when corrected to 20° gives I. P. = 10.05 (see part I).

Electrophoretically purified cytochrome from horse hearts (iron content 0.41%) gave a titration curve almost identical with that for cytochrome from the heart of a cow. This is of some importance, as Keilin and Hartree[5] do not state whether their amino acid analyses, from which our results diverge considerably, were carried out on horse or cow cytochrome. Our titration curves show that, as far as the basic amino acids are concerned, this is a matter of indifference.

(5) D. Keilin and E. F. Hartree, *Proc. Roy. Soc.* (London), **B122**, 298 (1937).

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Studies on Cytochrome c. IV. The Magnetic Properties of Ferric and Ferrous Cytochrome c

BY HUGO THEORELL*

The typical hemochromogen nature of ferrocytochrome c made it *a priori* very probable that the molecule contains covalent octahedral bonds (d^2sp^3), leading to diamagnetism of the substance, as had already been shown in connection with ferrous hemochromogens (Pauling and Coryell[1]). Less certain was the nature of the binding in the five different types of ferricytochrome, which, as we know, show spectral similarities with hemin (I), methemoglobin (II), and parahematins (III and IV); type V shows some similarity with ferrihemoglobin hydroxide. It was therefore of interest to investigate the magnetic properties of the different cytochromes and certain of their derivatives, in order to compare these with the results obtained by Pauling and his co-workers for hemoglobin and its derivatives.[2,3,4,5,6]

* The experiments described in this paper were performed during my stay in Pasadena in June, 1939.

(1) L. Pauling and C. D. Coryell, *Proc. Nat. Acad. Sci.*, **22**, 159 1936).

(2) L. Pauling and C. D. Coryell, *ibid.*, **22**, 210 (1936).

(3) C. D. Coryell, F. Stitt and L. Pauling, THIS JOURNAL, **59**, 633 (1937).

(4) D. S. Taylor and C. D. Coryell, *ibid.*, **60**, 1177 (1938).

(5) C. D. Russell and L. Pauling, *Proc. Nat. Acad. Sci.*, **25**, 517 (1939).

(6) C. D. Coryell and L. Pauling, *J. Biol. Chem.*, **132**, 769 (1940).