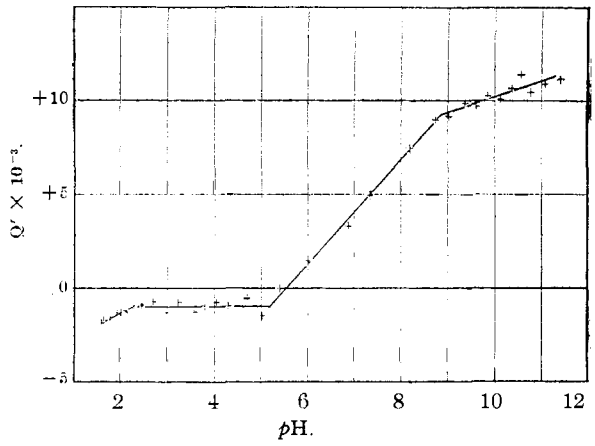


Fig. 2.—Apparent heat of dissociation, Q′, in the range 0–20°, plotted against *p*H.

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).

STOCKHOLM, SWEDEN RECEIVED OCTOBER 1, 1940

[CONTRIBUTION FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY OF THE CALIFORNIA INSTITUTE OF TECHNOLOGY, PASADENA, 824, AND FROM THE BIOCHEMICAL INSTITUTION OF THE MEDICAL NOBEL INSTITUTE, STOCKHOLM]

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).

The Experimental Investigations

On account of the large quantities of material required for measurements of the magnetic values by the Gouy method[1] employed in Pasadena, it was not possible, for practical reasons, to use electrophoretically purified cytochrome. The determinations were carried out on two different preparations of degrees of purity of 0.69 (prepn. 1) and 0.74 (prepn. 2), respectively. They did not, however, contain any foreign iron, so that the impurities affected only the size of the correction for diamagnetism. This was determined for the preparations in question after reduction with $Na_2S_2O_4$.

The experiments were carried out in tubes that were closed at the top with rubber stoppers, through which reagent solutions (acid, alkali, hydrosulfite, etc.) were introduced by means of injection with a syringe. Calibration was carried out by determining the diamagnetism for water against air. Corrections were made for the value of the tubes themselves (water against water), and for the diamagnetism of the reagents added and of the cytochrome. The measurements were carried out at both 10 and 14 amperes (7640 and 8830 oersted) for the sake of control. The temperature was 22 ± 1°.

In two series of experiments the molar magnetic susceptibility for ferricytochrome (prepn. 2) at *p*H values between 0.7 and 8.8 was determined. The *p*H measurements were carried out with a Beckman glass electrode *p*H meter.

The results are given in Tables I and II.

Experiment 1.—2.45 grams of ferricytochrome (prepn. 2) was dissolved in water to a volume of 25 ml. Small volumes of 2 *N* hydrochloric acid were injected and mixed rapidly with the cytochrome solution, after which small quantities were withdrawn for *p*H determination. Corrections for the dilution by the addition of hydrochloric acid and for the change of volume resulting from the withdrawal of the samples for *p*H measurement were carried out. The preparation contained from the outset small, analyzed quantities of Na^+ and Cl^-, which was taken into account in connection with the calculation of the concentration of sodium chloride and hydrochloric acid in the solutions. Corrections for the diamagnetism of the cytochrome were made with 0.45 mg. per 0.005 molar cytochrome solution according to expt. 2.

TABLE I

*p*H	Millimoles of cytochrome per liter	Molarity of NaCl	Molarity of HCl	$\chi_m \times 10^6$
4.75	5.00	0.066	0.031	3,350
4.34	4.95	.066	.051	3,670
3.87	4.90	.065	.071	3,970
3.35	4.85	.064	.091	4,900
2.80	4.80	.063	.110	6,840
2.50	4.78	.063	.120	7,780
2.20	4.75	.063	.130	8,690
1.97	4.73	.062	.140	9,320
1.79	4.70	.062	.150	10,100
1.52	4.66	.062	.170	10,910
1.30	4.61	.061	.190	11,680
1.14	4.56	.060	.210	12,490
1.02	4.51	.059	.230	12,850
0.72	4.43	.058	.330	13,060

When the experiment was finished, there was added alkali to *p*H 1 and 1 ml. 20% $Na_2S_2O_4$ (it is difficult to get a complete reduction below *p*H 1). As was to be expected, the cytochrome on reduction became diamagnetic. Carbon monoxide was now bubbled in through a syringe nozzle and allowed to go out through another, and mixed with the solution during a period of ten to fifteen minutes. The resulting ferrocytochrome carbon monoxide compound (bands at 563 and 530) was found to be diamagnetic.

Experiment 2.—2.45 grams of prepn. 2 was dissolved in water to 25 ml. volume. At first 0.3 ml. of 4.7 *N* sodium hydroxide was introduced; *p*H was then = 8.8; ΔW was determined. After this 0.25 ml. of 20% $Na_2S_2O_4$ solution was added, whereupon complete reduction took place, and ΔW was again determined. Since the ferrocytochrome certainly does not contain any odd electrons, it was possible, after correction for the diamagnetism of the existing electrolytes, to obtain the correction for the diamagnetism of the cytochrome itself. After this the *p*H was reduced by successive injections of 6 *N* hydrochloric acid to 1.20, determinations being made after every injection. In this way we obtained several values for the diamagnetism of ferrocytochrome at different *p*H values. The solution (*p*H 1.20) was taken out of the tube and further hydrochloric acid was added till *p*H was 0.80. On shaking with air the ferrocytochrome was rapidly oxidized to form ferricytochrome. The solution was put back into the tube and ΔW was measured. The *p*H was then successively raised again by additions of 4.7 *N* sodium hydroxide, and measurements of ΔW and *p*H were carried out. The following values were obtained: for the diamagnetism of the ferricytochrome "II" at 10 amperes, recalculated for the concentration 0.005 *M*, at *p*H 8.8, −0.45 mg.; *p*H 4.4, −0.43 mg.; *p*H 2.5, −0.46 mg.; average −0.45 mg. At *p*H 1.2 we obtained −0.26 mg., but this diverging value was undoubtedly conditioned by some autoxidation through traces of air. The value −0.45 mg. corresponds approximately to what is generally obtained for a diamagnetic protein in this concentration.

The determination of χ_m for ferricytochrome at different *p*H values gave the results listed in Table II.

TABLE II

*p*H	Millimoles of ferricytochrome per liter	Molarity of NaCl	Molarity of HCl	$\chi_m \times 10^6$, average of values at 10 and 14 amperes
0.80	4.61	0.113	0.334	12,790
0.96	4.57	.156	.288	12,400
1.19	4.53	.198	.243	11,920
1.51	4.48	.240	.198	10,780
2.15	4.44	.281	.154	8,360
2.75	4.42	.302	.131	6,650
3.50	4.40	.322	.110	4,410
4.37	4.36	.362	.067	2,580
8.8	4.94	.066	0	3,340

We see from these experiments that the iron atom in neutral ferricytochrome (type III) is bound to the surrounding nitrogen atoms by essentially covalent bonds, since it contains only one odd electron. The values obtained for the molar mag-

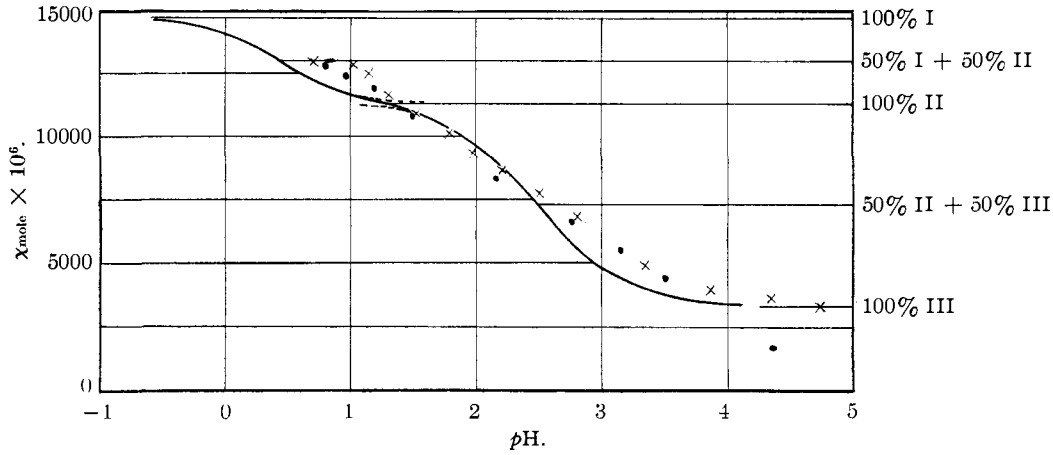


Fig. 1.—Spectrophotometric curve and magnetometric points plotted on a comparable scale.

netic susceptibility, 2580–3340 × 10^{-6}, lie close to that found by Coryell, Stitt and Pauling[3] for ferrihemoglobin cyanide (2610 × 10^{-6}). χ_m = 3300 × 10^{-6} gives 2.81 Bohr magnetons, while theoretically one odd electron would give 1.73 Bohr magnetons, plus some contribution from the orbital motion of the electron. The difference of 1.08 magnetons seems big, but is probably to be explained as being due to an abnormal orbital contribution. The difference cannot be explained by a heme–heme interaction, since the cytochrome molecule contains only one heme.

A determination of χ_m at *p*H 13.5 gave the value 1900 × 10^{-6}, close to the theoretical value for one odd electron. Russell and Pauling[5] have obtained similar values for imidazole-ferrihemoglobin. It would thus seem as if type V gives a somewhat lower value for χ_m than type III. Type IV certainly gives the same value as type III, as at *p*H 8.8 a blend of III and IV occurs, and χ_m = 3340 × 10^{-6} was obtained as the result of a very careful measurement with very small corrections for diamagnetism.

In types I and II the iron atom is, on the other hand, bound with essentially ionic bonds. Measurements were not taken at lower *p*H values than 0.7, as type I cannot in any case be obtained pure, even if so much hydrochloric acid is added that the cytochrome is disintegrated. Moreover, the corrections for the diamagnetism of the hydrochloric acid are especially high at extreme degrees of acidity. It is, however, obvious that χ_m, at *p*H 0.7, already begins to approach the theoretical value for five odd electrons, 14,700 × 10^{-6}, corresponding to 5.92 Bohr magnetons. This was *a priori* to be expected, as type I gives a purely acid hematin spectrum. Pauling and Coryell[1] and Cambi and Szegö[7] found somewhat lower values than 5.92 for crystalline hemin, while Haurowitz and Kittel[8] found higher values. Ferricytochrome c of type I thus contains, in accordance with the appearance of the absorption spectrum, five odd electrons for the iron atom.

Type II agrees, as regards the spectrum, very closely with acid ferrihemoglobin (see part II). At *p*H 1.4 there is practically speaking only type II in the solution. At this *p*H, according to the magnetic measurements, χ_m is 11,300 × 10^{-6}.

For the explanation of this value we find interesting analogies with ferrihemoglobin. Coryell, Stitt and Pauling[3] have found that acid ferrihemoglobin, Hb^+, shows a maximum of magnetic susceptibility of χ_m 14,000 at *p*H 6.5–7. Below these *p*H values χ_m begins to sink again to a value that has been extrapolated by Coryell and Pauling[6] as 12,570 × 10^{-6}. The explanation that is given for the phenomenon is that a heme-linked acid group, probably an imidazole group in histidine, is titrated in this region with *pK* 5.30. The rather uncertain value 12,570 × 10^{-6} is not far away from 11,300 × 10^{-6}, which was obtained for ferricytochrome c of type II. This form exists only in such a strongly acid solution that imidazolium ions must occur as in ferrihemoglobin below *p*H 5.3.

The curve for the magnetic susceptibility may be compared with the spectrophotometric curve by redrawing the latter on such a scale that 100% type III lies on the same level as χ_m = 3300, 100% type II at χ_m = 11,300 and 100% type I at χ_m = 14,700 (see Fig. 1).

(7) L. Cambi and L. Szegö, *Rend. ist. lombardo sci.*, **67**, 275 (1934).
(8) F. Haurowitz and H. Kittel, *Ber.*, **66**, 1046 (1929).

One may see from the figure that, although the curves on the whole have a similar course, certain divergences occur; thus, for example, the magnetic curve rises more evenly in the acid direction, as compared with the spectrophotometric curve. We shall not venture any opinion as to whether the difference is due to experimental error or is real.

Ferricytochrome Fluoride.—As type II, both spectrally and as regards its magnetic susceptibility, is very similar to acid ferrihemoglobin, it was of interest to determine also the magnetic susceptibility of the fluoride compound. This investigation could be carried out only at constant *p*H and with varying fluoride concentration, as the fluoride compound of ferricytochrome exists only within a narrow *p*H range near *p*H 3. Since the pK' of hydrogen fluoride is 3.14, the determinations were carried out by adjusting a ferricytochrome solution to this *p*H, with hydrochloric acid, and then adding successive portions of a solution containing potassium fluoride (4 molar) and hydrochloric acid (2 molar), with a calculated *p*H of 3.14. It was not possible to measure the *p*H of this solution or of its mixtures with the cytochrome solution, as hydrogen fluoride attacked the glass electrodes. The values listed in Table III were obtained.

TABLE III

FERRIC CYTOCHROME + KF AND HCl, AT CONSTANT *p*H 3.14

Millimoles of ferricytochrome c per liter	Milliequivalents of fluoride ions per liter	$\chi_{molar} \times 10^6$	$K = [II][F^-]/[IIF]$
8.15	0	5,500	....
8.91	14.5	6,650	0.0132
7.75	56	8,270	.0176
7.67	76.5	9,060	.0163
7.59	94.5	9,660	.0154
7.44	132	10,230	.0170
7.28	168	10,400	(.0203)*
7.02	242	10,640	(.0264)*
		Average 0.0159 ± .0015	

* The two last values of K were rejected, as the ionic strength here was so high that there was reason to fear that the equilibrium between types III and II had been changed.

The equilibrium constant K was calculated in the following way. At *p*H 3.14, according to the spectrophotometric measurements (see part 2), types III and II occur in the constant relation 86:14 = 6.14. As it proved impossible to add so much fluoride that the maximal value for χ_m at saturation of the cytochrome with fluoride was reached, this maximal value was postulated as 14,700, corresponding to 5 odd electrons. Coryell, Stitt and Pauling[3] found the value $14,660 \times 10^{-6}$ for ferrihemoglobin fluoride.

From this assumption the following equations are obtained

$$[III]/[II^+] = 6.14 \quad (1)$$

$$[II^+] + [III] + [IIF] = C \quad (2)$$

$$([II^+] + [III]) \times 5500 + [IIF] \times 14,700 = \chi_m \times C \quad (3)$$

from which we get

$$[IIF] = \frac{C(\chi_m \times 10^6 - 5500)}{9200}$$

$$[II] = \frac{C - [IIF]}{7.14}$$

$$[F^-] = [F^-]_{Total} - [IIF]$$

It is interesting to note that the value obtained for K_{II^+,F^-} agrees within the limits of error with the values that Lipmann[9] and Coryell, Stitt and Pauling[3] obtained for ferrihemoglobin fluoride. Here again, then, we find a striking similarity between ferricytochrome type II and ferrihemoglobin. We may, however, draw attention to one difference: if the hydroxyl concentration in a solution of Hb^+F^- is increased, F^- is ousted by OH^-: in the case of II^+F^-, on the other hand, F^- is ousted on increasing (OH^-), not by OH^- itself, but by the sixth nitrogen atom around the iron atom, so that type III arises, which thus does not correspond to HbOH. This may be seen also from the magnetic values. While HbOH gives $\chi_m = 8350 \times 10^{-6}$, probably corresponding to 3 odd electrons,[3] type III gives $\chi_m = 3000 \times 10^{-6}$, one odd electron.

Ferricytochrome cyanide was studied in preparation 1 in 0.005 molar solution with 0.67 N sodium hydroxide. χ_m was determined both before and after addition of 28 mg. of potassium cyanide, 156 mg. of potassium cyanide, and 38 mg. of $Na_2S_2O_4$, respectively. After the addition of potassium cyanide the spectrum already described (part 2) appeared; after the addition of $Na_2S_2O_4$, on the other hand, the spectrum 555, 525 of ferrocytochrome - cyanide - hemochromogen appeared. The latter substance was assumed to be diamagnetic,[10] and on this assumption the following figures were obtained for the former:

Ferricytochrome, type V: 1900×10^{-6} c. g. s. u.

Ferricytochrome, type V + 28 mg. KCN: 2300×10^{-6} c. g. s. u.

Ferricytochrome, type V + 156 mg. KCN: 2300×10^{-6} c. g. s. u.

(9) F. Lipmann, *Biochem. Z.*, **206**, 171 (1929).

(10) This assumption, in itself very plausible, was confirmed also by the calculation of the diamagnetism of the electrolytes and the protein.

The values show that the iron atom in the cyanide complex contains one odd electron and is combined by octahedral covalent bonds to the six surrounding nitrogen atoms.

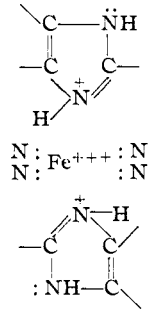
The carbon monoxide compounds of ferrocytochrome above *p*H 13 and below *p*H 3 were found to be diamagnetic.

Discussion of the Results

The titration curves showed that the imidazole nitrogen atoms of two of the three histidine molecules in cytochrome c are not titrated in the normal way. This can be explained most easily by assuming that two histidine molecules are bound to the iron atom. Both the hemochromogen-forming groups of the cytochrome would thus be imidazole groups of histidine. We proceed now to examine this theory in relation to the results of other experiments.

Regarding the constitution of the five spectral forms of ferricytochrome the following is to be said.

Form I agrees spectroscopically with "hemin c," the only difference being that in hemin c the peptide linkages binding the protein component to the cysteine residues (*cf.* part 1) have been dissolved by hydrolysis. Thus in strongly acid solutions both the hemochromogen-forming N atoms are dissociated from the Fe atom. The binding between the Fe^{+++} atom and the four N atoms in the porphyrin is, according to the magnetic measurements, essentially ionic, and the formula may be written



Type I

If *p*H is raised to 1.5 we get type II, which shows the following similarities with acid ferrihemoglobin, Hb^+.

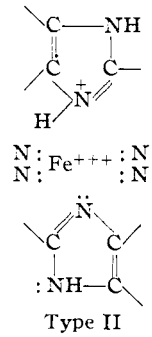
1. Hb^+ and II show the same absorption spectrum, with the difference that the band of type II is shifted 5–7 mμ in the short-wave direction in relation to Hb^+. This displacement is due to the fact that Hb^+ has two vinyl side chains in the porphyrin, while these have been saturated in the cytochrome, probably by addition of cysteine hydrosulfide.

2. The magnetic susceptibility is about as high in II as in Hb^+ in acid solution, according to Coryell and Pauling's calculation,[6] thus rather lower than the theoretical susceptibility for five odd electrons.

3. Hb^+ and II both give fluoride compounds with very similar absorption bands. The only difference is, as before, a displacement of 5–7 mμ in the short-wave direction, as compared with Hb^+.

4. One cannot add enough fluoride to a ferricytochrome II solution to convert all the cytochrome to II F, but on the assumption that 100% II F would give the same χ_m as Hb F, 14,700 × 10^{-6} c. g. s. u., one gets the same value for the dissociation constant of II F as has been determined by other authors for Hb F.

The arguments adduced thus all speak in favor of a complete analogy between the constitutions of acid ferrihemoglobin and type II. The latter thus may be described by the same formula as that assumed for acid ferrihemoglobin below *p*H 5.25



Type II

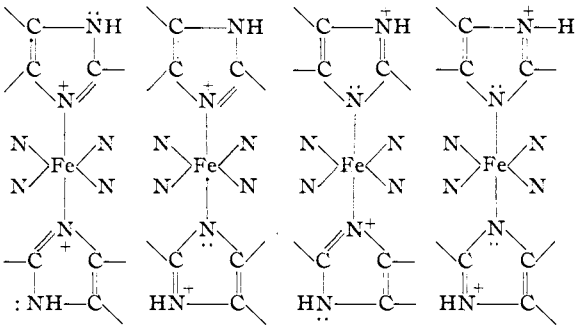
In the transition from type I to type II an acid group would thus be titrated; this is not, however, open to experimental confirmation by titration on account of the high degree of acidity.

If the *p*H is raised a proton is split off in both Hb^+ and II, with $pK_{Hb^-} = 5.25$ (Coryell and Pauling) and $pK_{II} = 2.50$ (Theorell and Åkesson). The difference between Hb^+ and II is that in Hb^+ there is supposed to exist either no binding or only a weak binding of one of the two imidazole groups to the iron atom on account of the unfavorable position for electrostatic coördination of this imidazole group (Conant[11]); in cytochrome II, on the other hand, both imidazole groups are

(11) J. B. Conant, *Harvey Lectures*, **28**, 159 (1932–33).

presumably in favorable position for coördination with the iron atom. When the second imidazole group also is linked to iron, the essentially ionic bonds become essentially covalent bonds. One equivalent more is titrated in ferricytochrome (pK 2.5) than in ferrocytochrome (see part 3), which would thus keep the imidazole group bound below pH 3, although more loosely, so that it can be ousted from the iron atom by carbon monoxide.

Thus ferricytochrome type III, which, physiologically, is the important one, probably has the mutually resonating constitutions



Type III

The interpretation of the constitution for types IV and V is rather more uncertain. The following facts may be adduced in this connection.

1. The spectrophotometric measurement at 650 mμ gives a dissociation constant $pK_{\text{III-IV}} = 9.35$, with $n = 1.0$.

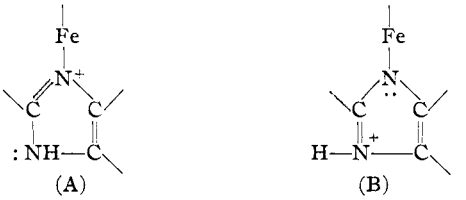
2. The titration curve shows that in the pH range 8–10.6 two equivalents more are titrated in ferric than in ferrous cytochrome. The curves for ferric and ferrous intersect at approximately pH 9.6. This value is only approximate on account of the small angle between the two curves.

3. Type IV forms no cyanide compound, nor does ferrocytochrome give any carbon monoxide compound in the corresponding pH range. From this we may conclude that all six coördination positions of the iron atom in type IV are still taken up by nitrogen atoms. The transition from type III to IV thus does not imply an addition of hydroxyl ion.

These observations can be explained by the assumption that the imino groups of the imidazole rings are titrated in this region, one with pK 9.35, the other with pK 9.85. The difference, about 0.5 pH, seems to correspond well to that expected, in view of the rather large distance between the two imino groups with corresponding weak interaction between them. Type IV would thus actually comprise two slightly different forms, IVa and IVb, one for each titrated imino group. It is, however, curious that only IVa (pK 9.35) is spectrophotometrically operative. But, on the other hand, the difference in light absorption between III and IV is in general so small that it was only possible to measure it in the red. It is quite conceivable that IVa and IVb give the same light absorption at 650 mμ so that a possible difference escapes observation.

The above interpretation is supported by Russell and Pauling's[5] demonstration of an acid dissociation constant pK 9.5 in the covalent imidazole-ferrihemoglobin complex. The dissociation constant is considered by these writers to correspond to the titration of the imino group in the imidazole.

This interpretation, however, presupposes that the two resonating structures



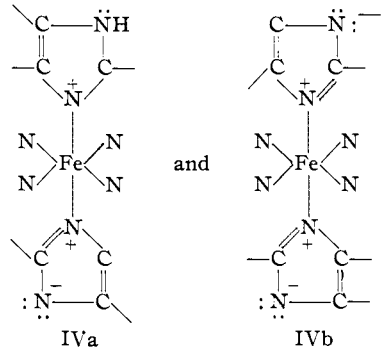
(A) (B)

in cytochrome c, just as in imidazole-ferrihemoglobin, do not contribute nearly equally to the normal state of the molecule, but that form A is for some reason more stable and thus predominates over B. In this way the difference in pK between 6.65, for Hb^+, with about equal contribution of A and B, and pK 9–10 for ferricytochrome and imidazole-ferrihemoglobin, is explained by the weakly acid character of the imino group in structure A. That a displacement of pK for the histidine of cytochrome has taken place is proved by the titration curves (see part III), which show that the histidine groups are not titrated within their normal range between pK 5.5 and 8.5. As regards imidazole-ferrihemoglobin, as has already been mentioned, Russell and Pauling have demonstrated that the imino group in the imidazole should show a pK of 9.5. This means that two of the three histidine-imidazole groups in the ferricytochrome molecule are titrated in the acid region with pK 2.5 and 0.44, one proton being added each time to the tertiary nitrogen atom, the bond with the iron atom being broken, while in the alkaline region the imino groups are titrated with pK 9.35 and 9.85. This would explain the

flat course of the titration curves within the *p*H range 5.5 to 8.5, where only one histidine-imidazole per molecule is titrated, namely, the one not heme-linked.

In **ferrocytochrome,** on account of the electrostatic interaction with the ferro-atom, the same imino groups presumably should be titrated at a higher *p*H value than in ferricytochrome, probably in the region of *p*H 11. This seems also to be the case, as the difference between the titration curves of the ferrous and ferric cytochromes seems to diminish again above *p*H 10.5. The difference was determined very carefully at *p*H 10.69 (see part III) as 0.7 equivalent.

The forms IVa and IVb would thus show the constitution



IVa IVb

With these forms resonate the forms without separated electric charges, in which the N atoms of the imidazole groups have their normal covalence.

Type V, finally, gives a cyanide compound, and ferrocytochrome gives, within the same *p*H range, above 13, a compound with one molecule of carbon monoxide. This is an indication that one of the imidazole groups here is split off from the iron atom. If we assume that a hydroxyl group enters into the sixth coördination place of ferricytochrome in place of an imidazole group, type V would be expected to show analogy with alkaline ferrihemoglobin, HbOH. There is a striking similarity in the absorption bands of the cyanide compounds of the two.

It may seem strange that **ferrous cytochrome** shows the same spectrum throughout the whole *p*H scale. But this is connected with the fact that the four linkages from the ferro-atom to the 4 porphyrin-nitrogen atoms are covalent throughout the scale, which is the only necessary condition for the appearance of a hemochromogen spectrum (Pauling and Coryell[1]).

That no visible change in the spectrum occurs below *p*H 3 or above *p*H 13 does not, then, mean that the constitution of ferrous cytochrome is independent of *p*H. This cannot be the case, for the formation of CO-compounds and the rapid oxidation of ferrous cytochrome by oxygen outside the *p*H limits 3–13 show a change that probably consists in a loosening of the linkage of the one imidazole group to the iron atom.

The author is indebted to Dr. Linus Pauling for advice and for the facilities provided in his laboratory, and to Dr. John T. Edsall for kindly reading the proofs.

General Summary of Papers I, II, III and IV

Cytochrome c from the heart of the cow and the horse was electrophoretically purified to a hemin-iron content of 0.428%, after which it was electrophoretically uniform. Analyses of iron, sulfur and amino acids showed whole-number proportions between all constituents that could be determined with sufficient accuracy. The amino acid analyses showed interesting features. The molecule probably contains 96 amino acids = $2^5 \times 3,^{11}$ and thus fits in this respect into Bergmann and Niemann's multiple system; no representative of this class with molecular weight about $^1/_3 \times$ 35000 has previously been reported. However, the number of lysine residues, 22, does not correspond to the Bergmann–Niemann system. The amino acid composition of cytochrome c is unusual, and diverges markedly from that of hemoglobin and other proteins. Especially noteworthy is the high lysine content (24.7%) of cytochrome, and its low content of histidine (3.3%). It appears to contain a number of free $-$COOH and NH_2 groups, suggesting that the molecule consists of several polypeptide chains with free NH_2 and COOH groups at each end.

Ferricytochrome shows five different spectral forms; the reversible equilibria between them are a function of *p*H. The optical properties of the five forms have been examined spectrophotometrically. Characteristics of the spectrum of each form which are significant for the interpretation of its constitution are pointed out.

Titration curves showed that the dissociation constants for the histidine-imidazole groups of the cytochrome molecule lie for the most part outside the normal titration region (*p*H 5.5–8.5). The explanation is probably that *two of the three imidazole groups in cytochrome constitute the hemochromogen-forming groups.* This theory was found

to be in harmony with magnetic measurements on cytochrome and its derivatives, with spectrophotometric and titrimetric determinations on cytochrome, as well as with the investigations of Pauling and his co-workers on the constitution of hemoglobin. We see here a striking analogy with hemoglobin, which is also supposed to contain 2 histidine-imidazole groups in the vicinity of each iron atom (Conant,[11] Wyman[12]). One main difference between hemoglobin and cytochrome c, as regards the incorporation of the iron atom in the molecule, is that in hemoglobin one imidazole group is in a favorable position for coördination with the iron atom, whereas the other is not. Thus one of the six octahedral valences of the iron atom is left free for addition of oxygen, carbon monoxide, etc. In cytochrome c, on the other hand, both imidazole groups are in such a favorable position for coördination with the iron atom that a very firm compound is formed. Only in strongly alkaline or strongly acid solutions are one or both of the imidazole groups freed from the iron atom, after which compounds with carbon monoxide (to Fe^{++}) or CN^- and F^- (to Fe^{+++}) can be formed.

The heme of the cytochrome is thus built into the protein component in a manifold way: by means of thioether bindings from the side chains of the porphyrin to the protein, and by means of two histidine-imidazole groups strongly bound to Fe on each side of the flat heme disc. Thus the heme group appears to be built into a crevice in the protein molecule. This explains why cytochrome c is not autoxidizable, since oxygen can never approach the iron atom, and why no CO-compounds or cyanide compounds are formed at physiological *p*H values.

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

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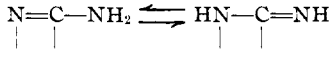
[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, SIENA HEIGHTS COLLEGE, AND FROM THE RESEARCH LABORATORIES OF THE INSTITUTUM DIVI THOMAE]

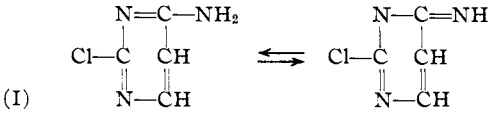
Ultraviolet Absorption Spectra of Nitrogenous Heterocyclic Compounds. III. Effect of *p*H and Irradiation on the Spectrum of 2-Chloro-6-aminopyrimidine

BY SISTER MIRIAM MICHAEL STIMSON, O.P., AND JOHN R. LOOFBOUROW[1]

The change in the absorption of adenine with *p*H[1a] was accounted for by tautomerism of the amidine type



In adenine there are four nitrogen atoms which have a potentially labile hydrogen. With the exceptions of the 1,6 and 6 positions the possible tautomers may be expected to be all equal in absorption characteristics since in every case the —N=C< chromophore is within the ring and hence is approximately equal. In the shift involving the 1,6 and 6 positions the resonator is the free >C=N— in which the weight of the attached hydrogen is negligible. Brode[2] points out that the effect of a weight on a vibrating body is to decrease the frequency of oscillation and consequently of the absorption. Therefore a change in the position of a selected resonator from the 1,6 to 6 atoms should be detectable. We, therefore, investigated 2-chloro-6-aminopyramidine (I), since in this compound the introduction of a chlorine in the 2 position permits tautomerism involving only the 1,6 and 6 positions.



(I) (II)

Experimental

Materials.—2-Chloro-6-aminopyrimidine was prepared according to the method of Johnson and Hilbert,[3] and separated from its isomer by steam distillation and repeated recrystallization from glass distilled water. Spectra were obtained with concentrations of 1.25 and 1.29 mg./liter; 4 and 2 cm. cells were employed. Kolthoff buffer tablets were used to adjust the *p*H and the comparison cell contained the corresponding buffer at the same concentration.

Method.—The details of the techniques employed were given in Part I.

Results and Discussion

2-Chloro-6-aminopyrimidine shows two bands between 2200–2900 Å. as do also 6-aminopyrimi-

(1) Present address: Massachusetts Institute of Technology.

(1a) Loofbourow and Stimson, Part I, *J. Chem. Soc.*, 844 (1940).

(2) Brode, "Chemical Spectroscopy," John Wiley and Sons, Inc., New York, N. Y., 1939, p. 124.

(3) Johnson and Hilbert, THIS JOURNAL, **52**, 1152 (1930).