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

Studies on Cytochrome c. II. The Optical Properties of Pure Cytochrome c and Some of Its Derivatives

BY HUGO THEORELL AND Å. ÅKESSON

Some years ago we found that ferricytochrome c shows different absorption spectra in acid, neutral, and alkaline solution.[1] Recently four different absorption spectra, dependent on the *p*H of the solution, were distinguished, and the dissociation constants of the different types were determined.[2] It has now been found that still another type exists in strongly acidic solution, so that ferricytochrome c shows not less than five different absorption spectra, which we may call I, II, III, IV, and V in order from acid to alkaline solutions. In connection with the attempts to elucidate the question of the linkage between the iron atom and nitrogenous "hemochromogen-forming" groups in cytochrome c by means of spectrophotometric, magnetometric, and titrimetric methods, we thought that a thorough investigation of these five types would be of interest. A study of the compounds of ferricytochrome and ferrocytochrome with agents such as hydrocyanic acid, fluoride ion, and carbon monoxide and their dependence on *p*H was expected to give further information on the same subject. The purification of the cytochrome to the iron content of 0.43% necessitated a reinvestigation of the light absorption in the ultraviolet region.

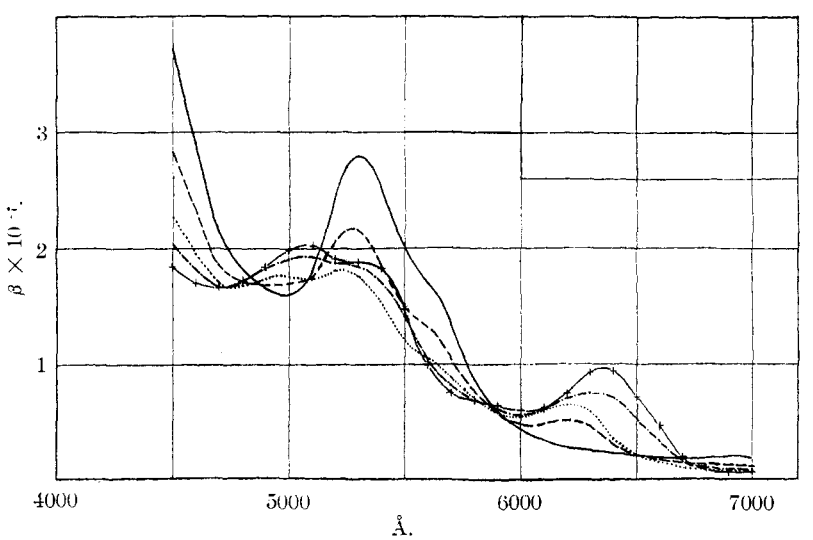


Fig. 1.—Absorption spectra of ferricytochrome c, at different *p*H values: ——— 7.0; - - - 2.43; - - - - 1.59; — · — · — 0.47; +–+–+ –0.3.

The Dissociation Stages in Acid Solution

In the acid region the dissociation constants $pK'_{I\text{-}II}$ and $pK'_{II\text{-}III}$ are located so close together that at intermediate *p*H values the forms I, II, and III may be expected to be present in the solution at the same time. Even where this is the case a quantitative estimation of each of them can be carried out spectrophotometrically by the following procedure. If light absorption curves are determined at suitable hydrogen ion concentrations it is generally possible to find "isobestic" wave lengths, where two of the components give the same absorption coefficient and the third component a different one. Thus the ratios

$$\frac{[I]}{[II] + [III]} = q_1 \text{ and } \frac{[I]}{[I] + [II]} = q_2$$

can be determined for different *p*H values from data for two isobestic wave lengths. As [I] + [II] + [III] = C_{Total}, the values of [I], [II], [III], $pK'_{I\text{-}II}$, and $pK'_{II\text{-}III}$ are then easily calculated.

To find suitable isobestic points absorption curves at different *p*H were determined in the region 4500–7000 Å. The results are given in Fig. 1 and Table I.

It is remarkable that cytochrome c is stable for some minutes even in 3 *N* hydrochloric acid, as estimated by neutralization after that period and determination of some absorption coefficients. In 2 *N* hydrochloric acid it is stable for hours as regards its optical properties. Of course this does not exclude the possibility of changes in the protein part of the molecule, which might not exert any influence on the optical properties.

The curves show the following isobestic points: A, $\beta_I = \beta_{II}$, β_{III} different: 4700, 5250, 5570, 5800,

(1) H. Theorell, *Biochem. Z.*, **285**, 207 (1936).
(2) H. Theorell and Å. Åkesson, *Science*, **90**, 67 (1939).

TABLE I

ABSORPTION COEFFICIENTS, β, OF FERRICYTOCHROME c, AT DIFFERENT pH VALUES

Final concentration of cytochrome c in all of the experiments = 4.24×10^{-7} mole per ml. pH varied by addition of HCl.

Wave length, Å.	pH 7.0	$\beta(=1/c \times 1/d \times \ln I_0/I) \times 10^{-7}$ 2.43	1.59	0.47	$-0.3(2\ N$ HCl)
4500	3.71	2.83	2.27	2.04	1.84
4600	2.91	2.31	1.95	1.82	1.70
4700	2.14	1.85	1.70	1.67	1.67
4800	1.84	1.72	1.68	1.72	1.69
4900	1.66	1.68	1.75	1.82	1.83
5000	1.60	1.70	1.76	1.91	1.99
5100	1.81	1.76	1.75	1.93	2.03
5200	2.47	2.07	1.81	1.87	1.91
5300	2.80	2.16	1.76	1.85	1.88
5400	2.50	1.87	1.51	1.71	1.83
5500	2.03	1.49	1.20	1.41	1.48
5600	1.70	1.32	1.06	1.03	0.998
5650	1.57	...	...	...	...
5700	1.34	1.03	0.879	0.812	.754
5800	0.871	0.770	.700	.703	.692
5900	.594	.568	.580	.635	.647
6000	.452	.492	.548	.562	.607
6100	.351	.493	.596	.609	.622
6200	.285	.517	.647	.702	.750
6300	.262	.471	.588	.756	.930
6400	.242	.309	.356	.718	.939
6500	.219	.220	.219	.532	.720
6600	.196	.178	.163	.342	.471
6700	.189	.143	.103	.167	.192
6800	.185	.137	.087	.109	.105
6900	.202	.137	.086	.100	.069
6950	.201	...	...	...	...
7000	.191	.123	.086	.092	...

and 6800 Å. The wave length 4700 Å. was found to be the most suitable for determining the ratio [III]/[I] + [II]. B: βII = βIII, βI different: 5090 and 6500 Å. The wave length 6500 Å. was used for the measurements.

At 4840 Å. there happens to exist a 3-fold isobestic point, which may be of value occasionally for the determination of the concentration of cytochrome c in a neutral or acid solution of unknown pH. The β for I, II, and III is here = 1.72×10^7 sq. cm./mole.

Determinations of the light absorption coefficients were carried out at 6500 and 4700 Å. with pure ferricytochrome c in solutions containing 3.65×10^{-4} mole of cytochrome, 0.02 mole of sodium chloride per liter, and varying amounts of hydrochloric acid. The results are given in Tables II, III and IV below. As even $3N$ hydrochloric acid does not give quite 100% type I, the value of $\beta_{I,\ 6500\ Å.}$ had to be found by extrapolation. The most probable value was found to be = 0.781×10^7 sq. cm./mole.

It is seen from the results that the two acid dissociation stages do not overlap to any appreciable extent. At pH 1.35 1.7% I, 94.5% II, and 3.8% III are present in the solution. It is not necessary to correct the dissociation constants for the coexistence of three forms in this narrow range of pH. All other pH values give either one or a mixture of at the most two spectral types of cytochrome c.

The determination of $pK_{II\text{-}III}$ carried out before type I was discovered[2] gave the correct value 2.50 because the wave length used in these experiments (5300 Å.) happened to lie very close to an isobestic point (5250 Å.)

Determinations of the Dissociation Constants in the Alkaline Range.—2.036×10^{-6} mole of pure cytochrome c in 7.0 ml. of water was first acidified by addition of 0.10 ml. of 3.0 N hydrochloric acid in the presence of oxygen in order to oxidize every trace of ferrocytochrome; 0.176 ml. of 1.82 N sodium hydroxide was added bringing the pH to 5.02. The light absorption coeffi-

TABLE II

TYPES I, II WAVE LENGTH 6500 Å.

Molarity of HCl	3.0	2.0	1.0	0.5	0.25	0.125	0.05	0.02	0.015	0.01
pH	−0.48	−0.30	0.0	0.32	0.64	0.95	0.135	1.82	2.11	2.39
$\beta \times 10^{-7}$	0.757	0.739	0.665	0.551	0.393	0.270	0.212	0.201	0.202	0.203
% I	95.9	92.8	80.0	60.3	33.0	11.7	1.7	0	0	0
% (II + III)	4.1	7.2	20.0	39.7	67.0	88.3	98.3	100	100	100
log [I]/[II] + [III]	1.37	1.11	0.60	0.18	−0.31	−0.88	−1.77	$-\infty$	$-\infty$	$-\infty$

TABLE III

TYPES I, II AND III, 4700 Å.

pH	1.02	1.35	1.62	1.82	1.98	2.11	2.26	2.39	2.53	3.01	3.41	3.62	7
$\beta \times 10^{-7}$	1.62	1.64	1.67	1.68	1.72	1.75	1.81	1.90	1.93	2.05	2.10	2.11	2.14
% (I + II)	100	96.2	90.3	88.4	80.7	75.0	63.5	46.2	40.4	18.3	7.7	5.8	0
% III	0	3.8	9.7	11.6	19.3	25.0	36.5	53.8	59.6	81.7	92.3	94.2	100
log [I] + [II]/[III]	$+\infty$	+1.404	0.907	0.882	0.621	0.477	0.241	−0.065	−0.168	−0.650	−1.079	−1.210	$-\infty$

TABLE IV

TYPES III AND IV, 6500 Å.

*p*H	5.02	7.02	7.39	7.68	7.76	8.09	8.55	8.85	9.13
$\beta_{6500} \times 10^{-7}$	0.2065	0.2073	0.2071	0.2052	0.2050	0.2006	0.1836	0.1730	0.1550
% III	100	100	100	98.5	98.3	95.1	82.7	74.8	61.5
% IV	0	0	0	1.5	1.7	4.9	17.3	25.2	38.5
log [III]/[IV]	.....	.....	.....	1.818	1.763	1.288	0.680	0.473	0.203
*p*H	9.41	9.71	10.01	10.30	10.59	10.85	11.09	11.3	
$\beta_{6500} \times 10^{-7}$	0.1347	0.1158	0.0972	0.0825	0.0808	0.0748	0.0713	0.0717	
% III	46.7	32.8	19.0	8.2	7.0	2.6	0	0	
% IV	53.3	67.2	81.0	91.8	93.0	97.4	100	100	
log [III]/[IV]	−0.057	−0.312	−0.630	−1.049	−1.124	−1.573			

TABLE V

TYPES IV AND V, 6599 Å.

Buffer	Glycine + NaOH					NaOH			
*p*H	11.01	11.30	11.50	12.12	12.60	12.90	13.21	13.55	13.80
$\beta_{6500} \times 10^{-7}$	0.0723	0.0726	0.0730	0.0789	0.0914	0.1231	0.1347	0.1477	0.1441
% IV	100	99.6	99.1	91.2	74.1	30.9	15.1	0	0
% V	0	0.4	0.9	8.8	25.9	69.1	84.9	100	100
log IV/V		2.4	2.0	1.04	0.457	−0.35	−0.75		

cient was determined (6500 Å., d = 1.996 cm.) and then the *p*H was increased stepwise by the addition of sodium hydroxide from a microburet, and the corresponding light absorption values were determined. The small dilution due to the addition of sodium hydroxide was corrected for. The concentration of sodium chloride was thus kept at the same value as in the determinations of the titration curve (see paper III of this series).

The results are given in Table IV.

$pK'_{IV\text{-}V}$ was determined in solutions of cytochrome c in glycine and sodium hydroxide, or in sodium hydroxide of different *p*H values, which were determined by means of the hydrogen electrode. The spectrophotometric measurements were carried out at 6500 Å.

The absorption coefficient of type V was taken as 0.1459, the average of 0.1477 and 0.1441.

Figures 2 and 3 show the logarithms of the ratios [I] : [II], [II] : [III], and so on, plotted against the *p*H. The angle of the lines should be 45° if n = 1, that is if 1 H^+ is titrated at each step. This fits fairly well for the step II–III (n = 1.14) and exactly for the step III–IV (n = 1.0), whereas $n_{I\text{-}II}$ is 1.53 and $n_{IV\text{-}V}$ is 1.64. The fact that these latter slopes are larger than 1 may be due to the extremely acid and alkaline reactions in these cases.

The dissociation constants were determined graphically, the following values being obtained: $pK'_{I\text{-}II}$ = 0.42; $pK'_{II\text{-}III}$ = 2.50; $pK'_{III\text{-}IV}$ = 9.35 and $pK'_{IV\text{-}V}$ = 12.76.

The whole "spectrophotometric titration curve" is shown in Fig. 4.

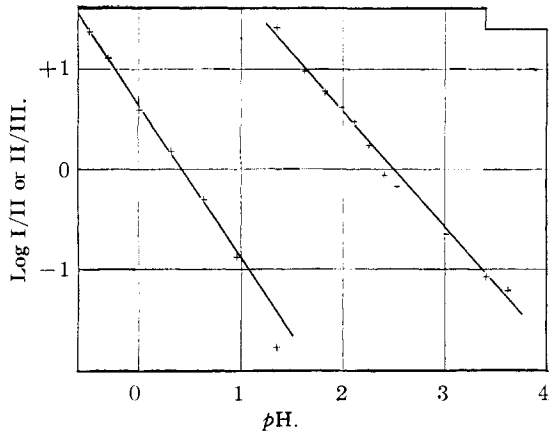


Fig. 2.—Log [I]/[II] and log [II]/[III] plotted against *p*H.

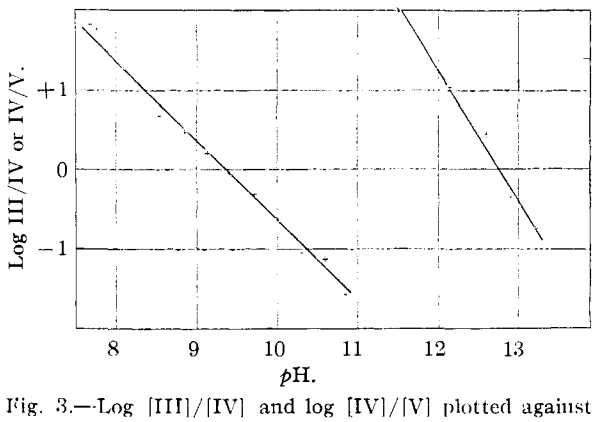


Fig. 3.—Log [III]/[IV] and log [IV]/[V] plotted against *p*H.

Spectroscopic Experiments with Cytochrome c and Fluoride Ion, Hydrocyanic Acid, Azide Ion, and Carbon Monoxide

Types I and II.—*The absorption spectrum of type I is the same as that of "hemin c" in acid solution.* This means that in strongly acidic solutions of cytochrome c *the iron atom is attached only to the four porphyrin nitrogen atoms.*

Raising the *p*H value to about 1.5 gives type II, which is spectroscopically similar to ferrihemoglobin (acid methemoglobin): Hb $^+$, bands: 630, (580), 540–500 mμ. Cytochrome "II": 625, (575), 535–500 mμ.

The order of intensity of the bands is the same in both cases. The only difference between the two spectra is in the displacement toward the blue of the cytochrome bands, compared with those of ferrihemoglobin. This difference can be attributed to the addition of SH-groups to the porphyrin vinyl groups in the cytochrome; a similar displacement toward the blue is found in all derivatives of hemin c, compared with protohemin. The α-bands of the pyridine-hemochromogens are for instance found at 550 and 557 mμ, respectively.

The Fluoride Compound of Ferricytochrome.—We have observed that type II gives a fluoride compound similar to ferrihemoglobin fluoride.

Cytochrome c fluoride is formed only in a fairly narrow range of *p*H, around *p*H 3. The reason is that at higher *p*H values type III, which gives no fluoride compound, predominates, so that only a small amount of type II is present in the solution, and at lower *p*H values the concentration of fluoride ions is very small because of the formation of undissociated hydrofluoric acid. The following experiment illustrates these points.

The fluoride compound of type II thus gives a strong band at 601 mμ and some very flat ones at 565 and 528 mμ, which may partly belong to type III. Ferrihemoglobin fluoride gives a very similar absorption spectrum, 607, (575), 500.

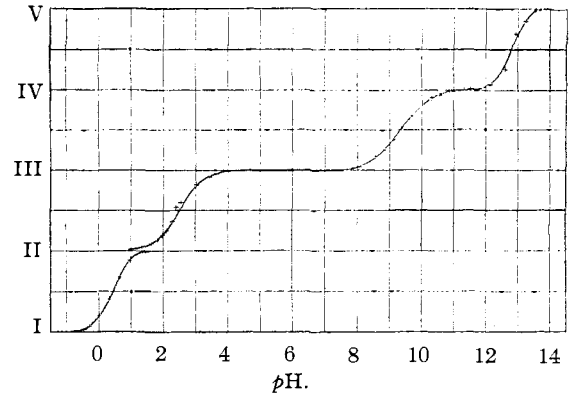
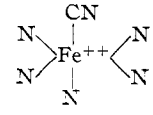


Fig. 4.—"Spectrophotometric titration curve" of ferricytochrome c.

Here we note again the displacement of the bands of the cytochrome derivatives toward the blue, *i. e.*, from 607 to 601 mμ.

Cyanide Compounds of Cytochrome c.—Types I, II, III, and IV of ferricytochrome do not give any compounds with cyanide, but if cyanide is added at *p*H not lower than 13 (type V) the two-banded spectrum 575, 536 mμ is replaced by a single broad band in the green part of the spectrum. Ferrihemoglobin cyanide shows practically the same absorption spectrum. If ferricytochrome cyanide is reduced by $Na_2S_2O_4$, sharp bands appear at 555 and 530 mμ, indicating the formation of a cyanide hemochromogen or ferrocytochrome cyanide, probably



Carbon Monoxide and Ferrocytochrome.—Dilute solutions of ferricytochrome of different *p*H values were introduced into tubes provided with side tubes containing some milligrams of sodium hydrosulfite. After the tubes were filled with pure carbon monoxide the $Na_2S_2O_4$ was mixed with the solution.

The following spectroscopic observations were made.

TABLE VI

Tube no.	Ml. cytochrome solution, 2 mg. per ml.	Ml. NaF, 1 *N*	Ml. HCl, 11.5 *N*	Ml. water	*p*H	Spectral bands	Cytochrome compound
1	2	0.25	0	0.25	6	(565), 530	III
2	2	.25	0.0125	.24	3	601, (565), 528	II F + III
3	2	.25	.025	.23	2	627, 601, (565), 528	II F + II
4	2	.25	.05	.20	1–1.5	627, (565), 535, 495 ?	II
5	2	.25	.1	.15	0.4	632, 540, 500	II + I
6	2	.25	.2	.05	0.1	635, 542, 505	I

1. *p*H = 1.16: at first the bands 550 and 520 appeared, indicating reduction to ferrocytochrome. Within about a minute these bands disappeared and new bands at 563 and 530 became visible. They were about equally dark; 530 was somewhat broader than 563. In appearance the bands closely resemble those of carbon monoxide hemoglobin (570, 537), having moved 7mμ toward the blue.

2. *p*H = 2.0: the bands 550 and 520, first visible after some minutes, were partly replaced by the bands 563 and 530, until 563 and 550 remained about equally dark.

3. *p*H = 2.5: at first 550, 520, then also 563, 530 appeared, 550 remained stronger than 563, that is, less of the CO-compound was formed than at *p*H 2.

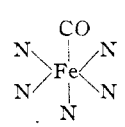
4. At *p*H = 3, 4, 10, 11, and 12 no formation of a CO-compound was observed.

5. *p*H = 13: a mixed spectrum of 550, 520, 563, 530 was observed immediately after adding $Na_2S_2O_4$.

6. *p*H = 14 (*N* sodium hydroxide): 563, 530 appeared immediately.

The only difference between the CO-compounds in acidic and alkaline solution seems to be that the addition of carbon monoxide in acid solution proceeds fairly slowly whereas the addition in alkaline solution is rapid.

The identity of the absorption spectrum of the CO-compound in acidic and alkaline solution renders it very probable that we are dealing with the same configuration in both cases



It was found recently by Keilin and Hartree[3] that the formation of a carbon monoxide compound of ferrocytochrome involves the addition of about 1 mole of carbon monoxide per atom of iron. However, these authors observed some carbon monoxide uptake (0.13 mole of CO/1 Fe) even at *p*H 7, which was believed to depend upon a certain degree of denaturation of the cytochrome. It was therefore of interest to repeat Keilin and Hartree's experiment with our practically pure preparation.

We measured the carbon monoxide uptake of ferricytochrome c in Warburg cups, on reduction with $Na_2S_2O_4$ at *p*H 7 and *p*H 13.3 (0.2 *N* sodium hydroxide). The cytochrome in each cup contained 0.250 mg. of cytochrome iron, which was determined spectrophotometrically (β_{550} of ferrocytochrome = 6.53×10^7 sq. cm./mole); 0.250 mg. of iron corresponds to 100 cu. mm. of carbon monoxide, if the ratio CO:Fe is = 1. The carbon monoxide uptake in ten minutes was found to be

*p*H = 7: 0 cu. mm.
*p*H = 13.3: 95 cu. mm.

Our pure preparation did not take up any carbon monoxide at all at *p*H 7. It was thus entirely free from denatured cytochrome, which was supposed to be responsible for the carbon monoxide uptake in Keilin and Hartree's preparation. For this reason it may seem advisable to use only electrophoretically purified cytochrome c in enzyme studies. At *p*H 13.3, after 95 cu. mm. of carbon monoxide had been taken up in ten minutes, the slow formation of a gas, probably carbon monoxide, was observed (7 cu. mm. in 60 minutes). The simplest explanation seems to be a slow destruction of the cytochrome at this high *p*H. This explanation also accounts for the fact that only 95 cu. mm. instead of 100 cu. mm. of carbon monoxide was absorbed.

Sodium azide did not cause any spectral changes of ferricytochrome c of any type.

Absorption curves of ferricytochrome at different *p*H values in visible and ultraviolet light were determined photoelectrically. The curves are seen in the figures.

Type I was not investigated because of its instability at acidities high enough to give I free from II.

Type II shows the following absorption maxima: 622, 525, 497, 395 and 275 mμ. The band at about 360 mμ, appearing in III–V is not present in type II.

Type III shows the following bands: 695 (sharp band, easily visible in fairly strong solutions), 655 (visible only in highly concentrated solutions), 565, 530, 408, 365 and 280 mμ.

Type IV: 565, 537, 408, 355 and 280 mμ. The most important difference between IV on the one hand, III and V on the other, is the low light absorption of IV in the red region, causing type IV to be bright red in color.

Type V: 565, 536, 412, 350 and 290 mμ. The absorption values in the ultraviolet may be somewhat uncertain, because some alteration of the protein part of the cytochrome probably takes

(3) D. Keilin and E. F. Hartree, *Proc. Roy. Soc.* (London), **B127**, 167 (1939).

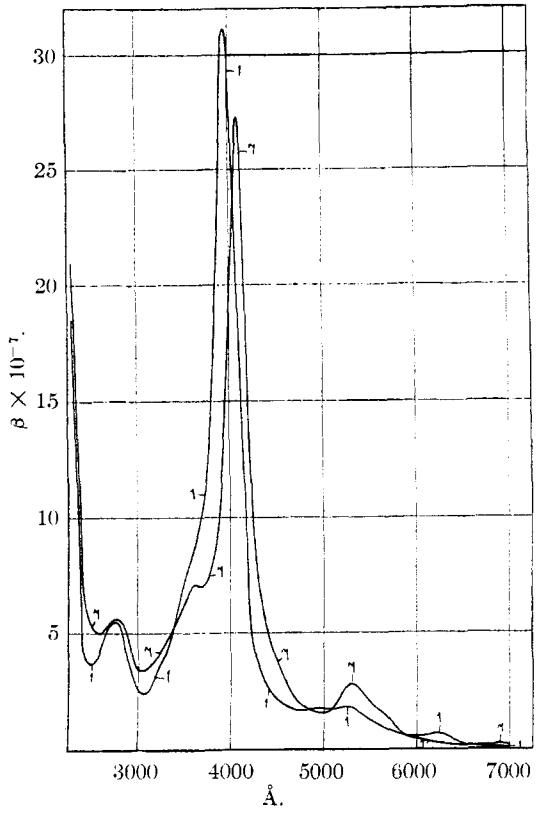


Fig. 5.—Light absorption of ferricytochrome c at *p*H 1 (92% type II + 8% I) and *p*H 7 (type III).

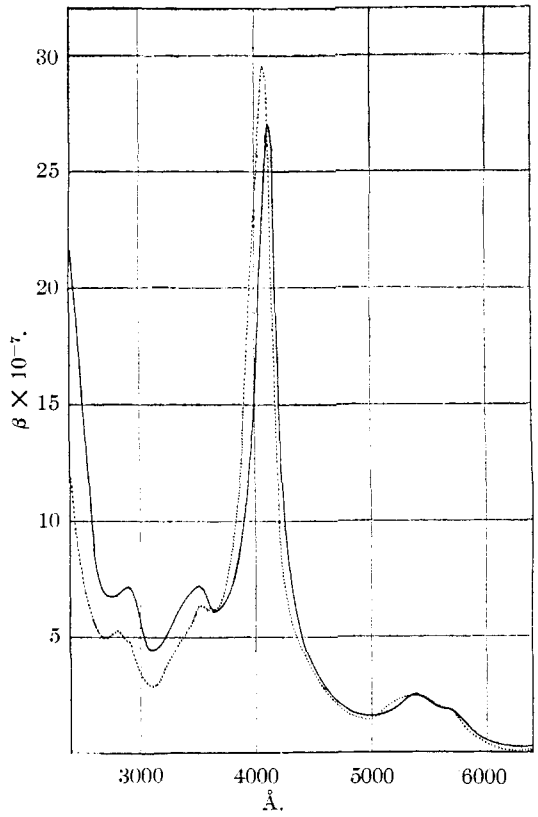


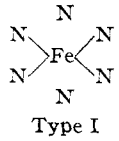
Fig. 6.—Light absorption of ferricytochrome c at *p*H 11.1 - - - - (type IV), *p*H 13.8 —— (type V).

place at this high alkalinity. Nevertheless the light absorption of type V at wave lengths below 360 mμ is considerably higher than for the other types.

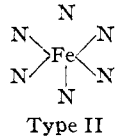
The ultraviolet absorption spectrum of type III does not differ significantly from that previously obtained with cytochrome c containing 0.34% iron.[1] This indicates that the electrophoretic purification does not remove any appreciable amounts of substances containing tyrosine or tryptophan.

Discussion of the Results

The investigation of the optical properties of cytochrome c directly suggests the following constitutions of the different types:

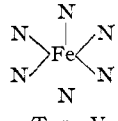


Type I



Type II

Types III and IV



Type V

The four porphyrin nitrogens are written to the right and left of the iron atoms; the two amino acid nitrogens directly above and below.

Types III and IV do not give any compounds with cyanide ion or fluoride ion. Ferrocytochrome does not give any addition compound with carbon monoxide within the range of *p*H where types III and IV of ferricytochrome exist. It thus seems reasonable to assume that in the *p*H range from 3 to 11, in addition to the four porphyrin nitrogen atoms, two nitrogen atoms are firmly attached to the iron atom, preventing the formation of any addition compounds. Types II and V have probably one amino acid nitrogen atom, as well as the four porphyrin nitrogen atoms, firmly linked to the iron, whereas the sixth nitrogen is split off, or only linked by a weak bond, so that CN^- (type V) or F^- (type II) can become linked with the iron. When type I, II or V is reduced, ferrocytochrome, with the typical hemochromogen bands 550 and 520 mμ, is formed, just as upon reduction of types III and IV. This means that both the hemochromogen-forming nitrogen atoms are then linked to the iron atom. However, below

*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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Studies on Cytochrome c. III. Titration Curves

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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).