ACTA CHEMICA SCANDINAVICA 20 (1966) 1476—1486

On the Heterogeneity of Beef Heart Cytochrome c

II. Some Physico-chemical Properties of the Main Subfractions (Cy I — Cy III)

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(1) The heterogeneity of monomeric beef heart cytochrome c previously described,[1] has been confirmed in the present study by moving-boundary electrophoresis in the pH range 8.0—11.2. The various subfractions are all strongly basic proteins, and their isoelectric points [pI(0°C)] in borate buffer, $\mu \approx 0.1$ were found to be Cy I: 10.78 (10.80), Cy II: 10.58 (10.60), Cy III: 10.36 (10.22) in the ferric (ferrous) form. The calculation of the true electrophoretic mobility of Cy I and Cy II is complicated by the fact that Cy I and Cy II are convertible to Cy II and Cy III, respectively, at pH $\gtrsim$ 9.3.

(2) From the curves of the mobility as a function of pH an estimate has been made of the number and nature of the ionizable groups which contribute to the difference in electrophoretic mobility of the subfractions. The difference in electrophoretic mobility of subfractions Cy II and Cy I is explained by the presence of one more negatively charged group (carboxyl) in Cy II than in Cy I since the latter contains one amide group more than the former. The difference in electrophoretic mobility of subfractions Cy III and Cy I is explained by the presence of two more negatively charged groups (carboxyl) in Cy III than in Cy I.

(3) The light absorption spectra of Cy II and Cy III reveal certain differences from those of Cy I characteristic of irreversible conformational changes.

(4) None of the subfractions Cy I—Cy III combine with carbon monoxide at neutral pH.

The heterogeneity of highly purified monomeric cytochrome c * from beef heart muscle was established in a previous study [1] on the basis of disc electrophoresis on polyacrylamide gel and chromatography on a cation exchange resin (Duolite CS-101). Following extraction with dilute sulphuric acid at pH 4.0 the hemoprotein was separated into four subfractions by disc electrophoresis (Cy I 89.0 %, Cy II 9.7 %, Cy III 1.1 %, and Cy IV 0.2 %); the main fraction (Cy I) being the most positively charged at neutral pH.

* The following abbreviation will be used: Cyt. c = cytochrome c.

The purpose of the present work is to give further details of the physicochemical properties of these subfractions.

MATERIALS AND METHODS

The following materials and methods were used in addition to those previously described:[1,2]

Chemicals. HCl (Suprapur ®) was obtained from E. Merck AG, Germany. Urea (reagent grade) was recrystallized twice from ethanol.

Moving-boundary electrophoresis. The electrophoretic mobilities of the subfractions were measured on unresolved cyt. c by the moving-boundary method in a Spinco Model H apparatus; an 11 ml cell being employed. Runs at 0 ± 0.5°C were mostly carried out in borate buffers of ionic strength 0.1;[3] a few runs were also made in phosphate buffers of the same ionic strength. The pH of each buffer was measured by the glass electrode at 20°C, and its value at 0°C was calculated from a reference curve, *i.e.* a plot of pH (20°C) *vs.* pH (0°C).

Cyt. c was concentrated by adsorption onto and elution from a short column of Duolite CS-101,[2] after being fully oxidized (reduced) by the addition of a minimum amount of potassium ferricyanide (sodium dithionite). The concentrated cyt. c solution thus obtained was dialyzed against 1 mM ammonia, and finally against 3 l of buffer selected for the electrophoresis experiment, for 24 h at 0°C. Before electrophoresis run, the cyt. c solution was diluted with this buffer to a concentration of approx. 7 mg/ml (ferric form) and 9 mg/ml (ferrous form). The migration of the boundaries was followed by using the tungsten lamp and a suitable red filter (Wratten No. 25),[4] and photographs were taken from time to time by using red-sensitive Polaroid plates (3000 ASA). The electrophoretic mobilities (u) were calculated from the descending pattern by measuring the distance moved by the components from the initial boundary [5] at one or more periods during the run.

Disc electrophoresis on polyacrylamide gel. In order to separate quantitatively the different subfractions (Cy I—Cy III) the preparative technique of disc electrophoresis [1] was performed. Each fraction eluted from the gel was re-run in an analytical column with the same technique to insure that no contamination had occurred during the cutting of the gel.

Spectrophotometry. Light absorption spectra were obtained by means of a Beckman DK-2 A recording spectrophotometer. For measurements of the absorbancy at a single wave-length a Beckman DU spectrophotometer was used.

The concentration of cyt. c was determined spectrophotometrically after reduction with sodium dithionite in 40 mM phosphate buffer, pH 6.9, using the specific extinction coefficient $E_{1\,\mathrm{cm}}^{1\,\%}$ at 550 mμ red = 23.94 [2] and a molecular weight of 12 130 (calc. on the basis of the known amino acid composition,[6] together with the contribution of the heme group [7]).

Fully reduced or oxidized cyt. c was obtained by the addition of a minimum amount of dithionite or ferricyanide, respectively. The spectra of reduced cyt. c were recorded immediately after the addition of dithionite.

The percentage of combination with carbon monoxide by the different subfractions was determined as described by Tsou,[8] except that the current of purified carbon monoxide (The Matheson Co., Inc., East Rutherford, N.J., U.S.A.) was passed through the cyt. c solution for 10 min *before* reduction with dithionite.

Amino acid and amide nitrogen analyses. Before these analyses, the cyt. c samples were adsorbed onto a column of Duolite CS-101 (sodium form, equilibrated with 40 mM sodium phosphate buffer, pH 6.9), and eluted with a sodium phosphate-chloride buffer, pH 7.5 of high ionic strength.[2] The hemoprotein was collected in a dialysis bag (impermeable to cyt. c) and subjected to electrodialysis at 4°C by placing the bag in a vertical column with an outlet which permitted a continuous slow flow of buffer throughout; a 40 mM sodium phosphate buffer, pH 8.0, was used.

For amino acid analyses portions (1—2 mg) were freezedried and dissolved in 0.6 ml of 6 N HCl, and the air removed from the solution by vacuum (pressure approx. 4 μm

Hg at 23°C). The vials were then sealed and heated at 110°C for 48 h. The hydrolysates were freed of excess HCl on a rotary evaporator, and the amino acids assayed with an amino acid analyser (Spinco, Model 120) by the method of Spackman *et al.*[9] Exactly the same amount of subfractions Cy I and Cy II was hydrolyzed, and the samples were analyzed on the same day. Tryptophan was determined on unhydrolyzed samples by the method of Bencze and Schmid.[10]

The amide nitrogen content was calculated from the amount of ammonia liberated by acid hydrolysis which was carried out in two ways: (*A*) Hydrolysis in conc. HCl at 37°C for 230 h; under these conditions 99 % of the amide-N of free asparagine is released and the amount of ammonia liberated by partial decomposition of the amino acids serine and threonine is negligible.[11] (*B*) Hydrolysis in 5 N HCl at 110°C for 6 h, 12 h, and 24 h in order to correct for the unknown contribution to the ammonia values by destruction of serine and threonine.[11,12]

The ammonia was estimated by a modification of the microdiffusion technique of Conway.[13] Thus, sulphuric acid was used instead of hydrochloric acid in the center well, and the glass lid covering the vessel was smeared with an alkaline fixative (made by boiling water-glass, water and KOH, 20:10:3, w/w).[14] The hydrolysate, containing conc. HCl or 5 N HCl, was diluted 1:10 or 1:5, respectively, and centrifuged to remove humin before assay. This dilution process was necessary in order to avoid contaminating the very dilute standard acid in the center well when the sample was introduced. Quantitative recoveries of added known amounts of ammonium chloride were obtained.

Correction for free ammonia in the unhydrolyzed cyt. c samples was made by determination of the ammonia liberated by mixing cyt. c solution with saturated potassium tetraborate in 10 % KOH (1:2, v/v) giving a final pH of 11.6.[15]

RESULTS

Moving-boundary electrophoresis of unfractionated cytochrome c

Unfractionated cyt. c, obtained in the monomeric form by gel filtration on Sephadex G-75, was examined. The preparation was considered to be 100 % pure since no unspecific protein could be traced by disc electrophoresis on polyacrylamide gel. As previously described,[1] this method revealed four subfractions of cyt. c, *i.e.* Cy I—Cy IV.

Free electrophoresis of cyt. c was complicated by the well known fact that this hemoprotein is difficult to obtain in either fully oxidized or fully reduced form except at pH < 4 and pH > 12 where ferro-cyt. c is rapidly and completely oxidized by molecular oxygen.[16,17] Thus, in neutral or slightly alkaline solutions a slow spontaneous reduction of ferri-cyt. c occurs [18] which is accelerated during electrophoresis.[19] On the other hand, it was also impossible to obtain 100 % reduced cyt. c since a small percentage of the hemoprotein is slowly oxidized during prolonged dialysis and electrophoresis even

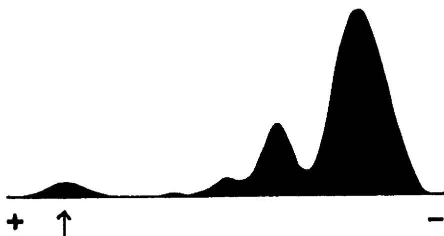

Fig. 1. Tracing of electrophoretic schlieren pattern (descending boundary) of monomeric beef heart cyt. c in the ferric form. Borate buffer of pH 9.04, $\mu \approx 0.1$; 0°C; $t = 51\,540$ sec. The arrow indicates the position of the ε-boundary as almost the same place as the initial boundary. From right to left the components are: Cy I, Cy II, Cy III and a small trace of Cy IV.

at 4 < pH < 12. This occurs in spite of the fact that none of the subfractions Cy I—Cy III (accounting for about 99.8 % of the total material) combine with carbon monoxide at neutral pH (see below). In borate buffer, however, the percentage of the unwanted form was always small, and no difficulties appeared in the interpretation of the electrophoretic pattern obtained either in the ferric or in the ferrous form.

(*a*) *Ferric form.* Ferricyanide has been widely used to obtain cyt. c in the fully oxidized form for enzymic,[20,21] chromatographic,[22–24] and electrophoretic studies.[25] Since this treatment did not alter the electrophoretic mobility of the various isolated subfractions, as determined by the disc electrophoresis technique (in the reduced form), cyt. c was completely oxidized by the addition of a minimum amount of ferricyanide (see methods).

Electrophoresis runs within the range pH (0°C) 8.0—11.2 revealed heterogeneity which was most clearly demonstrable in the lower pH range. Three components were clearly visualized and almost completely separated at, *e.g.*, pH (0°C) = 9.04 (Fig. 1); the percentage concentration of the various components agreed well with that found for Cy I—Cy III by means of disc electrophoresis.[1] Unfortunately, in most of the experiments the component Cy IV was difficult to localize with certainty due to its low concentration. The mobilities of the various components as a function of pH is shown in Fig.

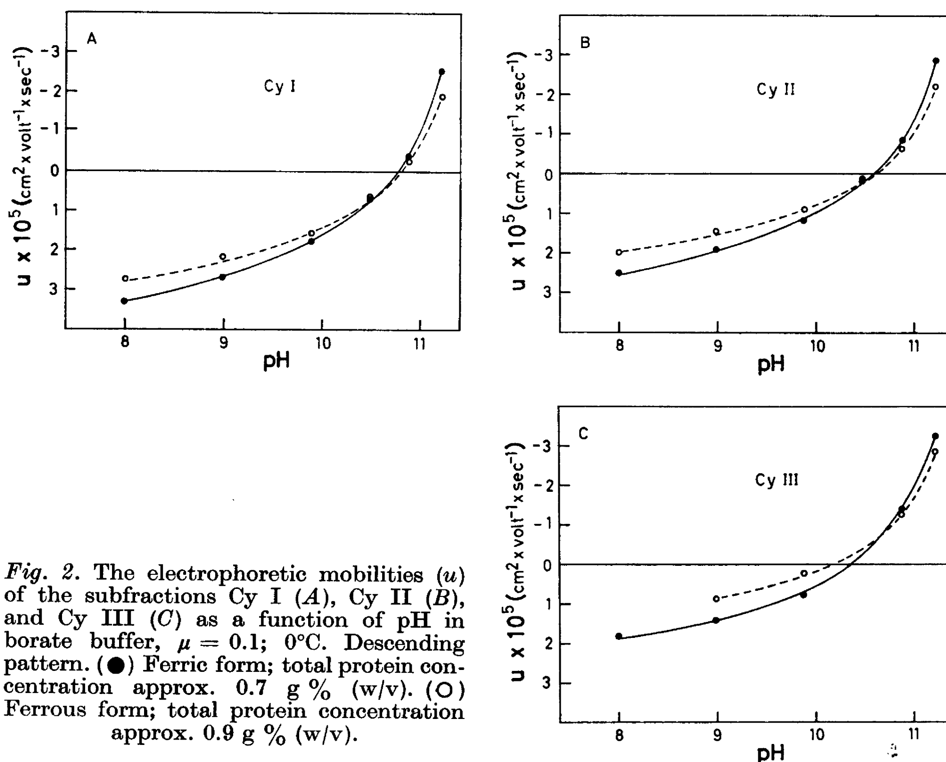

Fig. 2. The electrophoretic mobilities (u) of the subfractions Cy I (*A*), Cy II (*B*), and Cy III (*C*) as a function of pH in borate buffer, $\mu = 0.1$; 0°C. Descending pattern. (●) Ferric form; total protein concentration approx. 0.7 g % (w/v). (○) Ferrous form; total protein concentration approx. 0.9 g % (w/v).

2, A—C, and their isoelectric points are given in Table 1. It is worth mentioning here that the boundary compensation was repeated once during the electrophoresis run at pH 11.2, and that the localization of the component

Table 1. Isoelectric points (pI) of the different subfractions in borate buffer, $\mu \approx 0.1$; 0°C.[a]

	Cy I	Cy II	Cy III
Ferric form	10.78	10.58	10.36
Ferrous form	10.80	10.60	10.22

[a] Calc. from the pH-mobility curves given in Fig. 2, A—C.

Cy III was difficult to observe exactly near its isoelectric point due to interference by the ε-boundary. Its presence, however, was verified by observing the position of the boundaries by the naked eye at the end of the electrophoresis run.

(b) Ferrous form. Cyt. c is most conveniently obtained in the fully reduced form by means of sodium dithionite. This chemical agent could also be used successfully for the present purpose since it was found to have no effect on the stability of the subfractions Cy I—Cy III. Thus, when the various components, isolated by disc electrophoresis on polyacrylamide gel, were treated by dithionite and re-run in disc electrophoresis they still moved as homogeneous fractions with the original mobility. Therefore, the cyt. c solution was completely reduced by the addition of a minimum amount of solid dithionite (see methods).

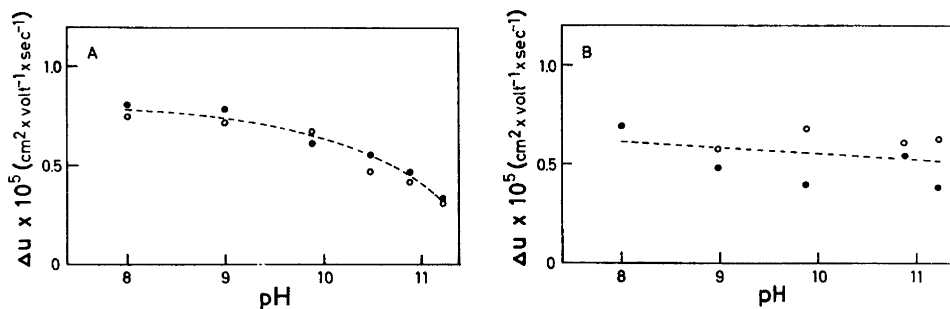

Fig. 3. The difference in electrophoretic mobility between Cy I and Cy II (*A*) and between Cy II and Cy III (*B*) as a function of pH. Data from Fig. 2. The straight line drawn in *B* was calculated by the least-square method. (●) Ferric form and (○) ferrous form.

When reduced cyt. c was run, however, a small component of ferri-cyt. c was observed moving ahead of the main peak of ferro-cyt. c (Cy I); it was most clearly seen from the ascending pattern at the lowest pH values. Otherwise the resolution into components was as good as in the case of ferri-cyt. c. The mobilities of Cy I—Cy III as a function of pH are shown in Fig. 2, A—C, and their isoelectric points are given in Table 1.

(c) Difference in electrophoretic mobility of the subfractions as a function of pH. The difference in electrophoretic mobility of Cy I and Cy II as a function of pH (Fig. 3, A) shows an excellent agreement between the two sets of results obtained in the ferric and in the ferrous form. On the other hand, the values obtained for the difference ($u_{\text{Cy II}}-u_{\text{Cy III}}$) is more widely scattered (Fig. 3, B) since the location of the Cy III boundaries was more difficult to state due to its low concentration.

(d) Effect of buffer anions. A few experiments were carried out in phosphate buffer, $\mu = 0.1$. The same resolution as shown in Fig. 1 was obtained in this buffer, but the ionic mobilities were lower than in borate buffer on the acid side of the isoelectric points (Table 2). The difference in mobility between the individual fractions, however, did not change.

Table 2. Comparison of the electrophoretic mobilities of subfractions Cy I and Cy II (ferrous form) in borate and in phosphate buffer of ionic strength 0.1; pH (0°C) = 8.0.

Buffer anion	Electrophoretic mobility ($u \times 10^5$ (cm^2 $\times$ volt^{-1} $\times$ sec^{-1})	
	Cy I	Cy II
Borate	2.74	1.99
Phosphate	1.40	0.64

(e) Effect of repeated electrophoresis runs in alkaline buffers. When the same cyt. c preparation was re-run for several times at increasing pH values, following concentration on Duolite CS-101,[2] a gradual increase in the percentage concentration of the minor subfractions was observed. This fact indicates that the minor subfractions may arise from Cy I by some sort of modification(s) in alkaline solution. Recent experiments with isolated pure Cy I have shown that this is actually the case, and further studies on the nature of this transformation will be published in a forthcoming paper.[15]

Physico-chemical studies of isolated subfractions

As previously reported,[1] a complete separation of subfractions Cy I—Cy IV is achieved under appropriate conditions by disc electrophoresis on polyacryl-

amide gel, and some physico-chemical properties of the isolated components thus obtained are given below. Unfortunately, the percentage of the component Cy IV was too small to allow accurate analytical data to be obtained, and thus this component will not be included in this study.

(a) Light absorption spectra. For examination of their optical properties, the different subfractions were dialyzed against 40 mM phosphate buffer, pH 6.9.

In the ferrous form, Cy I and Cy II revealed no difference in light absorption spectrum (visible region). On the other hand, from the difference spectrum (Cy III minus Cy I) in Fig. 4, A it is seen that Cy III reveals a slight hyperchromic red shift of the Soret peak in addition to a slightly higher absorbancy level with some minor peaks in the visible part of the spectrum. In the ferric form, however, both Cy II and Cy III revealed a spectrum different from that of Cy I in all regions. From the difference spectrum (Cy III minus Cy I) shown in Fig. 4, B—C, it is seen that the greatest changes were (i) a blue shift of the Soret peak, and (ii) a higher absorbancy level in the ultraviolet regions; the small negative peaks around 290.5 mμ and 230.5 mμ, respectively, are especially noteworthy (see discussion). Similar changes were found in the difference spectrum (Cy II minus Cy I), but here the changes were smaller than

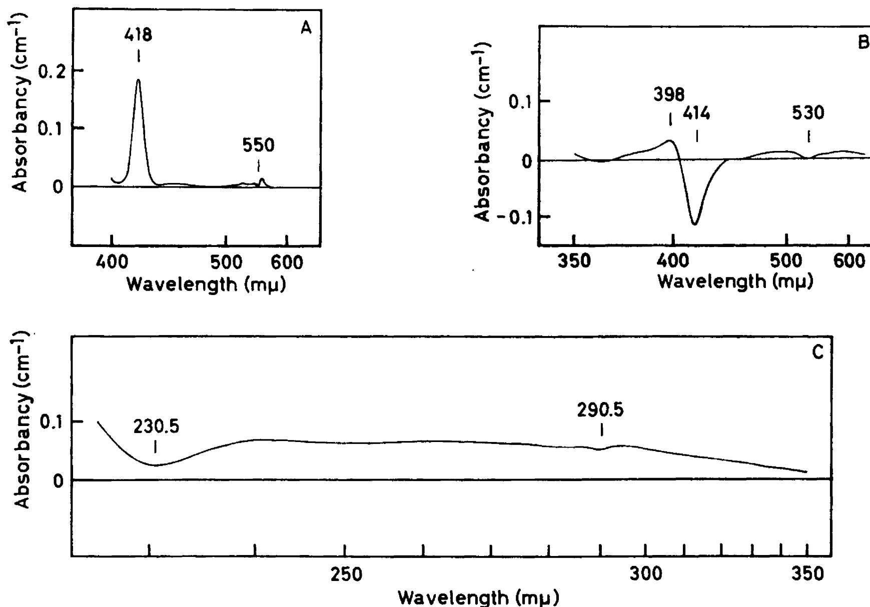

Fig. 4. Difference absorption spectra between Cy III and Cy I (Cy III minus Cy I) in 40 mM phosphate buffer, pH 6.9; 25°C.
A. Ferrous form (dithionite). The concentration of Cy I is 14.3 μM and that of Cy III is adjusted to give the same absorbancy at 550 mμ.
B and *C.* Ferric form (ferricyanide). The concentration of Cy I is 10.5 μM and that of Cy III is adjusted to give the same absorbancy at 530 mμ. This corresponds to an absorbancy at 550 mμ (reduced) of 0.306 (Cy I) and 0.263 (Cy III).

those shown in Fig. 4, B—C. In fact, these difference spectra were quite similar to those obtained for Cy I in aqueous urea (8 M) minus Cy I in phosphate buffer of pH 6.9. It has to be mentioned, however, that in the spectra shown in Fig. 4, the concentrations of Cy I and Cy III were adjusted to give the same absorbancy at 550 mμ (ferrous form) and at 530 mμ (ferric form); slightly different spectra might be expected if the concentrations of hemoprotein are based upon the same heme content.

Table 3. Comparison of the amino acid composition of subfractions Cy I and Cy II [a].

Amino acid	Molar ratio Cy I/Cy II [b]			No. of residues/ mole cyt. c [c]
	Expt. No. 1	Expt. No. 2	Expt. No. 3	
Lys	0.95	0.98	0.99	18
His	0.95	1.00	0.88	3
Arg	0.88	0.76	1.01	2
Asp	0.96	0.99		8
Thr [d]	0.97	0.95		8
Ser [d]	0.97	0.57		1
Glu	0.97	0.96		12
Pro	0.99	0.77		4
Gly	0.95	0.96		14
Ala	0.80	0.96		6
½Cys	0.93	—		2
Val	0.94	0.90		3
Met	1.05	1.04		2
Ile	0.95	0.97		6
Leu	0.96	0.98		6
Tyr	0.99	1.05		4
Phe	0.99	1.01		4
Trp [e]	~1.0			1
Amide-N [f]	1.12			8

[a] Determined from 48 h hydrolysates; for experimental details see text.
[b] Results are given only as relative values since the amino acid composition and sequence already have been determined (Ref. 6). The concentrations of hemoprotein are based on the same value of ε ($cm^{-1} \times mM^{-1}$) at 550 mμ (red) for both subfractions.
[c] Determined on unresolved cyt. c; Ref. 6.
[d] Uncorrected for decomposition.
[e] Calc. spectrophotometrically according to Bencze and Schmied; Ref. 10.
Calc. from Table 4.

(b) Affinity to carbon monoxide. In the ferrous form, neither of the subfractions Cy I—Cy III combine with carbon monoxide at pH 6.9.

(c) Amino acid and amide-N analyses. Subfractions Cy I and Cy II exhibited essentially the same amino acid composition within the error of the method employed (Table 3), except that Cy I contains one more amide group than Cy II (Table 4). The mean value of 8.40 amide-N/molecule for Cy I is in

Table 4. The amide nitrogen content of subfractions Cy I and Cy II.

	Number of amide-N/molecule	
	Cy I	Cy II
Hydrolysis procedure *A* [a]	8.46	7.89
Hydrolysis procedure *B* [b]	8.70	7.50
Mean value of procedure *A* and *B*	8.58	7.69
Corrected mean value [c]	8.40	7.51

[a] The values were calculated (least square method) from estimations on four different concentrations of the hydrolysates.
[b] The values were obtained by extrapolation to zero hydrolysis time (for details see text).
[c] Correction is made for the ammonia-N content of the unhydrolyzed cyt. c preparation. The value 0.18 μmoles NH_3/μmole cyt. c, estimated for Cy I, was used for both subfractions.

very close agreement with that previously recorded for unresolved cyt. c (*i.e.* mainly Cy I).[6,17]

Because only limited amounts of the pure Cy III were available, no amino acid and amide-N analyses were carried out on this subfraction.

DISCUSSION

The present electrophoretic studies confirm previous observations [1] that monomeric beef heart cyt. c is heterogeneous, containing subfractions of different electrophoretic mobilities and isoelectric points. In addition, the heterogeneity was demonstrated equally well in the ferric and in the ferrous form. The smallest component (Cy IV) observed by disc electrophoresis on polyacrylamide gel, however, was difficult to observe by moving-boundary electrophoresis due to its low concentration. Though the relative mobilities of the subfractions do not change, the absolute values were highly dependent on the buffer anion used. Thus, at the same pH and ionic strength, the cyt. c molecules carry a more negative charge in phosphate than in borate buffer, indicating that the borate ions are bound to a lesser extent than the phosphate ions.[26]

There is good agreement between the form of the mobility curve as well as the isoelectric point of ferri-Cy I (pI (0°C) = 10.78 in borate buffer, $\mu = 0.1$) and that obtained for unresolved ferri-cyt. c by Theorell and Åkeson.[17] In contrast to the observations made by Tint and Reiss,[4] the present study reveals that the electrophoretic mobility of cyt. c is dependent upon the state of oxidation-reduction (Fig. 2), which is in accordance with the acid-base titration studies of Theorell and Åkeson.[27] For subfractions Cy I and Cy II the difference in electrophoretic mobility of the oxidized and the reduced form as a function of pH showed an excellent parallelism between the two sets of data. The curves intersect at almost the same pH value (pH (0°C) $\approx$ 10.50). A slight discrepancy

was found between Cy III and Cy I (Cy II), but this difference can not be concluded to be significant since the location of the Cy III boundaries were more difficult to state due to its low concentration.

From the results published previously [2] and those presented in this paper it is clear that all subfractions (Cy I—Cy IV) represent monomeric forms of the hemoprotein, which reveal a difference in net charge; the main fraction (Cy I) being the most positively charged. The difference in electrophoretic mobility (Δu) of Cy I and Cy II at pH 8, *i.e.* 0.75×10^{-5} cm^2 $\times$ volt^{-1} $\times$ sec^{-1} for the oxidized form, is of the same order of magnitude as the difference in electrophoretic mobility of ferri- and ferro-Cy I at pH (0°C) = 8, *i.e.* $\Delta u = 0.56 \times 10^{-5}$ cm^2 $\times$ volt^{-1} $\times$ sec^{-1}. Since acid-base titrations have shown a difference of one equivalent between ferri- and ferro-cyt. c in the pH range 3.5—8.0,[27] and inasmuch as a linear relationship exists between the migration rate of a protein in free electrophoresis and its net charge,[28] it is reasonable to calculate that Cy I carries approx. one positive charge more or one negative charge less than Cy II at pH = 8. This calculation is in complete agreement with the demonstrated presence of one more carboxyl group in Cy II than in Cy I; the latter contains one amide group more than the former (Table 4). By increasing the pH of the buffer, however, the mobility curves of Cy I and Cy II suggest an approach to each other (Fig. 3, A). This phenomenon may, at least in part, be explained by the fact that Cy I converts to Cy II at pH $\gtrsim$ 9.3 (borate buffer, $\mu \approx 0.1$, 4°C).[15] On the other hand, there is a more constant difference in net charge between Cy II and Cy III over the pH range 8.0—11.2 (Fig. 3, B), since Δu as a function of pH is reasonably described by a straight line which is nearly parallel with the pH axis. Unfortunately, the points are widely scattered round this line since the localization of the Cy III boundaries were more difficult to state due to its low concentration. The line fits in well with a difference in net charge of one, *i.e.* the difference in electrophoretic mobility of Cy III and Cy I is explained by the presence of two more negatively charged groups (carboxyl) in Cy III than in Cy I.

In a preceding paper [2] all subfractions were stated to be in the native form. This conclusion was based primarily upon the fact that the unresolved cyt. c preparation did not combine significantly with carbon monoxide at pH 7. However, from the data presented here it is evident that all subfractions Cy II—Cy IV reveal an increasing degree of irreversible conformational* change as compared to the main subfraction (Cy I). This conclusion is based on the deviating absorption spectrum of Cy II and Cy III as compared to that of Cy I (Fig. 4). Thus, in the ferric form Cy II and Cy III reveal absorption spectra which are very similar to those obtained by exposing Cy I to different high concentrations of a strong denaturing agent, *e.g.* urea. The negative peaks at 290.5 mμ and 230.5 mμ of the difference spectrum (Fig. 4) are especially noteworthy, since the same peaks are seen in all types of difference spectra of Cy I where a conformational change does occur, *i.e.* in urea, in acid solution,[29] and at high temperatures. These problems will be discussed in further detail in a forthcoming paper.[15]

* The term *conformation* is used synonymously with the sum of the secondary and tertiary structures of the protein molecule.

Note added in proof. Since the acceptance of this manuscript for publication, my attention has been called to a recent review by Margoliash and Schejter (*Advan. Protein Chem.* **21** (1966) 113). These authors mention that preparations of horse and beef heart cytochrome c made by "acid extraction" contain, in addition to different polymers, monomeric molecules that have lost one or more of the 8 amide groups in the native protein. They claimed as many as 15 deamidated species of horse heart cytochrome c, but gave no details on the separation and the physico-chemical properties of these components. However, all the deamidated forms were interpreted to be artifacts *i.e.* to be a result of the purification procedure ("acid extraction").

Acknowledgement. I am grateful to Professor H. Theorell for his stimulating interest and discussions given during the course of this study and for his kind hospitality. Thanks are due to Docent Å. Åkeson for valuable discussions. It is also a pleasure to thank Mr. A. Baldesten for performing the amino acid analyses.

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Received February 25, 1966.