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On the Heterogeneity of Beef Heart Cytochrome c

I. Separation and Isolation of Subfractions by Disc Electrophoresis and Column Chromatography

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The heterogeneity of beef heart cytochrome c, obtained in the native, monomeric form by gel filtration on Sephadex G-75, has been studied, and the following results were obtained:

(1) By means of disc electrophoresis on polyacrylamide gel cytochrome c, extracted by dilute sulphuric acid at pH 4.0, was separated into four subfractions (Cy I 89.0 %, Cy II 9.7 %, Cy III 1.1 %, and Cy IV 0.2 %). A heterogeneity was also demonstrated by chromatography on Duolite CS-101, but five components were then consistently revealed. The subfractions separated by electrophoresis and chromatography were shown to be compatible with each other except that two of the chromatographic fractions demonstrated almost the same mobility on disc electrophoresis.

(2) Almost the same percentage distribution of subfractions was obtained whether cytochrome c was extracted by dilute sulphuric acid or by distilled water/saline-buffer. Prolonged autolysis of the minced muscle before extraction, however, resulted in a higher percentage of the minor subfractions (Cy II – Cy IV), but no conclusive evidence for interconvertibility was obtained.

(3) The distinctions so far observed between the various electrophoretic and chromatographic subfractions of cytochrome c is a difference in net charge; the main fraction (Cy I) being the most positively charged at neutral pH. Detailed chemical and physical properties are not yet established.

The preparation of highly purified cytochrome c** from beef heart muscle was described in a previous paper.[1] The hemoprotein was obtained in the native, monomeric form, as indicated by a low percentage of autoxidizable cyt. c (<0.5 %) and homogeneity in gel filtration. Since no heterogeneity for a preparation *fitting these criteria* has been reported so far, but on the contrary has been denied in a recent review by Margoliash and Lustgarten,[2]

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

further studies of this problem were undertaken. The present paper describes an electrophoretic technique and a chromatographic procedure, by which heterogeneity of the hemoprotein obtained by gel filtration can easily be demonstrated. Finally, evidence is presented to show that the subfractions obtained probably represent true *in vivo* variants, and not simply artefacts resulting from the preparation procedure.

MATERIALS AND METHODS

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

Sephadex G-200 powder was obtained from Pharmacia, Uppsala, Sweden.

Chemicals. Acrylamide monomer was obtained from Matheson, Coleman & Bell, Norwood, Ohio, U.S.A. and N,N′-methylene-bisacrylamide monomer from Eastman Kodak, Rochester & New York, U.S.A.

Cytochrome c was prepared from beef hearts by two alternative procedures: (*A*) By the procedure described earlier,[1] whereby cyt. c was extracted by dilute sulphuric acid at pH 4.0. (*B*) By a modification of procedure *A*; cyt. c was here extracted by distilled water/saline-buffer. Single beef hearts were freed from fat and connective tissue, minced and homogenized with an equal volume of distilled water in a teflon-glass Potter-Elvehjem homogenizer at 0°C. Solid NaCl was then added to give a final concentration of approximately 0.4 M. The homogenate was centrifuged (10 000 × *g*, 30 min, 4°C), the supernatant saved, and the sediment was extracted once more with an equal volume of a 60 mM sodium phosphate buffer, pH 7.5, to which solid NaCl was added to give 0.4 M. After re-centrifugation, the combined supernatants were immediately subjected to gel filtration on Sephadex G-75.[1] Some particulate material, migrating rapidly through the column, was discarded, and was followed by four coloured fractions: (1) A golden coloured fraction; (2) a hemoglobin fraction; (3) a myoglobin fraction; and (4) a cyt. c fraction; the separation of cyt. c from myoglobin was, however, far from complete. The effluent containing cyt. c was dialyzed and concentrated on a column of Duolite CS-101,[1] myoglobin and some other unspecific proteins passing through. The cyt. c eluted from this column usually demonstrated a ratio $A_{550\ red}/A_{280\ ox} \approx 1.0$, and this ratio was further increased to 1.22 – 1.23 by re-chromatography on Sephadex G-75.[1]

For purposes of comparison, the heterogeneity of cyt. c, purified from horse and rat heart, was also studied. The former was purchased from the Sigma Chemical Co., U.S.A. (type 3), and the latter was purified from albino rats of the Wistar strain by procedure *B* (see above). The animals were killed by exsanguination under ether anesthesia, and the hearts immediately removed for homogenization and extraction.

Disc electrophoresis on polyacrylamide gel

Electrophoresis on polyacrylamide gel was carried out according to Ornstein and Davis[3] and Broome,[4] with some modifications to fit the present purpose.

A 2,6-lutidine/glycine – KOH/glycine buffer system has been suggested for the separation of basic proteins,[5] and this system was successfully used in the present study; an even better resolution was, however, obtained by replacing K^+ with NH_4^+. The standard procedure, described in the following text, was found empirically to give optimal separation of subfractions.

Stock solutions. (*A*) NH_4OH-glycine buffer (pH 8.6):NH_4OH 1 N, 10 ml; glycine, 20 g; N,N,N′,N′-tetramethyl-ethylenediamine, 0.75 ml; water to make 100 ml. (*B*) NH_4OH-glycine buffer (pH 9.6): NH_4OH 1 N, 48 ml; glycine, 4.8 g; water to make 100 ml. (*C*) 40 mM ammonium phosphate buffer, pH 6.9. (*D*) Acrylamide solution: Acrylamide, 60 g; N,N′-methylene-bisacrylamide, 2.5 g; water to make 100 ml. (*E*) Riboflavin solution:[3] Riboflavin, 4.0 mg; water to make 100 ml. (*F*) The tray buffer (pH 8.3):[5] Glycine, 13.7; 2,6-lutidine, 38.2 ml; water to make 1 litre.

Preparation of lower (small-pore) gel (pH 7.2). For polymerization, the solutions A, D, E, and water were mixed (1:2:1:4,v/v) to yield a solution containing 15 % acrylamide and 0.6 % N,N'-methylene-bisacrylamide; the solution could be stored for days in darkness at 4°C until used. Entrapped air was removed, and glass tubes (internal diameter 4.8 mm) were filled with approximately 0.8 ml of this solution [3] and photopolymerized (a 40 W daylight fluorescent lamp was used). The gel started to polymerize within 1 min, but it was exposed to light for at least 30 min. When polymerization had proceeded for 10 min, the water layered on top of the gel was replaced by buffer containing solutions A, E, and water (1:1:6, v/v); this procedure was found necessary to avoid cone-shaped artefacts of the protein zones during electrophoretic separation. Reproducible elctrophoretic patterns were obtained when the gel was used within 12 h.

Preparation of upper ("spacer") gel (pH 9.4). Buffer B, buffer C, and distilled water were mixed (1:1:1, v/v), and a suitable amount of dry Sephadex G-200 power was added. After swelling and equilibration, the excess of buffer was removed, and a 0.3—0.5 cm layer of the gel was placed atop the lower gel; special care was taken to avoid trapping of air bubbles in the gel mixture.

Preparation of sample gel (pH 9.4). The cyt. c solution, dialyzed against 40 mM ammonium phosphate buffer, pH 6.9, buffer B and distilled water were mixed (1:1:1, v/v) and cyt. c was completely reduced by adding a minimal amount of solid dithionite. Dry Sephadex G-200 was added to this mixture, and the particles were allowed to swell for about 5 min. A layer of this gel was carefully placed atop the "spacer" gel, the height depending on the concentration of cyt. c in the sample. The upper part of the tube was then filled with tray buffer, and the procedure completed as described by Ornstein and Davis.[3]

Electrophoresis run. An apparatus, commercially available from Canal Industrial Corporation, Bethesda, U.S.A., and a power supply for maintaining a constant current, were used. The electrophoresis was run at room temperature at a current of 3.5 mA for 20—30 min (depending on the height of the sample gel); only 10 min were required for separation in the lower gel. One gel was run in each experiment.

Localization of cytochrome c in the gel. When electrophoresis was completed, the gel was immediately removed, and sectioned into two equal parts. One was stained for peroxidase activity by a benzidine-peroxide-nitroprusside reagent,[6,7] and the other for protein by a 1 % solution of Aniline Blue Black in 7 % acetic acid.[3]

Special care had to be taken to avoid damage of the gel during its removal from the glass tube; after rimming the gel, it was removed by applying hydraulic pressure from a water-filled rubber bulb.

Preparative electrophoresis. Electrophoresis was carried out as described above, but the internal diameter of the glass tube was 1.05 cm, and the electrophoresis was run at a current of 10 mA. The individual components of ferro-cyt. c, clearly visible to the naked eye, were obtained by cutting the gel with a razor blade; the relative height of the segments was 5/2/2/2 (Cy I/Cy II/Cy III/Cy IV). The cut gel sections were finely minced in a conical centrifuge tube, and cyt. c was eluted by a 40 mM ammonium phosphate buffer, pH 6.9. Enough buffer was added to give a total volume of 5 ml (fraction Cy I) or 2 ml (the other fractions), and the mixture equilibrated for 48 h at 4°C before centrifugation (10 000 × *g*, 15 min, 4°C). The concentration of cyt. c in the supernatant was assayed spectrophotometrically at 416 mμ after reduction with dithionite.

Alternative procedure. The disc electrophoresis technique described by Reisfeld *et al.*[8] for the separation of basic proteins and peptides, using the buffer system β-alanine/acetic acid-KOH/acetic acid, was also tried, but cyt. c migrated as a single component in this system.

Chromatography on Duolite CS-101

The chromatographic column, a piece of polyethylene tubing, was mounted within a cylindrical glass tube, special care being taken to get the column straight. The polyethylene tubing was supplied with a capillary outlet of glass, and a small piece of cotton wool was laid on this capillary. The column (1.4 × 16 cm) was packed and equilibrated with 40 mM ammonium phosphate buffer, pH 6.9, and cyt. c adsorbed onto the resin, as previously described for Duolite.[1] Just after the cyt. c solution had passed into the

column, the chromatogram was developed by the buffer used for equilibration of the column; flow rate 9—10 ml/h. To avoid photo-reduction or oxidation,[9] the column was supplied with a dark cover.

When the desired degree of separation into subfractions was obtained, the flow of buffer was discontinued while all the cyt. c was still on the column. The polyethylene tubing was carefully separated from the glass tube, and each zone of cyt. c was cut out by a razor blade, starting at the top of the column. The resin of each segment was suspended in a suitable volume of the elution buffer, packed into a column, and cyt. c eluted;[1] the eluate was collected into a volumetric flask (2 or 5 ml). By means of this procedure, the minor cyt. c components were obtained at reasonable concentrations.

Spectrophotometry. Absorption spectra were obtained by a Beckman DK-2 A recording spectrophotometer; otherwise a Beckman DU spectrophotometer was used. The concentration of cyt. c was determined spectrophotometrically after reduction with dithionite in 65 mM sodium phosphate buffer, pH 6.8, using the specific extinction coefficient $E^{1\%}_{1cm}$ at 550 mμ red = 23.94.[1] The percentage of reduced (oxidized) cyt. c was calculated from A_{550} and $A_{550\ red}$ using the ratio $E_{550\ red}/E_{550\ ox} = 3.63$.[1]

RESULTS

Cyt. c, purified by procedure *A*, was used unless otherwise stated. It was obtained in the native, monomeric form, as indicated by the low percentage of autoxidizable hemoprotein (<0.5 %) and the homogeneity in gel filtration.[1] However, disc electrophoresis on polyacrylamide gel or chromatography on a weakly acidic cation-exchange resin of the carboxylic acid type, *i. e.* Duolite CS-101, disclosed heterogeneity in this material.

Disc electrophoresis on polyacrylamide gel

A great many factors influence the migration pattern of proteins in disc electrophoresis,[3] but only the main problems concerned with the present study, will be dealt with.

(*a*) *The standard procedure* revealed the presence of one major and three minor subfractions of cyt. c, which will be designated by the numbers Cy I — Cy IV (Fig. 1). They could all be seen as pink zones by the naked eye if a sufficient amount of cyt. c was applied (*e.g.* 0.1 mg), and preparative disc

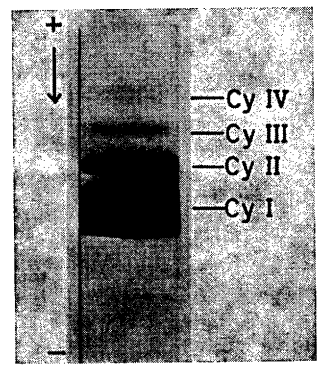

Fig. 1. Photograph of disc electrophoresis pattern of fully reduced cyt. c purified from beef heart muscle by procedure *A*. Aniline Blue Black staining revealed four zones: Cy I, Cy II, Cy III, and Cy IV; they were all visible as pink zones in the unstained gel and demonstrated a positive peroxidase staining. Approximately 0.1 mg cyt. c was applied. Standard procedure (for details see text).

Table 1. Percentage distribution of the subfractions of beef heart cyt. c isolated by preparative disc electrophoresis.

Material[a]		Percentage of total			
		Cy I	Cy II	Cy III	Cy IV
Cyt. c extracted by means of dilute sulphuric acid (procedure *A*)	Mean ($n = 4$)[b]	89.0	9.7	1.1	0.2
	Range	88.1-90.1	8.8-10.5	1.0-1.2	0.1-0.3
Cyt. c extracted by means of distilled water/ saline-buffer (procedure *B*)[c]	Mean ($n = 2$)[d]	87.1	11.4	1.3	0.2

[a] Approximately 1.5 mg cyt. c were applied in each electrophoresis run.
[b] n = number of assays on one preparation obtained from pooled beef hearts.
[c] The extraction was carried out 1 h after killing the animal.
[d] n = number of assays on one preparation obtained from a single beef heart.

electrophoresis gave their relative percentages as: Cy I 89.0 %, Cy II 9.7 %, Cy III 1.1 %, Cy IV 0.2 % (Table 1). A small fraction of unspecific protein could be traced travelling behind Cy IV when a larger amount of cyt. c was applied (*e.g.* 0.3 mg); this fraction stained with Aniline Blue Black, but not with the peroxidase stain for cyt. c. All the subfractions of cyt. c, however, gave a strongly positive reaction with the latter stain. Each subfraction appeared quite homogeneous and maintained its relative mobility when re-run on disc electrophoresis.

(*b*) *Effect of reducing and oxidizing agents.* A slow reduction of ferri-cyt. c occurred during the electrophoresis run. Thus, when fully oxidized cyt. c (obtained by ferricyanide) or a mixture of ferro- and ferri-cyt. c was subjected to electrophoresis, the same subfractions of ferro-cyt. c as described above appeared, but the zones were less distinct than with chemical reduction before electrophoresis. The reduction observed during electrophoresis, is in good agreement with that previously reported in paper electrophoresis experiments,[10] and with the spontaneous reduction which is obtained in faintly alkaline solution.[11]

Cyt. c was usually reduced by dithionite. The same electrophoretic pattern was, however, obtained when cyt. c was reduced by Pd-H_2.[12]

(*c*) *Effect of the composition of small-pore gel.* The porosity (*i.e.* the degree of cross-linking) of the lower gel was important for the relative mobilities of the subfractions, but not for their total number. This was observed in a series of experiments, where the concentration of acrylamide was held constant at 15 % and the concentration of N,N′-methylene-bisacrylamide ("bis") was varied. The lowest concentration of "bis" (0.1 %) gave a soft gel, and the highest concentration (0.6 %) a hard gel which required special care for its removal from the glass column; the harder the gel, the better was the separation of components Cy I — Cy III from each other. The hardest gel, which could be removed without disruption, was preferred in the standard procedure.

Residues in the gel of ammonium persulphate, commonly employed to initiate polymerization, were found to oxidize cyt. c during its migration in the gel. Riboflavin, however, did not produce this side effect, and was therefore preferred. In the standard procedure, no effect on the migration pattern attributable to the presence of catalyst residues, was observed. This was tested by removing any free catalyst by electrophoresis; the buffer used in preparing the gel, served as tray buffer.

Chromatography on Duolite CS-101

Heterogeneity of cyt. c was also demonstrated by chromatography on Duolite CS-101 (Fig. 2). As the solution passed into the resin column, oxidized cyt. c lodged as a dark zone at the top of the column and reduced cyt. c as a pink coloured zone just below (Fig. 2,a). The chromatogram was developed by 40 mM ammonium phosphate buffer, pH 6.9, and when approximately 40 ml had passed through, subfractions of ferro-cyt. c began moving slowly down the column. When about 675 ml had passed through, cyt. c had separated into six distinct zones (Fig. 2,b). The slowest migrating component (O) had a dark red colour, and consisted of 98 % oxidized cyt. c; in the other fractions (R-I to R-V) cyt. c was obtained mainly in the reduced form (Table 2). When the nearly colourless zones between the components (except R-I and R-II) had reached a minimum of 2 mm, the column was cut, and the fractions of cyt. c recovered by elution. Analytical data are given in Table 2. If the chromatogram was further developed, fraction R-V was difficult to localize on the column with the naked eye; on the other hand, fraction O was then obtained in the completely oxidized form.

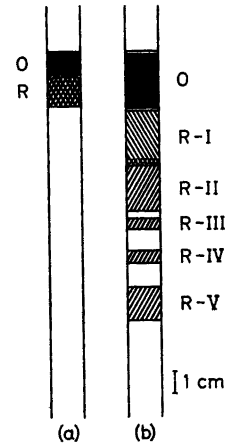

Fig. 2. Chromatography of beef heart cyt. c on Duolite CS-101. (a) At the start of the chromatography. As the cyt. c solution (approximately 70 mg in 15 ml) passed into the resin column, the oxidized form appeared to lodge as a dark red zone at the top of the column (O), and some of the reduced form as a pink coloured zone just below (R). (b) The migration pattern obtained after about 675 ml buffer had passed through (flow rate 9.3 ml/h). O = dark colour of ferri-cyt. c, and R = pink colour of cyt. c obtained mostly in the ferrous form. The R values[13] of fractions R-I and R-V were calculated to be 0.006 and 0.02, respectively. The separation of fraction R-II from R-I was not complete; no colourless zone was obtained between these two fractions. The great difference in colour intensity and the disc electrophoresis pattern (*cf.* Fig. 3), however, revealed the presence of two separate fractions. For details see text.

Table 2. Analyses of various chromatographic fractions of cyt. c obtained from the same experiment as in Fig. 2.

Fraction	Percentage of Σ fractions R-I to R-V	Percentage of total	Degree of reduction (%)	Disc electrophoresis[a]
R-I	67.0	52.1	80.3	Two components
R-II	26.1		86.2	Two components
R-III	2.8		70.8	Homogeneous
R-IV	2.7		68.1	Homogeneous
R-V	1.4		59.6	Homogeneous
O	—	47.9	1.9	Four components

[a] *Cf.* Fig. 3.

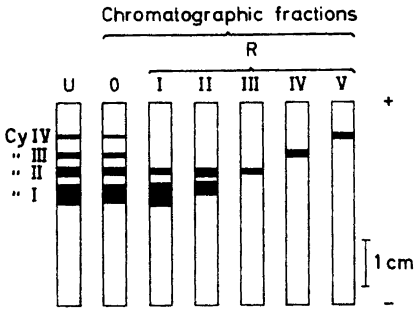

Fig. 3. Diagram of disc electrophoresis pattern of the cyt. c fractions obtained by chromatography on Duolite CS-101 (*cf.* Fig. 2). O: The fraction which consisted of 98 % oxidized cyt. c. R: Cyt. c fractions mainly in the ferrous form. U: The pattern obtained on unchromatographed material (*cf.* Fig. 1). Standard procedure; all fractions of cyt. c were fully reduced by dithionite before their electrophoretical examination.

In order to relate the individual chromatographic components to those previously separated by disc electrophoresis, each fraction was subjected to the latter procedure (Fig. 3). As to be expected, fractions R-I and R-II were electrophoretically heterogeneous, containing both Cy I and Cy II. Fractions R-III, R-IV and R-V, however, were electrophoretically homogeneous; R-III demonstrated the same (or almost the same) mobility as Cy II, and R-IV and R-V the same mobility as Cy III and Cy IV, respectively. The ferri-cyt. c fraction (O), when reduced, separated into the usual four subfractions on disc electrophoresis. Thus, the subfractions separated by disc electrophoresis and by chromatography on Duolite CS-101 were compatible with each other; the electrophoretically slowest component (Cy IV) showed the highest R value on chromatography, and the fastest component (Cy I) demonstrated the lowest R value.

The effect of the extraction procedure on the pattern of heterogeneity

For the purpose of comparison, and to exclude the possibility that the acid extraction (pH ≈ 4.0) had produced artefacts responsible for these

subfractions, cyt. c was extracted by distilled water/saline-buffer (procedure *B*). When subjected to disc electrophoresis, these cyt. c preparations all demonstrated the same heterogeneity as shown in Fig. 1, and the same relative amount of the individual components were obtained by preparative disc electrophoresis (Table 1).

Investigations into the interconvertibility of the subfractions

One of the criteria used to show that multiple forms of an enzyme represent in fact different molecules, is that each form, when separately put through the fractionation procedure again, should not be converted into the other forms. The subfraction Cy I, isolated by preparative disc electrophoresis, was therefore subjected to preparative procedure *B*. When re-run in disc electrophoresis, the component still moved as a single fraction with the originally mobility. Neither freezing and thawing nor prolonged storage (one week) at 4°C had any effect on the electrophoretic behaviour of the various subfractions.

Margoliash and Lustgarten[14] have shown that polymers of cyt. c are rapidly transformed to the monomeric form by means of 4 M urea. Therefore, in order to further exclude the possibility that the minor subfractions (Cy II— Cy IV) being polymers of cyt. c, the hemoprotein was pretreated by 4 M urea at 20°C for 1/2 h before the electrophoresis run. No effect was, however, observed on the migration pattern of Cy I to Cy IV following this treatment as compared with that obtained in the standard procedure.

The effect of autolysis on the pattern of heterogeneity. By using beef hearts, it has been impossible to reduce the time (1 h) which elapsed after killing the animal until the extraction could be carried out. Therefore, to get a clearer

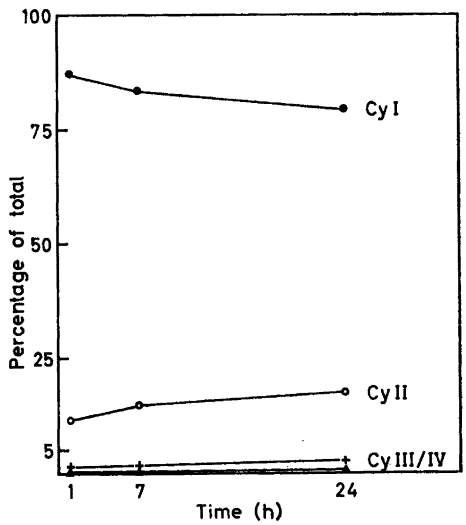

Fig. 4. Effect of autolysis on the percentage distribution of the subfractions of cyt. c isolated from beef heart muscle. The abscissa indicates the time which elapsed after killing the animal until the extraction (procedure *B*) was performed; the minced heart muscle was kept at 22°C from the time of the first extraction (1 h specimen). Symbols as in Fig. 1. The fractions were isolated by preparative disc electrophoresis, and each preparation was run in duplicate; approximately 1.5 mg cyt. c were applied in each electrophoresis run.

idea of the effect of autolysis,* the minced muscle was left at room temperature for some time until the extraction by distilled water/saline-buffer was performed (Fig. 4). It is seen that the longer the autolytic process was allowed to proceed, the higher was the percentage of the minor subfractions (Cy II – Cy IV).

Heterogeneity of rat and horse heart cytochrome c. When disc electrophoresis was used, cyt. c from rat heart revealed four subfractions and the commercial preparation of horse heart five subfractions; the latter preparation was not subjected to further purification before electrophoresis, and its content of modified cyt. c was unknown.

DISCUSSION

Since the introduction of cation-exchange resins in the purification of cyt. c,[15] separation of the hemoprotein into subfractions has been described by several authors. Thus, if the hemoprotein is extracted from beef or horse heart muscle by TCA according to Keilin and Hartree,[16] and chromatographed on Amberlite IRC-50 or XE-64, three fractions are usually obtained;[15,17–20] these are a small fraction of reduced cyt. c (I), and two fractions (II and III) of oxidized cyt. c. The minor component (III) is eluted only at a higher pH and ionic strength than fractions I and II, and it has been shown to represent a series of polymers of cyt. c as well as some denaturated cyt. c.[14] These components have been clearly shown to be artefacts resulting from the purification procedure (effect of TCA).[14,17,21] When the fractions I and II, however, were subjected to re-chromatography on Amberlite, no heterogeneity could be demonstrated except a different migration rate of the reduced and the oxidized form,[15,17,18,20,21] which has also been observed in electrophoresis experiments.[10,22] Inhomogeneity of cyt. c preparations, purified and crystallized from beef heart muscle following extraction with dilute sulphuric acid at pH 4.1, has further been demonstrated by Paléus and Theorell [23] by means of moving-boundary electrophoresis. The hemoprotein revealed three different peaks of the completely reduced form, all moving towards the cathode. The possible relationship of these components to the chromatographic fractions described above was not studied, but has later been suggested [24] since a further improved preparative method gave a crystalline preparation which appeared to be homogeneous on moving-boundary electrophoresis.[25] In the present study, however, the native, monomeric form of beef heart cyt. c of the completely reduced form has been separated into four subfractions by disc electrophoresis on polyacrylamide gel, and five by chromatography on Doulite CS-101. It has been shown quite clearly that this heterogeneity is not related to the chromatographic fractions already discussed.

The homogeneity in gel filtration indicated that the cyt. c preparation, purified by procedure *A*, contained only the monomeric form of the hemoprotein,[14] and the low degree of autoxidability (<0.5 %) that the hemoprotein was obtained in the native form.[26,27] To exclude the possibility that the het-

* Autolysis is here used in the sense of the complex chemical changes which occur in tissues after death.[33]

erogeneity could nevertheless be artefacts arising from the extraction with dilute sulphuric acid at pH 4.0 (procedure *A*), cyt. c was also purified by a milder method. However, extraction of cyt. c by distilled water/saline-buffer (procedure *B*) yielded the same pattern of heterogeneity on disc electrophoresis as that obtained in procedure *A*. Further, the resolution of cyt. c into its constituent subfractions is nearly the same in the two analytical methods used, *i.e.* disc electrophoresis and chromatography.

The effect of autolysis on the percentage distribution of the cyt. c subfractions is, however, of paramount importance. Prolonged autolysis of the minced muscle before extraction resulted in a higher percentage of the minor subfractions (Cy II — Cy IV) which may be interpreted as a result of a proteolytic or another chemical effect on the hemoprotein *per se*. At the present time, however, no conclusive evidence for interconvertibility has been obtained. Based on the fact that cyt. c of rat heart, extracted immediately upon its removal, gave the same pattern of heterogeneity, it is very likely that the electrophoretic and chromatographic components observed, represent true *in vivo* variants of the hemoprotein and not simply artefacts resulting from the preparation procedure.

The heterogeneity of cyt. c demonstrated in the present study, is partly in agreement with that previously described by Paléus and Theorell.[23] The divergence in the number of subfractions as well as their relative percentages may be due to: (1) The differences in the purification procedure. Thus, Paléus and Theorell [23] did not include "some small, coloured fractions" which were initially separated at low ionic strength during chromatography on CM-W cellulose in the final purification procedure. (2) The difference in the analytical methods. The quantity of the smallest component (Cy IV), demonstrated by disc-electrophoresis, may be too small to be detectable by moving-boundary electrophoresis as was the case in earlier studies on isoenzymes of lactic dehydrogenase.[28] Since the latter procedure requires a lot of time and material, the use of disc electrophoresis is preferable to test the homogeneity of a cyt. c preparation.

The results obtained thus far suggest the existence of multiple molecular forms of cyt. c, but it will be necessary to investigate other physical and chemical properties of these fractions, before it can be concluded with certainty that the fractions represent microheterogeneity as described for other hemoproteins, *e.g.* myoglobin.[29,30] In this connection it is, however, interesting to mention the observations recently made by Matsubara and Smith.[31,32] On the basis of the determination of the peptide pattern and the amino acid sequence of human heart cyt. c, obtained from a large pool of human hearts, they concluded the presence of two cytochromes in the approximate proportion of 9:1. The authors supposed [32] that the most likely hypothesis to explain this heterogeneity was that a genetic variant was present in the starting material. In the present study, however, the heterogeneity demonstrable by means of disc electrophoresis, was the same whether cyt. c was purified from a single or from pooled beef hearts.

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