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Studies on the Peroxidase Effect of Cytochrome c

II. Purification of Beef Heart Cytochrome c by Gel Filtration

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(1) A simple and well reproducible procedure for the large scale purification of cytochrome c is described. Final purification was obtained by two consecutive gel filtrations on Sephadex G-75.

(2) Cytochrome c was obtained in the native, monomeric form, as indicated by the low percentage of autoxidizable hemoprotein (< 0.5 %) and the homogeneity in gel filtration.

(3) A golden-coloured protein impurity, which is always observed during purification of beef heart cytochrome c, was easily and more completely removed by gel filtration than by chromatography on cation-exchange resins. This fact may explain why the ratios $A_{550\ \mathrm{red}}/A_{280\ \mathrm{ox}}$, $A_{550\ \mathrm{red}}/A_{550\ \mathrm{ox}}$, and $A_{550\ \mathrm{red}}/A_{535\ \mathrm{red}}$ are higher than those earlier obtained.

(4) Some physicochemical properties of the purified preparation are given.

Numerous procedures have been described for the large-scale preparation of cytochrome c.**[1] The purest preparations have been obtained by chromatography on synthetic cation-exchange resins of the carboxylic acid type (Amberlite IRC-50 or XE-64 and Duolite CS-101)[2–15] followed by repeated crystallizations as the final purification procedure.[10–14] Some of the steps involved in these procedures are rather tedious, and alternatives have therefore been sought.

Porath [16] and Flodin [17] found that cyt. c was efficiently separated from materials of larger size by gel filtration on Sephadex, but the degree of purification was not investigated, and the method was not applied to crude extracts of biological materials.

In some previous studies [18] gel filtration on Sephadex G-75 was used successfully for the purification of cyt. c in extracts from rat kidneys. The purpose of

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** The following abbreviations will be used: Cyt. c = cytochrome c; TCA = trichloroacetic acid.

the present report is to demonstrate that gel filtration is useful, even in large-scale work, in the final purification of cyt. c. The procedure yielded from beef heart muscle a preparation containing only the native, monomeric form with an even higher degree of purity than that obtained by chromatography on a cation-exchange resin, ammonium sulphate precipitation, and triple crystallization.[13]

MATERIALS AND METHODS

Cation-exchange resins. Amberlite XE-64 (purchased from Kebo AB, Stockholm, Sweden) was treated and converted into its ammonium form as described by Paléus,[13] except that 2 N HCl was used instead of 5 % H_2SO_4 in the intermediate procedure. After the removal of small grain particles (settling time approximately 3 min), the resin was dried. A. G. Duolite CS-101, 100—200 mesh ("Bio-Rex 70", from Bio-Rad Laboratories, Richmond, Calif., U.S.A.) was converted to the ammonium form by 1 M ammonia, washed repeatedly in distilled water (settling time approximately 5 min) and finally equilibrated with 40 mM ammonium phosphate buffer, pH 6.9. This buffer was also used for the equilibration of the column.

Sephadex. Sephadex G-75 (medium) powder was obtained from Pharmacia, Uppsala, Sweden.

Chemicals. Analytical grade reagents and glass distilled water were used throughout.

Electrolyte and buffer solutions. The columns of Amberlite XE-64 and Duolite CS-101 were equilibrated and washed with 2 mM ammonia and 40 mM ammonium phosphate buffer, pH 6.9, respectively. Cyt. c was eluted from the columns of Amberlite XE-64, Duolite CS-101 and Sephadex G-75 by means of 60 mM sodium phosphate, pH 7.5, to which NaCl was added with a final concentration of 0.4 M; thus, the same buffer was used for elution from all columns. The buffer will be referred to below as "the phosphate-chloride buffer".

Extraction of cytochrome c

Cyt. c was extracted from beef heart muscle by two different methods:

A) Dilute sulphuric acid. Fresh beef hearts were trimmed of visible fat and connective tissue, washed with distilled water and minced. Cyt. c was extracted by means of dilute sulphuric acid at pH 4.0,[19] and the extract was further treated as described by Paléus[13] before adsorption on Amberlite XE-64. A batch of convenient size was obtained from 3—6 kg muscle, and the values given below in the chromatographic procedures refer to such a batch.

B) Trichloroacetic acid. Cyt. c was prepared essentially according to Keilin and Hartree,[20] dialyzed against dilute ammonia, and stored at −20°C until used.

The preparation and operation of chromatographic columns

Concentration on Amberlite XE-64. Amberlite powder was added to the dilute extract of cyt. c (10 g dry resin/litre extract), and 1.5 h were allowed for the adsorbtion onto the resin.[13] The resin was collected by settling and extensively washed by gentle stirring for 5 min in 10 l of 2 mM ammonia; this procedure was repeated five times. The resin was finally suspended in about two volumes of 2 mM ammonia, and the resulting slurry poured into the chromatographic tube (diameter 8.6 cm) in small portions. The surface of the resin column was covered by a disc of porous polyethylene. The column was washed with 2 mM ammonia until $A_{280} \leq 0.030$.

Concentration on Duolite CS-101. The cyt. c solution was dialyzed against 40 mM ammonium phosphate buffer, pH 6.9, and passed at a rate of 1 ml/min through a short column of Duolite CS-101, 2.8 cm in diameter, pre-equilibrated with the same buffer. Under these conditions, the hemoprotein was completely removed from the solution; a slight excess of the resin was always used. The surface of the bed was protected as de-

scribed above, and the column was washed with the equilibration buffer (about 2 bed volumes). Cyt. c was finally eluted with the "phosphate-chloride buffer", and the recovery was almost quantitative.

Chromatography on Duolite CS-101. Oxidized and reduced cyt. c were separated by chromatography on Duolite CS-101 as to be described in a forthcoming paper on the heterogeneity of cyt. c.[21]

Gel filtration on Sephadex G-75. The column of Sephadex G-75 (6.25 × 35 cm) was packed according to Flodin [17] and equilibrated overnight by continuous flow of the elution buffer (see above). The concentrated cyt. c solution from the Duolite column was placed atop the bed, and the chromatogram was developed at an average flow rate of 7.3 ml/h. The effluent was collected at volumes of approximately 5 ml by means of a siphon balance. Each fraction was assayed for absorption at 280 mμ and 550 mμ; when necessary after dilution in 65 mM sodium phosphate buffer of pH 6.8 [13] (see below).

After use, the gel was removed from the column and regenerated by letting it sediment a few times in distilled water and finally in the elution buffer. Following this regeneration, the column was repacked and equilibrated as described above.

All concentrations and chromatographic procedures were carried out at 4°C.

Spectrophotometry and elementary analyses

Spectrophotometry. All spectrophotometric assays were performed in 65 mM sodium phosphate buffer, pH 6.8.[13]

The ratio $A_{550\ \mathrm{red}}/A_{280}$ was followed during the purification procedure. After measuring the absorbancy at 280 mμ, the absorbancy at 550 mμ was measured and solid dithionite was added until complete reduction was obtained. The readings were taken at small intervals around 550 mμ, and the highest value was used.

The amount of cyt. c in purified preparations was calculated from the absorbancy at 550 mμ red using $E\,{}^{1\ \%}_{1\ \mathrm{cm}}$ 550 mμ red = 23.94 (see results). During the purification procedure, the difference between the absorbancy at 550 mμ red (maximum) and 535 mμ red (minimum) was taken as a measure of the cyt. c concentration, since the absorbancy at 550 mμ red alone tends to give too high values in impure solutions. In all calculations it was assumed that the difference in the specific extinction coefficients at these wavelengths was $E\,{}^{1\ \%}_{1\ \mathrm{cm}} = 18.08$ (see results).

The amount of autoxidizable cyt. c was determined by the CO method described by Tsou,[22] 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.

For the measurement of light absorption coefficients the cyt. c solution was dialyzed extensively against 2 mM ammonia [1] (five changes). The concentration of cyt. c was determined as dry weight (105°C to constant weight).

A Beckman DU spectrophotometer with a slit width of 0.017 mm and quartz cells of 1.002 cm light path were used for the determination of the light absorption coefficients, and whenever possible, all measurements were made in the region of minimum relative error, *i.e.* at absorbancies between 0.2 and 0.7.[23] Absorption spectra were measured by using a Beckman model DK-2A recording spectrophotometer.

Determination of nitrogen and iron. Cyt. c was dialyzed against 0.0001 N hydrochloric acid in quartz distilled water for 24 h (five changes) to remove any non heme iron from the preparation. The concentration of cyt. c was determined as dry weight (105°C to constant weight). The iron was determined according to the sulphosalicylic acid method,[24,25] and the nitrogen content according to the Kjeldahl method after hydrolysis with 4 M H_2SO_4 at 110°C for 40 h before combustion.[26]

Dialysis. All dialyses were carried out in Viscora casings, impermeable to cyt. c, at 4°C.

RESULTS

Extraction with dilute sulphuric acid

Data from one preparation will be described in detail (Table 1).

A) Initial purification. Cyt. c, when eluted from the column of Amberlite XE-64 with the "phosphate-chloride buffer", moved down the column as a heavy pink band, increasing in size as it descended. Cyt. c was collected in a total volume of 413 ml, and dialyzed until chloride-free against large volumes

Table 1. Summary of purification of beef heart cytochrome c.

Main step	Volume (ml)	A_{550} red/A_{280}	Amount of cyt. c [b]		Comments
			(mg/ml)	(total mg)	
1. Extraction	12 416 [a]	0.06	—	—	—
2. After concentration on Amberlite XE-64	413	0.39	1.43	590	—
3. After dialysis	481	0.54	1.22	587	—
4. After concentration on Duolite CS-101, 1st time	50.4	0.74	11.63	586	Column: 2.8 × 9.1 cm. 0.9 mg cyt. c was collected during adsorption and washing.
5. After gel filtration on Sephadex G-75, 1st time	Main peak 120	1.20	4.70	564 [c]	Indicated by arrows in Fig. 1.
	Other fractions 42	0.56	0.18	8 [c]	Was omitted in the following procedure.
6. After concentration on Duolite CS-101, 2nd time	12.2	1.22	46.14	563	Column: 2.8 × 4 cm. 0.3 mg cyt. c was collected during adsorption and washing.
7. After gel filtration on Sephadex G-75, 2nd time	Main peak 109	1.24 [d]	4.73	515	Indicated by arrows in Fig. 2.
	Other fractions 87	1.14	0.55	47	—

a) The starting weight of minced beef hearts was 3.6 kg.
b) The amount of cyt. c was calculated spectrophotometrically as described in the text.
c) 11.3 mg cyt. c were used for analyses and are not included in these values.
d) A_{550} red/A_{280} ox = 1.26; fully oxidized cyt. c was here obtained by ferricyanide.

of 2 mM ammonia and finally against 40 mM ammonium phosphate buffer, pH 6.9. After the removal of an uncoloured precipitate by centrifugation (3000 × *g*, 30 min, 4°C), the cyt. c was adsorbed onto a small column of Duolite CS-101. A golden-coloured material appeared in the effluent during this procedure. The column was washed with about two bed volumes of the equilibration buffer, and cyt. c was then eluted with the "phosphate chloride buffer" (586 mg in 50.4 ml); $A_{550\ red}/A_{280} = 0.74$.

B) Final purification. This was achieved by passing the hemoprotein twice through a column of Sephadex G-75. The concentrated solution of cyt. c from the column of Duolite CS-101 was transferred directly to a column of Sephadex G-75, and eluted with the "phosphate-chloride buffer". Cyt. c moved slowly down the column as a homogeneous zone, and was eluted as a nearly symmetrical peak (Fig. 1). Most of the impurities moved ahead of the cyt. c peak, and the collected fractions displayed a golden colour. No heme compounds

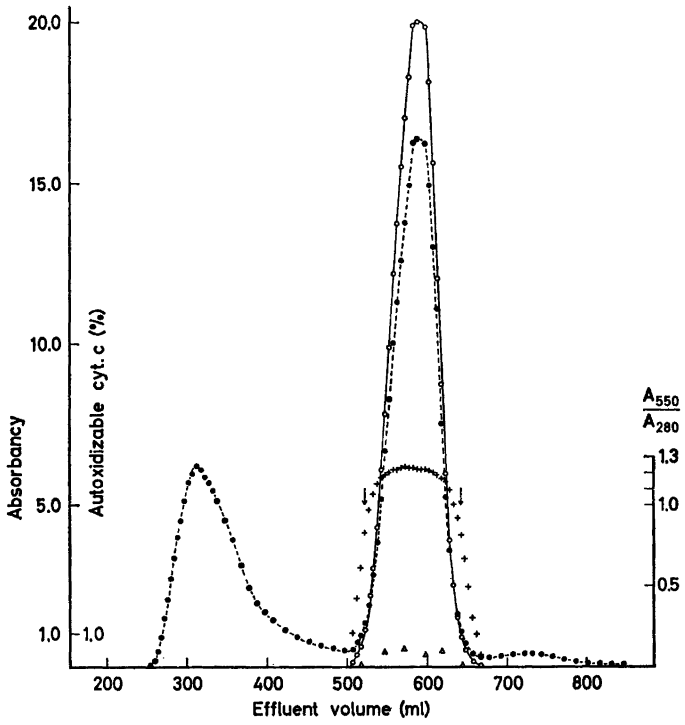

Fig. 1. The elution pattern obtained when the partly purified cyt. c preparation was subjected to gel filtration on Sephadex G-75 (gel filtration I). Column: 6.25 × 35 cm. ●, A_{280}; ○, $A_{550\ red}$; +, the ratio $A_{550\ red}/A_{280}$; and △, autoxidizable cyt. c. The fractions between the arrows were used for re-chromatography (Fig. 2). Note that cyt. c is eluted as only one symmetrical peak, that there is a very low percentage of autoxidizable cyt. c in all fractions (< 0.5 %), and that the first peak of unspecific protein demonstrated a golden colour.

could be detected as pyridine hemochromogen,[27] but TCA gave a slightly golden-coloured precipitate, and a nearly colourless supernatant. A small peak of uncoloured protein appeared just behind the cyt. c peak. A_{280} and $A_{550\ red}/A_{280}$ in Fig. 1 give evidence that cyt. c is contaminated with some unspecific protein. Nevertheless, the most concentrated fractions demonstrated a ratio $A_{550\ red}/A_{280} = 1.22-1.23$. The amount of autoxidizable cyt. c was very low in all fractions (< 0.5 %). The pooled fractions of the main peak (indicated by arrows in Fig. 1), together 564 mg cyt. c in 120 ml, had the ratio $A_{550\ red}/A_{280} = 1.20$. This solution was dialyzed against 2 mM ammonia and then against 40 mM ammonium phosphate buffer, pH 6.9, and concentrated on a small column of Duolite CS-101 (Table 1). Cyt. c was eluted by means of the "phosphate-chloride buffer" in a total volume of 12.2 ml with a small increase in purity ($A_{550\ red}/A_{280} = 1.22$). Re-chromatography on Sephadex G-75 gave a cyt. c preparation with a still higher degree of purification. The fractions of the main peak (indicated by arrows in Fig. 2), 515 mg cyt. c in 109 ml, gave the ratios of $A_{550\ red}/A_{280} = 1.24$ and $A_{550\ red}/A_{280\ ox} = 1.26$ (fully oxidized cyt. c was obtained by ferricyanide). These ratios could not be significantly increased by re-chromatography on Sephadex G-75, nor by chromatography on Duolite CS-101. By the latter procedure, however, a fraction of fully oxidized cyt. c was separated.

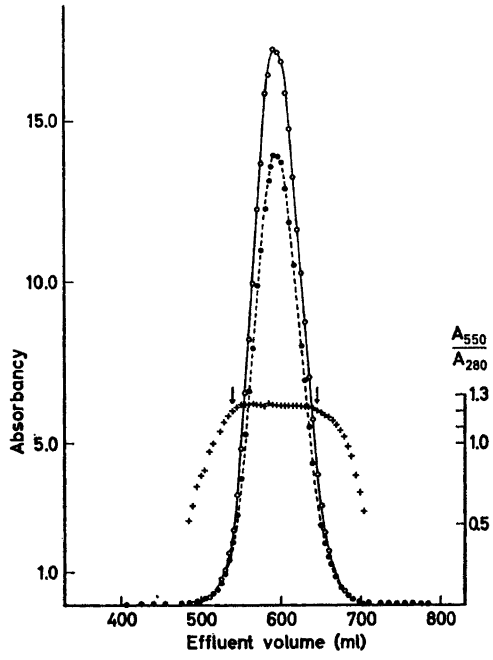

Fig. 2. Re-chromatography on Sephadex G-75 of the main peak from gel filtration I subsequently concentrated on Duolite CS-101 (gel filtration II). Symbols as in Fig. 1. Note the constant level of the ratio $A_{550\ red}/A_{280}$.

C) Elementary composition and spectrum. The fraction of ferri-cyt. c, obtained by chromatography on Duolite CS-101, was assayed for some physicochemical properties (Table 2). The contents of iron and nitrogen were determined in duplicates at three different protein concentrations, and were found by the least square method to be 0.43(8) % and 16.13 %, respectively, giving N/Fe = 147/1 and a molecular weight of 12 750.

The spectra of the oxidized and reduced hemoprotein were closely similar to those given by other authors. The specific extinction coefficient of the pure reduced protein at 550 mμ was, however, found to be considerably higher, $E^{1\,\%}_{1\,cm} = 23.94$. Based on an iron content of 0.43(8) % and a molecular weight of 12 750, the millimolar extinction coefficient of the reduced protein at 550 mμ was calculated to be ε ($cm^{-1} \times mM^{-1}$) = 30.53. Some other spectral properties are given in Table 2 and compared with those earlier reported in the literature. It is interesting to observe that the ratios $A_{550\ red}/A_{280\ ox}$, $A_{550\ red}/A_{550\ ox}$, $A_{550\ red}/A_{535\ red}$, and $A_{590\ ox}/A_{590\ red}$ are higher than previously reported. The ratios suggest that the hemoprotein has been obtained at a higher degree of purity. Cation-exchange resins consistently failed to remove quantitatively the golden-coloured protein, travelling in front of the cyt. c during gel filtration.

Table 2. Some physicochemical properties of beef heart cytochrome c.[a]

Property [b]	Exptl. value [c]	Earlier reported values
Fe content (%)	0.43(8) ($n = 6$)	Range: 0.34 – 0.47 [1]
N content (%)	16.13 ($n = 6$)	Range: 14.83 – 15.37 [1]
N/Fe	147/1	Range: 137/1 – 143/1 [1]
Mol.wt.	12 750	Range: 12 000 – 15 600 [1]
$E^{1\,\%}_{1\,cm}$ at 550 mμ red	23.94 ($n = 3$)	22.6 [13]
ε ($cm^{-1} \times mM^{-1}$) at 550 mμ red	30.53 ($n = 3$)	27.7[7]; 27.2[9]; 26.9[20]; 28.3[30]; 29.9[33]; 29.5[34] *d*
$E_{550\ red}/E_{280\ ox}$	1.26 ($n = 6$)	1.17[2]; 1.17[3]; 1.14[9]; 1.20[13]; 1.29[10]; 1.25[16]
$E_{550\ red}/E_{550\ ox}$	3.63 ($n = 6$)	3.24[9]; 3.31[15]
$E_{550\ red}/E_{535\ red}$	4.09 ($n = 6$)	3.82[2]; 3.86[9]
$E_{590\ ox}/E_{590\ red}$	7.29 ($n = 6$)	5.78[9]; 5.85[13]

a) The fraction of oxidized cyt. c, obtained by chromatography on Duolite CS-101, was used.
b) Full experimental details are given in the text.
c) n = number of assays on this preparation.
d) Basis of calculation: Iron content, Ref.[7,9,19]; sulphur content, Ref.[32]; titration with succinate, Ref.[33]; titration with NADH, Ref.[34]

Extraction with trichloroacetic acid (TCA)

For the purpose of comparison, cyt. c, purified according to Keilin and Hartree,[20] was subjected to gel filtration on Sephadex G-75. The preparation was first concentrated on a column of Duolite CS-101, and by this procedure some unspecific proteins and denatured cyt. c were removed; the former were not adsorbed onto the column, and the latter was not eluted by the "phosphate-chloride buffer". The eluate, with $A_{550\ red}/A_{280} = 1.0$, was transferred to the Sephadex column and chromatographed. Cyt. c now separated into subfractions (Fig. 3). The minor fractions, eluted in front of the main peak, demon-

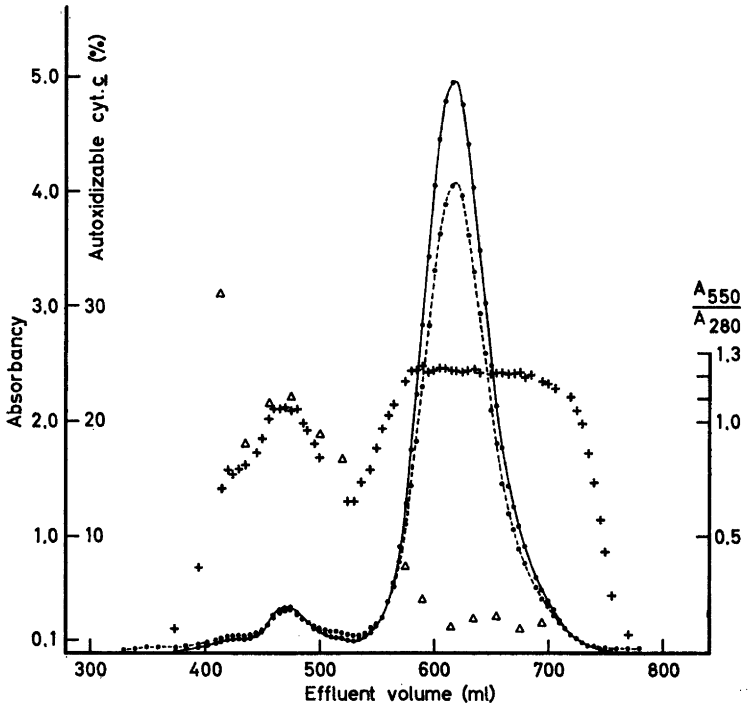

Fig. 3. Elution pattern of cyt. c, prepared according to Keilin and Hartree [20] and concentrated on Duolite CS-101, when subjected to gel filtration on Sephadex G-75. Symbols as in Fig. 1. Note that some minor fractions of autoxidizable cyt. c are eluted in front of the main peak, which has only a low percentage of autoxidizable cyt. c.

strated a high percentage of autoxidizable cyt. c, and probably represent a series of polymers of the monomeric form (the main peak).[28,29] It is noteworthy that these fractions were not removed by Duolite CS-101.

DISCUSSION

In the classical procedures for the large-scale purification of cyt. c from beef heart,[19,20] extraction with acids was used. Since TCA [20] has been shown to result in the formation of some denaturated cyt. c and to produce a series of highly autoxidizable polymers,[14,28,29] this procedure is no longer recommended. Extraction with dilute sulphuric acid [19] has been considered to be a more gentle procedure than the TCA method,[1,13] and the following facts all support this view: (1) In the present study cyt. c was almost quantitatively eluted from a column of Duolite CS-101 in conditions where a part of the TCA prepared cyt. c still remained adsorbed onto the resin; it was eluted only at a higher pH and ionic strength. (2) On gel filtration cyt. c moved as a homogeneous fraction, demonstrating only a minimal amount of autoxidizable cyt. c (< 0.5 %), in conditions where some autoxidizable fractions were clearly separated from the non-autoxidizable fraction of a TCA preparation (Fig. 3). In all probability, the homogeneous fraction corresponds to the monomeric fraction.[29] (3) Ferro-cyt. c is practically non-autoxidizable at pH $\gtrsim 4.0$ in the presence of chloride and sulphate ions,[20,30,31] but autoxidizable in the presence of trichloroacetate ions.[31] (4) The same chromatographic pattern was obtained when cyt. c was extracted by dilute sulphuric acid and by distilled water/saline buffer.[21] These facts all indicate that the extraction of cyt. c with dilute sulphuric acid at pH ≈ 4.0 is a harmless procedure.

The advantages of the present purification procedure are its simplicity and reproducibility and the high degree of purity of the product. Comparative experiments with Sephadex G-50 and G-75 demonstrated that cyt. c is more retarded by the latter, and this gel was therefore preferred for the present purpose; most of the impurities travelled in front of the cyt. c peak. Further, the Sephadex column used has a high capacity, ferro- and ferri-cyt. c exhibit the same migration rate, and the volume of the preparation remains conveniently low throughout the whole procedure. Finally, only two buffers have to be prepared, and ammonium sulphate precipitation and crystallization for the final purification can be avoided. The complete separation of the golden-coloured proteinaceous impurity as a fraction in front of the cyt. c peak (Fig. 1), is of paramount importance. This material was not completely removed by Duolite CS-101 or by Amberlite XE-64, which may possibly explain the higher ratios for $A_{550\ \mathrm{red}}/A_{280\ \mathrm{ox}}$, $A_{550\ \mathrm{red}}/A_{550\ \mathrm{ox}}$, and $A_{550\ \mathrm{red}}/A_{535\ \mathrm{red}}$ in this investigation as compared with those earlier reported (Table 2).

Columns of Duolite CS-101 have previously been used to concentrate solutions of cyt. c.[10,13] The resin in the ammonium form has been equilibrated with, and the cyt. c solution to be concentrated dialyzed against, a 50 mM ammonium phosphate buffer, pH 7.0. These conditions, however, unfortunately result in a considerable loss of the specific protein (29 % as calculated from the data given by Paléus[13]). The reduced form of the hemoprotein is lost during this procedure, especially during the washing of the column. Under the conditions of the present study, the resin retains almost quantitatively the ferric as well as the ferrous form of cyt. c, though it holds the former more firmly. Only a minimal loss of cyt. c occurs (< 0.2 %), and the procedure is therefore recommended for a more quantitative yield.

The yield was calculated to be very high. The final, main fraction with $A_{550\ red}/A_{280\ ox} = 1.26$ and $E^{1\ \%}_{1\ cm}$ 550 mμ red = 23.94 accounted for 87 % of the material eluted from the Amberlite. The cyt. c fractions, not pooled during the gel filtrations I and II, could be concentrated on Duolite and rechromatographed on Sephadex, which raised the total yield of highly purified cyt. c to 96 %. The method described by Paléus [13] gave a yield of 31 % of three times crystallized cyt. c ($A_{550\ red}/A_{280\ ox} = 1.20$ and $E^{1\ \%}_{1\ cm}$ 550 mμ red = 22.6).

Different assays have been used in the present study to test the purity of the cyt. c preparation; the determination of the ratios $A_{550\ red}/A_{280\ ox}$, $A_{550\ red}/A_{550\ ox}$, and $A_{550\ red}/A_{535\ red}$, and the assay of the iron content.

(1) The simplest procedure to test a cyt. c preparation will be to measure the ratio $A_{550\ red}/A_{280\ ox}$.[2,3,9,10,13] This ratio, when used as a criterion of purity, requires strictly defined conditions. Various types of monochromators may differ by as much as 3—4 % in absorbancy of the same solution.[1] The pH of the test solution will also influence the ratio. At pH$\gtrsim$ 9.4 tyrosyl groups in cyt. c will ionize,[32] and this results in decreased absorbancy at 280 mμ, and hence an increase of the ratio $A_{550\ red}/A_{280\ ox}$.

The ratio $A_{550\ red}/A_{280\ ox} = 1.26$ obtained in the present study is higher than those earlier reported (Table 2) except that of Hagihara *et al.*[10] ($R = 1.29$). No information was, however, given about their spectrophotometer and the buffer used, and the degree of purification is therefore difficult to compare with the values obtained by the present procedure.

A high degree of purification is also indicated by the ratios $A_{550\ red}/A_{550\ ox} = 3.63$, $A_{550\ red}/A_{535\ red} = 4.09$, and $A_{590\ ox}/A_{590\ red} = 7.29$, which are all considerably higher than those reported in the literature (Table 2).

(2) The iron content (0.43(8) %) remained, however, within the range earlier obtained.[1] Thus, at a high degree of purification this criterion of purity seems to be of limited valüe. This conclusion is in agreement with statements of earlier investigators.[2,9,30]

The extinction coefficients of cyt. c are usually based on dry weight or iron determinations. There are, however, considerable variations in the published values. The value of ε ($cm^{-1} \times mM^{-1}$) at 550 mμ red = 30.53 obtained in the present study is higher than those earlier reported, but in good agreement with $\varepsilon = 29.9$ [33] and $\varepsilon = 29.5$ [34] which are based on enzymic titrations. This confirms that a high degree of purification is obtained by the present procedure.

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