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Potentiometric and Other Studies on Preparations of Cytochrome *c* from Ox- and Horse-Heart Muscle

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Cytochrome *c* obtained from ox- and horse-heart muscle by the well-known procedure of Keilin & Hartree (1945) has an iron content of about 0.34 % (w/w) and a fairly well-defined E'_0 value, constant over a range of pH values. Treatment of this material by means of chromatography on a resin column (Paléus & Neilands, 1950; Neilands, 1952; Margoliash, 1954*a, b*) appears to provide cytochrome *c* of a purity not yet attained by other methods [0.43–0.46 % (w/w) Fe]. In the course of a study of oxidation–reduction potentials of various metallo-porphyrin systems by potentiometric titration, it seemed of interest to compare the E'_0 value of this highly purified cytochrome *c* with that of the '0.34 %' iron preparation.

While we were preparing the cytochrome, our attention was drawn to the need for closer investigation of the methods of dialysis, by the observation that a non-cytochrome, haem-protein precipitate, present in copious amounts after dialysis of ox-heart preparations against sodium chloride, was not found after dialysis against ammonium hydroxide.

This observation, which does not seem to have been reported previously, seemed to be of importance, as it was obvious that the impurity was incorporated into cytochrome preparations when using ammonium hydroxide; this procedure is now common for preventing loss of cytochrome through the dialysis sac, and has been accepted as a standard alternative to the procedure of Keilin & Hartree (1952).

Details are given of the potentiometric titration apparatus. These include the nitrogen purification system which is designed to take into account the often-neglected finding of Keilin & Hartree (1943) that oxides of nitrogen may be generated by passage of nitrogen over heated copper. Other trace gases which interfere with many metallo-porphyrin systems are also eliminated in the purification system used.

MATERIALS AND METHODS

Cytochrome c preparations

Samples with a '0.34 % iron content'

Fresh ox or horse hearts were treated usually in 2–3 kg. batches according to the method of Keilin & Hartree (1945). The preparations were then dialysed against either 0.5 % (w/v) NaCl or 0.01 N-NH₄OH. In two cases, preparations (c) and (d), the material was divided after the final washing with saturated (NH₄)₂SO₄; one half of each was dialysed against 0.5 % (w/v) NaCl and the other against 0.01 N-NH₄OH. All dialyses were carried out in Visking seamless cellulose tubing at 4° until sulphate-free.

The dark, non-cytochrome precipitate (*P*) left after NaCl dialysis was removed by centrifugation and the supernatant filtered to provide 'NaCl'-cytochrome *c*. The precipitate (*P*) was washed free of cytochrome *c* with 0.5 % (w/v) NaCl. The small amount of residue obtained in the case of NH₄OH dialysis was removed by filtration and discarded. The filtrate provided 'NH₄OH'-cytochrome *c*.

For testing the effect of various conditions of dialysis, one preparation was divided at the pre-dialysis stage into six portions which were then dialysed respectively against

distilled water, 0.5, 1, and 2% (w/v) NaCl, 1% (w/v) ammonium acetate, 0.01N-NH₄OH; each was dialysed for four days with four changes of the particular dialysis liquid. The precipitates (*P*) were collected by centrifugation in graduated tubes and the volumes were compared.

Samples with a 'high iron content'

Ox heart. Cytochrome *c* obtained from a 5 kg. batch of ox-heart mince was dialysed against 0.01N-NH₄OH, filtered and freeze-dried; 0.5 g. of this 'NH₄OH'-cytochrome, preparation (*a*) (Table 1), which contained 0.33% iron was dissolved in the minimal amount of 0.1M potassium phosphate buffer pH 7.0 and placed on a column of Amberlite IRC-50, prepared according to Neilands (1952). Elution of the non-cytochrome fraction (*N*) with the above buffer and of the cytochrome *c* with saturated ammonium acetate was carried out following the method of Neilands. No attempt was made to collect separately the reduced and oxidized cytochrome. A pink band, similar in colour to cytochrome *c*, remained about 2-3 cm. from the top of the column, together with a narrow brown band at the top. Elution with 0.5N-NH₄OH removed only part of the pink band; the brown band was moved only very slowly. Very little of the remaining material was eluted with 5% (v/v) 0.880 sp.gr. NH₄OH. Finally, 5% (w/v) KOH effected removal of both these fractions. The cytochrome *c* was dialysed against five changes of 0.01N-NH₄OH. In view of the findings of Margoliash (1954*a, b*), which were published at this time, it was decided to take the added precaution of removing any modified cytochrome *c* that might have been present. The dialysed material was placed on a column of the ammonium salt of Amberlite IRC-50 and eluted with 0.25M ammonium acetate adjusted to pH 9.6 with NH₄OH. This treatment yielded two fractions only, a small fast-running cytochrome *c* band which was probably the same as fraction I of Margoliash; the second large fraction moved as one band with the front mostly reduced and the tail mostly oxidized. This second fraction, 'resin'-cytochrome, preparation (*a*) (Table 1), dialysed against 0.01N-NH₄OH, contained 0.44% (w/w) iron.

As a final check to ensure that no modified cytochrome *c* remained, a portion of the second fraction was placed on a column of the ammonium salt of Amberlite IRC-50 at pH 7.0. It was eluted cleanly in one slow-moving band by means of 0.25M ammonium acetate at pH 7.0.

Horse heart. Cytochrome *c* from 6 kg. of horse-heart mince after NaCl dialysis and removal of non-cytochrome precipitate (*P*) was freeze-dried and the major part treated by the two-column resin procedure of Margoliash, to yield, after dialysis, preparation (*e*), having an iron content of 0.43%, which was used for E'_0 value determinations. A small portion was treated by the method of Neilands in order to compare qualitatively the respective amounts of the non-cytochrome fraction (*N*) obtained.

All the above cytochrome preparations, and the non-cytochrome fractions isolated in the course of purification, were stored at -15°.

Iron analysis

The method of Drabkin (1941) was used with slight modification. Several samples of A.R. 'pure' iron wire were found unsatisfactory as standards. Molecular

extinction values were found to be too low and the colour fading was significant. A grade of iron rod as supplied for spectrographic standardization (Johnson and Matthey, London) proved entirely satisfactory; the colours were still stable after 3 months at room temperature and the extinction coefficients agreed with the values of Drabkin to $\pm 0.1\%$. Optical densities were read at 510 m μ .

No trouble was encountered in colour development with the 'resin'-cytochrome or the 'NaCl'-cytochrome. With the less pure 'NH₄OH'-cytochrome samples, however, troublesome frothing occurred, particularly during the first heating and there was always a slight turbidity in the final solution. These difficulties were more noticeable still when dealing with the non-cytochrome impurities, precipitate (*P*) and fraction (*N*). As a control check, known amounts of iron were added to each of the samples; where the above difficulties were encountered, recoveries of only 90-96% were obtained, whilst the 'NaCl'- and 'resin'-cytochromes gave 100% iron recoveries. Corrections were made on the basis of these iron recoveries to the iron content values obtained. Comparisons with the above method were made using the iron oxide residues remaining after ignition at 550° for the dry weight estimations (Keilin & Hartree, 1945). Difficulty was experienced, however, in completely dissolving the iron oxide off the walls of the platinum crucible with hot N-HCl, and low results were obtained.

Nitrogen purification

Nitrogen as supplied by British Oxygen Co. Ltd., already de-oxygenated by passage over heated Cu at some unspecified temperature, was quoted as containing not more than 0.002% (v/v) O₂. Further purification was carried out in the following system:

(a) Traces of CO were first removed by passage through a column packed with K₂Pd(SO₄)₂ on granulated silica gel.

(b) Oxides of N₂ were effectively removed by means of granular activated charcoal (Keilin & Hartree, 1943).

(c) At this stage any further traces of O₂, CO, CO₂, SO₂ and possibly unsaturated hydrocarbons coming through from preceding stages were removed with an ammoniacal soln. of CuCl.

(d) Although the NH₃ vapour pressure at stage *C* was very low, complete elimination was ensured by passage through two wash bottles fitted with sintered glass disks and filled with N- and 0.1N-H₂SO₄, followed by drying over granulated silica gel, and then over CuSO₄-activated charcoal.

(e) The purified gas was stored over 0.1N-H₂SO₄ in a bottle of adequate dimensions (20 l.).

Great care was found necessary to eliminate oxygen leakage into the system. Refrigeration-grade Cu tubing was used to connect the N₂ supply to the purification train and the purification train to the electrode vessel in order to provide flexible coiled couplings; all-glass connexions were used elsewhere. The cone and socket joints used for separating sections were sealed with good quality stopcock grease and were spring-loaded. The Cu tubing was silver-soldered to standard brass tapers sealed with Apiezon W100, and spring-loaded. Maintenance of a pressure of 10-15 cm. Hg throughout the system also helped to prevent diffusion of O₂ into the system.

Titration vessel and magnetic stirrer

In Fig. 1 the main features of the titration vessel and magnetic stirrer are indicated. The size of the vessel (*V*) was found very suitable for titrating 10 ml. samples but could be used for smaller or larger volumes. Two bright Pt electrodes were sealed through the vessel wall and connexion to the saturated KCl bridge (*K*) was made through a closed, ungreased ground-glass tap (*T*). Purified N_2 was introduced into the electrode vessel by repeated evacuation and flushing. During titration a slow gas-flow was maintained over the surface at about 7 cm. water pressure.

Fig. 1. Titration vessel and magnetic stirrer used for potentiometric measurements.

The magnetic stirrer (Fig. 1) consisted of two small permanent Alnico magnets (*M*) wedged into thin brass cylinders (*C*). The inner cylinder was brazed to a 1.5 mm. diameter stainless steel shaft. A small stainless steel hemisphere (*H*) was used to support the weight on the base of the glass envelope (*E*) with minimum friction. Stirring was effected by rotating the outer magnet concentrically around the inner one by means of a variable motor and pulley system. This arrangement enabled smooth operation even at very slow speeds and provided a complete seal against the atmosphere. The Perspex paddle (*P*), friction-fitted to the shaft, was readily interchangeable; this stirrer lends itself to use in electrode vessels of various sizes and shapes.

Titrating agent, mediator and cytochrome samples

A solution of the sodium salt of anthraquinone-2-sulphonic acid (British Drug Houses Laboratory Reagent) was used at concentrations to give a titre of about 0.5 ml. with 10 ml. of 10^{-4} M cytochrome *c*. The anthraquinone solution

was reduced with H_2 and platinized asbestos (British Drug Houses Ltd.), then washed free of H_2 with purified N_2 , filtered and stored under N_2 . The reduction apparatus, containing a sintered glass filter, storage vessel and micro-burette, was all-glass and was interconnected with ground-glass joints, spring-loaded, so that transference of the solution from one section to the other under pressure of purified N_2 could be made as required.

The mediator, 2,6-dichlorophenolindophenol (British Drug Houses, indicator), was used in all titrations at a final concentration of 2×10^{-6} M. The cytochrome *c*-mediator solutions were buffered in each case to a final concn. of 3.3×10^{-2} M potassium phosphate at pH 6.4. The mediator at this pH level was found to give $E'_0 = 0.273$ V; at the relative concentrations used, calculation shows that the presence of the mediator would increase the values for cytochrome *c* by less than 0.2 mv.

The above cytochrome solutions were always checked just prior to the commencement of electrometric titration; where necessary, adjustment to full oxidation was made by cautious addition of 0.01 M- $K_3Fe(CN)_6$, the end-point being judged by direct-vision spectroscopy. All experiments were carried out at $25^\circ (\pm 0.1^\circ)$ in a thermostatically controlled room. Complete titration curves were obtained in all but the one case of ox-heart ' NH_4OH '-cytochrome and E'_0 values were assessed by graphical and mathematical analysis, including the rectification method of Reed & Berkson (1929).

Electrometer

The use of a 3S4-type radio receiving valve in place of a special electrometer valve, as suggested by Hay (1951), was found to be quite satisfactory when incorporated into a modified DuBridge-Brown (1933) circuit. The above unit was used in conjunction with a vernier potentiometer (Cambridge Instrument Co. Ltd., London) and a short-period galvanometer having a deflexion of 430 mm. at 1 m. for $1 \mu A$.

Calomel reference electrode

Saturated KCl calomel electrodes were made using a variety of methods including that of Hills & Ives (1951); agreement within less than 1 mv was obtained. The electrodes were frequently checked against one another and also against standard buffer solutions using a quinhydrone electrode. The standard value of 0.2443 V at 25° recommended by Dole (1941) was used.

Absorption spectrophotometry

Samples were measured in both the oxidized and reduced states in a Beckman spectrophotometer Model DU over the range of wavelengths of 620 to 480 m μ . Preliminary measurements were made on each preparation to ensure that optical density readings did not exceed 0.6 when the cytochrome solutions were made up to a final phosphate buffer concentration of 0.067 M at pH 7.4. The complete oxidation of samples was ensured by the addition of a slight excess of $K_3Fe(CN)_6$; at the concentrations used this reagent has no significant absorption over the above range of wavelengths. Oxidation was avoided for reduced samples by sealing the optical cells, which had been completely filled with cytochrome solution, after addition of a slight excess of solid $Na_2S_2O_4$. Measurements at different wavelengths were made on reduced preparations

only when the absorption at 550 $m\mu$. became constant, usually after 10–15 min. For the comparison between cytochrome samples in the reduced condition in the presence of pyridine, absorption measurements were made at final concentrations of 20% (v/v) pyridine and 0.1N-NaOH.

In order to permit direct comparison of curves, irrespective of actual cytochrome concentrations, the logarithmic function of optical density is plotted against wavelength (Figs. 2, 3). For convenience in comparing the α -bands of Fig. 3 the characteristic curves have been brought close together.

RESULTS

Non-cytochrome precipitate (P)

It was found by dry-weight estimations of the salt-free products obtained by dialysis of the ox-heart cytochrome extract against 0.5% (w/v) sodium chloride, that the soluble partially pure 'NaCl'-cytochrome was present to the extent of only five times the amount of the precipitate *P*. In the course of many preparations it was consistently found that ox-heart material gave far greater quantities of precipitate *P* than horse heart; quantitative estimation on the latter material showed the 'NaCl'-cytochrome to be present to the extent of forty times that of the non-cytochrome residue.

showed a faint diffuse band at 640 $m\mu$. Reduction with $\text{Na}_2\text{S}_2\text{O}_4$ produced a haemochromogen spectrum with a diffuse α -band at 558 $m\mu$. and a barely perceptible β -band at 525 $m\mu$. The addition of 20% aqueous pyridine to the alkaline solution of the residue, followed by reduction, produced an intense typical pyridine haemochromogen spectrum with bands at 558 and 525 $m\mu$. It is interesting to note that precipitate *P* after solution in 0.01N ammonium hydroxide and dialysis against the same solvent did not show any appreciable loss of haem pigment over a period of seven days at 4°.

Effect of varying conditions of dialysis

In the case of ox-heart preparations the large amount of precipitate *P* led us to examine the effect of changing the conditions of dialysis; as shown in Table 2, precipitation decreases with increase of salt concentration. Although the greatest amount of precipitate was obtained against distilled water, it was found that after the third day, when the concentration of ammonium sulphate became low, the cytochrome began to pass through the sac. Mention has already been made that very little precipitation occurred against ammonium hydroxide; it was of particular interest to find that re-dialysis of such preparations against 0.5% (w/v)

Table 1. *Iron contents (%) of cytochrome c preparations and of impurities separated from them*

The small letters in parentheses following the iron values denote products obtained from the same batch at the pre-dialysis stage of preparation.

Source	NaCl-dialysed	NH_4OH -dialysed	Resin-purified	Precipitate (<i>P</i>)	Fraction (<i>N</i>)
Ox heart	0.30 (<i>b</i>)	0.33 (<i>a</i>)	0.44 (<i>a</i>)	1.09 (<i>b</i>)	0.19 (<i>a</i>)
	0.28 (<i>c</i>)	0.30 (<i>c</i>)	—	0.54 (<i>c</i>)	—
Horse heart	0.32 (<i>d</i>)	0.33 (<i>d</i>)	0.43 (<i>e</i>)	0.89 (<i>d</i>)	—

Table 2. *Variation in amount of precipitate with altered conditions of dialysis*

Dialysis fluid ...	Water	NaCl			1% ammonium acetate	0.01N- NH_4OH
		0.5%	1.0%	2%		
Amount of residue (precipitate <i>P</i>)	++++	+++	+++	++	+	—
Final pH	4.4	5.3	5.4	5.3	6.6	10.8

Precipitate *P* was obviously not homogeneous and consisted of at least two different coloured components; although protein and haem were definitely identified, no free porphyrin was detected by fluorescence tests both on the solid and in solution. The iron content varied considerably, as can be seen from the three examples (Table 1). Within the pH range 4–9, precipitate *P* was insoluble in water. In more alkaline solution (0.1N-KOH) a diffuse double band at 600 $m\mu$. and another diffuse band at 545 $m\mu$. were observed (Hartridge reversion spectroscopy). A suspension of the residue in 0.5% (w/v) sodium chloride

sodium chloride induced little further precipitation. This was in marked contrast to the direct dialysis against sodium chloride, where during the initial stages quite high concentrations of ammonium ions were present.

Non-cytochrome fraction (N)

The origin of this fraction from resin purification of ox-heart ' NH_4OH '-cytochrome is described above. As eluted from the column by 0.1M phosphate buffer at pH 7.0, the fraction *N* possessed a mid-brown colour and showed absorption bands at 640 $m\mu$. (strong, diffuse); 595 $m\mu$. (faint, narrow);

550 $m\mu$. (very faint, diffuse); after addition of potassium ferricyanide: 640 $m\mu$. (strong); 595 $m\mu$. (very faint, broad). Reduction with $\text{Na}_2\text{S}_2\text{O}_4$ produced a broad, diffuse double band with more intense regions of absorption at 595 and 570 $m\mu$. merging into a strong band at 550 $m\mu$.; a very faint and narrow band was also found at 520 $m\mu$. Reduction in the presence of a final 20% (v/v) concentration of pyridine gave a typical intense haemochromogen spectrum with bands at 558 and 525 $m\mu$. The iron content was 0.19% (Table 1).

Ox-heart 'NaCl'-cytochrome also gave during resin purification a haem-containing fraction *N* but in much smaller amounts. Paléus & Neilands (1950) obtained under similar conditions a non-cytochrome fraction with an iron content of 0.04% (w/w). No identification of the nature of the iron compound was reported by these workers.

A small amount of fraction *N* was obtained from horse-heart 'NH₄OH'-cytochrome using the ammonium salt of IRC-50; slightly more could be separated by means of the potassium salt using the method of Neilands (1952). Again, addition of pyridine and $\text{Na}_2\text{S}_2\text{O}_4$ produced a definite band at 558 $m\mu$. and a faint band at about 525 $m\mu$.; there was also a weak band at 550 $m\mu$. Fraction *N* was also obtained from horse-heart 'NaCl'-cytochrome, but in still smaller amounts.

Oxidation-reduction potentials

In order to check the apparatus and reagents to be used in the evaluation of the cytochrome samples, preliminary runs were made by titrating the mediator (2:6-dichlorophenolindophenol), at a concentration of 10^{-4} M in 0.067 M phosphate buffer at pH 6.40, with reduced anthraquinone-2-sulphonate. Throughout the titrations stable potentials were rapidly obtained and the results fitted closely to the symmetrical curve of the standard electrode equation in which $n=2$ and $E'_0=0.273$ v (25°).

Table 3. *Oxidation-reduction potentials of various samples of cytochrome c*

E'_0 values are quoted in v, at 25° and pH 6.4.

Cytochrome sample	NH ₄ OH-dialysed	NaCl-dialysed	Resin-purified
Ox heart	0.272	0.271	0.255
Horse heart	0.269	0.268	0.255

The resin-purified cytochrome *c* from both ox- and horse-heart preparations gave stable potentials within 5–10 min. and produced very good titration curves which corresponded to the theoretical electrode equation in which $n=1$ and $E'_0=0.255$ v

(Table 3, Fig. 4). Both the 'NaCl' and 'NH₄OH' horse-heart preparations showed a slight drift of potential beyond about 50% reduction, but symmetrical curves were obtained in close agreement with the assumption that $n=1$. Some drift of potential in the later stages of reduction also occurred with the ox-heart 'NaCl'-cytochrome titrations, but again the end-point was definite and there was no difficulty in accurately assessing points of inflexion. In the case of ox-heart 'NH₄OH'-cytochrome, however, the potentials drifted so seriously at about the 0.24 v level that it was impossible to obtain a direct determination of the end-point. Up to this stage the potentials were stable and gave a smooth, $n=1$ type curve and a point of inflexion could be assessed. It is of interest that this 'NH₄OH'-dialysed ox-heart cytochrome, the most impure of the series, was the only specimen in which a light-coloured flocculent precipitate appeared during titration. As can be seen from Table 3, with all specimens other than the resin-treated products, E'_0 values consistently close to 0.270 v were obtained.

Spectrophotometric results

Over the range of wavelengths studied all samples showed, within the limits of experimental error, identical positions for band maxima and minima, viz., α -band, 550 $m\mu$.; β -band, 520 $m\mu$.; minimum, 535 $m\mu$. The isosbestic points of the oxidized and reduced forms for each sample were also found to be identical, viz., 557, 542, 526 and 505 $m\mu$. Absorption curves for the three grades of ox-heart cytochrome in the reduced condition are shown in Fig. 2; comparison of these curves reveals little difference in the region 560–500 $m\mu$. Significant deviations do occur at longer wavelengths, and in general correspond closely with the level of purity of the sample. Very little difference, however, was observed between all curves of the oxidized samples. Similar results not illustrated here were obtained for the horse-heart series. For later discussion various ratios of light absorption at different wavelengths are presented in Table 4.

Addition of pyridine to the three grades of ox-heart cytochrome, under conditions suitable for haemochromogen formation, produced a considerable broadening of the α -absorption band on its longer-wavelength slope only in the case of the 'NH₄OH'-cytochrome (Fig. 3); no peak or shoulder at 558 $m\mu$. was discernible in any of these spectrophotometric curves.

In addition to the optical density ratios quoted in Table 4, the following values of the ratio $E'_{550\text{ }m\mu}(\text{reduced})/E'_{280\text{ }m\mu}(\text{oxidized})$ were measured for the resin-purified preparations: ox heart, 1.14; horse heart, 1.22.

Fig. 2. Comparison of characteristic absorption spectra of reduced cytochrome *c* samples from ox-heart muscle. (Curve *A*: 0.30% Fe, 'NH₄OH'-dialysed prep. (c); curve *B*: 0.28% Fe, 'NaCl'-dialysed prep. (c); curve *C*: 0.44% Fe, 'resin'-purified prep. (a).)

Fig. 3. Effect of alkaline pyridine on the characteristic absorption spectra of reduced cytochrome *c* samples from ox-heart muscle. (Curves *A-C* refer to same preparations as shown in Fig. 2; 20% (v/v) pyridine and 0.1 N-NaOH at final dilutions.)

Table 4. Optical-density ratios of various cytochrome *c* samples

		The small letters in parentheses in the first column denote products obtained from the same batch at the pre-dialysis stage of purification. In the second column the terms 'NH ₄ OH' and 'NaCl' refer to the method of dialysis of preparations; 'Resin' refers to material finally purified on Amberlite IRC-50.									
Source	Preparation	% Fe	550 mμ. (reduced)	520 mμ. (reduced)	550 mμ. (reduced)	535 mμ. (reduced)	550 mμ. (oxidized)	590 mμ. (reduced)	590 mμ. (oxidized)	550 mμ. (reduced)	590 mμ. (reduced)
Ox (c)	NH ₄ OH	0.30	1.74	3.38	1.94	2.07	2.93	1.93	17.1	17.1	17.1
Horse (d)	NH ₄ OH	0.33	1.76	3.63	2.07	2.10	3.22	2.48	25.4	25.4	25.4
Ox (c)	NaCl	0.28	1.77	3.73	2.10	2.18	3.25	3.13	31.7	31.7	31.7
Horse (d)	NaCl	0.32	1.77	3.84	2.18	2.16	3.40	3.57	42.3	42.3	42.3
Horse (e)	Resin	0.43	1.77	3.84	2.16	2.19	3.48	4.00	47.0	47.0	47.0
Ox (a)	Resin	0.44	1.76	3.86	2.19	2.19	3.24	5.78	53.7	53.7	53.7

Autoxidation

Autoxidation was qualitatively tested by allowing cytochrome samples of 10^{-4} M concentration and pH 6.4 to stand in air, with occasional shaking, after reduction with slight excess of reduced anthraquinone-2-sulphonate. A striking effect of the presence of impurities was found with the ox-heart series, where the ' NH_4OH '-cytochrome became completely oxidized within 30 min., the ' NaCl '-cytochrome took about 6 hr., whereas the 'resin'-cytochrome of the same preparation was still largely reduced even after 24 hr.

Similar results were obtained with the horse-heart series, except that the ' NH_4OH '-cytochrome was not oxidized as rapidly as the corresponding ox-heart cytochrome; it was nevertheless oxidized more rapidly than either of the ' NaCl '-cytochromes.

DISCUSSION

The results show that an iron-containing impurity was associated with cytochrome *c* in the final precipitation prior to dialysis in the method of preparation as described by Keilin & Hartree (1952). This impurity dissolves in varying degree according to the method of dialysis, and is included in the final cytochrome preparation. The amount of precipitate *P* obtained under the different conditions of dialysis, as listed in Table 2, is an indication of the amount of this impurity included in such cytochrome samples. It appears that the effect of ammonium hydroxide was due more to the high pH value of 10.8 than to any specific effect of ammonium ions. At lower pH values incorporation of impurity was diminished, but increased ionic strength tended to reverse the effect. Precipitation was greatest with distilled water; sodium chloride, although not quite so effective, has the well-known advantage of preventing loss of cytochrome by adsorption by and passage through the Visking tubing. There appears to be no objection to dialysis against ammonium hydroxide after removal of the non-cytochrome precipitate *P*, e.g. if sodium chloride is to be removed prior to freeze-drying.

The impurity, isolated after sodium chloride dialysis as non-cytochrome precipitate *P* and also from the resin column as non-cytochrome fraction *N*, consisted of protohaem together with protein. Both horse- and ox-heart preparations gave similar results; precipitate *P* was about eight times greater from ox heart than from horse heart. It is interesting to note that the haem content of precipitate *P*, calculated from the iron content, varied between 6 and 12% (w/w), a range of values consistently higher than in either myoglobin or haemoglobin. From the light-absorption data of ox-heart preparation (c), under conditions in which non-

cytochrome haem is converted into pyridine haemochromogen, we calculate that the ' NH_4OH '-cytochrome contained 15% of its total iron as haem impurity.

The iron content of cytochrome *c* which has been so widely used as a criterion of purity can be shown to be of doubtful value. For instance, in the case of horse-heart preparation (d), calculation shows that if the precipitate *P* of iron content 0.89% were completely included in the corresponding ' NaCl '-cytochrome (0.32% iron), the product would contain 6% of foreign iron and would have a total iron content of 0.33%. This latter figure, in fact, agrees exactly with the iron content for the ' NH_4OH '-cytochrome of this preparation. Then again, the ox-heart ' NH_4OH '-cytochrome of preparation (c), as mentioned above, was calculated to contain 15% of foreign iron and yet had a total iron content of only 0.30%; this figure also agrees with the value calculated from the foreign iron content on the basis of inclusion of a 0.54% iron content impurity.

The results leave no doubt as to the presence of protohaem in all our preparations, except perhaps the resin-purified samples. Although there is evidence for combination between the haem and protein impurities, some linkage of the haem with cytochrome *c* cannot be discounted; Zeile & Gnant (1940) have shown that there is combination under alkaline conditions between protohaem and the protein of cytochrome *c*. Certainly, once the impurity is brought into solution by ammonium hydroxide dialysis, prolonged dialysis against sodium chloride causes only very slight re-precipitation. It is doubtful if electrophoresis would separate protohaem firmly bound to the cytochrome protein. Theorell & Åkesson (1941) reported the presence sometimes of 1–4% non-cytochrome iron even in their purest (0.43% iron) electrophoretic preparations. The impurities similarly separated by Tint & Reiss (1950) were found to contain no iron. The possibility of incorporating foreign iron makes it difficult to understand the close relationship reported by a number of workers between iron content and the activity of cytochrome *c* samples in respiration systems.

The electrometric results, with regard to stability and titration curve symmetry, followed closely the incorporation of iron-containing non-cytochrome impurity. The E'_0 values themselves were all well within the range of values published by a number of workers. We found, however, a consistent difference of about 15 mv between the resin-purified and the other cytochrome preparations from both horse- and ox-heart muscle. The difference appears to be due to the presence of modified cytochrome *c* possessing a higher E'_0 value, rather than to the non-cytochrome impurities, as the values for the less pure grades of cytochrome were almost

identical. Margoliash (1954*b*) has found 10–25 % of modified cytochrome in preparations prior to resin purification. The electrometric results show that it is always necessary to consider a complete titration curve in assessing an E'_0 value for cytochrome *c* rather than one or even several isolated values.

Fig. 4. Oxidation-reduction titration at pH 6.4 of resin-purified cytochrome *c* samples. Ox heart ○, ●, ▲; horse heart +. Solid line is theoretical curve for $E'_0 = 0.255$ v, $n = 1$.

Increase in haem-protein impurity was accompanied by a parallel increase in light absorption in the reduced cytochromes from about 560 $m\mu$. towards the red end of the spectrum, with a maximal effect at about 590 $m\mu$. The characteristic absorption curves of three ox-heart preparations are arranged in Fig. 2 in order of increasing purity, commencing with the most impure at the top. An identical result, not shown, was obtained with the corresponding horse-heart samples. The two ratios given in columns (5) and (6) of Table 4 show large variations according to the degree of purity; this table is also of interest in showing that some of the often used ratios of maxima and minima have very limited value as criteria of purity.

The molecular extinction value, $\epsilon_{550 m\mu} = 27.2 \times 10^3$, obtained for ox-heart 'resin'-cytochrome on an iron basis is slightly higher than, but in good agreement with, that determined by Paléus & Neilands (1950), and the one calculated by Tint & Reiss

(1950). We feel disinclined, however, to accept as final any values yet reported, as there is still uncertainty regarding the presence of impurities or modified cytochrome in even the best preparations so far obtained; e.g. Margoliash (1954*b*), during the resin purification of horse-heart cytochrome, has obtained some evidence for the existence of fractions having iron contents considerably in excess of 0.465 %. Our results add further weight to the opinions expressed by such workers as Theorell & Åkesson (1941) and Paléus & Neilands (1950) that there is need for the use of criteria of purity other than those based solely on iron content.

SUMMARY

1. The E'_0 values at 25° and pH 6.4 of both ox- and horse-heart cytochrome *c* purified by ion-exchange resin column chromatography were found to be 0.255 v; less pure preparations gave values higher by about 15 mv.

2. A haem-containing impurity was consistently found in relatively large amounts in the final cytochrome *c* precipitate before dialysis according to the method of Keilin & Hartree (1945). The extent of incorporation of this impurity into the final cytochrome preparations varied with the conditions of dialysis. The most impure cytochromes were obtained by dialysis against ammonium hydroxide; this effect appears to be due to the alkalinity rather than to any specific action of ammonium ions.

3. The purest preparations of reduced cytochrome *c*, obtained by purification on an ion-exchange resin column, showed insignificant autoxidation. The autoxidation of the less pure samples appeared to be largely dependent upon the level of haem impurity; the most impure cytochrome solutions became fully oxidized in air within 30 min.

4. Spectrophotometric results have been used to assist in determining the degree of purity of the various cytochrome *c* preparations.

5. Evidence has been presented which throws considerable doubt upon the value of the iron content as a criterion of purity.

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The *in vitro* Enzymic Hydroxylation of Steroid Hormones

3. AN INVESTIGATION OF THE ROLE OF FUMARATE AND PYRIDINE NUCLEOTIDES IN THE ENZYMIC 11 β -HYDROXYLATION OF 11-DEOXYCORTICOSTERONE*

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Hayano & Dorfman (1953) have claimed 'specificity and absolute necessity for fumarate' for the 11 β -hydroxylation of 11-deoxycorticosterone (DOC) when using 'residue preparations of twice washed ox-adrenal homogenates obtained at 5000 g'. More recently (Hayano & Dorfman, 1954) they have made the same observation with acetone-dried 'homogenate residues' as a source of the hydroxylating enzyme. We have investigated the 11 β -hydroxylation of DOC using ox-adrenocortical mitochondria suspensions prepared in sucrose solution (Brownie & Grant, 1954*a*). With these suspensions 11 β -hydroxylation of DOC occurs readily provided that there is concurrent oxidation of a member of the Krebs tricarboxylic acid cycle. If the simultaneous oxidation of succinate, for instance, is inhibited by malonate, 11 β -hydroxylation does not occur. However, on addition of α -oxoglutarate to the malonate-blocked system, 11 β -hydroxylation of DOC proceeds. From these observations we concluded that there is no absolute and specific necessity for fumarate for 11 β -hydroxylation by intact ox-adrenocortical mitochondria. We also concluded from other evidence presented (Brownie & Grant, 1954*a*) that 11 β -hydroxylation by intact mitochondria requires concurrent oxidative phosphorylation, for which members of the tricarboxylic acid cycle are oxidizable substrates.

In order to investigate the differences between our results and those of Hayano & Dorfman (1953), and in order to obtain further information on the cofactor requirements of the 11 β -hydroxylating enzyme system, we have performed experiments with deliberately damaged mitochondria. The results of these experiments are now reported.

A preliminary account of this work has been communicated to the Biochemical Society (Brownie & Grant, 1954*b*).

EXPERIMENTAL

Enzyme preparations, materials and methods

Suspensions of ox-adrenocortical mitochondria were prepared as already described (Brownie & Grant, 1954*a*; Brownie *et al.* 1954). These will be referred to as 'fresh mitochondria'. Fresh mitochondria were subjected to three types of damaging treatment: (a) They were dispersed in 9 vol. of glass-distilled water and incubated in air with shaking at 37° for 0.5 hr. These preparations will be referred to as 'pre-incubated mitochondria'. (b) Pre-incubated mitochondria were mixed with 1/9 vol. of 1.54 M-KCl to assist subsequent sedimentation, kept for 5 min. at room temperature and centrifuged at 5000 g. The sedimented material was finally dispersed in 9 vol. of 0.154 M-KCl. In this way water-soluble material released on pre-incubation of the mitochondria is removed, and consequently this preparation will be referred to as 'pre-incubated washed mitochondria'. (c) Fresh mitochondria were acetone-dried as described by Drysdale & Lardy (1953).

Diphosphopyridine nucleotide (DPN) was prepared from yeast by the method of LePage (1949*a*) and purified by counter-current distribution (Hogeboom & Barry, 1948). The material obtained was not less than 75% pure as assayed spectrophotometrically following reduction by Na₂S₂O₄ (LePage, 1949*b*). Triphosphopyridine nucleotide (TPN) was prepared from sheep liver by a modification of the method of LePage & Mueller (1949) and purified by gradient elution from an ion-exchange resin column of Dowex-1-formate. The material obtained ranged in purity from 60 to 70% as assayed by the method of Horecker & Kornberg (1948) with isocitric dehydrogenase prepared according to Straub (1942). We are indebted to Professor H. A. Krebs, F.R.S., and to Dr D. D. Davies for providing details of the method of preparation of TPN and for a

* Part 2: Brownie, Grant & Davidson (1954).