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Preparation of Cytochrome c with the Aid of Ion Exchange Resin

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A certain phase of the research in our cytochrome c program called for the preparation, from different sources, of relatively large quantities of this pigment in a form as pure as possible. We found, in following the previously published procedures for the electrophoretic purification of cytochrome c (Theorell and Åkeson [1]) that the project would soon develop into a rather formidable task. Therefore, in the course of our preparative work, we have been particularly interested in the introduction of any new technique which might augment the effectiveness of the original method.

The first highly purified cytochrome c was obtained in 1935 by Theorell [2]. In this and in subsequent methods (Theorell [3]; Keilin and Hartree [4]) advantage was taken of the extreme stability of the enzyme. These methods gave preparations containing 0.34 % iron. Another outstanding property of the enzyme, the very basic character of its protein (I. P. = 10.05)[1], was finally used by Theorell and Åkeson [5] in 1939 when they employed electrophoresis at different pH levels to obtain a preparation containing 0.43 % iron. Keilin and Hartree [6] later obtained cytochrome of the same order of purity by precipitation with ammonium sulfate at pH 10.

In our experiences with cytochromes from different animal sources, the application of the electrophoretic technique as the final step always leads to a higher iron content but cannot be depended upon, except possibly for horse cytochrome, to give material containing as much iron as that originally described by Theorell and Åkeson [1]. In addition, electrophoresis is very time consuming and not well adapted for large-scale isolation work.

It was considered possible that the exceedingly high isoelectric point of the cytochrome might render its separation possible on a cation exchange resin. This technique has been tested and developed with cytochrome prepa-

rations from various animal sources. In all cases it has led to the rejection of a golden-colored impurity, protein in nature, and the separation of the original cytochrome into several fractions, each of which contain higher levels of iron.

PREPARATION METHODS

The first cytochrome c preparation tried was one from salmon hearts, prepared according to Keilin and Hartree [6]. It contained 0.24 % iron as determined by the sulfosalicylic acid method of Lorber [7]. As cation exchanger we chose the new, white, bead-form, carboxylic acid Amberlite IRC—50 *. The resin was dried at room temperature and pulverized in a mortar to give a fine, white powder. The powder was made into a slurry with water and poured into a glass tube of inner diameter 0.7 cm to a height of 20 cm. The column was then washed with liberal quantities of 5 % H_2SO_4, water and 5 % ammonia. Finally, the resin was conditioned with 0.1 M ammonium hydroxide-ammonium acetate solution, pH 9.0, until both influent and effluent had the same pH and the same optical density at 280 mμ. Ammonium acetate was chosen as the agent for controlling the pH on the column since this salt quickly evaporates during the dry weight determinations which are required for finding the per cent. iron in aliquots of the effluent.

The cytochrome sample was introduced in a 0.15 ml volume in aqueous solution containing about 15 mg of dry weight, allowed to flow in to the column and washed with the acetate solution. The cytochrome c lodged at the surface of the resin, where it formed a brick-red layer. A golden-colored material quickly appeared in the effluent. The latter fraction could be precipitated with either trichloroacetic acid or four volumes of acetone and had a very high optical density at 280 mμ. Prolonged washing of the column with 0.1 *M* ammonium hydroxide-ammonium acetate solutionof pH 9.0 did not elute the cytochrome nor cause any 280 mμ-absorbing material to appear in the effluent. When the pH of the ammonium acetate solution was adjusted to 10.8 by the addition of more ammonium hydroxide, the cytochrome migrated down the column. The elution rate appeared to be much higher for the smaller particles of the resin. Consequently, in all subsequent work, the Amberlite was first pulverized to pass a 0.075 mm mesh screen.

The colored band was collected in a few ml and dry weight and iron determinations carried out. The iron content was 0.420 %. A repeat experiment starting with the same crude cytochrome from salmon gave the figures 0.447 %

* Obtained from the Rohm and Haas Co., Philadelphia, U.S.A., kindly supplied to us by J. Troedsson, Nordiska Armaturfabrikerna AB, Linköping.

and 0.461 % iron respectively for the front and tail fractions of the colored zone in the effluent.

It was then of considerable interest to determine the precise elution-point pH of cytochrome. Such information might: 1) enable one to refine the method so as to increase its resolving power and 2) indicate whether or not the elution-point pH coincides with the isoelectric point of the protein.

A column was prepared as above and after loading with a sample of salmon cytochrome the alkalinity of the ammonium hydroxide-ammonium acetate solution was increased in intervals of 0.1 pH unit, starting with pH 9.0. The golden-colored impurity soon appeared in the effluent and was saved for future investigation. At pH 9.9 the lower portion of the cytochrome band increased in color and on continued washing a new red band could be seen to leave the original red zone at the top of the column. The latter was easily eluted with 0.5 % ammonium hydroxide. Chromatography of dog-heart and chicken heart cytochromes at pH 9.0 also gave a three component mixture. In all these experiments the first band to appear in the effluent had the spectrum of reduced cytochrome while the second band had the spectrum of the oxidized form. The small amount of pigment which remained at the top of the column was eluted with 0.5% ammonium hydroxide and had the oxidized-type spectrum.

When it became apparent that the use of ion exchange resin in the above way for the purification of cytochrome c would resolve the pigment into several fractions, we decided to next study cow heart cytochrome. This is generally considered to be the most readily available source of cytochrome c. The run with cow heart cytochrome is described below in detail.

The primary product was obtained by the method of Keilin and Hartree and had an iron content of 0.34 %. After it had been obtained as a dry, salt-free powder by lyophilization, the material was dissolved in 0.1 *M* ammonium hydroxide-ammonium acetate solution, pH 9.0, to give a clear solution containing 112.7 mg per ml. At first some difficulty was experienced in washing the column free from very finely divided particles of resin. However, if the dry, powdered resin was first suspended in the ammonium acetate solution in a beaker, stirred, the supernatant liquid decanted after several minutes and this process repeated several times, this difficulty disappeared. The bottom of the chromatography tube was closed with a one-hole rubber stopper; on the top of the stopper a layer of glass wool and a layer of powdered asbestos prevented the particles of Amberlite from escaping from the tube. The resin, a column 2.0 × 15 cm, was prepared by washing for several hours each with 5 % H_2SO_4, water, 5 % ammonium hydroxide and 0.1 *M* ammonium hydroxide-ammonium acetate solution, pH 9.0. The effluent was checked for correct pH and the absence of any appreciable optical density at 280 mμ.

Exactly 4.5 ml (dry weight 507 mg) of the above cytochrome solution was placed on the resin and the column developed with 0.1 *M* ammonium hydroxide-ammonium acetate solution, pH 9.0. The golden-colored impurity, which was brownish in more concentrated solution, could be almost exclusively confined between the 22 and 37 ml marks in the effluent. This fraction contained a dry weight of 101 mg. The per cent. iron was 0.040 and the optical density ratio $\frac{550\ m\mu}{280\ m\mu}$ was 0.072. These figures plus the fact that in the reduced solution the handspectroscope showed only a very faint band at 550 mμ shows that the amount of cytochrome in this material must have been very small. As mentioned above, the addition of trichloroacetic acid or four volumes of acetone to this fraction produced a heavy precipitate. The first cytochrome band to emerge was collected between the 182 ml and 482 ml marks in the effluent. It had the spectrum of reduced cytochrome and was labelled Fraction I. This band was immediately followed by another (Fraction II) of oxidized cytochrome and was collected in a volume of 500 ml. Finally, Fraction III could be stripped off the top of the column with 0.5 % ammonium hydroxide to give a 50 ml solution of oxidized cytochrome.

The next problem was the concentration of the cytochrome from these rather dilute solutions. Direct lyophilization was ruled out as impractical. Attempts to remove the buffer by evaporation under reduced pressure always led to the formation of a certain amount of insoluble cytochrome, possibly foam denatured. Some success was had by re-adsorbing the material on a fresh column of Amberlite IRC-50. A more effective process proved to be the addition of 20 % trichloroacetic acid to pH 3.5 in the cold followed by centrifugation after the solutions had stood at 4° C for several hours. This isolated all the color and yielded the cytochrome as a red precipitate which dissolved at once in water. Concentrated solutions of each fraction were thus obtained and dialyzed overnight against flowing 0.1 % ammonium hydroxide.

The three fractions were then analyzed for dry weight and iron. The results are given in Table 1.

Table 1. Yield and iron content of three cytochrome c fractions separated with ion exchange resin.

Fraction	Dry weight mg	Relative % of each fraction	Fe content %	Fe content mg
I	66.2	27.2	0.401	0.27
II	155.4	63.9	0.466	0.72
III	21.8	8.9	0.353	0.08
Total	243.4	100.0		1.07

The dry weight of the impurity, 101 mg, when added to the 243.4 mg of cytochrome, gives a recovery of $\left(\frac{344.4}{507} \times 100\right)$ or 67 %. All three fractions reported in Table 1 gave different iron analyses. An admixture of the three fractions would have an iron content of $\left(\frac{1.07}{243.4} \times 100\right)$ or 0.44 %. The iron content of the impurity fraction added to the cytochrome iron, 1.07 mg, leads to a total iron recovery of 11.1 mg or $\left(\frac{1.11}{1.75} \times 100\right) = 63$ %. Much of the unrecovered dry weight and iron was probably contained in the effluent which emerged between the impure fraction and the reduced cytochrome band. This fraction had a certain small amount of density at 280mμ and 550 mu (after reduction).

Fraction II of Table 1 appeared, from the iron content, to be relatively pure cytochrome. Therefore, the optical density of this material was measured in a Beckman Model DU spectrophotometer at various wavelengths for the reduced pigment (carried out according to Tint and Reiss[8]) and at 280 mμ for the oxidized form. This enabled us to calculate the light absorption constants recorded in Table 2. It is obvious that Fraction II, the main component, is a very highly purified substance. A short run in the electrophoresis apparatus of Tiselius at pH 10.5 did not reveal the presence of any contaminating material.

Table 2. Some physical constants for fraction II of beef cytochrome c prepared with ion exchange resin.

Cytochrome c (form)	Wavelength (mμ)	$E^{1\%}_{1cm}$	e_{1cm} *	Extinction ratios and MW
Oxidized	280	19.15	22 940	$\frac{e_{550}}{e_{280}} = 1.17$ MW from Fe analysis (0.466 %) = 11 980
				$\frac{e_{550}}{e_{535}} = 3.82$
Reduced	520	12.91	15 466	
»	535	5.87	7 035	$\frac{e_{520}}{e_{535}} = 2.20$
»	550	22.41	26 882	

* Molar extinctions per gram atom of iron.

DISCUSSION

The separation of cytochrome into several fractions by chromatography on cation exchange resin could be most easily explained if there should exist cytochromes with different degrees of basicity. Or, on the other hand, a single cytochrome might enter into complex formation with other constituents of the medium. In the future it would be of interest to study the comparative electrophoretic behaviour of all the different fractions and their reconstituted mixtures. Zeile and Reuter [10] claimed that oxidized, but not reduced cytochrome, could be adsorbed on kaolin.

There is a rather striking agreement between the constants reported for Fraction II and those calculated by Tint and Reiss [8] for a beef preparation which they believed to be 81 % pure. These authors reported, per gram atom of iron, $e^{1\text{ cm}}_{550\text{ m}\mu} = 26.400 \pm 560$; $e^{1\text{ cm}}_{535\text{ m}\mu} = 7\,030 \pm 140$; $\frac{e_{550\text{ m}\mu}}{e_{535\text{ m}\mu}} = 3.76 \pm 0.11$. Corrected for purity the $E^{1\text{ cm }550\text{ m}\mu}_{1\%}$ was 21.38 $\pm$ 0.34 and the $E^{1\text{ cm }535\text{ m}\mu}_{1\%} = 5.69 \pm 0.08$. However, we do not feel that we should claim the isolation of pure beef cytochrome c. This is partly because we have not made a sufficiently large number of preparations and partly because of the fact that such claims should be substantiated by several criteria of purity. This would certainly include analyses for several elements, such as Fe, N and S; electrophoresis at different pH levels; a maximum extinction coefficient at 550 mμ and maximum ratio $\frac{e_{550\text{ m}\mu}}{e_{535\text{ m}\mu}}$; and maximum activity in an enzyme assay. Since reduced cytochrome c has such sharp maxima and minima, accurate spectrophotometric constants could only be derived with an apparatus having a narrow spectral interval.

While making cytochrome preparations from different animals, Tint and Reiss [9] observed that the same or similar impurities always contaminated the different cytochromes. As mentioned above, we have made a similar observation. Since this impurity has such a low ratio $\frac{e_{550\text{ m}\mu}}{e_{280\text{ m}\mu}}$ this figure should be of value as a general check on the freedom of cytochrome preparations from this impurity.

Chromatography on the ion exchange resin is a method which can be easily scaled up to handle any desired quantity of material at a negligible cost. The same column can be washed and used for successive preparations.

SUMMARY

Preparation of cytochrome c by a new method, chromatography on ion exchange resin at controlled pH, has been described. The method has been tested with salmon, dog, chicken and beef-heart cytochromes. A separation

of the cytochrome into different fractions has been observed. A preparation of beef heart cytochrome has been obtained which gave the following constants: Fe = 0.466 %, $E^{1\ \%}_{1\ cm}{}^{550\ m\mu} = 22.41$; $e^{1\ gram\ atom\ Fe/liter}_{1\ cm}{}^{550\ m\mu} = 26\,882$; $\frac{e_{550\ m\mu}}{e_{535 m\mu}} = 3.82$. The measurements for light absorbtion were carried out with a Beckman model DU spectrophotometer.

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REFERENCES

1. Theorell, H., and Åkeson, Å. *J. Am. Chem. Soc.* **63** (1941) 1804.
2. Theorell, H. *Biochem. Z.* **279** (1935) 463.
3. Theorell, H. *Ibid.* **285** (1936) 207.
4. Keilin, D., and Hartree, E. F. *Proc. Roy. Soc., London* **B 122** (1937) 298.
5. Theorell, H., and Åkeson, Å. *Science* **90** (1939) 67.
6. Keilin, D., and Hartree, E. F. *Biochem. J.* **39** (1945) 289.
7. Lorber, L. *Biochem. Z.* **181** (1927) 391.
8. Tint, H., and Reiss, W. *J. Biol. Chem.* **182** (1950) 397.
9. Tint, H., and Reiss, W. *Ibid.* **182** (1950) 385.
10. Zeile, K., and Reuter, F. *Z. physiol. Chem.* **221** (1933) 101.

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