ACTA CHEMICA SCANDINAVICA 17 (1963) S110–S116

Investigation of a Fraction Separated from Beef-Heart Cytochrome *c*

R. W. HENDERSON* and S. PALÉUS

Medicinska Nobelinstitutet, Biokemiska avdelningen, Karolinska Institutet, Stockholm, Sweden

Amino-acid analysis of the non-pigmented fraction (X) obtained from 0.3 % Fe content beef-heart cytochrome *c* by electrophoretic separation (Theorell and Åkeson[1]) has been carried out. Fraction (X) has been found to have a nitrogen content of 8.9 %. The high non-protein content has been partly explained by the presence of carbohydrate. There appears to be no identity between fraction (X) and globin from either myoglobin or haemoglobin.

Cytochrome *c* of 0.34 % iron content was first prepared from heart-muscle by Theorell[2,3] using the technique of acid extraction followed by one of several alternative fractionation procedures. A modification involving the use of trichloroacetic acid to obtain a product of similar iron content was introduced by Keilin and Hartree[4,5].

Theorell and Åkeson[1] separated electrophoretically a colourless fraction from the pigment so obtained and by this means increased the iron content to 0.43 %. Resin column chromatography of cytochrome *c* (Paléus and Neilands[6]) may also be used for this purpose. The colourless fraction (X) so removed has been generally considered to be protein in nature and the view was early expressed (see *e. g.* Lemberg and Legge[7]) that it was likely to be associated with cytochrome *c in vivo.*

Margoliash[8] obtained evidence that globin from myoglobin and also globin from haemoglobin would combine with resin column purified cytochrome *c*. This along with their similarity in behaviour during paper electrophoresis led Margoliash to the view that the fraction (X) was globin from myoglobin present as an artifact of the acid-extraction procedure. Evidence has however been obtained by Henderson and Rawlinson[9] that fraction (X) is present in cytochrome

* Present address: Russell Grimwade School of Biochemistry, University of Melbourne, Victoria, Australia.

c obtained by neutral extraction and zinc precipitation of myoglobin and haemoglobin.

It was clear that amino-acid analysis would assist in the identification or otherwise of the fraction in question with globin. This was accordingly carried out.

MATERIALS AND METHODS

Cytochrome c was prepared from minced beef-hearts by the method of Keilin and Hartree[4]. After the final precipitation with trichloroacetic acid the preparation was dialyzed free from sulphate against 0.5 % sodium chloride. The precipitated material was filtered off; the cytochrome was then dialyzed until chloride free against 0.005 M ammonium hydroxide and freezedried. Iron content of many preparations were 0.3 % ± 0.04 %. The two column resin treatment of Margoliash[10] was used to obtain cytochrome of 0.43 % Fe content from part of one of the above preparations.

Fraction (X). In order to minimize denaturation with consequent insolubility resulting from resin-column procedures this fraction was separated using (i) a Spinco Model H Electrophoresis instrument*. 1 % (w/v) cytochrome *c* solutions in phosphate buffer pH 7.2 and 0.1 ionic strength were run for several hours according to the size of cell used. At the conclusion of the run the colourless boundaries (Fig. 1) which had separated toward the anode were removed together by suction constituting fraction (X). This material was then either dialyzed until salt-free and freeze-dried or stored at −10°C. (ii) A Perkin-Elmer Model 38 Electrophoresis instrument was also used. In this apparatus 2 % (w/v) cytochrome *c* in 0.02 M phosphate buffer pH 7.3 and 0.15 M KCl, 0.2 ionic strength was used. In all cases the cytochrome *c* was in the oxidized condition.

Amino-acid analysis. About 2.5 mg samples of salt-free freezedried fraction (X) were weighed and hydrolyzed 46 hr at 110°C in a sealed and evacuated tube. Isoluble humin formed was removed by centrifugation and washed twice with dist. water. The washings were added to the hydrolysate which after evaporation to dryness over KOH was analysed according to Moore *et al.*[11]

Nitrogen content of fraction (X) was determined by a modified Dumas method[12].

Carbohydrate estimation was carried out by a modification due to Shetlar and Masters[13] of the thymol-sulphuric acid reaction method which it was reported was not significantly influenced by the presence of protein. Recovery of glucose added to resin treated cytochrome *c* was found to be 96 %.

Spectrophotometry. A Beckman Model DK2 and a Zeiss Model PMQII Spectrophotometer were used.

RESULTS AND DISCUSSION

Electrophoresis. A typical electrophoretic pattern obtained with 0.3 % Fe content beef-heart cytochrome *c* is shown in Fig. 1. The non-pigmented material (X) formed three boundaries (a, b and c) which moved toward the anode at a rate equal to or greater than that at which the cytochrome moved toward the cathode (Table 1).

Fractions (a) and (b) represent 90–95 % (by determination of areas under the curves — means of values from each limb) of the total non-pigmented material (X) separated. In some preparations there was more equality in the amounts of (a) and (b) present. From the similarity of the sum of the amounts of these two fractions present, however, in different cytochrome *c* samples it seemed likely that (b) was split from (a) during preparation. The ratio of cytochrome *c* to the

* We wish to thank Docent A. Åkeson for his assistance in the use of this apparatus and particularly for making available to us the cell assembly which he has devised and which enables long runs to be carried out with minimal trouble due to gassing at the electrodes.

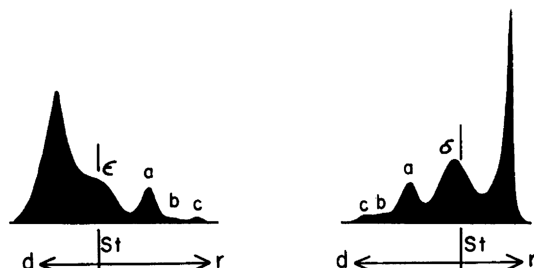

Fig. 1. Electrophoresis patterns of 0.3 % beef-heart cytochrome *c*. 2 % (w/v) cytochrome *c* in 0.02 M PO_4 buffer pH 7.3 and 0.15 M KCl, 0.2 ionic strength. Run 120 min, potential gradient 4.82 V. cm^{-1}. (St.), starting points; (ε) and (δ), stationary salt boundaries on sides where cytochrome *c* descending (d) and rising (r), respectively; (a), (b) and (c), the three non-pigmented boundaries which together constituted fraction (X).

total non-pigmented fraction (X) was estimated to be 100 : 25–33. Fraction (X) thus represents 20–25 % of 0.3 % Fe content cytochrome *c*, which is consistent with the value expected from a comparison of iron-contents before and after electrophoresis.

The electrophoretic mobilities determined for the cytochrome *c* are within the range of values already reported by Theorell and Åkeson[1] and Tint and Reiss[14]. Differences in shape and mobility of cytochrome *c* boundaries between the two limbs of the cell were reported by Theorell and Åkeson[1]. Tint and Reiss[14] considered that these differences were due to the use of solutions of 0.1 ionic strength and could be obviated by increasing the ionic strength to 0.2. The boundaries shown in Fig. 1 are typical of many obtained in solutions of 0.2 ionic strength. Considerable differences are apparent in the patterns from opposite limbs of the cell. Furthermore the mobilities (Table 1) of the rising cytochrome *c*

Table 1. Electrophoretic mobilities (cm^2 $volt^{-1}$ $sec^{-1} \times 10^{-5}$) of fractions separated from 0.3 % beef-heart cytochrome *c*, 0.02 M phosphate buffer pH·7.3 ionic strength 0.2 (including 0.15 M KCl). Means of a number of determinations except in the case of electrophoretically separated cytochrome *c* where the values are from a single run. Lettering as in Fig. 1.

Fraction	Limb of cell in which cytochrome *c* descending	Limb of cell in which cytochrome *c* rising
Cytochrome *c*	2,4	2,8
Fraction (a)	3,0	3,0
Fraction (b)	4,9	4,6
Fraction (c)	5,9	5,7
Electrophoretically separated cytochrome *c* re-run	2,3	2,8

show small but consistently higher values than those for the descending pigment. There was some evidence in the fractions (a), (b) and (c) also of higher mobilities where the components were rising and out of contact with oppositely moving protein. In the case of the already electrophoretically separated cytochrome *c* run in 0.2 ionic strength buffer (not shown) only one peak was apparent in each limb of the cell and the patterns were much more similar than those obtained when fraction (X) was present. The rising cytochrome *c* still showed, however, a higher value than that of the descending pigment (Table 1).

It would seem likely therefore that the observed differences are due in some measure both to the formation of dissociable salt complexes between the various fractions present as suggested by Theorell and Åkeson[1] plus salt concentration and pH changes which may occur throughout the cell during electrophoresis (see *e. g.* Longsworth[15]).

No attempt was made to separate the three fractions (a), (b) and (c) and they were removed from the cell together. There was no absorption in the visible region by fraction (X) either before or after addition of dithionite. Protohaem was not present in detectable amounts under conditions of pyridine ferrohaemochromogen formation.

Amino-acid analysis. Mean values of a number of amino-acid analyses of fraction (X) are listed in Table 2. The nitrogen content (8.9 %) of fraction (X) indicates a protein content of about 60 % which is consistent with the recovery of 62 g amino-acid/100 g unhydrolyzed sample.

The amino-acid values (Table 2) have therefore been multiplied in one column by the factor $\frac{100}{60}$ in order to facilitate comparison with reported analyses of beef myoglobin, haemoglobin and cytochrome *c*. The results do not agree closely with any of the comparison proteins listed and in particular fraction (X) was found to have a markedly lower histidine content than either beef myoglobin or haemoglobin.

Carbohydrate was detected qualitatively in 0.3 % beef-heart cytochrome *c* by the micro aniline acetate test as outlined by Feigl[18].

Light absorption curves obtained by application of a modified thymol-sulphuric acid reaction method of carbohydrate estimation (Shetlar and Masters[13]) to a number of samples are shown in Fig. 2.

It was found that the absorption peak position of 0.3 % Fe content beef-heart cytochrome *c* (507–509 mμ) was practically identical with that for glucose alone and also for resin-column treated cytochrome *c* of 0.43 % Fe content to which glucose had been added. Fructose under the same conditions gave an absorption maximum at 510–511 mμ. Other carbohydrates listed by the above authors have maxima at wavelengths still further removed from that for glucose.

A curve (not shown) was obtained with fraction (X) which was almost identical in shape with that for glucose. As expected the absorption peaks at 550 mμ and 596–597 mμ, due almost certainly to the haem prosthetic group of the cytochrome *c*, were completely absent.

The carbohydrate content of fraction (X) calculated as glucose was found to be 8 %. This compared with 1.5 % for 0.3 % Fe content cytochrome *c*.

Table 2. Results of amino-acid analysis of fraction (X) from beef-heart cytochrome *c* compared with similar analyses of the apo-proteins of several other pigments. (Figures in parentheses in first column refer to the number of determinations from which the values were obtained.)

	g amino-acid/ 100 g fraction (X) from 0.3 % Fe content beef-heart cytochrome *c*	Results from first column $\times \frac{100}{60}$ *i. e.* g amino-acid/ 100 g protein	Adult beef myoglobin g/100 g protein[16]	Adult beef haemoglobin g/100 g protein[16]	Beef-heart cytochrome *c* 0.43 % Fe content g/100 g protein[17]
Cysteic	Trace		—	—	2.6
Aspartic	6.9 (3)	11.5	10.0	11.9	9.7
Threonine	3.3 (3)	5.1	3.3	4.8	7.4 (Threonine + Serine)
Serine	1.8 (3)	3.0	3.1	5.2	
Glutamic	10.2 (3)	17.0	13.9	7.9	13.6
Proline	2.7 (2)	4.5	2.5	3.8	3.5
Glycine	2.5 (3)	4.2	5.1	3.8	9.0
Alanine	4.0 (3)	6.6	8.1	10.3	4.6
Valine	3.5 (3)	5.8	6.1	10.4	3.0
Methionine	Trace		1.9	1.4	2.3
Iso-Leucine	3.7 (3)	6.2	3.9	0	6.3
Leucine	4.9 (3)	8.2	12.8	15.2	6.3
Tyrosine	1.1 (3)	1.8	2.3	2.1	5.3
Phenylalanine	3.5 (3)	5.8	5.9	6.6	4.2
Lysine	8.4 (2)	14.0	15.4	11.5	20.7
Histidine	2.0 (2)	3.3	11.5	8.1	3.7
Arginine	3.4 (2)	5.7	2.5	4.2	4.6

It is of course obvious that this estimation takes no account of non-protein which is inert in the reaction. The above values compared with the high non-protein content of about 40 % calculated from the nitrogen content, indicate the presence of non-reactive material.

It is clear from the results that resin-column treatment and electrophoresis remove much of the carbohydrate. Even, however, in the case of the product from the former treatment which usually has a slightly higher Fe content than the latter there remained (Fig. 2) an absorption at 507—509 mμ which in terms of glucose amounted to 0.5 %.

It is of interest that about 3 % reducing sugar was found after acid hydrolysis in one of the early 0.3 % Fe content cytochrome *c* preparations of Theorell[3].

Preliminary results with 0.3 % Fe content horse-heart cytochrome *c* have established that this material also contains carbohydrate but it appears to be present in lesser amount than in the corresponding beef-heart preparations.

Although it is not known yet whether the carbohydrate or non-protein material is distributed between the three fractions (a), (b) and (c) or associated with any one exclusively, its relatively high content makes it appear unlikely that it would

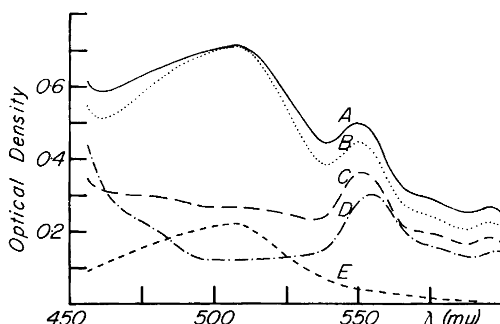

Fig. 2. Light absorption curves obtained from the complex formed by application under standard conditions, of the thymol-sulphuric acid method of carbohydrate estimation (Shetlar and Masters[13]) to the following samples: *A.* 0.3 % Fe content beef-heart cytochrome *c* (4.5 mg); *B.* 0.43 % Fe content resin-column treated beef-heart cytochrome *c* (prepared from part of the same batch used to obtain curve *A*) 2.5 mg, + 40 μg glucose; *C.* As for curve *B* without glucose addition; *D.* As for curve *C* without thymol addition; *E.* 40 μg glucose.

be restricted to the minor fraction (c). Furthermore there is some evidence that fraction (b) arises from fraction (a) during preparation. In this event the non-protein (carbohydrate) moiety would be a constituent of fraction (a) plus (b). In any case and irrespective of consideration of distribution of the non-protein (carbohydrate) component, the amino-acid analysis shows that globin from either myoglobin or haemoglobin if present at all could only be a very minor constituent of 0.3 % Fe content beef-heart cytochrome *c* or of fraction (X), which may be separated from it.

Addendum May 8, 1963: The electrophoretic mobility of beef apo-myoglobin I has recently been determined under similar conditions to those given in Table 1 above. The major apo-Mb fraction (comprising more than 70 % of the preparation) showed an electrophoretic mobility of 5.1×10^{-5} cm^2 $volt^{-1}$ sec^{-1}, thus moving at a rate approaching twice that of what is usually the main component of fraction (X).

Acknowledgements. The authors are indebted to Prof. H. Theorell for his advice and encouragement. One of us (R. W. H.) is especially grateful to Prof Theorell and his staff for their kindness during his stay at the Medicinska Nobelinstitutet when part of this work was carried out; also to the *Rockefeller Foundation* and the *National Health and Medical Research Council* (Commonwealth of Australia) for travel grants. It is a pleasure to thank Ing. A.-M. Fridén for performing quantitative aminoacid analyses. The authors wish to express their thanks for the support of this work to *Magnus Bergvalls Stiftelse* and *Statens Medicinska och Naturvetenskapliga Forskningsråd.*

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. Keilin, D. and Hartree, E. F. *Biochem. J.* **39** (1945) 289.

6. Paléus, S. and Neilands, J. B. *Acta Chem. Scand.* **4** (1950) 1024.
7. Lemberg, R. and Legge, J. W. *Hematin Compounds and Bile Pigments,* Interscience, New York and London, 1949, p. 348.
8. Margoliash, E. *Biochem. J.* **56** (1954) 529.
9. Henderson, R. W. and Rawlinson, W. A. In Falk, J. E. Lemberg, M. R. and Morton, R. K. *Proceedings of the Haematin Enzyme Symposium, Canberra,* Pergamon Press, London, 1961, Pt 2, p. 370.
10. Margoliash, E. *Biochem. J.* **56** (1954) 535.
11. Moore, S., Spackman, D. H. and Stein, W. H. *Anal. Chem.* **30** (1958) 1185.
12. Kirsten, W. In Wilson, C. L. and Wilson, D. W. *Comprehensive Analytical Chemistry,* Elsevier Publishing Company, Amsterdam, 1960, Vol. 1 B, p. 486.
13. Shetlar, M. R. and Masters, Y. F. *Anal. Chem.* **29** (1957) 402.
14. Tint, H. and Reiss, W. *J. Biol. Chem.* **182** (1950) 385.
15. Longsworth, L. G. In Bier, M. *Electrophoresis Theory, Methods and Applications,* Academic Press, New York, 1959, p. 91.
16. Timmer, R., van der Helm, H. J. and Huisman, T. H. J. *Nature* **180** (1957) 239.
17. Ehrenberg, A. and Theorell, H. *Acta Chem. Scand.* **9** (1955) 1193.
18. Feigl, F. *Spot Tests in Organic Analysis,* Elsevier, Amsterdam, 1960, p. 426.

Received March 27, 1963.