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Zone Electrophoresis on Cross-linked Polyvinylalcohol Applied to the Keilin-Hartree Preparation of Cytochrome c

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Beef heart cytochrome c preparations according to Keilin and Hartree have been fractionated by electrophoresis in columns of particulate polyvinylalcohol gel. A major and a minor component of similar chemical and physical properties were isolated.

Since cytochrome c preparations even following crystallization may be inhomogeneous [1] it was of interest to delineate the characteristics of a preparation made according to the method of Keilin and Hartree. Column electrophoresis on polyvinylalcohol gel was chosen for the purification. During the course of this study Gregolin and Singer [2] in an investigation on different commercial preparations of cytochrome c reported the interesting finding that different lots showed widely different activities when determined in different enzymatic tests. The authors attributed these differences in activity to the presence of varying amounts and possibly different kinds of modified cytochrome c. Gregolin and Singer also emphasized the difficulty of distinguishing the acid-modified cytochrome c protein from its native form by their absorption spectra. This observation is in agreement with that of Paul [3] in 1948 in his study of the stability of cytochrome c at extreme pH values. One of us (S.P.) has also observed that lyophilization of crystallized preparations depending on experimental conditions causes some degradation.

PREPARATION

Beef hearts were processed according to the method of Keilin and Hartree [4]. The procedure was strictly followed except for omission of the step in which the suspension is shaken with a few drops of chloroform. After dialysis against 0.5 % NaCl the precipitate was removed by centrifugation. The solution was then dialyzed against 0.02 M sodium acetate buffer (pH 5.0) and at the same time concentrated by keeping a reduced pressure outside the bag.

ELECTROPHORETIC TECHNIQUE

Supporting medium. Block-polymerized cross-linked polyvinylalcohol *, of water regain 11.6 (kindly supplied by Dr. P. Flodin, Pharmacia, Uppsala) was allowed to swell in distilled water for at least 10 h. The block was cut into small pieces and mixed with dry ice and agitated in a Waring blendor. The ground gel was then suspended in a large volume of acetate buffer, 0.05 M with respect to sodium acetate, pH 5. The suspension was stirred for 15 min. The large particles, sedimenting at a high speed, were removed. The finer particles were then also allowed to settle and the supernatant discarded. The larger particles were ground once more and centrifuged. The fractions of fine particles were pooled and suspended in fresh buffer. After the particles had settled the supernatant was decanted. This washing procedure was repeated 3 times. The suspension was then ready for use. Packing of the column was then performed with a suspension containing about 10 volumes of free liquid per volume of swollen gel. No fractionation of the particles was considered necessary because the settled grain had superficially coalesced into what appeared to be an almost continuous gel phase. Upon shaking, however, the gel grains separated from each other.

Apparatus. The apparatus was essentially the same as described elsewhere [5]. It consists of an externally cooled column tube of glass (160 × 4 cm). A short "elution" column provided with a side arm is attached to the lower end of the main column. The column tubes are closed at the lower ends by porous plastic discs that permit passage of current and liquid.

Preparation of the column and application of sample. The main column tube is filled with gel suspension and the gel is allowed to settle. After a day or two a firm packing is obtained. The side arms of the short column is provided with long rubber tubings which are filled with buffer and clamped off. The short column is now filled with buffer supported by cellulose (Munktell cellulose powder, obtainable from Grycksbo Pappersbruk, Grycksbo, Sweden). The two columns are joined without introducing air and then connected to the electrode vessels [6]. A 25 ml sample of the concentrated cytochrome c solution (containing about 850 mg) was slowly introduced through a capillary tubing to form

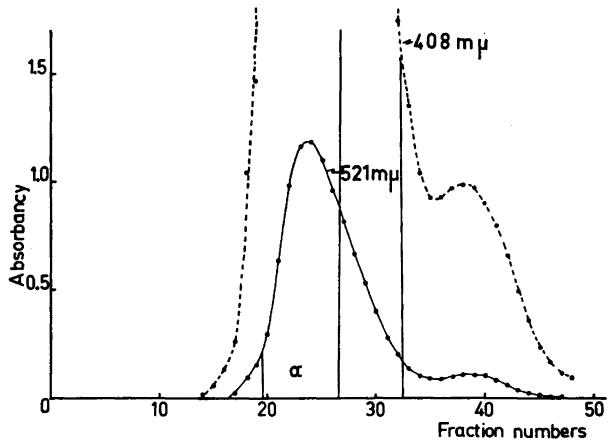

Fig. 1. Electropherogram of the Keilin-Hartree cytochrome c preparation. Fractions 27—32 were not further investigated because of the suspicion of the presence of a third component in this region. Fractions 32—45 were pooled, concentrated and subjected to another electrophoretic run (see Fig. 2).

* The synthesis of the gel substance will be published by Dr. P. Flodin, Pharmacia, Uppsala, Sweden.

a stable, strongly colored zone, about 2 cm of height, layered under the buffer and supported by the gel sediment.

Electrophoresis. 500 V were applied across the electrodes resulting in a current of 50 mA. When the colored zone had migrated into the gel (approximately 4 h after the start) the voltage was increased to 700 V. During the experiment the current varied between 90 and 95 mA. The buffer in the electrode vessels (each containing 8 l of buffer) was mixed every other day. The brown-red zone broadened considerably during the run, largely due to the higher rate of migration in the interior parts of the column. When the zone had passed through 150 cm gel bed it had become more than 10 cm wide with a diffuse portion trailing behind indicating the presence of a minor component of lower mobility. After 215 hours of electrophoresis, the elution from the bottom started. A peristaltic pump removed the buffer at a speed of 20 ml per hour. The colored material was quantitatively displaced from the column to a fraction collector. The fractions were analyzed in a Beckman DU Spectrophotometer at 408 and 521 mμ. The distribution of material is shown in Fig. 1.

After concentration of the fractions 32–45 (Fig. 1) to 1.7 ml this material was subjected to another electrophoretic run in the same buffer as before but this time in a small column (2.5 × 35 cm). After 62.5 h, elution was started and fractions were collected at 25 min intervals. The distribution obtained is given in Fig. 2. Fractions 20–27 (Fig. 1) were collected for further analysis.

Analysis. The iron content, determined by the Lorber method as described by Paul [3], was 0.45 % for the major component and 0.35 % for the minor one (hereafter labelled "α" and "β", respectively).

The nitrogen content (Dumas method) was found to be 15.61 % for "α" and 15.15 % for "β". These values should be evaluated with special regard to the recently published investigation of Åkeson [7] as to the nitrogen content of apomyoglobin and myoglobin. By using either the Kjeldahl or the Dumas method he found variable nitrogen values. After introduction of hydrolysis with 4 M H_2SO_4 for 40 h at 110°C before the combustion step of the Kjeldahl method, the nitrogen values became reproducible and also higher than found by the use of the ordinary procedure.

The amino acid analysis of the two fractions were made according to Spackman, Stein and Moore [8]. The result is given in Table 1.

Moving boundary electrophoresis in phosphate buffer of pH 7.1 and $\mu = 0.1$ did not further resolve "α". This fraction was crystallized according to Paléus [9]. $E^{1\,\%}_{1\,cm}$ at 550 mμ was found to be 22.5 for "α" and 24.0 for "β".

Ultracentrifugal analyses performed under identical conditions in the Spinco Ultracentrifuge Model E gave the same sedimentation coefficient, 1.6 S, for both components. Conditions, 0.1 M sodium acetate-acetic acid buffer of pH 5.0, 0.3 % protein; speed of rotation 59 780 rev./min. Temp. 20.0° and 20.2°C.

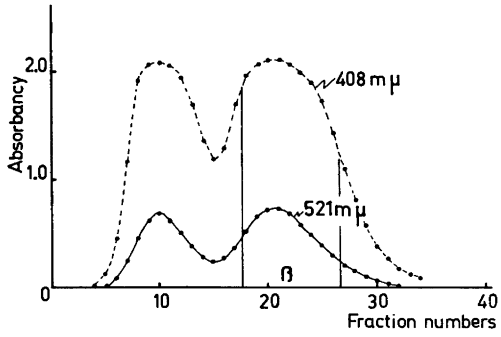

Fig. 2. Electropherogram of the material in fractions 32–45 on Fig. 1. Only fractions labelled by "β" were further studied.

Optical rotatory dispersion curves for the components in oxidized and reduced forms in 10^{-4} M solutions were obtained by the Rudolph Recording Spectropolarimeter. A difference between the "α" and "β" components could not be detected within the entire wave length range measured, 280—700 mμ.

DISCUSSION

The present investigation has shown the cytochrome c preparation of Keilin and Hartree consistently to contain at least two haemoproteins. The α-component corresponds undoubtedly to the recently [9] described preparation of crystalline beef cytochrome c (see Table 1). It is rather uncertain if the analytical nitrogen values of the α- and β-component indicate a difference in composition. The low iron content of "β" is remarkable when compared with the extinction coefficient at 550 mμ which was even somewhat higher for "β" than for "α". This discrepancy remains to be explained. The similarity in optical dispersion seems, however, to rule out extensive contrasting features in the tertiary structure. The result of the amino acid analyses shows that the two components may in fact be identical in amino acid composition within the limits of experimental error. The difference in electrophoretic behaviour may be related to some chemical modification of one or more ε-amino groups of lysine or the phenolic hydroxyl groups of tyrosine as recently discussed by Åkeson [7] in his investigation of the chemical differences between the myoglobin

Table 1. Amino acid composition of the α- and β-component of the Keilin-Hartree preparation.

Amino acid residue	Number per molecule				
	α-component		β-component		Crystalline cyt.c (Paléus) [9]
	Time of hydrolysis				
	30 h *		30 h	70 h	
Lysine	16.9	18.2	15.8	18.1	18
Histidine	2.8	3.1	2.7	3.0	3
Arginine	1.9	1.9	1.8	1.8	2
Cysteic acid	—	1.2			
Aspartic acid	7.6	7.9	7.3	7.3	8
Methionine Sulfone	—	—			
Threonine	7.2	7.1	6.9	5.5	8
Serine	0.9	0.9	1.1	1.1	
Glutamic acid	12.2	11.9	11.7	10.9	12
Proline	3.9	4.2	3.7	3.7	4
Glycine	13.3	13.9	12.6	13.3	15
Alanine	5.6	5.9	5.5	6.1	6
½ Cystine			1.3		2
Valine	2.6	3.2	2.8	3.1	3
Methionine	1.8	0.6	1.6	0.5	2
Isoleucine	5.5	6.0	5.1	5.8	6
Leucine	5.6	5.9	5.3	5.7	6
Tyrosine	3.7	2.7	3.3	3.2	4
Phenylalanine	3.7	4.0	3.3	3.7	4

* This hydrolysate was investigated by the use of two different analysers.

variants. We have found that lyophilization of a solution of the α-component yields a product which can be shown to be heterogeneous in the electrophoretic procedure described here. Repeated lyophilization resulted in the appearance of substantial amounts of components of decreasing mobilities. In a number of preparations where lyophilization had been avoided prior to electrophoresis the "α"- and "β"-components were obtained in approximately the same proportions. Repeated electrophoresis of purified components in small columns gave single zones. This suggests strongly that the minor component is not an artefact induced by the purification procedure here described.

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