ON THE MOLECULAR WEIGHT OF CYTOCHROME c FROM MAMMALIAN HEART MUSCLE

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Cytochrome c, a conjugated protein containing iron protoporphyrin IX as the prosthetic group, is an important intermediary catalyst in tissue respiration (see Oppenheimer and Stern (1)). It is water-soluble, readily extracted from certain animal and plant tissues as well as microorganisms, and stable in purified form. Although its structure and physicochemical properties, e.g. absorption spectrum, magnetic moment, and catalytic activity, have been extensively studied (see Lemberg and Legge (2)), only scanty information is available on its molecular size and shape (3–6). Some of the values quoted in these reviews refer to unpublished experiments, and no sedimentation or diffusion diagrams appear to have been presented thus far.

The object of the present study was to obtain quantitative information on the molecular weight and related parameters of ferricytochrome c from mammalian heart tissue. Highly purified preparations from several species (horse, beef, pig) were kindly placed at our disposal by Dr. H. Tint and Dr. W. Reiss of the Wyeth Institute of Applied Biochemistry, Philadelphia. These investigators have previously determined the electrophoretic homogeneity, spectrophotometric constants, and iron content of the same cytochrome preparations (7). The minimal molecular weight was calculated from these data ((7) p. 397) on the assumption that 1 atom of iron, in the form of a heme group, is present in 1 molecule of the conjugated protein.

In the present experiments, the molecular weight was derived from sedimentation velocity and diffusion measurements by means of Svedberg's formula. The values thus obtained are independent of any assumptions as to the iron or heme content of the cytochrome c molecule.

EXPERIMENTAL

Methods and Materials—The ferricytochrome c preparations employed in these experiments had been stored for approximately 2 years at about 4° in 0.14 M NaCl solutions, with small amounts of Merthiolate added as a

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The cytochrome concentration was approximately 1 per cent. Prior to study, these solutions were dialyzed in cellophane bags for 24 hours at 1° against a large volume of 0.1 M phosphate buffer, pH 7.73. The outside fluid was used as a solvent for dilution and as the supernatant solution in the diffusion experiments. The dialyzed cytochrome solutions were centrifuged. Only the beef preparation was observed to yield a sediment under these conditions, this material originally having shown the greatest amount of impurity (19 per cent). The pig material was originally 89 per cent pure, and the horse preparation was free from impurities, as far as could be determined. The Fe content of these three preparations was beef 0.368 per cent, pig 0.384 per cent, and horse 0.456 per cent, respectively. All the experiments were performed on the material in its ferri form. Aliquots from each experimental solution were submitted to Dr. Reiss, who determined the cytochrome concentrations spectrophotometrically.

An air-driven ultracentrifuge of the turret type (8) was used for the sedimentation velocity experiments, the optical system being of the Thovert-Philpot type. A General Electric air-cooled high pressure mercury arc lamp No. (BH-6) served as the light source, and Eastman Kodak spectroscopic plates, type 103F, were employed for photography in conjunction with a Wratten No. 195 gelatin filter, as recommended by Kegeles and Gutter (9).

The diffusion experiments were carried out at 20 ± 0.05° with a Klett electrophoresis apparatus, equipped with the Longworth schlieren scanning system (10), and a modified diffusion cell of the Lamm-Tiselius type (11). The cytochrome solutions were carefully stratified under the solvent columns with hydrostatic pressure. The diffusion boundaries were recorded at suitable intervals on Eastman Kodak No. 103F plates with a Corning No. 2412 red glass filter and a single filament tungsten source (General Electric No. T10).

The diffusion constants were calculated by means of the "maximal ordinate area" as well as the "second moment" methods.

The molecular weights were computed by means of Svedberg's equation (3) with Theorell's (5) value of 0.707 for the partial specific volume of cytochrome c. Axial ratios were calculated from the frictional ratio, f/f₀, with the aid of Perrin's equation for prolate ellipsoids of revolution (3).

Observations and Results

Sedimentation Velocity Experiments—The sedimentation rates of two of the ferricytochrome preparations (horse and pig) were determined at two

1 In addition to phosphate, the cytochrome solutions contained a small amount (less than 0.02 per cent) of sodium chloride.

2 Dr. H. Tint, personal communication.
different concentrations, while the third (beef) was examined at one concentration only. Due to the deep red color of the solutions, long exposure times (up to 4 minutes) were necessary, since the light emission of the mercury arc in the red region of the spectrum is very low. The slow sedimentation rate of the cytochrome c boundaries made it necessary to spin the solutions at high centrifugal speeds for relatively long periods of time. A certain amount of boundary spread due to diffusion was thus unavoidable.

### Table I

*Molecular Kinetic Data on Ferricytochrome c Preparations*

Solvent, 0.1 M phosphate buffer, pH 7.7.

<table>
<thead>
<tr>
<th>Species</th>
<th>Purity, per cent</th>
<th>Concentration, per cent</th>
<th>Sedimentation constant, $s_20 \times 10^3$</th>
<th>Diffusion constant, $D_{0} \times 10^7$</th>
<th>Molecular weight, $M_a$</th>
<th>Disymmetry coefficient, $f/h$</th>
<th>Axial ratio, $a/b$</th>
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<tr>
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<td>Mean</td>
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<td>13.3</td>
<td>15,600</td>
<td>0.93</td>
<td>(1.0)</td>
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</table>

* Value based on dilution factor; the spectrophotometric assay indicated a higher value.

An additional amount of blurring was caused by the long exposure time.

Visual observations in the absence of light filters showed that the color gradient coincided with the sedimenting boundaries; i.e., the sedimentation of the hemoprotein itself was recorded in these experiments. Furthermore, when the analytical centrifuge cell was inspected at the conclusion of the runs, a layer of colorless supernatant fluid was present on top of the red bottom layer.

The sedimentation data obtained in these experiments are summarized in Table I.

In earlier experiments performed on another preparation of horse cyto-
chrome c of high purity, it was found that reduction of the ferri to the ferro form by means of ascorbic acid at pH 7.1 did not alter the sedimentation rate significantly; for the ferri form a value of \( s_{20} = 2.0 \) S was obtained, while the ferro form yielded a value of \( s_{20} = 2.2 \) S, both measured at 1 per cent concentration.

The high degree of stability of the material is borne out by the fact that the sedimentation constant of the pure horse ferricytochrome c remained essentially the same after storage at about 4° for 2 years. The examination of a preparation of a lower degree of purity (75 per cent) indicated that the presence of colorless impurities does not materially influence the sedimentation rate of the cytochrome c (\( s_{20} = 2.4 \) S at 1 per cent concentration).

A representative sedimentation diagram is reproduced in Fig. 1.

**Diffusion Measurements**—The diffusion apparatus was calibrated by means of a 0.5 per cent solution of mannitol of high purity. The value obtained, \( \text{viz.} \ d_{20} = 0.55 \times 10^{-5} \) sq. cm. per second, for the diffusion coefficient is in good agreement with the value of \( 0.53 \pm 0.02 \times 10^{-5} \),
listed in the "International critical tables" (12). The diffusion rates of
the three cytochrome preparations were studied at two different concen-
tration levels. Representative diffusion diagrams are shown in Fig. 2.

It is evident from Fig. 2 that there is no appreciable skewing of the
boundaries, and hence little concentration dependence is to be expected.
Table I presents the diffusion constants for the three cytochrome prepara-
tions, the values listed being those obtained by the "maximal ordinate area"
method. Those obtained by the "second moment" method are closely
similar (for a further analysis of these data, see Farber (13)).

Molecular Parameters—Table I lists the molecular weights, dissymmetry
coefficients, and axial ratios of the three cytochrome c preparations ob-
tained in the manner indicated above. These values are subject to the
limitations mentioned by Neurath (14).

DISCUSSION

The results of the present experiments on highly purified cytochrome c
preparations from various mammalian species are in satisfactory agreement
with the previously reported values. Thus, Pedersen, in his unpublished
experiments cited by Paul (6), obtained a sedimentation constant, \( s_{20} = 1.83 \) S, on 0.22 to 1 per cent solutions of "pure cytochrome c," presumably
prepared from horse heart, over a pH range from 3 to 10. By inserting a
value of \( 11.3 \times 10^{-7} \) sq. cm. per second for the diffusion coefficient \( D_{20} \),
together with the above sedimentation constant, and a value of 0.702 for
the partial specific volume into Svedberg's equation, a molecular weight
of 13,200 was obtained. The close numerical agreement with our own
value for the molecular weight of pure horse heart cytochrome c (Table I)
is probably fortuitous. Adair (15) had previously obtained a value of
16,500 for the molecular weight of horse heart cytochrome c, containing
0.34 per cent Fe, by osmotic pressure measurements.

For beef heart cytochrome c, containing 0.33 to 0.34 per cent Fe, Theorell
(16) originally reported a molecular weight of 16,500, which was based on
a sedimentation constant of 2 S obtained by Pedersen (in unpublished
experiments), and a diffusion coefficient of \( 11.1 \times 10^{-7} \) and a partial specific
volume of 0.707, determined by himself. Subsequently, Polson (17) ar-
rived at a value of 15,600, with \( s_{20} = 1.89 \) S (unpublished measurements
by Andersson), \( D_{20} = 10.11 \times 10^{-7} \), determined by himself, and \( \bar{V} = 0.71 \)
(Theorell's value). Again, the identity of this value for the molecular
weight of beef heart cytochrome c with our value (Table I) must be re-
garded as a coincidence. Tint and Reiss ((7) p. 397) have calculated the
molecular weight of the cytochrome preparations studied here, based on
their Fe content and their purity as determined by electrophoresis, assum-
ing that each mole of the pigment contains 1 gm. atom of Fe. A compar-
ison of their values ((7) Table I, p. 388) with the data here reported (Table
I) shows that they are of the same order of magnitude, and hence provides further evidence that cytochrome c contains only one heme group per molecule.

Cytochrome c belongs to the smallest molecular weight class of proteins established by Svedberg and Pedersen (3). Although the molecular weight values for the three preparations differ somewhat, it cannot be stated with assurance that these differences are significant. However, Tint and Reiss ((7) p. 385) have reported small but significant differences in the electrophoretic mobility of these preparations, which would suggest that the cytochromes isolated from horse, beef, and pig heart tissues are not identical. This is also true for the hemoglobins of different animal species (2).

The molecule appears to be very nearly spherical in shape, as indicated by the fact that the dissymmetry coefficients are close to unity (Table I).

There is no a priori reason to assign to cytochrome c the molecular weight of 16,500, i.e. that of the hypothetical Hübner unit which represents one-quarter of the molecular weight of hemoglobin (68,000), just because cytochrome c, like the Hübner unit, contains only 1 hemin residue and thus only 1 Fe atom per molecule. The amino acid composition of the protein moieties of these hemoproteins displays significant differences (2). Although the heme of both compounds is iron protoporphyrin IX, the linkage of the prosthetic group to the protein components differs greatly in nature and stability. Thus hemoglobin and cytochrome c represent distinct and non-interconvertible entities.

**SUMMARY**

Sedimentation velocity and free diffusion measurements were performed on highly purified ferricytochrome c preparations from the heart tissues of three mammalian species (horse, beef, and pig). Neither the sedimentation velocity nor the diffusion rate exhibits concentration dependence. The shape of the molecule as derived from hydrodynamic data appears to be nearly spherical for each of the species studied. The molecular weight is of the order of 15,000.

The authors wish to express their indebtedness to Dr. H. Tint and Dr. W. Reiss of the Wyeth Institute of Applied Biochemistry for making available the cytochrome preparations and for the spectrophotometric assays. They also wish to thank Professor Kurt G. Stern for suggesting the problem and for his assistance in the preparation of the manuscript for publication.

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