Molecular Size and Subunit Structure of the Hemoglobins of Chironomus tentans*

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SUMMARY

The larval hemoglobins of the insect Chironomus tentans, present in solution in hemolymph, were demonstrated to be exclusively monomeric with a molecular weight of approximately 15,900. This finding was based on studies of sedimentation velocity at various concentrations and with the use of agents generally effective in subunit separation, together with determinations by sedimentation equilibrium. No evidence of subunit aggregation was obtained. Column filtration studies suggest that the hemoglobins are more asymmetrical than myoglobin, but not to the extent of greatly altering sedimentation velocity. The monomeric nature of the molecule establishes a direct correspondence of hemoglobins to globin genes.

The larval hemoglobins of the insect Chironomus, noted for their extraordinary oxygen affinity and sharply hyperbolic equilibrium curve, are generally considered to consist of two polypeptide chains with two heme groups. Svedberg and Eriksson-Quensel (1) estimated the molecular weight of hemoglobin from Chironomus plumosus at 31,400 with a sedimentation coefficient (s20,w) of 2.0. This hemoglobin they supposed to consist of paired globin subunits comparable to the 17,000 molecular weight subunits of vertebrate hemoglobins. Braunitzer and Braun (2, 3) have isolated individual hemoglobins of Chironomus thummi and split them into pairs of dissimilar polypeptide chain types by countercurrent distribution. The amino acid content of specific chains gave a derived molecular weight for dimeric combinations of approximately 31,000, in agreement with previous estimates. More recently, however, Braunitzer (4) has expressed doubt as to the dimeric structure of his material, which may have represented combined monomeric forms.

In studies of the multiple hemoglobins of Chironomus tentans (5), hybridization of extensively different genetic strains failed to produce unfamiliar electrophoretic components resulting from new dimeric combinations of polypeptide subunits. Furthermore, it was not possible among the hemoglobins of C. plumosus (6) and C. tentans (7) to obtain the separation of dissimilar units by electrophoresis in starch gels with 8 M urea, low pH, and mercaptoethanol combined, whereas vertebrate hemoglobins readily split into subunits under these conditions. Manwell (6) has concluded from such evidence that individual hemoglobins of C. plumosus, if dimers, must consist of identical subunits.

For the interpretation of past studies on the respiratory function of hemoglobins in Chironomus (reviews in References 8 and 9), as well as genetic studies now in progress, it has seemed essential that the molecular size and subunit structure of the material be clarified. The present determinations indicate the exclusive occurrence in C. tentans of a monomeric form with a molecular weight of approximately 15,900.

EXPERIMENTAL PROCEDURE

Preparation of Hemoglobin Samples—Solutions of hemoglobin were prepared from the hemolymph of larvae (20 to 25 mm) of C. tentans deriving from a collection at Madison, Wisconsin. This species was separated from other chironomids on the basis of markings, dentition, and stenogamic behavior. Numbers of larvae were washed in distilled water, cut open gently, and bled in 0.05 M KH2PO4 buffer either with or without 1/2 % K2FeCN6, depending on whether the study involved oxyhemoglobin or cyanmethemoglobin. The larval debris was removed by centrifugation at 40,000 x g for 30 min. The supernantant material was reduced in volume by the direct application of dry Sephadex (Pharmacia, type G-25), and filtered on two columns, 2.5 x 45 cm, packed with Sephadex G-100 and placed in series. Elution was carried out in an upward fashion with a KCN buffer at pH 7.0 and a flow rate of 20 ml per hour.

Samples from the well defined peaks of absorption at 420 mμ (cyanmethemoglobin) or 412 mμ (oxyhemoglobin) were collected and reduced in volume. Scanning with a Cary model 15
sulfate (IO, 11). In the present study, samples of Chironomus monomeric subunits can be induced readily with sodium lauryl tions, and the effect on molecular size was determined by ultra-

See Table I for a summary of sedimentation velocities of Chironomus hemoglobin at various concentrations.

Effect of Concentration

The possibility of a concentration-dependent association of subunits was studied by determining sedimentation velocity over a wide range of sample concentrations, with oxyhemoglobin in a 0.05 M KH$_2$PO$_4$ buffer at pH 7.0. For these determinations, the Spinco model E ultracentrifuge was employed at 52,000 rpm with a Kodak Wratten filter, No. 77A, and schlieren optics. Exposures were made at 8-min intervals on metallographic plates. All plate readings were made with a Nikon model 6C microcomparator.

Submit Dissociation

The dissociation of human hemoglobin into dimeric and monomeric subunits can be induced readily with sodium lauryl sulfate (10, 11). In the present study, samples of Chironomus hemoglobin, human hemoglobin, and sperm whale myoglobin were treated with sodium lauryl sulfate under identical conditions, and the effect on molecular size was determined by ultracentrifugation as before. The vertebrate materials were supplied by Mann Research, and all three pigments were put into solution in 0.05 M Tris-HCl at pH 7.0.

In each case, sedimentation velocity of the native protein was determined in a wedge cell, and an aliquot of the same sample, made 1% with sodium lauryl sulfate, was run in a standard cell in the same rotor. Centrifugation was carried out within 30 min of adding the sodium lauryl sulfate. The Spinco model E ultracentrifuge was used at 60,000 rpm, and schlieren patterns were recorded at 8-min intervals.

Sedimentation Equilibrium

A determination of apparent molecular weight was carried out by low speed sedimentation equilibrium studies with the ultracentrifuge. The Spinco model E machine was used with Rayleigh interference optics and was aligned by standard Spinco procedures. A Baird-Atomic filter, with a bandpass at 690 m$n$ and infinity blocking, was employed. Interference patterns were photographed on type 1-N spectroscopic plates (Eastman Kodak). The cell was a standard Beckman Epon-filled double-sector centerpiece, with an interference window, filled to a column length of about 3 mm.

Equilibria were established at three concentrations. The least concentrated sample had an absorbance of 5.4 at 420 m$m$ and was centrifuged at 12,000 rpm. If the extinction coefficient of Chironomus hemoglobin is assumed to be near that of human hemoglobin (12), this sample represents a concentration of about 0.75 mg per ml. On the basis of fringe numbers, the other samples were at 1.29 and 1.96 mg per ml. They were centrifuged at 14,000 and 12,000 rpm, respectively. Apparent molecular weights ($M_{app}$) were calculated by the method of Svedberg and Pedersen (13), as described by Richards and Schachman (14) and the whole column method (14, 15).

Gel Filtration

Chironomus hemoglobin and a number of proteins of known molecular weights were compared by filtration on a column of Sephadox G-100. A slurry of the gel with a phosphate-NaCl buffer at pH 7.0 was allowed to stand for 3 days and evacuated before pouring. A column (Pharmacia), 2.5 x 45 cm, was filled with the Sephadox G-100 gel in one pass, at an effective height of 10 cm. (The procedure yielded an over-all gel bed height of approximately 40 cm.) Equilibration and filtration of samples were carried out at 23º. A flow rate of approximately 30 ml per hour was maintained.

Each sample was applied as a 3-ml layer under a volume of eluent at the top of the column while the column system was closed. Samples consisted of a protein and sucrose mixture, together with 0.1% blue dextran (Pharmacia) as a visible reference material. Fractions (1 ml) were collected in a Packard fraction collector with drop-count control. Protein peaks were identified by the ratio of optical densities at 412:281 m$m$ as determined with the Beckman model DU-2 spectrophotometer.

RESULTS

Sedimentation Velocities

Table I summarizes the determinations of sedimentation velocity with varied concentration. In spite of the scatter of $c_{20,w}$ values, it is clear that over a wide range of concentrations, including an approximation of actual physiological concentrations, molecular size does not change consistently with concentration. In view of the difficulties encountered at high concentration (shadowing of the schlieren pattern) and low concentration (spreading of the peak), the determination at an absorbance of 9.2 might be considered of greatest accuracy, and this value (1.78) is close to that of myoglobin. Furthermore, the schlieren pattern of each sample (Fig. 1) is a single, sharp peak with no suggestion of a multicomponent system or a concentration-dependent equilib-

between monomeric and dimeric forms.

The treatment of Chironomus hemoglobins with sodium lauryl sulfate also failed to produce any modification of sedimentation velocity and apparent molecular size. Table II shows a con-

firmation of the expected effect of this chemical on the molecular size of human hemoglobin, reducing the aggregate form to sub-
units with a sedimentation coefficient near that of myoglobin. This separation into monomeric subunits was a sharp one under the conditions employed (Fig. 2A). No significant change was seen in the behavior of myoglobin, and no change whatsoever occurred in the insect material (Fig 2B). Here, also, it appears that the native Chironomus hemoglobin is already in the mono-
meric form and does not conform to a system of subunit separa-

<table>
<thead>
<tr>
<th>Sedimentation velocities of Chironomus hemoglobin at various concentrations</th>
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<tbody>
<tr>
<td>Concentration (mg/ml)</td>
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<tr>
<td>-----------------------</td>
</tr>
<tr>
<td>1.5</td>
</tr>
<tr>
<td>5.4</td>
</tr>
<tr>
<td>9.2</td>
</tr>
<tr>
<td>22.0</td>
</tr>
<tr>
<td>22.0</td>
</tr>
</tbody>
</table>

*Assuming an extinction coefficient of $1.6 \times 10^4$ at 575 m$m$ (12).
Estimates of Molecular Weight

On the basis of sedimentation equilibrium studies at three concentrations, as described previously, molecular weight of the Chironomus hemoglobin was determined according to two formulations. Estimates can be made by the method of Svedberg and Pedersen (13), by means of the equation

\[ M_{\text{app}} = \frac{2RT}{\omega^2(1 - \epsilon_p)} \frac{d \ln c}{dr^2} \]  

where \( \omega \) is the angular velocity of the rotor, \( \epsilon_p \) the partial specific volume of the protein, \( p \) the solution density, \( R \) the gas constant, \( T \) the absolute temperature, and \( c \) the concentration at different distances, \( r \), from the axis of rotation. A specific volume of 0.751 was used, since Svedberg and Eriksson-Quensel (1) have found most invertebrate hemoglobins to conform closely to this value.

Concentration at each point was determined by counting fringes from the meniscus. The concentration, \( c_m \), at the meniscus was calculated by the equation (14)

\[ c_m = c_0 - \frac{r_b^2 (c_b - c_m) - \int_{r_m}^{r_b} r^2 dc}{r_b^2 - r_m^2} \]  

where \( c_0 \) is the concentration of solute at the base of the column, \( c_b - c_m \) the observed difference in fringes from bottom to meniscus, and \( r_m \) and \( r_b \) are the distances of the meniscus and bottom from the center of rotation. The initial concentration, \( c_0 \), was obtained by a preliminary run with a double-sector synthetic boundary cell. Homogeneity and lack of aggregation within samples may be inferred from Fig. 3, which shows an essentially linear plot of log \( c \) versus \( r^2 \) in the most concentrated sample.

Molecular weights were also calculated from the interference

![Fig. 1. Sedimentation boundary of Chironomus hemoglobin showing single peak. Done at 13°, pH 7.0, absorbance = 9.2 at 412 m\( \mu \); 52,000 rpm; after 48 min.](image1)

![Fig. 2. Effect of sodium lauryl sulfate on the sedimentation of (A) human hemoglobin, (B) Chironomus hemoglobin. Lower samples with sodium lauryl sulfate, upper samples without. The pictures were taken at 8-min intervals at 60,000 rpm, and the direction of sedimentation is to the right.](image2)

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
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<tbody>
<tr>
<td>Human hemoglobin</td>
<td>10.5</td>
<td>12.0</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>12.0</td>
<td>12.0</td>
</tr>
<tr>
<td>Chironomus hemoglobin</td>
<td>12.0</td>
<td>12.0</td>
</tr>
</tbody>
</table>

**Table II:** The effect of sodium lauryl sulfate on molecular size

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sedimentation coefficient</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Buffer alone</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Buffer with sodium lauryl sulfate</td>
<td></td>
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</table>
patterns by the whole column method, by means of the equation of Lansing and Kraemer (15)

\[ M_{wp} = \frac{2RT}{\omega^2(1 - \eta_p)(r - r_p^2)} \left( \frac{c_h - c_m}{c_0} \right) \]  

Equilibrium concentrations, together with initial concentrations and estimates of molecular weight by both equations, are summarized in Table III.

Molecular weights determined by the distribution of protein over the whole column are in general agreement with those determined on a point-by-point basis within the same sample. Averaging within samples gives molecular weights of 15,700, 15,900, and 15,900. Thus, it appears that at every concentration, and without any indication of aggregation, we have been dealing with the single polypeptide of about 16,000 molecular weight described by Braunitzer and Braun.

With this evidence for a monomeric hemoglobin somewhat below myoglobin in molecular weight, the results of molecular filtration on columns of Sephadex G-100 take on greater interest. It may be seen from a plot of elution rate against molecular weight of a number of reference proteins and Chironomus hemoglobin (Fig. 4) that the latter moves as a slightly larger molecule than myoglobin. The discrepancy between molecular weight and apparent molecular size may relate to some asymmetry of the insect hemoglobin. Svedberg and Eriksson-Quensel inferred that the molecule is highly asymmetrical because the sedimentation velocity, \( s_{20,w} = 2.0 \), observed by them does not agree with a molecular weight of 31,400. In our study, however, sedimentation velocities of about 1.7 are consistent with the 15,900 molecular weight, and asymmetry would appear to be a minor factor. Binding on the Sephadex column cannot be ruled out as a possible basis for discrepancies in rate of migration.

### DISCUSSION

In comparisons of the functioning and the physicochemical properties of vertebrate and invertebrate hemoglobins, molecular size and the aggregation of subunits have been major considerations (16). It is clear, for example, that the characteristically tetrameric vertebrate hemoglobins have distinct heme-heme interactions and relatively low oxygen affinities. On the other hand, the monomeric hemoglobin of the hagfish and mammalian myoglobin have no heme-heme interaction and high oxygen affinities. Hemoglobin of the lamprey, Petromyzon marinus, exemplifies these alternatives most perfectly since it undergoes a

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**Table III**

Estimation of molecular weight of Chironomus hemoglobin by equilibrium studies

<table>
<thead>
<tr>
<th>Sample concentration (mg/ml)</th>
<th>( \omega )</th>
<th>( \omega - \omega_m )</th>
<th>Molecular weight (( \times 10^4 )) By equation 1</th>
<th>Molecular weight (( \times 10^4 )) By equation 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.7</td>
<td>3.91</td>
<td>1.02</td>
<td>1.58</td>
<td>1.55</td>
</tr>
<tr>
<td>1.3</td>
<td>6.78</td>
<td>5.50</td>
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<td>1.73</td>
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<tr>
<td>2.0</td>
<td>10.47</td>
<td>8.38</td>
<td>1.52</td>
<td>1.66</td>
</tr>
</tbody>
</table>

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reversible aggregation with increased concentration. The monomeric molecule has no heme-heme interaction and a hyperbolic oxygen equilibrium, while the aggregate form does give evidence of a heme-heme interaction and decreased oxygen affinity (17, 18).

Dimeric hemoglobins of invertebrates such as *Gastrophilus*, and supposedly *Chironomus*, have high oxygen affinities and hyperbolic equilibria. To some extent, the basis of these properties may be configurational, since both are reported to be distinctly nonspherical (1, 19). In the present study, however, it has been shown that *Chironomus* hemoglobin is largely or entirely monomeric, conforming to the type of myoglobin in structure as well as physicochemical behavior. The absence of heme-heme interactions is quite predictable on this basis alone.

These lines of evidence are compatible with observations on the genetic hybridization of *C. tentans*, in which hybrid individuals show a codominant electrophoretic pattern of hemoglobins. In *C. tentans*, the only member of the genus that can be mated by rigorous genetic procedures, the interpretation of genetic control becomes very simple. Although there are as many as 8 to 12 hemoglobins in individual larvae (5), each hemoglobin component is a single, unique polypeptide chain under the control of a single gene. The existence of multiple hemoglobins, then, implies a system of duplicate globin genes like that postulated in man (20) but numbering 8 to 12 to correspond to polypeptide chain types.

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REFERENCES
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