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The Extracellular Hemoglobin of the Earthworm, *Lumbricus terrestris*

DETERMINATION OF SUBUNIT STOICHIOMETRY*

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The giant extracellular hemoglobin of the earthworm, *Lumbricus terrestris*, has four major O_2-binding chains, *a*, *b*, and *c* (forming a disulfide-linked trimer) and *d* ("monomer"). Participation of additional "linker" chains L_1, L_2, and L_3 is necessary for the assembly of the ≈3,900+ kDa two-tiered hexagonal structure. We have determined the proportions of linker chains, trimer, and chain *d* in the hemoglobin by reverse phase high performance liquid chromatography which resolves all of the components and also permits simultaneous determination of the heme content. The proportions of components were determined by two independent procedures: integration of the absorbance peaks at 220 nm and amino acid analysis of the peak fractions. The results indicate that the weight proportion of linker chains is 0.163 ± 0.023. This value, together with molecular masses determined both by amino acid sequence analysis and by matrix-assisted laser desorption mass spectrometry, gives a molar ratio of *abcd* chains to linkers of 8:1, corresponding to the minimal unit $(abcd)_2 \cdot L$. This ratio suggests that 24 $(abcd)_2$ units and 24 linker chains form the complete structure with a total calculated mass of polypeptide of 3,975 kDa with hemes on chains *a*, *b*, *c* and *d* and on one linker. The calculated heme content is 3.1% not including carbohydrate. This accounts for a measured heme content of 3.0% on a polypeptide basis. Additional mass (≈133 kDa, 3.4%), attributed to carbohydrate, brings the total mass to 4,108 kDa with a minimum molecular mass/heme of 20,500 Da. The presence of equimolar quantities of three unique linker chains means that the apparent one-twelfth structural units seen by electron microscopy cannot all be identical.

The giant extracellular Hb of the earthworm, *Lumbricus terrestris*, has ≈200 chains that form a two-layered hexagonal structure of 3.9 ± 0.2 MDa (1–6). The sequences of the four major heme-binding chains, *a*, *b*, *c*, and *d*, have been determined (7, 8). Chains *a*, *b*, and *c* form a disulfide-linked trimer. Additional structural components, "linker chains," deficient in heme, are necessary for the assembly and integrity of the molecule (9).[1] The proportion of linker chains and heme content of the molecule has been controversial (10, 13, 16, 17). Direct measurement of the proportions of components resolved by reverse phase chromatography of the Hb of the related annelid *Tylorrhynchus heterochaetus* showed that the linker chains accounted for approximately 19% of the total 220 nm absorbance, a value suggesting 192 heme-binding chains and 24 linkers (11). Measurement of the heme:protein ratio in *Lumbricus* Hb by combination of heme and amino acid analyses gave 11.6% non-*abcd* chains (17). In contrast, Vinogradov and colleagues (9, 10, 12, 13) proposed a model in which the linkers comprise approximately one-third of the mass of the protein. The primary functional subunit in their model is a dodecamer $(abcd)_3$ with a molecular mass of ≈200 kDa. Twelve such units would then account for two-thirds of the total mass with the balance contributed by linker chains. "Mild" dissociation of the Hb in 4 M urea followed by gel chromatography in the absence of urea does indeed give an apparently homogeneous product of this mass (10). The much-reduced cooperativity of O_2 binding of the product led them to conclude that the complete two-layered hexagonal structure of the native Hb is required for full cooperativity (10). A different procedure, that of dissociation at pH 9.3, was used by Fushitani and Riggs (15) to isolate the trimer and chain *d*, which are then reassociated at neutral pH. The complex thus formed, *no larger* than $(abcd)_2$, displays an O_2 equilibrium *indistinguishable* from that of the native molecule at pH 6.8 (15). These data, together with the earlier measurement of the heme:protein ratio (17), suggests (15) that the primary functional subunit is a dimer of tetramers, $(abcd)_2$, not a dodecamer.

Resolution of this problem clearly requires that the proportions of the constituent chains be accurately determined. We report here determination of the stoichiometry of chains and heme content by new, unambiguous techniques that utilize high performance liquid chromatography, amino acid analysis, and matrix-assisted laser desorption mass spectrometry.

MATERIALS AND METHODS

Worms purchased from Wholesale Bait Co. (Hamilton, OH) were washed twice in water and anesthetized in 5% ethanol prior to bleeding. A dorsal incision anterior to the aortic loops was made to

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[1] The previous designation (10) for linker chains, Va, Vb, and VI, the components of subunits (D1A, D1B, and D2) was based on the assumption that they were dimers. Since they are not dimers and are distinct from globin chains, we use the nomenclature adopted for *Tylorrhynchus* Hb (11). We also use the *abcd* nomenclature adopted earlier (8). Chains *a*, *b*, *c* and *d* correspond, respectively, to chains IV, II, III, and I of Ref. 12. It seems less cumbersome to refer to $(abc)_2$ rather than $(IV\text{-}II\text{-}III)_2$. Although chain *d* has usually been described as the "monomer" chain (13), we avoid this term because it is dimeric (14, 15).

avoid cutting the intestine. Worms were bled into CO-saturated 0.1 M phosphate, pH 7.0, 1 mM EDTA containing the following protease inhibitors: leupeptin (0.5 mg/liter), pepstatin (0.7 mg/liter) (Boehringer Mannheim), and phenylmethylsulfonyl fluoride (0.2 mM) (Sigma). The presence of a protease inhibitor in the coelomic fluid of *L. terrestris* (18) may afford some protection against proteolysis. Cellular and particulate debris were removed by low speed centrifugation. The hemoglobin was precipitated with 10% (w/v) polyethylene glycol (molecular weight 8,000; Sigma). The precipitate was collected by centrifugation at 10,000 × *g* for 20 min and then resuspended in CO-saturated 50 mM Tris, pH 7.5, 1 mM EDTA. The Hb was pelleted twice at 50,000 rpm for 1.5 h (Sorvall model OTD 75B, T-875 rotor, 243,200 × *g*) and stored at −80 °C. Storage at −80 °C was chosen to avoid the possibility of proteolysis. Hb from 12 individual worms was prepared in the same manner, except that each was bled into a separate 1.5-ml Eppendorf tube containing 0.5 ml of the same pH 7.0 buffer. All preparatory steps were performed at 0–4 °C.

Chromatography—Gel filtration was on a column of Ultrogel AcA 44 (LKB) of 80 × 2.5 cm, in CO-saturated 0.1 M borate, pH 9.3, 1 mM EDTA both at 4 °C and at room temperature.

All HPLC[2] was performed on Synchropak RP-P C_{18} reverse phase columns, 250 × 4.6 mm (SynChrom, Inc., Lafayette, IN), driven by a Beckman 332 gradient liquid chromatography system. Absorbance of the eluate was monitored at 220 nm with an Hitachi 100–10 spectrophotometer. Peak areas were estimated either by cutting out and weighing the chart paper corresponding to the peaks or by a Hewlett-Packard model 3390A integrator connected in parallel with the strip chart recorder. The two methods agreed to within 2%.

A linear gradient was used between H_2O, 0.1% trifluoroacetic acid and CH_3CN, 0.1% trifluoroacetic acid with a constant flow rate of 0.8 ml/min. Solvents were passed through 0.45-μm filters (Nylon 66, Schleicher & Schuell); samples were either filtered or centrifuged at low speed (<10,000 × *g*). Injection volumes were 20 μl for the more concentrated Hb solutions; a 180-μl loop was used for larger volumes. Fractions were collected from the HPLC and dried in a SpeedVac centrifuge under vacuum. Care was taken to ensure that the fractions collected corresponded closely to those determined by the 220 nm absorbance. Samples were vortexed midway through the drying process to help avoid losses on the walls of the vessel. It was imperative that the drying of these fractions be accomplished immediately; delays resulted in decreased solubility of the dried product.

Gel Electrophoresis—SDS-polyacrylamide gel electrophoresis (see Fig. 2*b*) was carried out on fractions collected from HPLC (see Fig. 2*a*). An acrylamide gradient was used between 15% (bottom) and 10% (top), pH 8.8. The stacking gel was at pH 6.8. Samples were run with Tris-glycine buffer, pH 8.3, at a constant 100 V. Gels were stained with Coomassie Brilliant Blue R (Sigma). Molecular mass markers (Kit no. MW-SDS-70L, Sigma) were: 66 kDa (bovine serum albumin), 36 kDa (glyceraldehyde-3-phosphate dehydrogenase), 29.0 kDa (carbonic anhydrase), 24 kDa (trypsinogen), 20.1 kDa (soybean trypsin inhibitor), and 14.2 kDa (α-lactalbumin). Integrated spot densities were obtained with a videodensitometer developed by the Clayton Foundation, United States patent 5194949. This instrument obtains 63,000 optical density measurements that cover the entire gel simultaneously.

Protein and Heme Calibration—To determine whether protein was retained on the HPLC column, and particularly whether some chains were preferentially retained, amino acid analysis was performed for both the individually collected fractions and the unchromatographed native Hb. The fractions were dried as described above. Comparison of total protein in the two analyses showed that protein recovery from HPLC is better than 93%. The amino acid analyses were used to correlate the peak areas (integrated 220 nm absorbance) with the actual quantity of protein present.

SDS-polyacrylamide gel electrophoresis of the heme-containing fraction showed that no protein coeluted with the heme. This is a necessary precaution because chain L_1 does coelute with heme on some reverse phase columns.

Calibration of the heme peak areas was made with a series of injections of varying amounts of a hemin stock solution (0.85 mM, $\epsilon_{mM} = 50$ at 390 nm (19), covering the range of heme concentrations of the Hb experiments. A linear least squares fit of the resulting peak areas to the quantity of heme injected was used to estimate the quantity of heme present in the Hb chromatograms (Fig. 1).

Amino Acid Analysis and Sequencing—Amino acid analysis was performed by the University of Texas Protein Sequencing Center with an Applied Biosystems model 420A-03 instrument with the hydrolysis option (model 4204-H). Amino acid sequencing was performed with an Applied Biosystems model 477A Sequencer. Amino acid analyses were performed in triplicate with norleucine as internal standard. No corrections were made for losses of tryptophan and cysteine or decreased yields of other amino acids.

Mass Spectrometry—Protein samples isolated by reverse phase HPLC were subjected to analysis on a matrix-assisted laser desorption time-of-flight mass spectrometer constructed at Rockefeller University and described elsewhere (20, 21). The mass spectra were collected by adding individual spectra obtained from 200 laser shots. Protein samples were prepared for laser desorption mass analysis (20–22) as follows. The laser desorption matrix material (sinapinic acid) was dissolved in 0.1% trifluoroacetic acid, acetonitrile 2:1 (v/v) to a concentration of 50 mM. A 20 μM aqueous solution of the protein was then added to the matrix solution to give a final protein concentration of approximately 2 μM. A small aliquot (0.5 μl) of this mixture was applied to the metal probe tip and dried at room temperature with forced air. The sample was then inserted into the mass spectrometer and analyzed. Horse heart myoglobin or bovine carbonic anhydrase II was used to calibrate the mass spectra. Reduction of both the trimer *abc* and the minor trimer T_2 was carried out in 50 mM NH_4HCO_3, 50 mM dithiothreitol, pH 8.0, for 1 h. A 1-μl aliquot of the mixture of reduced products (20 μM/component) was added to 9 μl of 50 mM sinapinic acid in 0.1% trifluoroacetic acid/acetonitrile, 2:1 (v/v). The pH of the resulting solution was lowered by the addition of 2 μl of 2.5% trifluoroacetic acid/acetonitrile, 2:1 (v/v) because matrix-assisted laser desorption using sinapinic acid as a matrix is ineffective at pH values close to neutrality.

RESULTS AND DISCUSSION

Stoichiometry

Reverse phase chromatography (HPLC) of native Hb, monitored at 220 nm (Fig. 2*a*), resolves five distinct protein components and the completely dissociated heme. The three major linker chains L_1, L_2, and L_{3c}[1] are completely separated from chain *d* and the disulfide-linked *abc* trimer. Minor components L_{3a} and L_{3b} are also present. Each of the major linker chains have been characterized by SDS-electrophoresis, NH_2-terminal amino acid sequencing, amino acid analysis, and determination of the molecular masses by mass spectrometry (Fig. 2, *a* and *b*, and Tables I–V). Quantitative analysis is given in Table I for the fractions obtained in the chromatography shown in Fig. 2*a*. Chains L_1 and L_2 have mobilities on SDS-gel electrophoresis (Fig. 2*b*) which are consistent with their masses determined by mass spectrometry. However, L_3 has a slightly lower mobility than expected. A possible explanation is that L_3 has almost twice the proline content (5.7 mol %) found in L_1 and L_2 (3.1 and 3.4 mol %, respectively). A high proline content has been proposed to explain the anomalously high apparent molecular weight of other proteins, for example, DNA ligase I (Pro, 7.8 mol %), on SDS-gel electrophoresis (23).

The proportions of components in four chromatograms were analyzed by two independent procedures: integration of absorbance at 220 nm and amino acid analysis (Table II). The computational procedure is given in Table III. The linker chain L_1 corresponds to chain Va (subunit D1A) of (9) on the basis of NH_2-terminal sequence (17), but the correspondence of L_2 and L_3 with the previously reported linker chains Va (subunit D1B) and VI (subunit D2) (9) is uncertain[1] because the latter chains have not been well characterized. Although chain L_2 may correspond to chain VI (subunit D2) of (9) on the basis of molecular mass, we cannot identify chain L_3 with previously reported chains. The fraction of the total mass which can be ascribed to linker chains, determined from the integrated 220-nm absorbance (Table III), is 0.163 ± 0.023. This result might be in error because of preferential loss of components on the column or differences in extinction coef-

[2] The abbreviation used is: HPLC, high performance liquid chromatography.

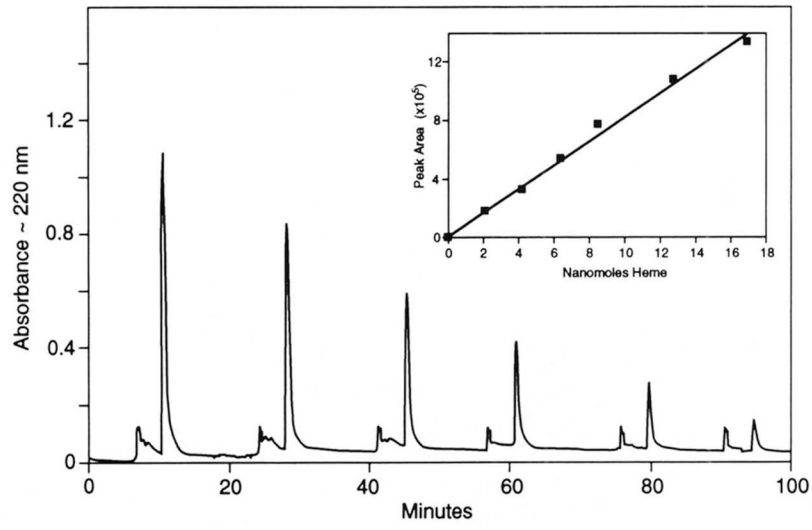

FIG. 1. **Calibration of peak area to moles of hemin.** Aliquots (20–2.5 μl) of a stock solution (0.85 mM hemin) were injected onto a reverse phase C_{18} column and eluted isocratically with 55% H_2O, 45% acetonitrile, 0.1% trifluoroacetic acid. Areas are in arbitrary units. The *inset* shows a linear least squares fit of the integrated areas as a function of mol of hemin.

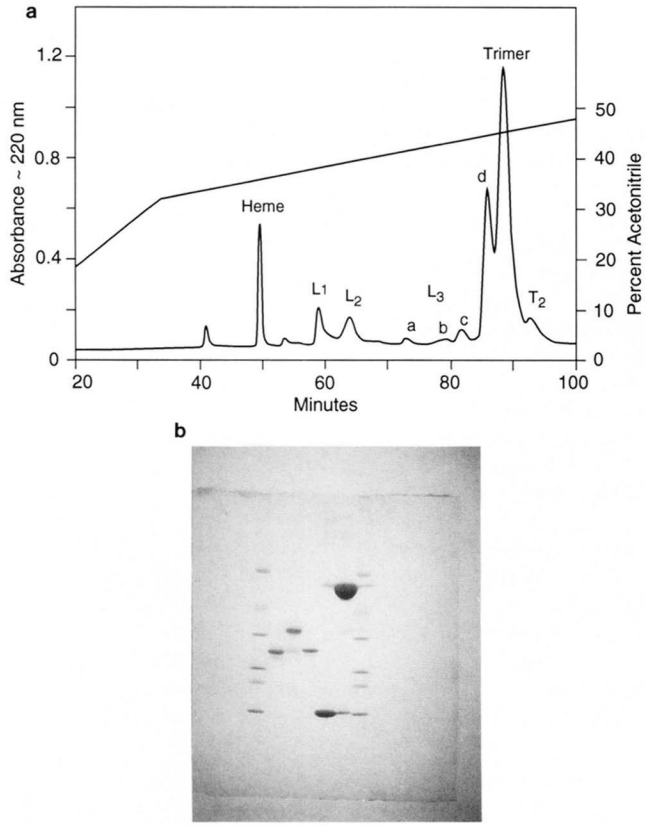

FIG. 2. *Panel a*, HPLC of Hb from *L. terrestris*. Hb (15 μl, ≈ 1 mM heme) was applied to the column and eluted with a linear gradient between H_2O and acetonitrile, both 0.1% in trifluoroacetic acid. Flow rate: 0.8 ml/min. The pre-heme peak appears to be a non-protein artifact in the sample buffer; it contained no amino acids. The minor peak at 54 min appears to be a degradation product because it increases in amount upon prolonged storage of Hb samples at 4 °C. T_2 is a minor trimer (see *Heterogeneity* under "Results and Discussion"). *Panel b*, SDS-polyacrylamide gel electrophoresis of fractions recovered from HPLC of Hb from *L. terrestris* (as in *panel a*). A nonreducing gradient gel between 10 and 15% (*top* to *bottom*) was used with 25 mM Tris, 192 mM glycine, pH 8.3, and stained with Coomassie Blue R-250. *Far right* and *far left lanes*, molecular mass markers, 66, 36, 29.0, 24, 20.1, and 14.2 kDa; *second lane*, L_1; *third lane*, L_2; *fourth lane*, L_{3c}; *fifth lane*, chain *d*; *sixth lane*, *abc* trimer. *All* of each HPLC fraction was loaded. The mobility of L_{3c} is anomalous; the apparent molecular weight is higher than that determined by mass spectrometry (see "Results and Discussion.").

TABLE I

Comparison of amino acid analysis and integrated peak areas for the HPLC of the Lumbricus *Hb sample in Fig. 2a*

Chain	Weight	Weight fraction	Area[a]	Area fraction
	μg			
L_1	13.0	0.0658	513,150	0.0505
L_2	10.2	0.0517	600,110	0.0591
$L_{3a,b,c}$	4.54	0.0229	250,880	0.0247
d	43.9	0.222	2,350,500	0.231
(*abc*)	126.0	0.638	6,448,380	0.635
Total	198.0		10,163,020	

[a] Arbitrary units.

ficients of the components at 220 nm. The first possibility was explored by collection, pooling, and rechromatography of the eluted components (data not shown). This procedure resulted in no significant change in the proportions of components and, together with an overall recovery better than 93%, indicates that no significant preferential loss occurred. The second possibility is that the 220-nm absorbance is misleading because of differences in the extinction coefficients of the components. This was examined by amino acid analysis of each fraction (Table II). The close correspondence of the proportions obtained by the two methods (correlation coefficient > 0.999) indicates that 220 nm absorbance is a satisfactory measure of proportions within the errors of measurement.

These results differ by roughly a factor of 2 from the proportions of linker chains estimated by densitometry of Coomassie Blue-stained gels (9, 13, and other references in 10). However, it is well established that Coomassie Blue staining differs greatly for different proteins, and extinction coefficients of the dye-protein complexes can vary by a factor of 10, depending on the positive charge carried by the protein and other factors (24). Even when interfering SDS is removed, the extinction coefficients of *different* proteins vary greatly after Coomassie Blue staining (25). Since linker chains do differ greatly from chains *a*, *b*, *c*, and *d* and may be unrelated (26), the extinction coefficients of the corresponding complexes with Coomassie Blue can also be expected to differ. Differences between our preparations and those of Vinogradov and co-workers (9, 10, 13) might conceivably be responsible for the differences in proportions of linker chains, but we have performed HPLC on fresh preparations supplied by them and find that the linker chain proportions are essentially the same as those we report here. Quantitative densitometry of the bands in Fig. 2*b* showed that Coomassie Blue staining resulted in an overestimate of the proportion of linker chains

TABLE II

Weight and area fractions and heme content for a quadruplicate set of HPLC and amino acid analysis experiments

Heme and iron content values are the weight percent of total heme and protein, calculated from the heme peak area as calibrated in Fig. 1 and from the peak area:protein ratio determined from the amino acid analysis. The data for experiment 1 are from Table I.

Chain	Exp. 1		Exp. 2		Exp. 3		Exp. 4		Averages[a]	
	Weight	Area	Weight	Area	Weight	Area	Weight	Area	Weight	Area
L_1	0.0658	0.0505	0.0587	0.0584	0.040	0.0499	0.0600	0.0521	0.0561 ± 0.01	0.0527 ± 0.004
L_2	0.0517	0.0591	0.048	0.0672	0.0731	0.0755	0.0528	0.0490	0.0564 ± 0.010	0.0627 ± 0.010
$L_{3a,b,c}$	0.0229	0.0247	0.0514	0.0597	0.0260	0.0296	0.008	0.0356	0.0271 ± 0.017	0.0374 ± 0.014
d	0.222	0.231	0.165	0.183	0.186	0.179	0.189	0.233	0.191 ± 0.021	0.207 ± 0.027
(*abc*)	0.638	0.635	0.677	0.631	0.675	0.667	0.690	0.631	0.670 ± 0.021	0.641 ± 0.016
% Heme	2.72		2.69		2.90		3.31		2.91 ± 0.26	

[a] Values given are ± 1 S.D., $n = 4$.

TABLE III

Average area fractions and predicted polypeptide number and heme content for the hemoglobin of L. terrestris

Values given are the average area fractions and weight percent heme for the four data sets in Table II combined with five additional chromatograms for which amino acid analyses were not performed. Errors given are ± 1 S.D., $n = 9$.

Chain	Area fraction	Experimental[a]	Model
L_1	0.057 ± 0.015	8.6 ± 2.2	8
L_2	0.062 ± 0.015	8.1 ± 1.8	8
$L_{3a,b,c}$	0.044 ± 0.015	7.5 ± 1.8	8
d	0.196 ± 0.023	47.7 ± 5.6	48
(*abc*)	0.642 ± 0.029	49.0 ± 2.2	48
ΣL_i	0.163 ± 0.023	24.2 ± 3.4	24
$\Sigma abcd$	0.837 ± 0.037	195 ± 9	192
% Heme	3.04 ± 0.25	199 ± 22	200

[a] A molecular mass of 3,900 kDa has been assumed for polypeptide mass of the native Hb, and sequence-derived masses for chains *a*, *b*, and *c*, and mass-spectrometry derived mass of chain *d* (see Table V) are used for subunits. The experimental values were calculated by the following equation.

$$\text{No. chains } i \text{ per molecule Hb} = \frac{(\text{peak area})_i}{\sum_i (\text{peak area})_i} \times \frac{\text{Molecular mass of Hb}}{\text{Molecular mass of chain } i}$$

by at least 60% (data not shown). We conclude that staining of polyacrylamide gels cannot be used to provide accurate information on stoichiometry. Although high proportions of linkers were estimated by direct analysis of amino acid compositions (27), the proportions calculated are suspect because the errors given are as large as the values themselves.

Heme Content

The complete dissociation of the heme during reverse phase chromatography (Fig. 2*a*) makes possible the determination of the heme and chain proportions from *the same chromatogram*. This procedure avoids all potential errors that may arise from isolation and analysis of heme and protein in separate experiments. The heme peak was directly calibrated with a known sample of heme (Fig. 1). The results (Table III) indicate a heme content of 3.04 ± 0.25%. These values depend on amino acid analysis and do not include the additional mass attributed to carbohydrate and should not be confused with values dependent on determination of dry weight. We have used the isolated trimer fraction as a control. The heme content measured for the trimer was 3.58%, which is only 2.3% higher than the calculated heme content of 3.50%.

The *abc* trimer, chain *d*, and linker chains have also been separated by gel chromatography at alkaline pH (Fig. 3). The peak in the 280/570 nm absorbance ratio between the trimer and chain *d* fractions reflects the presence of non-heme protein. If one assumes (i) that the extinction coefficient of heme at 570 nm is the same for chain *d* and trimer and (ii) that the extinction coefficient at 280 nm for the protein is the same for each component, then the proportions of non-heme chains can be determined.[3] This rough calculation, based on Fig. 3, can account for approximately two-thirds of the linker chains. A second experiment with the gel chromatography carried out at 4 °C, followed by HPLC (data not shown), resulted in 11.5 nmol of heme; a total of 8.0 nmol of *a*, *b*, *c*, and *d* chains; and 10.1 nmol of linker chains. The difference, 11.5 − 8.0 = 3.5 nmol, should give the heme content of the linker chains and indicates that 34.7% of the linker chains have heme. These results are consistent with the earlier report that subunit D2 (13) has heme, and with the recent measurement of the CO and NO binding kinetics of a linker chain fraction containing heme (28).

Although our determination of 3.04% heme is close to that predicted by the model (3.11%, see below), substantially lower values have been obtained by others (16). We suggest that these low values have resulted from a combination of heme loss, perhaps exacerbated by proteolysis, and by intrinsic difficulties with the determination of protein mass by dry weight. Shishikura *et al.* (29) reported that chain *c* (= III in

[3] With these assumptions the approximate proportion, *f*, of heme-free linker chains in a chromatographic fraction is given by

$$f = 1 - \frac{A_{570}}{A_{280}}\left(\frac{A_{280}}{A_{570}}\right)^* \quad \text{(Eq. 1)}$$

where

$$\left(\frac{A_{280}}{A_{570}}\right)^*$$

is measured in a selected region of the chromatogram known to be composed entirely of *abc* trimer or chain *d*.

TABLE IV

NH_2-terminal sequences of linker chains, L_1, L_2, $L_{3a,b,c}$

Chain	Sequence[a]
L_1[b]	Ala-Ser-Asp-Pro-Tyr-Gln-Glu-Arg-Arg-Phe-Gln-Tyr-Leu-Val-Lys-
L_2	Leu-Phe-Ser-Asp-Gln-Leu-Asp-Pro-Ala-Leu-Ala-Ala-(*N*)-Ala-Phe-
$L_{3a,b,c}$	Asp-Ser-Pro-Pro-Ala-Gln-Ser-His-Asp-Glu-Ile-Ile-Asp-Lys-Leu-

[a] *N* designates unidentified residue.
[b] Complete sequence is given in Ref. 26.

TABLE V

Molecular masses of chains of L. terrestris *hemoglobin*

Chain	From sequence	From mass spectrometry	Difference
a	17,525[a]	19,386 ± 15[b]	+1,861
		19,221 ± 10	+1,696
		18,901 ± 15	+1,376
b	16,254[a]	16,248 ± 5	−6
c	17,289[a]	17,290 ± 5	+1
d	16,131[c]	15,989[d]	−142
abc trimer	51,068	52,868 ± 50	+1,800
abcd	67,057[e]	(68,857)[f]	
L_1	25,847[g]	27,728 ± 15	+1,881
L_2	(30,063)[h]	32,251 ± 20	(+2,188)
L_{3c}	(23,229)[h]	24,919 ± 10	(+1,691)
$L_{average}$	26,380	28,300	(+1,920)

[a] From sequences in Ref. 8.

[b] The heaviest, major component of chain *a* was used in the calculations in the text.

[c] From sequence in Ref. 7. See Footnote *d* below.

[d] Mean of the following preparations: (i) standard HPLC (Fig. 1), 15,900 ± 4; (ii) gel chromatography (Fig. 4), 15,986 ± 4; (iii) components d_1 and d_2 resolved on a different HPLC column: d_1, 15,991 ± 4 and d_2, 15,988 ± 4. A minor component d_3, 15,958 ± 4, was not included in the average. The mean value, 15,989, has been used in all calculations of protein mass.

[e] This value is the sum of the sequence-derived average masses of chains *a*, *b*, and *c* and the average mass spectrometry derived mass of chain *d*. See Footnote *d* above.

[f] Sum of masses of *abc* trimer and chain *d*.

[g] From sequence in Ref. 26.

[h] Calculated on the assumption that chains L_2 and L_3 have the same proportion of presumed carbohydrate as chain L_1.

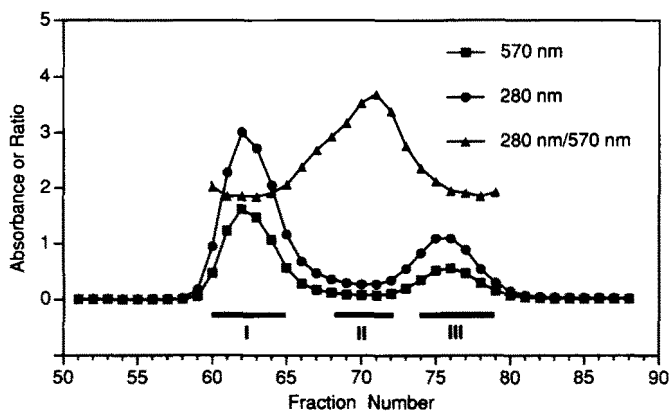

FIG. 3. **Gel chromatography of HbCO from *L. terrestris* on an AcA44 Ultrogel column with 0.1 M borate, pH 9.3, 1 mM EDTA, saturated with CO at room temperature.** Flow rate: 4 ml/h. Fraction size: 1.5 ml. Sample applied: 0.3 g. Room temperature. Fraction I is largely trimer, fraction II contains the linker chains, and fraction III is largely chain *d*.

Ref. 29) *lacked* heme, but this chain has been shown to have the stoichiometric quantity of heme (17) and, as a dimer, to bind oxygen cooperatively (15). Their preparation of the *abc* trimer (29) was converted to the CN-Met derivative at pH 7.0, reduced with dithiothreitol at 4 °C for 3–5 h, and then chromatographed at low pH. Since chains *a* and *b* did *not* lose heme, it appears that conversion to CN-Met Hb and low pH chromatography demonstrated that heme loss from chain *c* occurred more readily than from chain *a* or *b*. Heme is known to transfer from human Met Hb to serum albumin, especially at low pH (30). If the linker chain heme and half of chain *c* heme were lost, the percent heme would drop to 2.5%, typical of reported values (16).

The second factor favoring low heme values is the dry weight determination. Virtually all errors in dry weight measurements *increase* the apparent mass because it is impossible to remove all bound ions even by prolonged dialysis against distilled water. For example, this procedure fails to remove organic phosphates from human Hb (31). Procedures such as those described by Nozaki (32) may be necessary for accurate results.

Carbohydrate

Mass spectrometric analysis of the intact trimer (*abc*) yielded intense signals for the singly, doubly and triply protonated molecular ions of (*abc*) as well as of chain *d* which was present as an impurity in the analyzed HPLC fraction (Fig. 4*A*). The molecular mass of the trimer (*abc*) was determined to be 52,868 ± 50 Da. This value is 1800 Da higher than the molecular mass (51,068 Da) derived from the known sequence (see Table V). The molecular masses of individual chains *a*, *b*, and *c* were determined after complete reduction of the trimer with dithiothreitol (Fig. 4*B*). The results (Table V) show that the measured molecular masses of chains *b* and *c* agree closely with the masses calculated from the known sequences. In contrast, chain *a* is heterogeneous, having three components (*a1*, 19,386 ± 15 Da; *a2*, 19,221 ± 10 Da; and *a3*, 18,901 ± 15 Da) with significantly higher molecular masses than that calculated from the known sequence (17,525 Da). This confirms the earlier report (29) that the carbohydrate of the trimer is only on chain *a*. The differences between these values suggest that *a1* has one more hexose than *a2*, which

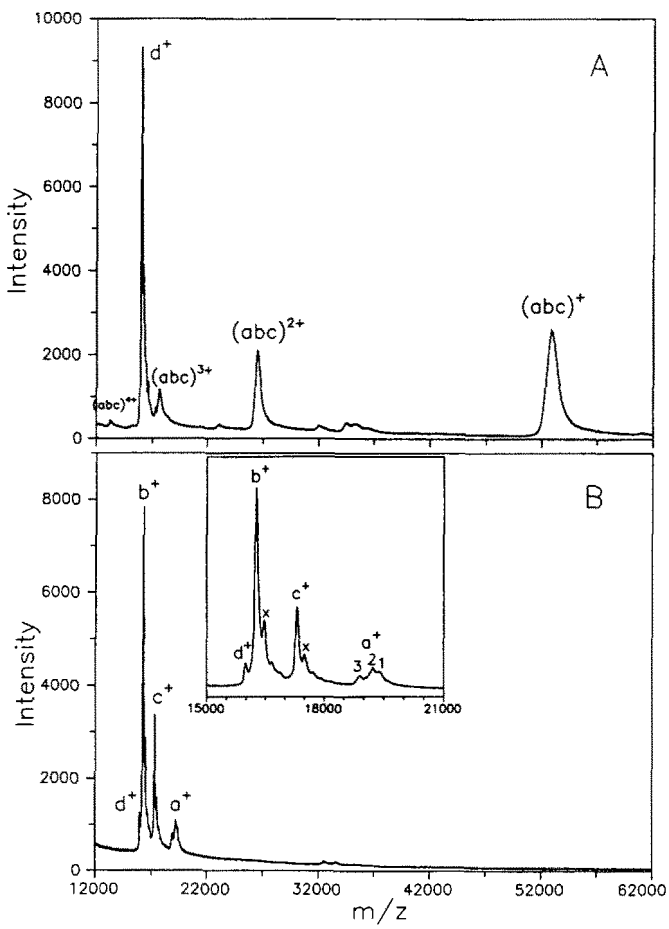

FIG. 4. **Matrix-assisted laser-desorption mass spectra of *abc* trimer (*panel A*) and chains *a*, *b*, and *c* (*panel B*) obtained after complete reduction of the trimer.** The *inset in panel B* displays the expanded region from 15,000 to 21,000 Da. Peaks denoted with (*X*) correspond to photochemically formed protein matrix adduct ions (20).

has two more than *a3*. The differences between these masses and the sequence-derived mass may be accounted for on the basis that *a1*, *a2*, and *a3* have, respectively, 11–12, 10–11, and 8–9 hexoses. The total excess mass for an $(abcd)_2 \cdot L$ unit (see Table V) is 3.4%, which is substantially greater than the 2.0% carbohydrate estimated earlier for *Lumbricus* Hb (29, 33). The first analysis of carbohydrate of the intact Hb indicated a 1:1 molar ratio of mannose to *N*-acetylglucosamine (33); the later analysis (29) gave a 9:1 ratio. The discrepancy between these analyses has not been addressed. *N*-Acetylglucosamine is usually bound to an asparagine in the sequence Asn-*X*-Ser/Thr, where *X* is any amino acid except proline (34). No such sequence exists in chain *a*, *b*, or *c*, but one is present in chain L_1 (26). This raises the possibility that the *N*-acetylglucosamine reported in chain *a* might come from a contaminating linker chain. Table V compares the molecular masses based on amino acid sequences with their masses determined by mass spectrometry. The data show that L_1 has about 7.2% of mass additional to that of the polypeptide. We shall assume for the present discussion that L_2 and $L_{3a,b,c}$ have the same proportion of additional mass. (This is a minor consideration. Even if we were to assume that chains L_2 and $L_{3a,b,c}$ have *no* carbohydrate, the same overall stoichiometric conclusions would hold.) The HPLC data (see above) indicate that L_1, L_2, and L_3 with its variants are present in equimolar amounts, so that the average molecular mass of the linker chains is 26,380 Da for *the polypeptide alone*, and 28,300 Da from the mass spectrometry measurements. The extra non-polypeptide mass is presumed to be carbohydrate (see below).

Heterogeneity

Isoelectric focusing shows *Lumbricus* Hb to be heterogeneous (33). Sequence analysis of chain *d* revealed the presence of major and minor components (7). Our results suggest at least four sources of heterogeneity: (i) variation in carbohydrate content of chain *a*; (ii) multiple chains *d*; (iii) three variants of linker chain 3: *a*, *b*, and *c*; and (iv) a minor trimer (T_2).

Chain a—The chain *a* results can be interpreted in terms of three components which have different numbers of sugars. The same set of three components has been found in a second Hb sample prepared 2 years earlier.

Chain d—We have isolated three components of chain *d* by HPLC (Table V), two of which had the same molecular mass (average 15,989 ± 4) and one about 30 Da smaller. However, the mass calculated from the amino acid sequence of the major component of chain *d* (7) is 142 Da higher. This difference cannot be explained by proteolytic cleavage of either the NH_2-terminal or COOH-terminal residue because the mass difference does not correspond to either of the reported residues (7). This raises the possibility that the sequence is incorrect.

Linker Chains—Examination of Hb from individual worms (Fig. 5) shows that substantial variation can occur in the linker chain L_3. The pooled Hb from ≈3,000 worms shows three major components of linker chains which we have designated in order of HPLC elution as L_1, L_2, and L_{3c}. Two minor peaks L_{3a} and L_{3b} elute between L_2 and L_{3c}. Worm 1 showed a pattern similar to that of the pooled Hb, but L_{3c} is absent from worm 2, and L_{3a} and L_{3b} are prominent. In worm 3, L_{3c} is reduced to half the proportion found in the pool, but L_{3b} is prominent. The chromatograms also show evidence of several additional minor components the nature of which has not been investigated. One possibility is that the linker chains are all interchangeable in the full assembly as suggested for the linkers of the Hb from *T. heterochaetus* (11). A second possibility is that L_1, L_2, and L_3 serve unique roles in assembly and that L_{3a}, L_{3b}, and L_{3c} are interchangeable among themselves. The amino acid sequence has been determined for L_1 (26). L_2 has a unique NH_2-terminal sequence; those of L_{3a}, L_{3b}, and L_{3c} are all the same, at least for the first 15 residues (Table IV). The fact that L_1, L_2, and L_3 (with its variants) are present in equimolar quantities especially in individual worms makes it extremely unlikely that L_1, L_2, and L_3 themselves are variants. If L_2 and L_3, for example, had evolved from L_1, there would be no obvious reason for them to be equimolar.

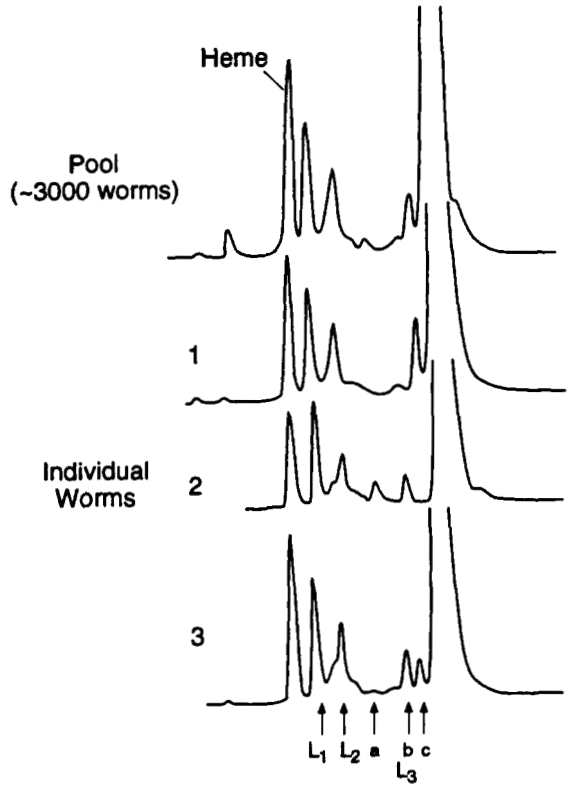

FIG. 5. **HPLC of Hb from the pooled sample and from individual worms.** Relative amounts of L_{3a}, L_{3b}, and L_{3c} vary from worm to worm, but the overall proportions of L_1, L_2, and L_3 taken together remain constant.

Minor Trimer—Fig. 2*a* shows a minor trimer component (T_2) with a mass of 53,300 ± 100 Da, which is significantly higher than the sum of the masses of chains *a*, *b*, and *c*. Reduction of T_2 with dithiothreitol gave chains *a*, *b*, and *c* that had the *same* masses as found for the major component (data not shown). This finding can be explained by assuming the presence of a small disulfide-linked molecule (430 ± 100 Da) which would be dissociated upon reduction.

Assembly of Subunits

The polypeptide mass of an *abcd* unit is 67,057 Da.[4] The HPLC data (Table III) indicate that this mass is approximately 83.7% of the total polypeptide mass, which would then be 80,116 Da (67,057/0.837). This is the polypeptide mass of one *abcd* unit together with the mass of linker chains associ-

[4] We have used the sequence-derived masses for chains *a*, *b*, and *c* and the mass spectrometry derived mass for chain *d* (see Table V). The difference between the sequence- and spectrometry-derived masses of chain *d*, 142 Da, suggests either that all of the samples subjected to mass spectrometry had suffered proteolytic losses of this mass or that the sequence (7) is incorrect. Since this difference does not correspond to the residue weights of either the NH_2- or the COOH-terminal residues of the reported chain *d* sequence, and because chain *d* appears not to have carbohydrate (29), we have chosen to use the mass spectrometry-derived mass in calculations (see Table V).

ated with it on the basis of the *measured* proportions. The total linker chain mass is determined to be 16.3% of this total or 13,059 (80,116 × 0.163). Since this value is within 1% of half of the estimated average linker chain mass (26,380/2 = 13,190 Da), we conclude that the primary stoichiometric unit is $(abcd)_2$·L, or one (*abcd*) dimer/linker. This unit, with a polypeptide mass of 160,232 Da (80,116 × 2), would bind 8⅓ hemes (8 on $(abcd)_2$ and one on one of the three linkers, total heme mass of 5,137.5 Da) to give a total polypeptide mass (with heme) of 165,370 Da.[5] Thus the experimental polypeptide mass/heme is 19,228 Da (160,232/8.333), which is only 1.2% higher than the value determined earlier by a combination of the pyridine hemochromogen method for heme and amino acid analysis (17). This close correspondence supports the accuracy and utility of the hemochromogen-based analysis which has been criticized recently as being unreliable (16) even though internal controls (analysis of chains *a*, *b*, *c*, *d*, and trimer)[6] demonstrated the accuracy of the procedure to an average of better than 1.5% (17). Furthermore, the percent heme on this basis (616.5/(19,228 + 616.5) × 100) is 3.11, in close agreement with the value of 3.04% determined directly (see above).[7]

An accurate molecular weight for the native Hb is required for the calculation of the number of polypeptides in the molecule. Published values obtained with different techniques and preparations differ, however, by as much as 50% (referenced in 6). Similarly, reported values of the diffusion coefficient (D) for the Hbs of various species of related annelids also differ greatly (33, 35–40). Examination of these data shows that the highest values of D were obtained with preparations of ground or homogenized worms, a procedure guaranteed to expose the hemoglobin to proteases. This suggests that proteolytic degradation may be partly responsible for the low molecular weights and high diffusion coefficients, provided that the original Hbs have similar molecular weights and shapes. This assumption is supported by the finding that all extracellular Hbs of annelids have a similar appearance and dimensions in electron micrographs and have similar patterns of components in polyacrylamide gel electrophoresis (41, 42). Proteolytic cleavage of the first 16 residues of chain *a* of *Lumbricus* Hb has been documented (8). A minor heterogeneous, modified trimer with a mass of 45.3 ± 0.3 kDa (mass spectrometry) has been isolated from an older preparation along with a variety of smaller peptides, one with a mass of 7,996 Da (data not shown). This peptide together with the modified trimer add up to the mass of the original trimer. The most recent determinations of the molecular weight of Hb from *Lumbricus* cluster between 3.8 and 4.1 × 10^6 (Table VI) and suggest a probable value of the molecular mass near 3,900 kDa. Although masses of 3,500–3,600 kDa have recently been adopted (10, 27), these values do not agree with published experiments (Table VI).

Electron micrographs show that the molecule has a hexagonal bilayer structure (2, 3) that suggests 12 subunits, a conclusion supported by the D_6 symmetry found by x-ray diffraction of crystals (44) and by electron microscopy (45). This does not necessarily mean, however, that such a unit is an isolatable subunit. This possibility depends on the relative free energies for the dissociation of the different interfaces. The total mass of an $(abcd)_2$· L unit increases from 165,370 Da with 8⅓ hemes to 171,152 Da (with both heme and carbohydrate) by using the mass spectrometry-derived masses of the chains (68,857 × 2 + 28,300 + 8.333 × 616.5 = 171,152). This difference, attributed to carbohydrate, is 3.4% of the total mass (171,152–165,370)/(171,152 = 0.0338). Two units of 171 kDa can be accommodated *exactly* in a one-twelfth subunit of a 4,108-kDa molecule. Such a molecule would be composed of 216 chains and 200 hemes and would have a minimum molecular weight on a heme basis of 20,500 and 3.0% heme.

TABLE VI

Some determinations of the molecular mass of earthworm hemoglobin

MDa	Method	Ref.
3.78 (3.68)[a] ± 0.17	Sedimentation equilibrium	14
3.86 ± 0.09	Sedimentation equilibrium	43
3.95 ± 0.15	Low angle x-ray scattering	4
4.3[b]	Sedimentation velocity/diffusion	40
4.1[c]	Sedimentation velocity/diffusion	33, 40
3.8 ± 0.3	Electron microscopy	6
3.98	Model (no carbohydrate)	Present study
4.11	Model (with carbohydrate)	Present study

[a] This low value assumes the partial specific volume, $\bar{v}$, to be 0.733 from (33). Use of $\bar{v}$ = 0.740, however, measured both by Svedberg and Pedersen (39), and more recently by Gros (40), raises the value to 3.78 MDa.

[b] This value was calculated from $s^0_{20,w}$ = 60.6 S, $\bar{v}$ = 0.740 and $D^0_{20,w} = 1.3 \times 10^{-7}$ cm²/s (40).

[c] This hybrid value was obtained from $s^0_{20,w}$ = 58.9 S (33) and the D value of Ref. 40 and an early value of $\bar{v}$ = 0.733 (33). This $s^0_{20,w}$ value is lower and the D value higher in the data of (33) than in the later experiments of Gros (40), suggesting that some dissociation and/or proteolysis may have occurred.

Our model, based on $(abcd)_2$·L, differs from the model based on the $(abcd)_3$ dodecamer (13) which would have a molecular mass of 213,969 Da (with heme) calculated from the mass spectrometry-derived masses of the *abc* trimer and chain *d* (Table V). Twelve dodecamers in the latter model would have a mass of 2,568 kDa. If our linker chain proportions are correct, the total mass would be 2,568/0.837 or ≈3,068 kDa which is only about three-quarters of the best estimates of the mass of the complete molecule. Alternatively, if one adopts the proportions of linker chains (one-third) suggested for the dodecamer model (10), a molecule of Hb would have a mass of ≈3,852 kDa (3/2 × 2,568). Although this is close to the experimental values, a linker chain content of 33% is completely inconsistent with our experimental results.

The dodecamer (10) $(abcd)_3$, was obtained by dissociation in 4 M urea at neutral pH followed by gel chromatography during which the urea was removed, and complex reassociation processes presumably occurred on the column. This procedure provides no proof that the dodecamer represents a structural unit in the original Hb. The dodecamer may be compared with β chain subunits isolated from human Hb which form tetramers (46), yet β_4 is clearly not a subunit of the native Hb. Although the dodecamer may correspond to a structure in the Hb, several lines of evidence support the conclusion that $(abcd)_2$·L is the basic structural unit. (i) The stoichiometric results indicate $(abcd)_2$·L. (ii) Light scattering measurements provide evidence that the CO derivative of the trimer is a dimer, $(abc)_2$, at neutral pH (15); it is probably

[5] The value calculated from the sequence-derived mass of $(abcd)_2$, 134,114 Da, together with the *estimated* average mass of linkers, 26,380 Da (Table V) gives a calculated value for $(abcd)_2$·L of 165,631 Da, which is within 0.2% of the value based on the measured proportions.

[6] The apparent error of 10.8% shown for chain *a* in Table 1 of Ref. 17 can be attributed entirely to the proteolytic loss of the first 16 residues which have a mass of 1,957 Da, corresponding to an 11.1% loss (8).

[7] The value given in Ref. 16 for the percent heme from the data in Ref. 17 is the weight ratio of heme to polypeptide multiplied by 100, not the percent heme (100 × (g of heme/(g of polypeptide + g of heme))).

also present at high pH (33).[8] (iii) Chain *c* by itself forms a dimer that is highly cooperative in O_2 binding (15). Such cooperativity implies the existence of highly specific interfaces that are likely to exist also in the intact Hb, implying that the *abc* unit should exist *in pairs*. (iv) The complex of $(abc)_2$ with d_2 has an oxygen equilibrium that is *indistinguishable* from that of the native Hb at pH 6.8 and is *no larger* than $(abcd)_2$ (15), whereas the dodecamer binds O_2 with decreased cooperativity[9] (10). (v) The proposed primary unit and stoichiometry is consistent with the measured effect of Ca^{2+} on oxygen binding, which is essentially the same for the trimer, trimer + chain *d*, and the whole Hb (15). Oxygen binding data on native Hb (47) indicate that one O_2-linked Ca^{2+} is bound per trimer oxygenated at pH 7.4. This corresponds to 48 Ca^{2+}/molecule, a value essentially the same as the measured *total* content of *tightly* bound Ca^{2+}, *i.e.* 49.5/molecule, measured after exhaustive dialysis against 10 mM EDTA (48). Although this correspondence has been interpreted to suggest that all Ca^{2+} is O_2-linked (47), the results with Hbs from *Amphitrite* (49, 50) and *Eisenia* (51) Hbs indicate the presence of two sets of Ca^{2+} binding sites, one structural and tightly bound, the other O_2-linked and less tightly bound. It now seems likely that the allosteric calcium in *Lumbricus* Hb is additional to the 49.5 calciums tightly bound.

Finally, the careful experiments done by David and Daniel (52) on the Hb of a related earthworm (family Lumbricidae, species unidentified) provide strong support for our model. They isolated a 10 S component by dissociation at alkaline pH followed by isolation on Sephadex G-150 at neutral pH. This component has a molecular mass of 163,000 Da and contains eight hemes. They conclude that the native molecule is composed of 24 of these units on the basis of their determination of the molecular mass of the native Hb by sedimentation equilibrium, 3.84 MDa.[10] Their 10 S unit appears to correspond closely to our $(abcd)_2 \cdot L$ unit in the Hb of *L. terrestris*, both in mass and in heme content.

The calculated numbers of linker chains, trimer, and chain *d* in a molecule of Hb of polypeptide mass 3.9 MDa is given in Table III. A striking feature of these results is that the three linker chains are present in equimolar amounts. This finding raises the intriguing possibility that each may have a specific, unique function in the giant assemblage. Since there are eight of each kind of linker, the conclusion is inescapable that the apparent one-twelfth subunits seen in electron microscopy cannot all be identical. How can this finding be rationalized in terms of the D_6 symmetry found by x-ray crystallography (44) and by electron microscopy (45) to a resolution of 6 Å? Each apparent one-twelfth subunit of the two-layered hexagonal molecule should have two linkers. Although L_2 and L_3 differ in mass (Table V) by about 30%, distribution of pairs of linkers in each molecule as four $L_1 \cdot L_1$ complexes and eight $L_2 \cdot L_3$ complexes would be consistent with the stoichiometry determined and give the same mass in each one-twelfth unit to within 2%. It appears that the present x-ray data could probably accommodate this small difference if the linker chain structures were very similar. The location of the linker chains within the apparent one-twelfth subunits is not known. However, electron micrographs (12, 45) show that the part of the subunit closest to the central hole comprises a distinct mass of just the right dimensions to accommodate two linker chains.

All extracellular Hbs of annelids appear to have similar structures. It is likely that they also have proportions of linker chains close to what we have determined for the hemoglobin of the terrestrial oligochaete, *L. terrestris*: 16%. Non-heme chains comprising a similar proportion of the protein were found in the hemoglobin of the marine polychaete, *Marphysa sanguinea* by Chew *et al.* (35) who appear to be the first to suggest that these chains might serve as linkers in the assembly of the molecule. Another example is the determination of the linker chain proportion (19%) in the hemoglobin of the polychaete, *T. heterochaetus* (11). A similar proportion of linker chains (18%) also appears to be present in the chlorocruorin of the annelid, *Eudistylia vancouverii* (54). Although the authors (54) estimated the linker chain proportion to be 26% on the basis of Coomassie Blue staining, analysis of the HPLC chromatogram of their Fig. 5 shows that the well separated linker chain fractions comprise only about 18% on the basis of 214-nm absorbance.

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[8] A prominent electrophoretic component with an apparent mass of 117 kDa obtained at pH 9 in nondenaturing gels (33) may be $(abc)_2$ which has a total mass, with heme, of 108 kDa.

[9] Although the dodecamer preparations (10, 28) were reported to contain only traces of linker chains, our HPLC examination of the *same* samples shows that they have 30–40% of the linker chain content of the original Hb. This suggests that functional heterogeneity might explain the lower cooperativity.

[10] Re-examination (53) of these sedimentation data showed a small concentration dependence. Extrapolation to zero concentration yielded a weight-average molecular weight of $(4.4 \pm 0.2) \times 10^6$.

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