Articles

Subunit Structure of Hemocyanin from the Gastropod *Levantina hierosolima*†

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ABSTRACT: Hemocyanin from the terrestrial snail *Levantina hierosolima* (Gastropoda, Pulmonata) was studied. A M_w of 10.4×10^6 was determined for the native 100S molecule by sedimentation equilibrium. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis gave one band with a mobility corresponding to a M_w of 360000. The molecular weight of the polypeptide chain was determined to be 334000 by sedimentation equilibrium in 6 M guanidine hydrochloride and 0.1 M 2-mercaptoethanol. Analysis of the copper content gave 0.229%, corresponding to a minimal weight per two copper atoms of 55000. We conclude that a 100S molecule of *Levantina* hemocyanin is composed of 30 polypeptide chains, each of which contains six binuclear copper centers for binding oxygen. These findings constitute a fundamental departure from the 20 polypeptide chain eight functional unit model currently accepted for gastropod 100S hemocyanin.

Hemocyanins are multisubunit proteins that function as oxygen carriers in arthropods and molluscs (Van Holde & Van Bruggen, 1971; Lontie & Witters, 1973; Wood, 1980; Van Holde & Miller, 1982; Ellerton et al., 1983). Each oxygen binding site involves two copper atoms and in the molluscs, cephalopods and gastropods, is associated with a protein mass of 50000 daltons. Electron microscopy and hydrodynamic studies have shown that gastropod hemocyanin can exist in a number of discrete states of aggregation, 100, 60, 20 and 11 S, that have been identified as 1 (whole), $^1/_2$, $^1/_{10}$, and $^1/_{20}$ molecule (Van Holde & Van Bruggen, 1971; Bloomfield et al., 1967). The 11S species, which corresponds to the polypeptide chain, has in turn been shown to consist of eight functional units, similar in size, each carrying a binuclear copper center (Gielens et al., 1977; Van Breemen et al., 1977). On the basis of the aforementioned results, a model for 100S gastropod hemocyanin has emerged and become widely accepted. According to this model, the 100S molecule is composed of 20 polypeptide chains, each consisting of eight functional units (Van Bruggen, 1983).

Over the years, much data on the molecular weight of the 100S molecule, the polypeptide chain, and the functional unit of gastropod hemocyanin have accumulated. The molecular weight of the 100S native molecule has been determined to be $\sim 9 \times 10^6$ [*Archachatina marginata*, 9.08×10^6 (Wood et al., 1971); *Busycon canaliculatum*, 8.8×10^6 (Quitter et al., 1978); *Helix pomatia*, 8.95×10^6 (Pilz et al., 1970); *H. pomatia* α, 8.91×10^6 (Wood et al., 1971); *H. pomatia* β, 8.95×10^6 (Wood et al., 1971); 9.02×10^6 (Berger et al., 1977)]. The molecular weight determined for the polypeptide chain was ~350000 [*B. canaliculatum*, 300000 (Quitter et al., 1978); *H. pomatia* α, 365000 (Siezen & Van Bruggen, 1974) and 330000 (Pilz et al., 1974); *H. pomatia* β, 350000 (Berger et al., 1977)]. A M_w of 55000 has been determined for *H. pomatia* hemocyanin functional unit by limited trypsinolysis (Gielens et al., 1975; Van der Laan et al., 1981). Examination of the molecular weights reported up to 1981 and listed above reveals a serious contradiction. While the accepted model for 100S gastropod hemocyanin predicts a polypeptide chain with a M_w of ~450000 [$(1/20) \times 9 \times 10^6$ or 8×55000], the observed molecular weight is ~350000. Attention to this inconsistency was focused by Van Holde and Miller in their 1982 review on hemocyanins (Van Holde & Miller, 1982).

Recently, two studies on *H. pomatia* hemocyanin, aimed at solving the above-mentioned inconsistency, have been published. Wood et al. (1985) determined a M_w of 450000 for the polypeptide chain by sedimentation equilibrium. Taken with the value 8.95×10^6 determined earlier for the molecular weight of the whole molecule (Wood et al., 1971), the result is consistent with 20 polypeptide chains per 100S molecule. Herskovits and Russell (1984), using light scattering, determined M_w of 7.55×10^6 and 3.72×10^5 for the 100S molecule and the polypeptide chain, respectively. The two studies confirm the 20 polypeptide chains per 100S molecule feature of the accepted model; they are, however, in contradiction with each other.

Hemocyanin from the snail *L. hierosolima* has been previously studied in our laboratory with regard to its spectroscopic (Shaklai & Daniel, 1970, 1972; Klarman et al., 1972; Shaklai et al., 1978) and oxygen binding (Er-el et al., 1972; Klarman et al., 1975; Shaklai et al., 1975) properties. In this study, a structural investigation of *Levantina* hemocyanin was undertaken. Our findings lead us to propose a new model for the subunit structure of 100S gastropod hemocyanin, different from the one generally accepted.

Materials and Methods

Preparation of Hemocyanin. Terrestrial snails *L. hierosolima* (Gastropoda, Pulmonata) were gathered from Yaar Hanassi, a forest near Jerusalem. The shell covering the location of the heart was imbibed with HCl (diluted 1:4) until it almost completely dissolved. The residual thin layer was peeled off with a pincet, and a puncture in the pericard was made. The animal was placed in a small glass funnel over a cooled test tube and left to bleed for 5 min. The hemolymph was gel-filtered through a Sephadex G-75 column (1.6 × 6 cm). A preparation buffer consisting of 0.1 M sodium ace-

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tate–acetic acid, 10 mM $MgCl_2$, and 0.1 mM phenylmethanesulfonyl fluoride, pH 5.7, was used for elution. Passage through the column was completed in less than 5 min, and a new column was used for each snail. Emphasis was placed on carrying out this first isolation step as quickly as possible in order to avoid possible proteolytic degradation.

Pooled hemocyanin eluates from several snails were centrifuged for 90 min at 232000g. The precipitate was dissolved in preparation buffer and centrifuged again. Dissolution of the resulting blue pellet in a small amount of buffer gave a concentrated (~50 mg/mL) solution of purified hemocyanin.

Concentration Determinations. Hemocyanin concentrations were measured by absorption spectroscopy with a Cary 118 spectrophotometer. Absorption coefficients were determined as described elsewhere (Ilan & Daniel, 1979). At 278 nm, values of $A^{1\%}_{1cm}$ of 14.5 in 0.05 M glycine–NaOH buffer, pH 8.8, and of 13.6 L g^{-1} cm^{-1} in 6 M guanidine hydrochloride containing 0.02 M tris(hydroxymethyl)aminomethane (Tris), pH 8.8, were obtained.

Amino Acid Analysis. Amino acid analysis was carried out as described by Spackman et al. (1958) in a Durrum-500 amino acid analyzer. Samples were hydrolyzed with 6 M HCl in evacuated sealed tubes for 24, 48, and 72 h at 110 °C. Cysteine and cystine were determined as cysteic acid in performic acid oxidized samples of the protein (Hirs, 1956). Tryptophan was determined spectrophotometrically in 0.1 M NaOH as described by Beaven and Holiday (1952).

Copper Determination. Copper was determined with 2,2′-biquinoline after reduction with cysteine hydrochloride (Felsenfeld, 1960) and by atomic absorption spectroscopy. The latter determinations were carried out in three instruments: Varian Techtron AA-5, Perkin-Elmer 403, and Perkin-Elmer 5000 with Zeeman correction equipped with HGA-500 graphite furnace.

Polyacrylamide Gel Electrophoresis. Sodium dodecyl sulfate gel electrophoresis was performed on 5% polyacrylamide gels as described by Weber et al. (1972) in the presence of 1% (w/v) sodium dodecyl sulfate and 1% (v/v) 2-mercaptoethanol. The samples were heated for 7 min at 90 °C before their application to the gels. Samples containing ferritin were heated for 15 min at 60 °C. Molecular weights were estimated from band mobilities with a calibration curve obtained with four protein markers. The markers were egg white ovalbumin (45 000), bovine serum albumin (66 000), horse spleen ferritin (220 000), and hog thyroid thyroglobulin (two bands with close mobility, average M_w 330 000).

Ultracentrifugation. Ultracentrifugation was performed with a Beckman Model E ultracentrifuge equipped with electronic speed control using an An-D rotor. Sedimentation velocity was performed at 20 °C or at a temperature close to it, with schlieren phase-plate optics. Sedimentation coefficients were corrected to $s_{20,w}$ in the usual way (Svedberg & Pedersen, 1940). Sedimentation equilibrium studies were performed by using the short-column meniscus-depletion technique (Yphantis, 1964), with interference optics. The fringe pattern was read at 0.1-mm intervals on photographs taken immediately on reaching speed and after attainment of equilibrium. Local slopes of the plot of ln c vs. r^2, $d(\ln c)/dr^2$, c being the concentration and r the distance from the axis of rotation, were determined by least-squares fitting of the base line corrected concentration distribution at equilibrium and used to calculate the weight-average molecular weight at r, $\bar{M}_{w,r}$, according to

$$\bar{M}_{w,r} = 2RT/[\omega^2(1 - \bar{v}\rho)]\ d(\ln c)/dr^2$$

where R is the gas constant, T is the absolute temperature, ω is the angular velocity, $\bar{v}$ is the partial specific volume, and ρ is the density of the solution. For the calculation of the molecular weight in 6 M guanidine hydrochloride solution, $\bar{v}$ in the expression for $\bar{M}_{w,r}$ has to be replaced by ϕ', the apparent specific volume of the protein in dialysis equilibrium with the solvent (Casassa & Eisenberg, 1964). The values of $\bar{v}$ and ϕ' were experimentally determined from density measurements made with a Digital Densimeter DMA-02 (Anton Paar K.G., Graz, Austria) [for details, see Ilan & Daniel (1979)].

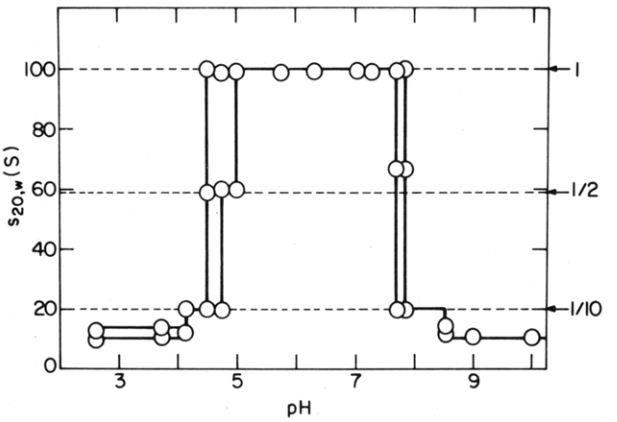

FIGURE 1: Dependence of sedimentation coefficient of *Levantina* hemocyanin on pH. Protein concentration was 0.7–0.9 mg/mL. Buffers, about 0.1 M, were used in the pH ranges indicated: glycine–HCl (2.5–3.6), acetate (3.6–5.7), phosphate (5.7–7.3), Tris-HCl (7.3–9.0), and glycine–NaOH (above 9.0). The dashed lines indicate predicted sedimentation coefficients for 1 (whole), $^1/_2$, and $^1/_{10}$ molecules of gastropod hemocyanin by the Kirkwood theory (Bloomfield et al., 1967).

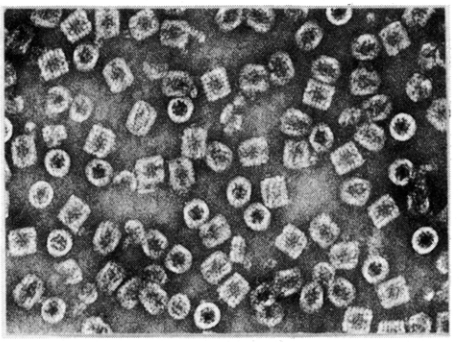

FIGURE 2: Electron micrograph (96000×) of negatively stained native *Levantina* hemocyanin.

Electron microscopy was carried out with the technique of negative staining (2% uranyl acetate). Observations were made with a Jeol-Jem 100B electron microscope.

Results

Aggregation States of Levantina Hemocyanin. The dependence of the sedimentation coefficient of *Levantina* hemocyanin on pH is presented in Figure 1. It is seen that the native 100S molecule is stable between pH 5.0 and pH 7.6. Outside the stability range, at more acidic and at more alkaline pH values, dissociation into species with sedimentation coefficients of ~60, 20, and 11 S occurs. An additional species with a sedimentation coefficient of ~14 S is sometimes observed.

Native 100S Molecule. Figure 2 is an electron micrograph of a negatively stained preparation of *Levantina* 100S hemocyanin. Two projections of the molecule are seen: a rectangular profile (370 × 340 Å) with six parallel layers and a circular profile with a diameter equal to the short side of the rectangle.

The molecular weight of the 100S native molecule was determined by meniscus depletion sedimentation equilibrium.

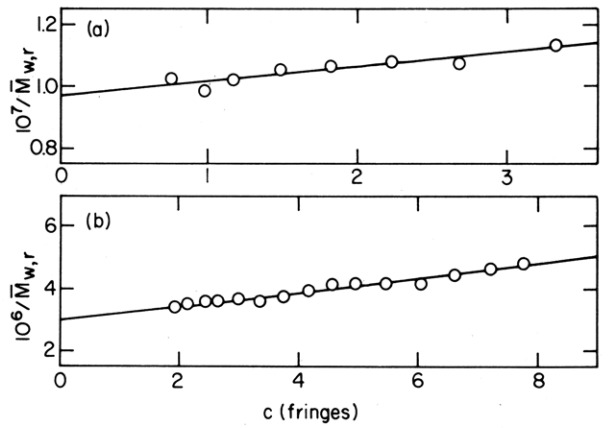

FIGURE 3: Sedimentation equilibrium of *Levantina* hemocyanin. (a) Reciprocal weight-average molecular weight of native 100S hemocyanin as a function of protein concentration. Protein concentration was initially 1.2 mg/mL in 0.1 M acetate buffer, pH 5.7, containing 0.01 M $MgCl_2$ and 10^{-4} M phenylmethanesulfonyl fluoride. Conditions: speed, 2435 rpm; temperature, 17.8 °C. (b) Reciprocal weight-average molecular weight of hemocyanin in guanidine hydrochloride as a function of protein concentration. Protein concentration was initially 1.3 mg/mL in 6 M guanidine hydrochloride, containing 0.1 M 2-mercaptoethanol and 0.02 M Tris, pH 7.6. Conditions: speed, 11 965 rpm; temperature, 20.3 °C.

Point-by-point weight-average molecular weights were found to be dependent on protein concentration. The molecular weight was therefore obtained by extrapolation to zero concentration of a plot of the reciprocal weight-average molecular weight against concentration. Two experiments gave $\bar{M}_w = 10.28 \times 10^6$ (Figure 3) and 10.46×10^6. Taken together, the data from the two experiments give for the molecular weight of *Levantina* 100S native hemocyanin a value of 10.4×10^6. A value of $\bar{v} = 0.729$ mL/g, experimentally determined by us in water at 20.1 °C, was used in the calculation.

Polypeptide Chain. Figure 4 presents typical results of sodium dodecyl sulfate–polyacrylamide gel electrophoresis of *Levantina* hemocyanin. The electrophoretic pattern shows a single band with a mobility slightly lower than that of thyroglobulin. The same pattern was obtained with hemolymph from a live animal and with hemocyanin that was prepared by rapid gel filtration. A M_w of 360 000 was estimated from the band mobility by extrapolation of a linear calibration curve obtained with standard protein markers.

A rigorous determination of the molecular weight of the polypeptide chain was carried out by sedimentation equilibrium in solution containing 6 M guanidine hydrochloride, 0.02 M Tris, and 0.1 M 2-mercaptoethanol. A linear extrapolation to zero concentration of a plot of the reciprocal of the weight-average molecular weight against concentration was carried out. Two experiments gave $\bar{M}_w = 3.38 \times 10^5$ (Figure 3) and 3.30×10^5. Taken together, the data from the two experiments give $\bar{M}_w = 3.34 \times 10^5$. For this calculation, we used a value for the apparent specific volume ϕ' of 0.713 mL/g, experimentally determined by us in 6 M guanidine hydrochloride and 0.02 M Tris at 20.0 °C.

Copper Content and Amino Acid Composition. Determination of copper gave 0.229 ± 0.003% (w/w) (Table I). From this result, we calculate for the molecular weight per two copper atoms a value of 55 000 ± 1000. The amino acid composition is given in Table II. Noticeable is the low content of methionine.

Discussion

Levantina hierosolima belongs to Gastropoda, subclass Pulmonata, order Stylommatophora, and thus is taxonomically closely related to *H. pomatia* (Ellerton et al., 1983). This relationship is reflected in the similarity of the hemocyanins from the two snail species. The features seen in the electron micrographs of *Levantina* hemocyanin are characteristic of gastropod hemocyanins in general and *H. pomatia* hemocyanin in particular. Like *Helix*, the pH dissociation of *Levantina* hemocyanin reveals aggregation states of 100, 60, 20, and 11 S. The amino acid composition of *Levantina* hemocyanin (Table II) is similar to that reported for hemocyanin from *H. pomatia* (Ellerton et al., 1983).

The molecular weight determined by us for *Levantina* hemocyanin, 10.4×10^6, is higher by more than a million than any reported for *H. pomatia* or other gastropod 100S hemocyanin. On the background of the taxonomically close relationship between *L. hierosolima* and *H. pomatia* and the similarities noted above for hemocyanins from the two snails,

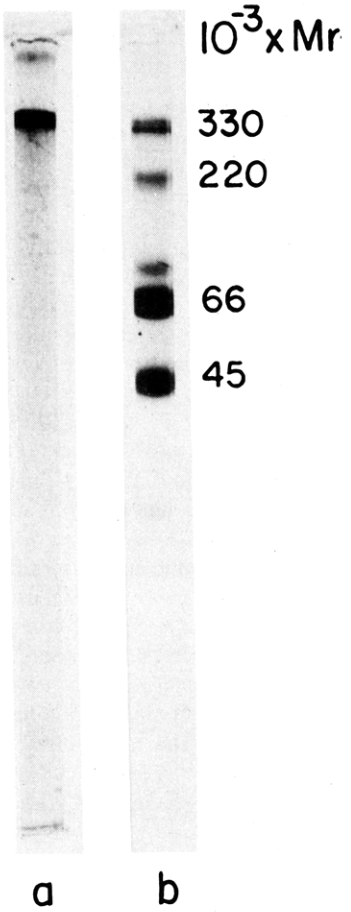

FIGURE 4: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of *Levantina* hemocyanin. Samples contained 15 μg of purified hemocyanin (a) and mixture of protein markers (b).

Table I: Copper Content of *L. hierosolima* Hemocyanin

method	no. of determinations	% Cu (w/w)[a]
chemical[b]	5	0.226 ± 0.005
atomic absorption[c]	3	0.23 ● 0.01
atomic absorption[d]	5	0.229 ± 0.008
atomic absorption[e]	3	0.232 ± 0.004
av		0.229 ± 0.003

[a] Determined from the ratio of copper to protein concentrations in salt-free hemocyanin solutions. Copper concentration was determined by reference to copper standard solutions. Protein concentration was determined by dry weight measurement. [b] With 2,2′-biquinoline. [c] Using a Varian Techtron AA-5 spectrophotometer. [d] Using a Perkin-Elmer 403 spectrophotometer. [e] Using a Perkin-Elmer 5000 spectrophotometer.

Table II: Amino Acid Composition of *L. hierosolima* Hemocyanin[a]

amino acid	wt %	amino acid residues/M_w 55 000
Lys	4.42	19.0
His	6.37	25.6
Arg	6.42	22.6
Asp	11.56	55.3
Thr	4.67	25.4
Ser	4.34	27.4
Glu	12.05	51.4
Pro	5.25	29.8
Gly	2.87	27.7
Ala	4.13	32.0
$^1/_2$-Cys	1.42	7.6
Val	5.34	29.7
Met	0.708	3.0
Ile	4.42	21.5
Leu	9.05	44.0
Tyr	6.38	21.5
Phe	7.22	27.0
Trp	3.37	10.0

[a] The entries for threonine and serine were calculated by backextrapolation to zero time of the values obtained for 24, 48, and 72-h hydrolysates; those for valine, isoleucine, and leucine are values of maximal recovery.

there is no basis to attribute the difference in the molecular weights determined for the 100S hemocyanins to the difference in species. It is rather due, in our opinion, to difficulties inherent in the molecular weight determination of proteins of very high molecular weights. One of the problems encountered is the nonideal behavior of the protein. This problem becomes more serious with an increase of the protein concentration. Among the different methods available for the molecular weight determination of very large proteins, the method of meniscus-depletion sedimentation equilibrium is most suitable. This method involves protein concentrations of 0.25–2 mg/mL (one to eight fringes), whereas in the other methods the protein concentrations needed are much higher (1–5 mg/mL for light scattering and 5–50 mg/mL for small-angle X-ray scattering). The seriousness of the nonideality problem is borne out by the concentration dependence of the apparent molecular weight of *Levantina* hemocyanin in our experiments (Figure 3). We would like to draw attention to the fact that a concentration dependence of a comparable magnitude (a drop of 10% over four fringes) has been found for the apparent molecular weight of 39S *Callianassa californiensis* hemocyanin (1.7×10^6) by Roxby et al. (1974). For the *Callianassa* 39S hemocyanin, as for *Levantina* 100S hemocyanin, an extrapolation to zero concentration was needed in order to obtain the molecular weight.

The molecular weight of the polypeptide chain was obtained by sedimentation equilibrium in 6 M guanidine hydrochloride and involved the determination of the isopotential apparent specific volume in this solvent. The molecular weight was also estimated from sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Naturally, we place more confidence in the value 334 000 determined by sedimentation equilibrium, which is a thermodynamic method of determination of absolute molecular weight. The identity of the electrophoretic pattern of purified hemocyanin used in this study with that of hemocyanin in hemolymph electrophoresed immediately after bleeding of the animal indicates unequivocally that we have determined the molecular weight of the intact polypeptide chain and not of a proteolytic fragment. These results make it very unlikely that the molecular weight is in the vicinity of 450 000 as suggested from the existing model of gastropod hemocyanin. Support for this conclusion comes from a recent

Table III: Summary of Molecular Weight Data for *L. hierosolima* Hemocyanin

M_w of native molecule	10.4×10^6
M_w of polypeptide chain	3.34×10^5
min M_w per two copper atoms	5.5×10^4
no. of polypeptide chains per molecule	30
no. of binuclear copper centers per polypeptide chain	6

study (Avissar et al., 1986) where the size of the mRNA coding for hemocyanin in *L. hierosolima* was determined. The value obtained, 9.4 kb, is consistent with a polypeptide chain of molecular weight of about 350 000.

In this study, a value of 55 000 was determined for the mass per two copper atoms of *Levantina* hemocyanin. We are aware that this value is in conflict with the concensus value, 50 000, that has become accepted for molluscan hemocyanin (Van Holde & Van Bruggen, 1971; Ellerton et al., 1983). A special effort was therefore made to establish our value. Two independent methods were used in the determination of copper. Further, in these experiments, we carried out a dry weight measurement for each determination. We would like to point out that the high value for the molecular mass per two copper atoms obtained here for *Levantina* hemocyanin is not without precedent in gastropod hemocyanins. Makino (1971) has reported a value of 55 800 for *Dolabella auricularia* hemocyanin. For *Haliotis iris*, a value of 53 000 was found [Ellerton and Lankovsky, quoted by Ellerton et al. (1983)]. Lontie et al. (1965) reported for fresh *H. pomatia* hemocyanin a copper content of 0.239%, corresponding to a mass of 53 200 per two copper atoms. For preparations of the same hemocyanin dialyzed against ethylenediaminetetraacetic acid (EDTA), the values were 0.227, 0.224, and 0.223%, corresponding to 56 000, 56 700, and 57 000.

Our findings (Table III) allow us to calculate the number of oxygen binding sites per polypeptide chain of *Levantina* hemocyanin. The ratio of the molecular weight of a polypeptide chain to the minimal molecular weight per two copper atoms is 6.07, meaning that a polypeptide chain carries six binuclear copper centers for binding oxygen or six functional units. The molecular weights for the native 100S molecule and the polypeptide chain (Table III) are related by a ratio of 31.1. Taking into account the 5-fold symmetry of the molecule seen in the electron microscope, an acceptable value must be a multiple of 5, and hence 30.

The results of this study lead us to propose a model for 100S gastropod hemocyanin. The model consists of 30 polypeptide chains each containing six functional units. Considering the wealth of structural information accumulated over the years on gastropod hemocyanins, it is our duty to examine whether the model proposed here can be accommodated with the available evidence.

The first issue concerns the size of the functional units and their number per polypeptide chain. Limited trypsinolysis of *H. pomatia* hemocyanin yields multi functional unit fragments whose molecular weights are multiples of 55 000 (Gielens et al., 1975; Van der Laan et al., 1981), a value that is in complete agreement with our result for the molecular weight per two copper atoms. Electron microscopy of *H. pomatia* 11S hemocyanin has revealed a "necklace" structure (Siezen & Van Bruggen, 1974). The globular beads in the necklace have been identified with functional units. In this way, a direct count was possible of the number of functional units per polypeptide chain. In most cases, a cluster of seven or eight globules has been observed. Close scrutiny of the published micrographs reveals, moreover, one molecule, at least, with six globules [see Siezen & Van Bruggen (1974), plate III, frame at right bottom

corner]. The data from electron microscopy are not, therefore, unequivocal and cannot, in our opinion, be invoked to decide between models containing six, seven, or eight functional units per polypeptide chain.

The second issue concerns the structure of the native 100S molecule. Over the years, a number of models for hemocyanin have been considered. Among these, there is one discussed by Wright and Fish (1975), actually a modification of an earlier proposal by Van Bruggen (1968). In the original Van Bruggen model, 180 units were arranged in six layers inside the space contained between two coaxial cylinders, in such a way as to satisfy 5- and 10-fold symmetry about the cylindrical axis. The Wright and Fish modification consisted of requiring successive layers to be axially rotated by 18° with respect to each other. This helical form of the Van Bruggen model has been shown to be in reasonable agreement with results obtained on hemocyanin by electron microscopy, small-angle X-ray scattering, and three-dimensional image reconstruction[1] (Wright & Fish, 1975). According to our model, a 100S native molecule contains 180 functional units, the number of units assumed in the Van Bruggen original and modified models. The success of the helical form of the Van Bruggen model to account for the structural data on hemocyanin indicates, therefore, that the 30-chain six functional unit model proposed here is not an unacceptable model. It is clear, however, that a definite three-dimensional structure of the 100S hemocyanin molecule must await a solution by X-ray diffraction.

The third and last issue concerns the dissociation pattern of the native 100S molecule. As has long been known, and as shown in Figure 1, the dissociation is 100 S → 60 S → 20 S → 11 S. Electron microscopy and hydrodynamic theory identify the 60S species as $^1/_2$ of the 100S molecule and the 20S species as $^1/_5$ of the 60S species (Van Bruggen et al., 1962; Bloomfield et al., 1967). Taking our model into consideration, the dissociation scheme can be represented as $^1/_1$ (100 S; 30 chains) → $^1/_2$ (60 S; 15 chains) → $^1/_{10}$ (20 S; three chains) → $^1/_{30}$ (11 S; one chain). According to this scheme, the 20S species is a three-chain structure with an expected M_w of $\sim 1.0 \times 10^6$ ($3 \times 3.34 \times 10^5$). Early determinations of the molecular weight of the 19–20S species of *H. pomatia* hemocyanin gave 1.03×10^6 (Brohult, 1947), 0.89×10^6, and 0.99×10^6 (Lontie & Witters, 1966). Our model does not eliminate a two-chain dissociation species (predicted M_w $2 \times 3.34 \times 10^5$) as an intermediate in the 20S → 11S dissociation step. The molecular weight of the two-chain species studied by Siezen and Van Bruggen comes out as 730000 (Siezen & Van Bruggen, 1974) and 600000 (Pilz et al., 1974).

Mention should be made of the structural study carried out on hemocyanin from the whelk *B. canaliculatum* by Quitter et al. (1978). Using sedimentation equilibrium, they determined values of 8.8×10^6 and 3.0×10^5 for the molecular weights of the 100S molecule and the polypeptide chain, respectively (see the introduction). Their results also indicate 30 polypeptide chains per molecule and six functional units per polypeptide chain. The appreciable differences between their molecular weights and ours do not, however, allow us to invoke their results as a support for the model proposed here.

The 30 polypeptide chain six functional unit model proposed here for gastropod 100S hemocyanin constitutes a fundamental departure from the 20 polypeptide chain eight functional unit model. It is not possible, on the basis of the data presently available, to determine which of the two is the correct model. More work is needed, in our opinion, to solve this problem.

Registry No. Cu, 7440-50-8; O_2, 7782-44-7.

[1] The three-dimensional image reconstruction of Mellema and Klug (1972) has shown the structure of 100S gastropod hemocyanin to consist of three elements, the wall, the collar, and the cap. No differentiation into such elements exists in the Wright and Fish (1975) model.

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Structure and Activities of a Variant Ubiquitin Sequence from Bakers' Yeast†

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ABSTRACT: Ubiquitin is an extremely conserved protein, with an identical sequence throughout the animal kingdom. However, the gene sequence of the yeast protein [Ozkaynak, E., Finley, D., & Varshavsky, A. (1984) *Nature (London) 312*, 663–666] predicts three amino acid differences. This implies that some functions or binding interactions of ubiquitin are different in yeast and animal cells. In an effort to define these differences, ubiquitin has been purified to homogeneity from bakers' yeast and characterized. Amino acid analysis of the protein and the isolated tryptic peptides confirms the primary structure of this protein as predicted from the gene sequence. This result indicates that the gene sequenced is the transcriptionally active gene from yeast. The conformation of yeast ubiquitin is similar to human ubiquitin as judged by circular dichroism, sensitivity to trypsin, and Stokes radius. Yeast and animal ubiquitins show identical activities in supporting ubiquitin-dependent protein degradation and in the ATP–pyrophosphate exchange reaction catalyzed by the purified ubiquitin-adenylating enzyme. Thus, the three conservative amino acid differences between yeast and animal ubiquitins have very little effect on the structure of ubiquitin or its activity in the ubiquitin-dependent proteolytic system. These results suggest that at least some of the evolutionary pressure preventing sequence variation among animal ubiquitins stems from one or more of its nonproteolytic functions.

Ubiquitin is the most highly conserved protein known, with an identical sequence from animals as diverse as insects and humans (Gavalanes et al., 1982; Schlesinger & Goldstein, 1975). Even the protein from oat is identical in 73 of 76 residues (Vierstra et al., 1985, 1986). Recently, the gene sequence of yeast ubiquitin has been reported (Ozkaynak et al., 1984). The protein coded for by this gene has three conservative amino acid substitutions in the amino-terminal third of the molecule. The yeast gene sequence suggests that ubiquitin is synthesized as a polyprotein containing multiple copies of the mature protein, no intervening sequences, and a single asparagine as a carboxyl-terminal extension. The ubiquitin cDNA from humans also shows that ubiquitin is synthesized with a carboxyl-terminal extension (Lund et al., 1985), 80 amino acids in this case. Additionally, there are multiple, larger mRNA species present, which may indicate that the human protein is also synthesized as a polyprotein. Finally, there are multiple mRNA species of different sizes in *Xenopus* (Dworkin-Rastl et al., 1984). This has been shown to be due to the same arrangement of tandem repeats of the ubiquitin coding sequence, but there is no evidence for a C-terminal extension in this organism. Thus, even though the gene structure of these organisms is quite similar, the selective pressure that maintains absolute conservation of sequence in the animal proteins must be different from that in yeast.

One possible explanation for this difference is that the function(s) of ubiquitin may vary between yeasts and animals. Ubiquitin has been suggested to act as an immunostimulating peptide (Goldstein et al., 1975), a regulator of chromatin structure (Matsui et al., 1979) and the cell cycle (Finley et al., 1984), in the heat-shock response (Finley et al., 1984; Levinger & Varshavsky, 1982), in the structure and regulation of cell surface receptors (Siegelman et al., 1986), and as a cofactor in ATP-dependent protein degradation (Wilkinson et al., 1980; Wilkinson & Audhya, 1981). If one or more of the functions of ubiquitin are different between these organ-

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