corresponding to mol.wts. 55000 and 65000, there were a number of high-molecular-weight bands.

Detergent/polyacrylamide-gel analysis of the fragment in the supernatant after removal of proteolytic enzyme by gel filtration revealed that several bands were present. After chymotrypsin treatment there were two major bands, but after trypsin treatment there was a single major band of mol.wt. approx. 45000, and a number of fainter, minor bands. The major component in the supernatant obtained after trypsin treatment was partially purified by chromatography on DEAE-cellulose. The purified material sedimented as a single band with a sedimentation coefficient (at approx. 4mg/ml, uncorrected) of 6.5S. Calibration of the Sephadex G-100 column confirmed that this fragment had a molecular weight a little less than that of immunoglobulin G, and sedimentation-equilibrium experiments indicated mol.wt. approx. 120000. This fragment could therefore be a dimer or a trimer of the polypeptide chain observed in detergent/polyacrylamide-gel electrophoresis. It is noteworthy that this fragment had slightly shifted absorption maxima compared with native haemocyanin or tubes, having band maxima at approx. 340 and 545nm instead of at 346 and 570nm. Both fragment and dissociated tubes bound oxygen, although with a higher affinity than the native haemocyanin and without co-operativity.

Not all gastropod haemocyanins form tubes on digestion with proteolytic enzymes, at least under the present conditions. The β- but not the α-haemocyanin from Helix pomatia does (Van Breemen et al., 1975), but the haemocyanins from Buccinum undatum and Neptunea antiqua do not (E. J. Wood, unpublished work). Lymnaea haemocyanin easily forms tubes, and the present work shows that the fragment cleaved by proteolysis appears to possess somewhat different oxygen-binding domains, as judged by the absorption spectrum. Gielens et al. (1975) found that certain of the fragments resulting from the tryptic digestion of H. pomatia β-haemocyanin had different spectral properties. The significance of this difference in properties is not yet clear, although it does seem to suggest that these domains have different properties compared with the rest of the domains in the molecule. Little is known of the structures of the oxygen-binding domains in gastropod haemocyanins, the problem being exacerbated by microheterogeneity and by the possession of a subunit or subunits containing up to nine domains, but not separable after treatment with 6m-guanidinium chloride or succinic anhydride. Digestion with proteinases therefore represents an advance in the dissection of the native molecule to obtain functional subunits. The possibility exists that the fragment represents all or part of the ‘collar’ described by Mellema & Klug (1972), and whose properties are connected with the limitation of the polymerization of subunits in the formation of the whole, native molecule.


The Haemocyanin from the River Snail Viviparus viviparus (L.):
Some Properties and Subunit Structure

EDWARD J. WOOD and LINDSEY J. MOSBY

Department of Biochemistry, University of Leeds, 9 Hyde Terrace, Leeds LS2 9LS, U.K.

Gastropod haemolymph typically contains a high-molecular-weight haemocyanin in solution as the respiratory pigment, and in electron-microscopic appearance and physical properties the haemocyanins from the species from a variety of habitats are closely
Table 1. Dissociation and re-association of Viviparus haemocyanin

Haemocyanin solutions containing approx. 4 mg of protein/ml were dialysed against 0.1 M-Tris/HCl buffers containing the stated additions. The buffer pH was adjusted at the temperature at which it was used (20°C). Sedimentation coefficients were used to identify the schlieren peaks observed during sedimentation-velocity experiments, and the proportions of the different species present were calculated from the areas under the peaks with correction for radial dilution.

<table>
<thead>
<tr>
<th>Dialysis against</th>
<th>Percentage of</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>One-tenth molecules</td>
<td>Half molecules</td>
</tr>
<tr>
<td>pH 7.38, containing 10 mM-EDTA</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>pH 7.43, containing 10 mM-EDTA</td>
<td>~26</td>
<td>&lt;5</td>
</tr>
<tr>
<td>pH 7.50, containing 10 mM-EDTA</td>
<td>73.3</td>
<td>2.6</td>
</tr>
<tr>
<td>pH 7.80, containing 10 mM-EDTA followed by:</td>
<td>100</td>
<td>–</td>
</tr>
<tr>
<td>pH 7.30, containing 25 mM-CaCl₂</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

* Probably one-and-a-half molecules.

similar (Wood, 1973; Hall et al., 1975). Nevertheless, the oxygen-binding properties of the pigments are frequently very different in different species: for example, there may be a high or a low oxygen affinity, and there may be a normal or a reverse Bohr effect (Wood et al., 1976). In the course of investigating freshwater gastropod respiratory pigments we have studied the haemocyanin from Lymnaea stagnalis (Hall et al., 1975), which has a moderate oxygen affinity and a normal Bohr effect, the erythromycin from Planorbis corneus (Wood & Mosby, 1975), which has a high oxygen affinity and zero Bohr effect, and we now present data on the haemocyanin from the river snail, Viviparus viviparus (formerly Paludina vivipara). Viviparus appears to spend most of the time on the river bed in depths of up to 1 m of water. Lymnaea, on the other hand, is frequently found at or just beneath the surface of the water, although it is capable of diving deeper (see Jones, 1972).

Haemocyanin was purified from the haemolymph of specimens of Viviparus as described previously for Lymnaea haemocyanin (Hall et al., 1975). The copper-free apoprotein was prepared by treatment with KCN as described by Cohen & Van Holde (1964). Analytical ultracentrifugation was performed as described previously (Wood, 1973), and oxygen-equilibrium curves were obtained by the tonometric method of Rossi-Fanelli & Antonini (1958). Spectra and absorbance data in the oxygen-binding experiments were obtained with a Unicam SP.1800 spectrophotometer.

At pH values of 7.0 and below, the purified haemocyanin had a normal appearance in the electron microscope and sedimented in the analytical ultracentrifuge as a single major boundary (approx. sedimentation coefficient 100S), with a small amount (<5%) of more-rapidly sedimenting material. This latter is thought to be one-and-a-half molecules and higher polymers. When the pH was raised beyond pH 7.3 in the presence of EDTA, dissociation took place (Table 1). By comparison with other gastropod haemocyanins, the products of dissociation are believed to be one-half (~60S) and one-tenth (~18S) molecules. At pH values between pH 7.4 and 7.5 these co-existed with whole (~100S) molecules. As has been observed with other gastropod haemocyanins, the image of the schlieren phaseplate fell to the baseline between the peaks, indicating that whole molecules and their dissociation products were not in rapid equilibrium (see Wood & Peacocke, 1973). At pH values over 7.6, only one-tenth molecules existed, but the addition of Ca²⁺ along with lowering the pH to 7.3 caused complete re-association to whole
(and larger) molecules (Table 1). This sort of behaviour is typical of gastropod haemocyanins.

The spectrum of *Viviparus* haemocyanin was unusual. The native haemocyanin was more greenish than the typical bluish-grey of mollusc haemocyanins. In addition to the usual absorption bands at 278 nm (protein) and near 350 nm (copper-oxygen complex), broad bands in the visible region were observed at ~610 and 660 nm, instead of a single band at ~570 nm as is normally found. Removal of the copper from the protein by dialysis against KCN-containing solutions yielded an apoprotein which was green in colour (instead of the usual colourless), with a major band at 660 nm and a shoulder at ~610 nm. Subtraction of the spectrum of the apoprotein from that of the holoprotein revealed a band at 570 nm in the latter. It therefore appears that an unknown chromophore is attached to *Viviparus* haemocyanin, which is not removed by dialysis against KCN- or EDTA-containing solutions, nor is it left in the supernatant after sedimenting the protein by ultracentrifugation. Precipitation of the protein with trichloroacetic acid yielded a blue-green precipitate and a colourless supernatant. However, treatment of the apoprotein with cold acid/acetone (Rossi-Fanelli *et al.*, 1958) gave a white precipitate and a blue-green acetone supernatant. The structure and function, if any, of this chromophore remains to be elucidated. Preliminary amino acid analyses of *Viviparus* haemocyanin indicates an amino acid composition typical of gastropod haemocyanins in general.

The presence of the pigment did not interfere with the determination of the oxygen equilibrium. The data at present available indicate that *Viviparus* haemocyanin has a high oxygen affinity, although determinations are hampered by a tendency for the protein to undergo denaturation and precipitation on evacuation of the tonometer. At pH 7.8 in the presence of Ca$^{2+}$, the value for $p_{50}$, the partial pressure of oxygen at half saturation, was 4.2 mmHg (560 Pa), but the value for $h_{max}$, the maximum slope of the Hill plot, was approx. 1.5, indicating a comparatively low degree of co-operativity under these conditions. It may well be appropriate for an animal which commonly lives in deepish water to have a respiratory protein which is more myoglobin-like than haemoglobin-like. In contrast, the haemocyanin of *Lymnaea* showed a high value for $h_{max}$, (i.e. approx. 4.0; Hall *et al.*, 1975), similar to that found for the haemocyanins of terrestrial gastropods (Van Driel *et al.*, 1974). The relating of these different functional properties of haemocyanins from animals from diverse habitats will require a great deal more information on the nature of the oxygen-binding site and on the subunit structure of the protein than is presently available.

Jones, J. D. (1972) *Comparative Physiology of Respiration* p. 124, Edward Arnold, London