Essays in Biochemistry

Edited for The Biochemical Society by

P. N. Campbell

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Volume 16

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Biography

Edward J. Wood received his D.Phil. in 1971 from the University of Oxford. He has worked for The Wellcome Foundation in Beckenham, Kent, and for a spell was a lecturer in Biochemistry in the Medical School of the Royal University of Malta. He is now Senior Lecturer in the Department of Biochemistry, University of Leeds, and is mostly interested in gastropod respiratory proteins.

Professor Brenda E. Ryman graduated from Cambridge during the War, and after a spell at Glaxo Laboratories working in the research labs was seconded to Birmingham University. She eventually returned there to study under Professor Thorpe in the Medical School for the Ph.D. degree. Medical schools have been her main working place—the Royal Free Hospital Medical School from 1948–1972 (where under Professor W. J. Whelan she developed a strong interest in glycogen metabolism and its disorders), and Charing Cross Hospital Medical School from 1972 until the present, apart from study leave in Imperial College for a year in Sir Ernst Chain's department. From 1976 she has also been Mistress of Girton College, Cambridge, and has managed (not always very well, she says) to combine a career with marriage and a family.

David A. Tyrrell graduated from Queen Elizabeth College (University of London) in 1973. He proceeded to a Ph.D. in 1976 working in the Department of Biochemistry, Charing Cross Hospital Medical School, where he remained for a further two years—including six months in the U.S.A.—until joining the Radiochemical Centre, Amersham, in 1978.

James F. Tait graduated and took his Ph.D. in Physics at the University of Leeds. In 1948 he became a Lecturer in Medical Physics at the Middlesex Hospital Medical School. From 1958 he did research in endocrinology at the Worcester Foundation for Experimental Biology, Massachusetts, U.S.A. and then returned to this country as Joel Professor of Physics as Applied to Medicine at the Middlesex Hospital Medical School in 1970. His direct research interests have been in biophysical methodology, characterization of aldosterone, steroid dynamics and the dispersed cells of the adrenal cortex.

Sylvia A. S. Tait graduated in Zoology at University College, London. She joined the Courtauld Institute of Biochemistry at Middlesex Hospital Medical School working mainly on the biological activities of natural and synthetic oestrogens and later on the isolation of aldosterone. She continued in endocrine research at the Worcester Foundation for Experimental Biology, Massachusetts, U.S.A. and returned to the Middlesex Hospital Medical School in 1970 as a Research Associate on MRC Programme Grant. Her major field

of interest has been the biosynthesis and mechanism of control of steroid production by cells of the adrenal cortex.

Janet B. G. Bell graduated in Physiology and Biochemistry and obtained her Ph.D. at the University of Southampton. After lecturing at Brunel University, she spent several years as a Senior Research Fellow in the Department of Zoology, St. Bartholomew's Medical College studying the testicular steroidogenesis of man and other mammals. She is now working with Professor and Dr. Tait in the Biophysical Endocrinology Unit of the Physics Department, Middlesex Hospital Medical School, on the steroid metabolism of the different zones of the mammalian adrenal cortex.

Preface

Kind Sir, I've read your paper through,
And faith, to me 'twas really new!
ROBERT BURNS (1759–1796)

The unprecedented rate of growth in scientific effort, after the Second World War, encompassed major developments in such diverse areas involving biological phenomena as medicine, agriculture, microbiology, food science, environmental science and technology. The resulting growth in the body of knowledge of the chemistry and physics of the life sciences in general led quite naturally to a mounting literature both in the discipline of biochemistry itself and in a variety of other specialized areas.

The student and teacher of biochemistry became faced with the need to read increasing numbers of papers in more and more highly specialized areas. Some of the papers were "really new" in that they pioneered either new ideas or new experimental procedures, whilst many were concerned with testing the ideas or with further development of technique. Although specialist reviews of the various developing areas rapidly became available, the Committee of the Biochemical Society saw that there was a need for the advanced student to have available to him a series of Essays, setting out biochemical topics in a stimulating manner, which showed the background, the present state and possible future developments in the particular area, and which could moreover be read with pleasure and profit. It was from these thoughts that Essays in Biochemistry became a reality with the publication in 1965 of the first volume under the editorship of Peter Campbell and G. D. Greville.

The past fifteen years have seen a further expansion of the subject of biochemistry into all areas of biological sciences and the topics in the present volume indicate the diverse nature of the problems which the biochemist seeks to resolve. Haemoglobin and the haemoglobinopathies have been discussed extensively elsewhere and the Essay by E. J. Wood discusses the less well developed area of the ways in which oxygen is transported and stored by invertebrates. Further work in this area may lead to a better understanding of evolution. The Essay concerned with liposomes (B. E. Ryman and D. A. Tyrrell) stresses the potential uses, yet unresolved problems, in medicine, but at the same time the excitement of a topic developed in an interdisciplinary manner by, among others, physical chemists, pharmacologists and clinicians as well as by biochemists becomes evident. The more specialized nature of the

Essay on Steroid Hormone Production by Mammalian Adrenicortical Dispersed Cells (J. F. Tait, S. A. S. Tait and J. B. G. Bell) not only shows some of the ways in which the problems of steroid production are being tackled, but it also emphasizes some of the general aspects likely to be encountered when studies are made or cells removed from their normal pericellular environment, and the need to give great attention to the effects of the latter. Each of the topics has implications in other areas of study. We hope that this group of Essays will indeed be read with pleasure and profit.

This volume of Essays is only the second in which the writer has been involved as an Editor and it was only after considerable hesitation that the invitation to act as co-editor of the series was accepted. Essays in Biochemistry has achieved high standards under the editorship of Peter Campbell, with G. D. Greville, Frank Dickens and Norman Aldridge successively. Some degree of apprehension was naturally felt concerning the need to help maintain these standards and at the same time concerning the requirement for gaining the co-operation of authors who are likely to have, invariably, considerable individuality of thought. The second of the apprehensions was rapidly dispelled by the realization of how much time and effort is given by authors in describing their subjects in stimulating ways, and for this we are grateful. Encouragement was provided also by a number of individuals who indicated how much Essays was valued. The series is more likely to continue as a successful venture if the reader will suggest suitable topics for future volumes and will also offer constructive criticisms to the Editors. These will be welcomed and carefully considered.

August 1980 R. D. MARSHALL

Conventions

The abbreviations, conventions and symbols used in these Essays are those specified by the Editorial Board of *The Biochemical Journal* in *Policy of the Journal and Instructions to Authors* (revised 1976 *Biochem J.* **153**, 1–21 and amended 1978 *Biochem J.* **169**, 1–27). The following abbreviations of compounds, etc., are allowed without definition in the text.

ADP, CDP, GDP, IDP, UDP, XDP, dTDP: 5′-pyrophosphates of adenosine, cytidine, guanosine, inosine, uridine, xanthosine and thymidine
AMP, etc.: adenosine 5′-phosphate, etc.
ATP, etc.: adenosine 5′-triphosphate, etc.
CM-cellulose: carboxymethylcellulose
CoA and acyl-CoA: coenzyme A and its acyl derivatives
Cyclic AMP etc.: adenosine 3′:5′-cyclic phosphate etc.
DEAE-cellulose: diethylaminoethylcellulose
DNA: deoxyribonucleic acid
Dnp-: 2,4-dinitrophenyl-
Dns-: 5-dimethylaminonaphthalene-1-sulphonyl-
EDTA: ethylenediaminetetra-acetate
FAD: flavin-adenine dinucleotide
FMN: flavin mononucleotide
GSH, GSSG: glutathione, reduced and oxidized
NAD: nicotinamide-adenine dinucleotide
NADP: nicotinamide-adenine dinucleotide phosphate
NMN: nicotinamide mononucleotide
P_i, PP_i: orthophosphate, pyrophosphate
RNA: ribonucleic acid (see overleaf)
TEAE-cellulose: triethylaminoethylcellulose
tris: 2-amino-2-hydroxymethylpropane-1,3-diol

The combination NAD^+, NADH is preferred.

The following abbreviations for amino acids and sugars, for use only in presenting sequences and in Tables and Figures, are also allowed without definition.

Amino acids

Ala: alanine	Asx: aspartic acid or asparagine (undefined)	Cys or Cys: Cystine (half)
Arg: arginine		
Asn*: asparagine	Cys: Cysteine	Gln†: glutamine
Asp: aspartic acid		Glu: glutamic acid

* Alternative, $Asp(NH_2)$ † Alternative, $Glu(NH_2)$

Glx: glutamic acid or glutamine (undefined)
Gly: glycine
His: histidine
Hyl: hydroxylysine
Hyp: hydroxyproline
Ile: isoleucine
Leu: leucine
Lys: lysine
Met: methionine
Orn: ornithine
Phe: phenylalanine
Pro: proline
Ser: serine
Thr: threonine
Trp: tryptophan
Tyr: tyrosine
Val: valine

Sugars

Ara: arabinose
dRib: 2-deoxyribose
Fru: fructose
Fuc: fucose
Gal: galactose
Glc*: glucose
Man: mannose
Rib: ribose
Xyl: xylose

* Where unambiguous, G may be used.

Abbreviations for nucleic acid used in these essays are:

mRNA: messenger RNA
nRNA: nuclear RNA
rRNA: ribosomal RNA
tRNA: transfer RNA

Other abbreviations are given on the first page of the text.

References are given in the form used in *The Biochemical Journal*, the last as well as the first page of each article being cited and, in addition, the title. Titles of journals are abbreviated in accordance with the system employed in the *Chemical Abstracts Service Source Index* (1969) and its Quarterly Supplement (American Chemical Society).

Enzyme Nomenclature

At the first mention of each enzyme in each Essay there is given, whenever possible, the number assigned to it in *Enzyme Nomenclature: Recommendations (1972) of the International Union of Biochemistry on the Nomenclature and Classification of Enzymes, together with their Units and the Symbols of Enzyme Kinetics*, Elsevier Publishing Co., Amsterdam, London and New York, 1973: this document also appeared earlier as Vol. 13 (2nd edn, 1965) of *Comprehensive Biochemistry* (Florkin, M. & Stotz, E. H., eds), Elsevier Publishing Co., Amsterdam, London and New York. Enzyme numbers are given in the form EC 1.2.3.4. The names used by authors of the Essays are not necessarily those recommended by the International Union of Biochemistry.

Contents

The Oxygen Transport and Storage Proteins of Invertebrates

E. J. WOOD

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I. Introduction

During the course of evolution animals have developed a number of ways of transporting and storing oxygen, one of them being, in vertebrates, possession of a tetrameric haemoglobin in circulating corpuscles for oxygen transportation and a monomeric myoglobin in muscles for storage. A range of oxygen-binding proteins is found in invertebrate animals, many having very beautiful and complex architecture, and all having striking colours because of the presence of transition metal ions at the oxygen-binding site (Table 1).

Probably because of their colours, these proteins have long attracted the interest of biochemists and physiologists, and they had a very significant influence in leading Svedberg to propose that proteins had discrete molecular weights and that protein molecular weights were simple multiples of a

fundamental unit.[1] These ideas were important for the development of our understanding of protein quaternary structure.

The oxygen-binding centres of the respiratory proteins can involve either iron–porphyrin, iron, or copper (Table 1). It is doubtful whether any respiratory pigments with completely different centres remain to be discovered. Thus although the blood cells of tunicates contain vanadium (as "haemovanadin") it seems almost certain that this is not a protein complex,[2] nor is it capable of binding oxygen reversibly.[3]

TABLE 1

The oxygen-binding proteins of animals

Name	Oxygen-binding centre	Stoichiometry	Colour change oxy/deoxy	Mol. wt. per O_2 bound
Haemoglobin, myoglobin	Protoporphyrin IX–Fe(II)	$Fe:O_2$	Red/ red-purple	17 000
Invertebrate haemoglobin (erythrocruorin)	Protoporphyrin IX–Fe(II)	$Fe:O_2$	Red/ red-purple	17 000 (?)
Chlorocruorin	"Chlorohaem"†	$Fe:O_2$	Green/red	17 000 (?)
Haemerythrin	Non-haem Fe(III)	$2Fe:O_2$	Burgundy/ colourless	13 500
Haemocyanin	Non-haem Cu(II)	$2Cu:O_2$	Blue/ colourless	50 000 (molluscs) 75 000 (arthropods)

† Protoporphyrin IX in which a formyl group is substituted for a vinyl group at position 2.

In this review I have tried to illustrate the range of remarkable molecular forms of invertebrate oxygen-binding proteins and to give some sort of classification with a view to bringing out common features. I have also attempted, where possible, to correlate structure with function, and this aspect is rapidly expanding. The first sequence and X-ray data are now appearing and subunit structures are starting to be elucidated.[4] Apart from any aesthetic attraction of the molecular forms themselves, invertebrate respiratory proteins are of great interest as a study in evolution, as examples of highly elaborate self-assembly systems, as models for co-operative phenomena, and as subjects for the study of oxygen–metal–protein interactions.[5,6]

A. NOMENCLATURE

Haemocyanins (non-haem copper) and haemerythrins (non-haem iron) are sometimes abbreviated to Hc and He by comparison with haemoglobin (Hb).

Myohaemerythrin is the monomeric haemerythrin occurring in muscle. The large invertebrate haemoglobins are frequently called erythrocruorins and those containing chlorohaem, chlorocruorins (see Table 1). I shall refer to the former as "invertebrate haemoglobins" or simply "haemoglobins": I believe this is justified inasmuch as the oxygen-binding moiety is identical in all these, i.e. protoporphyrin IX–Fe.

II. Biological Distribution

Respiratory pigments are found throughout almost all phyla of the animal kingdom, becoming increasingly indispensable in the more highly developed and evolved groups. Even some ciliated protozoans such as *Paramecium* have a "myoglobin" in the cytoplasm, but curiously chordates such as *Amphioxus* appear to have no respiratory pigment at all.[7] It is possible to make calculations of the maximal body dimensions possible in the absence of a circulatory system based on measured rates of respiration and the diffusion coefficient of oxygen. Alexander[8] suggests that for a flatworm living in aerated water the maximum possible body thickness would be about 1·0 mm, and that for a cylindrical turbellarian a diameter of up to 1·5 mm would be possible. Of course not all animals live in fully aerated water, and any circulatory system may or may not contain a respiratory pigment to increase its oxygen-transporting capacity. Nonetheless the extremely low concentrations of respiratory proteins found in some invertebrates of considerable size has led to speculation that these proteins may have a function other than oxygen transport.

Figure 1 shows the distribution of respiratory proteins in the animal kingdom in very broad outline, but there are numerous exceptions and anomalies. Nematodes may have a haemoglobin in the body cavity and a chemically distinct myoglobin in the body wall. Many annelids have a giant haemoglobin or a chlorocruorin (both of molecular weight nearly four million) dissolved in the blood, and some have instead or in addition one or more smaller, often monomeric, haemoglobins in coelomic cells.[9] One minor genus of annelids is reported to have haemerythrin, but this pigment is in fact the least widespread of all the oxygen carriers and is characteristic of three phyla: the sipunculans, the priapulids and the brachiopods.

Arthropods may have either haemocyanins or haemoglobins and it is a matter for speculation how this situation has arisen in the course of evolution. Crustaceans such as crabs and lobsters possess large haemocyanins as do *Limulus*, the horseshoe crab, scorpions, spiders and isopods. On the other hand *Daphnia* and certain shrimps (*Cyzicus, Artemia*) have moderately large haemoglobins. Only the larval forms of certain insects (e.g., *Chironomus*) have haemoglobins and these are monomeric, and haemocyanins have never been reported in insects. Echinoderms have small, intracellular haemoglobins.

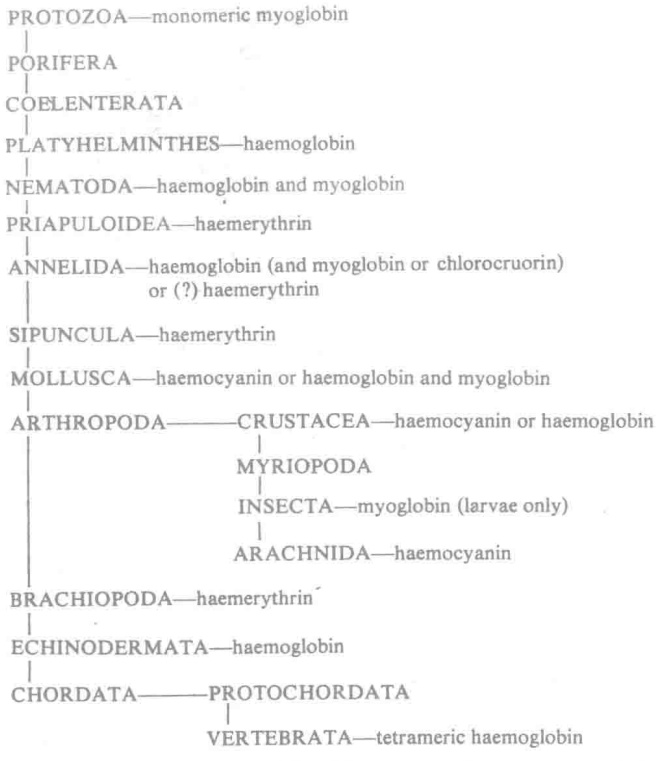

Fig. 1. Scheme showing the occurrence of the different types of respiratory protein in the animal kingdom.

Molluscs may possess either haemocyanins or haemoglobins, but bivalve molluscs, where they have a respiratory protein, have small (e.g., dimeric or tetrameric) intracellular haemoglobins. Gastropod haemocyanins are amongst the largest known protein molecules with molecular weights of nine million, and cephalopods (e.g., *Octopus*) have a haemocyanin of almost exactly half this size. However, some gastropods, notably the planorbid snails, have large haemoglobins instead. Again the reason for this curious divergence is not known. Typically, molluscs do not have an oxygen-binding protein in the great mass of their musculature but the exception to this is the muscles of the radula which contain a monomeric or more often a dimeric myoglobin. There does not appear to be a monomeric haemocyanin equivalent to myoglobin: gastropods have a radular myoglobin regardless of whether their circulating respiratory protein is haemoglobin or haemocyanin.[10,11] All vertebrates, with the exception of the cyclostomata, have a corpuscular, tetrameric haemoglobin

and a muscular, monomeric myoglobin. Lamprey haemoglobin is monomeric and corpuscular.

Svedberg[1] noted that with the exception of the small haemoglobin of *Chironomus* larvae, all the small respiratory proteins were intracellular and all the large ones were dissolved in the blood or haemolymph. One must suspect that if high concentrations of a blood respiratory protein became essential for oxygen transport, then because of the osmotic pressure exerted and of possible loss of pigment molecules from the capillaries, including renal loss, it became necessary either to encase the protein molecules in cells or to join low molecular weight functional units together to form giant molecules which are effectively "molecular corpuscles". Thus for snail (*Helix*) haemocyanin, if the molecules were small units each carrying one molecule of oxygen, the osmotic pressure of the haemocyanin in the haemolymph would be about 2500 Pa (25 cm H_2O).[8] This is similar to the recorded pressure in the contracting ventricle of the heart of *Helix*. In fact *Helix* haemocyanin consists of aggregates of about 160 oxygen-binding units, so that the colloid osmotic pressure is reduced by a factor of 160.

III. Haemocyanins

A. THE OXYGEN-BINDING SITE

Although the structures of the protein moieties of arthropod and mollusc haemocyanins show major differences, the oxygen-binding sites, while not identical, are similar. Most of the evidence on the structure of the copper–oxygen complex has been derived by spectroscopic methods. Sequence information for haemocyanins is not available and it is not certain to which ligands the copper ions are bound.

It has long been known that the binding site of haemocyanin contains two copper ions capable of binding one molecule of oxygen. The oxy form of the protein is blue ($\lambda_{max} \sim 570$ nm) and the deoxy form colourless, and in addition only the oxy form shows the characteristic absorption band in the ultraviolet at about 340 nm. It is generally accepted that the binding site is a binuclear copper(II) peroxo complex, while the deoxy form contains copper(I). As the oxy form shows no EPR signal it is assumed that the two Cu(II) atoms are dipole coupled, and magnetic susceptibility measurements confirm that oxyhaemocyanin is diamagnetic.[12] Resonance Raman and other spectral studies reveal that oxygen is in the form of peroxide (O_2^{2-}) and that the active site probably has μ-dioxygen bridged geometry.[13,14] Calculations of copper–copper distances based on such models give values ranging from 0·35 nm to 0·50 nm. If the copper–copper distance, calculated from the EPR spectrum of nitric oxide haemocyanin, of about 0·6 nm, is the same as in deoxyhaemo-

cyanin[15] this would imply a substantial movement of the copper ions upon oxygenation. Such a conformational change would be consistent with the oxygenation kinetics of haemocyanin.

Haemocyanin, like haemoglobin, also binds carbon monoxide but vibrational analysis shows that CO preferentially binds to one copper atom in each site with its carbon atom bound to a copper.[16] While oxygen binding is regulated by allosteric effectors, CO binding is not, and one may suppose that the protein can influence the binding of oxygen by altering the Cu–Cu distance.

The copper in haemocyanin is bound to the protein very tightly. It is not removable by chelating agents such as EDTA, and can be removed from the undenatured protein only by KCN. There is tentative evidence that histidine imidazole groups in the protein are responsible for binding the copper, and resonance Raman studies[13] indicate that the band near 340 nm in oxyhaemocyanin may be assigned to charge–transfer between N(imidazole) and Cu(II). There may in addition be another bridging ligand (Fig. 2). The 570 nm absorption band was assigned to peroxide → Cu(II) charge–transfer transitions.[14] The observed differences between the oxygen-binding sites of arthropod and mollusc haemocyanins may well be due to slight geometric alterations rather than to major structural differences in the sites.[13]

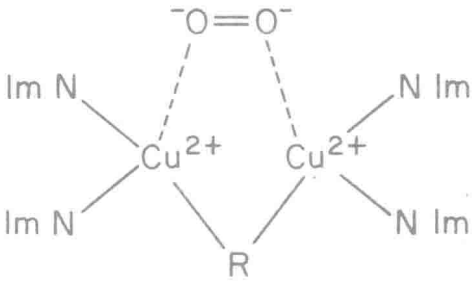

Fig. 2. Tentative structure for the oxygen-binding site of haemocyanin: the two tetragonal Cu(II) atoms are co-ordinated by histidine imidazole groups but another ligand, R, possibly phenolate, is also involved.

B. MOLLUSC HAEMOCYANINS

(*1*) *Quaternary structure of the protein*

Ultracentrifuge studies show that gastropod haemocyanins have a sedimentation coefficient of about 100 S, and those from cephalopods 50–60 S. The molecular weight of gastropod haemocyanins, calculated from accurate values for sedimentation and diffusion coefficients, is nearly nine million.[17] That for cephalopod haemocyanin is about half this value. Under the electron

microscope the molecules appear to be cylinders of diameter 35 nm, and height about 35 nm in gastropod haemocyanin and about 17 nm in cephalopod haemocyanins (Fig. 3). End-on views (circles) allow ten-fold rotational

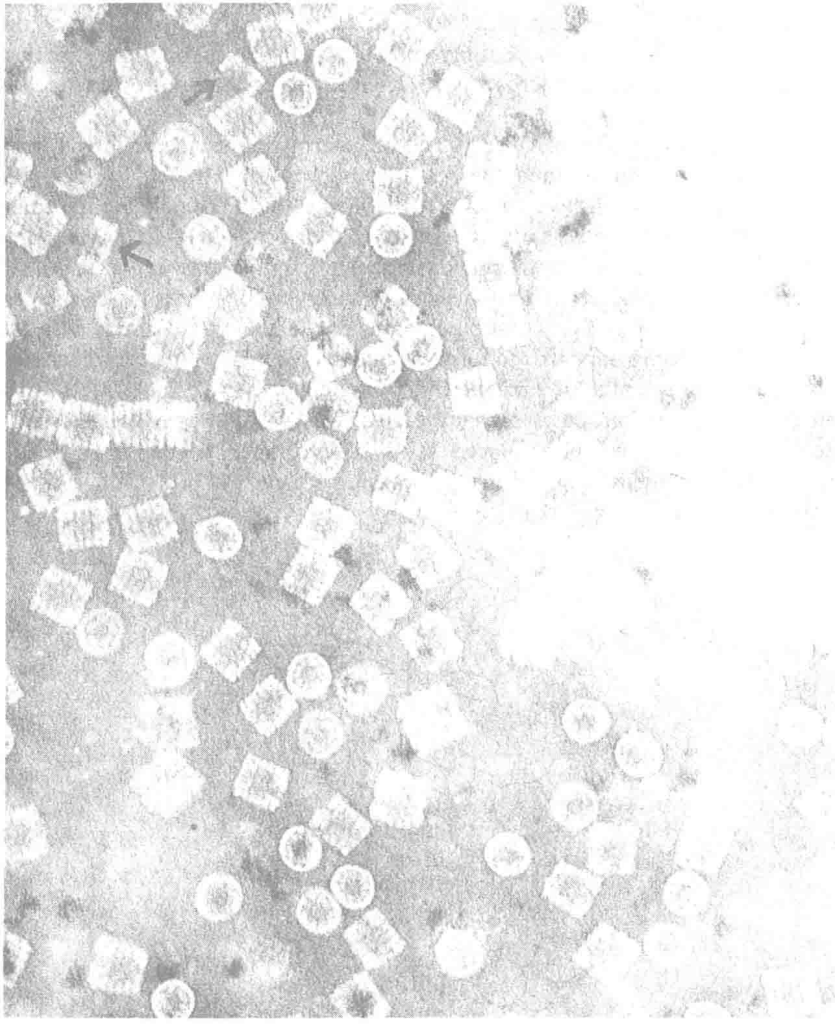

Fig. 3. Electron micrograph of negatively-stained haemocyanin from the freshwater snail, *Lymnaea stagnalis*. In end-on views of the molecule (circles) ten-fold symmetry is observed. Dimensions of the cylindrical molecule: 30 nm diam. × 32 nm height (approx.). Note the tendency of the molecules to stack: small peaks possibly corresponding to 1½ (130 S) and double (150 S) molecules are sometimes seen in ultracentrifuge (schlieren) pictures. Occasionally one-half molecules are seen (arrowed): cephalopod haemocyanins consist almost entirely of such molecules. (Electron micrograph taken by D. Kershaw, University of Leeds.)