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BRAIN VASCULAR VOLUME, ELECTROLYTES AND BLOOD–BRAIN INTERFACE IN THE CUTTLEFISH *SEPIA OFFICINALIS* (CEPHALOPODA)

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SUMMARY

1. Cephalopod molluscs have complex brains and behaviour, yet little is known about the permeability of their blood–brain interface. This paper presents studies on the brain fluid and electrolyte compartments of the cuttlefish *Sepia*, as a preliminary to characterization of the permeability of the blood–brain interface in this group.

2. *Sepia* is shown to be a satisfactory experimental animal, and techniques are described for anaesthesia, cannulation, and tissue and fluid analysis.

3. Our ionic analyses of body fluids are in general agreement with those of earlier workers. Analysis of brain tissue suggests that the extracellular space is 17–20 ml 100 g tissue^{-1} (i.e. 17–20 %).

4. Two large molecular weight tracers, ^{125}I-human serum albumin (HSA) and Blue dextran, give consistent values for the brain vascular volume of 3·5–5·5 %, slightly higher than in vertebrates.

5. The HSA results further confirm that the *Sepia* blood–brain interface is relatively tight to proteins.

6. Finally, we have shown that the fluid surrounding the brain, pericerebral fluid (PCF), is in relatively free communication with plasma.

INTRODUCTION

A blood–brain barrier is well developed in all vertebrate species that have been investigated (Bundgaard, 1982; Cserr & Bundgaard, 1984). The morphological site of the barrier in all groups except elasmobranchs is the brain capillary endothelium and choroid plexus epithelium, where the intercellular clefts are occluded by tight junctions (Brightman & Reese, 1969). In elasmobranch brain where the blood vessel endothelium is relatively leaky, the blood–brain barrier is attributable to tight junctions between glial cells, which here form a complete perivascular sheath (Brightman, Reese, Olsson & Klatzo, 1971; Bundgaard & Cserr, 1981).

Experiments were carried out at: The Marine Biological Association Laboratory, Citadel Hill, Plymouth PL1 2PB.

While the mechanism for the low passive permeability, and the associated carrier-mediated processes for selective transport of substances across the barrier, is well studied in vertebrates (Bradbury, 1979), the question as to why a barrier should be necessary at all has not been clearly answered. Hypotheses generally advanced centre on a greater requirement for homoeostasis of the micro-environment bathing neurones than that of most cells of the body, because of the extreme sensitivity of neuronal activity to the levels of ions and neuroactive agents in the surrounding medium (Davson, 1976; Abbott & Treherne, 1977; Cserr & Bundgaard, 1984). Thus a barrier might be necessary in species where the ionic composition of plasma makes it unsuitable as a neuronal medium, or where large fluctuations in plasma ionic composition occur.

However, the ubiquity of the barrier in vertebrate groups makes hypotheses concerning the function of the barrier difficult to test in these groups. By contrast, invertebrates show a wide variation in permeability of the blood–brain interface, from species with no diffusional restriction, to those with an effective barrier. Comparative studies in invertebrates are therefore likely to be instructive, since comparison of species with and without a barrier may be able to pinpoint aspects of brain or general physiology which necessitate the provision of a barrier. Moreover, differences in the barrier function of different regions within the nervous system in these lower animals can indicate more clearly than in vertebrates, which features of neuronal activity are most dependent on the presence of a barrier.

Among invertebrates previously studied, only members of certain arthropod groups (insects, arachnids) have an effective barrier; annelids and lower molluscs (gastropods, bivalves) do not (reviewed in Abbott & Treherne, 1977; Lane, Harrison & Bowerman, 1981). There is virtually no information on the permeability of the blood–brain interface in cephalopod molluscs, although early electron microscopic studies suggested that central neurones might be bathed directly by blood (Barber & Graziadei, 1967). Cephalopods are marine molluscs with blood composition close to that of sea water, and efficient blood ionic homoeostasis (Robertson, 1953); moreover, axonal and giant synapse activity can be maintained in a medium based on the animal's blood or sea water (Hodgkin & Huxley, 1945; Hagiwara & Tasaki, 1958). It could be argued that if the primary function of the blood–brain barrier is gross ionic homoeostasis, then a barrier would not be expected in cephalopods. If a barrier is nevertheless present in this group, in contrast to the situation in lower molluscs with simpler nervous systems, it could indicate an association between the necessity for a barrier and the complexity of central nervous system integrative activity. We have therefore aimed to establish whether a blood–brain barrier exists in cephalopods.

We chose to work on the cuttlefish, *Sepia officinalis*, although other cephalopods such as octopus and squid have been more extensively studied physiologically, since *Sepia* has several advantages as an experimental animal. These include availability, the ease of maintaining animals in good physiological state for days to weeks, the accessibility of blood vessels, and the large size, which makes procedures such as sampling of body fluids and cannulation relatively straightforward. However, the similarity of the structure of the nervous system, and of the complexity of behaviour possible in cuttlefish, octopus and squid, suggests that findings in *Sepia* are likely to apply to other cephalopods of the subclass Coleoidea.

Coleoid cephalopods have an elaborate central nervous system, supplied by an internal network of blood vessels (Barber & Graziadei, 1967). *Sepia* has a well-developed sensory apparatus, especially visual (Messenger, 1973), highly co-ordinated motor activity, and a complex learning and behavioural capacity (Sanders & Young, 1940; Boycott, 1961; Messenger, 1973), all features indicating a more highly specialized nervous system than that of lower molluscs.

In a parallel morphological study (Abbott, Bundgaard & Cserr, 1981) we have investigated the fine-structure of the layers at the blood–brain interface in the *Sepia* nervous system. The endothelium lining brain vessels is discontinuous, but is surrounded by a complete perivascular layer of glial processes. Horseradish peroxidase injected into blood, used as a tracer, is blocked at the level of the perivascular glia, indicating that this layer is an effective barrier to the penetration of proteins.

In this and the subsequent paper, we present evidence leading to the conclusion that the *Sepia* glial blood–brain barrier restricts the penetration of a range of non-electrolytes. This paper presents results of ionic analyis of fluids and tissues, and estimation of tissue vascular volumes. The methods used are described in detail, since no comprehensive account of experimental techniques used in this animal has been published. In the subsequent paper (Abbott, Bundgaard & Cserr, 1985*a*) a quantitative treatment is used to estimate the permeability of the blood–brain barrier, for comparison with the barrier of mammals. Some of the results have been presented in preliminary form (Abbott, Bundgaard & Cserr, 1981, 1982).

METHODS

Experiments were performed in May–July at the Marine Biological Association Laboratory, Plymouth, England.

Animals

Sepia officinalis (70–1900 g) of both sexes were trawled in coastal waters off Plymouth, and kept in circulating sea-water tanks in the laboratory, mean salinity 35·2‰, chlorinity 19·5‰ (in parts per thousand, ‰). Animals could be kept in good condition for several weeks, but were usually used within a few days of catching. They were fed on live prawns and crabs.

Anaesthesia

Animals were anaesthetized by immersion in sea water containing chloral hydrate (B.D.H. Chemicals) at a concentration of 0·2 %. They showed brief (*ca.* 30–60 s) periods of increased activity before becoming gradually quiescent over approximately 5 min. In early short-term isotope experiments (HSA-1, Fig. 2), animals were kept anaesthetized between tracer injection and sacrifice, with a maintenance dose of 0·075 % chloral hydrate in the sea-water bath, but in later and longer-term experiments, animals were allowed to recover in the interval and then reanaesthetized. Chloral hydrate was found to be a suitable anaesthetic as the depth of anaesthesia could be very precisely controlled, and recovery was rapid (2–10 min); moreover, anaesthesia could be repeated several times without apparent ill effect. To aid recovery, a vigorous stream of aerated sea water was allowed to flow over the gills.

Operative technique

Initially, experiments were performed with the anaesthetized animal ventral side uppermost, just covered by sea water, with the mantle cavity opened by cutting through the mantle muscle in the mid line (Fig. 1*A*). The sea water over the gills was vigorously gassed with 100 % O_2. However, this technique was not suitable for long-term experiments. Moreover, the abnormal orientation of the

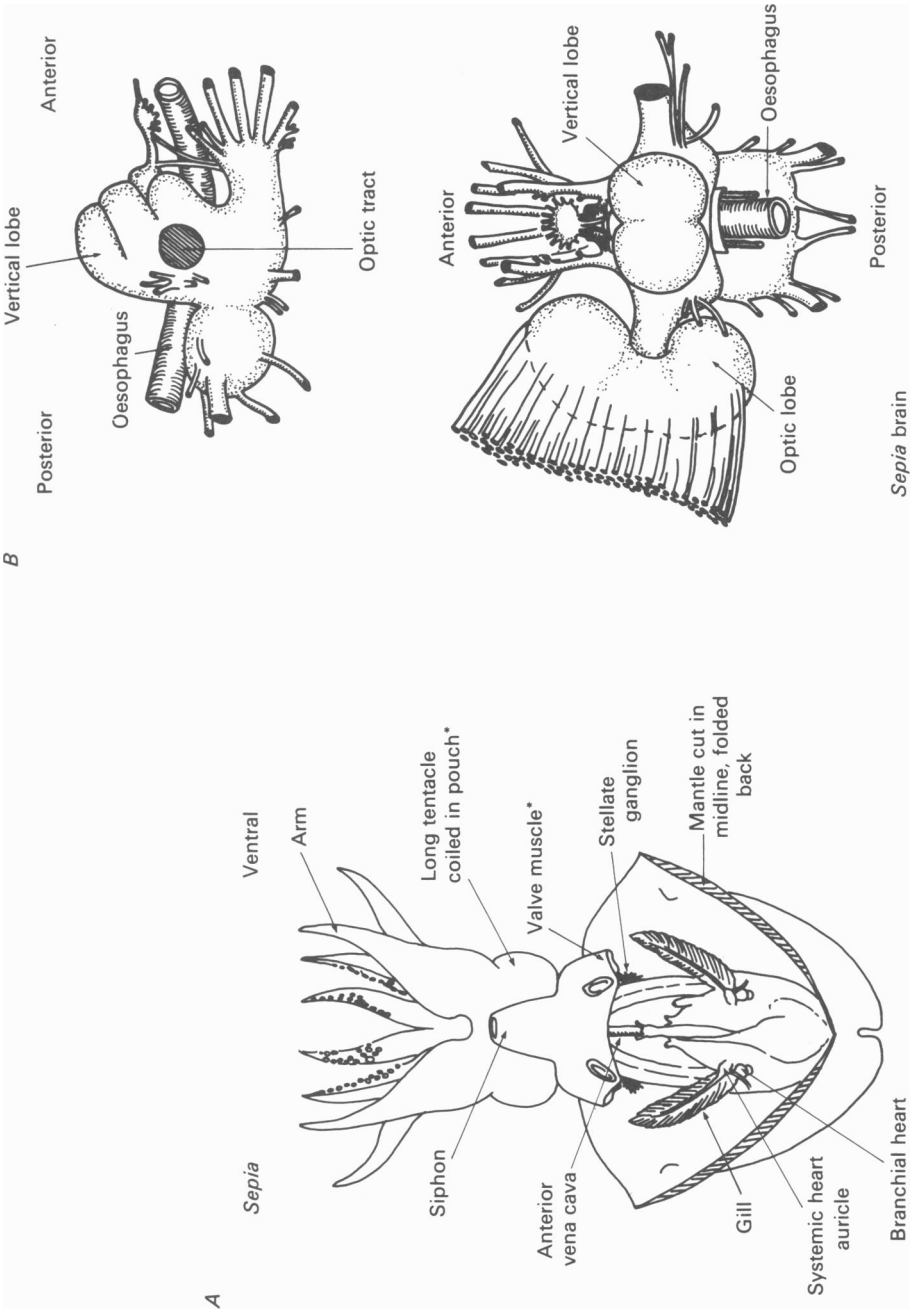

Fig. 1. *A*, ventral view of *Sepia*, mantle cut open in mid-ventral line to show the major organs visible in the mantle cavity. The source of the reference muscles (valve and tentacle) used in this study is indicated (*). Figure drawn after Tompsett (1939) fig. 5, using 'anterior vena cava' instead of 'cephalic vein' (see Wells (1978) fig. 2.6). *B*, *Sepia* brain lateral view with optic lobe removed (above) and dorsal view (below). Figure drawn after Tompsett (1939) figs. 56 and 57.

animal and shallow sea water in the experimental bath appeared to restrict the circulation to the head region in some animals. Therefore in later experiments the mantle was left intact, merely being held open to allow injection into the anterior vena cava (Fig. 1*A*), and the animal was allowed to float normally (dorsal side up) in sea water. At the end of the experiment, the animal was reanaesthetized.

Anatomy of vasculature, density and permeability of vascularization

Cephalopods have a closed circulation, with arterial and venous vessels (venous sinuses) connected by a capillary network in the tissues (Wells, 1978). Evans Blue albumin (1 % Evans Blue (Gurr; Hopkins and Wilkins, Chadwell Heath, Essex) in 5 % bovine serum albumin) made up in filtered sea water and injected intravenously, was used to show the anatomy of the blood system, and to gauge qualitatively the circulation time, density of vascularization and permeability of the vessels of different tissues.

Tissue and fluid samples

After anaesthesia, blood was taken either, (1) directly from the anterior vena cava with the mantle intact; or (2) (at the end of an experiment) from the vena cava or systemic heart auricle after slitting open the mantle cavity in the mid line. In a few animals, a fine (0·6 mm o.d.) polyethylene cannula filled with sterilized, filtered sea water was implanted under anaesthesia in the anterior vena cava and brought out on the dorsal surface of the mantle. Blood samples could then be taken without further anaesthesia over a period of 48 h. Blood samples were centrifuged at 12000 *g* for 6 min to spin down amoebocytes, and the supernatant plasma removed for analysis.

In addition to its internal vascular supply, brain is surrounded by a space within the cartilaginous skull, containing 'pericerebral fluid' (PCF). PCF samples were taken via a small hole in the dorsal mid line over the vertical lobe of brain, and treated in the same way as blood.

Animals were killed by severing the brain posterior to the vertical lobe, and samples of brain and muscle collected for analysis. Brain optic lobe and vertical lobe (comprising the vertical lobe proper and the underlying subvertical and superior frontal lobes, Fig. 1*B*, Sanders & Young, 1940) were selected as well-defined regions easy to dissect cleanly, and not contaminated by the contents of the oesophagus that runs through the centre of the brain. In addition, muscle (*valve muscle*: the outer collar muscle of the lateral valve flap associated with the siphon, Fig. 1*A* (Muscle O.C.F. in Tompsett, 1939, Fig. 23), and *tentacle muscle*: the base of the long (paired) tentacle) was analysed for comparison with brain. Optic lobes were carefully trimmed of optic nerve fibres. No attempt was made to remove adherent skin from the muscle samples, which in the case of the valve muscle was a significant fraction of the thickness. Sample weights in mg were typically 100–200 for the brain samples and 150–250 for muscle.

Estimation of tissue blood volume

Determination of the permeability of cerebral and muscle blood vessel walls to tracers requires an accurate estimate of the intratissue blood volume. Three methods were used (the third on brain only): (a) HSA-1, HSA-2. Human serum albumin (HSA) injection. (b) HSA-3. Human serum albumin perfusion. (c) Blue dextran perfusion. Sterilized, filtered sea water was used as the vehicle for injected or perfused tracer.

(*a*) *Human serum albumin injection.* Estimation of the distribution space of ^{125}I-human serum albumin at short times (5, 10, 30 min) after a single injection into the normally circulating blood stream. In the first series of experiments with ^{125}I-HSA (HSA-1), performed with animals kept anaesthetized and oxygenated during the experiment with the mantle cavity opened, isotope was injected into the left auricle. At the end of the experimental period, blood samples were taken from the right auricle and anterior vena cava. In later experiments (HSA-2), ^{125}I-HSA injection was into the vena cava without cutting the mantle, and return of the animal to sea water for the duration of the experimental period. The dose for HSA-1 and HSA-2 was 1–2 μCi/100 g body weight.

(*b*) *Human serum albumin perfusion, HSA-3.* Estimation of the distribution of ^{125}I-HSA introduced by continuous perfusion from a syringe into the dorsal aorta, which supplies the arterial system of the anterior part of the body (amoebocytes were first flushed out by pre-perfusion with filtered sea water). Both preperfusate and isotope solution contained chloral hydrate anaesthetic

at a concentration of 0·1 % and were aerated. The rate of perfusion (10 ml sea water over 2–3 min, followed by up to 25 ml isotope medium (0·2 μCi/ml) over 5–10 min) was designed to match the flow rate achieved by a perfusion pressure of 60–90 cm H_2O, within the range existing *in vivo* (assumed comparable to that of *Octopus* (Wells, 1978), 50–120 cmH_2O). The anterior vena cava was cut to allow drainage and collection of effluent perfusate from the head region.

(c) *Blue dextran perfusion.* As a check on the HSA methods, the larger molecule Blue dextran 2000 (mol. wt 2×10^6) molecular radius, *ca.* 105 Å was used to estimate the blood volume in brain. Blue dextran at a concentration of 2·4 g/50 ml was made up in filtered sea water containing 0·1 % chloral hydrate, dissolved by agitation for several hours at room temperature, filtered, and 20–25 ml perfused into the dorsal aorta following a protocol identical to (b) above, including preperfusion.

Tissue and fluid analysis

Measurements were made of electrolytes and water content, the osmolality of fluids and tank sea water, and the protein content of plasma and cell-free PCF. Na, K, Mg and Ca were determined in nitric acid extracts of tissues and fluids on a model 603 Perkin-Elmer Atomic Absorption Spectrophotometer (the Ca sample had 1 % lanthanum added). Cl was measured either with a Buchler–Cotlove chloridemeter (model 4-2000) on acid digests of tissues and fluids, or on fluid samples pipetted directly into acid buffer and measured on a Corning EEL 920 Cl meter. Fluid osmolality was determined by freezing point depression with an Advanced Osmometer, and protein was measured by the Lowry method (Lowry, Rosebrough, Farr & Randall, 1951).

For radioassay of ^{125}I-HSA (Radiochemical Centre, Amersham), tissues were briefly rinsed in two changes of sea water, blotted, transferred to tared scintillation vials, and reweighed; fluid samples were pipetted. Protosol (NEN) 0·5–0·8 ml was added (with 25–50 μl distilled water to speed tissue digestion), and samples were incubated at 40 °C for up to 24 h, with periodic shaking. The digested samples were frozen to reduce chemiluminescence, 10 ml Aquasol (NEN xylene-based scintillation cocktail) and 4 ml distilled water was added, and vials were shaken to gel the contents. Radioactivity was determined in a Packard Tricarb 2450 Liquid Scintillation spectrometer, with external standard quench correction.

For analysis of Blue dextran 2000 (Pharmacia), tissue was weighed, homogenized in 3 ml distilled water, and centrifuged at 12000 *g* for 24 min. The optic density of the tissue supernatant and of the perfusion solution and venous effluent was measured in a Unicam SP 600 spectrophotometer at 625 nm. Correction for background tissue absorption was made from control experiments in which the perfusion medium contained no Blue dextran.

The percentage distribution 'space' of each tracer in a tissue was calculated from:

$$\text{distribution space} = \frac{\text{d.p.m./mg tissue wet weight}}{\text{d.p.m./}\mu\text{l plasma}} \times 100.$$

For injected tracer, the distribution space was calculated with reference to the terminal plasma concentration. For perfused tracer, the mean vascular concentration was used, expressed as the arithmetic mean of arterial (perfusion solution) and venous effluent concentrations. In calculating vascular space, no correction was necessary for circulating blood cells, since the (amoebocyte) haematocrit was less than 1 %.

Assay of radiochemical purity of ^{125}I-HSA

The radiopurity of stock isotope solution, of *Sepia* plasma, and of buffer extracts of brain, was assayed as described previously (Cserr, Cooper, Suri & Patlak, 1981). Briefly, samples were evaluated both by gel filtration on Sephadex G-200 and by precipitation of the protein-bound radioactivity with trichloroacetic acid (TCA).

RESULTS

Physiological state

Several criteria for acceptable physiological state of the animals during experiments were determined:

(*1*) *Blood colour. Sepia* has a convenient built-in indicator of physiological state in the colour of its blood. Haemocyanin, the copper-containing blood pigment, is blue

when oxygenated, colourless when deoxygenated. Animals that showed clear blue blood (especially in the branchial veins and branchial hearts) immediately after anaesthesia and during the experiment, were judged to be in an acceptable physiological state.

(*2*) *Regularity of heart beat.* This was most easily monitored in the branchial hearts, which beat at the same rate as the auricles and ventricle of the systemic heart, usually a steady 15–25 beats per minute in a healthy anaesthetized animal. Healthy preparations also showed vigorous peristaltic waves in the major veins, including the anterior vena cava and branchial veins.

(*3*) *Circulation time.* In preliminary experiments it was found that dyes injected into the vena cava or systemic heart auricle could be seen returning in the anterior vena cava after periods of 15–60 s, but return was delayed up to 2 min or more in some animals. Circulation time appeared to be prolonged, and venous return from the anterior end of the body impeded, if the head and tentacles were not fully floating. For this reason, in subsequent experiments animals were kept fully submerged, especially when cannulation of the dorsal aorta was involved.

(*4*) *Lightness of anaesthesia.* Animals which were anaesthetized lightly recovered most rapidly, and maintained a good physiological state. Suitable lightness of anaesthesia was judged by reflex response to pinching of the arm tips, presence of muscular activity in the lateral valves controlling water entry into the mantle cavity, presence of muscle tone sufficient to support the head when fully floating dorsal side up in sea water, and fully retracted long tentacles.

(*5*) *Rapid recovery from anaesthesia.* Rapid recovery (usually 2–10 min in good conditions) from anaesthesia after blood sampling, judged as the time taken to the first contraction of the mantle sufficient to propel fluid from the siphon, indicated a healthy physiological state.

Animals with mantle intact during the experimental period, allowed to float dorsal side up, were generally in better physiological state than those with the mantle cut open. Animals with a chronic implanted cannula tolerated the cannula well, but amoebocytes generally accumulated around the tip and blockages occurred within 4–48 h.

Anatomy of the vasculature and density of vascularization

The general layout of the vascular system as described by Tompsett (1939) was confirmed. The density of tissue vascularization could be assessed qualitatively by the depth of staining after dye injection. In brain an elaborate network of vessels could be seen with the dissecting microscope within the exposed vertical and optic lobes, and large vessels on the surface of the brain were occasionally seen, but as these appeared to be closely adherent to the cartilaginous skull, they were generally removed in dissection. The brain parenchyma outside the clearly defined vessels was not diffusely stained by Evans Blue albumin.

Muscle was relatively poorly vascularized, apart from the lateral valve muscle (outer collar), and a region of the mantle around the stellate ganglion. The mainly muscular long tentacles were particularly poorly vascularized, apart from a small (arterial) vessel near the core of neural tissue, and another (vein) at one side. The mantle muscle showed diffuse staining with Evans Blue albumin after prolonged (2 h)

TABLE 1. Electrolyte content of *Sepia* plasma (PL) and pericerebral fluid (PCF) and of tank sea water (SW) expressed as A, mM and B, mmol kg H_2O^{-1}

A (mM)	Na	K	Mg	Ca	Cl
PL	464± 8 (11)	10·5±0·1 (6)	51·2±0·3 (6)	9·4±0·1 (5)	480±4 (12)
PCF	464± 8 (10)	10·9±0·2 (6)	50·2±0·2 (5)	9·5±0·1 (5)	490±7 (12)
SW	522±10 (5)	10·4±0·1 (2)	55·8±0·6 (3)	10·1±0·3 (3)	531±4 (14)
Standard SW, expected	483	10·2	55·1	10·5	563
B (mmol kg H_2O^{-1})					
PL	521± 8 (11)	11·7±0·2 (6)	57·4±0·4 (6)	10·5±0·1 (5)	538±5 (12)
PCF	520± 9 (10)	12·2±0·2 (6)	57·0±0·3 (5)	10·6±0·1 (5)	548±8 (12)
SW	528±10 (5)	10·5±0·1 (2)	56·4±0·6 (3)	10·2±0·3 (3)	537±5 (14)
Standard SW, expected	488	10·4	55·7	10·6	570

Electrolyte concentrations in mmol kg H_2O^{-1} calculated assuming water content of plasma is 0·892 g ml^{-1} and sea water 0·9986 g ml^{-1} (Robertson, 1953; Barnes, 1954). Values are means ±S.E. of mean with number of observations in parentheses. Standard SW, expected for sea water of mean chlorinity 19·5‰.

TABLE 2. Ratio of electrolyte concentrations (observed concentration in plasma [PL] or pericerebral fluid [PCF])/(theoretical concentration in plasma [PL theory]) where the theoretical concentration is predicted using the Donnan ratio for *Sepia* plasma

	Na	K	Mg	Ca	Cl
Donnan ratio*	1·022	1·016	1·048	1·187	0·978
[PL theory]	540±10 (5)	10·7±0·1 (2)	59·1±0·7 (3)	12·2±0·3 (3)	526±4 (14)
[PL]/[PL theory]	0·964	1·098	0·971	0·861	1·024
[PCF]/[PL theory]	0·963	1·140	0·964	0·874	1·043
[PL]/[PL theory]*	0·925	2·052	0·981	0·905	1·051

* From Robertson (1953). Concentrations in mm kg H_2O^{-1}. Values for [PL theory] calculated as the product, (Donnan ratio) × (concentration in sea water); values are means ±S.E. of mean with the number of observations in parentheses.

circulation of the dye. Powerful vasoconstriction of mantle vessels, which showed up as a white band approximately 3 mm wide in the otherwise dyed muscle, occurred either side of the cut when the mantle was slit in the mid-ventral line.

Fluid and tissue analysis

Tables 1–3 present the results of electrolyte and other analyses on fluid and tissue samples. Results from Robertson's earlier studies on *Sepia* (1953) are presented for comparison (see Discussion).

From the electrolyte analyses of Tables 1 and 3, the Na and Cl spaces of the tissues can be calculated ((tissue ion concentration in mmol kg wet $weight^{-1}$/plasma ion concentration in mM) × 100 %). Table 3 B presents values for brain and muscle Na and Cl space and for tissue extracellular space, estimated on the assumption that the true extracellular space is 0·76 × Cl space. The validity of this assumption is considered in the Discussion.

TABLE 3. Analysis of *Sepia* vertical lobe (v.l.), optic lobe (o.l.), valve muscle (v.m.) and tentacle muscle (t.m.). A, water and electrolyte content. B, Na and Cl spaces, calculated from [(concentration in tissue)/(concentration in plasma)] × 100 %, and extracellular space (e.c.s.), estimated as 0·76 × Cl space

	A				B		
	H_2O	Na	K	Cl	Na space	Cl space	E.c.s.
v.l.	77·52 ± 0·26 (10)	102 ± 9 (10)	180 ± 4 (10)	128 ± 8 (10)	22·1 ± 2·0	26·6 ± 1·7	20·2 ± 1·3
o.l.	78·02 ± 0·11 (10)	99 ± 5 (10)	182 ± 6 (10)	110 ± 2 (10)	21·2 ± 1·1	22·9 ± 0·4	17·4 ± 0·3
v.m.	80·00 ± 0·35 (6)	155 ± 12 (6)	121 ± 2 (6)	176 ± 9 (6)	33·4 ± 2·6	36·6 ± 1·9	27·8 ± 1·4
t.m.	78·23 ± 0·15 (13)	125 ± 10 (6)	130 ± 5 (6)	114 ± 3 (6)	26·8 ± 2·1	23·8 ± 0·6	18·0 ± 0·5

Units are for water, ml 100 g wet wt.$^{-1}$; for electrolytes, mmol kg wet wt.$^{-1}$; and for tissue space, ml plasma 100 g wet wt.$^{-1}$. Values are means ± S.E. of mean with number of observations given in parentheses.

Radiochemical purity of test compounds

The purity of ^{125}I-labelled albumin (HSA) was analysed by gel filtration and by precipitation of labelled protein with TCA. Samples were collected from *Sepia* killed 30 min or less after isotope injection. With gel filtration, the bulk of the radioactivity in pooled samples of plasma and of brain chromatographed as a single peak with the same mobility as the stock albumin injection solution. In both brain and plasma there was an indication of a smaller molecular weight component (second peak), however the total amount of this component was small (< 15 % of total tissue radioactivity and a smaller fraction of total plasma radioactivity). A high degree of radiochemical purity of the labelled albumin was also indicated by results with TCA precipitation. Only 5·5 % of total sample radioactivity was unbound in the ^{125}I-HSA stock solution and 3·8 % in plasma.

Tissue vascular volume

Tissue blood volume as estimated by the three techniques for brain tissue, and less comprehensively for muscle, is shown as histograms in Fig. 2. The consistency of the results using different methods and molecules of different size, is evidence that both HSA and dextran are largely confined to the blood space at short times, and are therefore reliable indicators of vascular volume. Mean (± S.E. of the mean) values for vascular space were 3·4 ± 0·1 (n = 29) for vertical lobe, 5·6 ± 0·2 (n = 23) for optic lobe, 2·2 ± 0·3 (n = 7) for valve muscle, and 0·4 ± 0·3 (n = 24) for tentacle muscle.

Impermeability of the blood–brain interface to protein

In order to confirm the low permeability of cerebral vessels to albumin, it was necessary to have accurate knowledge of the activity of isotope in blood during the experimental period. The plasma activity curve for ^{125}I-HSA is shown in Fig. 3. The results of Fig. 2, together with the relatively slow decline in plasma ^{125}I-HSA concentration up to 30 min indicate that cerebral vessels have relatively low permeability to albumin.

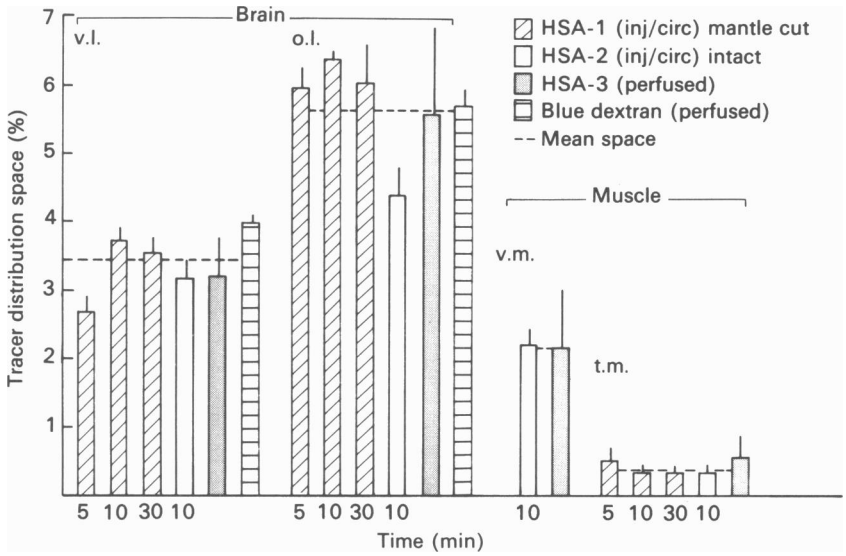

Fig. 2. Tissue vascular space for brain vertical lobe (v.l.), brain optic lobe (o.l.), valve muscle (v.m.) and tentacle muscle (t.m.) estimated as percentage distribution space for two intravascular probe molecules, radiolabelled HSA and Blue dextran. For detailed protocols, see text. Values are means ±S.E. of the mean.

Relation of PCF to blood and anatomy of PCF compartment

The PCF-containing cavity above the vertical lobe was found to be in continuity with the space around the optic lobe (the 'optic sinus' of Tompsett, 1939). In all the fluid analyses presented in Table 1, cell-free PCF composition is not significantly different from plasma.

After ^{125}I-HSA injection into the normally circulating blood, the level of activity in PCF follows that of the plasma (taken from the anterior vena cava and left auricle) very closely, although with a delay (5–15 min, Fig. 4), suggesting that the two fluids are in communication. In experiments to perfuse HSA or Blue dextran through the dorsal aorta, the PCF tracer concentration after 5–10 min was significantly less than that of the perfusion solution (Blue dextran, 62·9±6·05% ($n = 5$); $P < 0·01$; HSA 54·7±3·05 ($n = 6$), $P < 0·001$), and slightly less than that of the effluent collected from the vena cava (Blue dextran 71·7±2·72 ($n = 5$); HSA 73·8±3·73 ($n = 6$), percentage compared with perfusion solution). The difference between venous effluent and PCF was significant for HSA ($P < 0·01$) but not for Blue dextran. Bearing in mind the limited volume perfused in these experiments compared with the large volume of the plasma–PCF, it is unreasonable to expect complete mixing in the PCF compartment during the perfusion. However, the results suggest that the PCF may be a relatively stagnant pool, possibly in parallel rather than in series with the major route from dorsal aorta to vena cava through brain.

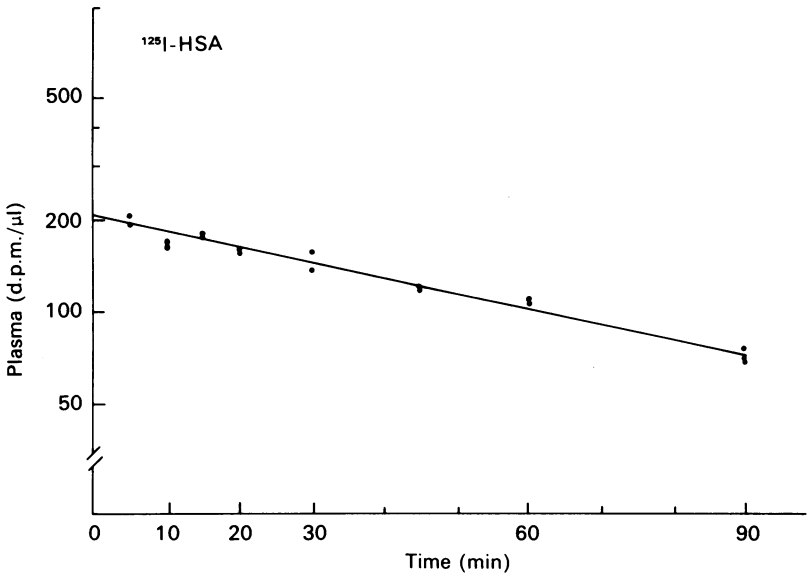

Fig. 3. Plasma radioactivity curve for ^{125}I-HSA. Data from a single animal, method HSA-1.

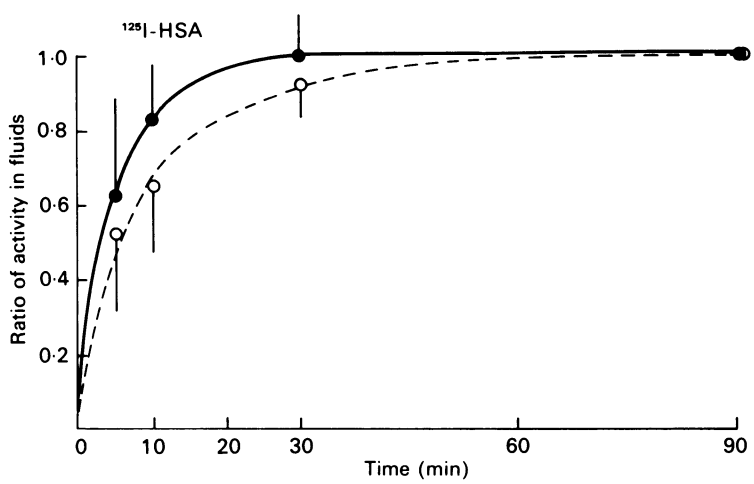

Fig. 4. The ratio of ^{125}I-HSA activity in plasma from different sampling sites, vena cava, auricle and PCF. Filled circles and continuous lines: vena cava/auricle. Open circles and dashed lines: PCF/auricle.

DISCUSSION

Sepia as an experimental animal

This study represents the first comprehensive physiological investigation on *Sepia*, and optimal conditions for experimentation had therefore to be established by trial and error. We have shown that the animal can be adequately and reversibly anaesthetized with chloral hydrate, that intravascular injection can be achieved with

minimal interference, and that the animals can be maintained in a healthy state during experiments lasting up to 48 h. Animals with the mantle cavity intact, and kept floating in sea water in their normal orientation were judged to be in better physiological state than those with mantle opened and kept ventral side up. Circulation time of 15–60 s in healthy animals is similar to the 30–120 s reported for *Octopus* of 1 kg weight (O'Dor & Wells, 1984). (Blood-flow measurements in individual cephalopod tissues have not been reported in the literature.) We conclude that *Sepia* is a suitable experimental animal for physiological studies.

Analysis of fluids

Our analyses of sea water taken from the Plymouth *Sepia* tank are approximately as would be expected for sea water of mean chlorinity 19·5‰ except that [Na] is slightly higher and [Cl] lower. The plasma and PCF values are not significantly different from each other. This similarity suggests that PCF is in direct continuity with the blood system, or, if separate, is separated by a very permeable layer, unlike the vertebrate cerebrospinal fluid or extradural fluid. Isotope studies further confirm this view (see below).

Robertson (1953) showed that ions in *Sepia* plasma are actively regulated, by demonstrating a consistent difference between the ionic composition of plasma and of plasma dialysed against sea water. The protein content of our samples is comparable to that of Robertson; if we assume that the plasma proteins have similar physicochemical properties in the two studies, then using the Donnan distribution ratios established by Robertson for the passive distribution of ions between *Sepia* plasma and its dialysate, it is possible to calculate the composition of a plasma dialysate expected from our sea water analysis ([PL theory] in Table 2). When compared with our plasma and PCF analysis, it is clear that the composition of these fluids is not determined by simple Donnan distribution, but must be the result of active transport. Our ratios ([PL] observed/[PL theory] calculated) are similar to the ratios given by Robertson (except for K) and confirm that active regulation of all ions occurs. Leakage of K from amoebocytes is a serious source of contamination of plasma samples in invertebrates (Potts & Todd, 1965; Brady, 1967). Presumably, this explains the raised [K] found in plasma by Robertson.

Estimation of osmolality and protein content (Table 4) are also consistent with values in the literature; thus Robertson (1953) found *Sepia* plasma to be isosmotic with sea water, and to contain protein in the range 6·8–10·9 g%.

Tissue extracellular space

From the determinations of plasma and tissue electrolytes, the extracellular space of *Sepia* brain was estimated on the assumption that the extracellular space is 0·76 × Cl space. In frog brain, careful comparison of Cl space and extracellular space as established by extracellular isotopic tracers (Bradbury, Villamil & Kleeman, 1968) showed that the true extracellular space is close to 0·76 × the measured Cl space. This is also close to the best estimates in mammalian brain (Bradbury, 1979). At the start of this study, virtually no information was available for invertebrate brain, so we took the value of 0·76 × Cl space as an estimate of the true extracellular space in *Sepia* brain. The suitability of this assumption is supported by experiments with ^{51}Cr-EDTA in the isolated brain of *Sepia* and squid (*Alloteuthis*), in which the blood–brain barrier

TABLE 4. Osmolality and protein content of *Sepia* fluids and tank sea water

	Osmolality (mosM)	Protein content (g 100 ml^{-1})
Plasma	1026 ± 12 (20)	9·4 ± 0·8 (5)
Pericerebral fluid	1024 ± 20 (13)	9·5 ± 0·9 (4)
Sea water	1025 ± 4 (12)	—

Values are means ± S.E. of mean with number of observations in parentheses.

was bypassed (Abbott, Bundgaard & Cserr, 1985*b*). These experiments indicate that the size of the extracellular space is of the order of 17–20 % (17–20 ml 100 g tissue^{-1}). The figures for *Sepia* were, optic lobe, 17·4 ± 0·4 % ($n = 14$) and, vertical lobe, 20·6 ± 2·0 % ($n = 9$). These estimates are comparable to the figures in the present paper Table 3B), and very similar to the extracellular space estimates for vertebrate brain (Bradbury, 1979).

Studies on vertebrate muscle indicate that the true extracellular space is between 0·45–0·8 × the Cl space (Schmidt-Nielsen, Renfro & Benos, 1972). Since the *Sepia* muscles examined in the present study have large extracellular spaces (N. J. Abbott & M. Bundgaard, unpublished observation), we expect the true extracellular space to be at least 0·7 × the Cl space. For simplicity, the figure of 0·76 × Cl space (used for brain) was also applied to muscle.

Tissue blood volume

While total vascular volume has been estimated as 5·8 % (*Octopus*, Martin, Harrison, Huston & Stewart, 1958) blood volume has not previously been measured in individual cephalopod tissues. Of the techniques used here, each has advantages and disadvantages. For example, perfusion into the dorsal aorta (^{125}I-HSA, method (b); Blue dextran, method (c)) from a closed syringe introduces an unknown pressure which could cause artificial vasodilatation. In an attempt to minimize this, the perfusion rate was adjusted to mimic that achieved by a pressure head of approximately 60–90 cmH_2O, estimated to be within the normal physiological range of blood pressure. Blue dextran, is a safer intravascular marker than albumin because of its larger size. However, dextran is known to show variable stickiness to cells and surfaces (Stalker, 1967) so that some adsorption by the blood vessel wall may have occurred. In spite of these theoretical objections, the results are relatively consistent, both between HSA and dextran, and among the different methods for introducing HSA (Fig. 2). This indicates that the shortcomings of the methods are probably insignificant in practice. The general pattern that emerges shows marked differences in the blood volume of brain and muscle, with the size of blood space being in the order brain optic lobe > brain vertical lobe > valve muscle ≫ tentacle muscle.

The 3·5–5·5 % blood volume in cephalopod brain is slightly higher than in most adult vertebrates (1–2 %, Bradbury, 1979), but is significantly less than the 10–12 % blood volume of crustacean cerebral ganglion (Abbott, 1970). The differences could be related to the different oxygen carrying capacities of the blood in the different animal groups (Altman & Dittmer, 1970) and the presence of large vessels in the tissue core in the invertebrate groups; in vertebrate brain, many major vessels run on the surface, and are not included in brain analysis.

The higher vascularity of the optic lobe (visual processing and analysis) compared to the vertical lobe (higher functions such as memory and learning) may reflect the dominance of the visual modality in cephalopod behaviour. Higher vascularity is found in synaptic areas than in axonal regions or areas containing cell bodies without a synaptic input, in cephalopods, decapod crustacea, and vertebrates (Abbott, 1971; Bradbury, 1979; N. J. Abbott & M. Bundgaard, unpublished observations).

The greater vascularity of valve muscle compared with tentacle muscle no doubt reflects the fact that regular rhythmic activity of the valves is required for ventilation of the mantle cavity, whereas the long tentacles are kept coiled in pouches below the eyes and are only extended briefly and infrequently in prey capture.

Low permeability of the blood–brain interface to protein

The distribution space for ^{125}I-HSA in brain at 30 min is not significantly different from that at 10 min, indicating a negligible loss of this tracer from the brain vascular space. At 30 min a small fraction of the ^{125}I-label within brain has apparently dissociated from the protein molecule (see radiochemical purity, above); moreover the half-time for clearance of HSA from the blood is 60 min (Fig. 3), so there is some fall in blood concentration over this period. Both these factors will tend to cause an artificially high distribution space for HSA at longer times. The fact that the brain HSA space nevertheless remains low at 30 min clearly indicates that the blood–brain interface has negligible permeability to this molecule.

Permeability of the blood–muscle interface

In tentacle muscle, the ^{125}I-HSA distribution space at 30 min is not significantly different from that at 10 min, indicating that any leakage of albumin from the blood vessels is undetectable in this tissue over the experimental period. However, the very small blood volume in tentacle muscle, and correspondingly low levels of radioactivity involved, mean that the resolution of the technique is poor. Therefore no conclusions about the permeability of tentacle-muscle blood vessels to protein can be drawn from these experiments. In valve muscle, the isotope results are less complete, but there is a suggestion from the diffuse staining at 2 h by Evans Blue albumin that this tracer may have access to the interstitial space. Further studies are needed to confirm this observation.

Relation of PCF and blood

The experiments and analyses presented here show that *Sepia* PCF behaves like a blood compartment, albeit a poorly mixed one, and therefore is not analogous either to vertebrate cerebrospinal fluid or extradural fluid in terms of composition. However, it may perform analogous functions as a buoyant compartment and volume buffer within the cranial cavity. Tompsett (1939) illustrates a large 'optic sinus' in *Sepia* draining into the anterior vena cava via the optic veins. We have shown that the PCF over the vertical lobe is in continuity with this optic sinus. Tompsett's figures also suggest that part of the circulation through the brain would by-pass the optic sinus, but the input to the sinus is not clearly shown. In histological material, we have observed that some venous channels in *Sepia* brain open to PCF (N. J. Abbott & M. Bundgaard, unpublished observation), suggesting a contribution to PCF from cerebral venous blood.

We conclude that *Sepia* is a suitable animal for experimental studies of the blood–brain interface and brain micro-environment in Cephalopods. The estimation of the extracellular volumes of the vertical and optic lobes of brain, and determination of the tissue vascular volumes in this paper form an essential basis for further studies of the blood–brain barrier permeability.

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REFERENCES

ABBOTT, N. J. (1970). Absence of blood–brain barrier in a crustacean, *Carcinus maenas* L. *Nature* **225**, 291–293.

ABBOTT, N. J. (1971). The organization of the cerebral ganglion in the shore crab, *Carcinus maenas*. I. Morphology. *Zeitschrift fur Zellforschung und mikroskopische Anatomie* **120**, 386–400.

ABBOTT, N. J., BUNDGAARD, M. & CSERR, H. F. (1981). Fine-structural evidence for a glial blood–brain barrier to protein in the cuttlefish, *Sepia officinalis*. *Journal of Physiology* **316**, 52–53*P*.

ABBOTT, N. J., BUNDGAARD, M. & CSERR, H. F. (1982). Experimental study of the blood–brain barrier in the cuttlefish, *Sepia officinalis L. Journal of Physiology* **326**, 43–44*P*.

ABBOTT, N. J., BUNDGAARD, M. & CSERR, H. F. (1985*a*) Tightness of the blood–brain barrier and evidence for brain interstitial fluid flow in the cuttle fish, *Sepia officinalis*. *Journal of Physiology* **368**, 213–226.

ABBOT, N. J., BUNDGAARD, M. & CSERR, H. F. (1985*b*). Comparative physiology of the blood-brain barrier. In *The Blood–Brain Barrier in Health and Disease*, ed. SUCKLING, A. J., RUMSBY, M. G. & BRADBURY, M. W. B. Chichester: Ellis Horwood.

ABBOTT, N. J. & TREHERNE, J. E. (1977). Homeostasis of the brain microenvironment: A comparative account. In *Treatment of Ions and Water in Animals*, ed. GUPTA, B. L., MORETON, R. B., OSCHMAN, J. L. & WALL, B. J., pp. 481–510. London: Academic Press.

ALTMAN, P. L. & DITTMER, D. S. (1971). *Respiration and Circulation*. Bethesda, MD: FASEB.

BARBER, V. C. & GRAZIADEI, P. (1967). The fine structure of cephalopod blood vessels. II. The vessels of the nervous system. *Zeitschrift fur Zellforschung und mikroskopische Anatomie* **77**, 147–161.

BARNES, H. (1954). Some tables for the ionic composition of sea water. *Journal of Experimental Biology* **31**, 582–588.

BOYCOTT, B. B. (1961). The functional organization of the brain of the cuttlefish *Sepia officinalis*. *Proceedings of the Royal Society* B **153**, 503–534.

BRADBURY, M. W. B. (1979). *The Concept of a Blood–Brain Barrier*. Chichester: John Wiley.

BRADBURY, M. W. B., VILLAMIL, M. & KLEEMAN, C. R. (1968). Extracellular fluid, ionic distribution and exchange in isolated frog brain. *American Journal of Physiology* **214**, 643–651.

BRADY, J. (1967). The relationship between blood ions and blood-cell density in insects. *Journal of Experimental Biology* **47**, 313–326.

BRIGHTMAN, M. W. & REESE, T. S. (1969). Junctions between intimately apposed cell membranes in the vertebrate brain. *Journal of Cell Biology* **40**, 648–677.

BRIGHTMAN, M. W., REESE, T. S., OLSSON, Y. & KLATZO, I. (1971). Morphologic aspects of the blood–brain barrier to peroxidase in elasmobranchs. *Progress in Neuropathology* **1**, 146–161.

BUNDGAARD, M. (1982). Brain barrier systems in the lamprey. I. Ultrastructure and permeability of cerebral blood vessels. *Brain Research* **240**, 55–64.

BUNDGAARD, M. & CSERR, H. F. (1981). A glial blood–brain barrier in elasmobranchs. *Brain Research* **226**, 61–73.

CSERR, H. F. & BUNDGAARD, M. (1984). Blood–brain interfaces in vertebrates – a comparative approach. *American Journal of Physiology* **246**, R277–288.

CSERR, H. F., COOPER, D. N., SURI, P. K. & PATLAK, C. S. (1981). Efflux of radiolabeled polyethylene glycols and albumin from rat brain. *American Journal of Physiology* **240**, F319–328.

DAVSON, H. (1976). Review Lecture. The blood–brain barrier. *Journal of Physiology* **255**, 1–28.

HAGIWARA, S. & TASAKI, I. (1958). A study on the mechanism of impulse transmission across the giant synapse of the squid. *Journal of Physiology* **143**, 114–137.

HODGKIN, A. L. & HUXLEY, A. F. (1945). Resting and action potentials in single nerve fibres. *Journal of Physiology* **104**, 176–195.

LANE, N. J., HARRISON, J. B. & BOWERMAN, R. F. (1981). A vertebrate-like blood–brain barrier, with intraganglionic blood channels and occluding junctions, in the scorpion. *Tissue and Cell* **13**, 557–576.

LOWRY, O. H., ROSEBROUGH, N. H., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265–275.

MARTIN, A. W., HARRISON, F. M., HUSTON, M. J. & STEWART, D. M. (1958). The blood volumes of some representative molluscs. *Journal of Experimental Biology* **35**, 260–279.

MESSENGER, J. B. (1973). Learning in the cuttlefish, *Sepia*. *Animal Behaviour* **21**, 801–826.

O'DOR, R. K. & WELLS, M. J. (1984). Circulation time, blood reserves and extracellular space in a cephalopod. *Journal of Experimental Biology* **113**, 461–464.

POTTS, W. T. W. & TODD, M. (1965). Kidney function in the Octopus. *Comparative Biochemistry and Physiology* **16**, 479–489.

ROBERTSON, J. D. (1953). Further studies on ionic regulation in marine invertebrates. *Journal of Experimental Biology* **30**, 277–296.

SANDERS, F. K. & YOUNG, J. Z. (1940). Learning and other functions of the higher centres of *Sepia*. *Journal of Neurophysiology* **3**, 501–526.

SCHMIDT-NIELSEN, B., RENFRO, J. L. & BENOS, D. (1972). Estimation of extracellular space and intracellular ion concentrations in osmoconformers, hypo- and hyperosmoregulators. *The Bulletin of the Mount Desert Island Biological Laboratory* **12**, 99–104.

STALKER, A. L. (1967). The microcirculatory effects of dextran. *Journal of Pathology and Bacteriology* **93**, 191–201.

TOMPSETT, D. H. (1939). *Sepia*. *Liverpool Marine Biological Committee*. Vol. 32. Liverpool: University Press.

WELLS, M. J. (1978). *Octopus: Physiology and Behaviour of an Advanced Invertebrate*. London: Chapman and Hall.