URINE FORMATION IN THE LAMELLIBRANCHS: EVIDENCE FOR ULTRAFILTRATION AND QUANTITATIVE DESCRIPTION

By F. HEVERT

Institut für Allgemeine und Spezielle Zoologie der Justus Liebig-Universität, D-6300 Giessen, F. R. Germany and *Station de Biologie Marine, Arcachon, France*

SUMMARY

1. Physical and chemical parameters were measured in the Japanese oyster *Crassostrea gigas* to investigate whether the first step of urine formation in the lamellibranchs could be an ultrafiltration and to give a quantitative description.

2. The effective filtration pressure was not constant, but a function of time, oscillating between $31.7\,mmH_2O$ and $-3.8\,mmH_2O$. During the filtration, a separation of proteins took place: the protein concentration in the haemolymph was $17\,\mu mol\,l^{-1}$ and the average molecular weight was 141 000. In the filtrate, the protein concentration was $2\,\mu mol\,l^{-1}$ and the average molecular weight was 45 000. The marker substance inulin, applied *via* the gills, appeared successively within the haemolymph and the pericardial fluid. These findings establish the idea that the pericardial fluid is formed by ultrafiltration from the haemolymph.

3. The rate of filtration was found to be $0.4\,\mu l\,g^{-1}\,min^{-1}$ by quantitative analysis of the transport of the inulin. The coefficient of filtration was $4.5 \times 10^{-6}\,ml\,s^{-1}\,cm^{-2}\,mmHg^{-1}$.

INTRODUCTION

Among the molluscs, both cephalopods and gastropods are believed to filter a primary urine through the heart wall either into the pericardial cavity or directly into the kidney as in terrestrial pulmonate gastropods (Picken, 1937; Martin, Stewart & Harrison, 1965; Andrews & Little, 1971; Potts, 1975; Schipp & Hevert, 1981).

In the lamellibranch *Anodonta*, such ultrafiltration has also been demonstrated by measurements of hydrostatic pressure differences and a rough estimation of colloid osmotic pressure (Picken, 1937); and is considered feasible in other lamellibranchs by several authors (Potts, 1975; Mangum & Johansen, 1975; Florey & Cahill, 1977; Hevert, 1980). However, it is not generally agreed that the lamellibranchs have this mechanism. For instance, no evidence for ultrafiltration has been found in *Modiolus* (Pierce, 1970), and in five other lamellibranchs, including *Anodonta*, the hydrostatic

haemolymph pressure is too feeble to drive the filtration against osmotic for█ (Tiffany, 1972). But it is not clear how the pericardial fluid is formed in these species.

The present investigation examines whether the primary production of urine in the lamellibranch *Crassostrea gigas* involves ultrafiltration, and gives a quantitative description.

MATERIALS AND METHODS

Animals, preparation

Japanese oysters *Crassostrea gigas* were obtained from the 'bassin d'Arcachon', Atlantic coast, France, and maintained for at least 12 h in running natural sea water for osmotic equilibration. Fluid samples were collected from the pericardial cavity and the heart ventricle by the following procedure. The right (i.e. functional upper) valve and the right mantle and gills were removed to expose the heart-pericardial-complex. To avoid contamination, the upper surface of the pericardium was carefully dried and about 100 μl of pericardial fluid was withdrawn by means of puncturing the cavity with a fine glass pipette. Next, haemolymph was collected by opening the whole pericardium and puncturing the ventricle with another pipette.

Hydrostatic pressures

Simultaneous recordings of haemolymph pressure and pericardial cavity pressure were made with strain gauge pressure transducers (S17P, Sensotec, U.S.A.) connected with brass adapters to glass capillaries with outer tip diameters of about 0·1 mm. The whole system was filled with degassed sea water as pressure transmission medium.

The difficulty with every pressure measurement in biological systems is to obtain a tight fit of the transducer to the compartment of interest. Lamellibranch tissue is rather delicate and not very resilient, so the pericardium and heart were perforated with glass capillaries with conically shaped tips. After the puncture, a slight advancing of the capillary by means of a micro-manipulator gave a very good seal. Calibration marks were produced with the same measuring system by means of a standpipe to simulate known hydrostatic pressures. The frequency characteristic showed no depression of amplitude up to 6 s^{-1} and sensitivity and accuracy were better than 1 mmH$_2$O (= 9·81 Pa).

The signals were displayed by a bridge amplifier and a pen recorder.

Osmolality, colloid osmotic pressure

Osmotic concentrations were measured with a freezing point semi-micro osmometer OM 801 (Vogel, Germany) and in addition in some cases with a vapour pressure osmometer (Knauer, Germany). Both instruments use 50 μl samples and have theoretical resolution of 1 mosmol kg^{-1}. Colloid osmotic pressures were measured with a membrane osmometer (Knauer, Germany) using a synthetic 40 000 Da membrane.

Protein content

The micro biuret method with phenol reagent after Folin and Ciocalteu was used (Lowry, Rosebrough, Farr & Randall, 1951).

Inulin, application and determination

The right valve was removed and the fluid of the mantle cavity was completely replaced by 2·0 ml of sea water containing 900 mg l^{-1} inulin. Every 10 min, 20-μl samples were taken from mantle cavity, pericardium and heart. Determination of inulin was carried out after Führ, Kaczmarczyk & Krüttgen (1955).

Statistics and mathematical methods

Significance was measured with the paired *t*-test or a two way analysis of variance. To analyse the pressure oscillations, Fourier series were used. The Fourier coefficients were calculated with a computer programme which is a translation for the Hewlett-Packard 9815A from a programme by Jan Honcu, Liaz, Czechoslovakia.

For calculation of the quantitative parameters the following equations were applied:

$$m = m_0 \cdot e^{-k_{in}t},$$

$$k_{in} = \frac{\ln 2}{t_{m,50}},$$

$$h = \frac{m_0 \cdot k_{in}}{k_{ex} - k_{in}} \cdot (e^{-k_{in}t} - e^{-k_{ex}t}),$$

$$t_{h,max} \cdot k_{in} = \frac{1}{1 - k_{ex}/k_{in}} \cdot \ln \frac{1}{k_{ex}/k_{in}}.$$

Results are presented as arithmetic mean ± standard deviation, except in Table 1 where the results are arithmetic mean ± standard error of the mean.

RESULTS

Hydrostatic pressures

In 23 animals the mean systolic pressure was 32·5 ± 7·0 mmH$_2$O, with highest values in two animals of more than 100 mmH$_2$O. (Some preliminary measurements with hypodermic needles, $N = 6$, gave a mean of 26·7 ± 18·2 mmH$_2$O, max 37·0.) Surprisingly, in all 23 animals the diastolic pressure fell to 3·0 ± 2·0 mmH$_2$O, in contrast to the zero value found in previous studies for *Saxidomus* and *Mya* (Florey & Cahill, 1977). Pericardial pressure oscillated between −3·0 ± 2·8 mmH$_2$O, when the ventricle was in systole, and +3·0 ± 3·4 mmH$_2$O with the ventricle in diastole. This finding corresponds to Ramsay's hypothesis of the constant volume mechanism to fill the auricles (Ramsay, 1952; cf. also Florey & Cahill, 1977) and, as we will discuss later, seems to be important for the ultrafiltration.

The coupled cardiac and pericardial activities are rather similar and uniform (Fig. 1), so Fourier series analysis could be employed to calculate the typical average time course of one heart action and of one pericardial action ('action' means the lapse of pressure as a function of time from the beginning of systole at minimum pressure to the end of diastole). The effective difference between heart and pericardium is then given by substraction of the two calculated curves (Fig. 2).
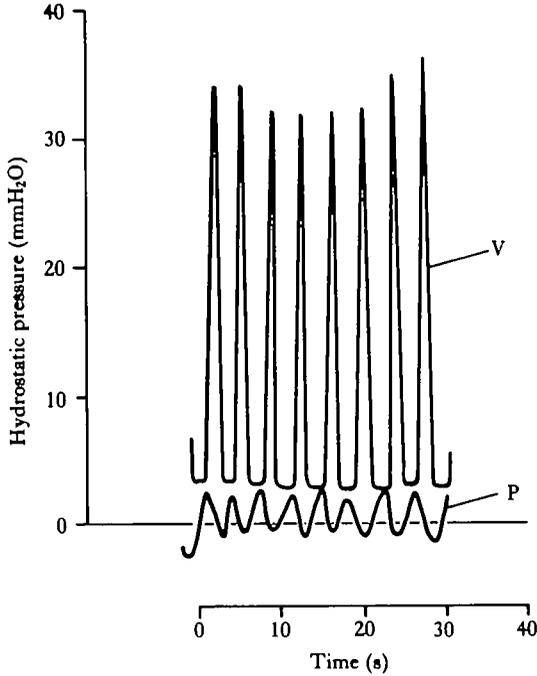
Fig. 1. *Crassostrea gigas*. Record of intraventricular (V) and intrapericardial (P) hydrostatic pressure.
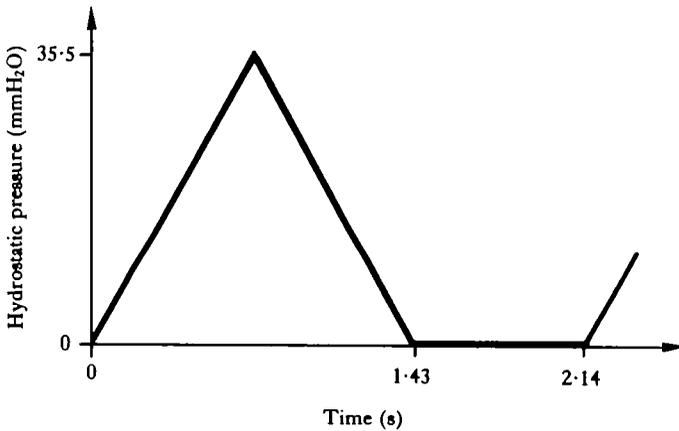

Fig. 2. *Crassostrea gigas*. Typical pattern of the pressure difference between heart and pericardium of one single beat. The diagram is the result of a Fourier series analysis of 400 beats.

Osmolality, colloid osmotic pressure and protein concentration

Table 1 summarizes the measurements of osmotic concentrations in pericardial fluid and haemolymph of 87 animals at different medium concentrations. The arithmetic mean of the osmolality of the haemolymph was a little higher (about $4\,mosmol\,kg^{-1}$) than the arithmetic mean of the osmolality of the pericardial fluid, as measured by freezing point depression, but this difference is not statistically significant

■ similar result was obtained using the method of vapour pressure depression.

Colloid osmotic pressure in haemolymph and pericardial fluid was then measured to see if this would produce an opposing force against the hydrostatic pressure as in vertebrates. Protein content was also measured, enabling calculation of the average molecular weight and, important for the osmotic active difference between the two compartments, the molar concentration of the proteins (Table 2). We have thus determined all physical driving forces which could account for fluid movement through the heart wall, as summarized in Fig. 3.

It can be seen that at systole there is an effective filtration pressure (EFP) of $31 \cdot 7 \, mmH_2O$ which could drive, i.e. ultrafilter, fluid from the heart into the pericardium. At diastole there is a much smaller EFP in the reverse direction. From these results and the time course of the heart action (see Fig. 2), the net movement of water and solutes can be estimated (Fig. 4). About ten times as much fluid will flow out of the heart during each heart beat as will flow back (Fig. 4).

Table 1. *Osmotic concentrations in pericardial fluid and haemolymph of* Crassostrea gigas *at different medium concentrations*

Medium	Osmolality ($mosmol \, kg^{-1}$)		Difference[*]	Number of animals	Significance of difference
	Pericardial fluid	Haemolymph			
902	$898 \pm 5 \cdot 9$	$901 \pm 4 \cdot 5$	+ 3	26	NS
954	$955 \pm 2 \cdot 9$	$957 \pm 2 \cdot 6$	+ 2	31	NS
1113	$1105 \pm 5 \cdot 5$	$1112 \pm 4 \cdot 3$	+ 7	12	NS
1358	$1350 \pm 5 \cdot 2$	$1354 \pm 5 \cdot 1$	+ 4	18	NS

The values are given as arithmetic mean ± standard error of the mean. All data from freezing-point osmometry.
[*] Haemolymph osmolality minus pericardial fluid osmolality.
NS, not significant.

Table 2. *Colloid osmotic pressure and protein concentration in pericardial fluid and haemolymph of* Crassostrea gigas

Parameter	Compartment	
	Pericardial fluid	Haemolymph
Colloid osmotic pressure (mmH_2O)	$0 \cdot 5 \pm 0 \cdot 9, N = 27$	$4 \cdot 3 \pm 1 \cdot 9, N = 27$
Protein content ($g \, l^{-1}$)	$0 \cdot 09 \pm 0 \cdot 08, N = 11$	$2 \cdot 41 \pm 0 \cdot 085, N = 9$
Average molecular weight of proteins	45 000	141 000
Protein concentration ($\mu mol \, l^{-1}$)	2	17

The values are given as arithmetic mean ± standard deviation, N = number of animals.

Quantitative aspects

A quantitative approach to the ultrafiltration was attempted by kinetic analysis using a compartment model to describe movements of the tracer inulin. In the model (Fig. 5), the marker substance is given with known concentration of $m_0 = 900 \, mg \, kg^{-1}$ into the mantle cavity. The actual concentration as a function of time is m. From here, it reaches the haemolymph by first order processes (probably diffusion) with the time dependent concentration h. From the haemolymph it is ultrafiltered into the pericardium,
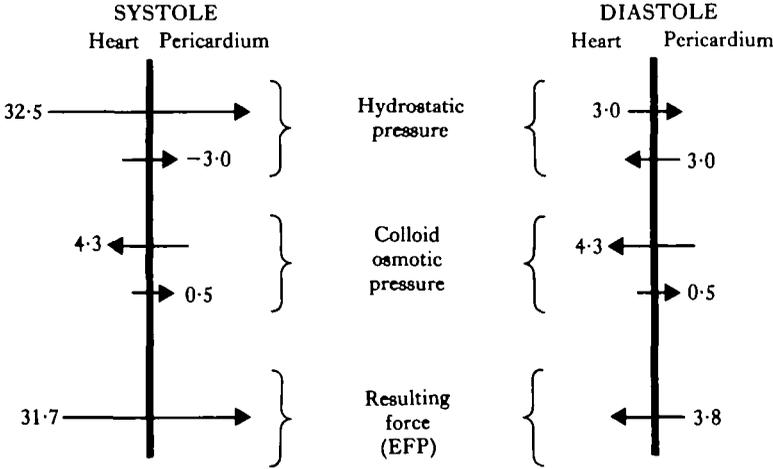
Fig. 3. Compilation of all physical driving forces for the fluid movement through the heart wall of *Crassostrea gigas*. The arrows indicate the direction of the resulting flow. Pressures are given in mmH_2O.
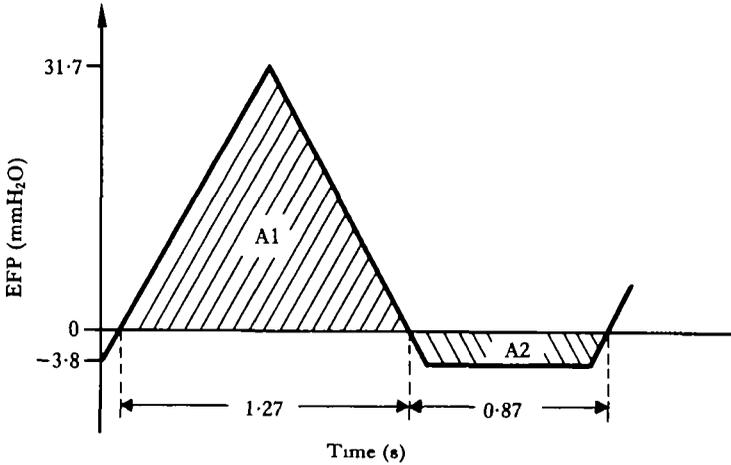

Fig. 4. Time course of the effective filtration pressure (EFP) in the heart of *Crassostrea gigas* during one heart beat. The area above the abscissa (A1) is about ten times as large as that below (A2).

where it has the concentration p, and it leaves the pericardium through the reno-pericardial duct towards the kidney. The coefficients which determine the velocity of the transports are called k_1, k_2, ..., k_5.

The mathematical description of this process leads to a system of three coupled differential equations (1–3):

$$\frac{dm}{dt} = -k_1 m + k_4 h \tag{1}$$

$$\frac{dh}{dt} = k_1 m - (k_2 + k_4)h + k_5 p \qquad m(0) = m_0,\ h(0) = 0,\ p(0) = 0 \tag{2}$$

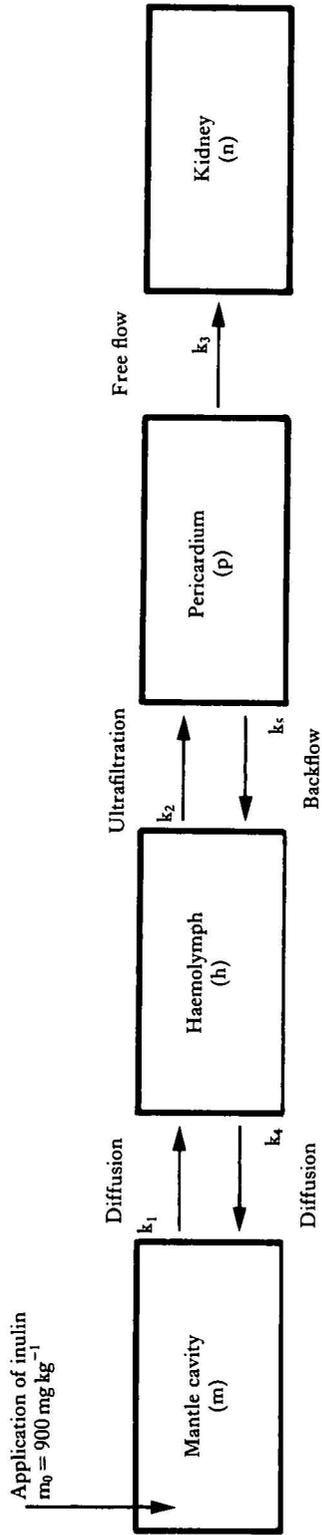$$\frac{dp}{dt} = k_2 h - (k_3 + k_5)p. \tag{3}$$

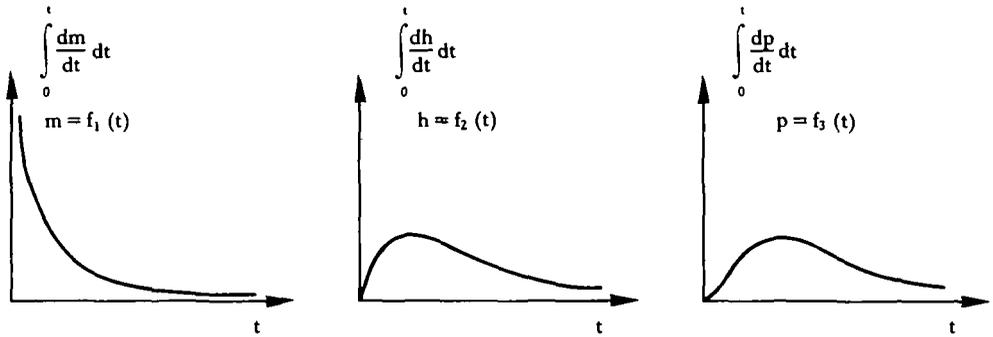Fig. 5. Hypothetical model of the tracer flow from mantle cavity to kidney.

Fig. 6. Integrals of equations 1, 2 and 3, generated with an analogue computer: expected concentrations of inulin in three compartments: mantle cavity, m; haemolymph, h; pericardium, p.

Theoretical solutions in the form $m = f_1(t)$, $h = f_2(t)$, $p = f_3(t)$ were found with an analogue computer and are shown in Fig. 6.

These equations predict that the tracer will disappear from the mantle cavity by simple exponential kinetics (equation 1), will pass through the haemolymph by Bateman kinetics (equation 2), and will pass through the pericardium with kinetics of higher complexity (equation 3) consisting of an overlay of the latter Bateman kinetics with another exponential elimination. In comparison with the concentration in the haemolymph compartment, the change of concentration in the pericardium is expected to be characterized by a lag phase at the beginning.

These predictions were tested by measuring the inulin concentrations in the three different compartments of 20 animals at 10-min intervals (this frequency was necessary to ensure sufficient accuracy in later calculation of parameters). The results are compared with the theoretically expected values in Fig. 7.

From these data, the following parameters could be determined:

(1) $t_{m,50} = 59$ min = half-life of the inulin concentration in the mantle cavity.

(2) $t_{h,max} = 62$ min = the time at which the inulin concentration in the haemolymph is at its maximum.

(3) $t_{p,max} = 123$ min = the time at which the inulin concentration in the pericardial fluid is at its maximum.

(4) $k_{in} = k_1 - k_4 = 0.0117$ min^{-1} = the coefficient which describes the entry of the inulin into the haemolymph.

(5) $k_{ex} = k_2 - k_5 = 0.0198$ min^{-1} = the coefficient which describes the exit of the inulin from the haemolymph.

k_{ex} is about twice as high as k_{in}, indicating that the outflux from the haemolymph must be coupled to a volume flow. This volume flow is nothing else but the ultrafiltration, and may be determined from the concentrations and the k_{in}/k_{ex} ratio to be 0.4 $\mu l\,g^{-1}$ min^{-1}, corresponding to about 23 ml day^{-1} for the whole animal.

Fig. 7. Inulin concentration in *Crassostrea gigas* in (A) mantle fluid, (B) haemolymph, (C) pericardial fluid. Values are arithmetic means ± s.D., curves are theoretically expected.

A

B

C

Time (min)

Inulin concentration (mg l^{-1})
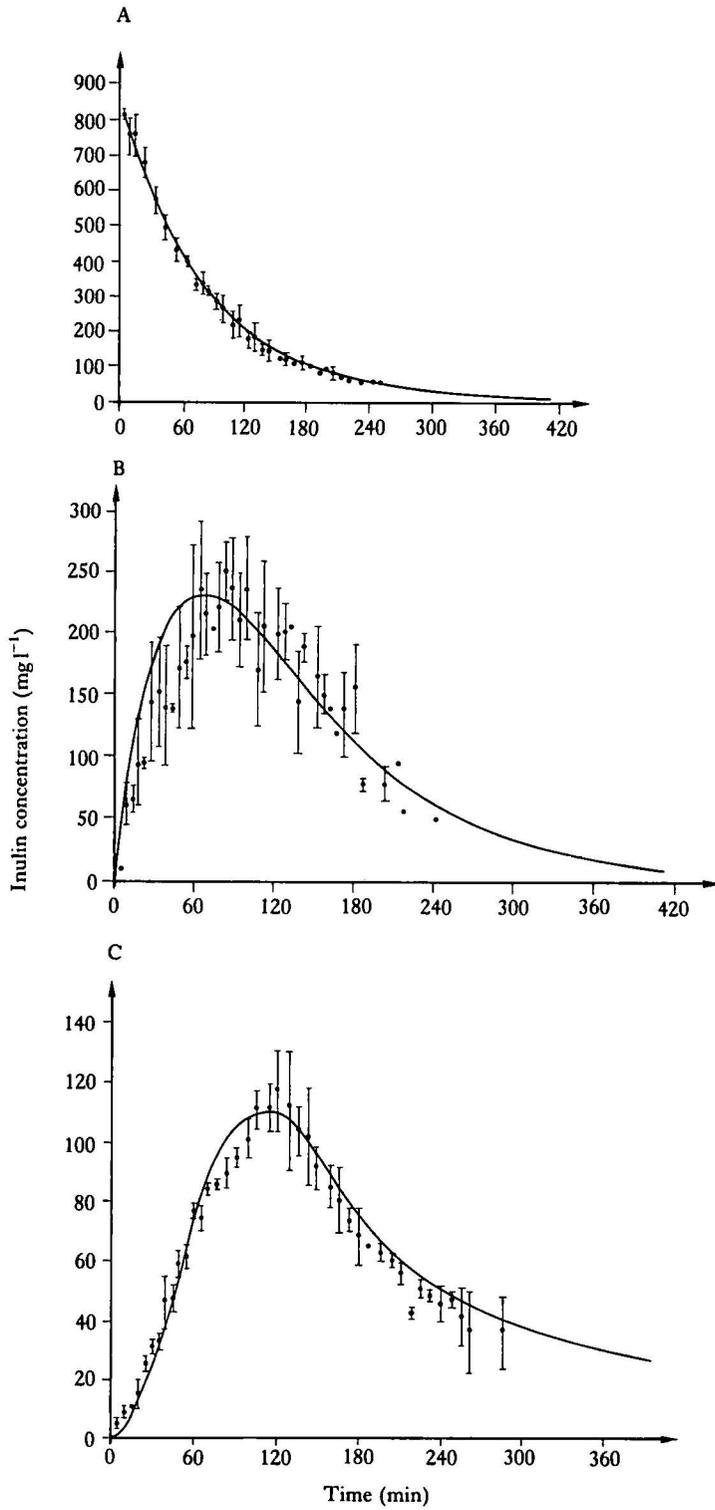
Evidence for ultrafiltration and remarks on the significance of osmotic measuring methods

Ultrafiltration between two biological compartments takes place if two conditions are satisfied.

(1) The hydrostatic pressure difference across the separating wall must exceed the opposing osmotic pressure.

(2) The partition must be permeable to water and small molecules but impermeable to molecules with the size of proteins.

This investigation shows clearly that both conditions are fulfilled at the heart-pericardial-complex of *Crassostrea*, so causing an ultrafiltration, i.e. a permanent volume flow from the haemolymph towards the pericardial fluid. Since the pericardial cavity is connected directly by the renopericardial duct to the kidney, we are forced to interpret this process by analogy to the vertebrates as the initial step in urine formation. The pericardial fluid is equivalent to the primary urine. These findings are in agreement with most studies on excretion in molluscs (cf. the extensive review of Potts, 1967), with the exception of that of Tiffany (1972). In five species he found the 'opposing osmotic force' to be at least twenty times greater than the hydrostatic pressure. His conclusion is, that 'therefore, ultrafiltration cannot take place across the ventricle of the heart into the pericardial cavity'.

The discrepancy can largely be explained by Tiffany's use of the van't Hoff equation to transform the measured freezing point depression into units of pressure, as this equation is only valid for 'ideal solutions' with very low concentrations (at most $10 \, \text{mmol} \, l^{-1}$) and containing only one kind of solute particles, and for systems separated by membranes which are only permeable to water (semipermeable membranes). Haemolymph and pericardial fluid are far from 'ideal' – the concentration is extremely high and they contain a complex mixture of solutes with different sizes, shapes and dissociation constants – and the heart wall is not a semipermeable membrane. All this leads to a marked over-estimate of the actual pressure.

In this study membrane osmometry was used to measure colloid osmotic pressure without any transformation. The synthetic membrane must be as similar as possible to the natural ultrafilter. Since almost nothing is known about the properties of this natural filter in molluscs, a membrane was selected with a nominal molecular weight cut-off lower than the estimated cut-off of the natural filter. In *Crassostrea* the average molecular weight of proteins was 141 000 within the haemolymph and 45 000 within the pericardial fluid. From pilot experiments with microdisc electrophoresis (F. Hevert & E. Pfeifer, unpublished observations) we know that the pericardial fluid contains more than one protein and therefore we are sure that the natural molecular weight cut-off is higher than 45 000 Da. So the synthetic 40 000 Da membrane used in our experiments would slightly overestimate the colloid osmotic pressure.

Quantitative aspects

The rate of filtration in lamellibranchs has been found by the method of fluid collection to be $3 \cdot 2 \, \mu l \, \text{min}^{-1} \, g^{-1}$ in *Anodonta* (Picken, 1937), and by inulin clearanc

be $0.34\,\mu\mathrm{l\,min^{-1}\,g^{-1}}$ in this species (Potts, 1954) and $0.17\,\mu\mathrm{l\,min^{-1}\,g^{-1}}$ in *Mytilus* (Martin, Harrison, Huston & Stewart, 1958).

In this investigation the method was not a simple inulin clearance but it investigates the movement of the tracer through the complete three-compartment system. This leads on the one hand to a dynamic view of the transmission of ultrafiltrable substances through the haemolymph and on the other hand it ensures that the amount of inulin which is not transported to the pericardium is not noticed. The rate at which an ultra-filtrable substance leaves the haemolymph is nearly two times higher than the rate at which it enters. From the biological point of view, this seems to make sense, because in that way no metabolic or foreign substance can accumulate in the haemolymph. If the inulin concentration is monitored not only within the haemolymph but also within the pericardial fluid it is possible to make use of the principle of the *korrespondierende Flächen* (Dost, 1968, 1970) and so to calculate very exactly the volume of fluid which is filtered towards the pericardium. It comes to $0.4\,\mu\mathrm{l\,min^{-1}\,g^{-1}}$, a value which is very similar to Pott's (1954) finding for *Anodonta*.

Table 3. *The coefficient of filtration of the heart wall of* Crassostrea *in comparison to other membranes*

Membrane	Coefficient of ultrafiltration ($\mathrm{ml\,s^{-1}\,cm^{-2}\,mmHg^{-1}}$)
Rana, endothelium of mesenterial capillaries	0.7×10^{-6}
Rana, glomerulus	2.9×10^{-6}
Crassostrea, heart wall	4.5×10^{-6}
Homo, glomerulus	7.8×10^{-6}

Data for *Rana* and *Homo* from Netter (1959) and Landis & Papenheimer (1963).

In the context of this discussion it is of interest to calculate from this volume flow and the pressure difference the specific coefficient of ultrafiltration. This coefficient is a measure of the physical properties of the filtering membrane such as thickness, pore diameter and pore geometry. In Table 3, the value for *Crassostrea* is compared to some values for other well known membranes. This compilation shows that the heart wall (or at least some parts of it) of *Crassostrea* actually has properties which are comparable to other known biological ultrafilters. The morphology and ultrastructure should now be studied.

REFERENCES

ANDREWS, E. & LITTLE, C. (1971). Ultrafiltration in the gastropod heart. *Nature, Lond.* **234**, 411–412.

DOST, F. H. (1968). *Grundlagen der Pharmakokinetik*. Stuttgart: Springer.

DOST, F. H. (1970). Transfer und Transit als pharmakokinetische Mengenbeziehungen in einem Multikompartiment-Modell. *Dt. med. Wschr.* **94**, 1713.

FLOREY, E. & CAHILL, M. A. (1977). Hemodynamics in lamellibranch molluscs: Confirmation of constant-volume mechanism of auricular and ventricular filling. Remarks on the heart as site of ultrafiltration. *Comp. Biochem. Physiol.* **57**A, 47–52.

FÜHR, J., KACZMARCZYK, J. & KRÜTTGEN, C. D. (1955). Eine einfache colorimetrische Methode zur Inulin bestimmung für Nieren-Clearance-Untersuchungen bei Stoffwechselgesunden und Diabetikern. *Klin. Wschr.* **33**, 729–730.

HEVERT, F. (1980). Salzbilanz und Ultrafiltration bei der Auster *Crassostrea gigas*. *Verh. dt. zool. Ges.* 304.

LANDIS, E. M. & PAPPENHEIMER, J. R. (1963). Exchange of substances through capillary walls. *Handbook of Physiology*, Section 2: Circulation Vol. II. American Physiological Society.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin Phenol Reagent. *J. biol. Chem.* **193**, 265.

MANGUM, C. P. & JOHANSEN, K. (1975). The colloid osmotic pressures of invertebrate body fluids. *J. exp. Biol.* **63**, 661–671.

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

MARTIN, A. W., STEWART, D. M. & HARRISON, F. M. (1965). Urine formation in a pulmonate land snail, *Achatina fulica*. *J. exp. Biol.* **42**, 99–123.

NETTER, H. (1959). *Theoretische Biochemie*. Stuttgart: Springer.

PICKEN, L. E. R. (1937). The mechanism of urine formation in invertebrates. II. The excretory mechanism in certain molluscs. *J. exp. Biol.* **14**, 20–34.

PIERCE, S. K. (1970). Water balance in the genus Modiolus. (Mollusca: Bivalvia: Mytilidae): osmotic concentrations in changing salinities. *Comp. Biochem. Physiol.* **36**, 521–533.

POTTS, W. T. W. (1954). The rate of urine formation in *Anodonta cygnea*. *J. exp. Biol.* **31**, 614–617.

POTTS, W. T. W. (1967). Excretion in the molluscs. *Biol. Rev.* **42**, 1–41.

POTTS, W. T. W. (1975). Excretion in gastropods. In *Excretion*, (ed. A. Wessing). *Fortschr. Zool.* **23**, 76–88.

RAMSAY, J. A. (1952). *A Physiological Approach to the Lower Animals*. Cambridge: Cambridge University Press.

SCHIPP, R. & HEVERT, F. (1981). Ultrafiltration in the branchial heart appendage of dibranchiate cephalopods: a comparative ultrastructural and physiological study. *J. exp. Biol.* **92**, 25–35.

TIFFANY, W. J. (1972). Aspects of excretory ultrafiltration in the bivalved molluscs. *Comp. Biochem. Physiol.* **43**, 527–536.