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THE FATE OF GLOMERULAR FILTRATION MARKERS INJECTED INTO THE HAEMOLYMPH OF THE AMPHIBIOUS CRAB *HOLTHUISANA TRANSVERSA*

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SUMMARY

1. Three glomerular filtration markers injected into *Holthuisana transversa* disappeared from the haemolymph at very different rates. Polyethylene glycol 4000 disappeared at a rate *ca.* 5 times faster than Cr-EDTA and *ca.* 15 times faster than inulin. Inulin and Cr-EDTA were excreted by the crab but only 5% of the injected PEG 4000 left the body.

2. Inulin injected into the excretory organs was reabsorbed into the haemolymph.

3. The reliability of commonly used glomerular filtration rate markers as indicators of filtration in decapods is discussed.

INTRODUCTION

It is generally accepted that the primary urine of decapod crustaceans is a filtrate of the haemolymph, formed as a result of a hydrostatic pressure differential across the coelomosac wall of the antennal organs. In crayfish, an osmotic pressure differential caused by formed bodies may also contribute to filtration (Riegel, 1977). The glomerular filtration rate (GFR) markers commonly used in the study of renal function in vertebrates have also been used with apparent success in aquatic decapods (e.g. labelled inulin (Binns, 1969), Na-diatrizoate (Riegel *et al.* 1974), Na-iothalamate (Ramamurthi, 1977)) whilst freshwater crayfish can also filter much larger molecules (e.g. low and high molecular weight dextrans and human serum albumin, although filtration of the latter two compounds is restricted (Kirschner & Wagner, 1965)). It is likely that the pores of the filtration site are larger than in vertebrates and that the molecular weight of the common GFR markers is unlikely to affect the rate of filtration.

Recent studies on lower vertebrates indicate that certain GFR markers may not give reliable measurements of extracellular space or plasma clearance as they do not fulfill the necessary requirements (Beyenbach & Kirschner, 1976; Hickman, Newcomb & Kinter, 1972; Schmidt-Nielsen, Renfro & Benos, 1972). These are (*a*) the marker should not penetrate cells or bind to plasma proteins, (*b*) it should enter the excretory organ only by filtration from the blood and (*c*) it should not be reabsorbed after filtration. In *Salmo gairdneri*, reabsorption of inulin, polyethylene glycol and Na-iothalamate occurs from the urinary bladder (Beyenbach & Kirschner, 1976) and

inulin is also absorbed from the bladder of the lizard *Trachydosaurus rugosus* (Braysh[illegible] & Green, 1972). Few similar studies have been carried out on decapod crustaceans. Kirschner & Wagner (1965) found that inulin and low molecular weight dextran were excreted at similar rates by the crayfish *Pacifastacus* whilst the marine crab *Carcinus* excretes ^{14}C-inulin, ^{51}Cr-EDTA and ^{131}I-Na-diatrizoate at essentially similar rates (Riegel *et al.* 1974). However, in the course of recent studies on water balance in the amphibious, Australian, arid-zone crab *Holthuisana transversa* Martens the author observed anomalies between rates of clearance measured with different markers. In view of this, further experiments were designed to determine the suitability of the markers concerned (^{3}H-inulin, ^{14}C-polyethylene glycol (PEG) 4000 and ^{51}Cr-EDTA) for measurement of filtration and urine flow in this crab and to shed further light on the behaviour of GFR markers in decapods generally.

MATERIALS AND METHODS

Materials

The three GFR markers used – [^{3}H]inulin, [^{14}C]polyethylene glycol 4000 and [^{51}Cr]EDTA – were obtained from the Radiochemical Centre, Amersham. Inulin and PEG were dissolved in crab Ringer's solution before injection whilst EDTA, which was received as a sterile aqueous solution, was injected as such. Crabs were collected at Bourke and Gulargambone, NSW, and maintained in the laboratory as described previously (Greenaway & MacMillen, 1978). Experiments were carried out at 25 °C in a temperature-controlled waterbath.

Methods

Tritium and ^{14}C-labelled markers were counted in a Packard model 3314 liquid scintillation counter using either Instagel (Packard Instruments) or Biofluor (New England Nuclear) as liquid scintillants. Samples of haemolymph and injection fluid were dispensed into 1 ml of water and mixed before addition of 10 ml of liquid scintillant. Where water samples were counted, the sample size was 1 ml and no correction for differential quenching between the two sample types was found to be necessary. Tissue samples and homogenates were dissolved in Soluene 350 (Packard Instruments) at 50–60 °C before the addition of scintillation fluid and sample counts were quench-corrected using an internal standard technique. Samples containing ^{51}Cr-EDTA were counted in a Packard Autogamma counter. Each sample was made up to 2 ml with distilled water. Tissue samples were treated similarly.

Isotopes were injected through a small hole drilled in the carapace over the pericardium, leaving the hypodermis intact to prevent bleeding. The crab was held vertically in a clamp and the needle of an Agla micrometer syringe positioned in the hole, by means of a micromanipulator, and sealed in place with silicon high vacuum grease. The needle was then advanced into the pericardial cavity and material injected slowly at a dose of 1–2 μl/g wet weight of crab. As the needle was withdrawn pressure was applied to the grease which effectively sealed the opening and prevented leakage.

After injection, the crabs were left for 1 h to allow dispersal of the injected material throughout the haemolymph. Clearance from the haemolymph was then followed by removing samples of haemolymph from the base of a leg after various time interv[illegible]

Table 1. *The mean rates of clearance* (±S.E.) *of injected GFR markers from the haemolymph*

Marker	Rate constant 100 h^{-1}	$T_{\frac{1}{2}}$ (h)	Weight range (g)	n
^{14}C-PEG 4000	4·9 ±0·56	16·4 ± 2·04	14·62–20·82	10
^{51}Cr-EDTA	0·89 ± 0·069	102·2 ± 12·4	12·99–30·64	32
^{3}H-inulin	0·32 ± 0·036	281·9 ± 43·0	13·51–27·23	16

Rate constants for the three markers differed significantly ($P < 0·001$).

and assaying 10μl samples for radioactivity. The rate constant for clearance was calculated from the slope of the plot ln % radioactivity remaining in the haemolymph versus time. To measure the rate of appearance of isotopes in the external medium, crabs were placed in 100 ml or 150 ml of artificial tapwater (ATW) (Greenaway, 1980) and 1 ml (^{3}H and ^{14}C) or 2 ml (EDTA) samples were taken at regular intervals for measurement of radioactivity. In some experiments, the opercula covering the urinary apertures were removed surgically and the openings sealed with superglue (Selleys Astrabond) or silastic to determine whether or not the injected markers appeared in the urine.

To determine the location of injected PEG 4000 the injected crabs were killed by placing them briefly in the freezing compartment of a refrigerator. The required tissues were then dissected out and prepared for liquid scintillation counting.

To test the possibility of reabsorption of markers from the bladder the opercula covering the excretory apertures were removed with a scalpel and cannulae were inserted into the holes and sealed in place with superglue. The markers were then injected into the excretory organs (30 μl each side) and the distal ends of the cannulae sealed with plasticine. The crabs were placed in water and samples of haemolymph taken at 1 h, and at various times thereafter, and assayed for radioactivity. In a few cases some of the marker was apparently forced directly into the haemolymph as very high levels of radioactivity were apparent in the one hour sample. In these cases the one hour values were taken as baseline measurements.

RESULTS

Rates of clearance

The mean rates of clearance of labelled inulin, PEG and EDTA from the haemolymph differed significantly, the clearance of PEG being 5·5 times faster than EDTA and 15·3 times faster then inulin (Table 1). Each marker was thus treated differently within the tissues of the crab. This phenomenon is examined below.

Loss of GFR markers to the water

Crabs were injected either with inulin, PEG or EDTA and then placed in a small volume of water. The appearance of the radioactive label was followed over a long period during which time bacterial breakdown of the ^{14}C- and ^{3}H-labelled markers was minimized by changing the external medium and thoroughly cleaning each container, daily. Each marker substance appeared in the water in pulses at relatively long time intervals (Fig. 1). This periodic release of markers suggested elimination via the gut or excretory organs as discharge from both organs is periodic. To test the latter

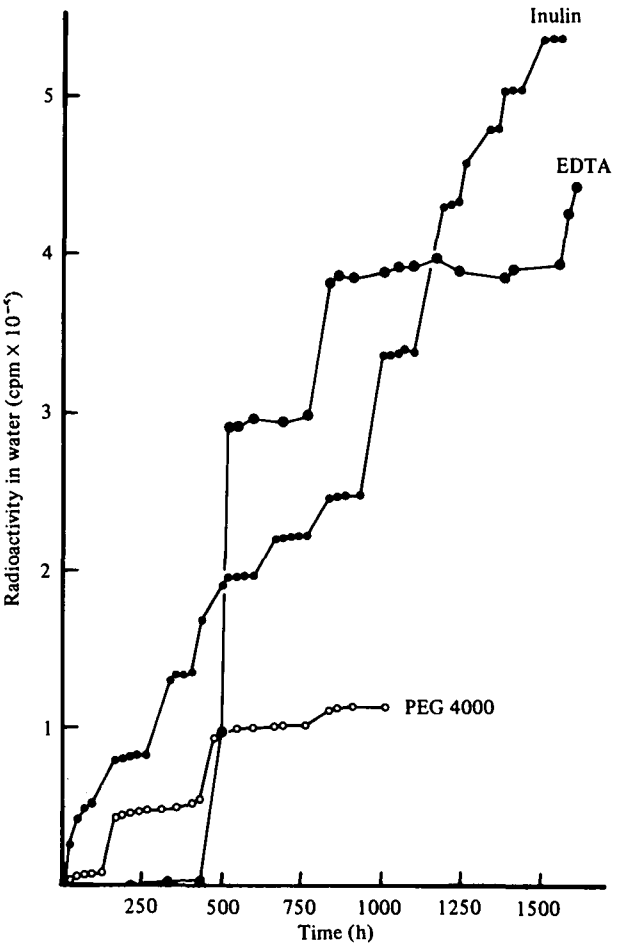

Fig. 1. Pulsatile release of GFR markers into the water following their injection into the haemolymph. A typical set of data for a single animal, in each case, is presented.

hypothesis experiments were carried out in which the urinary apertures were sealed prior to injection with ^{3}H-inulin or EDTA.

Following injection with EDTA the level of radioactivity in the water did not rise above background. Injection of ^{3}H-inulin was followed by the appearance of small amounts of radioactivity in the water for several days (total appearance approximated to 5% of radioactivity injected in 160 h), but thereafter loss ceased. This initial loss of ^{3}H may not represent loss of inulin as such but rather the passive elimination of inulin decomposition products or, alternatively, the ability of the crab to metabolize a small fraction of the injected polymer. In either case, the label could be lost as tritiated water across permeable surfaces of the body and would account for the pattern of loss observed. The experiments were of sufficient duration that several pulses of release of radioactivity might have been expected during their timecourse

Table 2. *Rate constants for the clearance of injected GFR markers from the haemolymph and for their appearance in the water*

Marker	Clearance rate const. 100 h^{-1} (n)	Appearance rate const. 100 h^{-1} (n)	*P*
^{14}C-PEG 4000	4·9 (10)	1·56 (4)	0·01 <P> 0·02
^{51}Cr-EDTA	0·89 (32)	1·69 (5)	<0·001
^{3}H-Inulin	0·32 (16)	0·29 (5)	0·7 not sig.

In the absence of significant release of the injected markers, after blockage of the excretory apertures, they must normally be released only in the urine.

The rates of clearance from the haemolymph, of labelled GFR markers, have been compared with their rates of appearance in the external medium over a similar time period (Table 2). Mean values for elimination by several animals were used to smooth out the normal stepwise appearance of radioactivity in the medium. This enabled the use of regression analysis to determine the initial slopes of the plots ln % radioactivity in water v time. Values for inulin clearance and appearance were not significantly different and it follows that inulin cleared from the haemolymph appeared in the water via the urine. The rate of appearance of EDTA was significantly greater than the rate of clearance from the haemolymph (Table 2). However, it is likely that this is an artifact of the small sample size used in the EDTA appearance experiments as two of the animals used had unusually high rates of clearance from the haemolymph. Additionally, virtually all the EDTA injected into the haemolymph was ultimately collected in the external medium (Fig. 2) and EDTA, like inulin, must pass into the water in the urine.

Polyethylene glycol 4000 behaved rather differently; the rate constant for its appearance was significantly lower than for clearance (Table 2). Additionally, only about 5 % of the injected label was recovered in the water after 1000 h. Only a small amount of injected PEG, then, can enter the water via the urine and the major portion must be retained within the tissues (but not in the haemolymph). To test this conclusion, the crabs used in the long-term clearance experiment for PEG (Fig. 2) were homogenized and samples of the homogenate prepared for liquid scintillation counting. Although radioactivity in the haemolymph was negligible (*ca.* 0·1 % of total counts in the crab), the level in the total tissues was very high and accounted for the bulk of the radioactivity injected. In one crab, levels of radioactivity were measured in several tissues and organs (leg muscle, carapace, digestive gland, gills, gut). The gills contained by far the largest amount of radioactivity (*ca.* 6 % of the counts injected) but the bulk of the PEG was stored in other tissues which have not been identified.

Reabsorption of markers from the excretory organ

It is apparent that both EDTA and inulin are cleared from the haemolymph into the excretory organ and eliminated in the urine with no significant storage in other tissues. However, the mean rate of clearance of inulin is significantly lower than for EDTA (Table 1), possibly due to the different sizes of the two molecules (inulin = 5000 and Cr-EDTA = 362 M.W.) or, alternatively, to reabsorption from or secretion into the excretory organ of one or both of the markers. The possibility of reabsorption from the bladder, of inulin and EDTA, was tested. Inulin or Cr-EDTA was injected into the excretory organs which were then sealed.

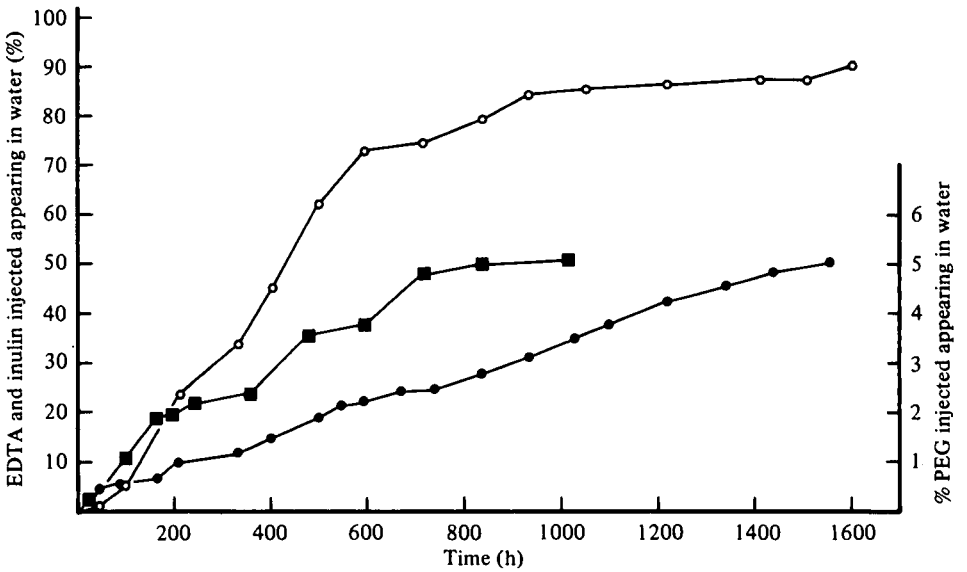

Fig. 2. Appearance of GFR markers in the water over a long time period. Data points represent means for 4 (PEG 4000) or 5 animals (EDTA, inulin). Open circles represent EDTA, closed circles represent inulin, squares represent PEG 4000.

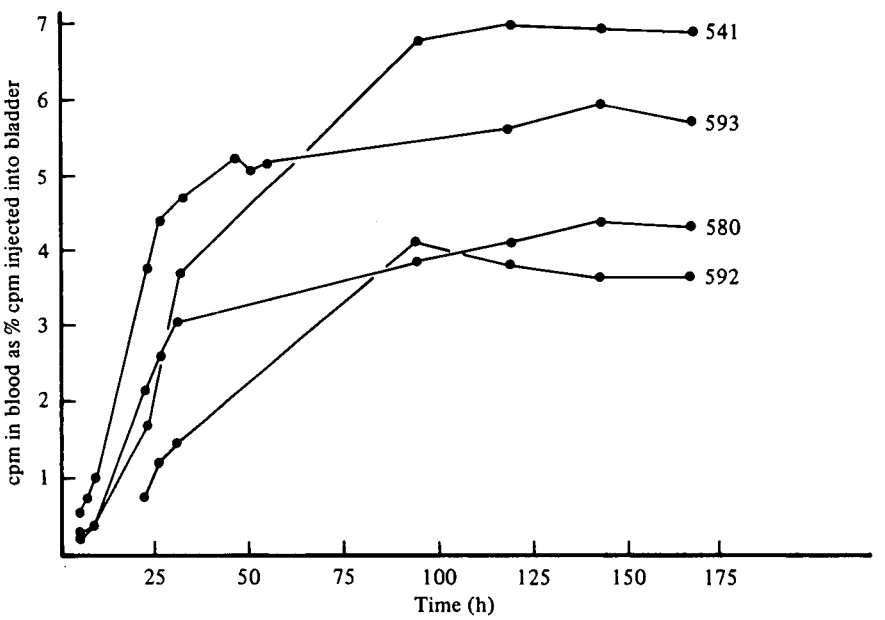

Fig. 3. The appearance of ^{3}H-inulin in the haemolymph following its injection into the bladder. Data for 5 individuals are presented.

Table 3. *Frequency of urine release. Data determined from appearance of ^{3}H-inulin in the water over a period of* 1560 *h*

Crab no.	Weight (g)	Mean Frequency (h)
631	13·34	149·7 ± 24·5 S.E.
635	13·69	158·8 ± 17·8
638	15·39	134·6 ± 13·0
640	14·54	320·3 ± 16·2
650	14·28	130·7 ± 16·3
$\bar{x}$	14·25	178·8

Inulin was detectable in the haemolymph within a few hours of injection into the excretory organ. The level rose rapidly at first but reached a plateau after about 40–50 h (Fig. 3). Clearly inulin is reabsorbed from the excretory organ of *Holthuisana*. The plateau level must represent an equilibrium between clearance from the haemolymph and reabsorption from the urine and occurs at a level equivalent to reabsorption of 4–7% of the inulin injected. No significant amounts of radioactivity appeared in the haemolymph following injection of EDTA into the excretory organs indicating that EDTA is not reabsorbed from the urine.

The duration of these experiments was *ca.* 160 h, rather less than the normal interval of urine release (Table 3). Consequently, it is unlikely that blockage of the excretory organs for this period caused any interference to the normal pattern of renal function.

Frequency of urination

Markers injected into the haemolymph appeared in the external medium via the urine. The frequency of urine release can, therefore, be gauged by the frequency of release of radioactivity during the long-term experiments described above. Inulin is most appropriate for this purpose as it is cleared slowly and can be monitored for a long time (Table 3). It is not possible to determine, in most cases, whether the excretory organs discharge synchronously or asynchronously. Synchronous discharge would result in a longer time lapse between discharges and may account for the pattern shown by crab 640 in which urine release occurred at a frequency half that of the other crabs studied.

DISCUSSION

The three GFR markers used in this study each behaved differently after injection into the haemolymph. A small proportion (5%) of ^{14}C-PEG injected was passed to the external medium in the urine, but the bulk was rapidly cleared from the haemolymph and sequestered in the tissues. Consequently PEG overestimates the true rate of filtration. Inulin passes out in the urine, but a certain amount is also reabsorbed from the excretory organ and passed back into the haemolymph. Inulin, therefore, underestimates the true rate of filtration of haemolymph. Chromium labelled EDTA is cleared from the haemolymph and passes out in the urine. There is no indication that EDTA is sequestered by the tissues or reabsorbed from the excretory organs and, consequently, it is likely to provide the most reliable estimate of filtration. The rate of clearance of EDTA is 0·885% haemolymph volume h^{-1} (Table 1) equivalent to 3·08 $\mu l\ g^{-1}\ h^{-1}$. Urine flow rate has been estimated at 0·196 $\mu l\ g^{-1}\ h^{-1}$ (Greenaway, 1980)

implying a mean U/H ratio of 15·7. U/H ratios in freshwater decapods are normally less than three although values as high as 31 have been obtained for freshwater crayfish maintained in 50% seawater (Bryan & Ward, 1962). Stress imposed by handling may also cause abnormally high ratios in crayfish (Riegel & Kirschner, 1960). The marine/terrestrial crabs *Gecarcinus lateralis* and *Cardisoma guanhumi* kept on sand moistened with seawater had U/H ratios close to unity (Harris, 1977). It remains possible that EDTA may enter the excretory organs in *Holthuisana* by secretion as well as filtration, thereby accounting for the elevated U/H ratio. Alternatively, the apparent high degree of reabsorption of primary urine may be real and represent an adaption to terrestrial life while water elimination during immersion may be predominantly extrarenal, as suggested previously (Greenaway, 1980). The anatomy of the excretory organs precludes collection of reliable samples of final urine (Greenaway, 1980) and it is thus impossible at present to distinguish between these two alternatives.

Studies of comparative clearance of markers by decapods (Kirschner & Wagner, 1965; Riegel *et al.* 1974) suggest that the filtration sites freely pass relatively large molecules (up to 20000 MW) including commonly used marker substances. Additionally, measurements of inulin concentrations along the length of the excretory organ of the crayfish do not suggest that reabsorption or secretion occur (Riegel, 1971). On the basis of existing evidence, the treatment of injected marker substances by *Holthuisana* is unique amongst decapods. However, in view of the very small number of comparative studies available such a conclusion is premature and many more studies are required to determine the reliability of the common glomerular markers in studies of excretion in crustaceans.

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REFERENCES

BEYENBACH, K. W. & KIRSCHNER, L. B. (1976). The unreliability of mammalian glomerular markers in teleostean renal studies. *J. exp. Biol.* **64**, 369–78.

BINNS, R. (1969). The physiology of the antennal gland of *Carcinus maenas* (L.). II. Urine production rates. *J. exp. Biol.* **51**, 11–16.

BRAYSHER, M. & GREEN, B. (1972). The absorption of inulin by cloacas and bladders in reptiles and the chicken. *Comp. Biochem. Physiol.* **43A**, 199–241.

BRYAN, G. W. & WARD, E. (1962). Potassion metabolism and accumulation of ^{137}Caesium by decapod Crustacea. *J. mar. Biol. Ass. U.K.* **42**, 199–241.

GREENAWAY, P. (1980). Water balance and urine production in the Australian arid-zone crab *Holthuisana transversa J. exp. Biol.* **87**, 237–246.

GREENAWAY, P. & MACMILLEN, R. E. (1978). Salt and water balance in the terrestrial phase of the inland crab, *Holthuisana* (*Austrothelphusa*) *transversa* Martens (Parathelphusiodea: Sundathelphusidae). *Physiol. Zool.* **51**, 217–29.

HARRIS, R. R. (1977). Urine production rate and water balance in the terrestrial crabs *Gecarcinus lateralis* and *Cardisoma guanhumi*. *J. exp. Biol.* **68**, 57–67.

HICKMAN, C. P., NEWCOMB, E. W. & KINTER, B. (1972). Anomalous behaviour of ^{3}H- and ^{14}C-labelled inulin and ^{3}H-labelled polyethylene glycol in incubated kidney tissue of the winter flounder, *Pseudopleuronectes americanus*. *Bull. Mt. Desert Isl. biol. Lab.* **23**, 47–50.

KIRSCHNER, L. B. & WAGNER, S. (1965). The site and permeability of the filtration locus in the crayfish antennal gland. *J. exp. Biol.* **43**, 385–95.

RAMAMURTHI, R. (1977). urine production in the shore crab *Hemigrapsus nudus* (Dana). *Ind. J. Exp. Biol.* **15**, 480–82.

RIEGEL, J. A. (1971). Excretion, Arthropoda. In *Chemical Zoology VI* (ed. M. Florkin and B. T. Scheer). London: Academic Press.

RIEGEL, J. A. (1977). Fluid movement through the crayfish antennal gland. In *Transport of water and Ions in Animals* (ed. B. L. Gupta, R. B. Moreton, J. L. Oschman and B. J. Wall). London: Academic Press.

RIEGEL, J. A. & KIRSCHNER, L. B. (1960). The excretion of inulin and glucose by the crayfish antennal gland. *Biol. Bull. mar. Biol. Lab. Woods Hole*, **118**, 296–307.

RIEGEL, J. A., LOCKWOOD, A. P. M., NORFOLK, J. W. R., BULLEID, N. C. & TAYLOR, P. A. (1974). Urinary bladder volume and the reabsorption of water from the urine of crabs. *J. exp. Biol.* **60**, 167–81.

SCHMIDT-NIELSEN, B., RENFRO, J. L. & BENOS, D. (1972). Estimation of extracellular space and intracellular ion concentrations in osmoconformers, hypo-and hyperosmoregulators. *Bull. Mt Desert Isl. biol. Lab.* **23**, 99-104.