J. Exp. Biol. (1970), 53, 425–443
With 7 text-figures
Printed in Great Britain

THE REGULATION OF CALCIUM AND MAGNESIUM IN THE BRACKISH WATER POLYCHAETE *NEREIS DIVERSICOLOR* O.F.M.

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(*Received* 29 *April* 1970)

INTRODUCTION

The mechanisms by which both vertebrates and invertebrates control the concentration of sodium and chloride in their body fluids has been and still is the subject of intensive investigation. Less attention, however, has been paid to the regulation of divalent ions in animals, and almost all of the literature on the subject is concerned with terrestrial vertebrates (Wasserman, 1963; Bronner, 1964; Wacker & Vallee, 1964). Both calcium and magnesium ions are necessary co-factors in a number of physiological processes, including muscle contraction, active transport of ions, and mitochondrial metabolism.

Nereis diversicolor has been shown (Beadle, 1937; Ellis, 1937) to require the presence of calcium in the external medium to regulate its body volume, and these authors concluded that in the absence of external calcium the water permeability of the cuticle is increased. However, the experimental results could be equally well explained by the assumption that *N. diversicolor* is unable to regulate its body calcium in the absence of external calcium, and that the observed swelling results from the failure to remove water entering osmotically, being a consequence of the failure of metabolism generally.

This paper presents the results of experiments performed to determine how *N. diversicolor* controls the concentrations of calcium and magnesium in its body fluids, and will show that these animals have very restricted abilities to regulate these ions independently of changes in the environment. A future paper will describe investigations on the effects of calcium on water permeability and volume regulation.

METHODS AND MATERIALS

Specimens of *Nereis diversicolor* weighing between 0·3 and 0·8 g were collected on a number of occasions between October 1967 and October 1969, from the lower reaches of Christchurch Harbour, Hants., England. The chlorosity of the interstitial water at the collection site was measured on several occasions, and values fell between 38 and 59 mM/l. The animals were maintained in the laboratory in 10% sea water at 12 ± 1 °C and acclimated slowly to other salinities as already described (Fletcher, 1970). Each animal spent 6 days in the final acclimation salinity, the medium being changed twice. All experiments were conducted at $12 \pm 0{\cdot}5$ °C.

Tracer ^{45}Ca as the chloride at 2–5 Ci/g of calcium was obtained from the Radiochemical Centre, Amersham, Bucks., and was measured by β-scintillation counting,

the samples being dried on planchets. The counter was an I.D.L. type 663C and light-proof drawer unit type 723, equipped with a 1 mm thick plastic phosphor (Nuclear Enterprises Ltd, NE 102A), aluminized on the lower surface. Standards were always made up identically to the unknowns to avoid any errors due to self-absorption of the β particles, and contained a known proportion of the radioisotope used in the experiment.

To measure the concentrations of ions and ^{45}Ca in the whole body the animals were blotted dry, weighed to 1 mg, then dissolved in Analar concentrated nitric acid (2 ml/g of animal) with careful warming. The solutions were weighed and diluted as appropriate to measure ions. The densities of the nitric acid solutions were established in control experiments to enable the dilutions to be made volumetrically. To measure the quantity of ^{45}Ca in whole animals 0·25 ml of animal solution was dispensed on to a planchet with an autozero pipette ($\pm 1\%$) and dried on a hotplate. Standards were prepared containing a known proportion of the ^{45}Ca used in the experiment and 0·25 ml of nitric acid solution of non-radioactive animals from similar salinities.

Samples of coelomic fluid (about 50 mg) were taken from animals using glass cannulas made by drawing clean melting-point capillaries to a fine point. For the measurement of ions the cannulas were weighed before use and again containing the sample, thus establishing the weight of the sample to $\pm 0{\cdot}2$ mg. The cannulas were washed out with 1% nitric acid and the samples were diluted as appropriate. For determination of ^{45}Ca the cannulas were blown out on to weighed planchets which were immediately re-weighed to establish the weight of coelomic fluid ejected, to $\pm 0{\cdot}2$ mg. The samples were then spread by adding a few drops of acetone, and dried.

Chloride was measured with an Aminco Cotlove chloride titrator (American Instrument Co., Silver Springs, Md.), the titration vial containing between 1 and 2 μM of chloride. Sodium was measured using an EEL flame photometer, or a Unicam SP 900 emission spectrophotometer, the latter instrument having been modified to record its results with a Beckman pen recorder. Potassium, calcium and magnesium were all measured with the SP 900 and recorder. Standards were used which contained as nearly as possible the same quantities of each cation as the samples, in 1% nitric acid; blanks for each ion were similar but omitted the ion concerned. The equipment was found to be substantially linear for all ions, and interferences were small except that sodium raised the background for magnesium significantly and enhanced the emission of that ion by about 5%, under the conditions employed. Measurements of calcium by emission and absorption techniques agreed to $\pm 2\%$, and since the former was more sensitive it was used routinely. Standards for cation measurements were prepared by diluting molar solutions of the chlorides. Molar calcium chloride was prepared by dissolving weighed calcium carbonate in a slight excess of 2 N hydrochloric acid, and diluting to the final volume. All reagents were Analar grade.

When cell-free coelomic fluid was required the coelomic fluid samples from 12 or more animals were pooled in a Pyrex tube of 3 mm bore and centrifuged until the supernatant was clear (10 min at 300 rev/min).

Determination of the density, total solids, organic solutes, inorganic solutes, and water content of coelomic fluid samples after centrifugation were made in triplicate. Samples of cell-free coelomic fluids were dispensed into weighed constricted lyophylizing tubes which were weighed again. The 0·25 ml autozero micropipettes used

were standardized to 0·1 % with distilled water. The lyophylizing tubes were chilled in liquid nitrogen, and their contents were freeze-dried until the tubes reached room temperature containing a gas pressure of less than 10^{-3} torr, when they were allowed to fill with dry nitrogen and sealed. The sealed section was washed externally, dried, weighed and then opened. It was heated in a muffle furnace to constant weight, thus ashing all organic compounds at 350 °C, cooled and weighed. It was finally washed out, dried and re-weighed, thus completing the necessary information.

Ultrafiltrates of cell-free coelomic fluids were prepared using Visking $\frac{1}{4}$ in cellulose dialysis tubing. The tubing, in 4 in lengths, was soaked in dilute nitric acid and then in several changes of distilled water for 12 h. One end of each piece was closed by two knots and mounted in a Pyrex apparatus which allowed a large surface to volume ratio. The centrifuged coelomic fluid was placed inside the membrane and ultrafiltered through it by atmospheric pressure into a reduced pressure created by a water filter pump.

Electrical potentials across the body walls of the animals were measured by inserting a fine spear-shaped glass cannula into the coelomic cavity. The cannulas were about 0·7 mm maximum outside diameter, tapering to 0·2 mm at the tip and 0·5 mm at the distal end where they were attached to a fine Polythene tube 9 in long leading to a chlorided silver electrode. The elasticity of the animals' body walls held the cannulas in place and provided good seals; in no case was leakage of coelomic fluid observed unless the cannula had been displaced. However, to ensure good insulation the animals' body surface was dried and locally smeared with silicone grease before insertion of the cannula. The animals were allowed to swim freely in a small glass dish containing flowing diluted sea water. The whole cannula was filled with 3 M-KCl, and the animal's bathing medium was grounded by a low-impedance Ag/AgCl/3 M-KCl electrode. The potentials were measured with a high-impedance cathode follower connected to a Beckman 100 mV pen recorder. The recorder was set to zero with the cannula in the bathing medium before insertion into the animal. It was checked for blockage by checking its resistance, and the potential then recorded. If the potential failed to return to within 1 mV of zero immediately after withdrawal of the cannula the results were discarded. In order to reduce the pick-up of stray mains potentials the input of the cathode follower was shunted with a capacitance of 0·22 μF (leakage resistance > $10^{9}\Omega$), giving a time constant of about 0·2 s with a typical cannula impedance of about 1 MΩ. The system was calibrated by a reference potential of 42·1 mV. The cannula was placed in a small dish containing a fluid of similar composition to coelomic fluid, the reference electrode was placed in an appropriate bathing medium, and the reference potential was connected between the two using agar bridges made with the respective media and Ag/AgCl/3 M-KCl electrodes.

All glassware was rinsed in 1 % nitric acid and then in distilled water after washing and then dried. All solutions were made up with distilled water. 150% sea water was prepared by dissolving suitable quantities of weighed salts in sea water. Sea water containing additional calcium was similarly prepared, and sea water containing reduced calcium was made by adding sea water to a similar volume of artificial sea water from which the calcium chloride had been omitted. The quantities of salts used were those of Hale (1965).

Whole-body calcium content

The whole-body calcium concentrations of individual worms acclimated to a range of salinities between 150% sea water and 0·5% sea water were measured by the techniques already described, and the results are shown in Fig. 1. Each point represents the mean of 12 animals, and vertical bars are used to represent the standard deviations. The calcium content of the media were also measured. It will be noticed that the calcium content of the 150% sea water was below the theoretical level, and those of the 10% and 5% sea waters were above the theoretical level. It is observed that the whole-body calcium content declines in parallel to the calcium concentration in the medium as far as a chlorosity of 270 mM/l, and thereafter levels off at about 1·3 mM of calcium/kg of animal at lower salinities, showing a substantial degree of regulation.

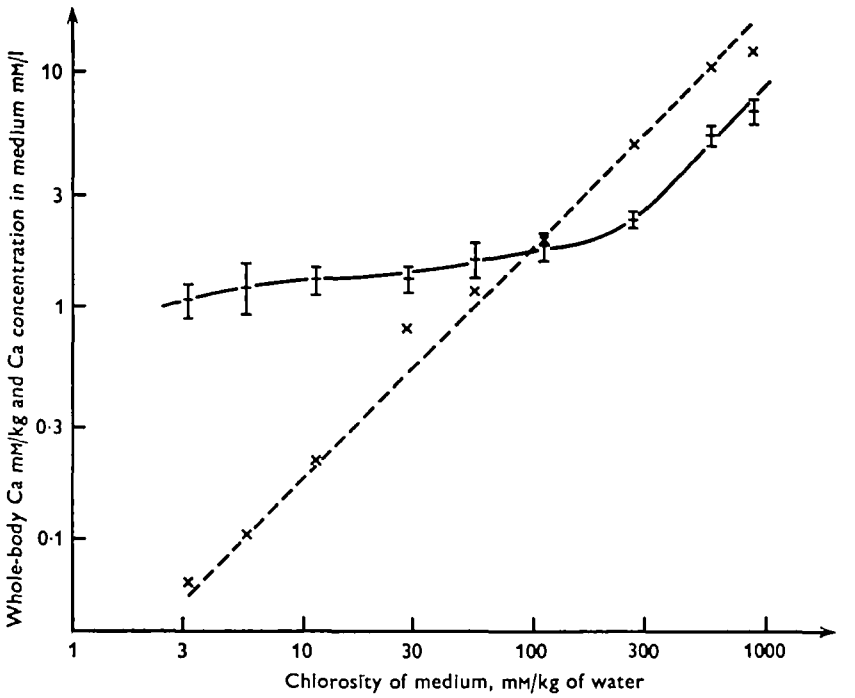

Fig. 1. Whole-body calcium content and calcium concentration in the medium as a function of the chlorosity of the medium. ×, Calcium concentration in the medium; — — —, calcium concentration in diluted standard Copenhagen sea water; ‡, calcium concentration in the animals, mean ± standard deviation.

Concentration of calcium in the coelomic fluid

The calcium concentrations in the coelomic fluid of worms acclimated to a similar range of salinities were measured on a number of occasions by the techniques already described. The results of similar measurements showed considerable disagreements which paralleled variations in the calcium concentrations of the acclimation media. This was further investigated by measuring the calcium concentrations in the coelomic fluids of animals acclimated to media containing artificially elevated or reduced calcium levels, and it was found that the calcium concentration in the coelomic fluid

was directly proportional to that in the medium at a given salinity, within experimental errors. Thus the results have been expressed as the ratio of the calcium concentrations in the coelomic fluid (mM/l) to the calcium concentrations in the medium (mM/l), and

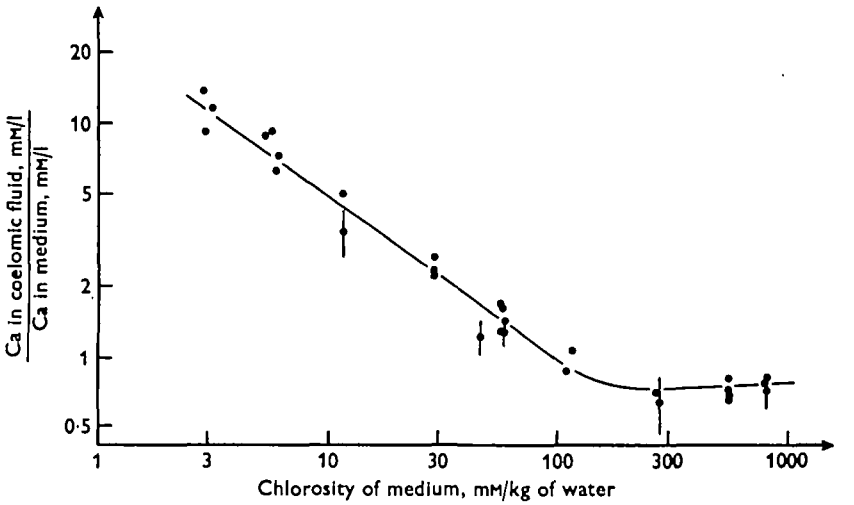

Fig. 2. Concentration of calcium in the coelomic fluid relative to calcium concentration in the medium as a function of the chlorosity of the medium. Each point is the mean of ten or more animals; some typical standard deviations are shown by vertical bars.

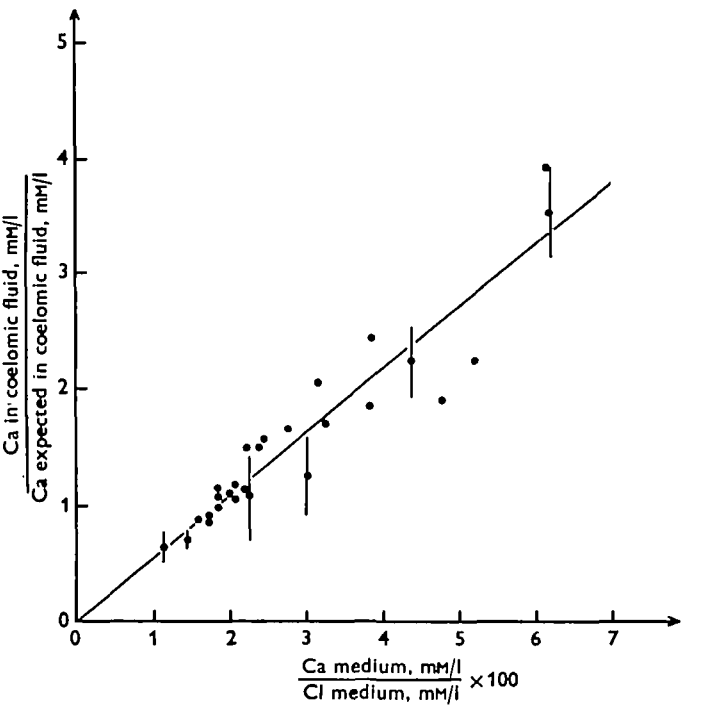

Fig. 3. The effect of varying the concentration of calcium relative to chloride in the medium on the calcium concentration in the coelomic fluid. Each point is the mean of ten or more animals; some typical standard deviations are shown by vertical bars.

are plotted in Fig. 2 as a function of chlorosity. Some representative standard deviations are shown as vertical bars. Each point is the mean of 12 or more animals, but since some of the observations were made on pooled samples not all the standard deviations are known. Some of the points have been slightly displaced horizontally to allow all the results to be included.

To show the dependence of the calcium levels in coelomic fluid on the external calcium levels the results have also been re-plotted in a different way (Fig. 3). The ordinate is the ratio of calcium observed in the coelomic fluid to that which would be

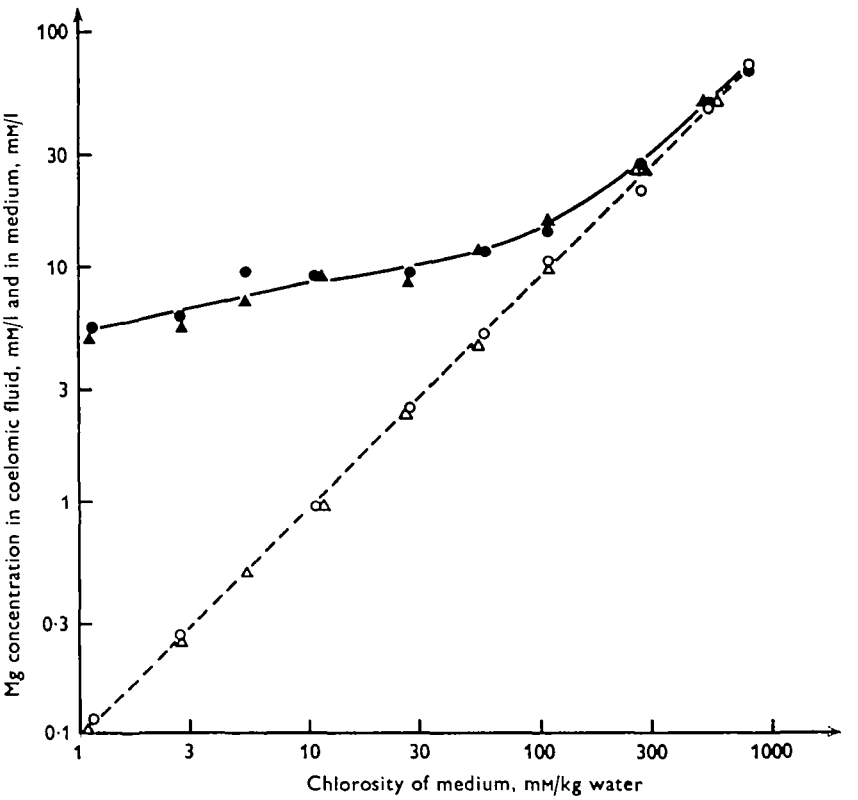

Fig. 4. Concentration of magnesium in the coelomic fluid (closed symbols) and in the medium (open symbols) as a function of the chlorosity of the medium, showing the results of two different experiments. Each point is one determination on pooled samples from ten or more worms. The broken line indicates the magnesium concentration of diluted standard Copenhagen sea water.

expected according to Fig. 2 if the medium had contained calcium at $1{\cdot}92 \times 10^{-2}$ times the chlorosity of the medium. This is the ratio in standard Copenhagen sea water. Again a few representative standard deviations are shown by vertical lines. The results clearly show that the concentration of calcium in the coelomic fluid is the concentration in the medium multiplied by a 'concentration factor' which is a function of the chlorosity of the medium only, and is shown in Fig. 2. The animals have little if any ability to correct for variation in the ratio of calcium to chloride in their environment.

Concentration of magnesium in the coelomic fluid

The concentrations of magnesium in the coelomic fluid have also been determined and are shown in Fig. 4. Since the SP 900 emission spectrophotometer is less sensitive when measuring magnesium than it is for calcium, determinations could only be made on pooled samples of coelomic fluid, and no standard deviations are available. However, the experiment was repeated and both sets of results are shown, using different symbols. No experiments were conducted to determine whether the animals are able to regulate magnesium independently of changes in the ratio of magnesium to chloride in the medium, but since magnesium is concentrated by a similar degree to calcium the mechanism is probably similar, and such regulation is unlikely.

Concentrations of ions in cell-free and in ultrafiltered coelomic fluid

Since only free ions in the coelomic fluid will be able to exchange directly across the body wall, the concentrations of ions in pooled centrifuged samples of coelomic fluid from 30 or more animals were determined, and were expressed in the relevant form, mM/kg of water, using data on the water content of centrifuged coelomic fluid determined as already described. The composition of the acclimation media were also determined and expressed in the same way. In order to determine what proportion of each ion was protein-bound in the body fluids of animals from each salinity, and the extent of any Donnan effects caused by charges on the proteins, aliquots of the same pooled coelomic fluid samples were ultrafiltered as already described, and the ionic composition of the ultrafiltrates was determined. As the concentrations of all

Table 1. *Concentrations of ions in diluted sea water, mM/kg of water*

Sea water %	Cl	Na	K	Ca	Mg	Anion deficit m-equiv/kg of water	Ionic strength equiv/kg of water
150	817·0	731·6	14·79	12·22	74·2	102·4	1·031
100	545·0	475·2	9·70	9·82	50·6	60·6	0·681
50	277·2	241·5	5·03	5·06	25·6	30·5	0·346
20	109·7	99·09	2·12	2·52	9·90	16·3	0·143
10	58·22	50·65	1·08	1·25	5·22	6·44	0·0727
5	27·56	24·50	0·509	0·514	2·52	3·52	0·0350
2	10·80	9·589	0·206	0·215	0·957	1·34	0·0136
1	5·271	4·669	0·107	0·121	0·483	0·713	0·00676
0·5	2·776	2·433	0·0576	0·0694	0·250	0·353	0·00354
0·2	1·148	1·005	0·0342	0·0371	0·103	0·171	0·00150

major ions were required to calculate the ionic strength of the various fluids, determinations were made of potassium, sodium, chloride, magnesium and calcium. Sulphate, carbonate, bicarbonate and phosphate are not easily determinable in small aliquots containing low concentrations, and their total concentrations in m-equiv/kg of water have been determined by the difference between the total measured cations and the total measured anions. All dilutions were to an accuracy of $\pm\frac{1}{2}\%$, and determinations of sodium and chloride were to $\pm\frac{1}{2}\%$. Determinations of calcium and potassium were reproducible to 1%, and magnesium to 2%.

The results for the acclimation media are given in Table 1, and for centrifuged coelomic fluid in Table 2. The packed-cell volume of the pooled coelomic fluids

ranged between 3·1 and 35% in an irregular manner, but higher values were usually obtained from animals from lower salinities. Despite this the concentrations of ions in the centrifuged coelomic fluids was remarkably similar to the concentrations in whole coelomic fluids, except that the potassium was a little higher in the latter and sodium and chloride a little higher in the former, showing that the cells contain more potassium and less sodium and chloride than the fluid. The results of measurements of the ionic composition of ultrafiltered coelomic fluid are given in Table 3. No results are available for animals from 50% or from 0·2% sea water. Sufficient coelomic fluid for two attempts at ultrafiltration was obtained in each case, but in these two instances the ultrafiltration membrane burst at each attempt. During ultrafiltration a certain amount of evaporation and condensation occurred at the reduced pressure, and this

Table 2. *Concentrations of ions in centrifuged coelomic fluid, mM/kg of water*

Sea water acclimated (%)	Cl	Na	K	Ca	Mg	Anion deficit, m-equiv/kg of water
150	771·2	762·1	31·99	8·95	77·7	196·2
100	504·2	487·1	24·30	7·84	53·2	129·2
50	240·2	263·6	16·94	4·59	27·9	105·2
20	191·9	206·0	12·63	2·70	17·0	66·14
10	144·4	164·0	10·93	1·70	11·7	57·33
5	135·7	154·9	10·35	1·95	9·91	53·31
2	118·0	140·8	9·408	1·90	9·18	53·37
1	96·31	118·1	10·25	2·36	7·58	51·93
0·5	77·82	97·78	8·408	2·48	5·99	45·30
0·2	56·90	80·05	8·970	2·37	6·15	49·15

Table 3. *Concentrations of ions in ultrafiltrates of coelomic fluids, mM/kg of water*

Sea water acclimated (%)	Cl	Na	K	Ca	Mg	Anion deficit, m-equiv./kg. of water
150	664·1	654·0	26·59	5·97	59·8	148·1
100	407·3	398·0	19·41	5·29	38·5	97·76
50	—	—	—	—	—	—
20	144·3	157·8	9·24	1·56	11·82	49·50
10	127·5	142·9	8·88	1·01	8·62	43·52
5	115·2	133·3	8·31	1·46	7·05	43·51
2	99·29	119·6	7·86	1·28	6·52	43·73
1	84·46	101·6	8·77	1·65	5·03	39·27
0·5	72·13	86·80	8·24	1·90	4·84	36·38
0·2	—	—	—	—	—	—

combined with the water in the membrane at the start resulted in dilution of the ultrafiltrate by up to 30%. This was corrected for by assuming that the product of the concentrations of sodium and chloride in the ultrafiltrate should equal the product of the concentrations of sodium and chloride in the centrifuged coelomic fluid (mM/kg of water), and the degree of dilution had been calculated on this basis. The use of the product avoids any complication by Donnan effects, and the only likely source of systematic errors lies in the possibility that some of the sodium or chloride may be bound. The ratio of each ion in the ultrafiltrate to its concentration in the cell-free coelomic fluid after applying this correction has been calculated and is given in Table 4.

The ratios for sodium and for chloride both vary randomly about 1 (means 1·001, 0·999) and at no time are significantly different from 1. Thus any Donnan effects due to charged molecules to which the membrane is impermeable are negligible, and no significant Donnan effects may be expected on other ions. Furthermore, the closeness of the sodium and chloride ratios to 1 implies that if either ion is bound significantly both must be bound in similar proportions. The potassium ratio is also close to 1, (mean = 0·979); the last value of 1·08 is outside the range of expected experimental

Table 4. *Ratio of concentrations of ions in the ultrafiltrates to concentrations in the centrifuged coelomic fluids, corrected for dilution*

Sea water acclimated (%)	Cl	Na	K	Ca	Mg	Anion deficit
150	1·002	0·998	0·967	0·776	0·896	0·878
100	0·994	1·006	0·983	0·831	0·893	0·931
50	—	—	—	—	—	—
20	0·991	1·009	0·964	0·763	0·916	0·986
10	1·007	0·993	0·926	0·671	0·893	0·865
5	0·993	1·007	0·939	0·878	0·833	0·955
2	0·995	1·005	0·988	0·794	0·839	0·969
1	1·010	0·990	0·985	0·804	0·764	0·871
0·5	1·022	0·978	1·080	0·842	0·890	0·885
0·2	—	—	—	—	—	—

errors and may represent slight contamination. The reason for the potassium ratio being otherwise less than 1 is possibly slight contamination with intact cells. The calcium ratio varies apparently randomly between 0·671 and 0·842 (mean = 0·795); some of the variation is probably real since the range is substantially greater than would be expected from experimental errors. Despite this it is clear that about 20% of the calcium is bound. The magnesium ratio varies between 0·764 and 0·916 (mean = 0·858), again seemingly random and not correlated with the calcium ratio. Thus about 14% of the magnesium in the coelomic fluid is bound. It is interesting to notice that between 3·1 and 12·2% of the unidentified anions are retained by the membrane, showing that the anion deficit comprises mainly small ions such as CO_3^{2-}, HCO_3^-, SO_4^{2-}, HPO_4^{2-}, $H_2PO_4^-$, and small organic acids.

Body-wall potential

The electrical potential across the body wall was measured as already described. Tracings of some typical records are shown in Fig. 5. It was observed that the potential did not alter significantly over a period of 15 min after the first half-minute except as the animal changed its activity, providing the cannula remained undisturbed. Thus potentials were usually recorded over a period between 1½ and 2½ min, and the average value after the first half-minute was taken as representative of the potential between the body fluid and the surrounding medium. The potentials showed marked variations between different animals acclimated to the same salinity, and altered according to the animals' activities, i.e. swimming, crawling or quiescent. The results are plotted in Fig. 6. It is to be observed that whilst there is only a small potential difference (inside negative) in high salinities, the potentials observed in salinities below 20% sea water are larger and rise to about 50 mV (inside negative) in 0·2% sea water. The cannula

was washed out by expressing about 2 μl of the 3 M-KCl between each reading to prevent accumulation of coelomic fluid in the cannula, and this caused the slight change in the zero observed in Fig. 5.

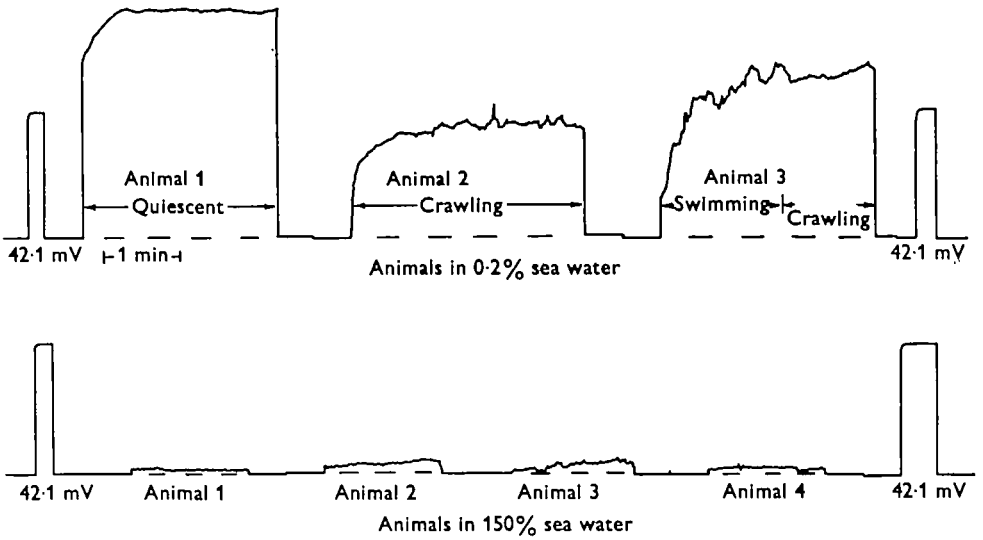

Fig. 5. Tracings of some typical chart recordings of electrical potential differences across the body wall.

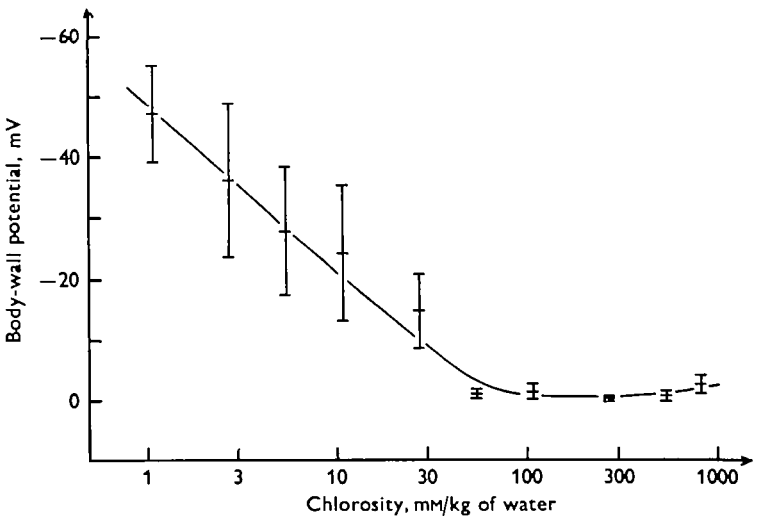

Fig. 6. Body-wall potentials (coelomic fluid relative to the medium) averaged over 1 min or more, plotted as a function of the chlorosity of the medium. Each point is the mean of 12 or more observations, ± the standard deviation.

Calcium fluxes

The rates of calcium influx into whole animals was measured by placing them into salinity similar to that which they were acclimated but containing ^{45}Ca at a concentration which ranged from 0·1 μCi/μM of Ca in 150% sea water to 4 μCi/μM of Ca in 0·5% sea water. The animals were removed after 2 h, or 1 h in salinities less than 2%

sea water, weighed, dissolved in nitric acid and prepared for counting as already described. Samples of the loading solutions were also taken at the beginning and end of each experiment and counted, together with suitable standards. The chlorosity and calcium content of the loading solutions were determined. The ^{45}Ca in the loading solutions declined by not more than 10%, and the means of the values at the beginning and end of the experiments were used in calculation of the results. All errors from counting were less than 2% S.D. or less than 1% S.D. for standards. The results, without correction for efflux of ^{45}Ca from the animals, are given in Table 5; each figure is the mean of 12 animals and standard deviations are given.

Table 5. *Rates of calcium influx*

Loading medium			Calcium influxes, mM/kg/h		
			Not corrected for efflux		Corrected for efflux
Salinity % S.W.	Chlorosity mM/kg aq.	Calcium mM/l	To whole body	To coelomic fluid	To whole body
150	744	13·07	0·371 ± 0·102	0·512 ± 0·127	0·392 ± 0·108
100	527	9·05	0·402 ± 0·045	0·675 ± 0·154	0·440 ± 0·049
50	276	3·14	0·157 ± 0·022	0·210 ± 0·049	0·168 ± 0·024
20	110	2·14	0·143 ± 0·022	0·140 ± 0·036	0·157 ± 0·024
10	52·3	0·977	0·107 ± 0·029	0·111 ± 0·035	0·119 ± 0·032
5	27·1	0·506	0·076 ± 0·019	0·071 ± 0·024	0·083 ± 0·020
2	12·1	0·219	0·043 ± 0·010	0·038 ± 0·022	0·046 ± 0·011
1	5·27	0·100	0·034 ± 0·010	0·035 ± 0·013	0·035 ± 0·011
0·5	2·61	0·048	0·025 ± 0·004	0·027 ± 0·008	0·025 ± 0·004

In the same series of experiments the activity of the coelomic fluid was measured using samples from additional animals, and the influx of calcium into the coelomic fluid was determined. These results are also given in Table 5; each figure is again the mean and standard deviation of 12 observations. It had been hoped to measure the total calcium on each planchet and thus determine rate constants for calcium exchange for each animal directly, but it was found that when the planchets were soaked in dilute nitric acid the calcium found in solution was rather low and variable. This was shown to be a genuine artifact in a control experiment in which known quantities of calcium were dried on to planchets, but it is not certain whether the artifact was incomplete dissolution of the calcium or dissolution of some substance from the planchet which suppressed the emission of calcium in the flame.

In order to correct the influx data for calcium efflux it was necessary to know how much of the calcium in the animals is free to exchange. This can be investigated by leaving animals in a loading solution containing ^{45}Ca until isotopic equilibrium is reached, and then following the rate of efflux of ^{45}Ca. This would lead to misleading results if the specific activity of calcium was not uniform throughout the animal, which would have taken an uncertainly long loading time. Thus the author chose to investigate calcium dynamics by following the rise in specific activity of whole-body calcium during loading.

Animals were acclimated to 100, 10 and 1% sea water, and were then placed in loading solutions of similar salinities, containing ^{45}Ca at similar concentrations to those used in the influx measurements. Ten animals were removed from each solution after suitable times, weighed and dissolved in nitric acid as already described. 0·25 ml of

each solution was taken for ^{45}Ca measurement, and 0·5 ml was diluted for measurement of total calcium with the emission spectrophotometer. Simultaneously samples of the loading solution were taken for similar measurements. The ratio of the specific activity of whole-body calcium relative to that of the medium was plotted. A typical influx curve is shown in Fig. 7. Within experimental errors all three sets of results can be represented by two pools of calcium, one exchanging slowly and the other exchanging more rapidly. The results are summarized in Table 6. The curves were fitted by inspection; the data was not considered to be sufficiently precise to justify more sophisticated treatment.

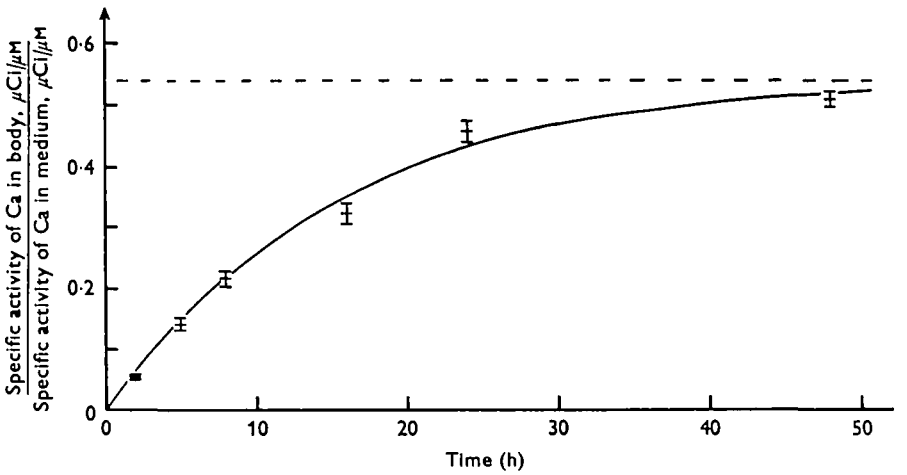

Fig. 7. Specific activity of body calcium relative to the medium as a function of time in 10 % sea water containing ^{45}Ca. Each point is the mean of 12 observations and the standard errors are shown. The curve drawn is for 46 % non-exchangeable, 54 % exchanging with a half time of 10·5 h.

The data in Table 6 has been interpolated and extrapolated for other salinities and used to calculate the true influx rates of calcium. The uncorrected influx figures in Table 5 were calculated using the linear approximation:

$$f' = \frac{AC_0}{A_0 t},$$

where f' = apparent influx rate, mM/kg/h,

A = radioactivity of animal, μCi/kg,

A_0 = radioactivity of medium, μCi/l,

C_0 = concentration of calcium in medium, mM/l,

t = time in loading medium in hours.

The accurate expression is:

$$f = \frac{-C}{t} \ln \left(1 - \frac{AC_0}{A_0 C}\right),$$

where f = true influx rate mM/kg/h, C = concentration of rapidly exchanging calcium in the animal, mM/kg.

It can readily be shown that

$$f = f' + \tfrac{1}{2}(f'^2[t/C]) + \tfrac{1}{3}(f'^3[t^2/C^2]) + \tfrac{1}{4}(f'^4[t^3/C^3]) + \ldots$$

Thus providing that $f't/C$ is less than 0·2, consideration of only the first three terms of the expansion gives a value for f which is within 0·22% of its true value. This was used in calculation of the corrected influxes. All the corrections were small (< 12%), the corrected whole-body influxes are also recorded in Table 5.

Table 6. *Calcium components and rates of exchange*

Acclimation salinity, % sea water	Rapid component	Slow component
100	4·3 ± 0·2 mM/kg $t_{\frac{1}{2}}$ = 8·0 ± 0·5 h	0·8 ± 0·2 mM/kg $t_{\frac{1}{2}} \gg$ 50 h
10	1·03 ± 0·05 mM/kg $t_{\frac{1}{2}}$ = 10·5 ± 0·5 h	0·88 ± 0·05 mM/kg $t_{\frac{1}{2}} \gg$ 50 h
1	0·62 ± 0·1 mM/kg $t_{\frac{1}{2}}$ = 22 ± 3 h	0·62 ± 0·1 mM/kg $t_{\frac{1}{2}}$ = 300 ± 60 h

Analysis of the results

Ionic fluxes are related to ionic activities rather than to concentrations, and thus ideally ionic activities should be measured rather than concentrations. The difference is not a trivial one as the activity of calcium in sea water is 0·22 times its concentration (Berner, 1965). Whilst suitable glass electrodes are available for measuring the activities of sodium and potassium in physiological solutions, the divalent ion electrodes are all too sensitive to monovalent ions and do not distinguish adequately between calcium and magnesium (Truesdel & Christ, 1967), and the same criticisms apply to other 'calcium electrodes', except the pressurized calcium stearate multilayer membranes described by Gregor & Schonhorn (1961), but these would be of no use in this context because of the large volumes necessary for an assay. Similarly the amplitude of the frog-heart contraction (McLean & Hastings, 1934) would be of no use, and in addition might respond to factors other than calcium in the coelomic fluid; also its range as an indicator is limited.

However, the activities of calcium and magnesium ions in various dilute solutions predicted by the Debye–Hückel equation agree very well with measured values up to 0·1 molal, using the values of 6 Å for the ionic radius of calcium and 6·5 Å for magnesium (Garrels, 1967; Hostetler, 1963), and the experimental values at higher ionic strengths are fairly self-consistent despite the variety of different methods used in their determination. Hence the ionic activities of magnesium have been calculated as the concentration in an undiluted ultrafiltrate multiplied by Garrel's (1967) 'best values' for the activity coefficients at the ionic strength of the fluid. Garrel's 'best values' for calcium were revised downwards slightly at high ionic strengths to incorporate Berner's (1965) value for sea water. This procedure neglects any binding of calcium as ultrafiltrable chelates such as citrate or phosphate, but if the concentrations of these are not much larger than they are in mammalian serum the errors will be negligible (Bronner, 1964, p. 374). The ionic strengths of the solutions were calculated assuming that half of the anion deficits were divalent ions and half were monovalent

ions. Since the anions not measured directly were probably mainly sulphate, carbonate, bicarbonate and in coelomic fluid samples phosphate and organic acids this assumption is also reasonable. If the anion deficit were entirely monovalent ions or entirely divalent ions this would not affect the calculated activity coefficients by more than 2%. The calculated ionic activities of the divalent cations are given in Tables 7 and 8.

Table 7. *Observed and predicted calcium activities, mM/kg of water in the coelomic fluid of* N. diversicolor

Acclimation media		Coelomic fluid ultrafiltrate, corrected for dilution	
Sea water (%)	Ca	Ca observed	Ca predicted*
150	3·73	2·20	4·74 ± 0·21
100	2·52	1·64	2·73 ± 0·08
50	1·37	0·967	1·41 ± 0·02
20	0·859	0·608	0·797 ± 0·034
10	0·523	0·408	0·576 ± 0·010
5	0·263	0·467	0·89 ± 0·14
2	0·136	0·478	0·99 ± 0·27
1	0·0866	0·616	0·85 ± 0·22
0·5	0·0541	0·745	1·12 ± 0·36
0·2	0·0313	0·670	1·46 ± 0·29

* The errors shown are standard errors of the mean resulting from variability of the measured body-wall potentials.

Table 8. *Observed and predicted magnesium activities, mM/kg of water in the coelomic fluid of* N. diversicolor

Acclimation media		Coelomic fluid ultrafiltrate, corrected for dilution	
Sea water (%)	Mg	Mg observed	Mg predicted*
150	22·4	20·2	28·5 ± 1·3
100	14·6	13·1	15·8 ± 0·4
50	7·75	7·17	7·99 ± 0·09
20	3·67	4·60	4·18 ± 0·15
10	2·33	3·34	2·57 ± 0·04
5	1·34	2·87	4·56 ± 0·72
2	0·613	2·68	4·47 ± 1·21
1	0·346	2·34	3·40 ± 0·88
0·5	0·194	1·94	3·72 ± 1·18
0·2	0·0867	2·06	4·05 ± 0·80

* The errors shown are standard errors of the mean resulting from variability of the measured body-wall potentials.

If an ion, of charge z, is in equilibrium between two aqueous solutions and a potential difference $\Delta\psi$ exists between the solutions, the ratio of the ionic activities in each solution will be given by the factor $\exp(-zF\Delta\psi/RT)$ where F is the Faraday, R is the gas constant, and T is the absolute temperature. On this basis the ionic activities of magnesium and calcium in the coelomic fluids of the animals which would be in equilibrium with the medium have been determined and are also given in Tables 7 and 8, using the measured body-wall potentials and calculated ionic activities in the media. The errors shown are only those resulting from the standard errors of the mean

body-wall potentials; these errors are large in low salinities, and swamp any other uncertainties.

It is to be noticed that both the activities of calcium and magnesium are similar to or less than the predicted equilibrium levels in the coelomic fluid. This shows that there is no need to postulate active uptake of either ion from the medium in any of the conditions studied. Rather, there must be removal of these ions from the body to maintain these concentrations, probably representing loss in the urine which these animals must produce to maintain their body volumes constant in low salinities. If any active uptake occurs it must be more than balanced by a higher non-diffusional loss rate.

No information has been obtained about the fluxes of magnesium in these animals, for lack of a convenient radio-isotope of magnesium. However, the similarity observed between the accumulation of magnesium and calcium suggests that both ions are accumulated by the same mechanism. The influx rates of calcium in Table 5 have been used to calculate the probable rates of urinary calcium loss. If the influx is assumed to be purely passive diffusional influx, the passive efflux will be the influx rate multiplied by

$$\exp(-zF\Delta\psi/RT) \quad \text{(Ca activity inside/Ca activity outside)},$$

and the non-diffusional efflux, which is presumably urinary, is the difference between the passive influx and efflux. This has been calculated and is given in Table 9. This procedure is valid whatever changes in permeability occur, providing there is no active uptake, exchange diffusion, solute drag, or coupling to any other fluxes. If half of the influxes were active uptake or exchange diffusion this would make the estimated urinary loss rates low or high by about 50%. The standard errors of the mean body-wall potentials contribute significantly to the uncertainty of these results especially in lower salinities, where an error of twice the standard error would nearly double the calculated urinary loss rates or reduce them to zero. However, these are extreme limits since the urinary calcium loss rates can neither be less than zero nor larger than the gross influxes.

Table 9. *Predicted urinary calcium loss rates*

Salinity, % sea water	Urinary calcium, mM/kg/hr*
150	0·199
100	0·161
50	0·049
20	0·054
10	0·031
5	0·034
2	0·022
1	0·009
0·5	0·007

* Predicted on the assumption that all the calcium influx is by passive diffusion.

DISCUSSION

The work reported here shows that the whole-body calcium concentration in *N. diversicolor* in high salinities (chlorosity > 100 mM/kg of water) rises nearly proportionally to the concentration of calcium in the medium to which the animal is

acclimated. Measurements of the calcium concentrations in ultrafiltrates of coelomic fluid allow the 'calcium space' in the animals to be determined; values lie between 650 and 800 ml/kg of animal. Since the sodium space is between 450 and 550 ml/kg (C. R. Fletcher, unpublished observations) and some of the sodium will be cellular, about half of the whole-body calcium must be cellular. From measurements of the rate of rise in specific activity of whole-body calcium in animals in a 100% sea water loading medium it was deduced that a mean of 84% (estimated maximum errors ± 4%) of the whole-body calcium is in a pool readily exchanging with the environment (Table 6), and thus a proportion of the cellular calcium is free to participate in this rapid exchange. The rates of calcium influx into the whole body (mM/kg of animal/h) and into the coelomic fluid (mM/kg of fluid/h) given in Table 5 are in accord with this model, and their ratio suggests that about 40% of the rapidly exchanging calcium is cellular.

The whole-body calcium in lower salinities remains nearly constant, and the calcium space is similar to the body volume, whilst the sodium space rises to about 600 ml/kg. Thus about half of the calcium is cellular in these lower salinities. The dynamics of calcium exchange indicate that about half of the whole-body calcium is free to exchange rapidly. This rapidly exchanging calcium cannot be equated with coelomic fluid calcium, however, since within experimental errors the rates of influx to the whole body and to the coelomic fluid are similar. A proportion of the coelomic fluid calcium must be slowly exchanging, and some of the cellular calcium must be rapidly exchanging. The slowly exchanging calcium in the whole coelomic fluid is probably protein-bound or cellular; the packed-cell volumes of the coelomic fluids of animals acclimated to 0·2% sea water averaged 32%.

The relative constancy of the calcium levels in the coelomic fluid is not maintained if the proportion of calcium in the bathing medium is altered relative to the major ions, and the animals have little if any ability to control variations of calcium concentration in their coelomic fluid under these conditions. Relatively little of the calcium or magnesium in the coelomic fluid is protein-bound, and the coelomic fluid proteins show no detectable Donnan effects. However, because of the electrical potentials across the body walls of the animals, calcium and magnesium ions inside are at a lower electrochemical potential than they are outside. Thus it is not necessary to postulate any active uptake of either ion to explain the coelomic fluid concentrations, and non-diffusional losses must be accounted for. Magnesium fluxes have not been measured, but the calcium fluxes are significant and have been used to predict the non-diffusional calcium loss rates, which are presumably urinary. The way in which the calcium concentration in the coelomic fluid changes with alterations in the ratio of calcium to chloride in the medium suggests that the body-wall potential is not significantly changed; it presumably arises as a result of the relative affinities of the active uptake mechanisms for sodium and chloride, and the permeability of the animals to these ions. Since the degree of concentration of magnesium is quantitatively similar to that of calcium the same mechanism probably operates in both cases.

Knowledge of the urinary calcium loss rate and the urinary flow rate enable conclusions to be drawn about the concentration of calcium in the urine. The water permeability may be deduced from the initial rates of swelling after transfer from one salinity to another, providing there is no immediate change in urine flow. If the urine

flow rate anticipated the swelling this would lead to underestimates of the permeability. The data reported by several workers for *N. diversicolor* (Beadle, 1931, 1937; Jørgensen & Dales, 1957; Fretter, 1955) may be interpreted to suggest urine flow rates in the more dilute media between 20 and 100 ml/kg/h, providing no changes in water permeability occur in low salinities. The evidence of Jørgensen & Dales on this point is ambiguous, but Smith (1964) has shown that no change in permeability to heavy water occurs in *N. succinea* and *N. limnicola*; and in the latter species isotopic measurements of permeability agreed with those deduced from swelling. Taking the upper limit for urinary calcium loss as the whole influx rate, and the lower limit of urine flow as 20 ml/kg/h, the concentration of calcium in the urine would be rather less than in the coelomic fluid. Since it is improbable that all the uncertainties would operate in one direction it is likely that the urine of these animals contains very much less calcium than the coelomic fluid, presumably as a result of resorption from the nephridial fluid.

The number of observations which have been used in drawing the preceding conclusions are such that they could not all be determined simultaneously or on the same group of animals, and the main work was spread out over more than a year. However, the level of calcium, magnesium and the monovalent ions do not vary significantly under constant conditions between September and March, and no experiments were performed outside this period. No animals were used which were obviously in spawning condition. The data of Tables 1–4 were obtained from pooled samples so no errors from this source are quoted, but the standard errors from this source should be similar to those where similar replicate observations are made on a similar number of individual animals; up to $\pm 5\%$ in high salinities and up to about $\pm 10\%$ in the lowest salinities. The uncertainties in low salinities are thus dominated by the variability of the body-wall potentials, which would probably not be reflected fully in variability of calcium levels as the latter would be set by the time-average of the potentials over about a day, rather than a minute or two.

These results have a bearing on Beadle's (1937) and Ellis's (1937) observations on the necessity of external calcium for volume regulation in *N. diversicolor*. When worms are transferred from 20% sea water to 20% calcium-free sea water they may be expected to lose half of the free calcium from the coelomic fluid in about 10 h, and neither author showed any significant disturbance of volume regulation over shorter time periods. The reduction in internal calcium could thus be the cause of failure of volume regulation, or it could be incidental to direct effects on water permeability which Beadle suggested as the cause of swelling. It is interesting to note that in similar work on *Procerodes* (*Gunda*) *ulvae* Beadle (1934) observed a delay before swelling commenced during which the animals showed obvious signs of distress, becoming motionless before swelling was observed. However, the effect of calcium removal on the permeability of epithelia is well established (Manery, 1966).

Whether the mechanism for regulating divalent cations is as simple in other invertebrates as it seems to be in *N. diversicolor* remains to be seen. Freshwater animals usually seem to maintain their body fluids at a lower electrical potential than the environment, and hypo-osmotic regulators at higher potentials (e.g. Smith, 1969, Evans, 1969), but it is not known if the potentials are large enough, particularly for animals living in soft fresh water. The situation in animals with calcareous exoskeletons may be further complicated by removal of calcium from the shell before moulting and

deposition in the new one (Robertson, 1957). Marine invertebrates usually concentrate calcium and magnesium to different degrees, and it is known that the low magnesium levels in the bloods of *Pachygrapsus crassipes* and *Carcinus maenas* are maintained by the secretion of magnesium into the urine, and this process is facultative (Riegel & Lockwood, 1961; Gross & Capen, 1966; Lockwood & Riegel, 1969).

The mechanism for calcium control in the higher vertebrates is a sophisticated one (Gaillard, Talmage & Budy, 1965), presumably because small effects of calcium levels on individual neurones in a complex central nervous system are integrated to give marked clinical effects; a fall of 30% in the blood calcium concentration in man induces convulsions, tetany and death. *N. diversicolor* must be much more tolerant of varying calcium levels, because of its much simpler nervous system.

SUMMARY

1. *Nereis diversicolor* tolerates changes in the concentration of calcium and magnesium in its coelomic fluid proportional to the concentrations in the medium between chlorosities of 100–1000 mM/kg of water.

2. In lower salinities both ions are maintained relatively constant providing that the ratios of these ions to chloride in the medium are similar to the ratios in sea water.

3. The ratio of the concentration of calcium in the coelomic fluid to the concentration in the medium is a function of the salinity of the medium but not of the calcium concentration.

4. Both calcium and magnesium are at lower electrochemical potentials in the coelomic fluid than in the medium, indicating that it is not necessary to invoke active uptake.

5. The rate of calcium influx is substantial.

6. In salinities below 10 mM of chloride/kg of water the urine must contain less calcium than the coelomic fluid.

7. The significance of these results is discussed.

I am indebted to the staff of the Department of Zoology and Comparative Physiology of the University of Birmingham where this work was performed during tenure of a Research Fellowship.

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