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# HYPO-OSMOTIC URINE IN *NEREIS DIVERSICOLOR*

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Although Beadle as early as 1937 suggested that the urine of *Nereis diversicolor* O. F. Müller might be of lower concentration than the body fluids when this worm is hyperregulating in low salinities, there has been no direct measurement of urine concentration to support this reasonable idea. Jørgensen & Dales (1957) studied osmotic water uptake and loss in *N. diversicolor* and calculated that this animal reduces its permeability to water by over 60% when in fresh water. On the assumption that chloride permeability was comparably reduced, they calculated that the observed rate of chloride exchange and the level of chloride concentration in the coelomic fluid were compatible with the production of a small volume of urine isotonic in chloride to the body fluid. However, Potts & Parry (1964) in reviewing this matter regarded the evidence of Jørgensen & Dales for reduced permeability to water as not well founded and, without presenting new data but by making the assumption that no changes in permeability occurred, drew up a balance-sheet for chloride exchanges which led them to postulate a reduction of chloride loss in low salinities by production of an hypotonic urine. Although there is no incompatibility between reduction of chloride loss by reduction of permeability to water (and hence urine volume) and by resorption of chloride from a consequently hypotonic urine, the fact that different authors can postulate these as alternative mechanisms on the basis of the same data makes it evident that measurements of urine concentration and volume would be useful in further attempts at analysis of the regulation of chloride and water in *N. diversicolor*. In a preceding paper (Smith, 1970*a*) there has been shown a correlation between chloride loss and weight loss compatible with the production of hypotonic urine by this species at low salinities, but this evidence is not conclusive. It has further been shown (Smith, 1970*b*) that *N. diversicolor* reduces its apparent permeability to water (as $D_2O$) in low salinities, which seems to confirm the view of Jørgensen & Dales (1957), but the urine volumes calculated were such as to make it probable that chloride resorption and production of a urine hypotonic in chloride to the body fluid can also take place.

In the present paper direct evidence is presented that the urine of *N. diversicolor* is hypo-osmotic to the coelomic fluid. The data are not so extensive as could be desired, but since I will be unable to continue work with *N. diversicolor* for several years, it seems best to record the hypo-osmoticity of the urine of this widely studied species, in order to expedite the studies of other workers.

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## MATERIALS AND METHODS

Worms were collected from a sandy substrate near the laboratory at Seili and adapted at 18–20 °C in the laboratory in plastic refrigerator dishes. Short segments of glass tubing were provided, in which the worms took up residence.

After unsuccessful attempts to collect urine by various methods it was discovered that if a specimen of *N. diversicolor* is merely placed on its back on dry filter paper and its ventral side blotted with a pad of facial tissue-paper, the cuticle is sufficiently hydrophobic to allow tiny hemispherical drops of urine to accumulate at the external nephridiopores. These are best seen if the worm is rolled slightly to one side so that the bulges ventral to the parapodia are seen in profile under a 6–12× dissecting microscope with oblique direct illumination. The urine droplets do not persist long, especially if the worm is active; they soon wet the cuticle and disappear, but with practice and a little luck several droplets may be picked up in sequence with a capillary. This is difficult if the worm is struggling but anaesthesia was avoided because the production of urine is more rapid in an active and turgid worm.

Collecting capillaries were drawn from hard glass tubing of *c.* 3 mm bore and 1 mm wall thickness (previously cleaned by boiling in distilled water) to an internal diameter of *c.* 85–105 $\mu$m. Such capillaries contain, in a sample 0·5 mm long, some 0·0028–0·0043 mm$^3$; smaller samples offer no special advantages and increase the chances of contamination. In capillaries of such dimensions, water droplets bounded by liquid paraffin on both sides can be moved only with difficulty. In practice, capillaries 3–5 cm long are held between the halves of a small split cork thrust into a 1 cm glass tube with slightly constricted tip, this in turn supplied with a rubber mouth tube. Control in picking up urine is achieved by taking 1–2 mm of liquid paraffin into the capillary tip. This liquid has a low enough capillary to permit control; a slight positive pressure keeps it at the capillary tip, and permits small increments of urine to enter as several droplets are touched in succession. When a sample 0·5–1 mm long has been taken, liquid paraffin is drawn in after the urine without entrapment of air until the urine is brought to the middle of the capillary. This is then reversed in its holder and the urine sample, with the small amount of oil now ahead of it, is forced to the other end of the capillary, and more liquid paraffin drawn in as the urine is returned to the capillary, mid-point. All air is thus excluded; this is necessary to prevent displacement of the urine sample during subsequent freezing and melting. Samples of media are easily collected, the capillary being half-filled with liquid paraffin and several short segments of medium alternating with paraffin drawn in, again without inclusion of air. Samples of coelomic fluid were taken in a tube of 3 mm bore drawn to a capillary tip; this tip was sealed in a flame, and the sample was centrifuged. From the resulting supernatant a sub-sample was taken in a 5 or 10 $\mu$l disposable pipette (Drummond 'Microcap') for chloride determination, and then a series of short segments were drawn up into liquid paraffin in a capillary as in the case of samples of medium. For some features of this procedure, including the split-cork capillary holder, I am indebted to Mr Jørgen Gomme, post-graduate student of the Zoophysiological Laboratory, Copenhagen.

Five determinations were made on each worm: melting points of urine, coelomic fluid, and medium; chloride concentrations of coelomic fluid and medium. Chloride determinations were made with an Aminco–Cotlove electrometric chloride titrator,

solid-state analytical model. Melting points were determined by a micro-modification of the comparative method of Gross (1954) using NaCl standards. In this method, standards and samples are quick-frozen and then allowed to melt in a chilled bath as the latter warms slowly. Osmotic concentration is expressed in terms of the equivalent concentration of NaCl.

Details of this method as adapted for micro-samples are as follows. A box of clear plastic containing 400 ml of 30% alcohol is placed in an insulating box of foam plastic with walls and bottom 5 cm thick, and covered with a lid 3 cm thick. This box rests on the stage of a dissecting microscope, and is provided with holes in the bottom and lid to allow samples in the bath to be viewed at 25× or 50× by transmitted substage illumination. Samples of urine, coelomic fluid, media and standards, all in separate capillaries in liquid paraffin, are fastened by rubber cement across a 1 cm hole in the centre of a piece of sheet brass in the following sequence: NaCl standards of 200, 150, and 100 mM/l; coelomic fluid, urine and medium of a worm adapted in a higher salinity; coelomic fluid urine and medium of a second worm adapted in a lower salinity; NaCl standards of 50 and 20 mM/l; distilled water. Such a 12-tube array occupies a band 4–5 mm wide, all in the same plane. Placing the tubes in regular sequence is necessary if confusion is to be avoided, and the use of samples from 2 worms adapted to different salinities reduces the chance of simultaneous melting of samples.

The alcohol bath is cooled in a deep-freeze, or by holding a block of dry ice at its surface, to 3–5 °C below zero. The set of samples on the brass holder is quick-frozen by immersion in 95% alcohol containing lumps of dry ice, and is then quickly transferred to the viewing bath, in which it is supported at about the middle depth. After removing $CO_2$ bubbles with a small brush, one views the frozen or partially melted samples at 25–50×; ice crystals are readily visible. The bath is allowed to warm without stirring. A temperature stratification develops, with the surface becoming 2–3 °C warmer than the bottom, but since all samples are in a 5 × 5 mm area at the same plane, no appreciable temperature differential exists among them.

As each standard or sample melts, the time of disappearance of the last ice crystal is recorded, the melt-time of the 200 mM/l NaCl standard being taken as 'time zero', and the elapsed time in seconds to each other melt worked out. The melt-times of the six standards are plotted on squared paper (abscissa, times; ordinate, NaCl concentrations), yielding a slightly curved line. At a room temperature of 18–20 °C, elapsed time between the melting of the first (200 mM/l) NaCl standard and that of the last standard (DW) is 500–700 sec. The melt-times of the unknown samples are then drawn from the abscissa to the curve, and the equivalent NaCl concentrations are read on the ordinate. Since it is common to miss the melt of one of the samples on the first melt-sequence, the set of samples is refrozen, the bath recooled with dry ice, and a second melt-sequence observed; the results are plotted as a fresh curve. Most final values are the average of the two determinations, a few of only one.

Certain precautions and comments regarding this method may be useful: the use of an unstirred bath was dictated by the fact that only 2 weeks were available at Seili, and no stirrer was at hand. However, the results seem as good with a stirred bath, and viewing is easier. But it is important, with an unstirred bath, to avoid occasional or irregular

movements that may upset the temperature stratification, and to mount all samples at the same plane, close together but not touching. It is most important to have all tubes in each set of 12 closely matched for size and wall thickness. A set of 12 tubes is selected by visual matching under the dissecting microscope before each set of standards and samples is made up. In order to eliminate systematic bias among standards, a fresh set of standards is made up for each array; drops of standards were placed under liquid paraffin in a glass dish with lacquered bottom, and were picked up in capillaries as were other samples. Since each group of samples from two worms was referred to its own set of standards, variations in warming rate and other variables were eliminated or randomized. Only those sets of determinations including melting points of coelomic fluid and urine from the same worm have been used. Samples of coelomic fluid for chloride determination were inadequate in the case of a few smaller worms. The smallest worm yielding a complete set of determinations weighed 152 mg; worms of 300–400 mg wet weight were easily handled.

## OBSERVATION AND RESULTS

### (1) *General*

Local Baltic Sea water ('merivesi') had a salinity about 17% of North Sea water, varying a little in the course of the study, with a chloride concentration of $95{\cdot}5 \pm 1{\cdot}6$ mM/l. This was diluted for purposes of adaptation to $30{\cdot}7 \pm 2{\cdot}3$ mM/l and to $9{\cdot}9 \pm 2{\cdot}4$ mM/l for several different lots of worms, using very soft water from a well on a granite hillside. Dilution of 'merivesi' to a chloride concentration of 5 mM/l resulted in a barely tolerable medium in which a number of worms died. Such very soft water is in contrast to the hard Northumberland pond water ($Cl^- = 0{\cdot}9$ mM/l; $Ca^{2+} = 1{\cdot}14$ mM/l) used successfully in earlier studies (Smith, 1970*a*, *b*) and emphasizes the importance of other ions when considering the adaptation of *N. diversicolor* to very low salinities as estimated by chloride concentration.

The urine produced by *N. diversicolor* seems, subjectively, to be copious; it is possible to collect only a small fraction of the urine emitted by an actively squirming worm, but enough is produced to wet the entire surface after the worm has been blotted dry. Urine droplets appear most readily when the worm is undergoing body movements, and their emission is favoured by body turgor as predicted by Beadle (1937) and Smith (1970*a*). However, it is probable that the volume of urine emitted under the stress of rough handling is far in excess of that produced by a resting worm, as calculated on the basis of apparent permeability to water (Smith, 1970*b*).

### (2) *Osmotic concentration of urine and coelomic fluid*

The results of determinations of osmotic concentration of urine and coelomic fluid of 38 worms are summarized in Table 1 and Fig. 1. At all concentrations of adaptational media tested the concentration of the urine is below that of the coelomic fluid, although the difference in the natural medium (Cl *c.* 95 mM/l) is hardly significant ($P < 0{\cdot}1$, $> 0{\cdot}05$). At chloride concentrations of *c.* 30 and 10 mM/l the urine is significantly hypo-osmotic ($P = 0{\cdot}001$), and also at *c.* 5 mM/l, although the results in the latter medium are few and may have been influenced by selective elimination

of weaker individuals. But the overall conclusion is clear: *N. diversicolor* produces a urine which becomes progressively more hypotonic as salinity is lowered, with some suggestion that the urine concentration might be even lower in a properly balanced medium of minimal salinity. Extrapolation of the curves of osmotic concentrations of urine and coelomic fluid (Fig. 1) suggests that urine is iso-osmotic with coelomic fluid in media of chloride concentration above 157 mM/l (28% of sea water).

Table 1. *Osmotic concentrations (as equivalent concentration of NaCl) of media, coelomic fluid and urine of* Nereis diversicolor, *and chloride concentrations of media and coelomic fluid.*

| | | Baltic Sea water | Dilutions of Baltic Sea water | | |
|---|---|---|---|---|---|
| | | Cl *c.* 95 mM/l | Cl *c.* 30 mM/l | Cl *c.* 10 mM/l | Cl *c.* 5 mM/l |
| Osmotic Concentration of Medium (as NaCl) | Mean | 101·2 | 36·8 | 18·0 | 5·8 |
| | S.D. | 12·7 | 4·6 | 4·3 | — |
| | % S.D. | 12·5% | 12·5% | 24·4% | — |
| Osmotic Concentration of Coelomic Fluid (as NaCl) | Mean | 178·0 | 150·9 | 130·2 | 119·6 |
| | S.D. | 24·8 | 22·4 | 16·4 | — |
| | % S.D. | 13·8% | 14·9% | 12·6% | — |
| Osmotic Concentration of Urine (as NaCl) | Mean | 150·3 | 90·3 | 78·3 | 59·5 |
| | S.D. | 35·7 | 33·2 | 37·4 | — |
| | % S.D. | 23·8% | 27·2% | 47·8% | — |
| Chloride Concentration of Medium (mM/l) | Mean | 95·5 | 30·7 | 9·9 | 4·8 |
| | S.D. | 1·6 | 2·3 | 2·4 | — |
| | % S.D. | 1·7% | 7·4% | 24·4% | — |
| Chloride concentration of Coelomic Fluid (mM/l) | Mean | 169·7 | 130·2 | 138·1 | 105·0 |
| | S.D. | 14·6 | 16·4 | 20·5 | — |
| | % S.D. | 8·6% | 12·6% | 14·8% | — |
| Number of worms used | | 10 | 12 | 12 | 4 |

(3) *Discrepancies between osmotic concentration and chloride concentration*

The above results are consistent with the view that the urine of *N. diversicolor* is hypotonic in chloride content to the coelomic fluid, but it is also clear from the data in Table 1 that the osmotic concentrations measured are higher than can be accounted for by chloride ion and its associated cations. An analysis of the major ions of the urine at low salinities is still needed. The expression of osmotic concentration in terms of equivalent NaCl does not imply anything about chloride concentrations as such. The measured chloride concentrations of coelomic fluid average 93·3% of the 'equivalent NaCl' osmotic concentration. For the media, measured chloride concentration ranges from 94·2% of 'equivalent NaCl' osmotic concentration in local Baltic Sea water, to 83·5% in a one-third dilution, and 55·5% in Baltic water diluted to a chloride concentration of 10 mM/l with well water. Thus, nearly half the osmotic pressure of this low salinity is accounted for by something other than chloride salts in the fresh water used as the diluant. Future studies of *N. diversicolor* in low salinities in nature should take account of all ions present in significant concentration, and not rely only on chloride values as a measure of salinity.

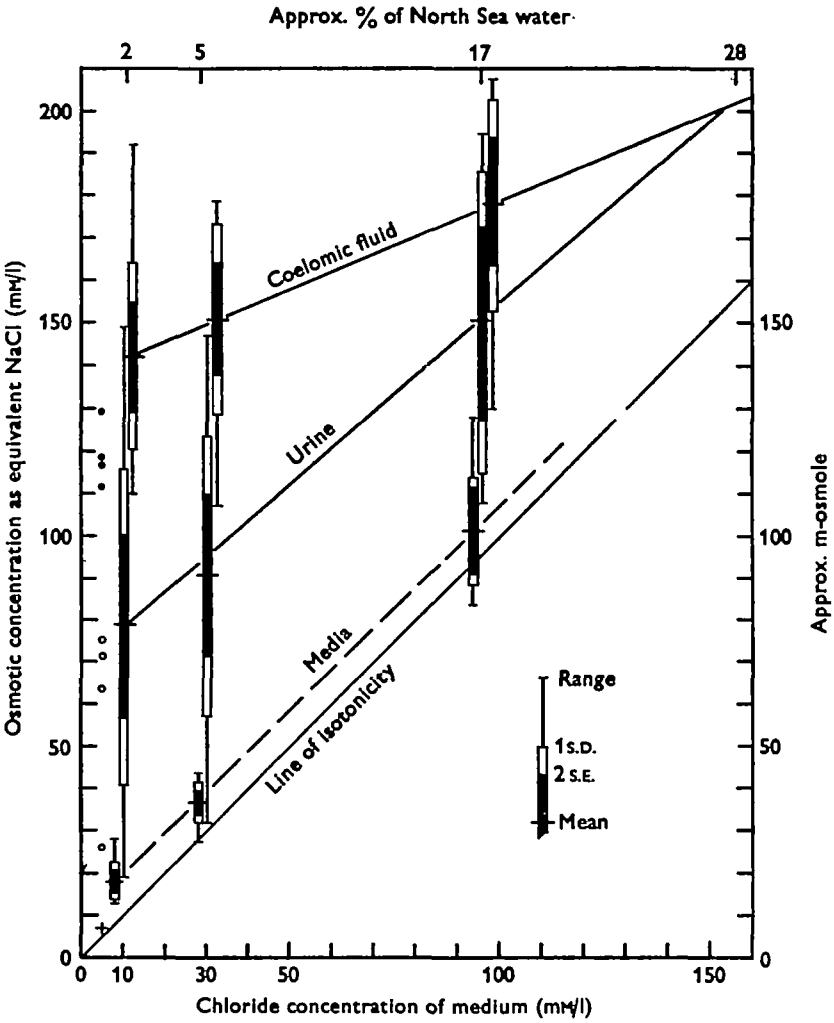


Fig. 1. Osmotic concentrations, expressed as equivalent concentration of NaCl, of media, coelomic fluid, and urine of *Nereis diversicolor* in different salinities. Separate points are shown for the most dilute medium (Cl *c.* 5 mM/l): ●, coelomic fluid; ○, urine; +, medium. Plotted by method of Dice & Leraas (1936), in which non-overlap of dark blocks representing ±2 standard errors indicates significant difference.

## DISCUSSION

Although the data relate to osmotic concentration rather than to chloride concentration, they support the view that recovery of ions from the urine is part of the chloride regulation of *N. diversicolor*, just as reduction of the osmotic pressure of the urine must be part of the osmoregulatory mechanism. Now that the collection of urine has been shown to be feasible, it is hoped that European workers will soon fill the gaps in our knowledge.

Any reference to Table 1 and Fig. 1 must raise the question of the extremely great variability in the osmotic concentrations of coelomic fluid and urine. Is this a true

intrinsic variability, or does it represent imprecision in the simple melting-point method used? In defence of the method, it may be noted that the variability in osmotic concentration of coelomic fluid (mean standard deviation ± 13·8%) is but little greater than that observed in the chloride concentration of coelomic fluid (mean standard deviation ± 12·0%) for the same groups of worms. The Cotlove chloridometer gives standard deviations of less than ± 1% on repeated identical samples, so that a large intrinsic variability of chloride concentration in coelomic fluid (standard deviations above 10%) seems to be characteristic of *N. diversicolor*, although the reasons for it are still obscure. But the mean standard deviation of the urinary osmotic concentration (± 32·9%) is so much greater than that of the coelomic fluid as to suggest that urine is either more variable than coelomic fluid, or that urine is more subject to contamination, evaporative loss, or modification in collection than is coelomic fluid. It is possible that the first urine collected is urine that has been thoroughly processed in a slow passage through the nephridial tubule, and so may be markedly different from coelomic fluid, but that urine collected later, and after much handling of the struggling worm, has moved rapidly through the tubule under considerable hydrostatic pressure, and so represents less-modified coelomic fluid. This point can and should be verified experimentally.

The brief study reported here indicates that *N. diversicolor* utilizes recovery of solutes from its urine as part of its osmoregulatory processes. In addition, it has been shown (Jørgensen & Dales, 1957; Smith, 1970*b*) to utilize the mechanism of reduction of urine volume by reduction of integumental permeability to water at low salinities. It is, furthermore, able to transport chloride inwards against substantial electrical and chemical gradients (Smith, 1970*a*); this active transport mechanism may well operate both at the body surface and in the nephridia. *N. diversicolor*, like other animals, does not regulate by any single mechanism, but is physiologically resourceful in utilizing a wide range of regulatory mechanisms.

SUMMARY

1. *Nereis diversicolor*, when regulating in water of low salinity, produces urine of lower osmotic concentration than the coelomic fluid.

2. The urine is significantly hypo-osmotic in 0·9–5·3% sea water (Cl = 5–30 mM/l), and slightly hypo-osmotic in natural Baltic Sea water (17% sea water; Cl = 95 mM/l).

3. The urine is probably iso-osmotic in 28% sea water (Cl = 157 mM/l) and higher salinities.

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