

THE MECHANISM OF OSMOTIC REGULATION IN
ARTEMIA SALINA (L.): THE PHYSIOLOGY
OF THE BRANCHIAE

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INTRODUCTION

In a previous paper (Croghan, 1958*b*) the very great osmo-regulating ability of *Artemia* has been described. It is clear that in concentrated media the animal must have very well-developed active mechanisms both for excreting NaCl and for taking up water to compensate for the passive movements of these substances into and out of the animal.

The staining of localized parts of Crustacea following immersion in dilute AgNO₃ solutions and subsequent reduction has been interpreted by Koch (1934) and Krogh (1939) as indicating the site of active ion uptake. But similar staining occurs in *Artemia* (Dejdar, 1930), and yet this animal in its normal hypertonic medium must be actively excreting NaCl (Croghan, 1958*b*). In this paper further observations on the branchiae of *Artemia* in relation to the mechanism of osmotic regulation are described.

MATERIAL AND METHODS

Adult *Artemia* as described previously (Croghan, 1958*a*) were used.

The basic silver-staining technique is very simple. Animals from a sea-water culture were used for most experiments. The living animal was washed in several changes of distilled water (for 1–2 hr. usually) to remove adherent chloride, and was then placed in 10⁻² M-AgNO₃ solution for 2–5 min. The animal was then given a prolonged wash in several changes of distilled water (1–2 hr. usually) to remove any adherent AgNO₃, and was placed in photographic developer (1. I.D. 13.) for 1–2 min. to reduce to the black metallic state any silver that had been taken up.

The methods used in studying the composition of the haemolymph and of whole animal have been described previously (Croghan, 1958*b*).

RESULTS

Histochemistry of the branchiae

The only parts of *Artemia* that showed silver staining were the branchiae (metepipodites) of the first ten pairs of phyllo-pods. The last (eleventh) pair of branchiae and the rest of the animal never stained. The first ten pairs of branchiae became white in the AgNO₃ solution. In the developer these branchiae became intensely black. Microscopic observation showed that the blackening was granular.

Staining occurred whether the medium from which the animal was originally taken was hyper-, iso-, or hypotonic to the haemolymph, although the intensity did tend to be somewhat greater when the external salinity had been higher.

Most observations were made using 10^{-2} M- AgNO_3 , but the most dilute solution used, 10^{-4} M- AgNO_3 , still gave a similar result, although the duration of exposure to get a comparable effect was much greater (> 20 min.).

The fact that the branchiae turned white in the AgNO_3 solution indicated that the silver ions had entered and been precipitated. Some of these white-gill animals were washed in distilled water and then soaked in 5% HNO_3 (30 min.), washed well with water and then developed. In another experiment some living animals were washed and placed directly in 10^{-2} M- AgNO_3 made up in 5% HNO_3 , and then washed thoroughly and developed. In both cases the branchiae were still blackened in the developer, suggesting strongly that the initial white precipitate is AgCl . The source of the chloride ion is of some interest. The period of washing in distilled water before transferring the animals to the AgNO_3 solution was varied from 3 min to 18 hr. Very prolonged washing resulted in some decrease of subsequent blackening, but the blackening was still considerable, and this suggested that the chloride was not derived directly from the medium, but was coming from the animal itself.

Close microscopical examination of isolated branchiae suggested that the black granular staining is very superficial. This was confirmed by watching branchiae becoming decolourized in Farmer's solution (potassium ferricyanide + hypo). The staining was still very superficial even when the violent treatment of development and subsequent Bouin fixation had caused the epithelium to retract away from the cuticle. This suggested that the black silver deposit was within the cuticle, which is only about 1μ thick. In some cases the epithelium had distorted and retracted so much that the thin blackened cuticle could be pulled off the branchia like a loose glove; the epithelium thus seen was not stained. Further, if detached stained branchiae were heated with saturated KOH solution in a boiling water-bath for 3-4 hr the appearance of the silver stain was not affected, although on subsequent removal of the silver with Farmer's solution, it was seen that the branchial epithelium had entirely disappeared leaving only the thin transparent cuticular ghost. These experiments indicate that the silver staining is confined to the thickness of the cuticle, and does not appear to affect the underlying epithelium.

The effect of metabolic inhibitors was studied. Animals were immersed in 15% ethyl urethane in distilled water for periods of up to 6 hr. They were then placed for 5 min. in 10^{-2} M- AgNO_3 made up in 15% ethyl urethane solution, washed thoroughly and developed. Other animals were immersed in sea water containing 2% sodium azide for 3-18 hr., and then washed for times varying from 1-30 min to remove azide (which would precipitate silver ions). They were then placed in 10^{-2} M- AgNO_3 for 5 min, washed and developed. With both urethane and azide the animals were completely and irreversibly motionless and apparently dead long before they were transferred to the AgNO_3 solutions. Yet in both cases the first ten pairs of branchiae were still very black. This suggests that the silver staining is a purely passive process.

All these results with the silver technique indicate that the cuticle over the first ten pairs of branchiae is permeable, as silver ions and subsequently developer could not otherwise enter. The silver ions diffuse into the permeable cuticle, meet chloride ions derived from the animal via the branchial epithelium, and form an AgCl precipitate within the cuticle. The whole phenomenon is purely passive. Over the last pair of branchiae and the rest of the external surface of the animal the cuticle must be sufficiently impermeable to prevent this from occurring.

The effects of a few other substances on the branchiae of *Artemia* have been studied. Some *Artemia* were put into a saturated solution of methylene blue for about 4 hr. After removal and rinsing, the first ten pairs of branchiae were seen to be markedly blue. Apart from a little dye that might have entered the gut lumen, no other part of the animal was appreciably coloured. Detached phyllopods were examined microscopically. At the edge of the flattened branchiae it could be seen that the dye had diffused across the very thin cuticle, and had caused a granular blue staining in the epithelial cells that form a layer 8–16 μ thick under the cuticle. Other *Artemia* have been exposed to saturated KMnO_4 solution for 5–15 min. The first ten pairs of branchiae had turned brown. The rest of the animal appeared unaffected. Microscopical examination showed that the epithelium under the cuticle of these branchiae had become brown and distorted, and in many cases had pulled right away from the cuticular sac. The observations with both these substances also strongly support the view that the permeability of the external cuticle is localized to the first ten pairs of branchiae. It is only here that the methylene blue and KMnO_4 can enter and affect the underlying cells.

The results with AgNO_3 , methylene blue and KMnO_4 are not direct evidence that active uptake or excretion is occurring, but they show where it could be occurring, as over the epithelium concerned with this process the cuticle must of course be permeable.

The fresh-water *Chirocephalus* also shows silver-staining of its branchiae (Dejdar, 1930; Panikkar, 1941 a), and Panikkar claimed from this that the branchiae were the site of ion uptake. In the present work two large specimens (12–15 mm.) were studied. They were treated with 10^{-2} M- AgNO_3 and developed in the way described previously for *Artemia*. The first ten pairs of branchiae became stained as in *Artemia*. The last pair of branchiae, which were smaller than the preceding ones, were not stained.

Experimental destruction of the osmo-regulatory mechanism

Further information about branchial function was obtained by studying animals whose branchiae had been 'burnt' with KMnO_4 as just described. The animals were immersed for 5 min. in saturated KMnO_4 solution, and then removed and washed in 25% sea water. Many animals survived, and the only sign of damage was the browning and distortion of the epithelium of the first ten pairs of branchiae. These animals were kept in 25% sea water, which is approximately isotonic with the haemolymph of normal animals (Croghan, 1958b). Many died during the first 24 hr., but a considerable number survived well for a week or more, swimming, feeding, and reproducing in an apparently normal manner. In these survivors

the damaged branchial epithelium underwent a further slow degeneration and blackening.

The physiology of active survivors was studied. After at least 24 hr. in 25% sea water they were transferred to other media. The limits of survival were very much more restricted than for normal animals and ranged from only about 10–75% sea water. The haemolymph osmotic pressure was measured after about 48 hr. in these media. The results are plotted in Fig. 1, and should be compared with those with normal animals (Croghan, 1958*b*, figs. 1, 2). The 'burnt' animals had completely lost the ability to osmo-regulate, and were very closely isotonic with the medium. The upper limit of survival is set by the maximum haemolymph concentration that the tissues can tolerate. This concentration is approximately that of 75% sea water, and is about the same as the haemolymph concentration of a normal animal in a nearly saturated brine.

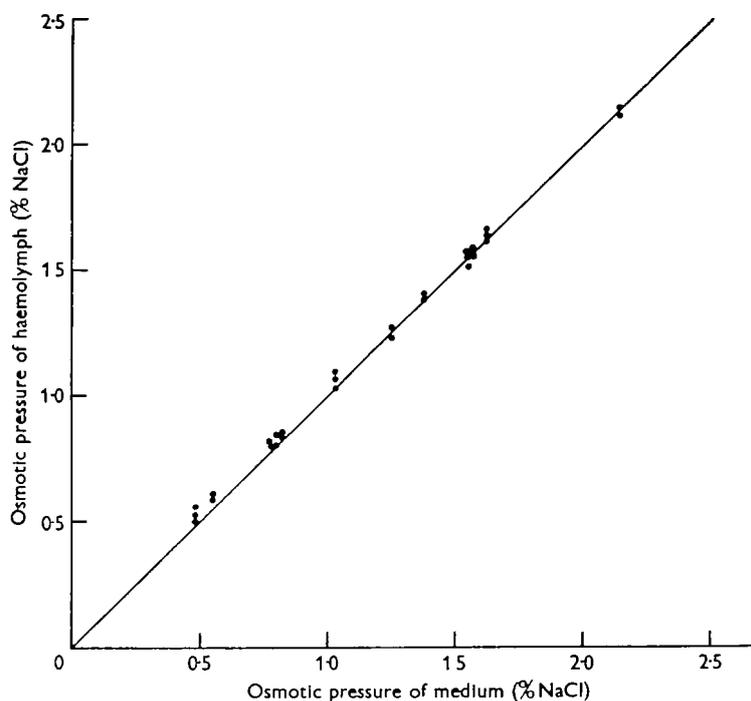


Fig. 1. The osmotic pressure of the haemolymph of animals treated with KMnO_4 . The diagonal line represents the condition of complete isotonicity between the haemolymph and the medium.

There appears to be no recovery of osmo-regulatory function; some 'burnt' animals that had survived actively for a week in 25% sea water and had then been transferred to 40% sea water became closely isotonic with their new medium within 24 hr.

The chemical composition of the haemolymph of some of the 'burnt' animals whose osmotic pressure is recorded in Fig. 1 was studied. 'Burnt' animals after

24 hr. in 25% sea water were placed in 50% sea water for 48 hr. Haemolymph samples were obtained from active animals with black or brown branchiae. Three samples, each derived by pooling the haemolymph from two animals, were obtained. The results are summarized in Table 1. Although the haemolymph osmotic pressure had risen and become isotonic with the medium, the ionic ratios were still very similar to those of normal undamaged animals (Croghan, 1958*b*, fig. 3), and very different from those of the medium. In another experiment, 'burnt' animals

Table 1. *The haemolymph composition of animals 'burnt' with KMnO₄*

Material	Osmotic pressure (% NaCl)	Na mM./l.	Cl mM./l.
50% sea water	1.55	228	264
Haemolymph groups			
1	1.58	244	210
2	1.52	238	200
3	1.55	238	210

that had been in 25% sea water for 24 hr. were transferred to 40% sea water to which a little solid MgSO₄ had been added. The final magnesium concentration in the medium was 64 mM./l. After 48 hr. in this medium, the haemolymph from a group of animals was pooled. The osmotic pressure had risen to become closely isotonic with the medium, but the haemolymph magnesium was not more than 1 mM./l. These observations indicate that although, following damage to the branchial epithelium, the haemolymph osmotic pressure becomes close to that of the medium, there is no great increase in indiscriminate permeability, as otherwise the haemolymph ionic composition would be the same as that of the medium. This is in keeping with the fact that the haemolymph of 'burnt' animals still looked like normal haemolymph, i.e. cherry-red with haemoglobin.

A series of observations was also made on the chemical composition of whole 'burnt' animals. The total water and chloride content were related to phosphate content as had been done with normal animals (Croghan, 1958*b*). 'Burnt' animals were kept in 25% sea water for 24 hr. The animals were then divided into two groups. One group was placed in fresh 25% sea water (= 0.83% NaCl) for a further 48 hr., and the other group was placed in 50% sea water (= 1.58% NaCl) for 48 hr. Then from each medium two groups of active animals with black or brown branchiae were taken, and the following quantities were determined: total water content, chloride content of the ash, phosphate content of the ash. The mean values of the chloride concentration in the total body water, and the chloride/phosphate and water/phosphate ratios in the two groups from 25% sea water were each taken as 100, and the mean values from the two groups of animals from 50% sea water were all expressed relative to this. The results are summarized in Table 2. It is evident that changes in haemolymph osmotic pressure are due more to changes in the total ion content of the animal than to water movements.

Table 2. *The chemical composition of whole animals 'burnt' with KMnO₄*

Medium	Whole animals		
Osmotic pressure	Cl in total body water	$\frac{\text{chloride}}{\text{phosphate}}$	$\frac{\text{water}}{\text{phosphate}}$
100	100	100	100
190.5	193	182.5	95

The conclusions from the preceding experiments are of interest. 'Burnt' animals lose the ability to osmo-regulate. In the more concentrated solutions the haemolymph osmotic pressure rises to become isotonic with the medium due to an increase in the total ionic content of the animal. This is not due to an increase in the indiscriminate permeability of the animal. It appears that the KMnO₄ treatment has destroyed the specific mechanism excreting NaCl against the concentration gradient. This, correlated with the fact that the visible damage is sharply localized to the epithelium of the first ten pairs of branchiae, and that it is only over these branchiae that the cuticle is appreciably permeable, indicates that this branchial epithelium is the site of the active transport of NaCl, which in undamaged animals maintains the haemolymph hypotonic to the more concentrated media. The same mechanism working the other way round is probably concerned with the uptake of NaCl that must occur in the dilute and hypotonic media.

Ontogeny of the regulatory mechanism

The nauplii of *Artemia* are also hypotonic to sea water (Croghan, 1958*b*), and yet in these young stages the limb buds are only just beginning to appear, and there are no branchiae. These nauplii, however, possess a large and curious structure: the neck organ, which is described and figured by Dejdar (1930). Dejdar correlated this structure with the adult branchiae. He considered that they are part of the same functional system, and that as the animal grows the branchiae form and replace the neck organ.

It is confirmed here that the silver staining of the nauplius is localized to the neck organ, and that it appears identical with that of the adult branchiae. Also, saturated KMnO₄ solution caused the epithelium of the neck organ to become brown and distorted and to pull away from the thin overlying cuticle, just as in adult branchiae. These reactions can, as in the branchiae, be interpreted as evidence that the cuticle over the neck organ is permeable. It is considered, therefore, that the neck organ is concerned in NaCl excretion in the nauplius and that it functions as such until the branchiae become functional. The neck organ then degenerates.

Some observations have been made on the process of replacement of the neck organ by the branchiae. The results are summarized in Table 3. The limb buds appear and develop in a sequence, the more anterior ones first. Branchiae appear and later develop the ability to stain with silver. The staining is 'all or none,'

Table 3. *The ontogeny of the branchiae*

Length of animal mm.	No. pairs limb buds	No. pairs branchiae	No pairs Ag-stained branchiae	Neck organ Ag-stained
0.5-0.8	0	0	0	+
1.0	4-5	0	0	+
1.7	8-9	5-6	1-2	+
2.0	10	6	3	+
2.5	11	8-9	4-5	+
3.0	11	10	6	+
3.8	11	11	7	+
6 (and upwards)	11	11	10	-

and it appears that at each moult one to two more pairs of branchiae can become stained, until only the last pair is left unstained. It seems that the branchial epithelium develops initially with an impermeable cuticle. Later the mechanism for NaCl excretion develops, and then at the next or a subsequent moult a permeable cuticle is secreted. There would appear to be an interesting co-ordination of two separate epithelial functions: NaCl excretion, and the secretion of a permeable instead of an impermeable cuticle.

DISCUSSION

Koch (1934) and Krogh (1939) have regarded the uptake of silver ions by Crustacea and Insecta as directly due to an ion transport mechanism. However, the *Artemia* results reported here show that the localized uptake and precipitation of silver is a purely passive process. The results indicate only that the cuticle at these sites is permeable. The experiments with methylene blue and KMnO_4 solutions confirm this. It is probable that this simple explanation also holds for the many other described examples of silver staining in the Crustacea and Insecta.

It is of interest that the eleventh pair of branchiae in *Artemia*, although apparently morphologically identical with the preceding pairs, is not affected by AgNO_3 , KMnO_4 or methylene blue, indicating that there is a differentiation of properties along the branchial series. A rather similar differentiation along the gill series has been observed in *Potamon* spp. by Ewer & Hattingh (1952). The structural basis of the localization of external permeability in *Artemia* has not been studied.

It seems clear that the cuticle over the first ten pairs of branchiae is the only appreciably permeable part of the external cuticle, and that the epithelium underlying this permeable cuticle is capable of actively excreting NaCl from the haemolymph into a hypertonic medium. This branchial excretory mechanism has been superimposed upon a basically fresh-water animal, and when this mechanism is destroyed the animal behaves like a fresh-water animal that has never evolved a hypotonic regulatory mechanism, and in which the upper concentration limit for survival is consequently low.

This regulatory mechanism in *Artemia* can be compared with those found in other animals that have evolved hypotonic regulation. It is very similar to that

found in the marine teleosts, in which Keys (1931) clearly demonstrated an excretion of chloride across the branchial epithelium. In both types the epithelium on the most permeable part of the external surface excretes NaCl against the concentration gradient. It is probable also that a similar mechanism operates in the marine palaemonids investigated by Panikkar (1941*b*).

These branchial mechanisms can be contrasted with that found in *Aedes detritus* larvae (Beadle, 1939; Ramsay, 1950). Here the same problem of excreting NaCl against a concentration gradient has been solved in a different way. The Malpighian tubules and rectal epithelium form an organ system that ultimately produces a concentrated fluid. In very dilute media, however, this rectal fluid can become hypotonic to the haemolymph. This may perhaps explain the prolonged survival of this animal in distilled water, which is a sharp contrast to *Artemia*.

The branchial excretory mechanism is not, however, the complete explanation of osmotic regulation in *Artemia*, and in a subsequent paper (Croghan, 1958*c*), the physiology of the gut will be considered in relation to osmotic regulation.

SUMMARY

1. The uptake of silver ions by *Artemia* has been investigated. The staining is localized to the first ten pairs of branchiae. There is no staining of the eleventh pair or of any other part of the animal. The uptake of silver is due to a purely passive precipitation of AgCl within the thickness of the branchial cuticle.
2. The effects of KMnO₄ and methylene-blue solutions have also been studied. Their effect is localized to the epithelium under the cuticle of the first ten pairs of branchiae.
3. It is concluded that all these staining reactions demonstrate that the cuticle over the first ten pairs of branchiae is the only part of the external cuticle that is appreciably permeable.
4. Animals whose branchial epithelium has been damaged by a brief exposure to saturated KMnO₄ solution have lost the ability to osmo-regulate. They are closely isotonic with their medium, and the range of external concentration tolerated is much restricted.
5. This isotonicity is not due simply to increased permeability, but is due to specific destruction of the mechanism normally excreting NaCl in hypertonic media.
6. Correlation of the physiological effects of KMnO₄ treatment with the sharp localization of damage, and the evidence for localized permeability indicates that the epithelium of the first ten pairs of branchiae is the site of active NaCl excretion in hypertonic media, and probably of active uptake from hypotonic media.
7. The ontogeny of this mechanism is traced. In nauplii the dorsal organ is apparently concerned in NaCl excretion. When the branchiae develop the dorsal organ degenerates.

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