IONIC FLUXES IN *ARTEMIA SALINA* (L.)

By P. C. CROGHAN

Department of Zoology, University of Cambridge, and Department of Biophysics, University of Edinburgh

(*Received* 10 *July* 1957)

INTRODUCTION

In previous papers (Croghan, 1958*a*, *b*, *c*, *d*) various aspects of the osmotic and ionic regulation of *Artemia salina* have been described. It is also of interest to investigate the dynamic aspects of the ionic fluxes in *Artemia* using radio-active isotopes. This paper contains the results of these studies.

THEORETICAL CONSIDERATIONS

In an animal maintaining its ionic composition in a steady state the total flux of any ion is the same in both directions, the influx from the medium being balanced by an identical efflux. This flux can be expressed as mM. of ion entering (or leaving)/l. of haemolymph/hr.

At zero time a small amount of a radio-active ion is added to the medium. The amount added is so small compared with the amount of inactive ion already present that the steady state is unaffected. The haemolymph activity should rise and reach an equilibrium value asymptotically (cf. Fig. 1). The slope at any point on such an exchange curve is clearly

$$\frac{dy}{dt}=\frac{mx}{c_0}-\frac{my}{c_i},$$

where x is the activity (defined as the count rate for a unit volume under standard conditions) of the medium and y the activity in the haemolymph, c_0 is the concentration (mM./l.) of the ion in the medium and c_i the concentration in the haemolymph, and m is the total flux (mM./l. haemolymph/hr.) of the ion.

Integrating

$$y=\frac{c_i x}{c_0}\,(1-e^{-mt/c_i}).$$

Now $c_i x/c_0=z$ (the activity of the haemolymph when the exchange is complete). Substituting and solving

$$mt=c_i \log_e [z/(z-y)].$$

On plotting $2{\cdot}3c_i \log_{10} [z/(z-y)]$ against t a straight line should be obtained, the slope of which is the total ion influx (m). If the course of the exchange is not followed to effectively infinite time, z can be calculated from $c_i x/c_0$.

Similar information can also be obtained from the converse type of experiment. The animal is soaked in the radio-active medium, and the rate of loss of the active

ion is then studied in an inactive, but otherwise similar, medium of effectively infinite volume. The rate of fall of haemolymph activity is clearly

$$\frac{dy}{dt} = -\frac{my}{c_i}.$$

Integrating

$$y = e^{-mt/c_i}.$$

On plotting $\log_{10}(y/z)$, where z is the haemolymph activity at the start of the experiment, against t a straight line falling with time should be obtained (cf. Fig. 2). When y/z has fallen to e^{-1} (0·37) of its original value, $m = c_i/t$. Thus the total ion efflux (m) can be easily found.

In the above analyses the animal is treated as a one-compartment system. This is justified by the fact that the volume of the haemolymph is apparently a large fraction of the total volume of the animal (Croghan, 1958*b*).

MATERIAL AND METHODS

Adult *Artemia* as described previously (Croghan, 1958*a*) were used. The experiments were done within the temperature range 19–24° C.

The isotopes ^{24}Na and ^{82}Br were obtained from Harwell in the form of neutron-irradiated $NaHCO_3$, NH_4Br, or NaBr. Standard counting equipment was used: a G.E.C. G.M.4 tube mounted in a lead castle, and Dynatron power pack, probe unit and scaler.

For the influx experiments groups of animals in 150–200 ml. of medium were used. At zero time about 50 mg. of an active salt were added. The amount added was insignificant compared with the amount of salts already present in the medium, and thus would not have affected the steady state. After various time intervals animals were pipetted from the medium, rinsed quickly in distilled water and dried with filter-paper, and the haemolymph was collected as described in Croghan (1958*b*). A haemolymph sample was measured out in a micropipette (vol. *c.* 1 μl.), transferred to a drop of water on a small planchet and dried under an electric fire. The count rate was then determined. A single animal gave enough haemolymph for a determination. A sample of the medium was also counted.

In the efflux experiments animals were 'loaded' by soaking them overnight in an active medium similar to the media used for the influx experiments, and then the rate at which activity was lost from the animals when they were washed in an inactive, but otherwise closely similar, medium was determined. In some experiments the loaded animals were transferred to a large volume of inactive medium and after various time intervals haemolymph samples were obtained and counted as in the uptake experiments. In most of the efflux experiments using ^{24}Na a simpler procedure was used. About twenty animals were pipetted into a thin-walled Pyrex tube (9 mm. diam.). The Pyrex tube had rubber bungs at both ends through which passed short lengths of 2 mm. diam. glass tubing. To the outer ends of these were attached fine rubber tubes that led out to a reservoir and to a waste bottle. The outflow tube had a bolting silk cap to prevent animals being carried through. The

tube was clamped in a V-saddle close under the window of the G.M. tube, and inactive medium was continuously passed through (*c.* 5 ml./min.). All the difficulties of taking haemolymph samples at intervals and counting them individually are avoided. One is working with the same large group of animals continuously and can measure their total activity at close time intervals. The procedure is not only more convenient but, judging by the close linearity of the results, more accurate. It has the further advantage that the nature of the washing medium can be easily changed if desired during the experiment.

Most of the experiments were done with ^{24}Na and a few with ^{82}Br or with both isotopes together. The irradiated bromide contained also the short-lived isotope ^{80}Br, but by the time the actual counting was carried out the activity of this had become negligible. When both ^{24}Na and ^{82}Br were present the sample was counted directly and then recounted immediately afterwards with a brass filter (110 mg./cm.^{2}) between the sample and the G.M. tube. The filter was calibrated separately with ^{24}Na and ^{82}Br samples and gave good separation. It was simple to set up two simultaneous equations and to solve them to find the separate activity of both ^{24}Na and ^{82}Br in a mixed sample.

All results were corrected for resolving time, background and decay. Sufficient counts were obtained to keep the statistical error well under $\pm 3\%$.

Chemical analyses were also made on media and haemolymph samples using the methods previously described (Croghan, 1958*b*).

RESULTS

Sodium flux

The extent of exchange of haemolymph sodium was studied. Some animals were placed in a NaCl solution containing tracer amounts of ^{24}Na and left for 12 hr. Three samples of haemolymph (each obtained from six animals) and a sample of the medium were taken, and the activity and sodium concentration determined in each. Each sample was analysed in duplicate or triplicate. The results are summarized in Table 1. The specific activity (counts per minute (c.p.m.)/mM. Na) of the haemolymph had risen and reached a value which, within the limits of accuracy, is the same as that of the medium. Thus the haemolymph sodium is rapidly and completely exchangeable with that of the medium.

TABLE 1. *Exchange of haemolymph sodium*

Material	c.p.m./unit volume	mM. Na/l.	c.p.m./mM. Na
NaCl solution	4272	592	7·22
Haemolymph samples	1198	164	7·30
	1180	160	7·37
	1162	162	7·20

The rate of uptake of ^{24}Na has also been studied in animals adapted to media ranging from 10 to 600% sea water. In these media the animals were in a steady

state. A little $^{24}NaHCO_3$ was added to the medium, and after various time intervals samples of haemolymph were taken from individual animals and their activity determined. A typical uptake curve is plotted in Fig. 1, which is for animals in sea water. The fluxes were calculated and expressed as mM. Na/l. of haemolymph/hr.

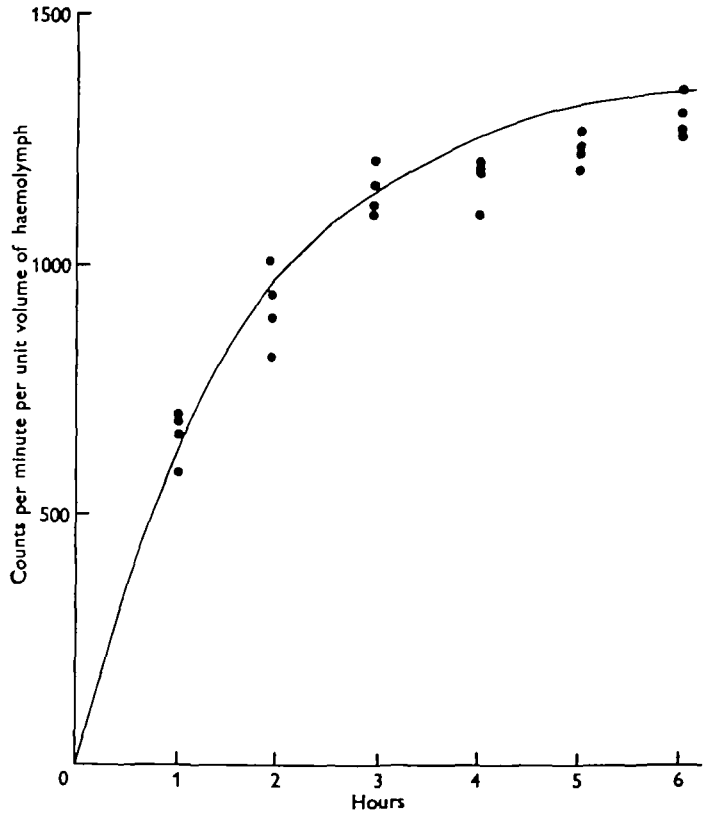

Fig. 1. Uptake of ^{24}Na from sea water. Each point represents a single animal.

The accuracy of the values from the more concentrated media tends to fall since the sodium concentration in the haemolymph is very much less than in the medium and thus the counting rate of the haemolymph samples is low. In addition, the influx appears much greater in the more concentrated media, making it more difficult to obtain accurate values.

The rate of loss of ^{24}Na has also been studied in animals adapted to media of various concentrations. In most of these experiments the total activity remaining in a group of animals while inactive medium was flowing past was determined at intervals. An example of a washing-out curve is given in Fig. 2, which is for animals being washed with sea water. On the assumption that all the body sodium is in the haemolymph, the fluxes were calculated and expressed as mM. Na/l. haemolymph/hr.

As both the influx and efflux experiments were carried out under steady-state conditions, the results should be the same. All the results are plotted in Fig. 3.

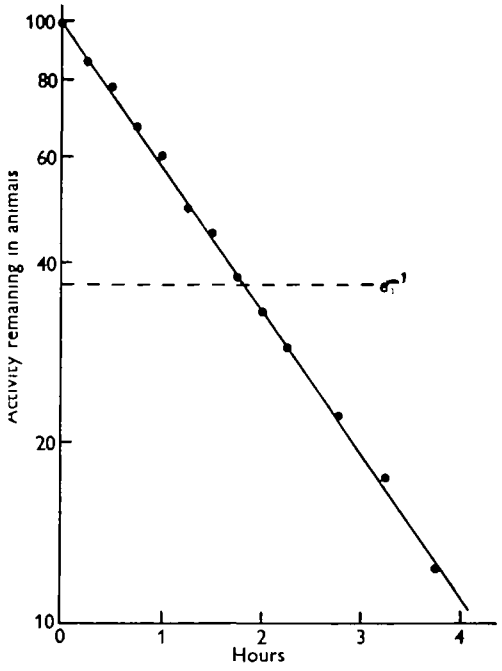

Fig. 2

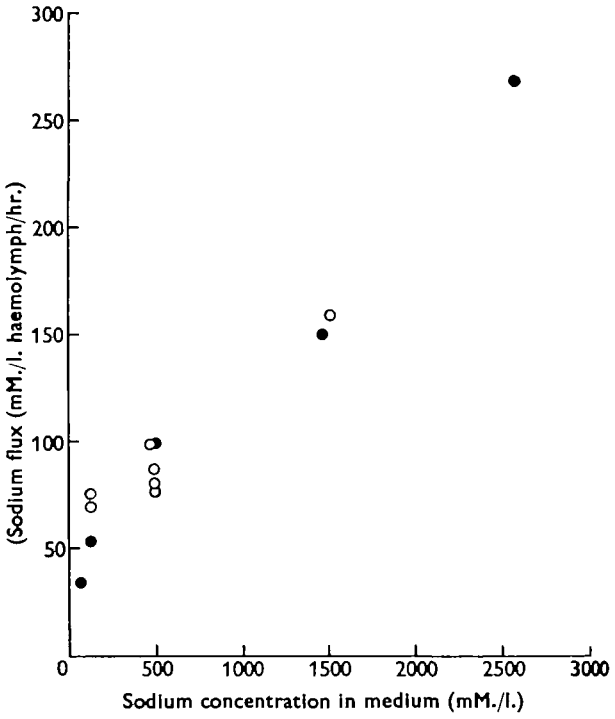

Fig. 3

Fig. 2. Loss of ^{24}Na from a group of animals washed with inactive sea water.

Fig. 3. Relation between the total sodium flux and the sodium concentration in the medium. Closed circles are influx experiments and open circles are efflux experiments.

Chloride flux

An attempt has been made to measure chloride flux using ^{82}Br as a label. The extent of exchange of bromide with chloride was studied. Some animals were transferred to sea water containing a small amount of $NH_4{}^{82}Br$. After 27 hr. two samples of haemolymph (each obtained from nine animals) and a sample of the medium were taken, and the activity and halide concentration determined in each. All analyses were done in duplicate. The results are summarized in Table 2. The specific activity (c.p.m./mM. halide) of the haemolymph had risen rapidly to a value which, within the limits of accuracy, is the same as that of the medium. In a further experiment animals were transferred from sea water to a NaBr solution containing some ^{82}Br. No chloride was present. After 27 hr. a haemolymph sample (obtained from seven animals) and a sample of the medium were taken and analysed as before. The results are also summarized in Table 2. They indicate that the haemolymph chloride is rapidly and completely exchanged with, and replaced by, bromide. These animals were still quite active.

Since the ratio Br/Cl in the haemolymph rapidly becomes the same as in the outside medium it is reasonable to consider that ^{82}Br is a satisfactory label for

TABLE 2. *Exchange of haemolymph chloride with bromide*

Material	c.p.m./unit volume	mM. halide/l.	c.p.m./mM. halide
Sea water	8430	585	14·40
Haemolymph samples	2170	147	14·75
	2260	152	14·85
NaBr solution	5610	514	10·90
Haemolymph sample	1254	117·5	10·70

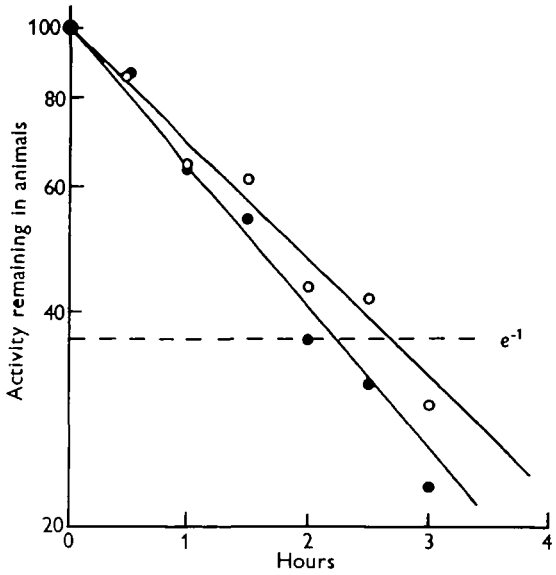

Fig. 4. Loss of ^{24}Na and ^{82}Br from animals washed in inactive NaCl solution. Closed circles refer to ^{24}Na and open circles to ^{82}Br.

chloride, and the results already quoted show that the haemolymph chloride exchanges rapidly and completely with that of the medium.

As the significance of a comparison of the fluxes of halide and sodium is much greater if they can be determined at the same time on the same group of animals, both ^{82}Br and ^{24}Na were used together. 30 mg. each of $^{24}Na^{82}Br$ and $^{24}NaHCO_3$ were added to 100 ml. of sea water. Animals were left in this medium overnight. The animals were then transferred to an inactive 500 mM./l. NaCl solution (closely isotonic with sea water). At intervals samples of the haemolymph (each obtained from five animals) were taken. The separate activities of ^{82}Br and ^{24}Na were determined in each sample. The residues of the haemolymph samples were then all pooled and the sodium and chloride concentrations were determined. The fall of haemolymph activity is plotted in Fig. 4, and the results are summarized in Table 3. The fluxes of both ions are very fast and of the same order, but the chloride flux is lower than that of sodium. It would be unwise to claim too much significance

for this difference, as there is always the possibility that the rate of bromide exchange is not quite the same as that of chloride.

TABLE 3. *Efflux of sodium and bromide from the haemolymph*

Ion	Time constant (hr.)	Haemolymph concentration (mM./l.)	Total efflux (mM./l. haemolymph/hr.)
Na	2·25	172	76·5
Cl	2·68	153	57

Ion influx in ligatured animals

Some animals from a sea-water medium were prevented from swallowing by being ligatured at the neck and anus with fine strands teased out of bolting silk. They were kept overnight in sea water. Then both unligatured and ligatured animals were transferred to a 516 mM./l. NaCl solution containing a little ^{24}Na and ^{82}Br. Haemolymph samples from individual animals were counted to obtain the separate activities of ^{24}Na and ^{82}Br in the haemolymph. The haemolymph residues were then pooled to form two samples: one from unligatured animals and the other from ligatured animals, and the sodium and chloride concentrations were determined. The ^{24}Na and ^{82}Br activities were also determined on a sample of the medium. There was no significant difference of sodium and chloride concentrations in the haemolymph as between the ligatured and unligatured animals. To make the ^{24}Na and ^{82}Br results comparable the relative activity of the haemolymph, $c_o y/x$, where y is the activity in the haemolymph, x the activity in the medium and c_o is the concentration of NaCl in the medium, is plotted in Fig. 5. There appears to be no significant difference between the two groups of animals.

Sodium efflux in sodium-free media

Animals were loaded by soaking in sea water containing ^{24}Na and the total activity remaining in a group of animals as inactive medium was flowing past was determined at intervals. In the experiment recorded on Fig. 6, the active animals were first washed with inactive sea water. Then the washing medium was changed to an erythritol solution (12·8%, closely isotonic with sea water). Later the washing medium was changed back to sea water. In the erythritol solution there was a very sharp sudden decrease in the efflux. The efflux increased sharply again when the medium was changed back to sea water. A similar sharp decrease in efflux has also been seen in distilled water. In similar experiments (Fig. 7) animals from a 25% sea-water medium (closely isotonic with the haemolymph) were loaded with ^{24}Na and then washed with inactive 25% sea water. On changing the washing medium to distilled water, there was again the sudden decrease in efflux.

Exchange of potassium

As high concentrations of potassium are rapidly toxic to *Artemia*, and as there appears to be a competitive effect between potassium and sodium ions (Croghan, 1958*a*), it was considered of interest to study the potassium exchange. Animals

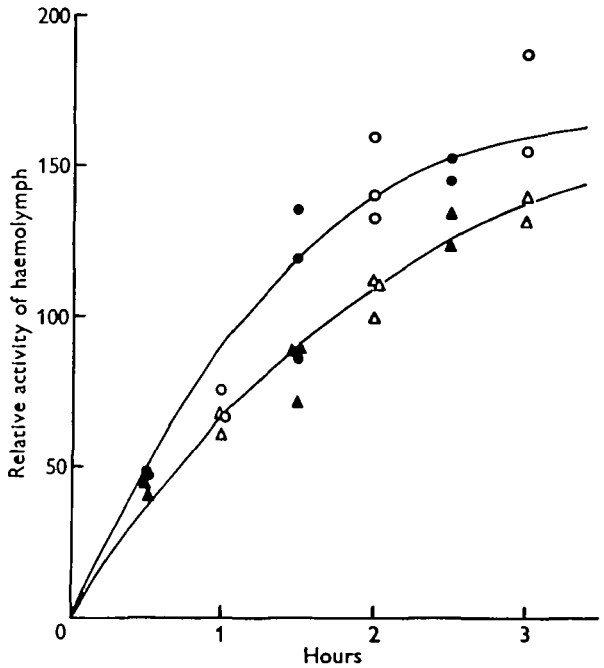

Fig. 5. Uptake of ^{24}Na and ^{82}Br from a NaCl solution. Uptake of ^{24}Na by unligatured animals, ●; uptake of ^{24}Na by ligatured animals, ○; uptake of ^{82}Br by unligatured animals, ▲; uptake of ^{82}Br by ligatured animals, △.

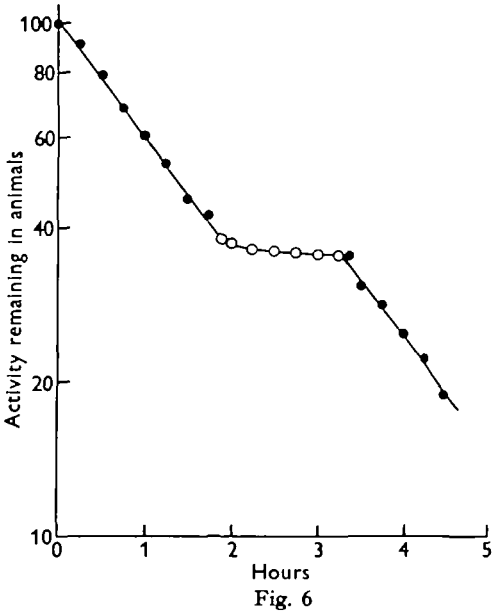

Fig. 6

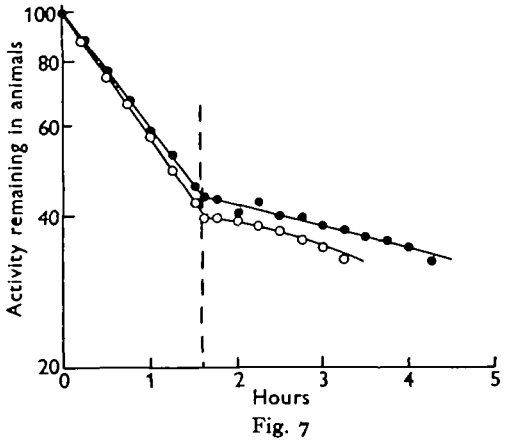

Fig. 7

Fig. 6. Loss of ^{24}Na from a group of animals washed with inactive media. Washing medium was sea water during time represented by closed circles and erythritol solution during time represented by open circles.

Fig. 7. Loss of ^{24}Na from two groups of animals (closed and open circles) washed with inactive media. Washing medium 25 % sea water until time represented by vertical broken line, when washing medium was changed to distilled water.

were taken from a sea-water medium and transferred to a 550 mM./l. solution of potassium benzenesulphonate. Haemolymph samples (each obtained from six to eight animals) were taken at the start and at 30 min. intervals, and the osmotic pressure, sodium, potassium and chloride concentrations were determined. By 30 min. all the animals were very moribund. The results are summarized in Fig. 8. All that is happening is a fairly rapid 1:1 exchange of potassium for sodium. The rapid rise in haemolymph potassium concentration easily explains the rapid toxicity of potassium rich media.

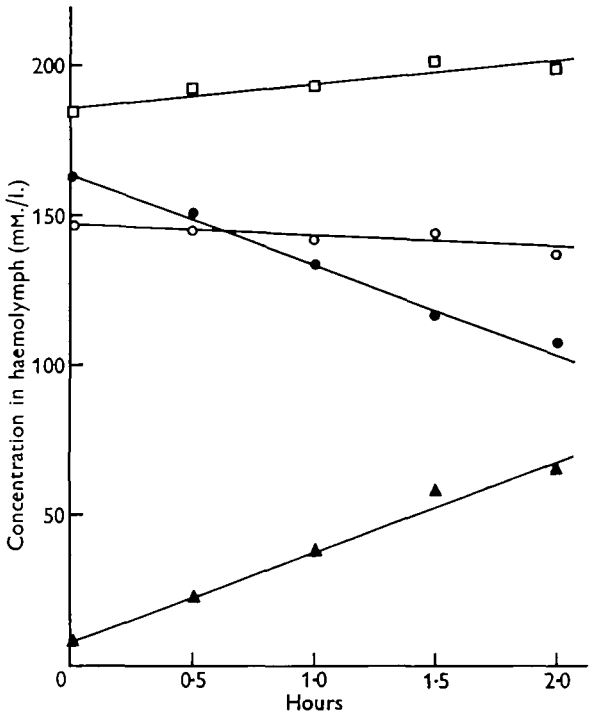

Fig. 8. Changes in haemolymph composition in a solution of potassium benzenesulphonate. Osmotic pressure, □; sodium concentration, ●; chloride concentration, ○; potassium concentration, ▲.

DISCUSSION

The first point of interest in the results is the extremely fast flux that has been found. We may compare these results from *Artemia* (weight about 7–10 mg. each) with those that have been found by tracer methods in other animals of comparable size. Holm-Jensen (1948) for *Daphnia magna* found an influx of 11 mM. Na/l. haemolymph/hr. (5·5 mM. Na/kg. animal/hr.), and for the little teleost *Lebistes reticulatus* an influx of 1·4 mM. Na/kg. animal/hr. From the data of Treherne (1954) it is possible to calculate that for *Aedes aegypti* the influx is about 1·1 mM. Na/l. haemolymph/hr. These other species are all freshwater forms. The flux in *Artemia* is enormously faster than in these other species.

It is of interest to consider the significance of the high ionic flux in *Artemia*. The sodium flux increases rapidly as the external concentration is raised and this indicates that the fast flux is dependent on a high concentration of this ion in the medium. The simplest explanation of these steady-state exchanges is that they represent diffusion movements and balancing active mechanisms. In a permeable animal, in a medium of a different concentration from the haemolymph, the ratio of the passive diffusion fluxes in both directions is: $m_i/m_o = c_o/c_i$, where m is the passive flux and c the concentration. The difference between the two passive fluxes $(m_i - m_o)$ is the net amount of ion that enters per unit time and has to be actively transported against the concentration gradient in order to maintain the steady state. It can be seen that the flux should increase proportionally to the increase of the concentration in the medium. The net influx $(m_i - m_o)$ should also increase similarly. The minimum thermodynamic energy required to excrete actively an amount equal to this net influx is $RT \log_e (c_o/c_i)$ cal./M. moved from c_i to c_o. With the maximum rate of exchange found in *Artemia* ($m = 268$ mM. Na/l. haemolymph/hr.; $c_i = 274$ mM. Na/l.; $c_o = 2570$ mM. Na/l.) this work would equal 320 cal./l. haemolymph/hr. In more dilute media this work would be much less, and would be zero when $c_i = c_o$ whatever the actual flux. These results can be compared with the total energy available from respiratory metabolism. Kuenen (1939), Eliassen (1952) and Gilchrist (1954) have all given data on oxygen uptake in *Artemia*. Using a value of 0·69 ml. oxygen/g. animal/hr. from Gilchrist (1954), and assuming that all the measured flux represents diffusion and balancing active transport, it can be calculated that, even with the highest rate of exchange observed, the minimum thermodynamic energy for NaCl excretion is only about 6% of the total available metabolic energy. Active transport of this magnitude could thus be well within the metabolic capabilities of *Artemia*.

The more detailed studies, however, have raised objections to the obvious and straightforward interpretation outlined above. The ligaturing experiments showed that there was little difference in the influx of ^{24}Na and ^{82}Br as between ligatured and unligatured animals. This strongly suggests that most of the rapid ionic fluxes are occurring across the outer surface of the body (branchiae?), and not appreciably across the gut epithelium. This is curious as it has been claimed (Croghan, 1958*d*) that most of the net entry of NaCl into the haemolymph takes place across the gut epithelium. This suggests that the flux across the external surface is so fast that it masks the contribution of the gut, and also raises doubts as to whether this flux across the external surface is really a simple diffusion process.

Further doubts arise when we consider the effect of changing the washing medium during efflux experiments. When the medium is changed from sea water or 25% sea water to a sodium-free medium the sodium efflux immediately drops to a very low value. This indicates that for sodium efflux to occur sodium ions must be present outside. It suggests that under steady-state conditions the influx and efflux of sodium are not independent processes but are a closely coupled 1:1 exchange between the medium and haemolymph, i.e. some type of exchange diffusion system. It appears that potassium ions in the medium can to some extent substitute for

sodium in this exchange process. The competitive effect between potassium and sodium in the medium (Croghan, 1958*a*) also supports this view.

Ussing (1947) and Levi & Ussing (1948) used the term 'exchange diffusion' for a system in which a fast exchange of an ion between two compartments in which it was not in electrochemical equilibrium could occur as a purely passive process (i.e. no work is done and no energy is required). They visualized a membrane impermeable to the ion concerned and containing scattered particles of an ion-exchange material with a high affinity for this ion. These particles move thermally between the inside and outside of the impermeable membrane. Ions attached to the exchange particles could exchange with the ions present in the solutions on either side of the membrane. The fluxes of this ion in both directions are not independent processes and a 1:1 exchange of this ion between the compartments on either side of the membrane should occur. It is not necessary to presuppose the existence of any special carrier for the above process, and any charged component of a cell membrane capable of thermal oscillation or rotation could presumably have this property.

Consider also a system in which, although the concentration of an ion is different, the electrochemical potential of an ion is the same on both sides of a membrane permeable to this ion alone (i.e. a Donnan equilibrium). If one of these ions moves thermally across the membrane, another similar ion must move across in the opposite direction, otherwise the system would move away from equilibrium. The fluxes of these ions in both directions are not independent and a 1:1 exchange of this ion would occur between two compartments in which the concentration of this ion was different. Such a situation would occur, for example, with the exchange of potassium between cell fluids and the cell medium. A model having such properties can be set up by separating two different concentrations of an electrolyte by a membrane of an ion-exchange material (i.e. either selectively anion or cation permeable). Such a model is likely to have biological analogues, as fixed charges are likely to be quite common in biological membranes.

Whatever the details of the actual exchange diffusion process concerned, it would be expected from Mass Law considerations that the flux would be proportional to the concentrations of the exchanging ion on either side of the membrane. The flux would increase with the concentration of the external medium, and when this medium is replaced by one in which the exchanging ion is absent the flux into this medium would immediately fall to a low value.

It appears probable that the main part of the fast flux in *Artemia* is due to exchange diffusion, although it is difficult if not impossible to obtain absolutely unambiguous evidence in such a small closed system. A net diffusion and active ion transport must also be occurring, but, if this is relatively slow compared to exchange diffusion, its contribution to the total rate of ion exchange would be masked. The exchange of chloride in *Artemia* appears to be nearly as fast as that of sodium. It is probable that both these ions are undergoing exchange diffusion. The simplest picture is to imagine a membrane with a mosaic of separated exchange diffusion sites.

Exchange diffusion itself can have no significance or value to the living system,

for as all ions of a given type are identical, and as no energy is required, the system can have no awareness that these ions are rapidly exchanging. For this reason no selective influences will operate on exchange diffusion as such. Exchange diffusion may be regarded as a harmless by-product of the properties of cell membrane constituents. The possibility of exchange diffusion, however, complicates the interpretation of tracer studies, especially in such systems as small intact animals, and great care is needed in interpretation.

SUMMARY

1. The ionic fluxes between the medium and haemolymph have been studied in adult *Artemia salina*, under steady-state conditions using ^{24}Na and ^{82}Br.

2. Extremely high fluxes have been found, the flux increasing markedly in the more concentrated media.

3. The results are interpreted as indicating that both the sodium and chloride in the haemolymph are undergoing rapid exchange diffusion with the sodium and chloride in the medium.

I wish to thank Dr J. A. Ramsay, F.R.S., for his interest and advice. I also wish to thank the Department of Scientific and Industrial Research for a maintenance grant.

REFERENCES

CROGHAN, P. C. (1958*a*). The survival of *Artemia salina* (L.) in various media. *J. Exp. Biol.* **35**, 213.

CROGHAN, P. C. (1958*b*). The osmotic and ionic regulation of *Artemia salina* (L.). *J. Exp. Biol.* **35**, 219.

CROGHAN, P. C. (1958*c*). The mechanism of osmotic regulation in *Artemia salina* (L.): the physiology of the branchiae. *J. Exp. Biol.* **35**, 234.

CROGHAN, P. C. (1958*d*). The mechanism of osmotic regulation in *Artemia salina* (L.): the physiology of the gut. *J. Exp. Biol.* **35**, 243.

ELIASSEN, E. (1952). Energy metabolism of *Artemia salina* in relation to body size, season rhythms, and different salinities. *Univ. Bergen Årb. naturv. R.* **11**, 1.

GILCHRIST, B. M. (1954). Haemoglobin in *Artemia*. *Proc. Roy. Soc.* B, **143**, 136.

HOLM-JENSEN, I. (1948). Osmotic regulation in *Daphnia magna* under physiological conditions and in the presence of heavy metals. *Biol. Medd., Kbh.*, **20**, no. 11.

KUENEN, D. J. (1939). Systematical and physiological notes on the brine shrimp, *Artemia*. *Arch. néerl. Zool.* **3**, 365.

LEVI, H. & USSING, H. H. (1948). The exchange of sodium and chloride ions across the fibre membrane of the isolated frog sartorius. *Acta Physiol. Scand.* **16**, 232.

TREHERNE, J. E. (1954). The exchange of labelled sodium in the larva of *Aedes aegypti* L. *J. Exp. Biol.* **31**, 386.

USSING, H. H. (1947). Interpretation of the exchange of radio-sodium in isolated muscle. *Nature, Lond.*, **160**, 262.