Ecophysiological responses to salinity changes in selected euryhaline amphipods with special reference to *Gammarus duebeni*.

by

Stephen R.L.Bolt.

A thesis submitted to the University of Southampton for the degree of Doctor of Philosophy.

Department of Oceanography
The University
Southampton
S09 5NH
U.K.

June 1982

UNIVERSITY OF SOUTHAMPTON

Faculty of Science
Oceanography.

ABSTRACT

DOCTOR OF PHILOSOPHY

Ecophysiological responses to salinity changes in selected euryhaline amphipods with special reference to Gammarus duebeni.

by Stephen R.L. Bolt.

Haemolymph and external medium sodium concentrations have been investigated in three euryhaline amphipods, Gammarus duebeni, Chaetogammarus marinus and Gammarus locusta. These were subjected to various salinity regimes, including sudden and cycling salinity changes. The permeability to water of these three species was investigated in various salinity regimes using THO as a marker, and of the three species, only G.duebeni demonstrated large and significant changes in apparent permeability to water.

Combining the haemolymph and medium sodium concentrations with the permeability to water, the bulk flow into and out of the animals was calculated showing a variation of responses in the three species. G. duebeni exhibiting the smallest bulk flow and G.locusta the largest.

The heart rate of G.duebeni was investigated, demonstrating that the heart rate of G.duebeni was not correlated to the observed permeability changes.

The urine clearance rates in G.duebeni were measured using ^{51}Cr E.D.T.A. in order to calculate the water fluxes into the animals. Using the haemolymph and medium sodium concentration results and the water fluxes found using ^{51}Cr E.D.T.A., the theoretical water permeabilities of G. duebeni were calculated and compared with the values obtained using THO. This comparison showed a good similarity in the changes of permeability to water using the two techniques and hence supported the hypothesis that the changes in permeability noted in G.duebeni using THO are indeed real.

In order to investigate possible mechanisms for such large changes in permeability, the potential difference (P.D.) across the body wall of G. duebeni was artificially controlled and the permeability to water monitored. These results demonstrated that changing the P.D. across the body wall does not appear to alter the permeability to water.

Two species of arctic amphipod, Gammarus setosus and Onisimus litoralis were studied and compared with the three British species. These two species showed large changes in apparent permeability to water, although they did not demonstrate the same pattern of change as found in G.duebeni.

The ecophysiological responses of G.setosus and O.litoralis to oil was also studied, showing that these animals appear sensitive to physical contact with oil while remaining apparently unaffected by the presence of dispersed oil.

In conclusion, the five amphipod species studied have demonstrated that osmoregulatory responses are correlated to the ecology of the species, and that in the more euryhaline species, a varying permeability to water appears to be an important facet of the osmoregulatory mechanism.

---oOo---

CONTENTS

---oOo---

ACKNOWLEDGEMENTS

I would like to express my appreciation to my supervisor, Professor A.P.M.Lockwood, for his support both during my research and in the preparation of this thesis.

My special thanks to Mr M.Dawson with whom most of the early work on the water permeability and haemolymph Sodium concentrations on Gammarus duebeni was carried out. The microprocessor controlled salinity system was developed in conjunction with Professor A.P.M.Lockwood, Mr M. Dawson and Mr N.W.Jenkinson. I would also especially like to acknowledge the invaluable help of Mr N.W.Jenkinson who patiently corrected my errors during my early attempts at microprocessor programming and who later modified the microprocessors to suit my varied requirements. My thanks also to the technical staff at the Department of Oceanography for their continued support.

The expeditions to the Canadian Arctic were financed jointly by The Winston Churchill Memorial Trust, The Natural Environment Research Council, Southampton University Convocation & Southampton University, Oceanography department. I should especially like to thank Dr N. Snow for his help and advice before and during my trips to the Canadian Arctic.

Finally, I should like to thank Mrs J. Watson for typing this thesis.

This thesis was supported by a Natural Environment Research Council studentship, Grant No. GT4/78/ALS/29.

---oOo---

LIST OF FIGURES

---oOo---

GENERAL INTRODUCTION

Fully marine invertebrate organisms inhabit a stable environment where the external medium is comparable with the internal osmotic concentration of their body fluids, causing few osmotic problems for the cells. Fresh water animals must maintain an osmotic gradient across the body wall so that the cells can operate at an acceptable ionic concentration. Organisms tolerating a variable salinity environment (euryhaline) face the unique problem of having to vary their regulatory responses as the external medium changes. These animals must be able to maintain their body fluids within concentration limits acceptable to the cells, which themselves must be capable of adapting to a range of body fluid concentrations. Euryhaline animals face loss of water and uptake of ions when the body fluids are hypotonic to the external medium and the loss of ions and uptake of water when the body fluids are hypertonic to the medium. This problem is heightened in small animals with a large surface area to volume ratio, where small changes in volume would cause large changes in concentrations. The development of a highly impermeable body surface to water and ion fluxes to alleviate this problem is impracticable for an aquatic animal which respires through part or all of the body surface since a highly impermeable surface would restrict gaseous exchange. Euryhaline animals regulate the loss and uptake of water and ions by actively transporting ions at the body surface and by controlling urine volume. Some species of animals in fresh and brackish water are able to control urine concentration, restricting ion loss to the external medium. Active transport of ions is energetically expensive. It would therefore be advantageous for fresh and brackish water animals to have reduced permeability to water and ions limiting ion loss and water uptake without too much reduction in respiratory efficiency. Animals experiencing salinity changes would benefit from the ability to restrict the passage of water and ions when large gradients between body fluids and external medium are present, and from relief of these restrictions when they are isotonic to the medium. This would necessitate a mechanism controlling the permeability

of body surface relating to the concentration gradient between the haemolymph and external medium.

Thus small euryhaline animals are faced with the problem of maintaining a relatively stable internal environment when exposed to an often unpredictable external medium concentration. This thesis is largely concerned with the possibility that some euryhaline amphipod species are able to vary the permeability to water in order to survive successfully in an osmotically stressful environment.

CHAPTER 1

HAEMOLYMPH CONCENTRATION AND APPARENT PERMEABILITY IN VARYING SALINITY CONDITIONS OF GAMMARUS DUEBENI, CHAETOGAMMARUS MARINUS and GAMMARUS LOCUSTA

A number of previous studies have examined the water fluxes in a range of marine (stenohaline) and brackish (euryhaline) species.

Smith (1967) reported a decline in the uptake of D_2O in the crab Rhithropanopeus harrisi when the salinity of the medium was reduced. A similar effect had previously been noted in the polychaete Nereis diversicolor by Jørgensen and Dales (1957).

Rudy (1967) reported no change in D_2O flux in the shore crab Carcinus maenas with varying salinity, but later work by Smith (1970) showed a change in Carcinus maenas similar to that noted in Rhithropanopeus harrisi. These species were apparently less permeable in fresh water when under osmotic stress than in sea water when relatively little stressed. However, there was no conclusive proof that the observed changes in D_2O fluxes were demonstrating actual changes in permeability.

Lockwood, Inman and Courtenay (1973) reported large changes in the half-time of exchange of THO when the euryhaline amphipod Gammarus duebeni was acclimated to varying salinities. They tentatively suggested on the basis of comparisons between flux studies and urine production rates that the THO fluxes reflect genuine permeability changes. It is one of the main concerns of this thesis to present further evidence to establish the validity of the permeability changes found in G. duebeni.

Amphipods form an ideal group for such ecophysiological studies, and also other aspects of osmoregulation, as they cover the full range from fresh water to fully marine environments. Three species have been studied, G. duebeni as an extremely euryhaline organism, Chaetogammarus marinus slightly less so and Gammarus locusta as the least euryhaline. All three species however, can tolerate some degree of fluctuations in external media. They are small, easily collected and simply maintained in laboratory conditions.

G. duebeni is found in a wide range of salinities from fresh water streams (Hynes, 1954) to 60% - 70% salt water rock pools in Norway (Davenport, 1979). The present work was carried out on populations from Totton Marsh, Southampton, England, where the animals are found in small pools on a salt marsh. They are subjected to large salinity changes, ranging from fresh water to 32‰ salinity. G. duebeni on Totton marsh are found in three localities (i) in drainage creeks where they are subjected to cycling salinity conditions as they are covered at each high tide, (ii) in near fresh water at the upper edge of the marsh in fresh water drainage channels where they only encounter saline conditions on the top of Spring tides, (iii) small pools at the extreme high water mark, where the animals are covered at Spring tides but can be subjected to extremes in salinity due to evaporation or precipitation between high Spring tides.

At high water during Spring tides the animals become widely distributed over the marsh, concentrating back to the pools as the tidal range decreases. This movement of G. duebeni during Spring tides ensures that physiologically distinct populations of G. duebeni are unlikely to occur on Totton marsh, even though several distinct habitats do exist. It also illustrates the necessity for flexibility in the ecophysiological responses in G. duebeni.

Chaetogammarus marinus (previously Marinogammarus marinus) is located well into estuarine reaches. (Spooner, 1947). The population used for the present study came from the intertidal zone on the muddy shore of Hayling Island, (near Portsmouth, Hampshire) under clumps of Fucus sp. The populations are covered during high tide, but are intermittently subject to fresh water run off and precipitation at low tides. C. marinus is capable of surviving low salinities for short periods, and was chosen as an example of an amphipod less euryhaline than G. duebeni.

Gammarus locusta is recorded by Spooner (1947) as being a species occurring at the seaward end of estuaries. It is generally considered to be less euryhaline than C. marinus, however, the population used for the present study

was found co-existing with the population of C. marinus under Fucus sp. on the intertidal zone on Hayling Island. This species was selected as being relatively stenohaline though still tolerating some measure of short term dilution to the external medium.

These three species, G. duebeni, C. marinus and G. locusta, show varying degrees of tolerance to salinity changes which are investigated in the present work by comparing and contrasting their ecophysiological responses to salinity stress.

MATERIALS and METHODS

Determination of Sodium concentration.

(i) Haemolymph collection.

The animals were removed from the experimental chamber, blotted dry and held firmly between thumb and forefinger. Haemolymph was extracted by means of a drawn out Pasteur pipette the tip of which was inserted into the dorsal surface. The sample was immediately transferred to liquid paraffin to minimise evaporation. It was possible to remove 1-5 µl of haemolymph from a 100mg animal. Slight pressure to the animal by squeezing gently with thumb and forefinger increased the volume of haemolymph collected. The animals were not used for any further experiments after haemolymph collection even though approximately 50% survived.

(ii) Sodium determination.

Aliquots of haemolymph (usually 1µl) were taken from the droplet under paraffin using a disposable microcap pipette and added to 5 or 10ml de-ionized water. These samples could be sealed and stored prior to analysis using an emission flame spectrophotometer (Pye Unicam SP900 or SP90). Sodium concentrations were determined by using a standard curve obtained using similarly treated known concentration of sodium chloride.

A microprocessor system R.C.A. (COSMAC CDP 1802) evaluation kit was developed for use with the SP900 to eliminate the necessity of diluting the samples. A constant-head device ran de-ionized water through the Spectrophotometer (Fig. 1.1) and an aliquot of sample (undiluted) was introduced directly to this de-ionized water. The signal from the spectrophotometer deviated from the baseline as the sample was analysed, returning to the baseline when the measurement was completed. The microprocessor integregated the area under the resultant curve to give a value which could be compared to a standard NaCl solution which had been introduced to the spectrophotometer in the same way as the sample. The

microprocessor was programmed to take repeated baseline readings initiating the integration when the signal from the SP900 deviated from the baseline and ending the integration when the signal returned. By repeatedly upgrading the baseline value, the microprocessor minimized inaccuracies introduced from baseline drift, displaying the result of the integration on to a teletype or V.D.U. (Visual Display Unit).

The microprocessor-controlled emission spectrophotometer system could be easily modified for use on the SP90 or any other similar spectrophotometer. This system omitted the dilution stage necessary in manual operation of the SP900, thereby increasing the speed of operation and the accuracy of the machine. No internal modification of the SP900 was necessary for this system. A program listing is given in the appendix.

Determination of Osmotic concentration.

Osmotic concentration of the haemolymph was determined using the Ramsey and Brown (1955) Cryoscopic method.

The original technique was modified by the use of a cooling coil to chill the chamber and the application of R/S freezing compound (aerosol) to freeze the sample prior to insertion in the cold alcohol bath. This procedure eliminated the use of dry ice as a coolant.

Microprocessor controlled salinity system.

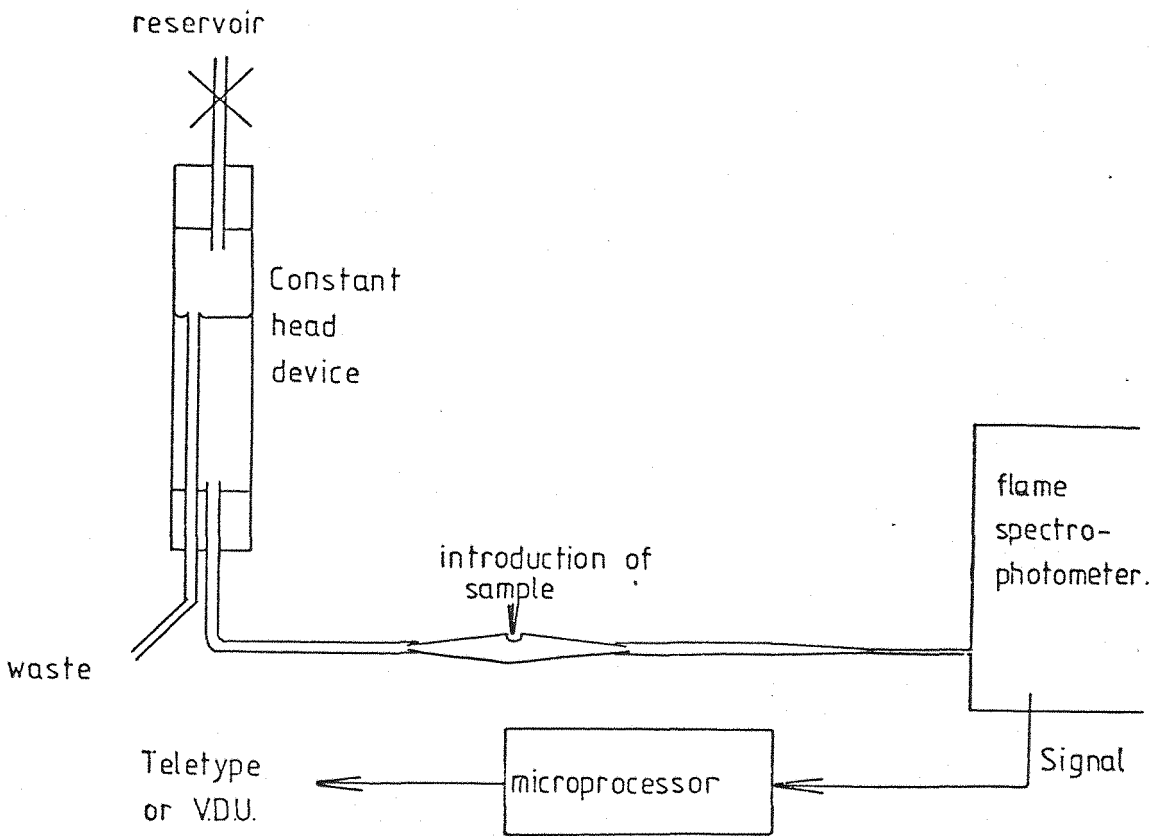
Lockwood and Inman (1979) developed a mechanical cycling salinity system. Their cycle was produced by rotating telfon discs creating a series of exponential curves which resulted in a curve with periods of flattening out during the increases and decreases in salinity.

These irregularities imposed limitations on the interpretation of results in experiments where animals were exposed to this salinity regime.

To eliminate these difficulties, Lockwood, et al (1981) have developed a microprocessor-controlled system based on the COSMAC CDP 1802. This system consists of a

Fig.1·1

Microprocessor assisted flame spectrophotometer.



chamber, a microprocessor, and two valves to control the flow of fresh and salt water into the chamber. The microprocessor is programmed with the required salinity curve. Every 30 seconds the microprocessor compares the salinity in the experimental chamber with the value of the stored curve at that point in the cycle. As a result, it switches on the solenoid delivering either fresh water or salt water to the experimental medium. (Fig. 1:2)

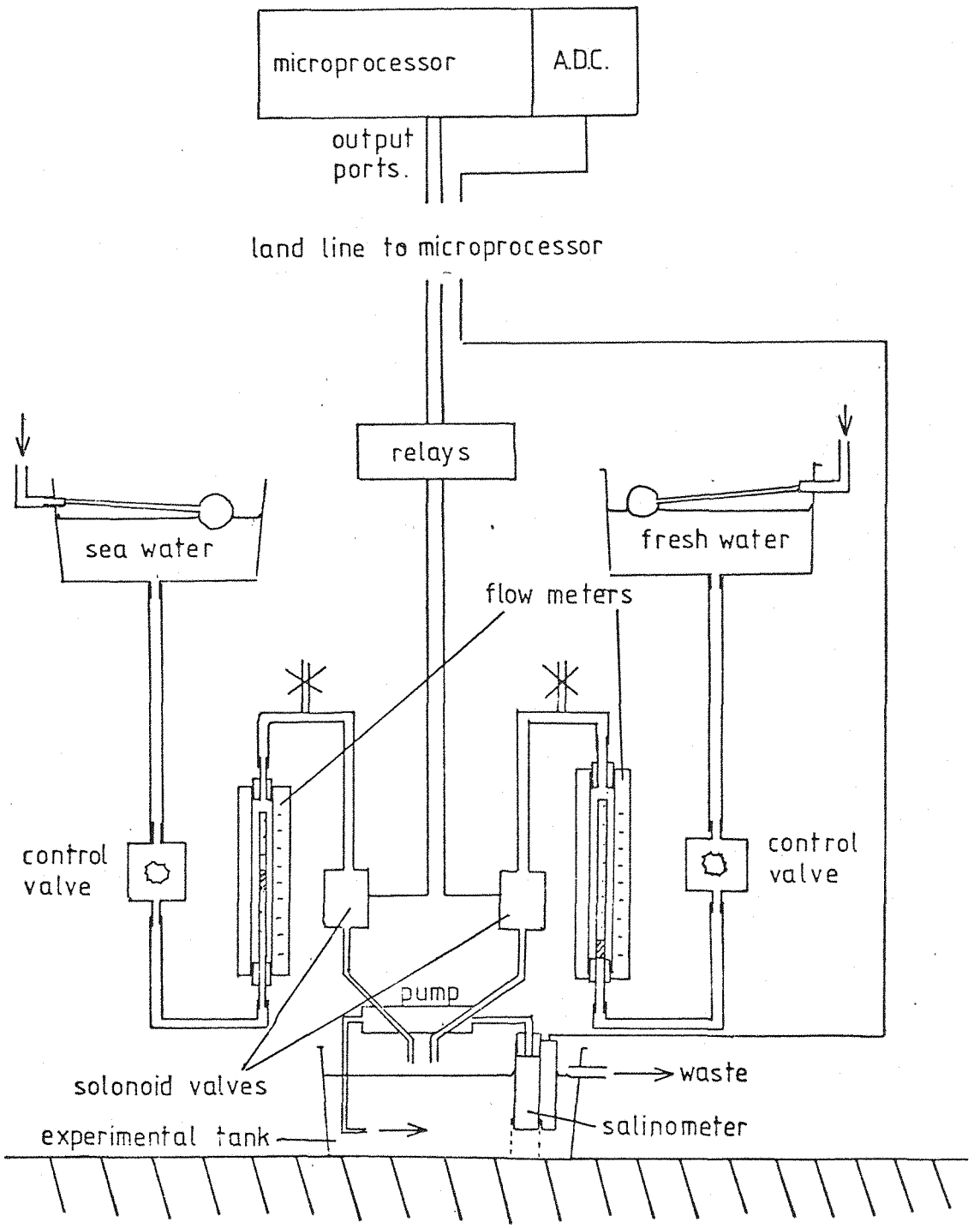
Mechanical salinity cycles often rely on accurately known, fixed flow rates into the experimental chamber. The advantage of a microprocessor-controlled feedback system is that the flow rates need only be maintained between arbitrary limits, the program automatically compensating for slight drifts in flow rates. By recording the salinity in the chamber on a suitable chart recorder any large changes in flow can be immediately noted and rectified.

Using a Mathematical model of the salinity in the chamber, assuming a fixed flow rate, fixed volume and perfect mixing, it was possible to predict the necessary flow rates and feasibility of the system before it was built. A comparison of the theoretical and actual curves shows a close similarity. (Figs. 1:3 & 1:4).

A volume of 4 litres was used in the experimental chamber necessitating an optimum flow rate of 120mls per minute through the chamber.

In the majority of cycling salinity experiments a sine wave curve was chosen for the lack of anomalies. In some experiments however, a saw-tooth curve was used to produce a linear increase and decrease in salinity. (Fig 1:4) The system could potentially be used to mimic actual salinity cycles measured in real situations, providing the change in salinity required is not too rapid.

Microprocessor controlled salinity system. Fig. 1·2



Mathematical prediction of the salinity cycle. Fig. 1·3

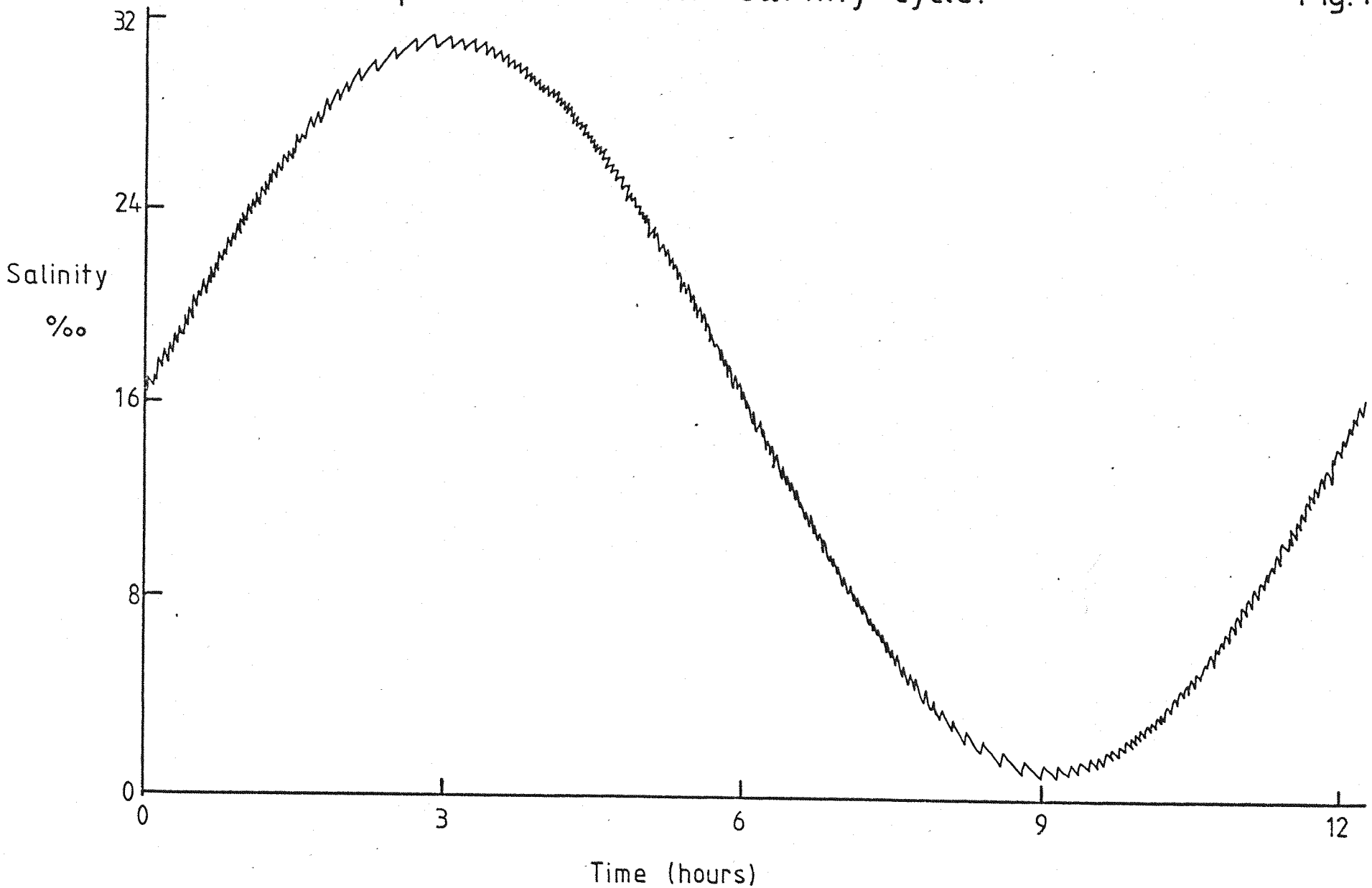


Fig. 1·4

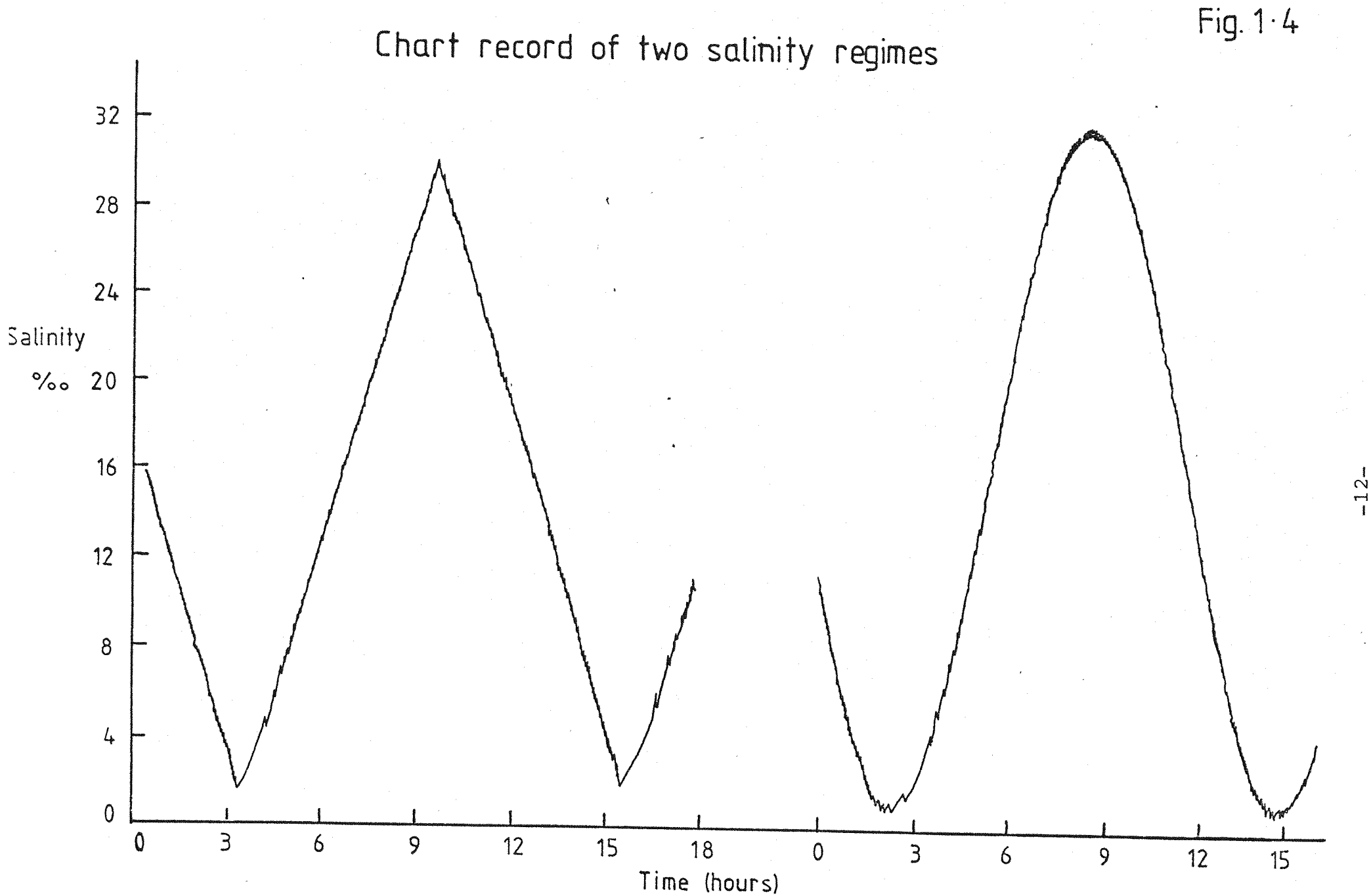


Chart record of two salinity regimes
Salinity ‰
32
28
24
20
16
12
8
4
0
0
3
6
9
12
15
18
0
3
6
9
12
15
Time (hours)

FLUX EXPERIMENTS

Tritiated water was used to determine water fluxes on the amphipods. Measurements of flux in both directions were made on animals in a steady state, but technical difficulties prevented outflux measurements in non-steady state systems. These methods were modified from Lockwood *et al*, 1973. Permeability to water of the amphipods was expressed as a half-time of exchange of tritiated water into or out of the animal.

(i) Influx

By comparing the amount of tritiated water taken up by the animal in a five minute loading period with the amount taken up when the animal is fully loaded and at equilibrium with the external tritiated water, the half-time of exchange of influx can be calculated.

Animals were transferred to tritiated water of the appropriate salinity and loaded for five minutes. They were then rinsed and individually unloaded in unlabelled medium for approximately 10 half-times of exchange (2hrs). Duplicate aliquots were taken, added to 4ml Liquid Scintillation cocktail, and counted in a Beckman Liquid Scintillation counter to give counts at time t (Ct). The animals were then re-loaded individually in the tritiated water for approximately 2 hours until equilibrium was reached. They were then rinsed and unloaded again. Duplicate aliquots were taken and counted to give counts at equilibrium (C_∞). During this experiment the only critical stage is the first five minute period of loading as it is during this period that the half-time of exchange is being measured. All the other stages are concerned with the animal when it is fully loaded or unloaded, making salinities unimportant provided that the water content of the animal remains constant.

It is possible to calculate the half-time of exchange using the following formulae, comparing the amount of tritium taken into the animal in 5 minutes at a given salinity (Ct) with that taken in at equilibrium (C_∞).

(1) $$k = \frac{1}{t} \times 2.3 \log_{10} \frac{C_\infty}{C_\infty - Ct}$$

(2) $$t_{\frac{1}{2}} = \frac{\log_e 2}{k}$$

Where k is the rate constant
Ct counts at time t
C_∞ Counts at equilibrium.

(ii) Outflux.

The outflux was measured by loading the animals with tritiated water at a given salinity, rinsing thoroughly, and transferring to unlabelled medium of required salinity. Samples were taken at intervals during unload (Ct) and when equilibrium (C_∞) was reached. This allows the amount of tritiated water left in the animal to be calculated by subtracting Ct from C_∞

This technique requires a considerable period of pre-loading before the $t_{\frac{1}{2}}$ can be derived. It would therefore be impractical to use this method in non-steady state experiments such as in the cycling salinity system, as the whole experimental chamber (4 litres) would need to be labelled with tritium, which would be running to waste at a rate of 120 ml per minute.

Animals were pre-loaded in tritiated water for approximately 10 half-times (2hrs). They were rinsed briefly in unlabelled medium and transferred to clean medium in a capped vial. Duplicate aliquots were taken at intervals until equilibrium had been reached.

The flux, as a half-time of exchange of tritiated water was calculated by plotting log (C_∞ -Ct). The half-time of exchange can be calculated using linear regression on the line y = mx + c where $t_{\frac{1}{2}}$ is the x co-ordinate corresponding to C-log2

where y = y co-ordinate
x = x co-ordinate
c = y intercept (constant)

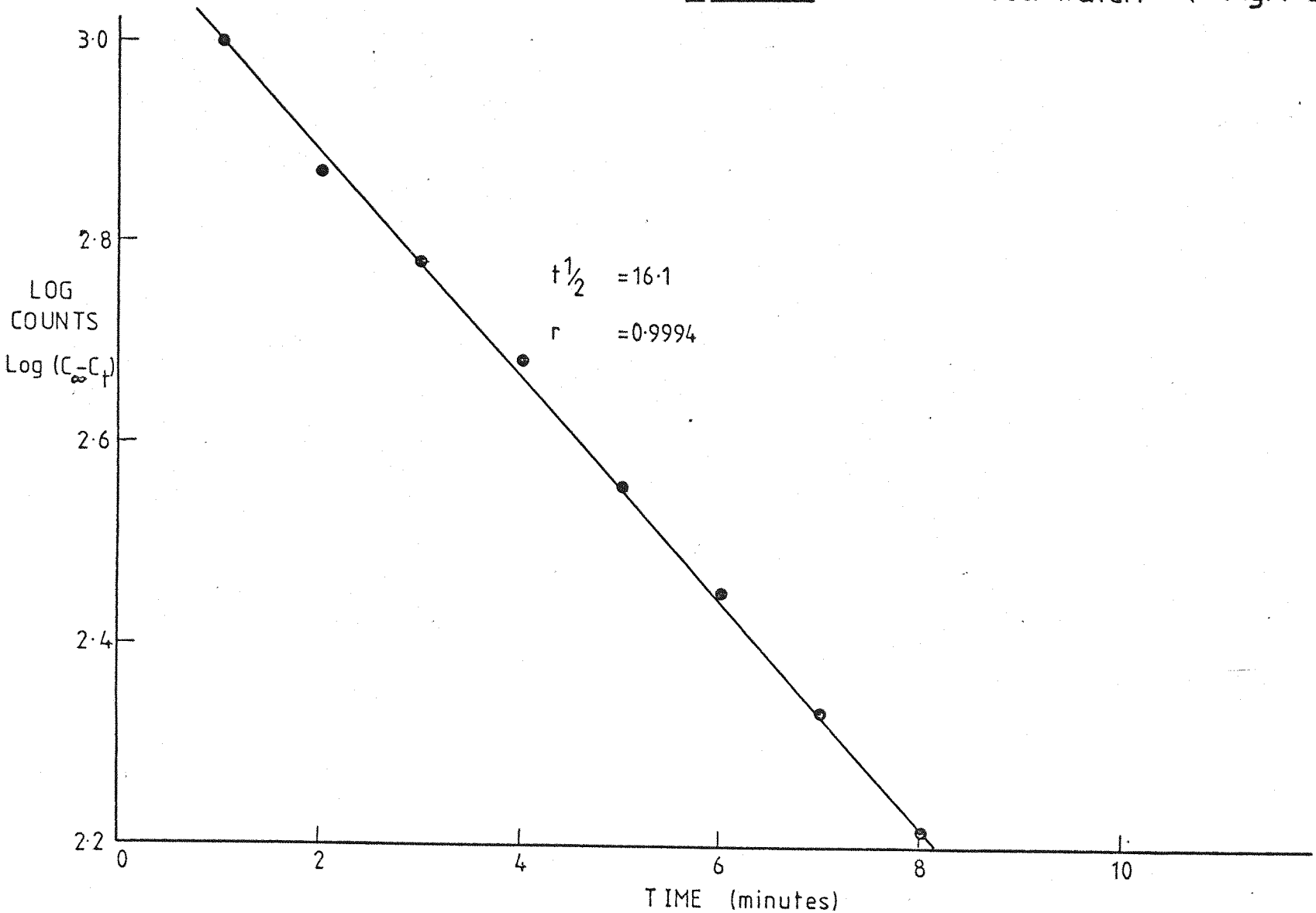
In outflux experiments where a large number of animals were used, it was practical to reduce the C_t samples to one duplicate, usually at t = 5 minutes. This was justifiable as

the regression coefficient of the log (C_∞ - Ct) against time was usually better than .99. (Fig. 1:5).

These techniques (Influx and Outflux) compare the counts at time t with counts at equilibrium. This enables counts per minute (cpm) to be used directly without the necessity of finding the efficiency of the counter using quench curves. It is assumed that as the sample taken at time t and at equilibrium is identically treated, the efficiency of the machine remains constant, thus eliminating the need to find disintegrations per minute (dpm).

During influx and outflux determinations, the vessels holding the animals were kept sealed as much as possible to minimise exchange of tritiated water with atmospheric water vapour. They were maintained, whenever possible, in a constant temperature water bath at 15°C.

Outflux from an individual G.duebeni in 100% sea water. Fig.1·5



RESULTS

Haemolymph concentrations and water fluxes

(i) Steady state.

Marine species usually have haemolymph fluids which are isotonic to the external medium. Organisms which are able to tolerate low salinities must be able to maintain their blood concentration above that of the surrounding environment. Beadle and Cragg (1940) examined the haemolymph concentrations of Gammarus duebeni acclimated to various salinities, demonstrating this animal's ability to maintain its blood concentration hypertonic at low salinities and isotonic or slightly hypertonic at high salinities (Fig.1:6). Gammarus duebeni is thus a hypertonic/isotonic regulator, contrasting with fully marine animals which, unable to regulate their body fluid concentration, are osmoconformers. Stenohaline animals are only able to tolerate a small dilution of the sea water before the drop in body fluid concentration becomes fatal.

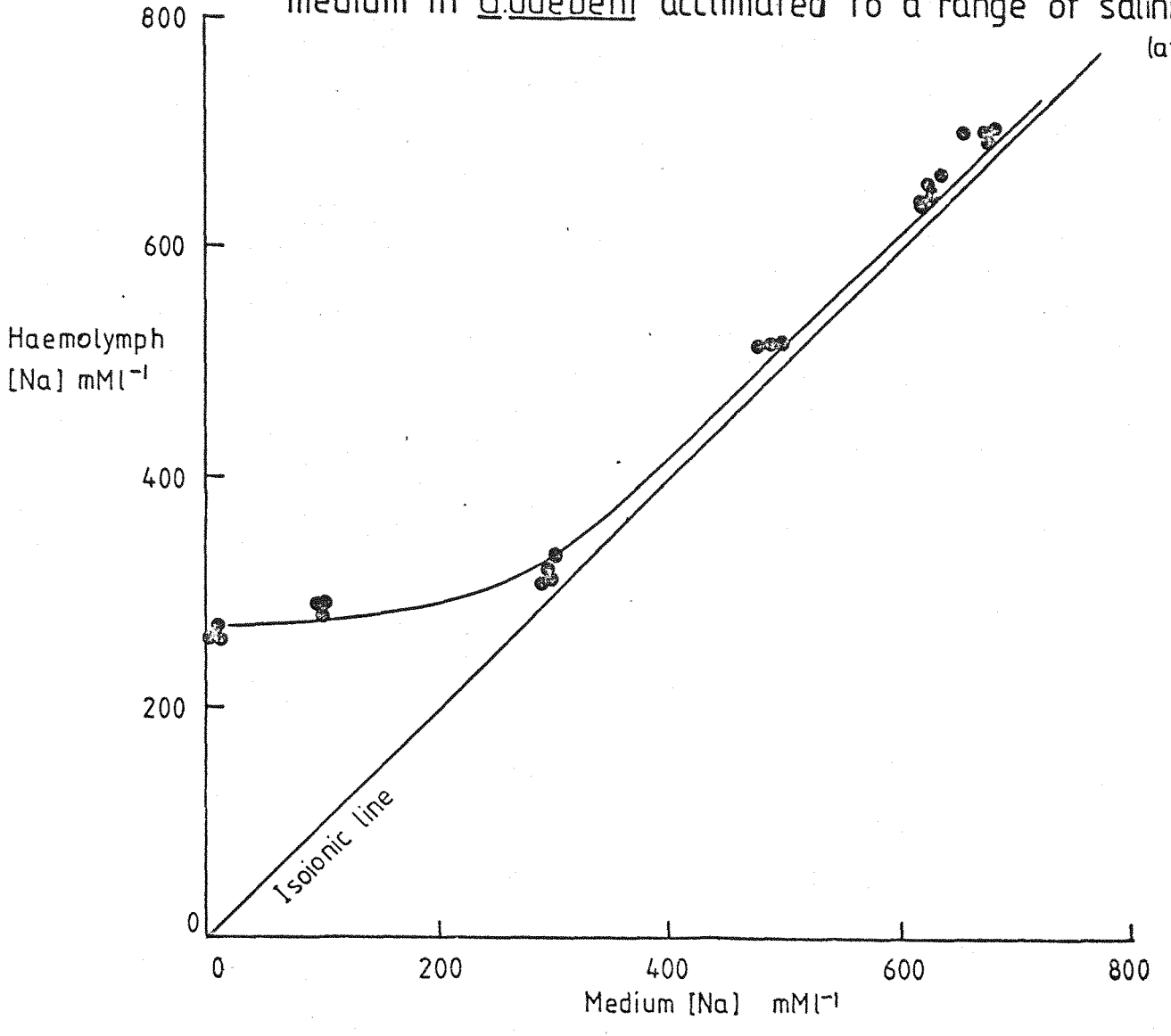
Chaetogammarus marinus and Gammarus locusta inhabit the intertidal zone where they are not subjected to long periods of low salinity, however precipitation and fresh water run-off at low tide necessitates their ability to tolerate low external concentrations for periods of up to four or five hours. Haywood (1970) looked at haemolymph concentrations of C. marinus and G. locusta acclimated to various salinities (Figs. 1:7 & 1:8), demonstrating that these species show a hypertonic/isotonic regulation similar to that found in G. duebeni. G. locusta was unable to maintain its body fluids strongly hypertonic in a low salinity medium and both species failed to survive at concentrations of less than 10% sea water. G. duebeni, C. marinus and G. locusta represent a graduation of responses to various salinities corresponding to their differing ability to survive in euryhaline conditions, which is reflected in the range of their habitats.

Changes in apparent permeability had been reported in decapod crustaceans with differing external medium concentrations. (Smith, 1967, 1970). Lockwood, Inman and Courtenay (1973) investigated

The relationship between the concentrations of sodium in the haemolymph and in the medium in G.duebeni acclimated to a range of salinities.

(after Lockwood 1964)

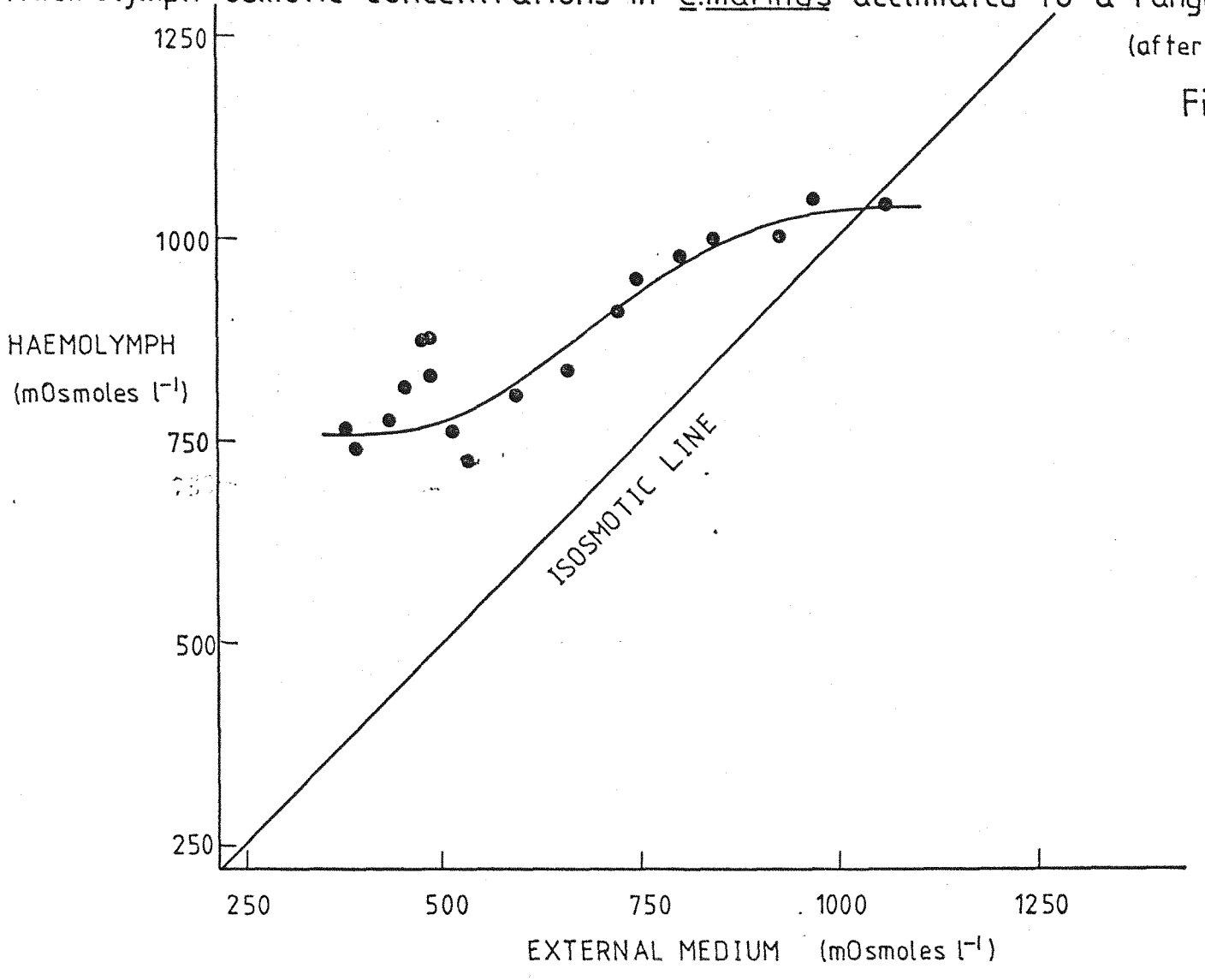
Fig. 1·6



Haemolymph osmotic concentrations in C.marinus acclimated to a range of salinities.

(after Haywood 1970)

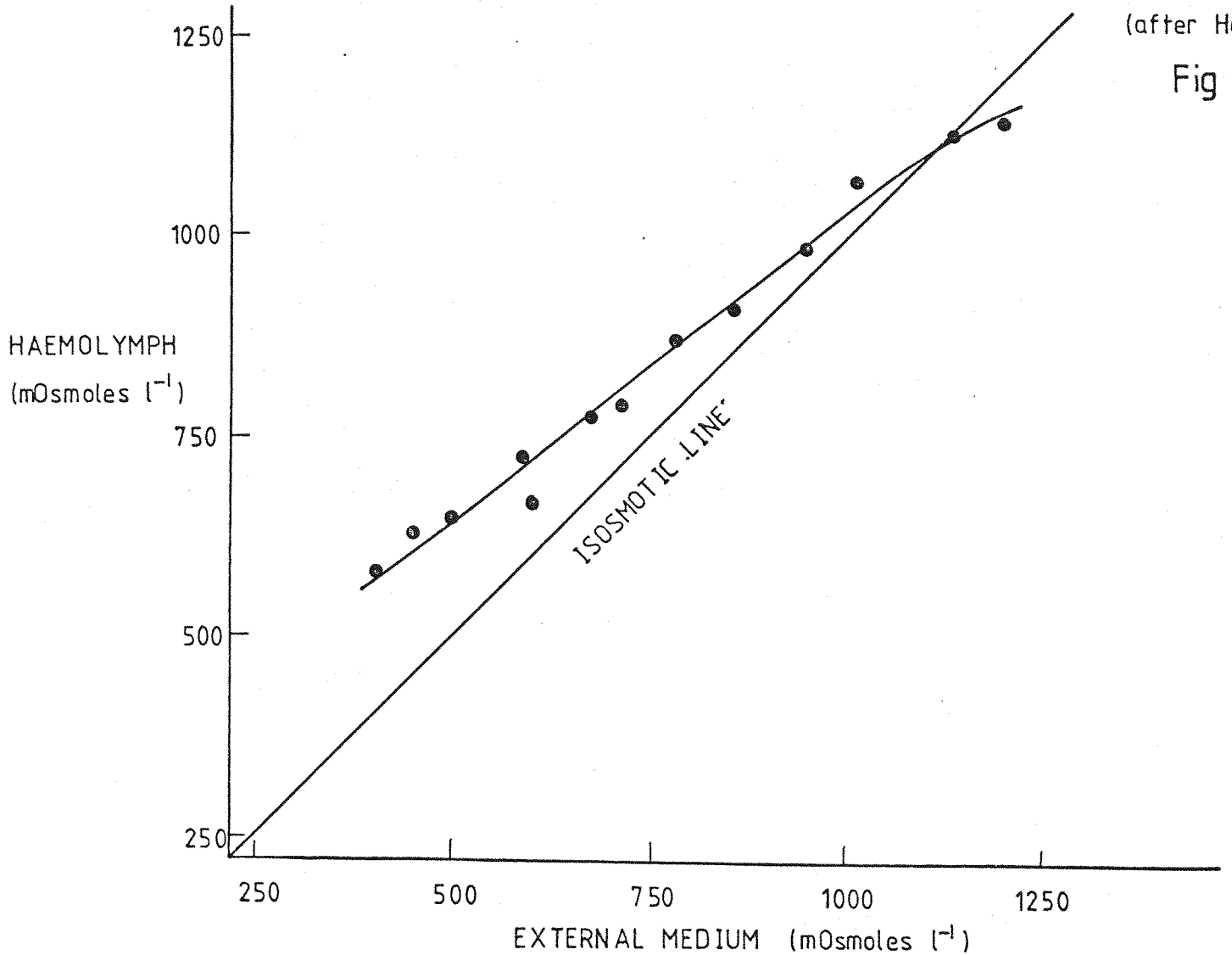
Fig.1·7



Haemolymph osmotic concentrations in Glocusta acclimated to a range of salinities.

(after Haywood 1970)

Fig 1·8



the apparent permeability of G. duebeni acclimated to varying salinities. This showed (Fig.1:9) a large change in apparent permeability, with a rapid half-time of exchange of tritiated water ($t_{\frac{1}{2}}$) of six minutes corresponding to a high apparent permeability at high salinities (60% s.w. and above), and gradually increasing to a $t_{\frac{1}{2}}$ of 16 minutes as the animal becomes more hypertonic at lower salinities.

Comparing the apparent permeability at various salinities, the high apparent permeability coincides with the animals' haemolymph concentration approaching isotonicity and the high apparent permeability with a large gradient between blood and medium. This can be demonstrated by plotting the half-time of exchange of tritiated water against the concentration gradient between the body fluids and medium in G. duebeni. (Fig.1:10).

The $t_{\frac{1}{2}}$ increases as the gradient increases, indicating a decrease in permeability as the osmotic gradient and hence osmotic stress increases. If the change in apparent permeability represents a real effect, then this decrease in permeability would alleviate the osmotic stress caused by the animal maintaining its blood strongly hypertonic to the medium.

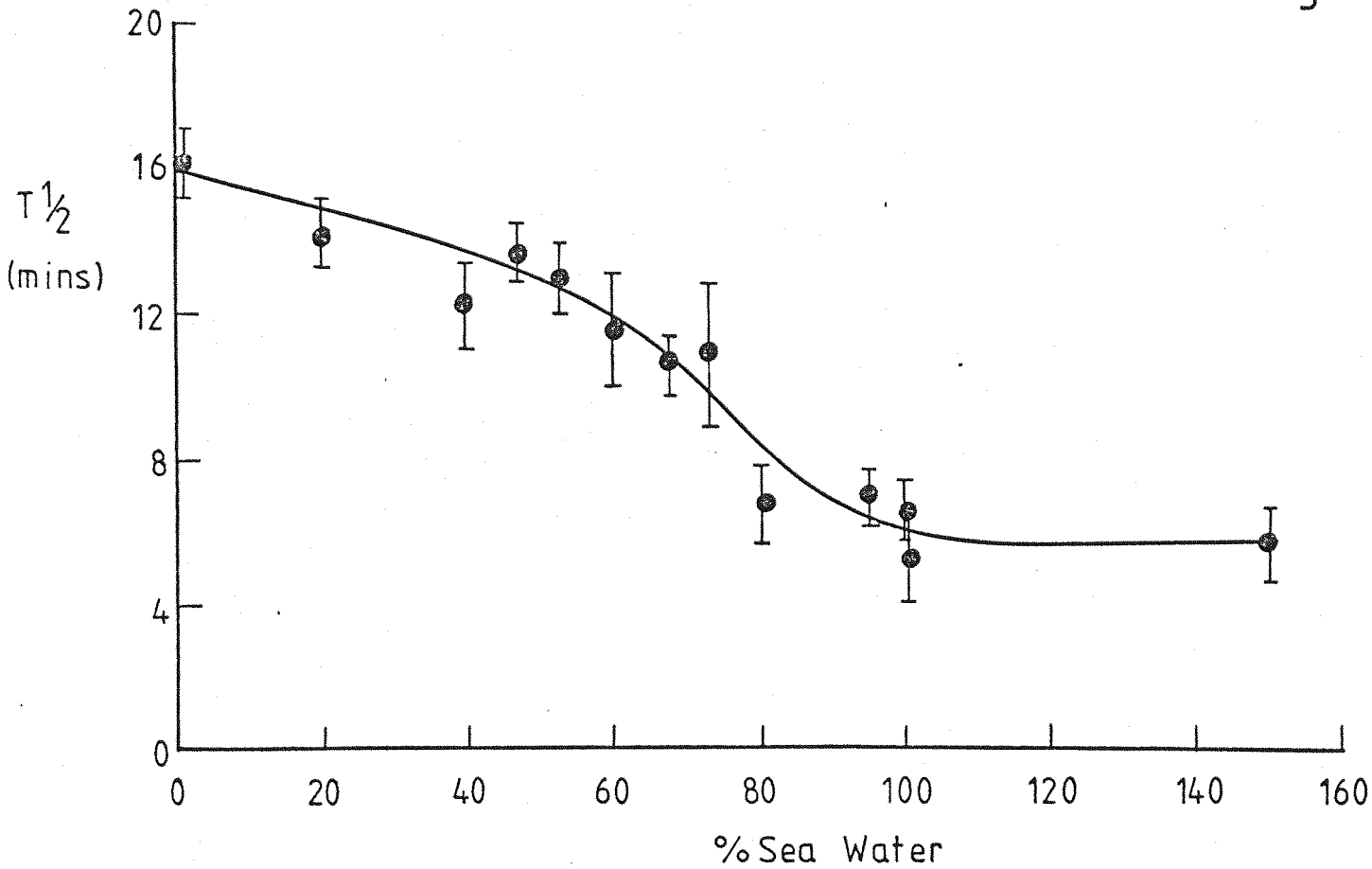
(ii) Non-steady state.

The environment in which G. duebeni is found can vary in salinity over a short period of time, for example, the shallow pools on Totton Marsh can be virtually fresh for long periods of time, changing to saline as a spring tide covers the marsh. To gain a better understanding of the osmoregulatory responses of G. duebeni, it is important to study the animal's responses when it is not at equilibrium with a stable external environment. Animals found in the drainage ditches filled with sea water every high tide and subject to fresh water drainage at low tide will experience a cycling salinity change. It is thus important to investigate the responses of these animals in a non-steady state regime, both in animals exposed to sudden changes in salinity and more gradually in a cycling salinity. For the cycling system, a repeated sine wave is used as an idealized cycle. This is not intended to mimic salinity cycles found in the habitat.

Half time for THO exchange in G. duebeni acclimated to various salinities.

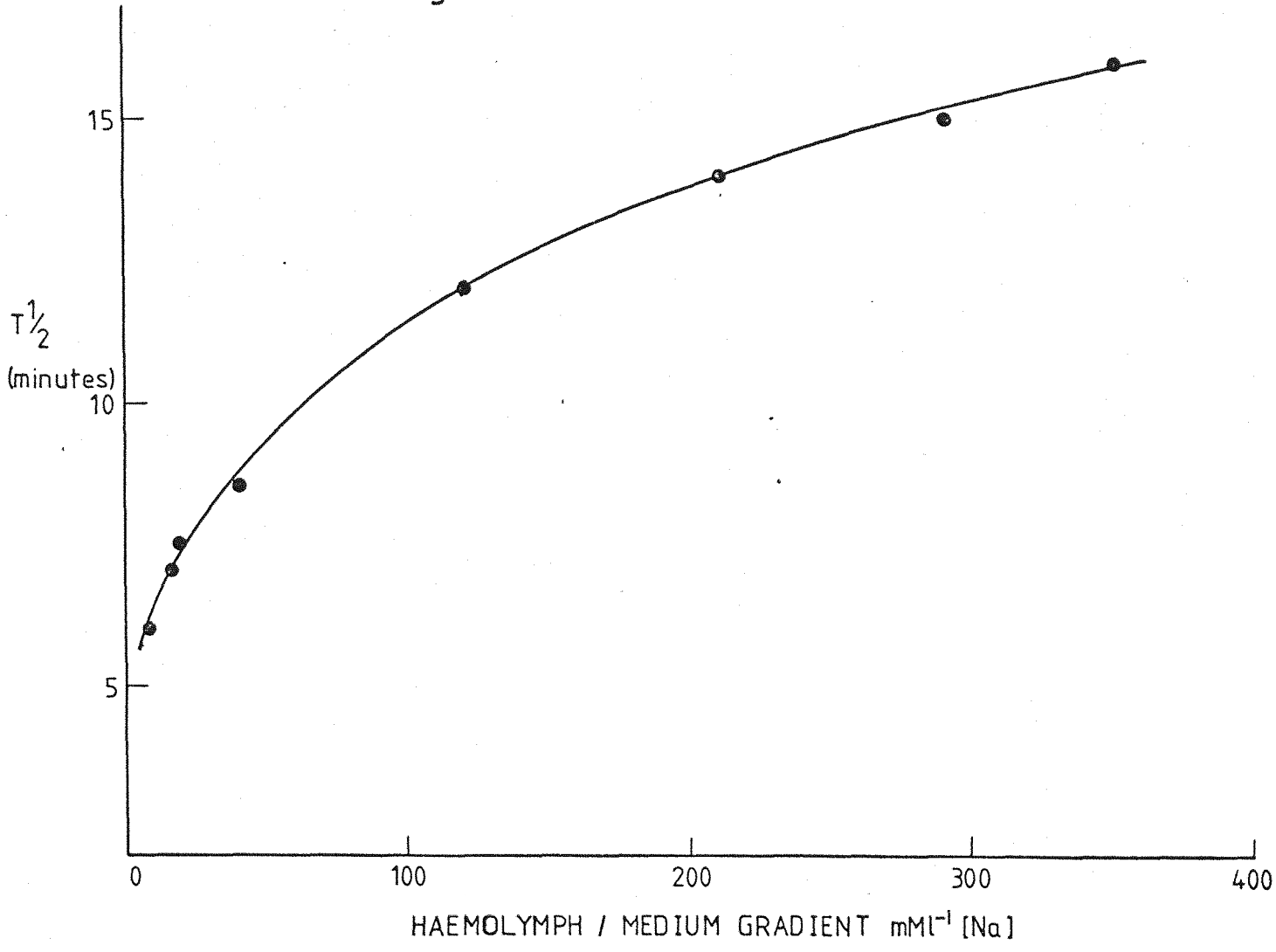
(after Lockwood, Inman & Courtenay 1973)

Fig. 1·9



$T\frac{1}{2}$ of exchange of THO against haemolymph/medium concentration gradient in G.duebeni acclimated to a range of salinities.

Fig 1·10



Lockwood and Inman 1979 measured water fluxes in G. duebeni, in non-steady states, transferring the animals rapidly from 2% s.w. to 100% s.w. (Fig.1:11). These preliminary results showed a different pattern of apparent permeability from those found in steady state values. The $t_{\frac{1}{2}}$ of exchange did not decrease to the value obtained for animals acclimated to 100% s.w. (6 minutes) until over twenty hours after the transfer. Similarly they found that animals subjected to a mechanically produced salinity cycle did not follow the pattern found in acclimated animals (unpublished).

Clearly further work is warranted to investigate this aspect of the physiological responses of G. duebeni, C. marinus and G. locusta in non-steady state salinities, including rapid changes and cycling salinities.

Haemolymph sodium concentration in a cycling salinity.

1. G. duebeni.

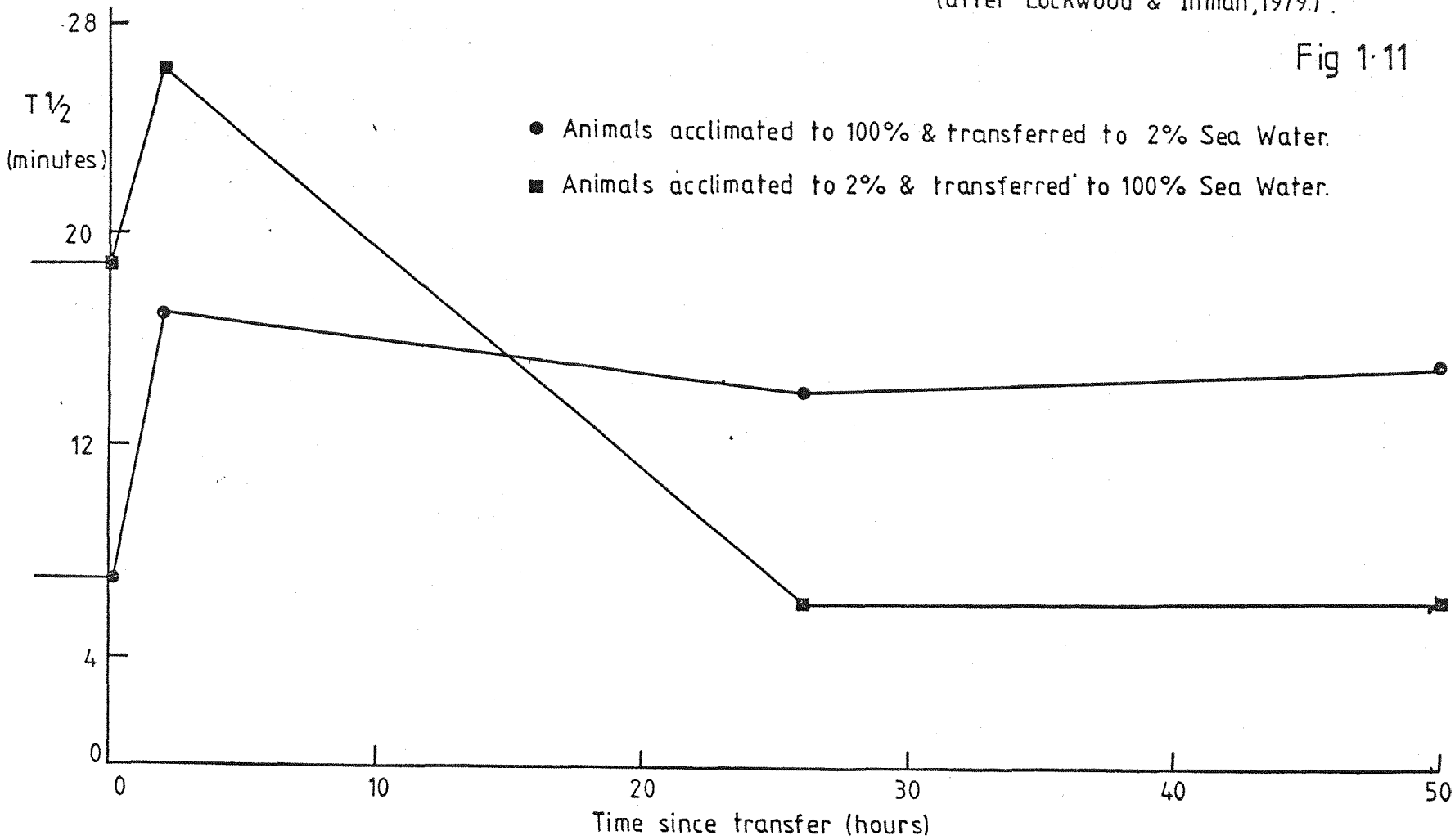
The microprocessor-controlled salinity cycle was programmed to provide a sine wave with a 12hr 25min. period and salinity limits of 31‰ and 1‰. G. duebeni were acclimated for the duration of at least four cycles. Haemolymph and medium samples were taken throughout the subsequent cycle and sodium concentration measured. The temperature was maintained at 15°C. In this regime G. duebeni was found to maintain its blood sodium remarkably constant throughout the cycle holding the blood sodium concentration at 295 ± 15mMl^{-1}Na. (Fig.1:12).

To compare the sodium concentration with the osmotic concentrations of the blood and medium, this experiment was repeated using the Ramsey cryoscopic technique. (Fig.1:13). Although the osmotic concentrations are not as consistant as the blood sodium concentrations due to experimental error, the results show that the isotonic and isionic points found by the two methods coincides at approximately 3½-4 hrs and at 8-8½ hrs into the cycle. Thus, blood sodium may be used as a measure of osmotic status.

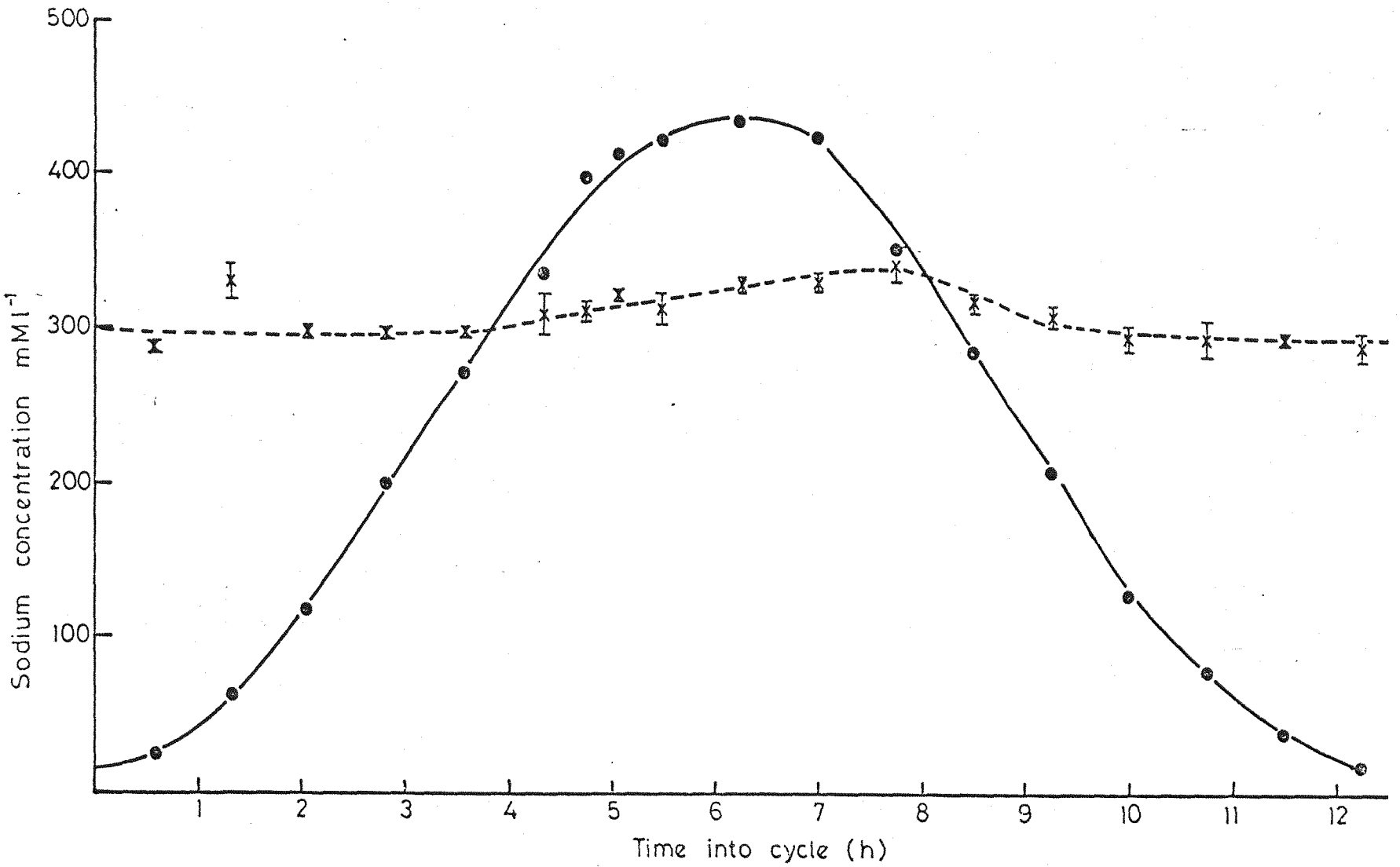
Half time for THO exchange (influx) in G.duebeni following a salinity change.

(after Lockwood & Inman, 1979.)

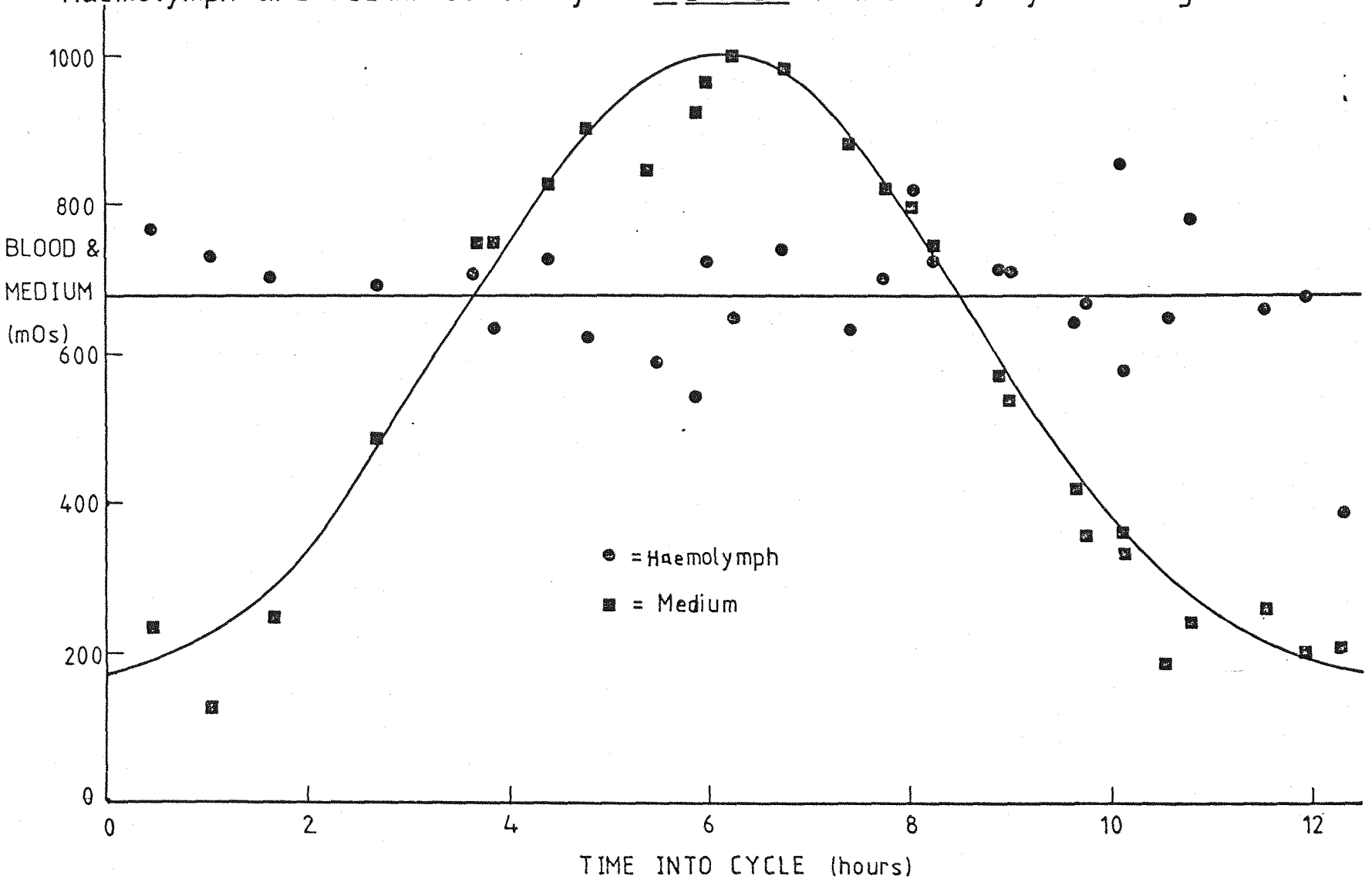
Fig 1·11



Haemolymph sodium concentration in G.duebeni exposed to the salinity cycle. Fig 1·12



Haemolymph and medium osmolality of G.duebeni in a salinity cycle. Fig 1·13



When the external medium concentration rises above 300mMl^{-1} [Na] the haemolymph is hypotonic to the medium. Thus, G.duebeni are hypotonic to the medium for approximately four hours in every 12hrs 25mins in the cycling salinity regime.

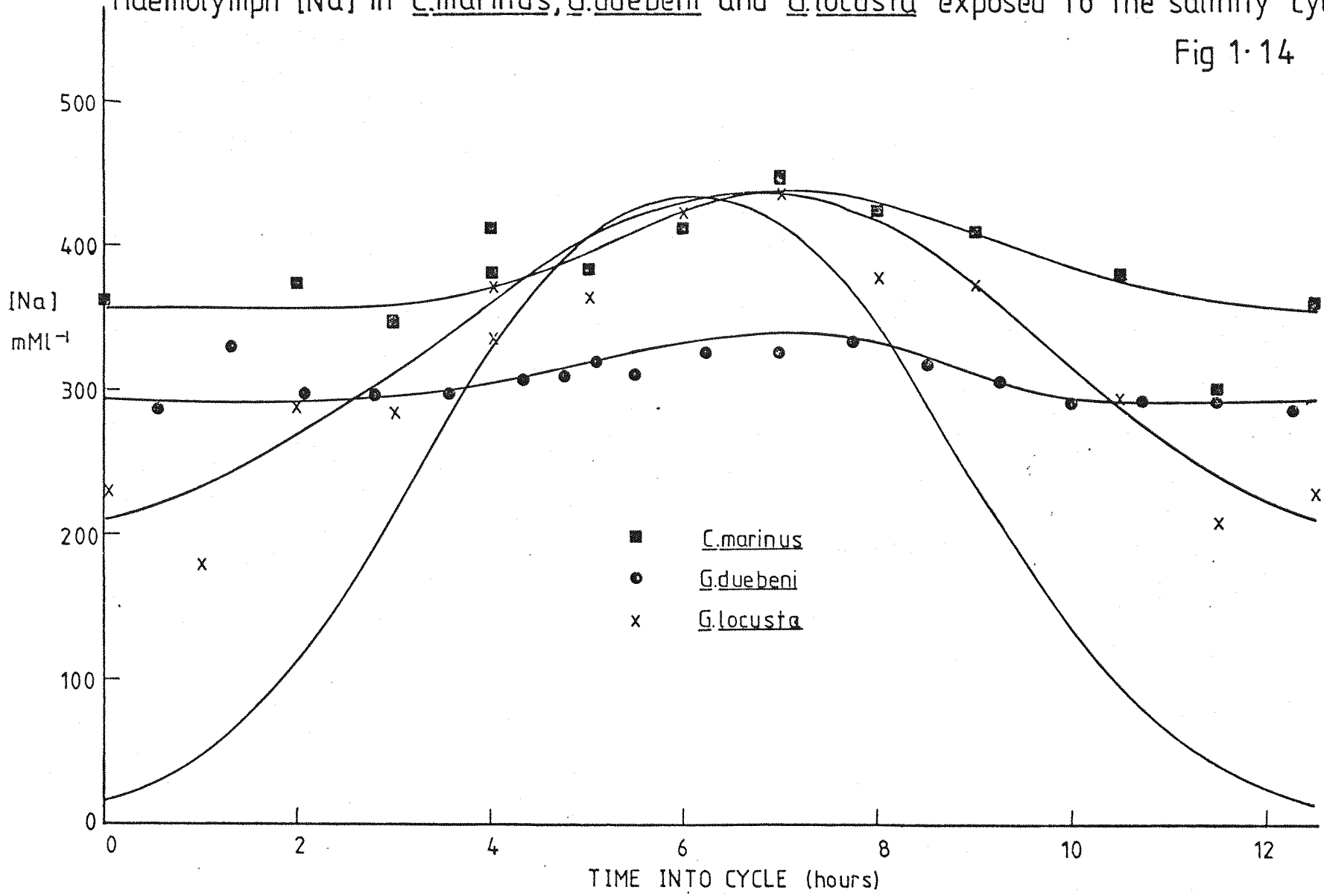
2. C.marinus and G.locusta.

C. marinus and G. locusta were acclimated to the same cycling salinity regime, haemolymph and blood samples taken and sodium concentrations measured (Fig.1:14). In contrast with G.duebeni, these species do not maintain their blood concentrations within narrow limits. C. marinus fluctuated from 350mMl^{-1} to 450mMl^{-1}, while G.locusta varied from 200mMl^{-1} to 450mMl^{-1}. C. marinus and G. locusta remain hyperionic or isionic throughout the cycle.

These three species exhibit a graduation of responses which correspond to their range of habitats and relative mortality in the experimental regime. G. duebeni, living in extreme conditions of changing salinity do not appear to be adversely affected by the cycling salinity and respond by maintaining a steady blood Na^+ concentrations in which the cells will have little osmotic stress. C. marinus is less able to maintain homeostatic conditions, suffering repeated changes in the fluids bathing the cells, which is reflected in an initial mortality of up to 15% as some of the animals fail to survive the changing conditions. G. locusta is even less adapted to such changes, and continued to die throughout the experiment. Both C.marinus and G.locusta appeared to vary on an individual basis, some animals of both species surviving for several weeks in the cycling regime.

Haemolymph [Na] in C.marinus, G.duebeni and G.locusta exposed to the salinity cycle.

Fig 1·14



Apparent permeability during a cycling salinity regime.

1. G. duebeni.

G. duebeni were exposed to the microprocessor controlled salinity cycle for 72 hrs or longer prior to the experiment. The half-time of exchange ($t_{\frac{1}{2}}$) was then measured by the influx of tritiated water on groups of individuals (usually 5) at different periods into the cycle. By taking readings through several cycles and superimposing them on to a single cycle, a comprehensive picture was built up. This technique assumes that the animals are acclimated to the cycle and respond similarly to each succesive cycle. (Fig.1:15). The results indicate that there are two distinct increases in apparent permeability; at approximately 4 hours and 8 hours into the cycle. At these times the $t_{\frac{1}{2}}$ dropped to 10 minutes and 5 minutes respectively. Comparisons of these rapid changes in $t_{\frac{1}{2}}$ with the haemolymph and medium concentrations suggest that the increase in apparent permeability coincides with the two periods of isotonicity. Conversely the apparent permeability decreases when the animals are hyper or hypotonic to the medium. The lowest permeability (highest $t_{\frac{1}{2}}$ of 24 minutes) occurs when G. duebeni is hypotonic to the medium. (Fig.1:15).

A more detailed study was made of the cycle during the period in which sudden changes in apparent permeability occurred. G. duebeni were acclimated to the salinity regime and apparent permeability and blood sodium measured over the six to ten hour period into the cycle. During this period, the animals were going from hypotonic to hypertonic to the medium, accompanied by a rapid change in apparent permeability.

Results from individual G. duebeni were plotted against time. (Fig.1:16). This shows a rapid transition from high $t_{\frac{1}{2}}$ (low apparent permeability) to low $t_{\frac{1}{2}}$ (high apparent permeability) as the animals approach isionicity from hypo-ionicity and a gradual return to a high $t_{\frac{1}{2}}$ (low apparent permeability) as the degree of hypertonicity increases. The $t_{\frac{1}{2}}$ drops rapidly from 16 minutes to 4 minutes subsequently returning gradually to 13 minutes. Thus when the animal is in transition from hypo-ionic to isionic, the change in apparent permeability is extremely rapid and in transition from isionic to hyperionic, the $t_{\frac{1}{2}}$ appears

Apparent permeability to water in G.duebeni under cycling salinity conditions.

Fig.1.15

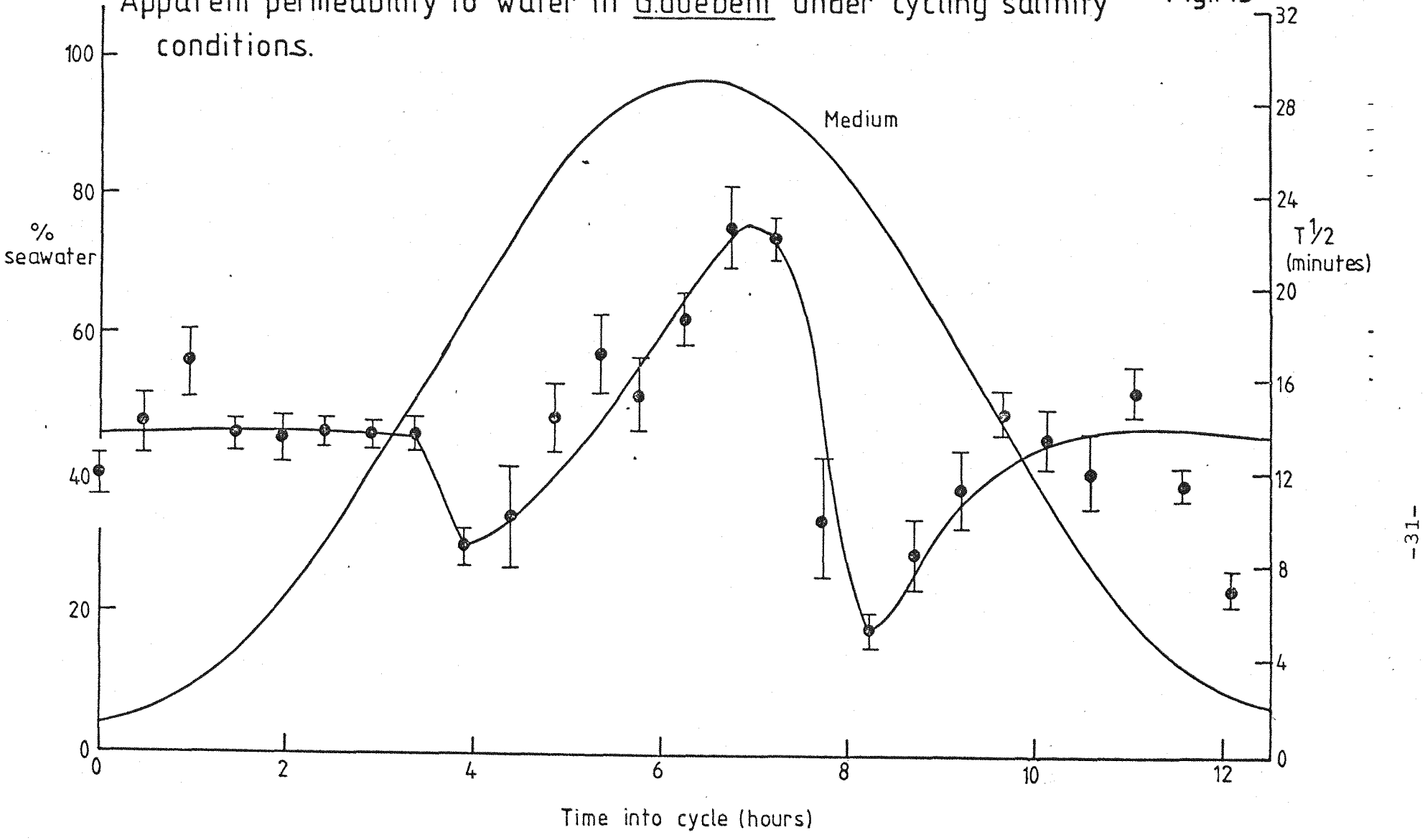
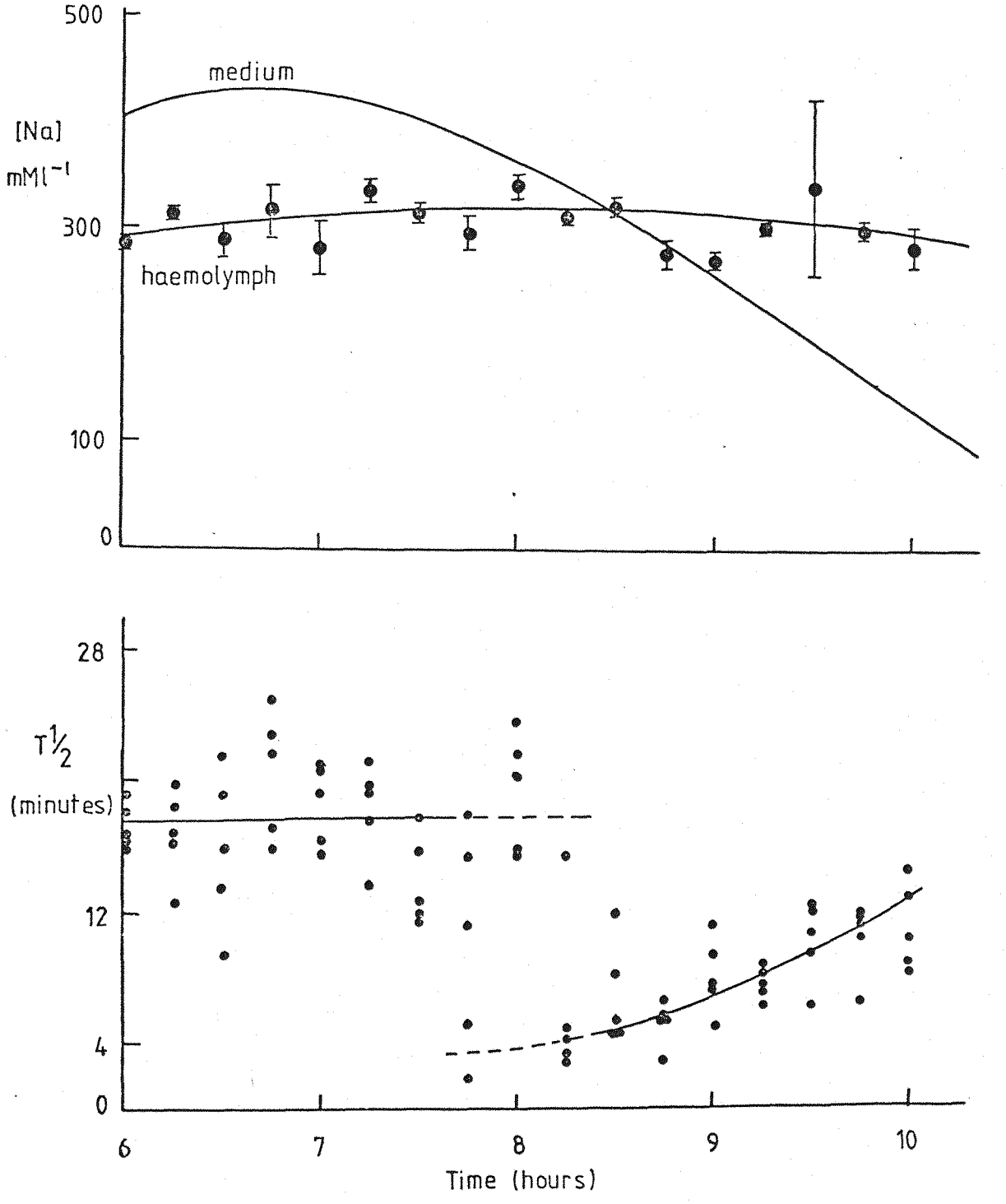


Fig 1·16

Half time of exchange of THO & haemolymph [Na] of G.duebeni in a cycling salinity.



proportional to the gradient between the haemolymph and external medium. Plotting the $t_{\frac{1}{2}}$ of exchange against blood/medium gradient over this period confirms a correlation during hypertonicity (Fig.1:17). A linear regression on the resulting line gives a regression coefficient of .993.

During hypotonicity the $t_{\frac{1}{2}}$ is not proportional to the gradient. Although there is individual scatter, there is no significant change in $t_{\frac{1}{2}}$ until the rapid change of $t_{\frac{1}{2}}$ as isionicity is reached.

2. C. marinus and G. locusta.

Comparative measurements of C. marinus and G. locusta were made during the complete salinity cycle. These results were plotted as the mean of 5 animals against time. (Fig. 1:18). C. marinus maintained a $t_{\frac{1}{2}}$ of 9 minutes ± 2 minutes throughout the cycle, while G. locusta exhibited a $t_{\frac{1}{2}}$ of 4 minutes ± 1 minute. This compares with G. duebeni which fluctuates from 5 to 24 minutes.

C. marinus and G. locusta do not change their apparent permeability during the salinity cycle. Thus these less euryhaline species do not respond in the same manner as G. duebeni to fluctuating salinities, being unable to maintain a constant haemolymph concentration or alter their apparent permeability to water. C. marinus is less permeable to water than G. locusta in the same conditions and is able to maintain blood sodium within narrower limits. This corresponds with their observed ecological tolerances.

Apparent permeability and blood sodium concentration of G.duebeni exposed to sudden and subsequent gradual salinity changes.

G. duebeni acclimated to the salinity cycle appeared to respond to isotonicity with a sudden decrease in $t_{\frac{1}{2}}$ of exchange (increase in apparent permeability). This observation was supported by designing an experimental regime which did not require cycling conditions, but still forced the animals hypotonic to the medium.

$T\frac{1}{2}$ of exchange of THO against haemolymph/medium concentration gradient in G.duebeni exposed to a cycling salinity system.

Fig 1·17

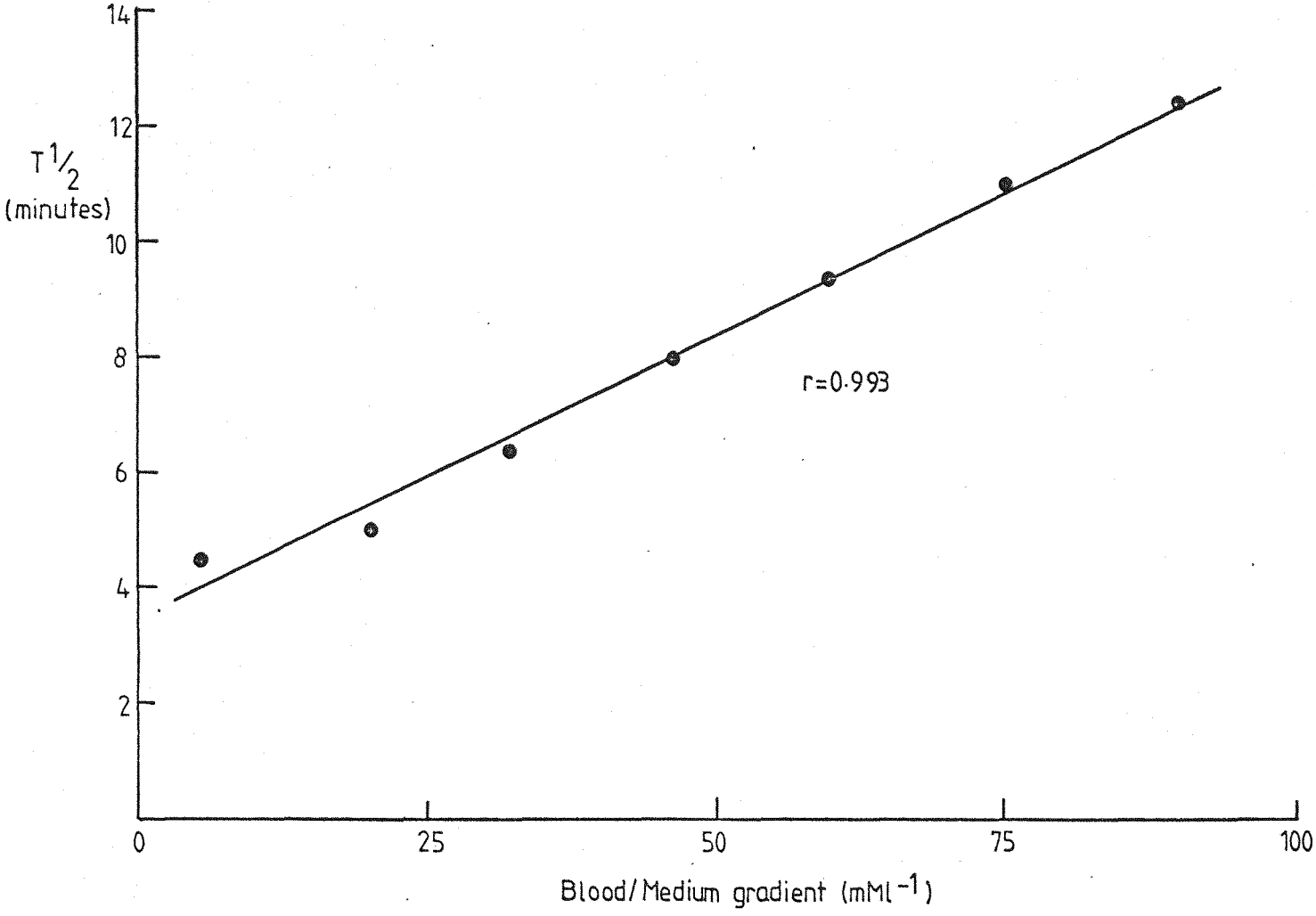
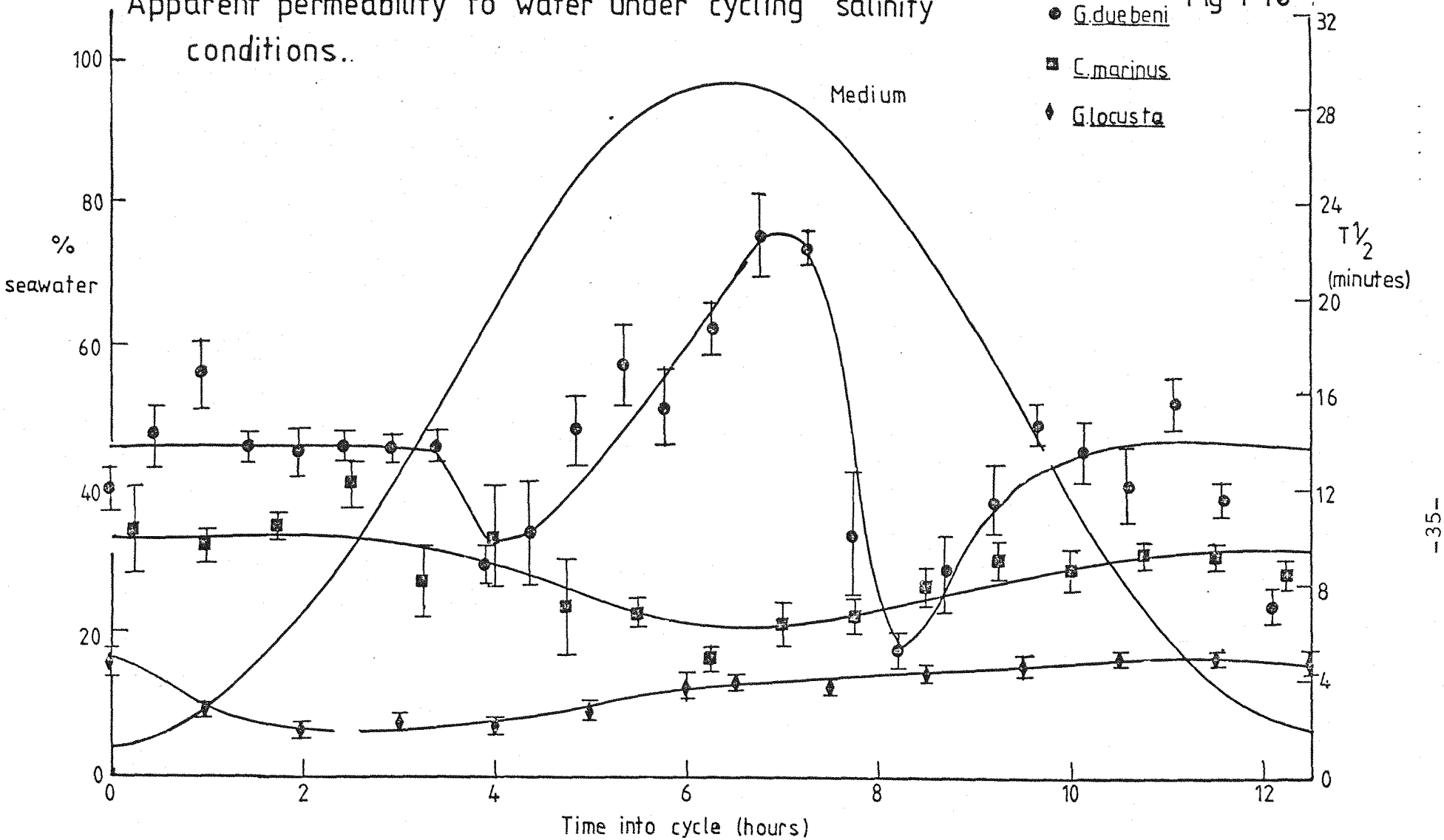


Fig 1·18

Apparent permeability to water under cycling salinity conditions..



G. duebeni were acclimated to 2% s.w. for 48 hours and then transferred to 100% s.w. The salinity was then reduced back to 2% over a six hour period using the microprocessor system which had been re-programmed to execute a linear salinity change. This experiment was designed to mimic the changes in the salinity cycling system without a repeated pattern.

Blood and medium concentration and apparent permeability were measured during this time period. The $t_{\frac{1}{2}}$ values of individual animals were plotted against time. (Fig.1:19).

These results show the same pattern of apparent permeability changes. In the period during which the haemolymph is hypo-ionic but approaching the isionic state the change in $t_{\frac{1}{2}}$ is rapid, while from isionic to hyperionic the change in $t_{\frac{1}{2}}$ is gradual.

Plotting the $t_{\frac{1}{2}}$ against the gradient between haemolymph and medium sodium concentration during hyperionicity again demonstrates a relationship between the gradient and $t_{\frac{1}{2}}$, appearing linear until the gradient increases above $100mMl^{-1}$ [Na] and slowly tailing off as the $t_{\frac{1}{2}}$ for acclimated 2% animals is reached. (Fig.1:20).

These results confirm that G. duebeni appears to be altering its permeability according to the relative blood and medium concentrations, irrespective of the absolute concentration of the salinity regimes.

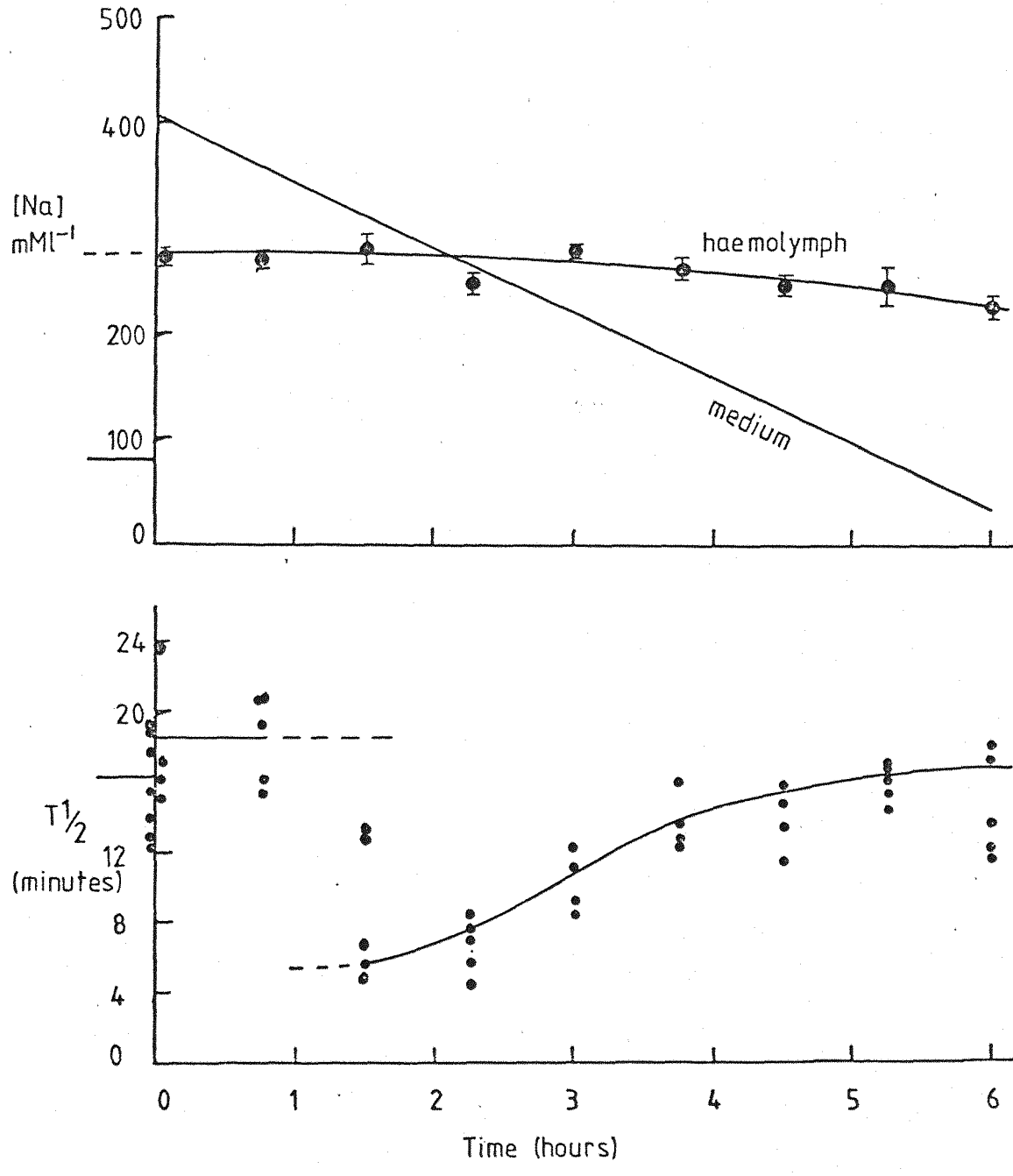
Apparent permeability and blood sodium in (i) G. duebeni exposed to a rapid change in salinity from 2% s.w. to 100% s.w.

(i) G. duebeni appears to be least permeable during periods of hypotonicity when the osmotic bulk flow is out of the animal. To investigate the high $t_{\frac{1}{2}}$ values during hypotonicity, G. duebeni were acclimated to 2% s.w. for 48 hours and transferred to 100% s.w. Blood and medium sodium concentrations and apparent permeabilities to water were measured for twenty-four hours after the change in salinity. Individual $t_{\frac{1}{2}}$'s were plotted against time. (Fig.1:21).

Immediately after the change in salinity the animals are forced strongly hypotonic. The haemolymph increases in concentration over a period of approximately 16 hours, until the animals become

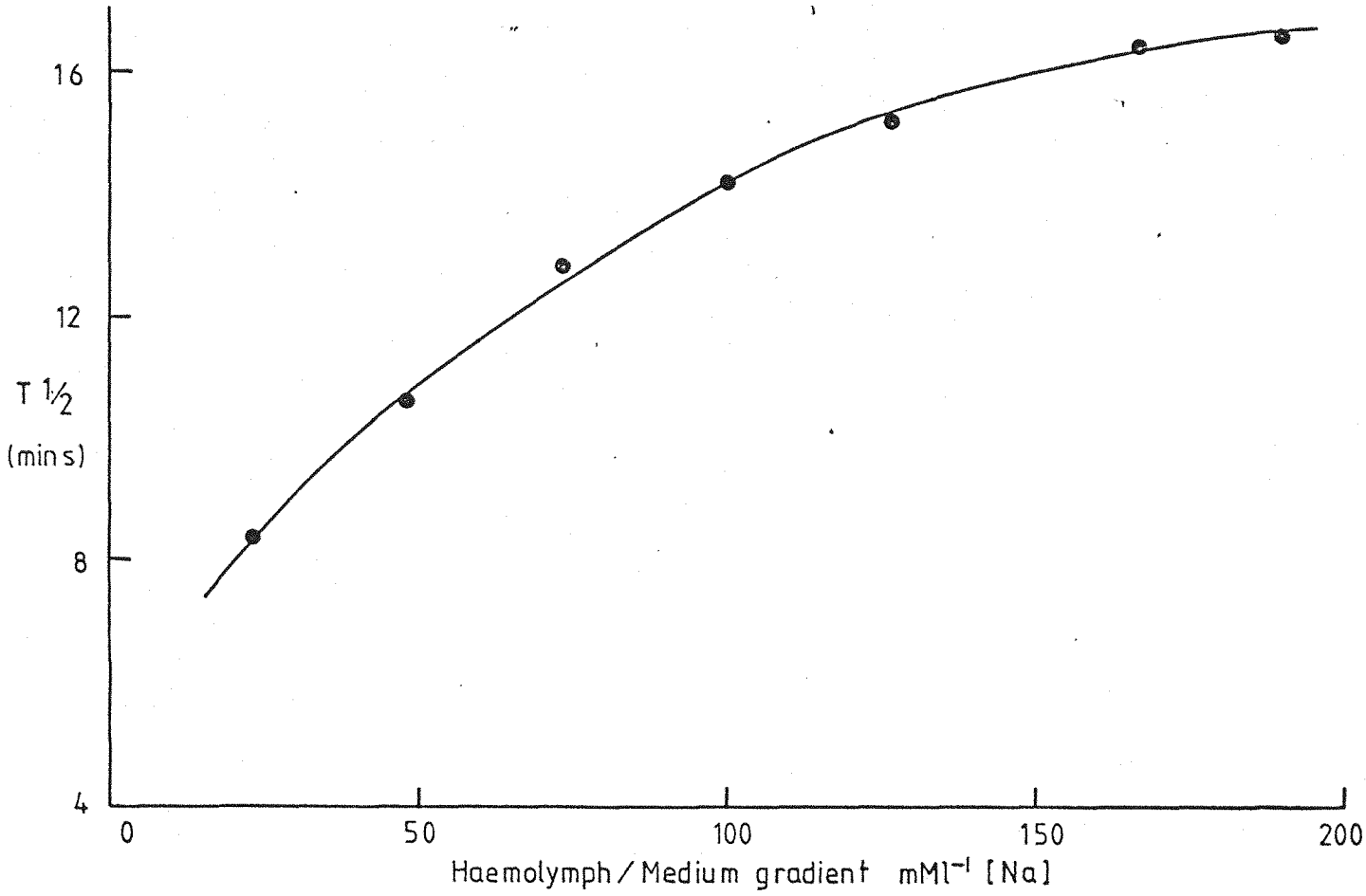
Fig 1·19

Half time of exchange of THO & haemolymph [Na] of G.duebeni in varying salinities.



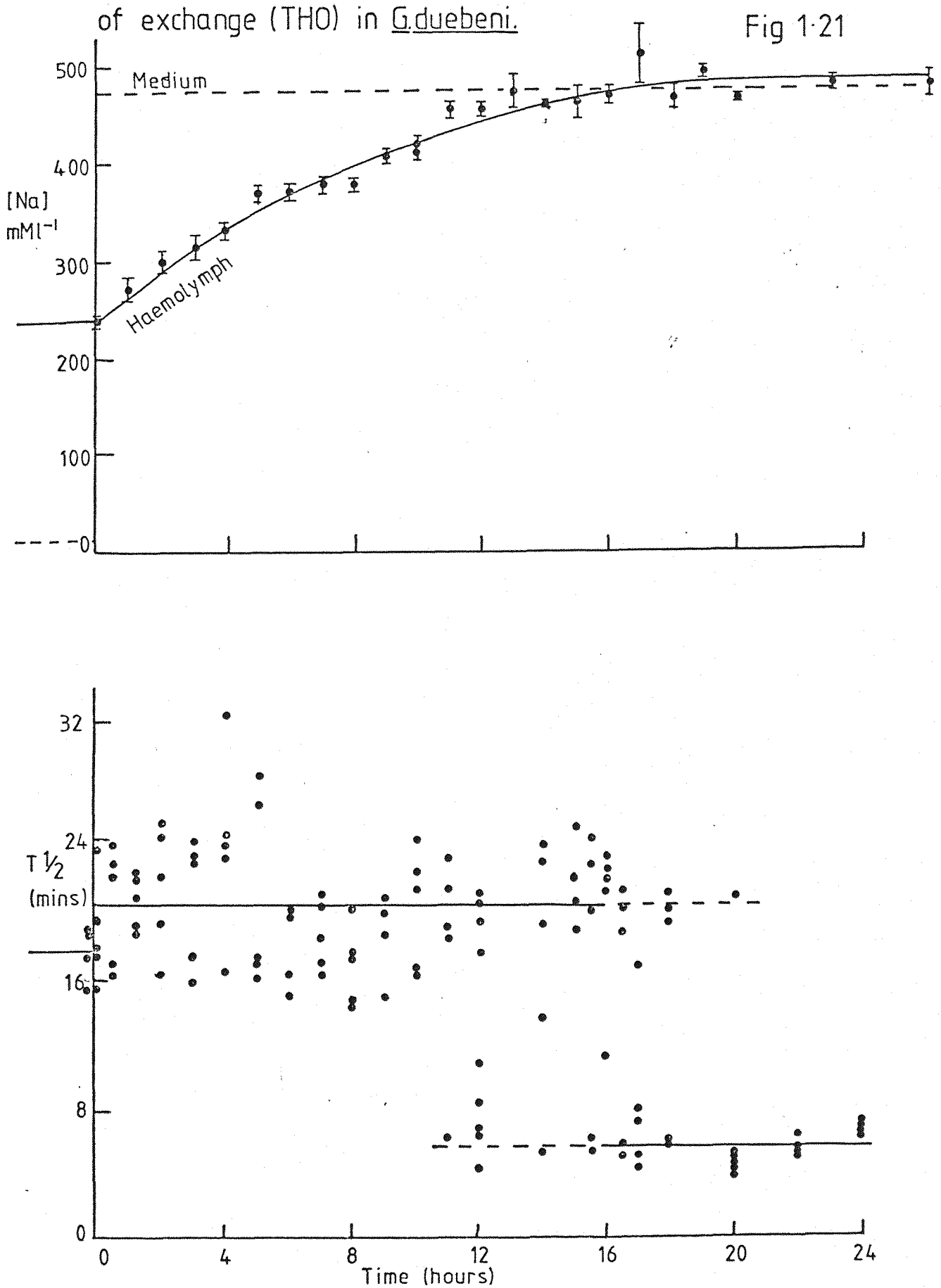
T ½ of exchange of THO against haemolymph/medium concentration gradient in G.duebeni. (see text).

Fig 1·20



Haemolymph and medium sodium concentration and half time of exchange (THO) in G.duebeni.

Fig 1·21



hypertonic to the medium. (Fig.1:21). The apparent permeability decreases slightly (increased $t_{\frac{1}{2}}$ of exchange) after the initial transference to 100% s.w., from approximately 18 minutes to 21 minutes, this latter value is maintained up to the isionic point, where there is a sudden increase in apparent permeability, the $t_{\frac{1}{2}}$ dropping to five minutes. There appears to be considerable individual variation in the time taken for animals to reach isionicity, causing some G. duebeni to have become permeable after only 11 hours and others to be still impermeable after nearly 20 hours. Unfortunately, it is not possible to measure apparent permeability and blood sodium on a single animal, and the exact time of change of $t_{\frac{1}{2}}$ cannot accurately be related to actual haemolymph/medium gradient. A rapid change from high $t_{\frac{1}{2}}$ (low apparent permeability) to a low $t_{\frac{1}{2}}$ (high apparent permeability) is indicated as only three individuals have been "caught" with intermediate $t_{\frac{1}{2}}$ values. The pattern of rapid change in apparent permeability as the animal approaches or reaches isoionicity is again present even though the haemolymph concentration is approaching that of the medium very slowly.

Immediately after the change in salinity, the $t_{\frac{1}{2}}$ appears to increase slightly, however there is a large individual scatter. To investigate this change further, the apparent permeability was taken immediately before the change of salinity, and 0-1 hour after the change. The half-times of exchange was compared on individual animals before and after the change in salinity using student-t test for paired samples. (Table 1:1).

TABLE 1:1

$t_{\frac{1}{2}}$ mins 2% s.w.	$t_{\frac{1}{2}}$ mins 2-100% s.w.
15.1	15.7
14.5	17.8
15.1	16.1
15.4	17.2
10.7	14.7
14.6	21.3
mean 14.3	17.1

t = -3.12 5 degrees of freedom

Significant at 0.05 level.

This result confirms an initial increase change in $t_{\frac{1}{2}}$ in G. duebeni when the external medium is rapidly changed from 2% to 100% s.w.

(ii) C. marinus and G. locusta.

C. marinus and G. locusta exposed to the salinity cycle were not forced significantly hypotonic to the medium. By transferring the amphipods from a low salinity to a high salinity, they are forced hypotonic and the $t_{\frac{1}{2}}$ of exchange are compared to that of G. duebeni during hypotonicity.

Animals were initially acclimated to 10% s.w. as they were unable to survive 2% s.w. for long periods, and transferred to 100% s.w. Apparent permeability was measured before and after the change in salinity.

TABLE 1:2

(i) C. marinus

	$t_{\frac{1}{2}}$ min	s.e.m.	Blood/ med. grad.	mMl^{-1} Na	n
10% s.w.	9.0	1.6	+320	Hypertonic	5
10-100% s.w.	6.1	1.0	-160	Hypotonic	6

(ii) G. locusta

	$t_{\frac{1}{2}}$ min	s.e.m.	Blood/ med. grad	mMl^{-1} Na	n
10% s.w.	4.21	0.24	+176	Hypertonic	5
10-100% s.w.	3.2	0.7	-305	Hypotonic	6

C. marinus and G. locusta do not exhibit changes in apparent permeability associated with hypotonicity demonstrated in G. duebeni. The half-time of exchange in C. marinus changing from 9.0 to 6.1 minutes, compared to G. duebeni which increases from 14.3 to 17.1. G. locusta also shows a small decrease in $t_{\frac{1}{2}}$ from 4.21 to 3.2 minutes.

Thus C. marinus and G. locusta do not exhibit large changes in apparent permeability even when they are forced hypotonic to the medium.

Investigation of the rate of change of apparent permeability in G. duebeni

G. duebeni acclimated to 2% s.w. and transferred to 100% s.w. are forced hypotonic. After approximately 16 hours isionicity is reached and the change in permeability appears extremely rapid. (Fig. 1:21). To investigate the rate of change of $t_{\frac{1}{2}}$, the

loss of tritiated water from individual animals was monitored. G. duebeni acclimated to 2% s.w. were transferred to tritiated 100% s.w. (50μCi ml^{-1}). They were transferred to unlabelled 100% s.w. after 15 and 15½ hours. Duplicate aliquots of the inloading medium were taken and counted at six minute intervals, and the $t_{\frac{1}{2}}$ of exchange (outflux) calculated for each 6 minute period. These results were expressed as $t_{\frac{1}{2}}$ against time for individual animals (Fig.1:22). Plotting $t_{\frac{1}{2}}$ against time, limits the resolution as each $t_{\frac{1}{2}}$ value is the average of a six minute period. By plotting log counts against time, (Fig.1:23) there is no such limitation. It is not possible to measure sodium concentration to predict the time after salinity shock that the change in apparent permeability is likely to occur. Many repeat experiments are needed to "catch" an animal in the process of change.

These results confirm earlier observations that the change in $t_{\frac{1}{2}}$ is rapid as the animal approaches isionicity with the medium. $T_{\frac{1}{2}}$ against time shows a rapid change in apparent permeability but log counts against time demonstrate the drop in $t_{\frac{1}{2}}$ as almost instantaneous.

Net flow calculation

If the half-time of exchange of water, the osmotic concentration of the blood, and the osmotic concentration of the medium are all known, it is possible to calculate the net fluxes in the animal. When the haemolymph is hypotonic to the external medium, the bulk flow of water is into the animal and if the blood is hypotonic, then the flow is outwards.

The net flow is calculated using the following formulae (Lockwood and Inman, 1973).

(1) $\frac{100\ 1n2}{t_{\frac{1}{2}}} = F$

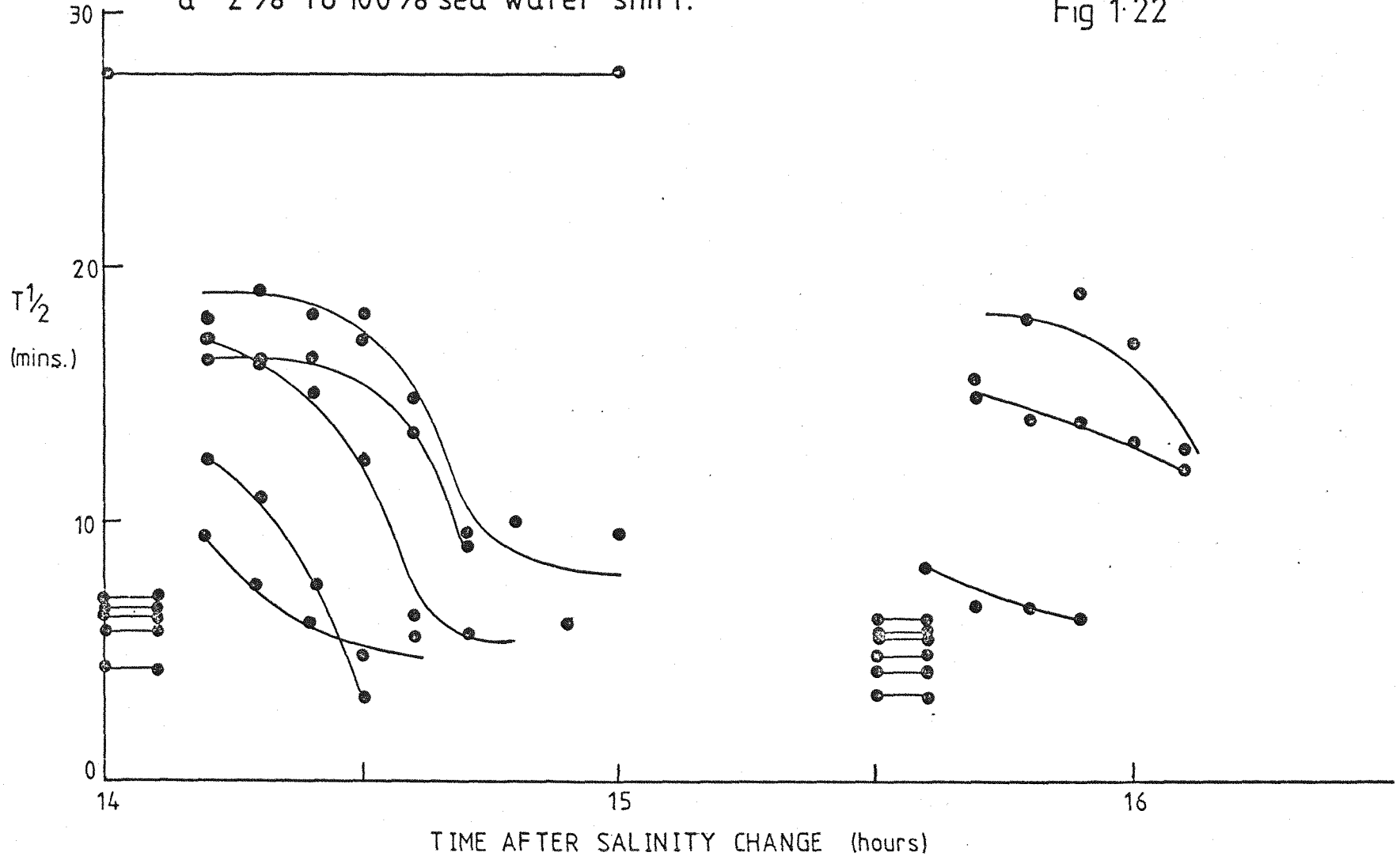
(2) $\frac{Mm - Ma}{Mm} F = Os$

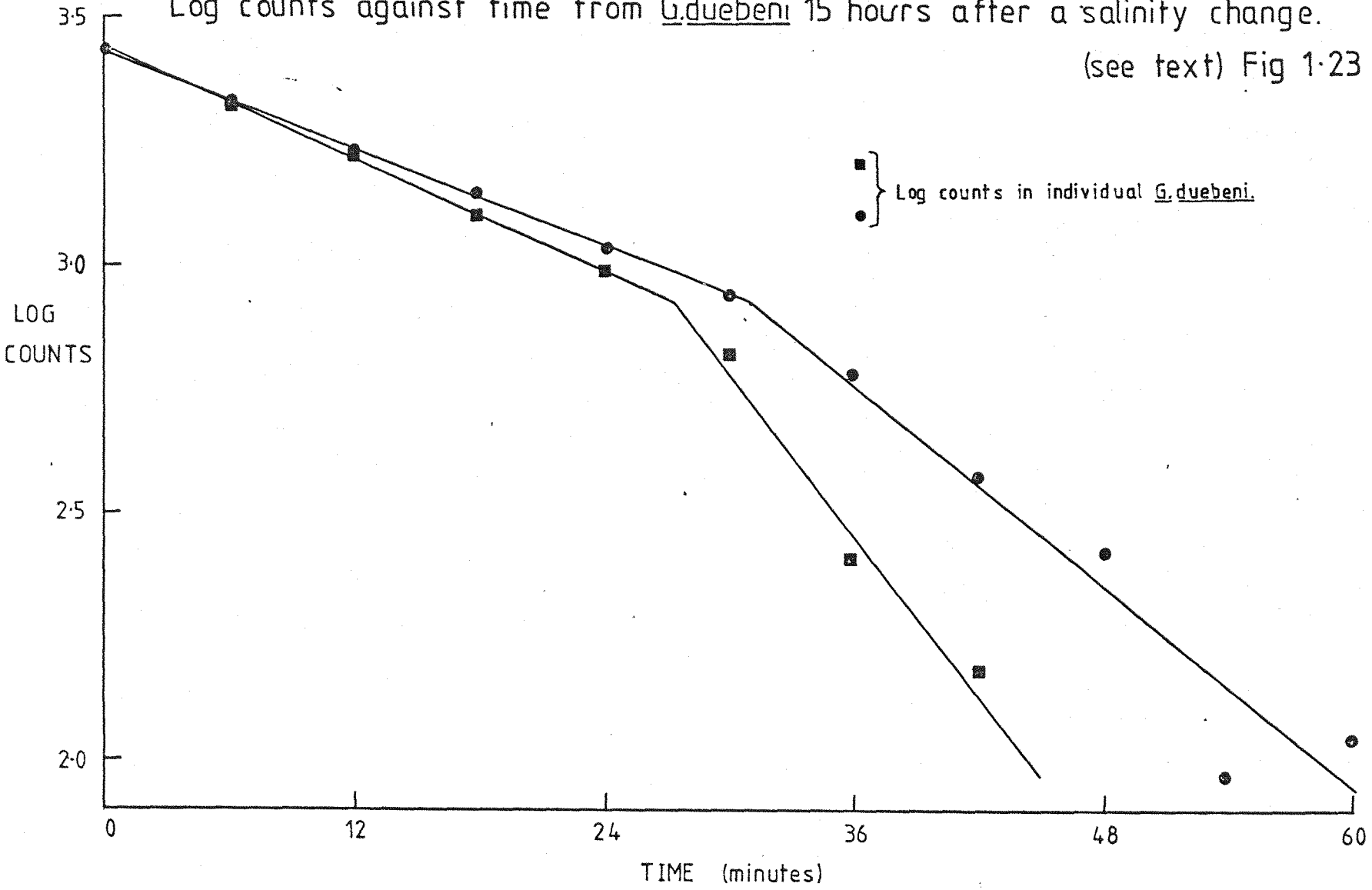
where Mm = mole fractions of medium
Ma = " " " blood
F = water flux
Os = net water flow

The mole fraction of blood and medium are calculated as:-

Time course of apparent permeability change as isotonicity is reached after a 2% to 100% sea water shift.

Fig 1·22




Log counts against time from G.duebeni 15 hours after a salinity change.
(see text) Fig 1·23
Log counts in individual G.duebeni.
3·5
3·0
2·5
2·0
LOG
COUNTS
0
12
24
36
48
60
TIME (minutes)

$$M = \frac{55.56}{55.56 + x}$$ where x is the osmolal concentration between 0 and 1.

This calculation enables the bulk flow of water to be estimated throughout experiments where $t_{\frac{1}{2}}$ and blood and medium concentrations were measured.

Calculating the net flow of water throughout the cycling salinity system in the three amphipods, G. duebeni, C. marinus and G. locusta shows (Fig. 1:24) clearly the different responses of these species. G. duebeni never exceeds the rate of 100% body water per day, while G. locusta peaks at a rate of over 300% body water per day. G. duebeni is the only amphipod to go hypotonic to the medium during the cycle, losing fluid at a maximum rate of 45% body water per day. If G. duebeni were unable to decrease its permeability during this period of isotonicity, and had a hypothetical $t_{\frac{1}{2}}$ of 5 minutes, then this rate increases to a rate of 80% body water per day. This demonstrates the effectiveness of reducing permeability as a mechanism to restrict water loss during a period of hypotonicity.

The difference between the three species is further emphasised by calculations of the % water uptake or loss of body water over the period of one cycle. This is done by integrating the area under the curve for each animal.

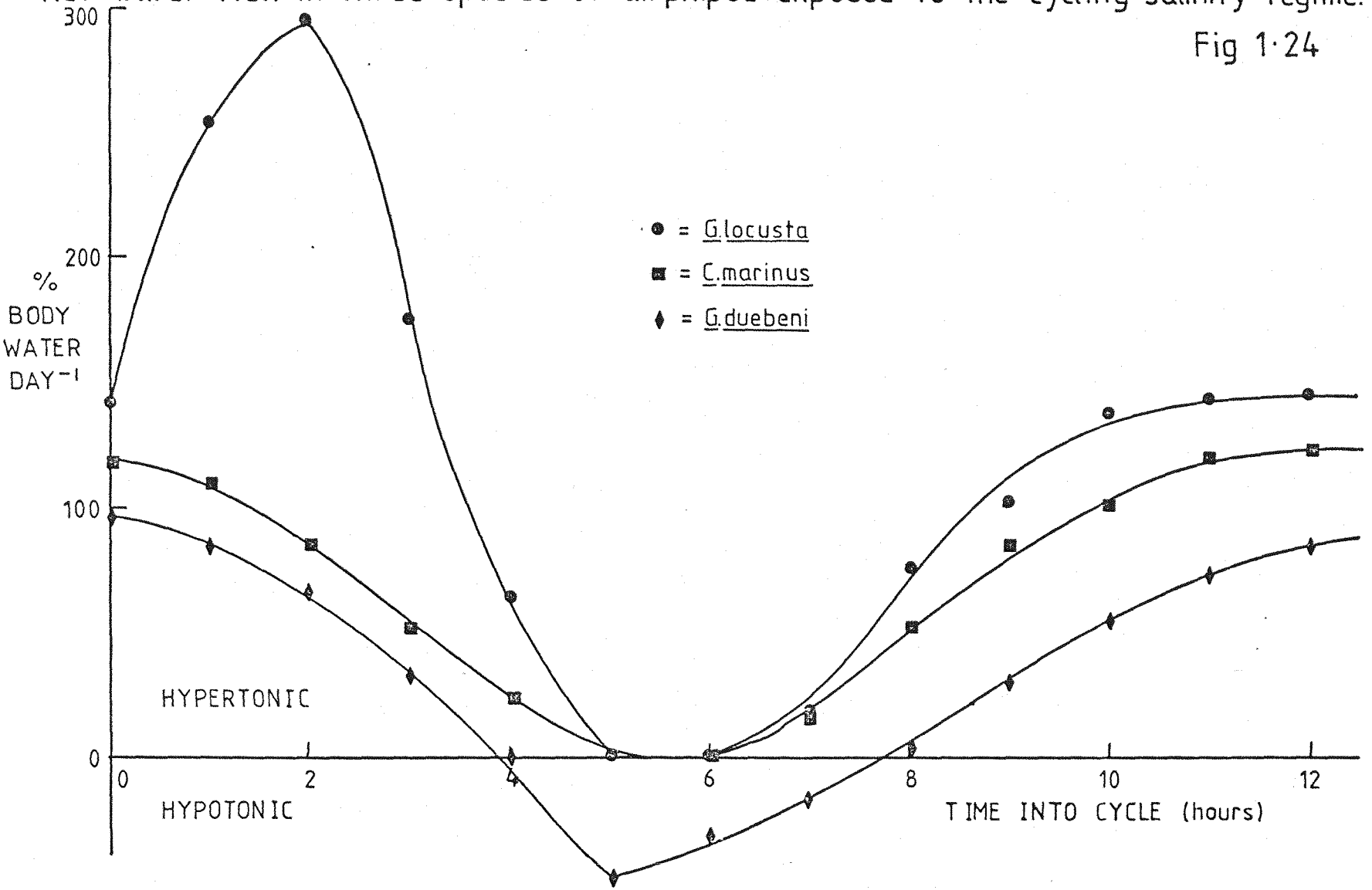
	% water uptake during 12 hr cycle
G. duebeni	+ 18.8 during hypertonicity -3.8 during hypotonicity Total uptake during cycle = 15% body water cycle^{-1}
C. marinus	+ 31.9% body water cycle^{-1}
G. locusta	+ 60.6% body water cycle^{-1}

Thus, during one cycle, the osmotic fluid turn over in G. duebeni is half that of C. marinus which is half that of G. locusta. These results dramatically illustrate the different responses of these animals to the osmotic stress of a salinity cycle.

It is assumed that these osmotic fluxes into the animal are matched by urine flow out of the animal if the volume of the animal is to remain constant.

Net water flux in three species of amphipod exposed to the cycling salinity regime.

Fig 1·24



DISCUSSION

The use of labelled water to measure permeability

The technique of investigating fluxes of labelled water to measure permeability to water has been subject to much discussion and criticism. The possibility of inaccuracy resulting from the presence of unstirred layers either side of the membranes has been proposed by Daintyand House (1966). They demonstrated the presence of unstirred layers on isolated frog skin.

Nernst (1904) put forward the theory of a thin static layer of liquid immediately adjacant to the surface of a solid object immersed in water whose concentration was not equal to that of the main body of water. If the permeability of this layer is very high compared to that of the object, then the permeability of the object measured by labelled water will be accurate. If, however, the permeability of the unstirred layer is low in relation to the permeability of the membrane, then the tritiated water measurement will underestimate the permeability.

Motais *et al* (1969) measured the permeability of teleost gills, both by the use of tritiated water, and by measuring osmotic fluxes. This allowed them to compare the two methods, to give the ratio Pos/Pdiff which should be unity if the two techniques agreed. *Anguilla anguilla* gills gave a Pos/Pdiff of 6.12 which, if due to unstirred layers, is a serious criticism of finding water permeability by using tritiated water. Motais *et al* (1969) postulate the presence of a mucous layer covering the gills, forming a "trapped" unstirred layer much thicker than otherwise expected.

Motais *et al* (1969) also state that tritiated water measurements of the half-time of exchange should ideally only be undertaken when there is no osmotic gradient accross the animal. They argue that the half-time of exchange measured in only one direction would not take into account the bulk flow into or out of the animal. For example, if the animal were hypertonic to the medium, the osmotic bulk flow would be into the animal, causing influx measurements to overestimate the permeability, and out-flux to underestimate it.

Comparing the apparent permeabilities in G. duebeni measured by influx to the $t_{\frac{1}{2}}$ measured by outflux when the animals are hypertonic to the medium shows no such discrepancy between the two techniques. Animals acclimated to 2% s.w. gave a $t_{\frac{1}{2}}$ of 17.6 ± 2.6 (n = 5) by influx, compared with 16.8 ± 2.5 (n = 16). (Data from Lockwood 1973). These results do not show a significant difference.

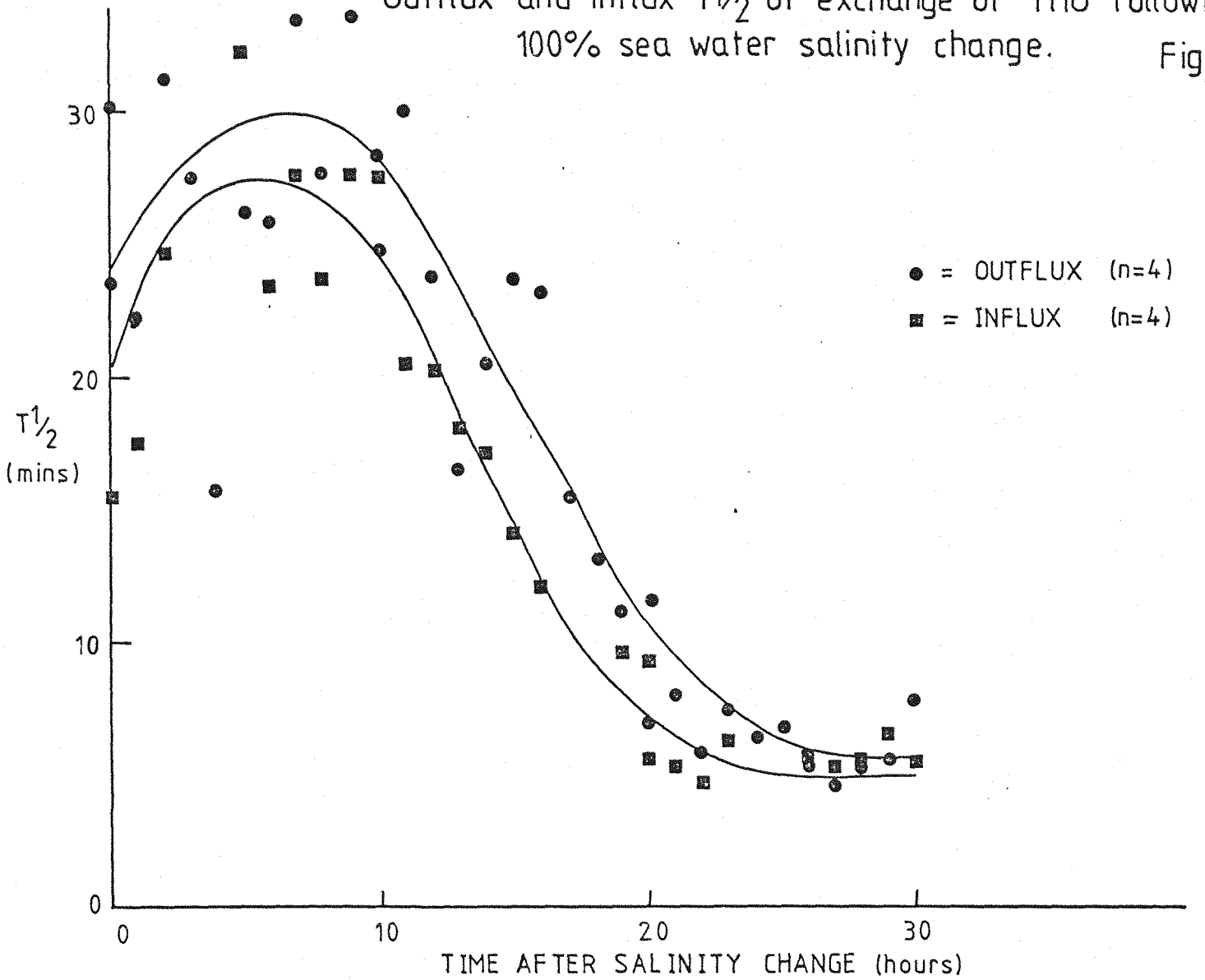
Calculations of bulk flow from osmotic concentrations and half-times of exchange of water show that the largest expected bulk flow of water in G. duebeni is a rate of 100% body water per day, while half-times of exchange of tritiated water vary from 4 minutes to 30 minutes. To see if the bulk flow could significantly affect the dynamic flow, the fastest bulk flow is compared with the least permeable condition, i.e. a bulk flow of 100% body water per day compared with a half-time of 30 minutes. Clearly the direction and magnitude of the bulk flow will have little effect on the measured half-time of exchange of tritiated water. This supports the experiment evidence of comparing influx and outflux with a hypotonic animal and can be further demonstrated by comparing the influx and outflux data during a period of hypotonicity.

G. duebeni were acclimated to 2% s.w. and transferred to 100% s.w. and the half-times of exchange measured by both influx and outflux (Fig.1:25). Each point represents the mean of 4 animals. This experiment again demonstrates that the dynamic fluxes are not affected by the bulk flow. In this experiment the influx data appears slightly more permeable during the period when the animals are hypotonic to the medium and the bulk flow would be out of the animal. If, therefore, the bulk flow were affecting the THO values, the influx data would show a lower permeability than the outflux data.

It is concluded from these experiments that an osmotic imbalance such as found in G. duebeni does not significantly affect the accuracy of the influx or outflux data.

Unstirred layers in and around the permeable surfaces could possibly affect the permeability results obtained by tritiated

Outflux and influx $T^1/_2$ of exchange of THO following a 2% to 100% sea water salinity change. Fig 1·25



water. These layers, if present, would tend to underestimate the permeability of the animals, however, the unstirred layers are unlikely to be responsible for the rapid and large changes in apparent permeability exhibited by G. duebeni. If bulk flow direction was responsible for some of the apparent permeability changes, it is unlikely that G. duebeni would exhibit large changes, while C. marinus and G. locusta, in a similar situation, do not. Further considerations of these problems will be discussed in Chapter II.

Investigations of the apparent permeability of G. duebeni indicates that these amphipods are apparently able to vary their permeability to water in osmotically stressful conditions. From the characteristics of the change in water fluxes demonstrated in G. duebeni it is important to consider the possible mechanisms of change of membrane permeability which could account for such rapid changes.

1. Workers with phospholipid bi-layers or artificial membranes (Fettiplace, 1978 , Griaziani and Livne 1972, Montal and Mueller 1972) have shown that different lipids and the degree of saturation in the lipid bi-layer can alter the permeability of an artificial membrane by an order of magnitude. Griaziani and Livne state that the similarity in water permeability characteristics may indicate a common mechanism of water transport in biological and artificial membranes.

These permeability mechanisms discussed by Griaziani and Livne may explain different permeabilities exhibited by different biological membranes or relatively long term changes in permeability which may occur in a single membrane. They are unlikely to account for the extremely rapid alterations found in G. duebeni, which apparently take place in less than five minutes.

2. The fluid mosaic model of membrane structure (Singer and Nicolson 1972) describes the membrane as resembling 'icebergs' of globular protein 'floating' in a 'sea' of lipid bilayer. The lateral movement of these proteins appear to be under the control of the peripheral membrane proteins and cytoplasmic elements.

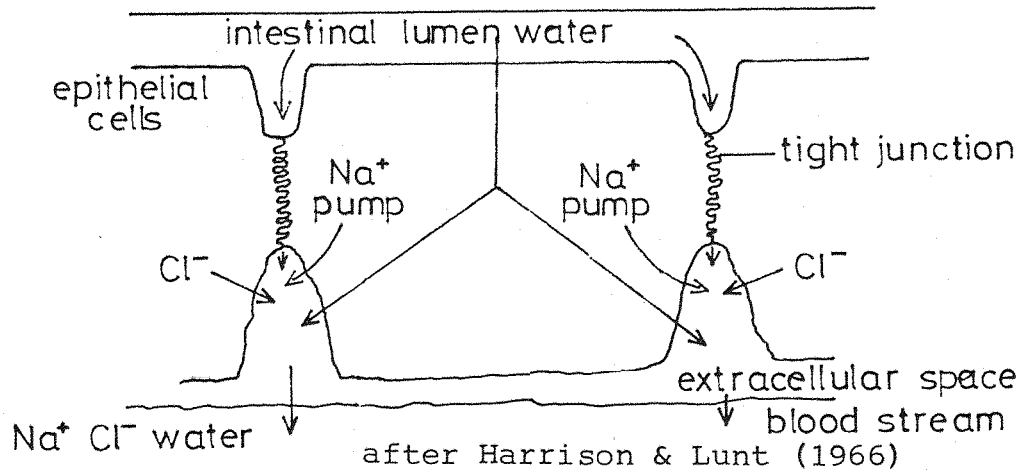
Some of these protein structures can form pores and channels through the lipid bi-layer. Gramicidins and Alamethicin are the best characterised channel or pore-forming ionophores. These ionophores are characterised by having a hydrophobic and a hydrophilic end. These molecules attach to the hydrophobic membrane by their hydrophobic actions, forming a hydrophilic oxygen lined pore through the membrane, thus allowing water to diffuse through the membrane, increasing the permeability of the membrane.

Alamethicin channel formation is dependent on the potential difference across the membrane, if there is no potential difference alamethicin does not form pores and enhance ion and water permeability, however as the voltage across the membrane is increased, a point is reached where a water-filled pore is formed, allowing diffusion across the membrane. This type of mechanism could possibly alter the permeability of the membrane extremely rapidly and could account for the observed changes in apparent permeability. Gerdenitsch, 1980 showed that the drug Isoptin (∝- isopropyl -∝- [(N-methyl-N-homoveratryl) - ⊃ γ aminopropy]-3, 4, - dimethoxypenylacetonitril) has a reversible effect on the permeability of Allium cepa cells. The Isoptin always increased the permeability of the cells. Gerdenitsch assumed that the Isoptin was forming pores across the membrane.

Further work on the effect of such chemicals on membranes is needed to investigate the possibility of reversible pore formation being responsible for the observed permeability changes in G. duebeni. The effect of P.D. across the body wall in an attempt to trigger pore formation is discussed later in the present study.

3. The epithelial cells of the small intestine of vertebrates show active re-absorption of water from the lumen. These cells are sealed by tight junctions, which tend to separate forming restricted extracellular channels. There is an active pumping of Na^+ into the channels below the tight junction generating an osmotic gradient across the junction. Water is drawn into the space, 50% through the tight junctions and the remainder from the cells. (Fig. 1:26).

Fig. 1:26



after Harrison & Lunt (1966)

It is possible for the permeability of the tight junctions to change but this change is unlikely to be quick enough to account for the observed change in G. duebeni.

It is concluded that the most likely mechanism for control of rapid changes in permeability is the PD controlled ionophore formation. This mechanism is further discussed and investigated in Chapter III.

CHAPTER 2

THE EFFECTS OF SALINITY AND TEMPERATURE CHANGE ON THE HEART RATE OF G. DUEBENI.

INTRODUCTION

G. duebeni has been shown to exhibit a change in apparent permeability when the external medium is altered. (Chapter 1). It is possible that this change in flux rates of THO could be caused by varying haemolymph flow through the gills. There are several mechanisms by which the animal could theoretically alter the flow rate through the gills (i) the blood flow might be restricted and hence alter the apparent permeability of the whole animal. (ii) the pleopod beat could vary, altering the rate of water flow over the gills. (iii) the heart rate might vary and thus affect circulation of haemolymph through the gills.

While it is not possible to measure the blood flow through the gills, pleopod beating and heart rate can be measured directly. A change of pleopod beating could alter the characteristics of the unstirred layers and hence apparent permeability. However, observation demonstrates that the pleopod beating is erratic, often stopping completely, even when the animal is acclimated to 100% s.w.

A change in heart rate would alter the blood flow through the gills affecting the apparent permeability. Cornell 1973 and Cornell (in Smith) 1976 demonstrated that changes in apparent permeability in the Atlantic spider crab Libinia emarginata was linked to changes in heart rate. The heart rate decreasing as the apparent permeability decreased and vice versa.

The rate at which fluid perfused through isolated gills approaches saturation with DHO from the medium is positively related to the flow rate of the perfusate. Thus a decrease in circulation through the gills following an osmotic shock could reduce the osmotic net flux of fluids and may represent a temporary adaptive response in Libinia emarginata.

Therefore the heart rate was investigated in G. duebeni so that any link between heart rate and apparent permeability to water would be demonstrated or rejected.

MATERIALS and METHODS

Animals were collected and maintained as described in Chapter 1.

Two methods of measuring the heart rate of G. duebeni were employed:

(i) Animals were placed individually in a perspex cell with a through flow of water. (Fig 2:1)

The cell was placed on a microscope stage, so that the heart rate could be counted directly.

The water was passed to the cell through heat exchange coils in a constant temperature water bath, so that the temperature could be maintained within the cell whilst the salinity was varied.

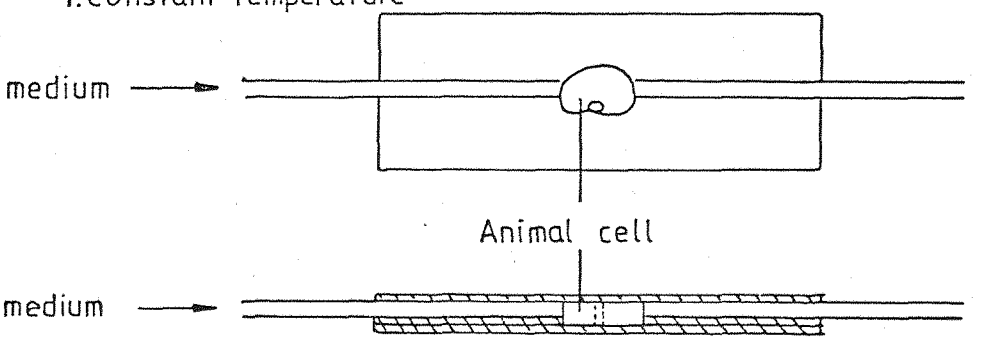
(ii) Single specimens of G. duebeni were mounted on double sided tape and placed in a container of the appropriate medium. (Fig 2:1) Care was taken to allow a window in the tape for observing the heart beat and that there was a portion of tape cut away to allow free movement of pleopods.

In both methods, a number of counts, usually 100, was timed using a stop watch and beats per minute calculated. With practice it was possible to count the heart rate accurately up to approximately 200 beats per minute.

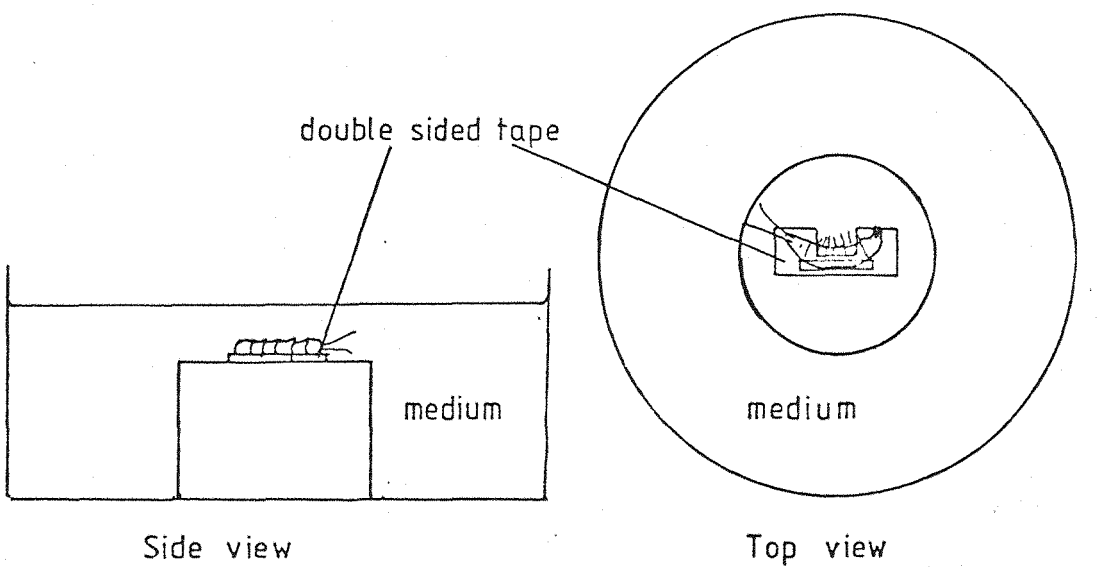
Fig 2·1

Heart rate apparatus.

1. Constant temperature



2. Varying temperature.



RESULTS

An animal was placed in the experimental flow cells and the heart rate measured every other minute.

After 45 minutes in 100% s.w. (32‰S) the medium was changed to fresh water for another 45 minutes and then returned to 100% s.w. (Fig. 2:2) A control experiment was also carried out with animals in 10% s.w. During the control experiment the flow was stopped momentarily at the 45 minute change-over period so that the animals experienced the same conditions as those exposed to salinity shock. These results show a large variation of measured heart rates, the two individuals plotted representing the limits of response shown in these conditions. (Fig 2:2)

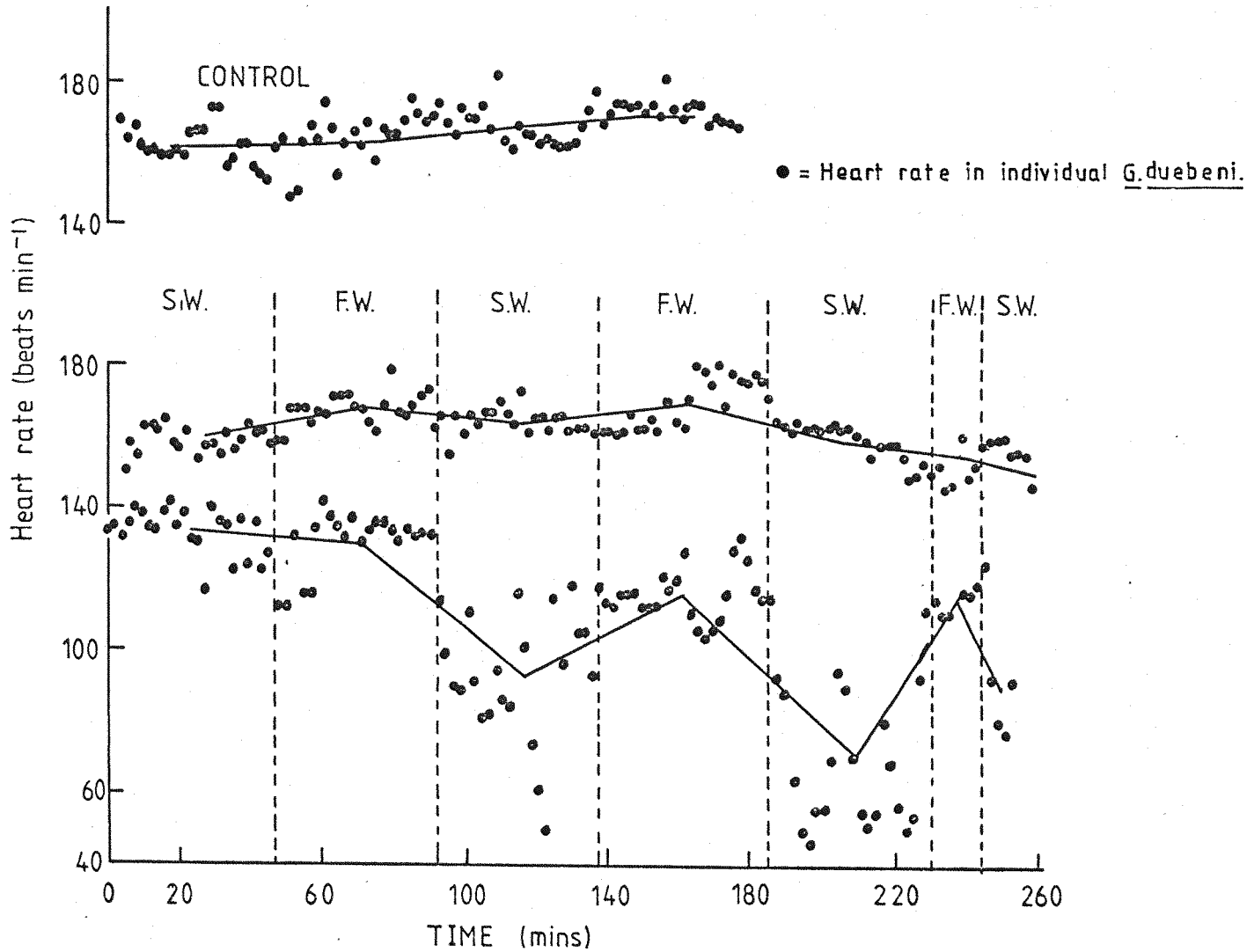
Control animals showed little change in heart rate if maintained in constant temperature and at constant salinity (Fig 2:2)

Animals pre-acclimated to 100% s.w. (Fig. 2:2) showed greater variability than the controls and although the individual variation is large, there is a tendency for the heart rate to be slower in 100% s.w. than in 2% s.w. If the heart rate were affecting the $t_{\frac{1}{2}}$ of the animal, a more rapid heart rate would be expected in 100% s.w. when the animals are more permeable, and a less rapid heart rate in the 2% s.w. where the animals are less permeable. Thus these results do not support the hypothesis that heart rate is correlated to apparent permeability.

In the above experiment the temperature was monitored $15 \pm 0.5°C$, however it is possible that even such a small change in temperature is affecting the animals' heart rate.

In order to demonstrate further that heart rate is not affecting apparent permeability, an animal was acclimated to 100% s.w. and mounted in the second heart rate apparatus (Fig. 2:2) Cold 100% s.w. (1-2°C) was added to the chamber and duplicate heart rate measurements taken at approximately 1°C intervals as the temperature in the chamber rose towards the ambient temperature. As the heart rate approached $200 min^{-1}$, the medium

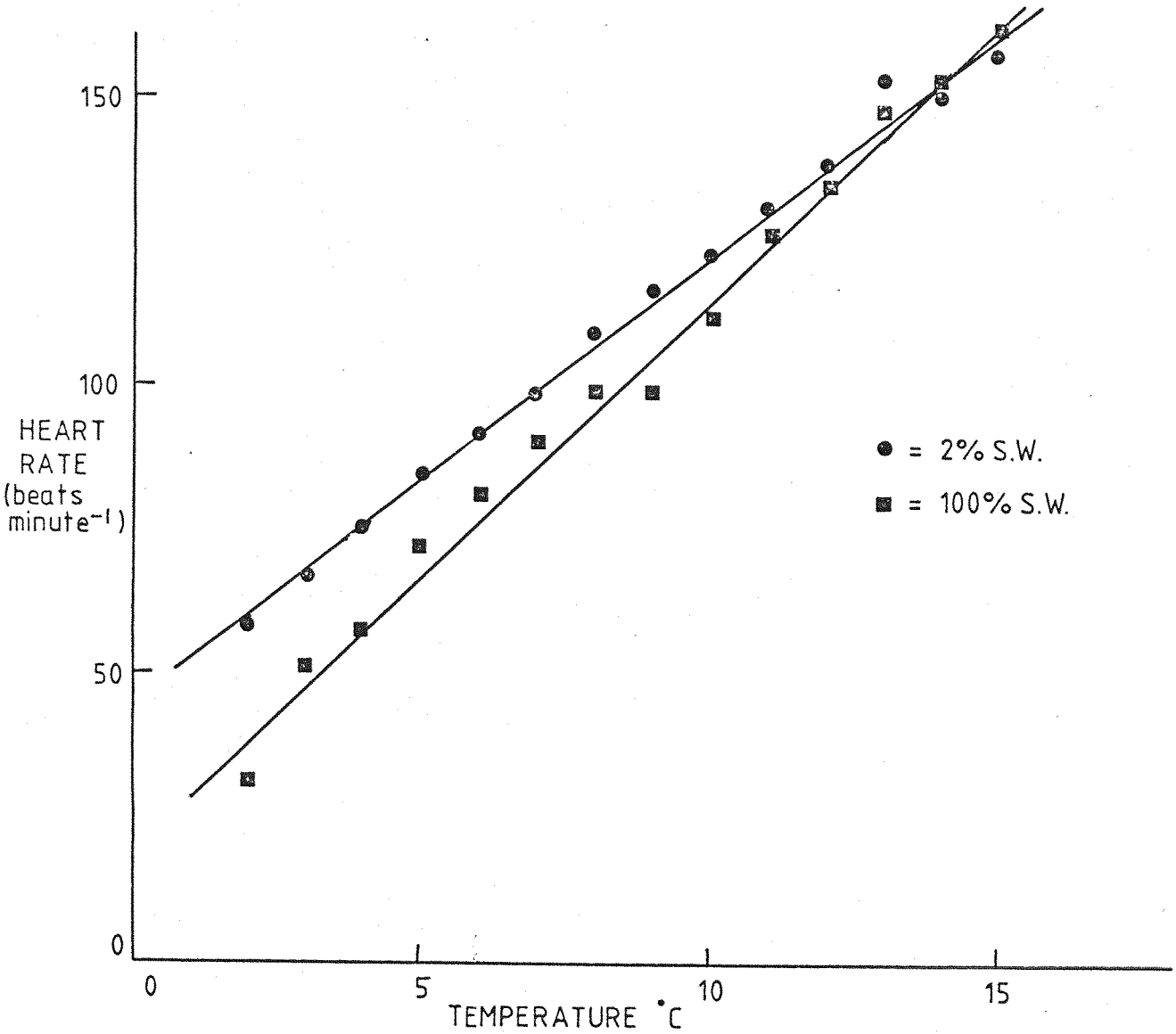
Heart rate in G.duebeni exposed to sudden changes in salinity. Fig 2·2



was drained and cold fresh water added. Heart rates were again measured as the temperature rose. By plotting the heart rate against temperature for two salinities (Fig. 2:3) the heart rates can be directly compared in conditions where the permeability of the animal would be different.

These results show a good correlation between temperature and heart rate, but no significant difference between the responses of the animals to salt and fresh water. In this experiment the animal in 2% s.w. appears to have a slightly higher heart rate than the animal in 100% s.w. As already stated, if heart rate was affecting the apparent permeability it should be higher in 100% s.w. than in 2% s.w. It may also be noted that at 15°C (the temperature chosen for all the permeability studies on G. duebeni) the heart rate of the animal in 2% s.w. is identical to that of the animal in 100% s.w. (152 beats per minute).

Heart rate against temperature in an individual G.duebeni. Fig 2·3



DISCUSSION

These results clearly show that there is no direct correlation between heart rate and permeability, although there is an extremely good correlation (r = .996) between temperature and heart rate. Thus G. duebeni does not behave in a similar fashion to Libinia emarginata, (Smith 1967) and it is unlikely that heart rate is affecting the apparent permeability by changing the blood flow through the gills. However, these results do not eliminate the possibility that the blood flow could be altered by vasoconstriction or vasodilation. Indeed recent work on the visual appearance of the gills of Gammarus duebeni (Dawson, 1982) indicate that a salinity change can alter the flow of haemolymph through the gills. When the animals are subjected to a sudden increase in external medim concentration, the gills become more opaque, and the flow of haemocytes through the blood vessels appears to cease. This experiment was carried out in different salinity regimes and can be compared with the half-time of exchange of THO.

TABLE 2.1

Salinity	$t_{\frac{1}{2}}$ mins *	Gill state
2%	15	Clear, heomocyte movement unobstructed.
2-100%	17	Opaque, haemocyte movement obstructed.
100%	6	Clear, haemocyte movement unobstructed.
100-2%	17	Clear, haemocyte movement unobstructed.

* Bolt, unpublished.

These preliminary results show that the gill state does not appear to be correlated to the half-time of exchange of THO. The only salinity change that caused a significant change to the observed gill structure is an increase in external medium concentration, which does not dramatically change the $t_{\frac{1}{2}}$ of exchange of THO. Initial experimentations injecting dye into the body cavity and timing the colouration of the observed gills confirms these findings (Dawson, 1982).

Thus, while these results need further investigation, it is not felt that such vasoconstriction is the cause for the significant permeability changes observed in G. duebeni.

CHAPTER 3

URINE CLEARANCE RATES IN G. DUEBENI EXPOSED TO VARYING SALINITIES

INTRODUCTION

Chapter 1 demonstrates that Gammarus duebeni shows a dramatic change in apparent permeability when exposed to differing salinities. These results were obtained using THO as a marker to measure water exchange rates. Until such changes in water permeability can be demonstrated by a different approach, the possibility that the permeability change found by THO is an artefact of the method used linger. Smith (1967) accepted this limitation and noted the necessity of using the term "apparent" when discussing permeability changes measured by the use of THO or DHO as a marker. Comparison of different amphipod species demonstrates that G. duebeni exhibits large and sometimes rapid changes in apparent permeability, whilst Chaetogammarus marinus and Gammarus locusta show no such changes when exposed to similar conditions. While this demonstrates that the change in apparent permeability in G. duebeni is not due to an experimental error in carrying out the technique, it still does not eliminate the possibility that unstirred layers or other artefacts could be present in G. duebeni and not in G. locusta or C. marinus, thus giving false permeability results.

To eliminate successfully the term "apparent" from the THO permeability results, permeability changes must be demonstrated in G. duebeni by another technique. THO experiments measure the dynamic fluxes of water into and out of the animal, such exchange of water in the presence of an osmotic gradient causes a bulk flow in the direction of the osmotic gradient. If this bulk flow is inwards, there must either be a build up of the internal pressure to equal the osmotic pressure or a method of excreting the excess fluid. It is assumed that this inward bulk flow is matched by an outward flow of urine. Thus theoretically measurement of urine flow and osmotic gradient should permit calculation of $t_{\frac{1}{2}}$ of water exchange for the animal.

This technique is only operable if the bulk flow of water is into the animal and the animal is urinating. Thus it is only possible when the osmotic concentration of the haemolymph of the animal is above that of the external medium.

By finding the urine flow in G. duebeni it is theoretically possible to determine the $t_{\frac{1}{2}}$ of exchange for that animal using a technique which does not involve THO as a marker. Thus if the urine flow can be monitored during a period of "apparent" permeability change, the change in THO $t_{\frac{1}{2}}$ can be confirmed or contradicted.

In order to measure the urine flow out of an animal, a radio-active marker is introduced into the body fluids so that the loss of marker via the urine can be monitored as a percentage of body fluids per unit time.

Markers of glomerular filtration rate (GFR) need to fulfil the following requirements to give reliable results.

(a) The marker should not penetrate into the cells or bind to the haemolymph molecules. This would limit free excretion into the urine and would hence cause underestimation of the urine rate.

(b) The marker should not be re-absorbed from the urine after filtration as this would also underestimate the flow of urine.

(c) The marker should only enter the urine by filtration. If active secretion into the urine occurred this would increase the excretion of the marker and overestimate the rate of urine flow.

In recent studies on the crab Holthuisana transversa, Greenaway (1981) investigated three GFR markers commonly used in higher vertebrates: ^{14}C Polyethylene glycol 4000, (PEG) ^{3}H Inulin and ^{51}Cr E.D.T.A. Greenaway concluded that the bulk of ^{14}C PEG is sequented in the tissues, underestimating the true rate of filtration and Inulin is partially re-absorbed from the excretory organ. ^{51}Cr E.D.T.A. however, appears to be cleared from the haemolymph and passed out into the urine. Greenaway found no evidence of ^{51}Cr E.D.T.A. sequented by tissues or of re-absorbtion from the excretory organ. He thus concluded that ^{51}CR E.D.T.A.

is likely to provide the most reliable estimate of filtration rate.

For these reasons, ^{51}Cr E.D.T.A. was selected as the most appropriate radio-active marker for the present study.

MATERIALS and METHODS

^{51}Cr E.D.T.A. was obtained from the Radiochemical Centre, Amersham, Bucks, the preparation contained 3-7mg ^{51}Cr E.D.T.A. ml^{-1} and 0.9-2.4mg sodium edetate BPC ml^{-1}. The specific activity provided was 1-2 mCi/mg Cr, and to obtain the required activity per unit volume it was necessary to concentrate the material 10:1 by freeze drying.

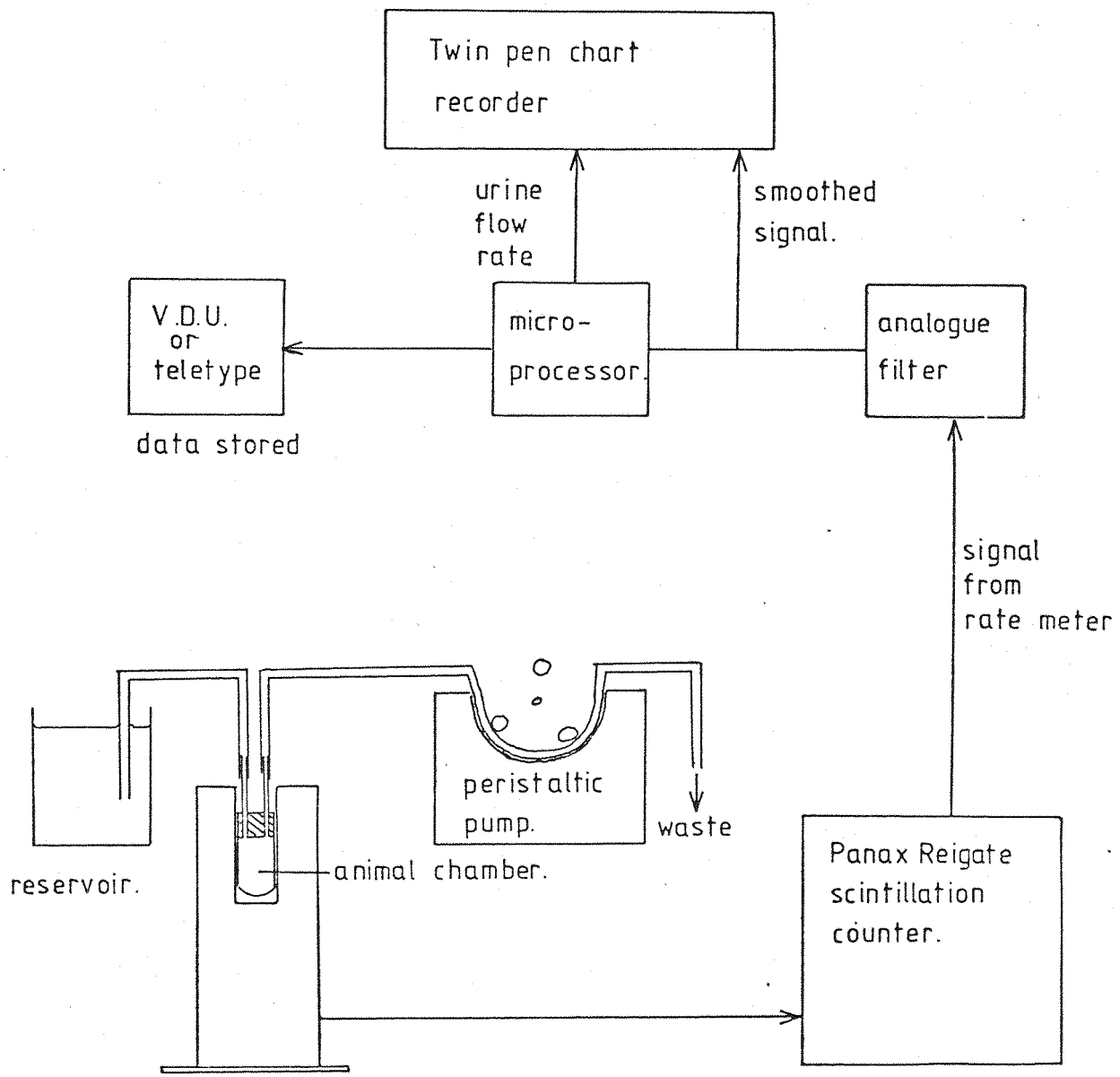
Animals were injected through the lateral surface between the cuticle segments with 1.5 µl of this concentrated ^{51}Cr E.D.T.A. using a fine drawn out pipette and oral pressure. It was not necessary to inject a known volume of radio-active material as the clearance rates are calculated as a % of body fluid volume. Approximately 10 animals were injected, allowed to recover for 2 hours and counted using a hand held scintillation counter. The four animals with the highest counts were chosen after any moribund animals had been discarded. The chosen animals were put in a cut down counting tube, which was placed in a well type scintillation counter with a through flow of medium to flush away any excreted ^{51}Cr E.D.T.A. The salinity in the chamber could be predetermined by controlling the salinity of the inflow resevoir. (Fig. 3:1).

It was necessary to count several animals in the experimental chamber to obtain a sufficiently high count to minimise the random fluctuations characteristic of radio-active disintegrations. The animals were observed at the end of the experiment and kept in clean 100% s.w. for several days. Any experiment carried out with an animal which subsequently died was ignored.

Two techniques for data processing were used to determine the % urine flow.

Fig 3·1

Apparatus to measure urine clearance rates.



(i) Data logging.

The signal from the Panax Reigate Scintillation Counter was sampled and recorded on to magnetic tape at one second intervals using a Super-8 data logger. At the end of the experiment this data was transferred to a Hewlett-Packard 20 bench calculator and a low pass mathematical filter employed. (L. Muir personal communication) in an attempt to filter out noise caused by non-uniform rates of radio-active disintegrations.

Equation 3:1 $$Yi = Y_{i-1} + (1 - \alpha)\, Xi$$

where Xi is input data
Yi is output data
α is the smoothing constant

A value of .9995 for α was found to give an acceptable degree of smoothing.

This method of smoothing the data proved unreliable due to mechanical problems with the data logger which could not be overcome.

The urine flow as a % of blood volume could be calculated from the smooth curve from the following equation:

Equation 3:2

$$\%\ \text{loss of blood volume per unit time} = \frac{2}{\Delta t \times n}\left(\frac{C_{i+n}-C_i}{C_{i+n}+C_i}\right) \times 100$$

where Δt is the time between points
C_i counts at time t
n = 1 if adjacent counts used
n = 2 if alternate counts used etc.

(ii) Microprocessor data handling

The previous method of using the data-logger and H.P. 20 was cumbersome as it took at least 8 hrs to process a single experiment. It was advantageous to re-design the data handling aspect of this technique and a COSMAC CDP 18S020 microprocessor was employed to this end. To overcome the problem of signal noise an electronic analog filter (Kemo) was purchased and used in place of the mathematical low pass filter. (Fig. 3:1).

This technique proved reliable and did not require the time consuming process of re-entering the data into a bench calculator, all the data smoothing and processing being carried out by the microprocessor as the experiment progressed.

A continuous signal was taken from the rate meter of the Panax Reigate and fed via a land line to the Kemo filter, which smoothed out much of the random fluctuations characteristic of nuclear disintegrations. This smoothed signal was fed to the COSMAC CDP 18S020 microprocessor for data logging. (Fig. 3:2)

The microprocessor was programmed to sample repeatedly and digitise the signal from the Panax Reigate. These digitised values were summed over a 60 second period and stored in memory. The microprocessor repeated this process, and took the mean of the two summations. The value was stored in memory and displayed on a V.D.U. or teletype. The process was repeated at intervals of 15 minutes. (Fig. 3:2)

The microprocessor also used these data to calculate the urine loss as a percentage loss of body fluid per unit time using equation 3:2

This was directly displayed on to a chart recorder to monitor the urine flow throughout an experiment. The smoothed signal from the Kemo filter was also displayed on a chart recorder to monitor the radio-activity of the animals during the experiment. It was possible to detect the death of an animal and subsequent high loss of ^{51}Cr E.D.T.A. without disturbing the animals. Any fatalities during an experiment meant the immediate abandonment of that experiment.

Knowing the osmotic concentration of the blood and medium by approximating 1 osmole = 500 mMl^{-1} sodium concentration, the molar concentration of water in the blood and medium is calculated by: Equation 3:3

$$Mm \text{ or } Ma = \frac{55.56}{55.56 + X}$$

where X = 0 to 1.0 osmole, blood or medium

Mm = Molar concentration of medium

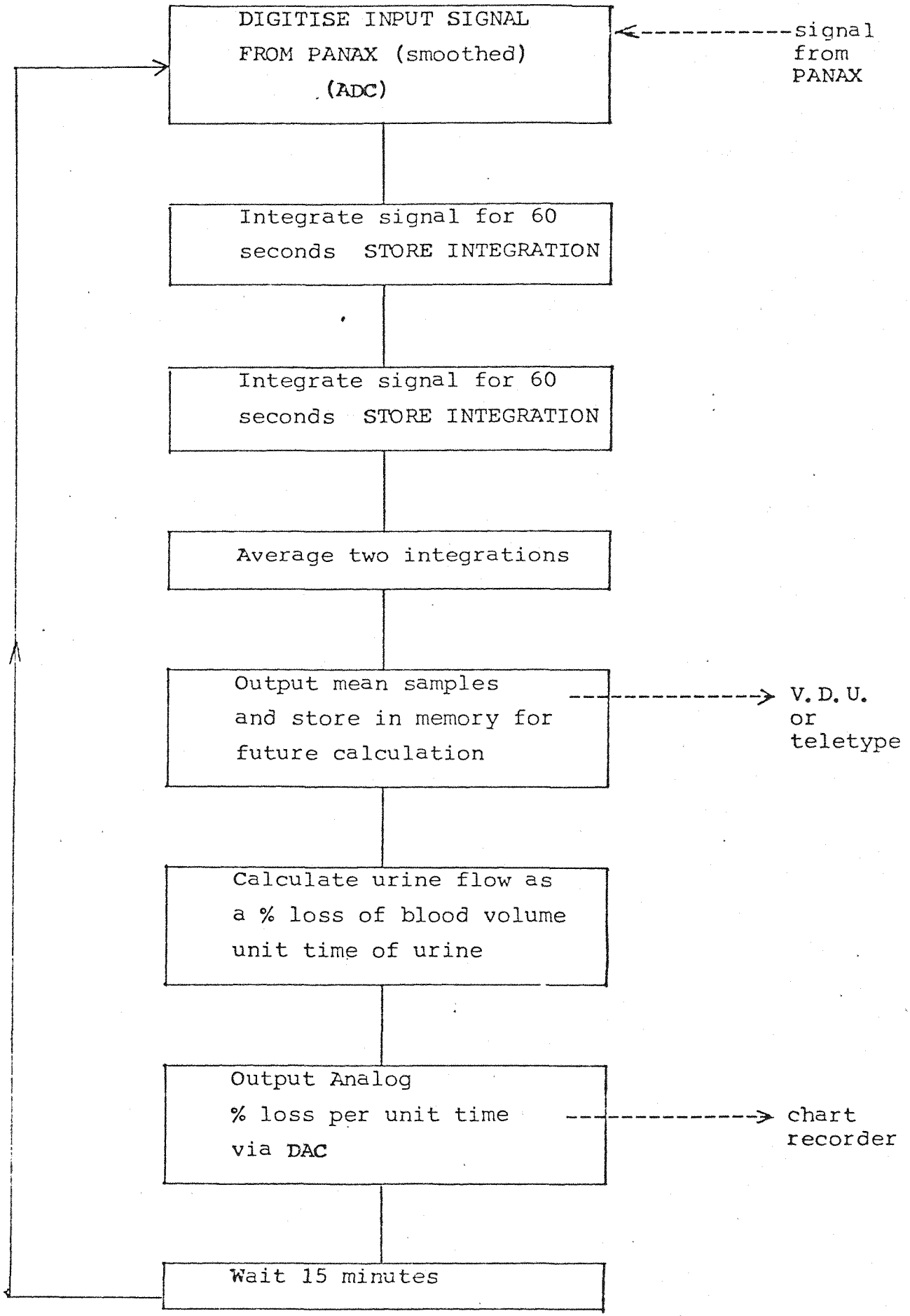
Ma = Molar concentration of blood

Using net flow equation from p42.

The theoretical $t_{\frac{1}{2}}$ of exchange of water is calculated from the urine flow.

Fig 3·2

Microprocessor data logger



Equation 3:4

$$\frac{\left(\frac{Mm - Ma}{Mm}\right) 100 \ln 2}{Os} = t\tfrac{1}{2} os$$

Where Os = % blood volume loss per minute.

Equation 3:5

$$Os = \frac{2}{\Delta tn}\left(\frac{C_{i+n} - C_i}{C_{i+n} + C_i}\right) X\ 100\ X\ 1440$$

(c.f. p. 66)

The $t\frac{1}{2}os$ can be compared directly with the half time of exchange as found by tritiated water ($t\frac{1}{2}$ THO) as both values refer to half time of exchange of blood volume. However, absolute values of both methods cannot be compared with confidence.

RESULTS

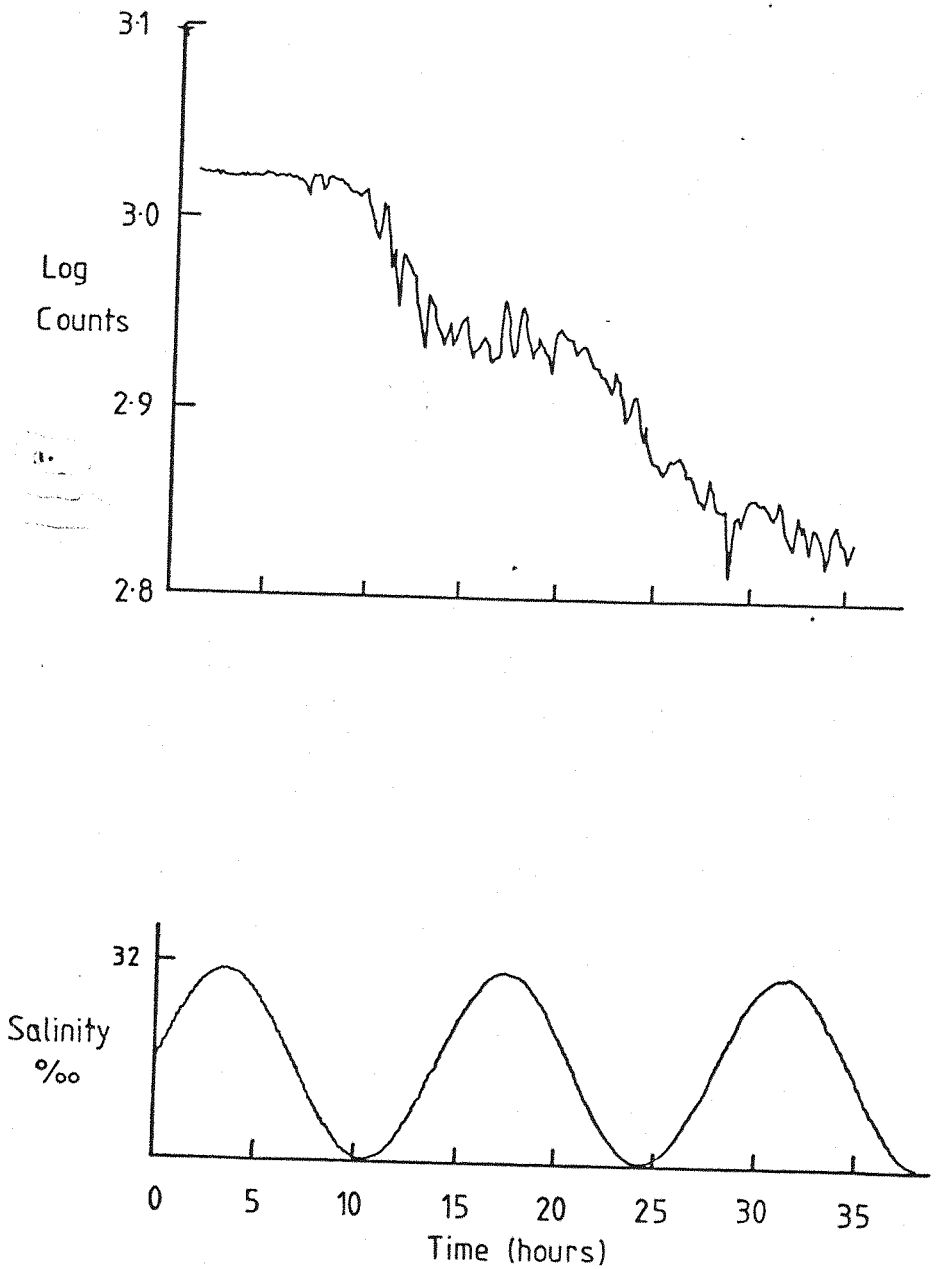
(i) Salinity cycle

G. duebeni were acclimated to the cycling salinity systems for four cycles (48 hrs +) and injected with ^{51}Cr E.D.T.A. They were allowed to recover in the cycling salinity for 2 hrs and then placed in the experimental counting tube, with the salinity cycle acting as the resevoir for through flow. (Fig. 3:1) Measurements of the output from the Panax Reigate rate meter were taken at one second intervals and stored on the Super-8 data logger. These data were later mathematically smoothed and plotted by the H.P. 20 bench calculator as log counts against time. (Fig. 3:3) This shows a single experiment which is representative of a series of experiments.

Although these preliminary results suffered from equipment problems, they clearly show a decline of the loss of urine during periods of high salinity and an increase in tracer loss at low salinities. This demonstrates G. duebeni are urinating when their haemolymph is hypertonic to the external medium and cease to urinate when their blood is hypotonic to the external medium.

Elimination of Cr EDTA by G.duebeni in a cycling salinity system.

Fig 3·3



From these data the half-time of exchange of water for G. duebeni can be tentatively calculated during the salinity cycle. First the urine flow out of the animal as a percentage of blood volume per day is calculated. Then, knowing the osmotic gradient, the theoretical half-time of exchange of water for the animal or $t_{\frac{1}{2}}$ calc can be estimated. Because there is no urine flow when the animal is hypotonic to the medium, a comparison of the calculated $t_{\frac{1}{2}}$ ($t_{\frac{1}{2}}$ calc) with the $t_{\frac{1}{2}}$ found using THO ($t_{\frac{1}{2}}$ THO) can only be made when the animal is hypotonic to the external medium. Unfortunately this eliminates the rapid changes in permeability which occur when the blood and medium are approximately isotonic (c.f. Fig. 1:15).

It must be emphasised that in comparing the calculated half-time of exchange of water with that found using THO, it is the pattern of change and not the absolute values which are important. However, during the salinity cycle $t_{\frac{1}{2}}$ calc and $t_{\frac{1}{2}}$ THO show a similar pattern of permeability change. (Fig. 3:4).

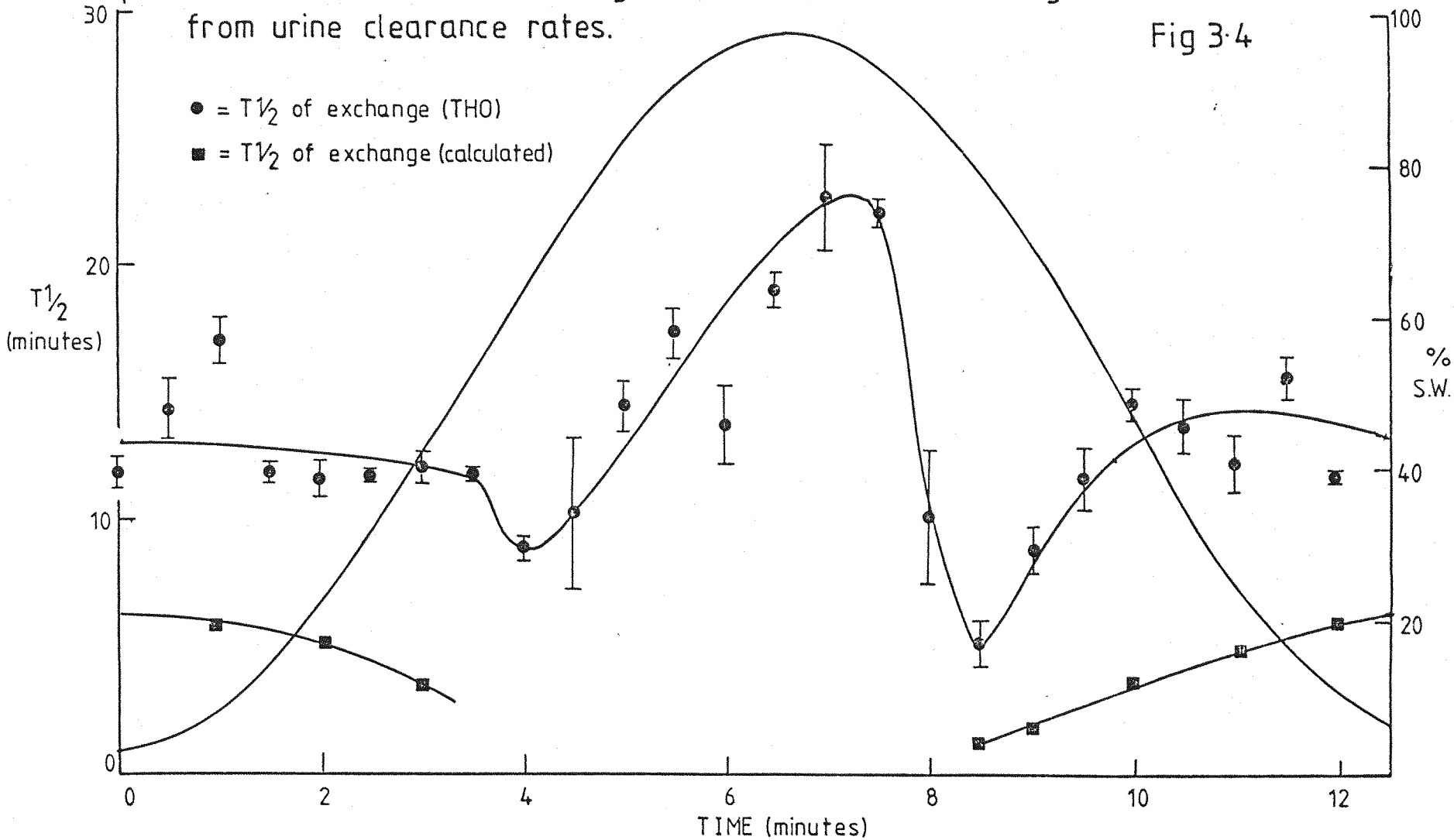
This initial experiment was exploratory and suffered from excessive data "noise" and hardware problems with the Super-8 data logger. However, the trend of permeability change is consistent in both $t_{\frac{1}{2}}$ THO and $t_{\frac{1}{2}}$ of water calculated using ^{51}Cr E.D.T.A. In both experiments, the apparent permeability of the animal to water increases as the animals haemolymph approaches isotonicity with the external medium, and decreases as their haemolymph becomes hypotonic to the external medium.

(ii) Apparent permeability to water as measured by THO showed that the rapid changes in apparent permeability occur when the animals blood is hypotonic to the medium approaching isoionic and vice versa. These conditions cannot be studied by urine flow as the animals do not urinate when the external medium is more concentrated than internal body fluids.

In order to compare further the calculated $t_{\frac{1}{2}}$ of water ($t_{\frac{1}{2}}$ calc) and $t_{\frac{1}{2}}$ THO, another period of significant change in apparent permeability is needed when the animal's haemolymph is hypertonic to the external medium, and hence it is urinating.

Comparison of half-time of exchange of water determined using THO and calculated from urine clearance rates.

Fig 3·4



Previous experimentation with THO (Chapter 1) has demonstrated that the experimental regime of acclimating G. duebeni to 2% s.w., transferring them to 100% s.w. and then using the microprocessor to reduce the salinity back to 2% s.w. shows a significant change in apparent permeability. (c.f. Fig. 1:19)

The animals are first forced hypotonic to the external medium, becoming isoionic and finally hypertonic, increasing the haemolymph to external medium concentration gradient until the external medium reaches 2% s.w. (c.f. Fig. 1:19). During the period of increasing blood/medium hypertonicity, THO studies indicated a decrease in apparent permeability from a $t_{\frac{1}{2}}$ of 6 minutes to a $t_{\frac{1}{2}}$ of 16 minutes. To study whether these changes are reflected in urine flow rates G. duebeni were acclimated to 2% s.w. injected with ^{51}Cr E.D.T.A. and subjected to the above regime. A second COSMAC CDP 1802 microprocessor was used to process the signal from the Panax Reigate counter, via an analog filter (Kemo) to smooth out excessive random noise. A control experiment was undertaken with G. duebeni acclimated to 2% s.w. injected with ^{51}Cr E.D.T.A. and transferred to 2% s.w. The signal from the Panax Reigate was processed as previously described. (Fig. 3:5).

The control experiment (Fig. 3:5) shows clearly why absolute values of $t_{\frac{1}{2}}$ obtained from this technique should not be used. There is a steady drift of $t_{\frac{1}{2}}$ in an animal at equilibrium, demonstrating a change in clearance rates of the tracer as the time from injection increases.

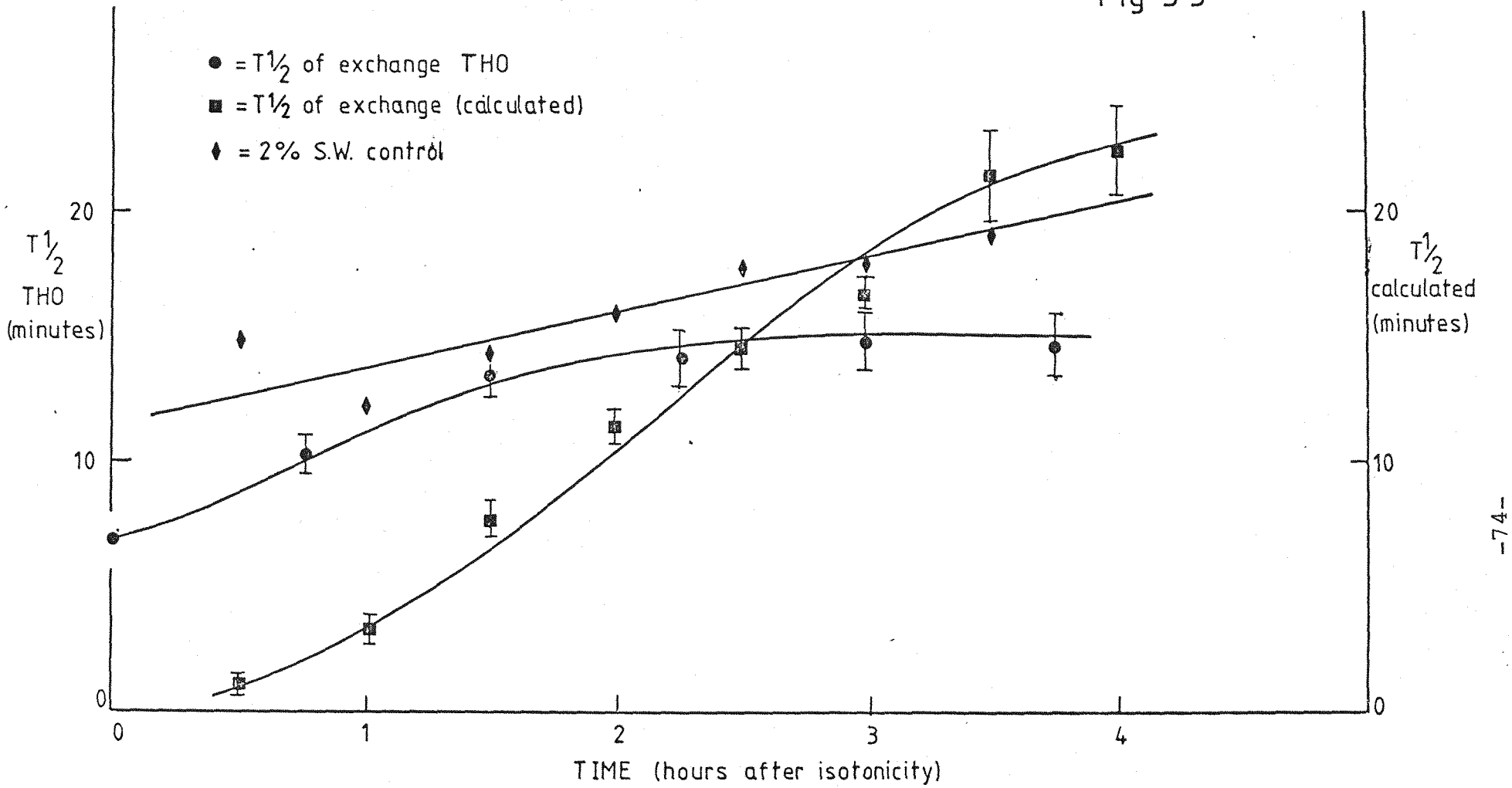
In the animals subjected to increasing blood/medium hypotonicity there is a large increase in $t_{\frac{1}{2}}$. (Fig. 3:5)

These results assume a urine to blood ^{51}Cr E.D.T.A. ratio (U/B) of 1 throughout the experiment. Lockwood and Inman 1973 have shown that the U/B ratio varies from 1.05 in 100% s.w. to 1.15 in 2% s.w.

Although the change in U/B ratio would affect the calculated $t_{\frac{1}{2}}$ value obtained from the urine loss results, the magnitude of the change in $t_{\frac{1}{2}}$ calc could not be explained by the change of U/B ratio as found by Lockwood. However, this U/B ratio could partially explain some of the discrepancy between the magnitude of change

Comparison of half-time of exhange of water determined using THO and calulated from urine clearance rates.(see text)

Fig 3·5



in $t_{\frac{1}{2}}$ THO and $t_{\frac{1}{2}calc}$.

One feature noted in this experiment is the high permeability calculated from urine flow around isoionicity, which, according to the calculations would lead to an animal of infinite permeability. This demonstrates that either there is some leakage of ^{51}Cr E.D.T.A. out of the injection wound, or the assumption that the urine flow equals bulk flow is not completely correct.

Animals in this experimental regime showed a consistant change in $t_{\frac{1}{2}}$ calc, exhibiting a larger change in $t_{\frac{1}{2}}$ than found by THO. However, if the slope of the 2% control is taken into account this change could be reduced to a similar magnitude as that of the change of $t_{\frac{1}{2}}$ THO.

In this experiment the absolute values of the $t_{\frac{1}{2}}$ calc and the $t_{\frac{1}{2}}$ THO appear to be similar. It must be emphasised that this is not significant as the time of the experiment after injection will alter the values of $t_{\frac{1}{2}}$ calc and thus it is the agreement of the pattern of change which is considered important.

These experiments show a definite change in permeability of G. duebeni which corresponds to that noted using tritiated water. However, only changes during hypertonicity can be compared, which unfortunately precludes the rapid changes shown by THO during periods when the animals are not urinating.

DISCUSSION

The technique of estimating the half-time of exchange of water using urine flow as an indirect indicator of bulk flow involves many assumptions. It is not intended as a technique to replace the use of tritiated water studies, or to give absolute value of $t_{\frac{1}{2}}$. The ^{51}Cr E.D.T.A. work was carried out in order to discover if the changes in $t_{\frac{1}{2}}$ observed in G. duebeni could be demonstrated by another method other than using THO.

The results from this chapter strongly suggests that the changes of $t_{\frac{1}{2}}$ observed in G. duebeni, using THO are real, by demonstrating that the calculated change for water, based on ^{51}Cr E.D.T.A. clearance are similar to those measured using THO. This evidence is considered in conjunction with the comparative studies on G. duebeni, G. locusta and C. marinus, where only G. duebeni exhibits radical changes in apparent permeability (cf. Chapter 1). If the results showing change in the $t_{\frac{1}{2}}$ of G. duebeni were artefacts of the experimental technique, it is likely that G. locusta and C. marinus would suffer from these same artefacts.

Thus there can be little doubt that the changes in apparent permeability measured by THO indicate an actual change in permeability of G. duebeni.

Furthermore, many of the criticisms of the THO technique have been shown to be unfounded. Chapter 2 clearly demonstrates that the heart rate of G. duebeni is unlikely to alter the permeability, while flux studies (Chapter 1) have shown that isosmosity between the haemolymph and the external medium is not necessary if dynamic fluxes are to be measured, as initially suggested by Motais (1969).

It is thus concluded that these urine studies, in conjunction with the comparative studies, have successfully achieved one of the main aims of this thesis, that is to obviate the necessity for the term "apparent" when discussing permeability changes measured using tritiated water as a marker.

CHAPTER 4

THE EFFECTS OF A POTENTIAL DIFFERENCE CHANGE ACROSS THE BODY WALL OF G. DUEBENI ON ITS PERMEABILITY TO WATER.

INTRODUCTION

Chapters 1-3 have been concerned with demonstrating that the euryhaline amphipod G. duebeni has differing permeabilities to water when exposed to varying salinities. In Chapter 1, various possible mechanisms were discussed, concluding that a protein pore formation across the membrane such as alamethicin (Harrison & Lunt, 1980) is the most plausible mechanism. These proteins could be aligned into pores across the membrane by a varying potential difference across that membrane. Thus a permeability change could in theory be triggered by variations in P.D. across the membrane.

Whilst it is difficult to disprove this hypothesis, if a significant change in permeability could be demonstrated by forcing a change in P.D. across the body wall, then this would lend considerable strength to the conclusion that P.D. controls permeability change.

Thus an experimental apparatus was designed to enable permeability to be measured while P.D. across the body wall is altered, approximating to the effect of a change of P.D. across a single membrane.

MATERIALS and METHODS

Microprocessor controlled voltage clamp.

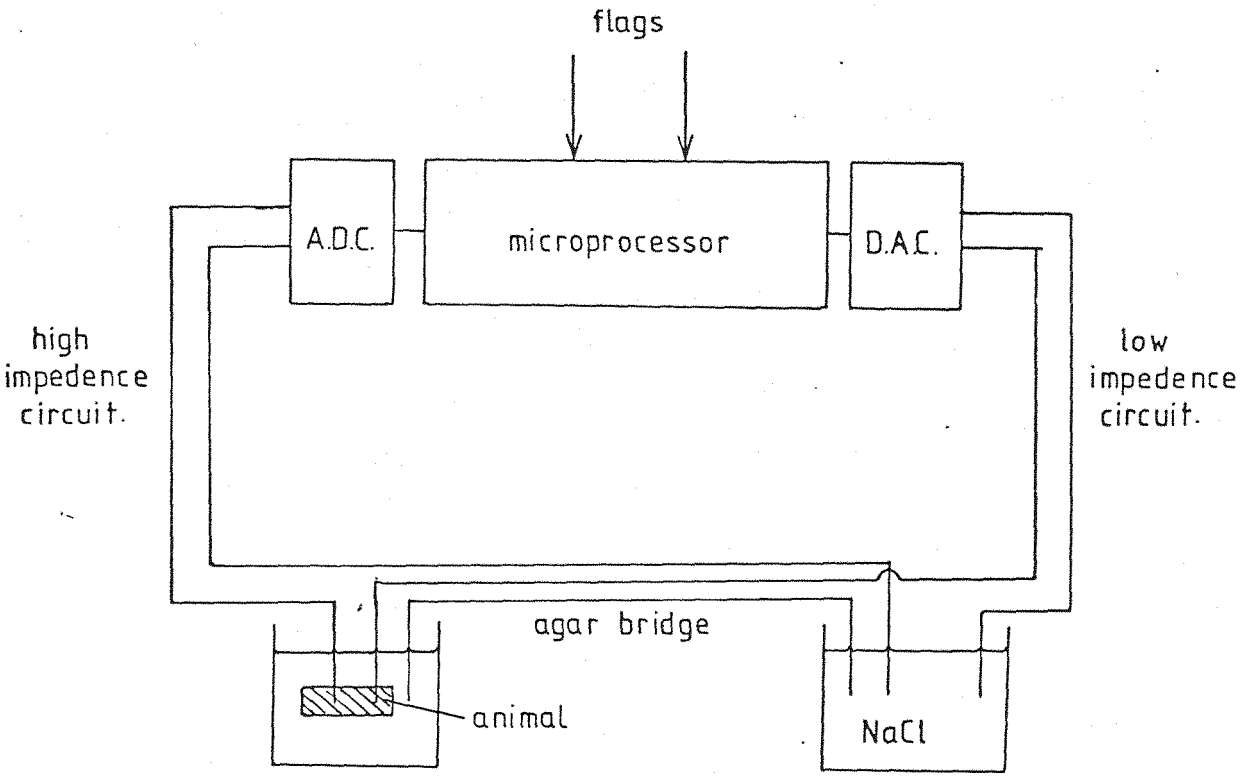
To investigate the effects of P.D. across the body wall of the amphipod, a mechanism to maintain a constant P.D. was required. This had to be capable of holding the P.D. within narrow limits even if the resistance of the body wall changed during the experiment. The system designed involved a feedback mechanism where the P.D. across calomel electrodes in a high impedence circuit is measured, and a separate low impedence circuit inputs the necessary voltage difference between the inside and outside of the animals. (Fig. 4:1).

The COSMAC CDP 18S020 microprocessor was modified (N. Jenkinson) to give a high resolution analogue output (12 byte digital to analogue (DAC) converter). This was used directly to input the voltage into the animal. Thus the microprocessor sampled the voltage from the calomel electrodes, compared this to a fixed stored value, and adjusted the output by the smallest increment, or decrement possible (.5mV). Due to the speed of operation of the microprocessor, this output voltage can be made to change rapidly over a range of -2.5 - +2.5 volts. (Fig. 4:2) even though the individual increments or decrements are very small.

In order to change the P.D. across the body wall without disconnecting the animal and reprogramming the stored P.D. value, two external "flags" are utilized into the program. This enables the stored P.D. value to be increased or decreased by making a circuit and switching on either flag 1 or flag 2 (Fig. 4:2). By monitoring the P.D. across the body wall on a chart recorder, it is possible to switch on the required flag for a second or two until the new required P.D. is reached, switching the flag off as the value is obtained. The microprocessor then holds the P.D. constant.

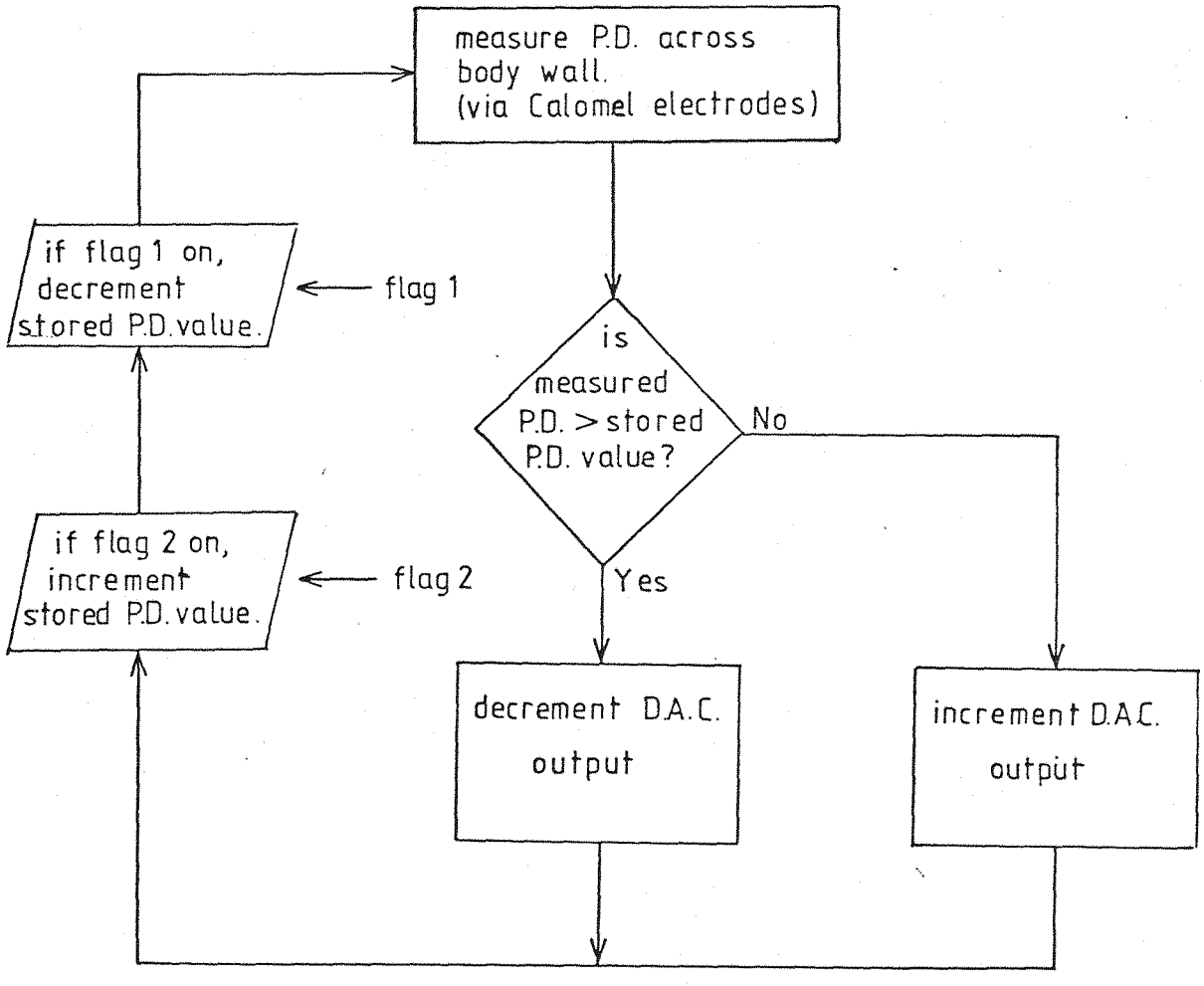
This mechanism proved flexible and accurate, the P.D. usually being maintained $\pm$ .5mV across the body wall throughout an experiment.

Microprocessor controlled voltage clamp. Fig 4·1



Flow diagram for microprocessor controlled voltage clamp.

Fig 4·2



Apparatus to introduce a voltage clamp across the body wall.

The animal was mounted on a rubber bung using double-sided tape so that it was immobilized but allowed free pleopod movement. (Fig. 4:3).

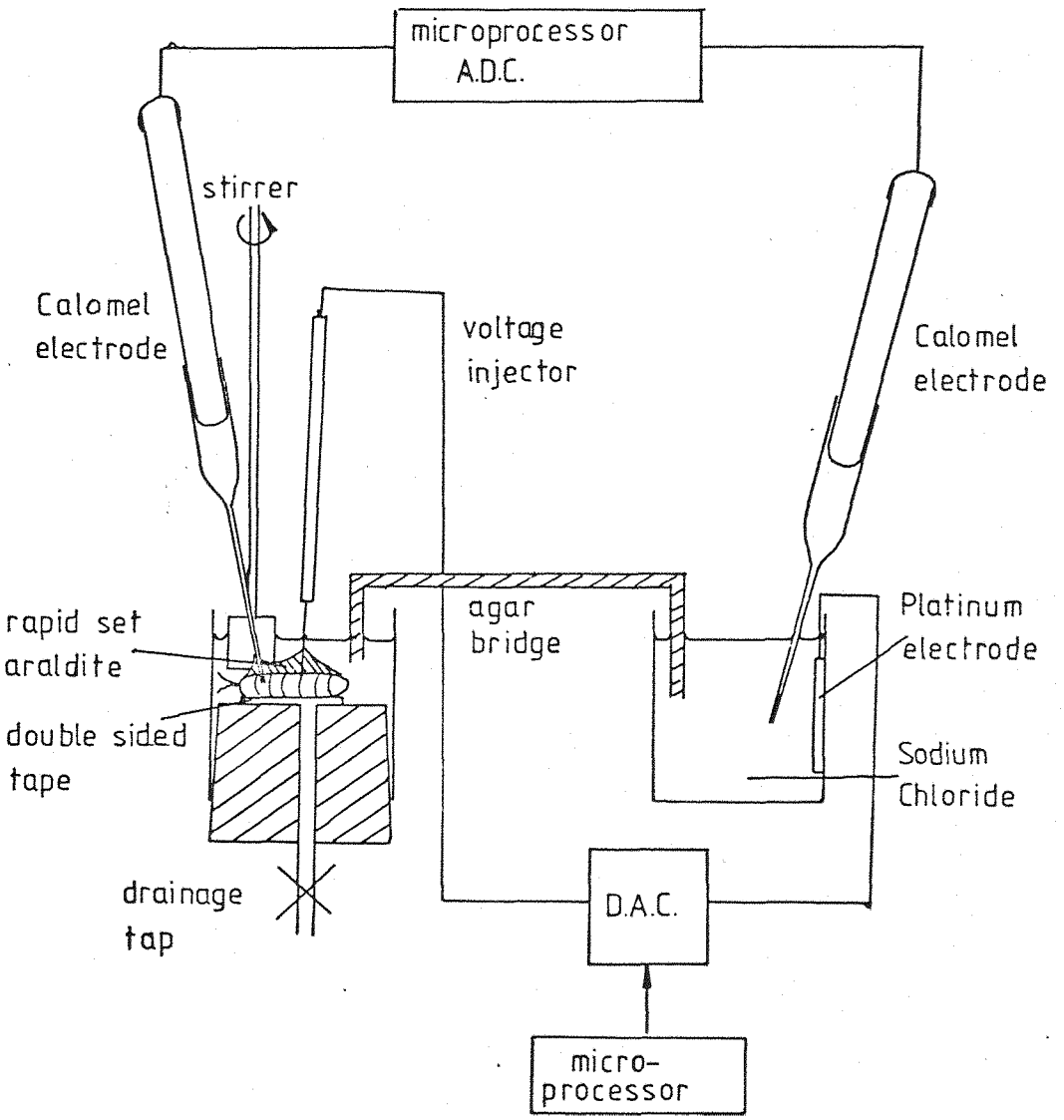
A calomel electrode was introduced to measure the internal P.D. and a low impedence electrode to adjust the internal P.D. The calomel electrode was modified to accept an agar/NaCl filled fine glass tip suitable for introduction between the lateral body segments. The voltage injector electrode was a fine piece of platinum wire, coated with varnish except for the tip which was introduced between the lateral segments of the animal. Both electrodes were attached to micromanipulators so that careful positioning was possible. A recurring problem during the experiments was the necessity of making a good electrical seal over these electrodes, so that even submerged under saline conditions, a P.D. could still be set up across the body wall. Many sealing compounds were tried, including various waxes, dental cement and varnish. However, the most consistantly successful compound was quick set araldite resin. Careful drying of the electrodes and body surface, followed by treatment with Silicon-based "Repelcote" appeared to enhance the sealing of the electrodes by araldite.

Half-time of exchange of THO in the P.D. apparatus.

Once the animal had been successfully mounted and the P.D. apparatus and pleopod beat was unhindered, $t_{\frac{1}{2}}$ for tritiated water was found using the outflux method (Chapter 1). Animals are loaded in the P.D. apparatus in the appropriate external medium concentration. During the loading period, the chamber was covered in "cling film" to minimise exchange of THO with atmospheric water vapour. The chamber was then drained, and the animals rinsed several times, the experimental vial was then filled with unloading medium, stirred using the mechanical stirrer and duplicate aliquots taken at one minute intervals.

One of the flags on the microprocessor could be switched to alter the internal P.D. during the experiment and any resultant change in permeability to water measured.

Voltage clamp apparatus. Fig 4·3



By plotting log ($C_\infty - Ct$) against time, where C_∞ is counted after the animal has reached equilibrium and Ct is counted at time t, the $t_{1/2}$ of exchange of the animals can be calculated. Any change in the slope of the line indicates a change in permeability of the animals.

RESULTS

(1) Experiment to measure P.D. across the body wall of G. duebeni.

Before the effects of changing the internal to external P.D. could be investigated, it was necessary to measure the "normal" P.D. across the body wall. Experiments were thus designed to attempt to artificially force a permeability change by changing the P.D. across the animal instead of the external medium concentration.

G. duebeni were acclimated to 2% s.w. and 100% s.w. for at least 48 hours prior to the experiment, individual animals were mounted in the P.D. approaches with only the calomel electrodes. The animals were allowed to settle down for 5 minutes. A reading was taken with the external medium identical to their acclimating medium. Animals from 100% s.w. were then exposed to 2% s.w. medium and vice versa. The animals were again allowed to settle for five minutes and a further reading taken.

TABLE 4:1

External medium % s.w.	P.D. (mV)	Standard deviation	n
100 - 100	+ 0.16	.96	5
100 - 2	+11.28	3.5	5
2 - 2	-11.05	2.7	6
2 - 100	- 4.5	4.4	4

The sign of the P.D. is given with reference to the body cavity of the animal.

These results show a negative P.D. in animals acclimated to 2% s.w. and a small positive P.D. in those acclimated to 100% s.w.

Only animals from 100% s.w. in 100% s.w. appear to have a P.D. of around OmV. It is thus possible for a low P.D. to correspond to high permeability.

These results largely agree with the values given by Lockwood, 1973 with the exception of the animals acclimated to 100% s.w. and transferred to 2% s.w. Lockwood found an internal P.D. of -11.8mV with reference to the external medium, whereas an internal P.D. of +11.28mV was recorded in this study.

(2) Changing the P.D. across the body wall without altering the external medium concentration.

To test the hypothesis that a change of internal to external P.D. could affect the permeability of an animal, the external medium concentration was maintained while the P.D. across the body wall was changed.

(a) Animals acclimated to 100% s.w. and maintained at 100% will have a half-time of exchange of approximately 5 minutes. Using the voltage clamp, the animals were held at 0mV, (the previously measured P.D. for animals in 100% s.w.) Outflux samples were taken for 5 minutes and the P.D. forced to +50mV for another 5 minutes, after which it was returned to 0mV.

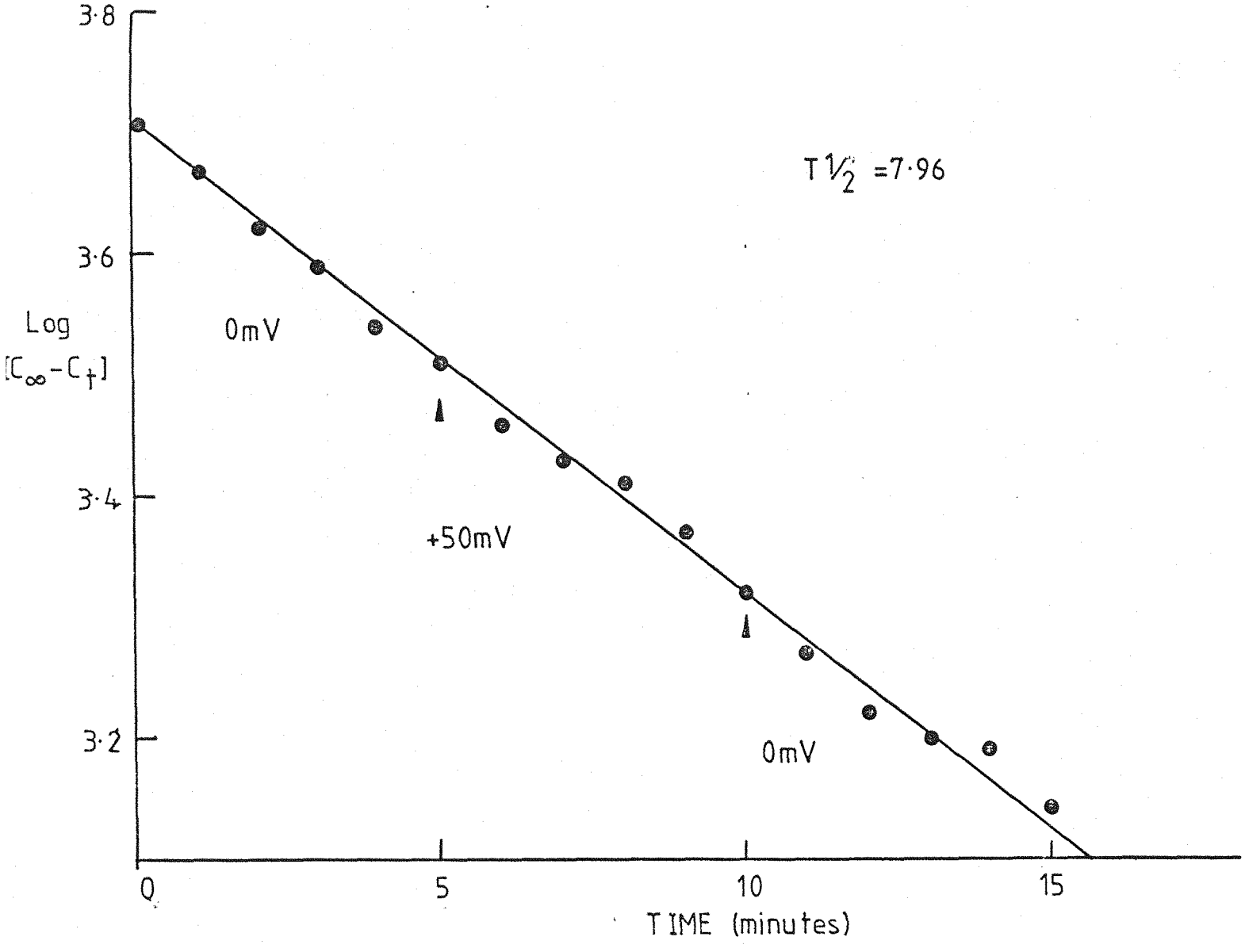
By plotting the log (C_∞ -Ct) for this entire period, the $t_{\frac{1}{2}}$ of exchange can be found throughout the experiment.

These results (Fig.4:4) shows that +50mV does not detectably alter permeability of the animal.

This change in P.D. was designed to emulate a salinity change from 100% s.w. to 2% s.w., where the P.D. would have changed from around 0mV to +11mV. A change in the $t_{\frac{1}{2}}$ from approximately 5 minutes to 15 minutes would have been expected. During this experiment, the $t_{\frac{1}{2}}$ remained at 7.96 minutes.

Calculating a linear regression coefficient on the plot of log (C_∞ - Ct) against time gives a value of -.998, effectively demonstrating the constancy of the $t_{\frac{1}{2}}$ throughout the experiment. This regime does not support the hypothesis of a P.D. controlled permeability change.

Log counts ($C_\infty - C_t$) against time. (see text) Fig 4·4

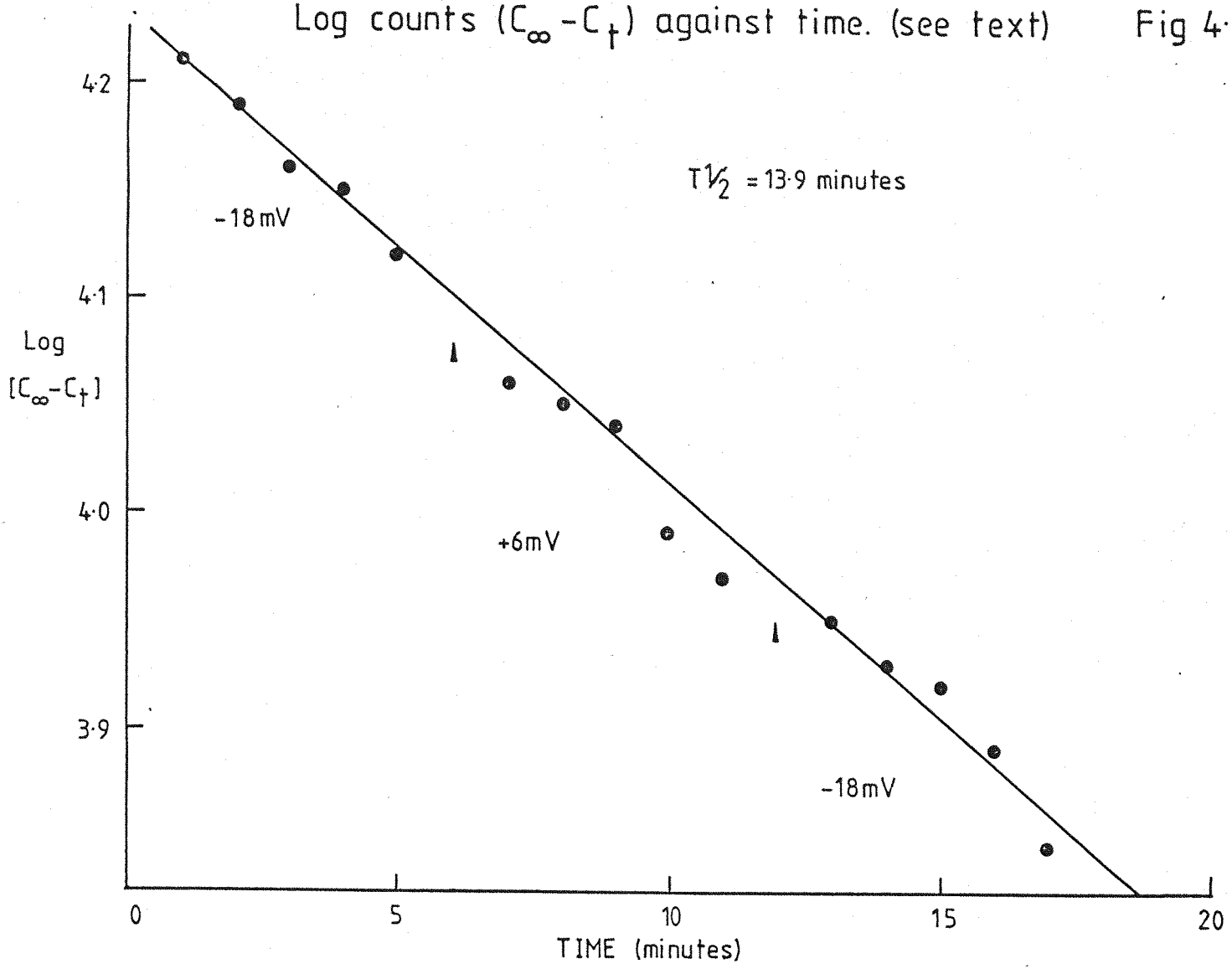


(b) A 2% s.w. acclimated animal was mounted in the P.D. apparatus and loaded with THO. During the first six minutes of unload, the animals had a clamped P.D. of -18mV, followed by a 5 minute period at 0mV, and finally back to -18mV. (Fig. 4:5).

The $t_{\frac{1}{2}}$ of exchange again did not change significantly during this experiment, remaining at 13.9 minutes with a linear regression coefficient of -.994. Consequently, this experiment also did not support the hypothesis of a potential difference controlled permeability mechanism.

These two experimental regimes are typical of a wide range of conditions tested, all of which showed no significant change of permeability related to a forced change of P.D. across the body wall.

Log counts ($C_\infty - C_t$) against time. (see text) Fig 4·5



DISCUSSION

These results clearly show that crudely forcing a change of potential difference across the body wall of G. duebeni does not significantly alter its permeability to water. Unfortunately, this does not prove or disprove that P.D. is directly or indirectly involved in the permeability changes observed in G. duebeni.

In order to explore the possibility further, it would be necessary to perform similar experiments on an isolated gill. This would be an extremely difficult experiment to perform, due to the small size of a single gill. If a much larger crustacean could be found which exhibits significant changes in apparent permeability, then further work on isolated gills could produce more definitive conclusions. Unfortunately, the larger the animal, the more stable its internal concentration, due to the larger volumes involved. It is thus unlikely that the larger crustacean will have evolved mechanisms to alter their permeability as dramatically as G. duebeni. This is demonstrated in the euryhaline crab Carcinus maenus which does not exhibit dramatic changes in permeability (Smith 1970), although it does alter slightly when exposed to different salinities.

In conclusion, further work on the affect of P.D. on permeability changes requires two conditions not yet available.

(i) A larger experimental animal which exhibits significant change in permeability related to salinity changes.

(ii) More elegant techniques to enable permeabilities to be measured on single gills.

CHAPTER 5

THE ECOPHYSIOLOGICAL RESPONSE OF THE ARCTIC AMPHIPOD GAMMARUS SETOSUS AND ONISIMUS LITORALIS

INTRODUCTION

Research on the amphipod Gammarus duebeni, Chaetogammarus marinus and Gammarus locusta has demonstrated their ability to live in estuarine conditions where the salinity can vary from 2°/₀₀ to greater than 35°/₀₀ (Spooner, 1947). The present study has been concerned with comparing and contrasting these amphipods and their ecophysiological responses to varying conditions of osmotic stress.

Two species of amphipod found in the Canadian Arctic, Gammarus setosus and Onisimus litoralis occur on the North coast of Baffin Island associated with salinity anomolies on the underside of the sea ice. (N. Snow - personal communication). Individuals of these species are located around and in brine channels formed on the under surface of the sea ice and hence provide excellent material for the study of salinity responses in a context different from the standard estuarine situation. (Buchanan et al, 1977), (L.G.L. Ltd, 1980).

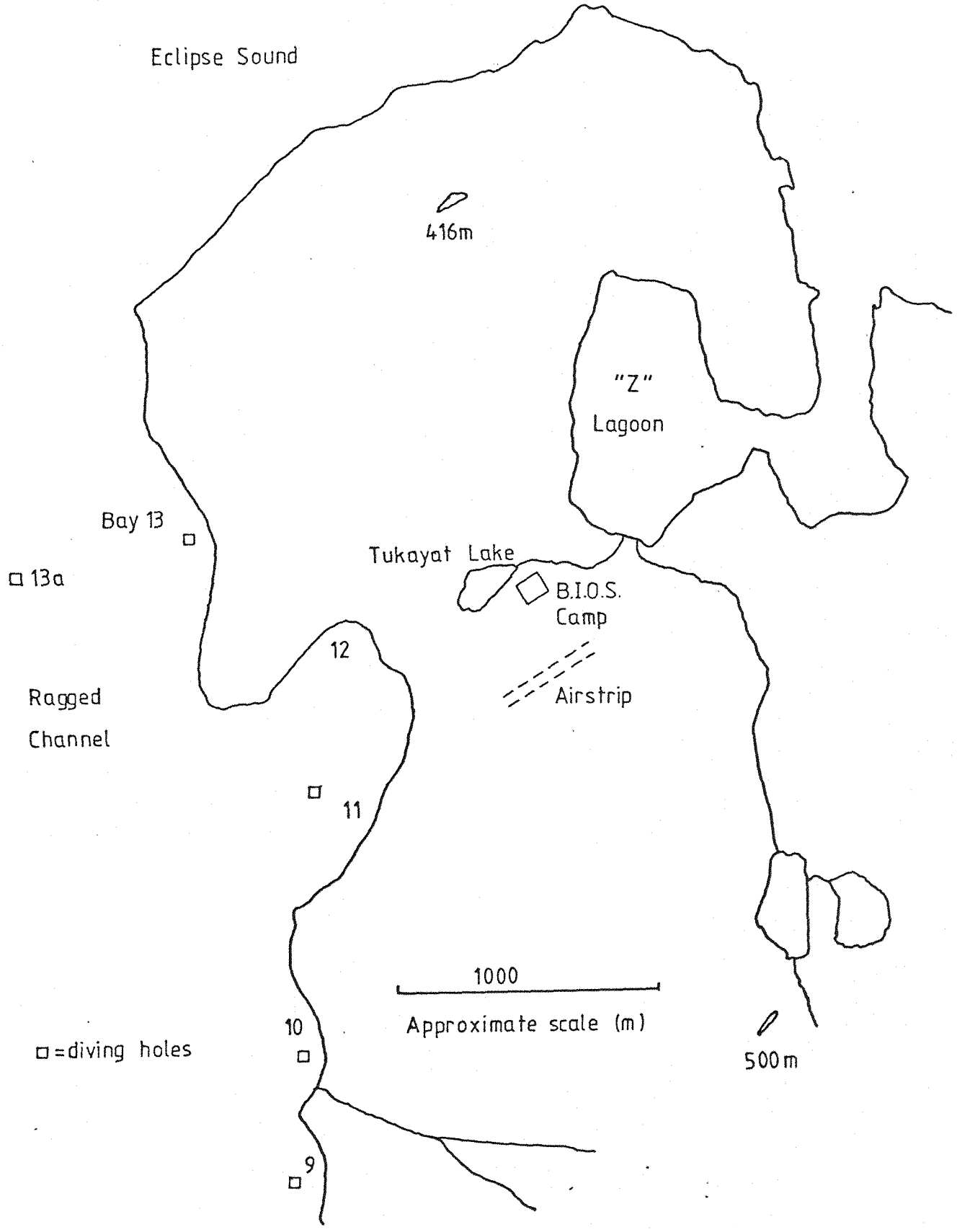
Work was carried out during two visits to the experimental B.I.O.S. (Baffin Island Oil Spill) camp in the Canadian Arctic - map ref. Lat. 72° 30'N Long 79° 50'N. The first visit was June/July 1980 and the second in May 1981. (Fig. 5:1).

The North coast of Baffin Island in the North West Territories consists of a ragged coastline of many fjords. The sea is ice covered for approximately nine months of the year with ice up to two metres thick. The ice begins to melt in June/July and is usually broken into drifting ice rafts by the beginning of August.

G. setosus and O. litoralis have been observed near the shore of the fjords around the experimental camp.

In addition to the work on salinity tolerance and the associated physiological responses of the amphipods to concentration variation of the external medium, a study was initiated on the effects of oil on the behaviour and tolerance of the animals.

Cape Hatt, Baffin Island, Northwest Territories.
Lat. 72° 30' Long. 79° 50'. Fig 5·1



MATERIALS and METHODS

Animals.

Onisimus litoralis were obtained by diving through the ice and were collected using a diving net. *Gammarus setosus* were obtained by diving and, once the ice had partially melted so that ice cracks and pools appeared, by hand net from these pools. Both species were maintained in the laboratory at 2°C.

Diving.

Dive holes were cut through the 1½-2m thick sea ice. Diving was carried out singly on surface to diver ropes, with a standby diver in attendance. Visibility was 35m+ and the water temperature was at -1.6°C. Diving periods were limited to a maximum of 4 hours under these conditions. Salinity profiles were made at the ice to water interface using a set of syringes which sampled simultaneously at five depths. (Fig.5:2). To ensure consistency of depth diving sampling, this device was held against the ice when samples were taken.

Toxicity and survival tests.

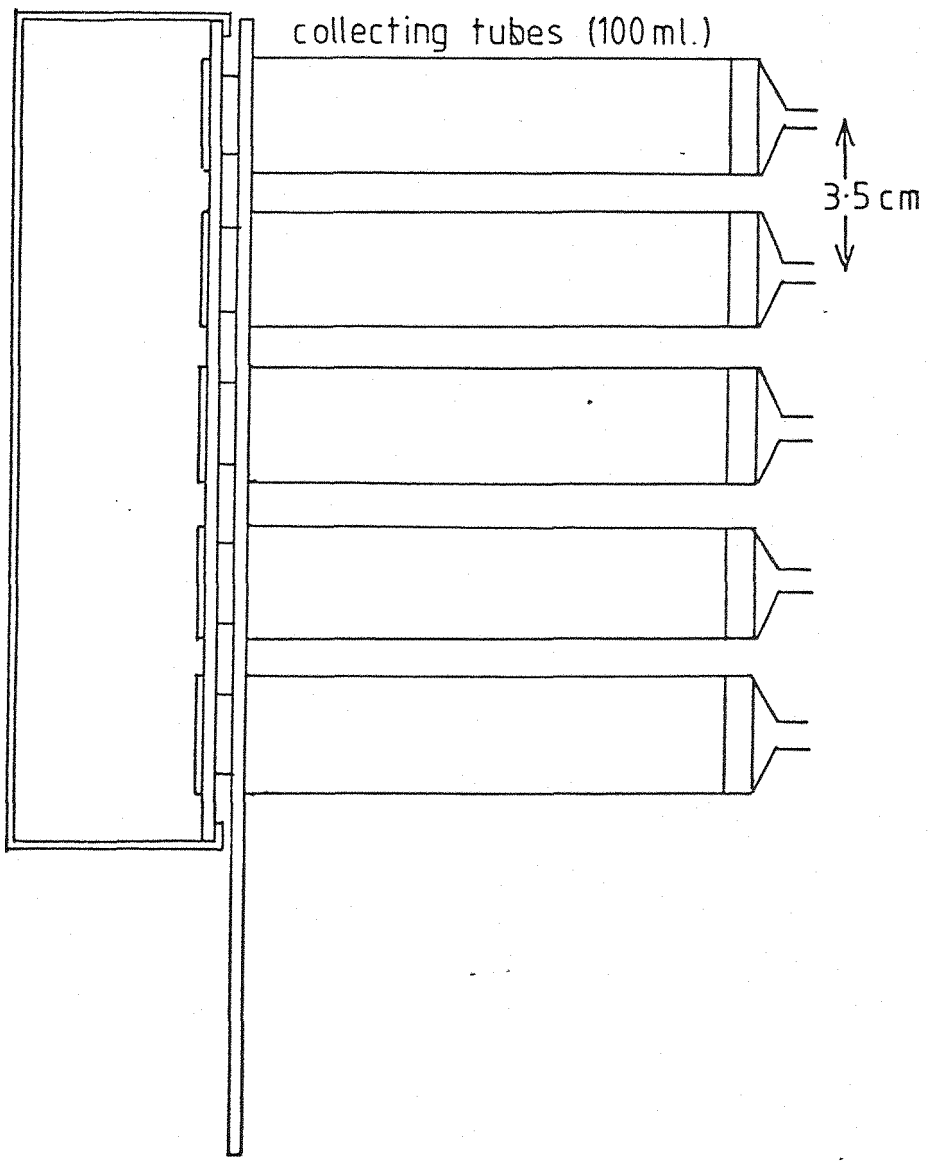
Throughout the experimental work in 1980, toxicity tests were carried out to find a realistic sub-lethal dose of oil and oil plus dispersant water soluble fraction (W.S.F.). Small *Onisimus litoralis* (3-4mm) were used as large numbers could be collected from the underside of the ice. Larger *O.litoralis* (up to 1.5cm) and *Gammarus setosus* (up to 1.5cm) were also used in smaller numbers. The water soluble fraction (W.S.F.) used was made by adding 1ml of Venezuelan crude oil to 100ml sea water. This was mixed periodically over a period of days and the W.S.F. drawn off by pipette. Oil plus dispersant (Esso Corexit 9527) was prepared as above with the addition of 1ml dispersant.

Samples of oil and oil plus dispersant W.S.F. were analysed by Dr Y. Vandermeulen, B.I.O. for determination of hydrocarbon concentration. The oil and dispersant W.S.F. was 231 $\mu g l^{-1}$ and the oil W.S.F. was 6.07 $\mu g l^{-1}$.

Chloride concentrations.

Haemolymph was collected from the amphipods and stored under liquid paraffin as described in Chapter 1. Chloride

Salinity profile collector. Fig 5·2



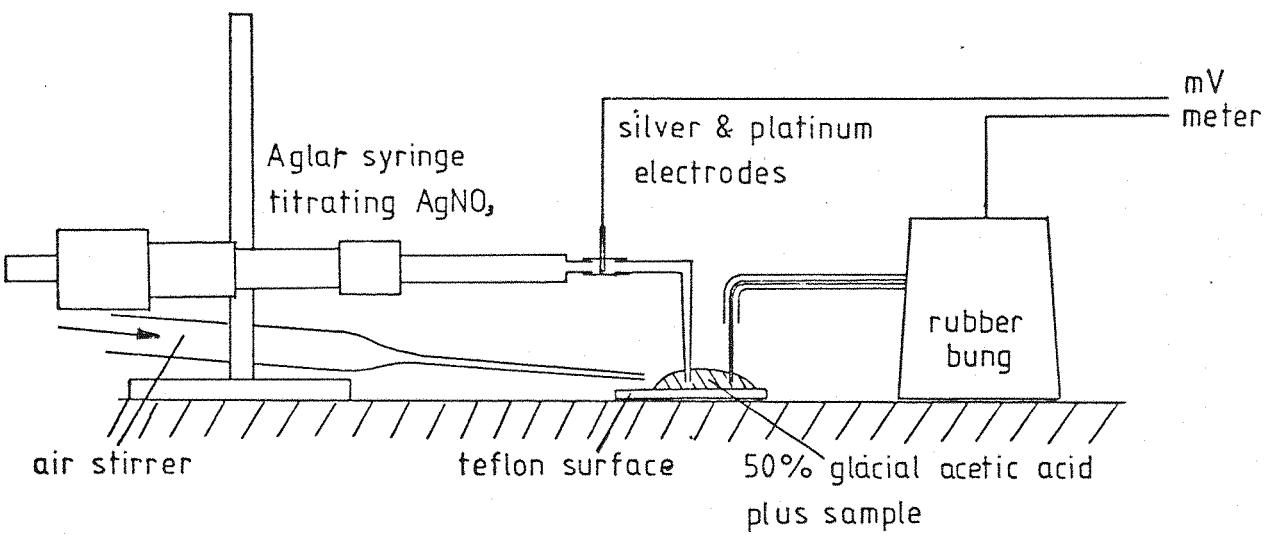
concentration was determined by a microtitration technique based on the second method described by Ramsey, Brown and Croghan, 1955) (Fig. 5:3). Briefly, 1µl of sample was added to 50µl of 50% glacial acetic acid, the potential difference (PD) across the electrodes measured, and Ag NO_3 added via an Aglar syringe until a sudden drop in PD was noted. During the titration the sample was stirred by a fine jet of air across the surface of the drop.

Using known NaCl solutions, a standard curve was constructed (Fig. 5:4). The concentration of an unknown sample was determined by interpolation of the standard curve.

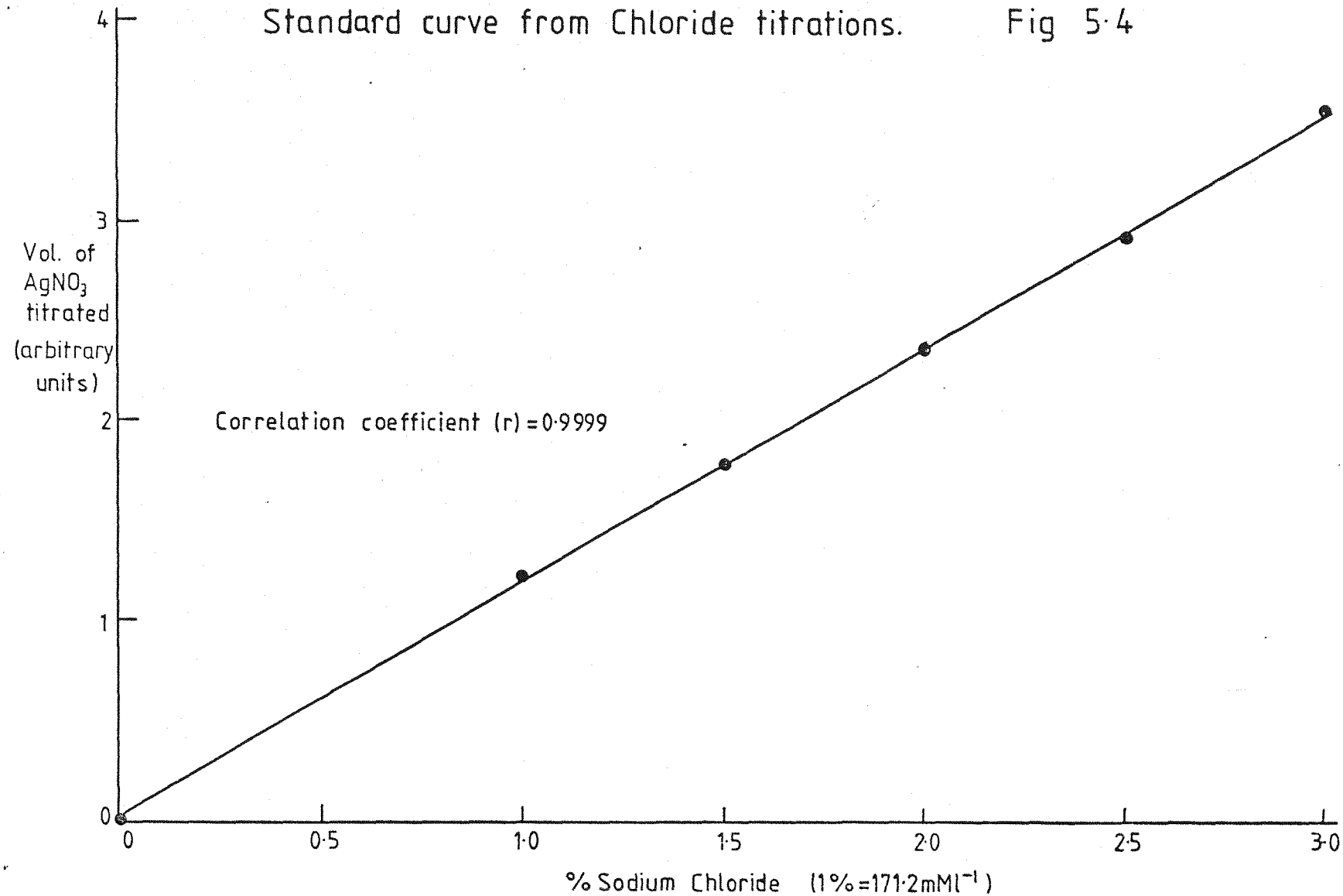
Permeability to water.

Water flux measurements made on the arctic amphipods were undertaken using the same technique employed with Gammarus duebeni (see Chapter 1). All measurements were of outflux and carried out at 2°C.

Chloride titration apparatus. Fig 5·3



Standard curve from Chloride titrations. Fig 5·4



RESULTS

Environmental Features

The sea ice consists mainly of ice formed the previous autumn. During the subsequent nine months, pockets of brine are formed and these migrate downward, becoming more saline until they are expelled out of the underside of the ice via brine channels. By this process sea ice becomes drinkable within one year if it does not melt during the summer thaw.

As the temperature rises in the spring (around June to July), the snow on top of the ice and the top layers of ice begin to melt. The fresh water thus formed percolates through the ice to form a fresh water layer between the ice and the colder sea water. Brine pockets are still migrating down through the ice, but must now pass through the fresh water between the ice and the sea water. This causes ice stalactites (Fig. 5.5) to form within the fresh water, probably due to the temperature drop as the brine mixes with the fresh water. These have been reported (Steele & Steele, 1979) to reach of up to 3m in Resolute Bay, and up to 6m (Paige 1970 and Dayton & Martin,1971) in the antarctic. At the sites investigated at Cape Hatt, the stalactites reached lengths of approximately 1m.

Diving observations show that the characteristics of the underside of the ice change as a daily basis and from location to location. The surface varied from being flat and featureless to being covered in ice crystal patterns and stalactites.

During the spring, as the snow cover melts, the light penetration increases resulting in an algal bloom on the soft spongy layer (1-5 cm thick) of the underside of the ice. This is visible as a greeny brown discolouration.

Salinity Profiles

Samples of under ice water were taken at each dive hole using the syringe profiler. A hand held refractometer (American optical) was used to determine the salinity from each collection syringe.

ICE STALACTITE

Fig 5·5

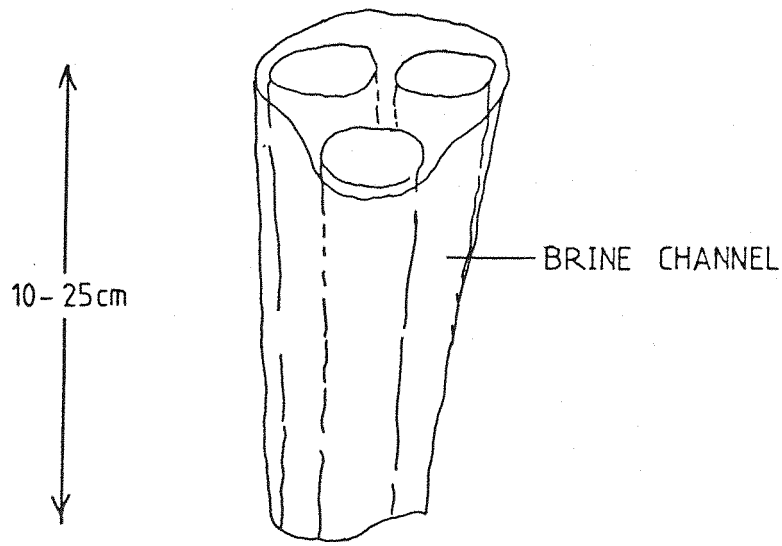


DIAGRAM OF ICE/BOTTOM REGIME

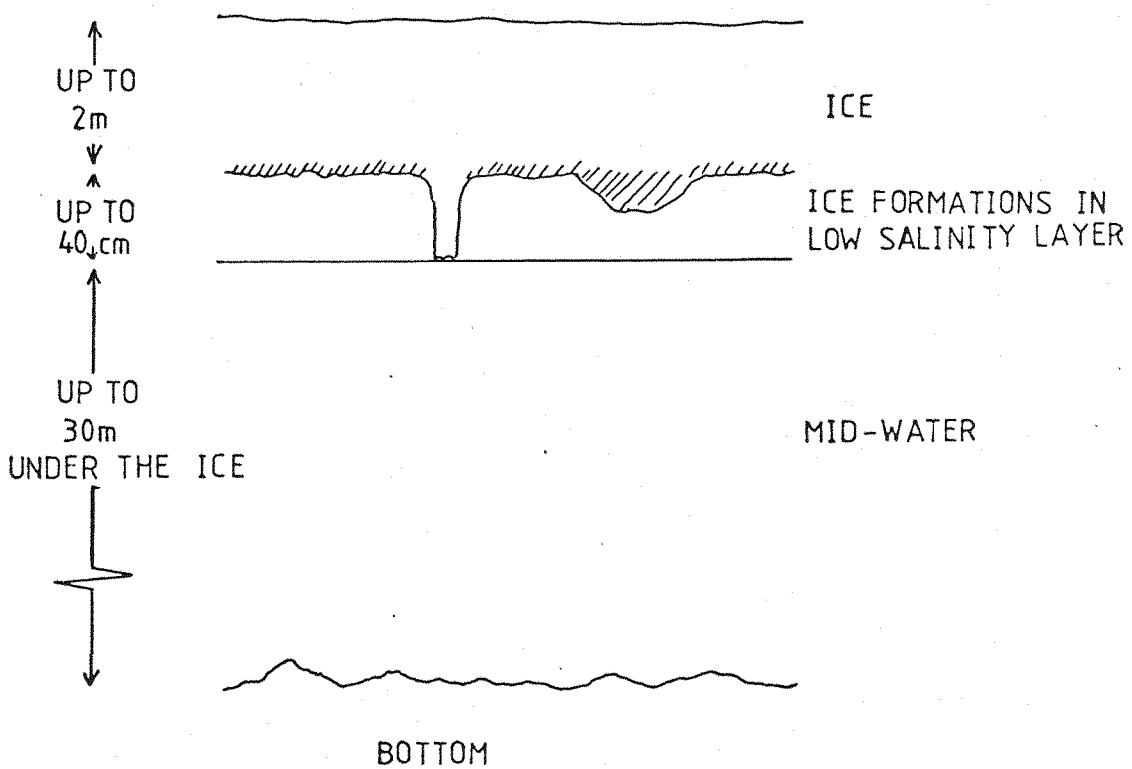


TABLE 5:1

Distance from the ice (cm)	Salinity °/oo						
	Bay 9	Bay 10		Bay 11/12	Bay 13		Bay 13a
0	2.0	2.0	3.8	1.0	5.0	3.8	10.5
3.5	6.3	4.0	3.0	1.5	14.5	3.8	12.0
7.0	23.0	21.5	5.0	2.0	28.5	16.3	20.25
10.5	31.8	32.0	13.0	5.0	32.5	30.3	29.3
14.0	32.0	32.0	27.0	6.0	32.0	31.0	29.3
17.5	-	-	26.5	-	-	-	
21.0	-	-	29.5	-	-	-	
24.5	-	-	30.0	-	-	-	
28.0	-	-	31.0	-	-	-	
31.5	-	-	32.0	-	-	-	

The mid-water salinity was recorded as uniformly 32°/oo at all depths encountered (up to 35m). The temperature of the mid-water was also uniform at -1.6°C.

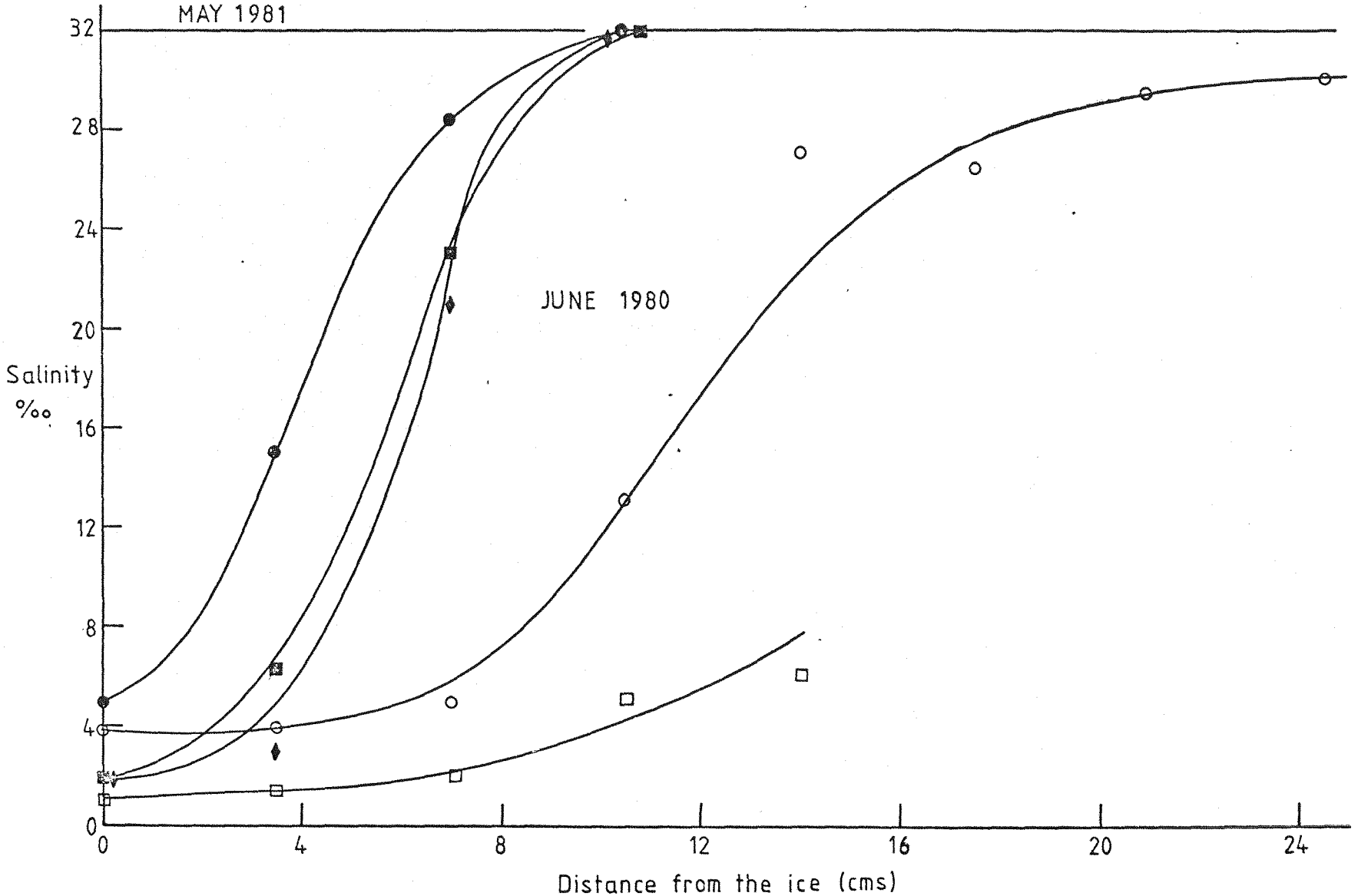
One of the stalactites from within the fresh water layer in Bay 10 was broken off and removed. After melting this had a salinity of 4°/oo (Fig.5:5).

By plotting the salinity profiles against distance from the ice (Fig. 5:6) it is apparent that the halocline is sharply defined being often only a few centimetres in thickness and can involve changes of salinity of up to 30°/oo. Clearly the amphipods freely entering and leaving this area of fresh water must be able to osmoregulate sufficiently to survive this rapid external concentration change.

During May 1981 there were no obvious salinity anomalies on the underside of the ice, which was reflected in profile measurements which gave a uniform 32°/oo (Fig.5:6).

This was due to diving taking place one month earlier than in 1980, before the snow lying on top of the sea ice had begun to melt. In June 1980, this snow was rapidly melting, producing the fresh water layer. By mid-June 1980 much of the ice had gone, leaving large quantities of fresh water on top of the cooler 32°/oo s.w.

A selection of salinity profiles from immediately below the ice. Fig. 5·6



On one collecting expedition (14.7.1980), from around the remaining ice, salinity profiles were taken by lowering a salinometer probe (Simpson Electric Co.) from a boat.

These results (Table 5:2) show that the fresh water layer is more substantial than those recorded earlier under the ice.

TABLE 5:2

1. Open water 0. 5m deep.

Temp °C	Depths m	Salinity °/oo
6°C	.5m	= 0°/oo

2. Open water by ice edge

Temp °C	Depths m	Salinity °/oo
3.1	0	0.2
1.9	1	1.6
1.2	2	27.2
0.4	3	29.4

3. In ice cracks near shoreline

Temp °C	Depths m	Salinity °/oo
2.7	0	0.2
0.7	1	7.2
0.3	1.5	8.3

4. In ice crack 100m from the shoreline

Temp °C	Depths m	Salinity °/oo
3.0	0	0.1
1.5	1	8.9

In all localities where salinities were measured, the surface water was 1°/oo. During diving operations in late July, a halocline/thermocline was visible at depth up to 10m.

Distribution of the animals

The two species of amphipod commonly observed under the ice were Gammarus setosus and Onisimus litoralis. Both species were found mainly on the undersurface of the ice, often associated with stalactile and other crystaline structures. They were also observed swimming in the mid-water and freely passing to and from the fresh water layer when present. O. litoralis were locally abundant.

The daily distribution of these two species appeared to vary considerably. The habitat preference also appeared unpredictable, with all the animals against the ice one day and the majority free-swimming in mid-water the next.

For approximately three months of the year, these amphipod populations are extremely euryhaline, while for the rest of the year, they encounter stable environmental conditions.

In the laboratory both G. setosus and O. litoralis survived up to a month in salinities down to 1‰. However, both species died within 1 hour if placed in de-ionized water or water taken from the fresh water lake.

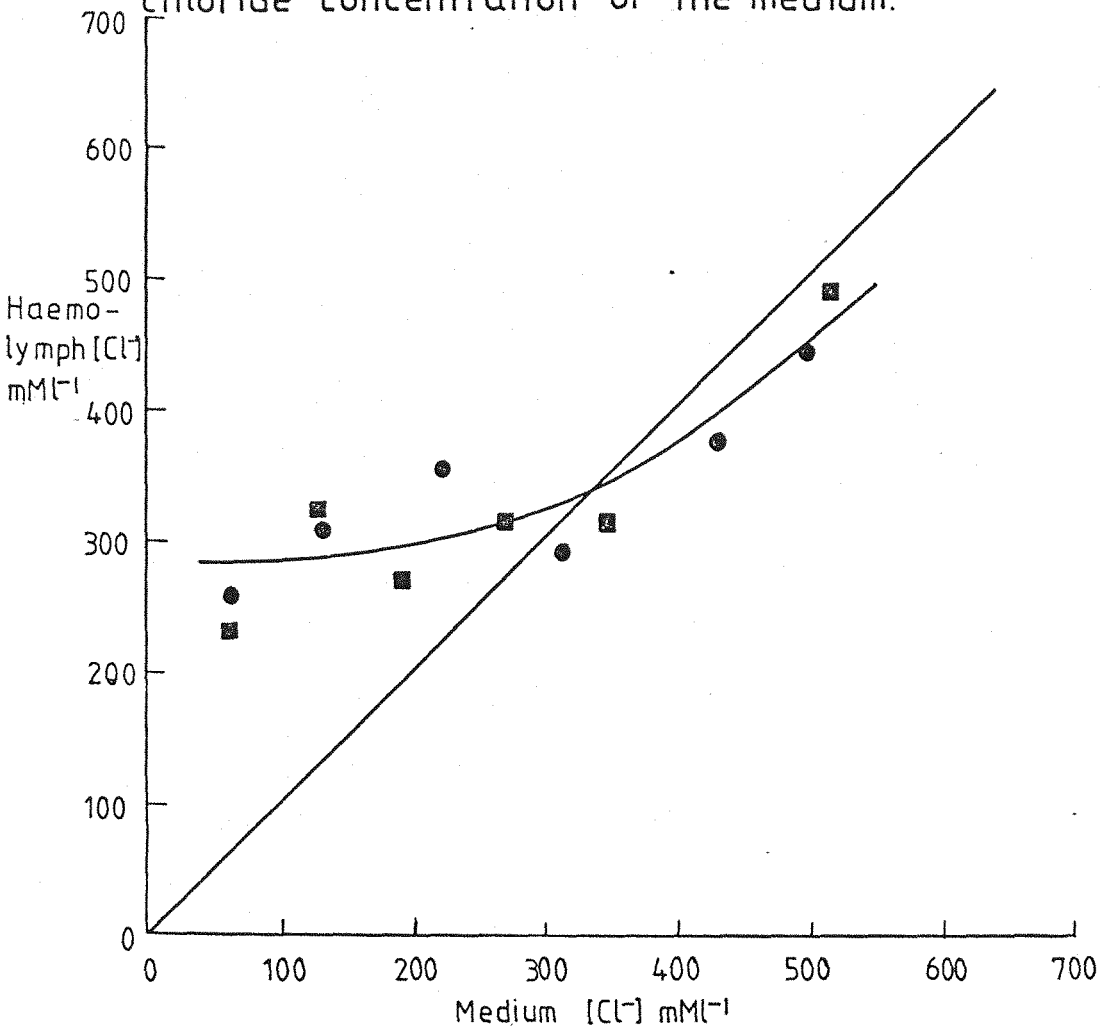
Haemolymph chloride concentrations.

G. setosus and O. litoralis were acclimated for at least 48 hours in various salinities and the haemolymph and external medium chloride concentration measured. (Figs. 5:7 and 5:8).

Both G. setosus and O. litoralis appear to maintain their haemolymph chloride concentration hypoionic to the external medium at high salinities. This was later confirmed in G. setosus by measuring the sodium concentration of the haemolymph at 100% s.w. on stored blood samples, where the sodium concentration was 30mMl^{-1} hypoionic to the external medium in 100% s.w.

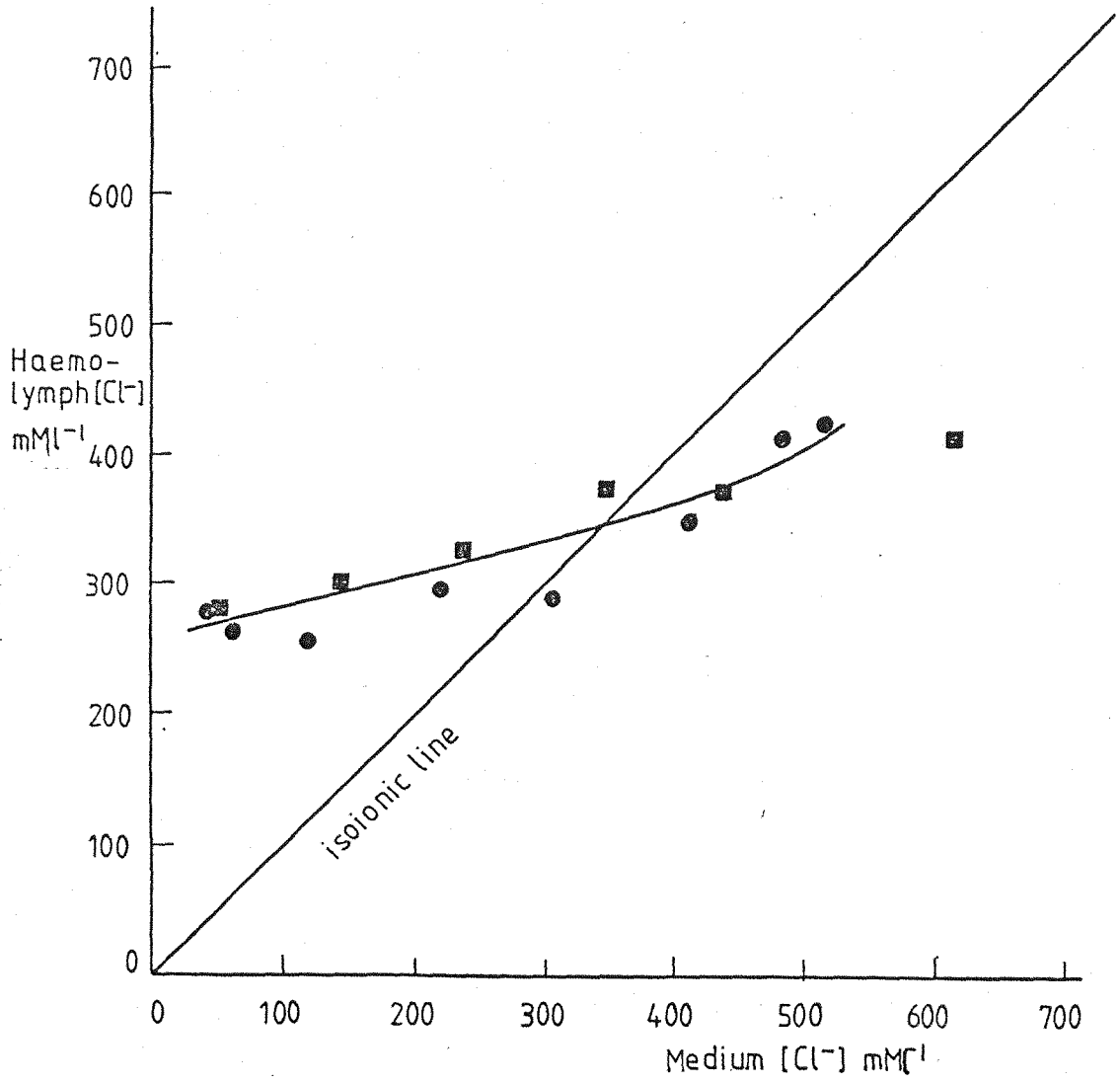
Comparison of these results with the chloride haemolymph concentration of G. duebeni (Fig.5:9) shows a lower chloride concentration in the arctic amphipods at high salinities. G. duebeni appears isionic with the external medium at chloride concentrations greater than 250mMl^{-1} while the G. setosus and O. litoralis are hypoionic to the medium.

The relationship between haemolymph chloride concentration in G.setosus and the chloride concentration of the medium. Fig 5·7



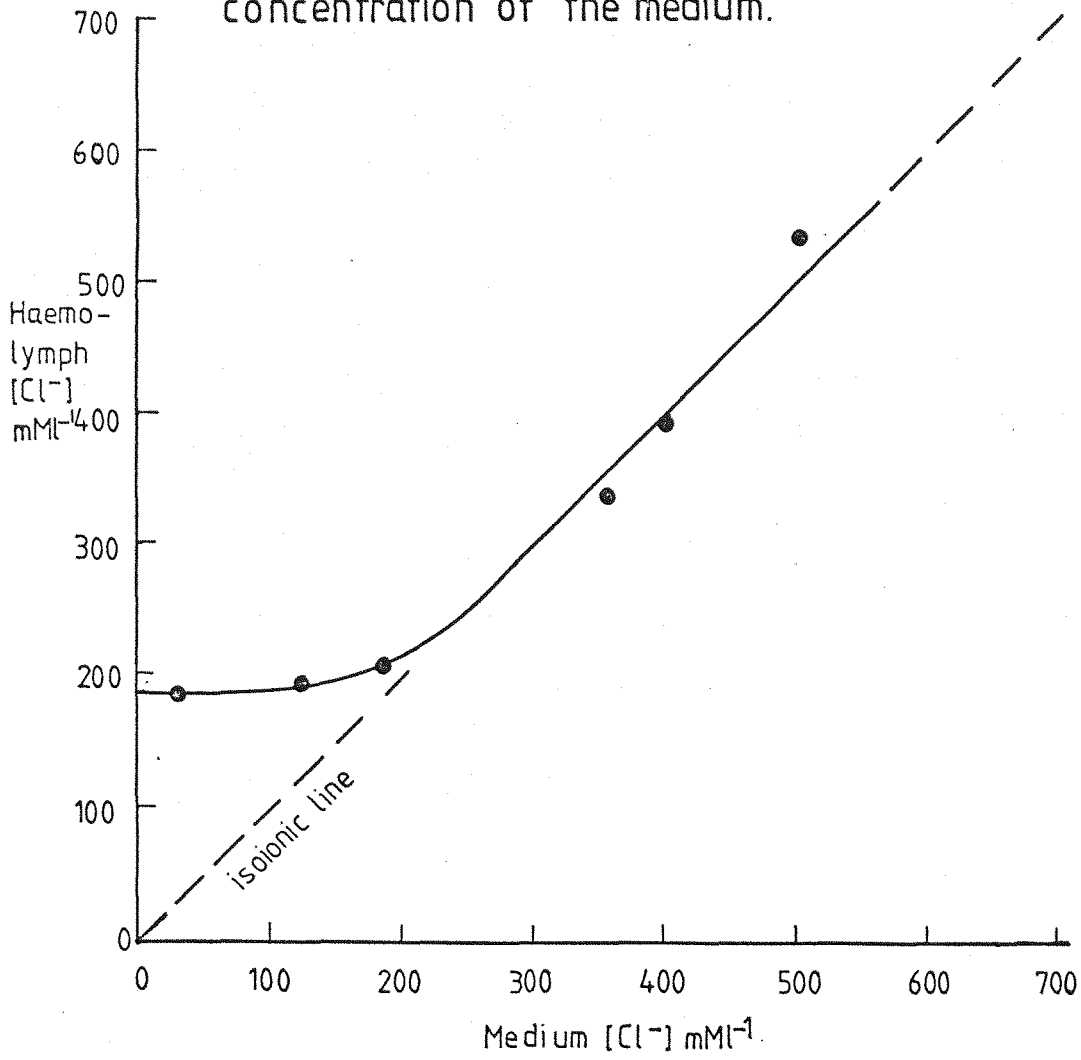
The relationship between haemolymph chloride concentration in O.litoralis and the chloride concentration of the medium.

Fig 5·8



The relationship between haemolymph chloride concentration in G.duebeni and the chloride concentration of the medium.

Fig 5·9



Responses to sudden changes in salinity in G. setosus.

For an animal which is freely travelling across a halocline, it is essential to be able to respond to the osmotic stresses of such a system. G. setosus was therefore acclimated to 2% s.w. and transferred to 100% s.w. and haemolymph chloride concentrations measured (Fig 5:10). Conversely they were acclimated to 100% s.w. and transferred to 2% s.w. (Fig.5:11) The animals from 2% s.w. became acclimated to 100% s.w. in less than 3 hours while animals transferred from 100% s.w. to 2% s.w. had reached dynamic equilibrium with the medium after 48 hours.

These results clearly demonstrate the animals ability to uptake ions which would rapidly increase their haemolymph concentration when placed in a high salinity from a low salinity. Conversely, they are able to retain chloride ions for a relatively long time on dilution of the external medium. (Fig. 5:11)

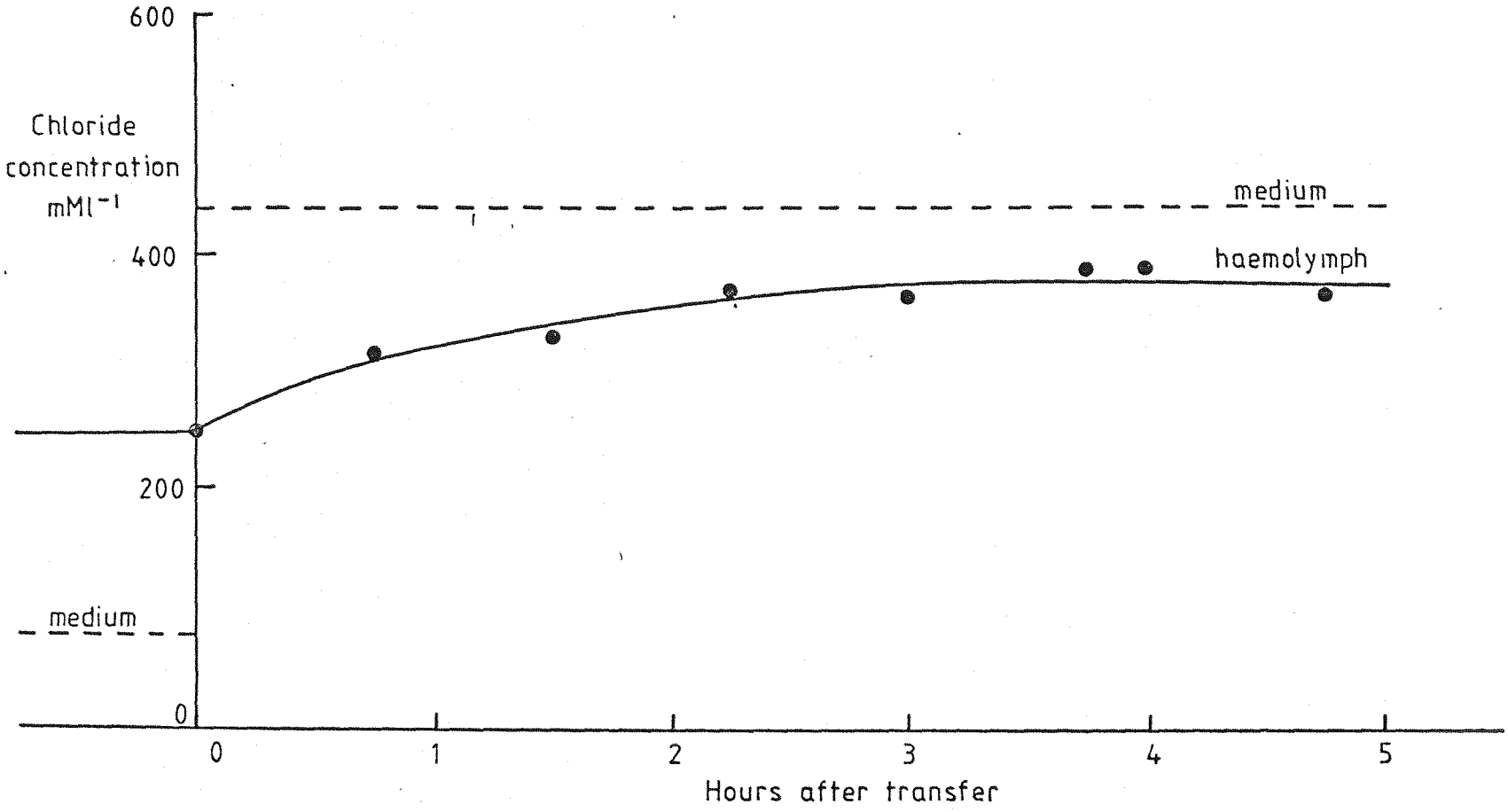
With these mechanisms it is possible to speculate that the animals could theoretically spend much of their time in the fresh water in the spring melt, dropping down into the mid-water for periodic "topping up" of body fluid ionic concentration. This tentative suggestion is supported by the observations that the amphipods are frequently seen swimming in the mid-water.

Individual amphipods have been observed "dropping" off the ice/water interface through the halocline into the mid-water.

G. setosus acclimated to 2% s.w. and transferred to 100% s.w. show a different response to that shown by G. duebeni in a similar situation. Whereas G. setosus becomes fully acclimated in water in less than 3 hours, G. duebeni takes up to 16 hours to reach dynamic equilibrium with the external medium. (c.f. Fig 1: 21). However, comparing the loss of ions following a 100% to 2% s.w. salinity change in these two species shows that G. duebeni (Lockwood & Inman, 1972)and G. setosus reach equilibrium with the external medium after approximately 36-48 hours.

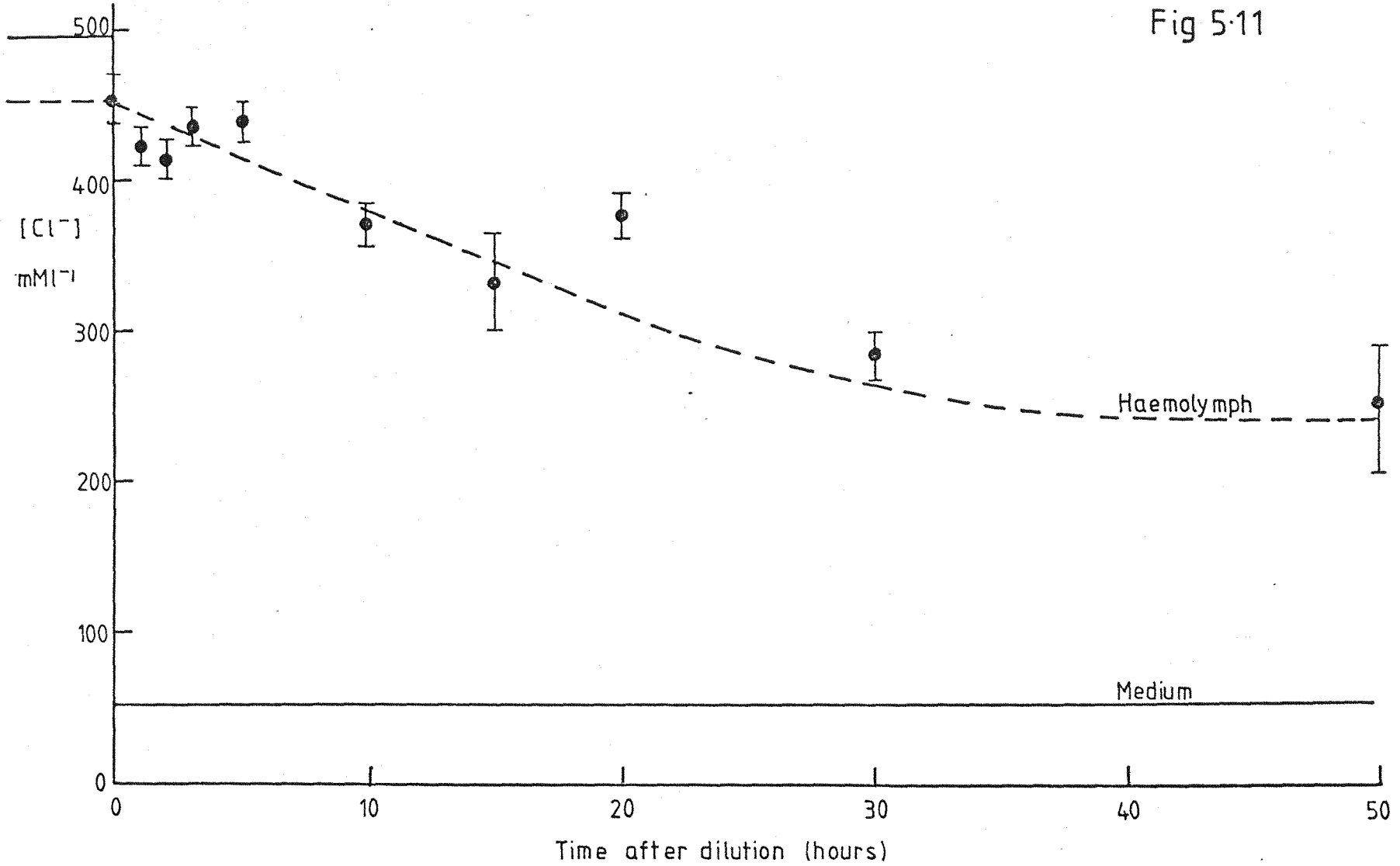
Fig 5·10

Haemolymph chloride concentrations in G.setosus following an increase in external medium concentration.



Chloride ion loss after a sudden dilution of external medium in G. setosus.

Fig 5·11



The permeability to water of the under ice amphipods.

While G. duebeni and G. setosus are both euryhaline, they differ considerably in their physiological responses to osmotic stress. One of the most interesting parameters measured in G. duebeni was its permeability to water which altered dramatically as the external salinity was changed. G. setosus found in the locality of the B.I.O.S. camp experience low salinity conditions for only a few months of the year, and it is interesting to know if this amphipod can also alter its permeability to water so that it can limit osmotic stress.

G. setosus were acclimated to 2% s.w. and 100% s.w. and the permeability to water measured using THO as a marker.

TABLE 5:3 Permeability of G. setosus acclimated to 100% s.w. & 2% s.w.

All permeability measurements at 2°C.

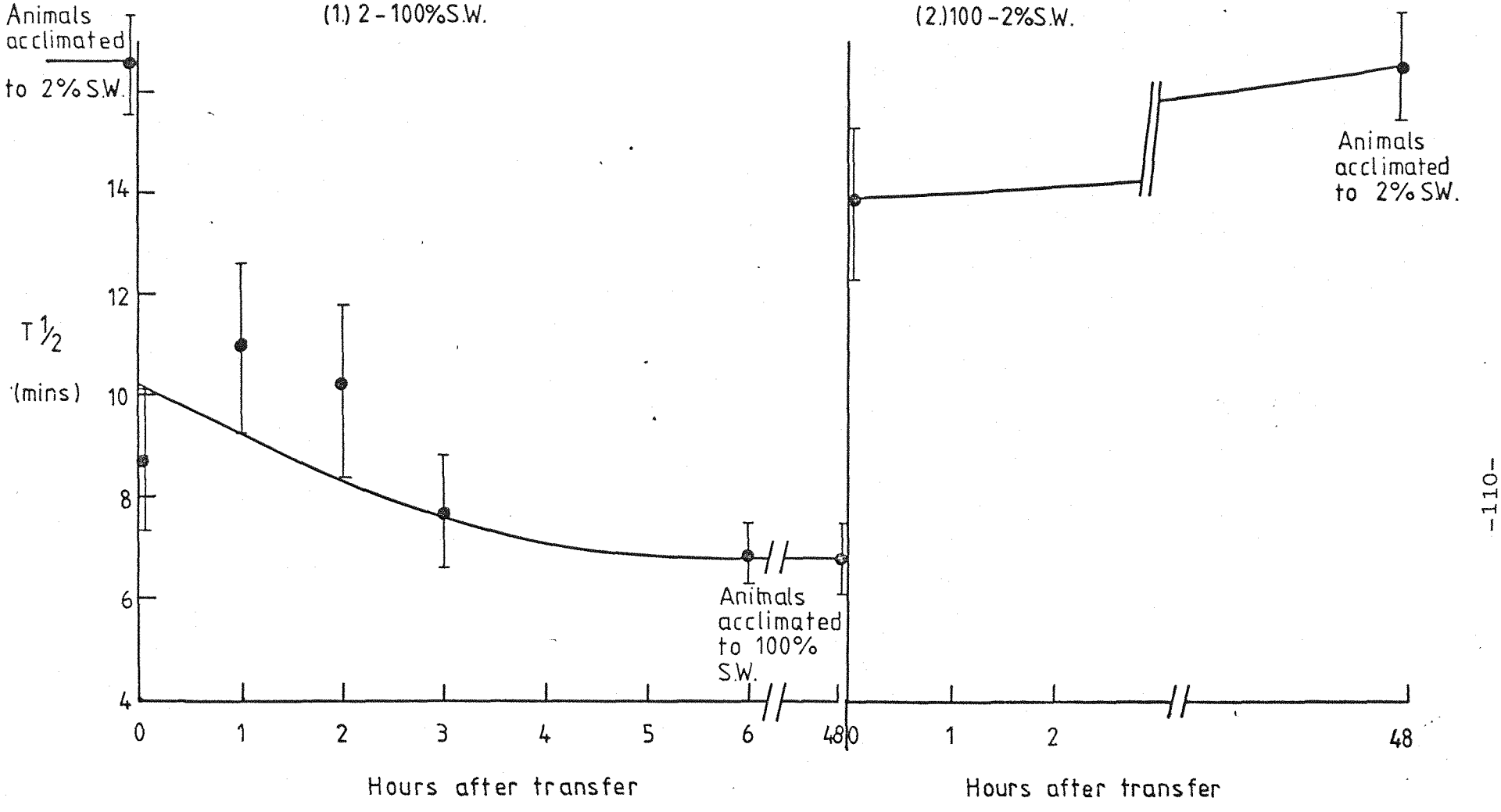
Salinity	$t_{\frac{1}{2}}$ mins	S.E.M.	n
100%	6.85	.74	6
2%	16.66	1.29	6

These results show similar trends to those found in G. duebeni. G. setosus are impermeable when acclimated to 2% s.w. ($t_{\frac{1}{2}}$ = 16.66 mins) and permeable when acclimated to 100% s.w. ($t_{\frac{1}{2}}$ = 6.85 mins).

Permeability readings were also taken after sudden changes in the external medium. Animals were acclimated to 2% s.w. and transferred to 100% s.w. Conversely, animals were acclimated to 100% s.w. and transferred to 2% s.w.

These results (Fig. 5:12) show a marked difference to response shown by G. duebeni. The animals transferred from 2% s.w. to 100% s.w. show a large initial increase in permeability (to $t_{\frac{1}{2}}$ = 10 mins) almost to the level found in animals acclimated to 100% s.w. ($t_{\frac{1}{2}}$ = 7 mins). In G. duebeni there is no such increase in permeability until the animals reach isotonicity with the external medium 16 hours after the salinity change. (c.f. Fig. 1:21).

The water permeability of G.setosus after sudden salinity changes. Fig 5·12



The high permeability found in G. setosus after the salinity change could explain the difference found in the time taken for the chloride ion concentration to reach equilibrium between G. deubeni (16 hrs) and G. setosus (3 hrs). If G. setosus needs to replenish its sodium chloride (NaCl) concentration by travelling from the fresh water layer into the salt water then this high permeability and fast uptake of NaCl would enable the animals to return to the fresh water after only a few hours.

The affect of the addition of oil and dispersed oil on G. setosus.

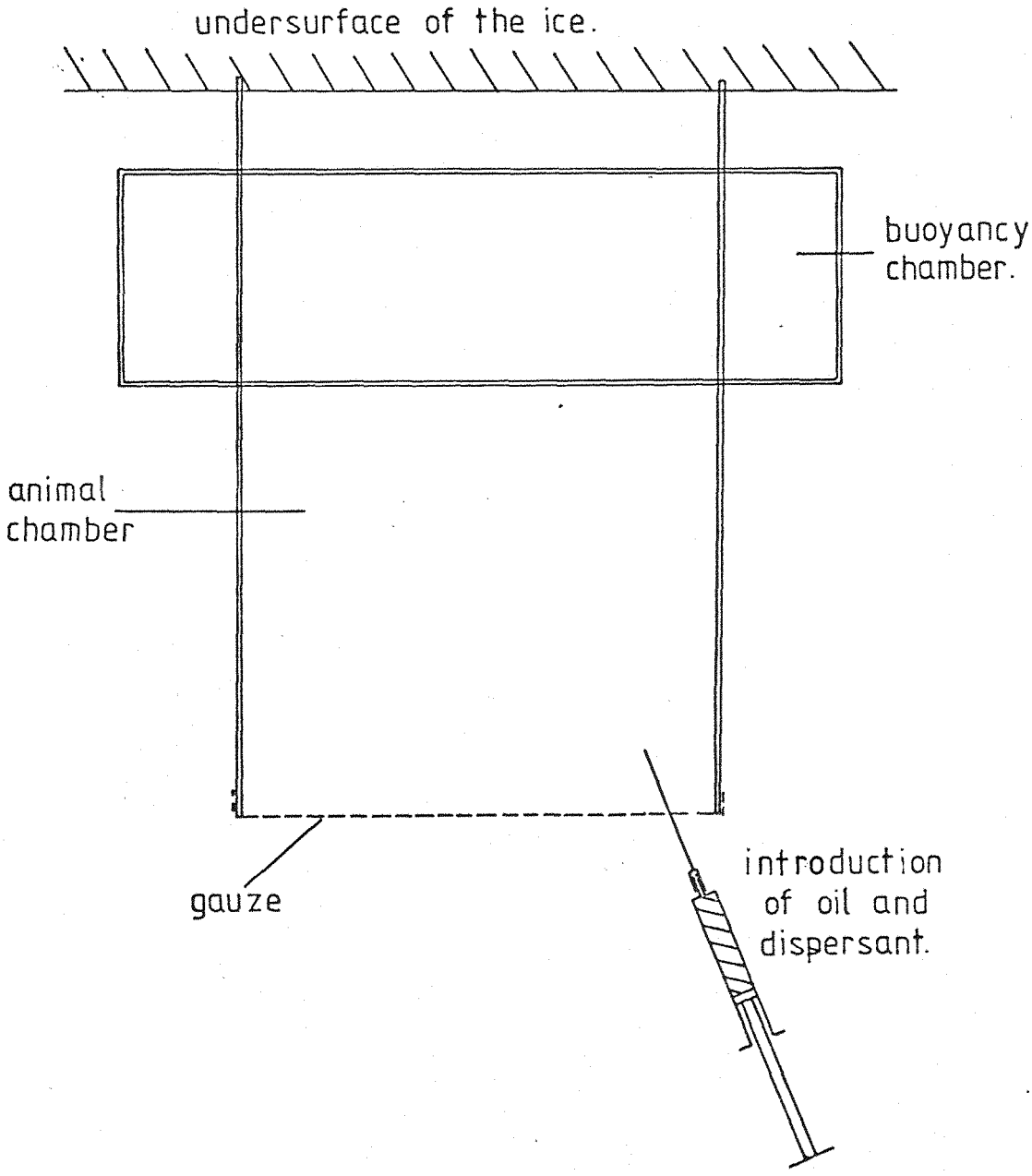
In order to understand the possible impact of an oil spill on the under ice organisms, the affect of contact of the oil and dispersed oil on the amphipods was studied. Animals living on the ice/water interface are a special case as their habitat coincides with the accumulation of oil from a spill. By contrast, most benthic or mid-water organisms are less at risk from physical contact as the oil floats on the surface of the water.

(i) In situ survival.

In order to study the animals response to free oil, perspex cells (Fig. 5:13) were placed on the underside of the ice well away from the dive hole, and 20 animals trapped in the cell. 2mls of oil was then introduced through the gauze using a syringe and the animals left for 24 hours. They were then collected and taken to the laboratory. All the animals were extremely heavily oiled and all were dead or moribund. Some individuals were so badly oiled that they resembled a drop of oil. This experiment was repeated with 1ml of crude oil and the animals were left for only 4 hours. There was still a high degree of oiling with subsequent mortality and direct observation showed that there appeared to be no active avoidance of the oil, animals often moving into the oil on the ice surface.

At this time weather deterioration and time limits forced further studies on the physical effect of oil to be carried out in the laboratory.

Under ice experimental chamber. Fig 5·13



(ii) Laboratory survival.

Animals were kept in 200ml of water at 2°C, and 200µl of oil and dispersants (10:1 ratio) added to the surface film. It was immediately noticable that the animals were making frequent trips to the surface of the medium, encountering the oil on the surface. Some individuals made repeated excursions through the oil, again indicating a lack of any active avoidance.

It was also obvious that the animals encountering dispersed oil were under much less physical stress as the oil did not form a film on the surface. Animals in dispersed oil did not "oil up". These individuals continued to feed and behave in a "normal" manner.

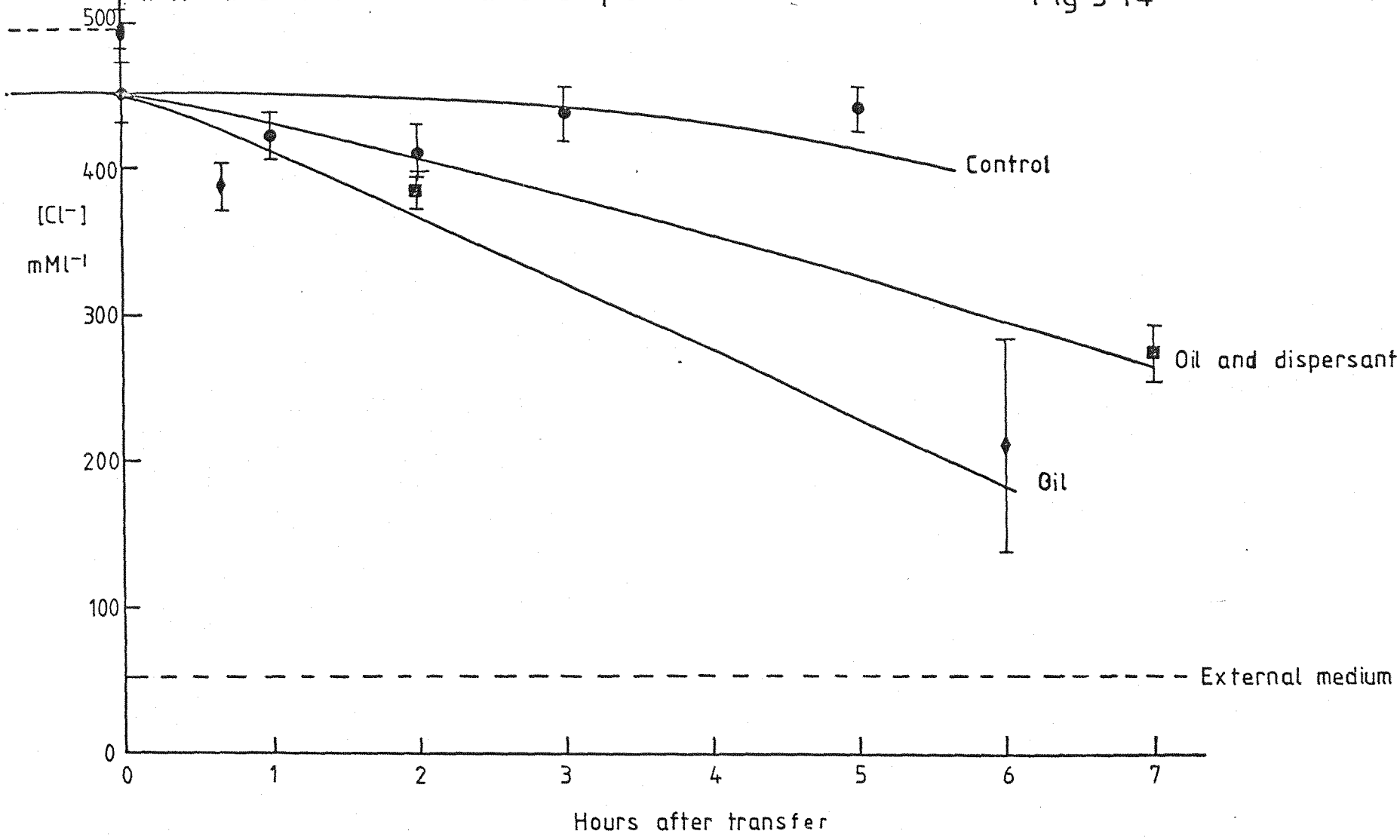
These results show that the amphipods were more affected by the physical presence of oil than by the dispersed oil. Work with the W.S.F. of the oil and dispersant has shown that they do not appear to be affected by low levels of oil and dispersant W.S.F. It is thus tentatively concluded that an untreated oil spill would have a much more immediate and detrimental effect on under ice populations than a dispersed oil spill.

This is demonstrated further by taking animals which have been in contact with oil and oil plus dispersants in 100% s.w., transferring them to 2% s.w. and measuring their loss of chloride ions after 6-7 hours. (Fig. 5:14). These results clearly show an increased chloride ion loss after dilution of the medium in the presence of dispersed oil. An even faster loss of chloride was demonstrated when the crude oil was present.

It was noted that the dispersant used (Korexit 9527) was not very efficient at 2°C, the oil separating out a little after the initial stirring. It is concluded that it is the reduced degree of physical oiling which increases the survival in the dispersed oil samples. These results confirm

Chloride ion loss after a sudden dilution of external medium in G.setosus with and without oil and dispersant.

Fig 5·14



the observation that dispersed oil reduces the detrimental effect of the oil simply by preventing the amphipods from coming into contact with the oil.

(iii) Permeability to water.

The addition of oil and dispersed oil to the animals appears to have little effect on the permeability of G. setosus to water in differing salinities. (Fig. 5:15).

The effect of oil and oil plus dispersant water soluble fraction on O. litoralis and G. setosus.

(i) Survival

Amphipods living in the vicinity of a spill are likely to come into contact with the water soluble fractions of the oil. It is thus useful to know the extent to which the W.S.F. affects these animals. Initially, it was thought that relatively low concentrations of W.S.F. would prove fatal to the amphipod populations. However, Table 5 : 4 shows that increasingly high concentrations of W.S.F. had no apparent effect on the amphipods. Only when 6ml oil and dispersant W.S.F. was added to 3ml 100% s.w. did 50% of the animals die in the first 48 hours.

These results show that only unrealistic levels of W.S.F. proved fatal in the short term.

(ii) Haemolymph chloride concentrations

Both Gammarus setosus and Onisimus litoralis were acclimated to varying salinities with the addition of oil and oil plus dispersant water soluble fractions (W.S.F.) and the chloride concentration determined. These results were superimposed on those obtained from animals not exposed to the W.S.F. (Figs. 5:15 & 5:14). The figures clearly show that the W.S.F. has no significant effect on the haemolymph chloride concentrations of acclimated individuals.

Water permeability of G.setosus at different salinities with and without oil and dispersant.

Fig 5·15

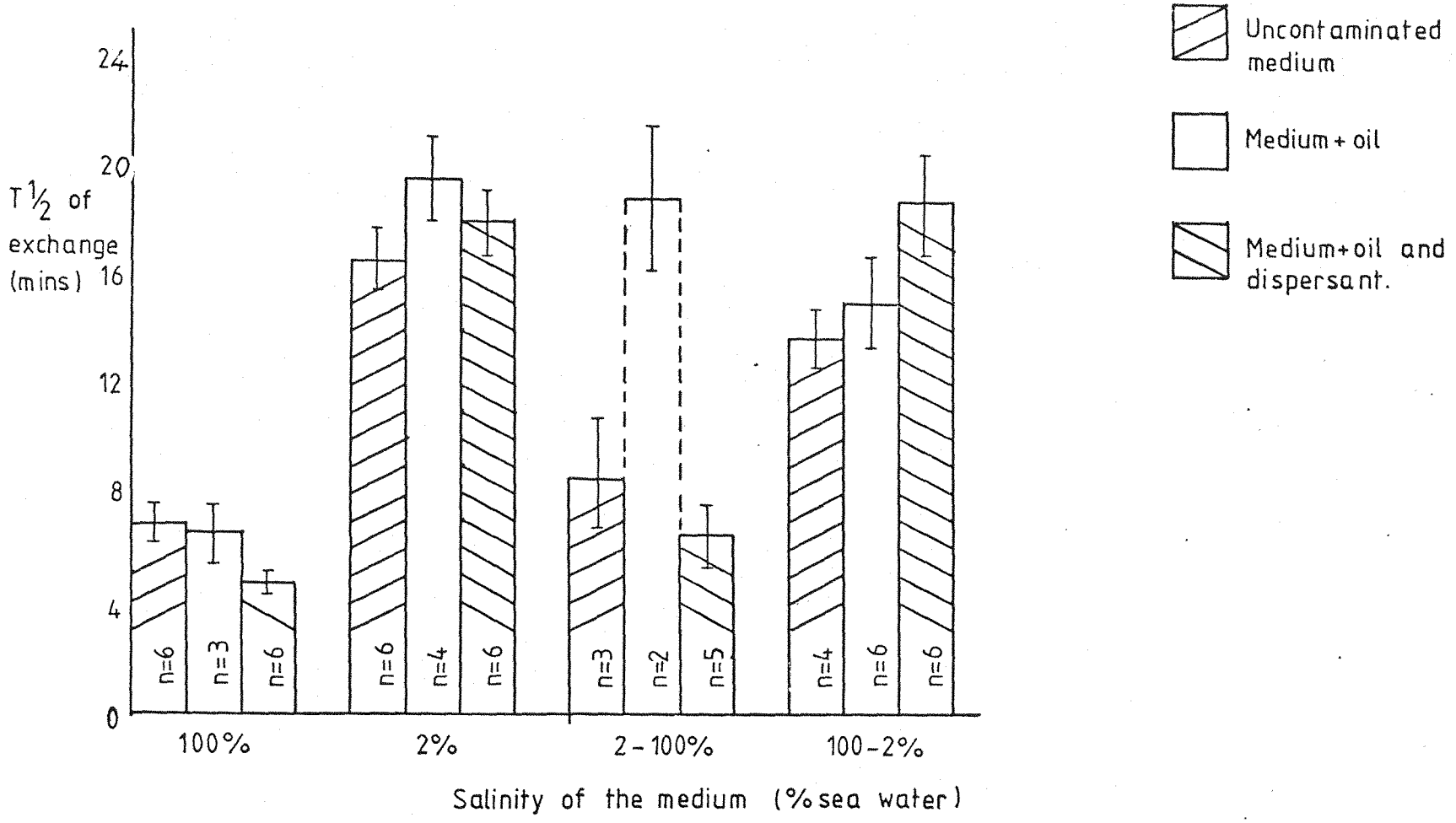
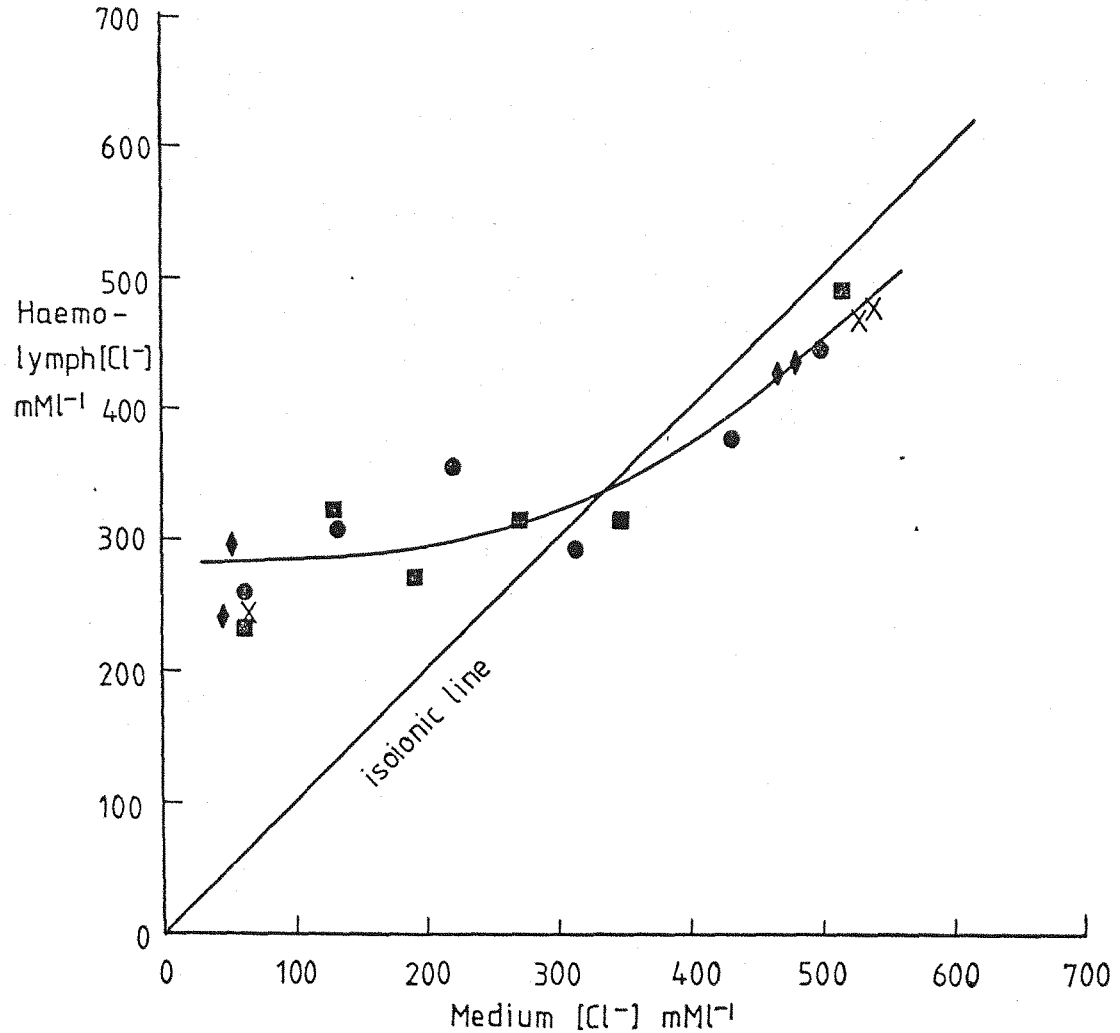


TABLE 5:4

Species	Medium % SW	WSF per 100ml	Results after at least 48 hours
10 *O. litoralis*	2%	50µl Oil	All survived
" "	2%	" " + disp.	" "
" "	2%	Control	" "
0 *O. litoralis*	2%	100µl Oil	
10 "	2%	100µl Oil + disp.	" "
" "	2%	100µl Control	" "
10 "	2%	200µl Oil	One fatality
" "	2%	" " + disp	All survived
" "	2%	200µl Control	One fatality
20 "	2% and 100%	.4ml Oil	One fatality
" "	" " "	.4ml Oil + disp	Mortality low
" "	" " "	.8ml Oil	Canabalism
" "	" " "	.8ml Oil + disp	accounting for the majority
" "	" " "	1.2ml Oil	of deaths
" "	" " "	1.2ml Oil + disp	
" "	" " "	1.6ml Oil	
" "	" " "	1.6ml Oil + disp	
2 *G. setosus*	" " "	.4, .8, 1.2, 1.6 ml Oil & Oil + disp.	
10 *O. litoralis*	" " "	1.6, 2.0, 2.4, 2.8, 3.2 ml Oil & Oil + disp.	
3 *G. setosus*	" " "	1.6, 2.0, 2.4, 2.8, 3.2 ml Oil & Oil + disp.	All survived
10 *O. litoralis*	100%	6ml Oil WSF + 3ml med.	Five survived
" "	"	6ml Oil WSF + Oil + disp + 3ml med.	One survived

The relationship between haemolymph chloride concentration in G.setosus and the chloride concentration of the medium.

Fig 5·16



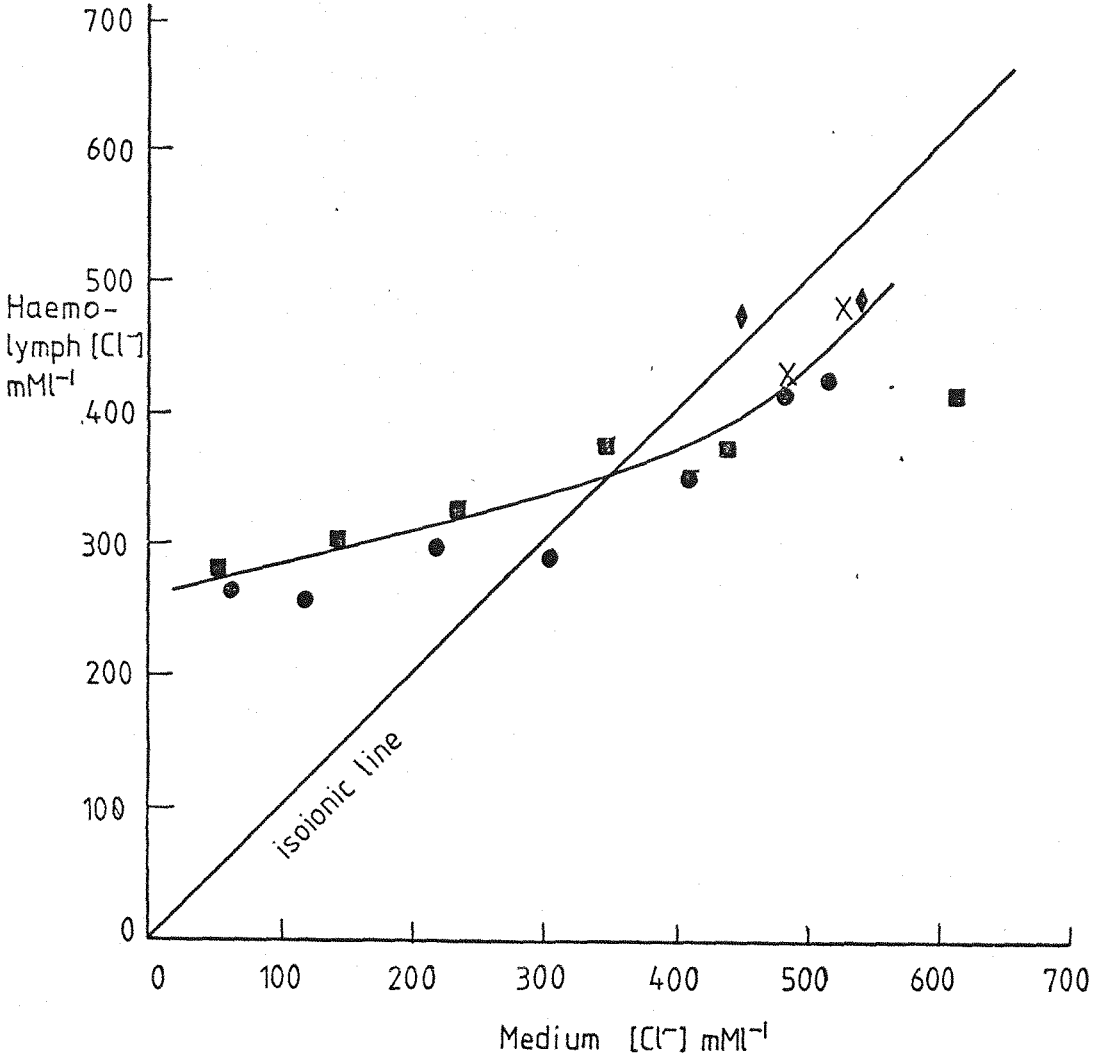
■ After 24hours

● After 48 hours

♦ 200µl W.S.F. 100ml⁻¹

X 3·2ml W.S.F. 100ml⁻¹

The relationship between haemolymph chloride concentration in O.litoralis and the chloride concentration of the medium. Fig 5·17



■ After 24 hours

● After 7 days

♦ 200µl W.S.F. 150ml⁻¹

X 3ml W.S.F. 100ml⁻¹

The effect of water soluble fractions on the heart rate of Gammarus setosus at different salinities and temperatures.

An investigation was undertaken of the effect of temperature, salinity and water soluble fractions of oil and dispersed oil on the heart rate of Gammarus setosus.

Individual animals were immobilised in the experimental medium and heart rate measured at varying temperatures with and without addition of oil and dispersant W.S.F.

(i) Temperature

In all experiments the heart rate rose by 19± 1) heart beats per minute for every 1°C increase in temperature. Linear regression of heart rate against temperature gave a correlation coefficient which varied from .995 to .999. (Figs. 5:18 & 5:19).

Comparison of these results with those obtained using G. duebeni shows a similar correlation between temperature and heart rate. However, G. setosus appears to have a faster heart rate for any given temperature. For example, at 10°C, Gammarus duebeni has a heart rate of approximately 115 beats per minute compared with G. setosus of approximately 150 beats per minute. This difference is easily explained as G. setosus is long term acclimated to lower temperatures. It would be interesting to know if the heart rate of G. duebeni increased if it were kept at 2°C prior to the experiments. G. setosus used varied from 200-250mg.

(ii) Salinity

There was no significant change in the heart rate of G. setosus when the salinity of the external medium was altered.

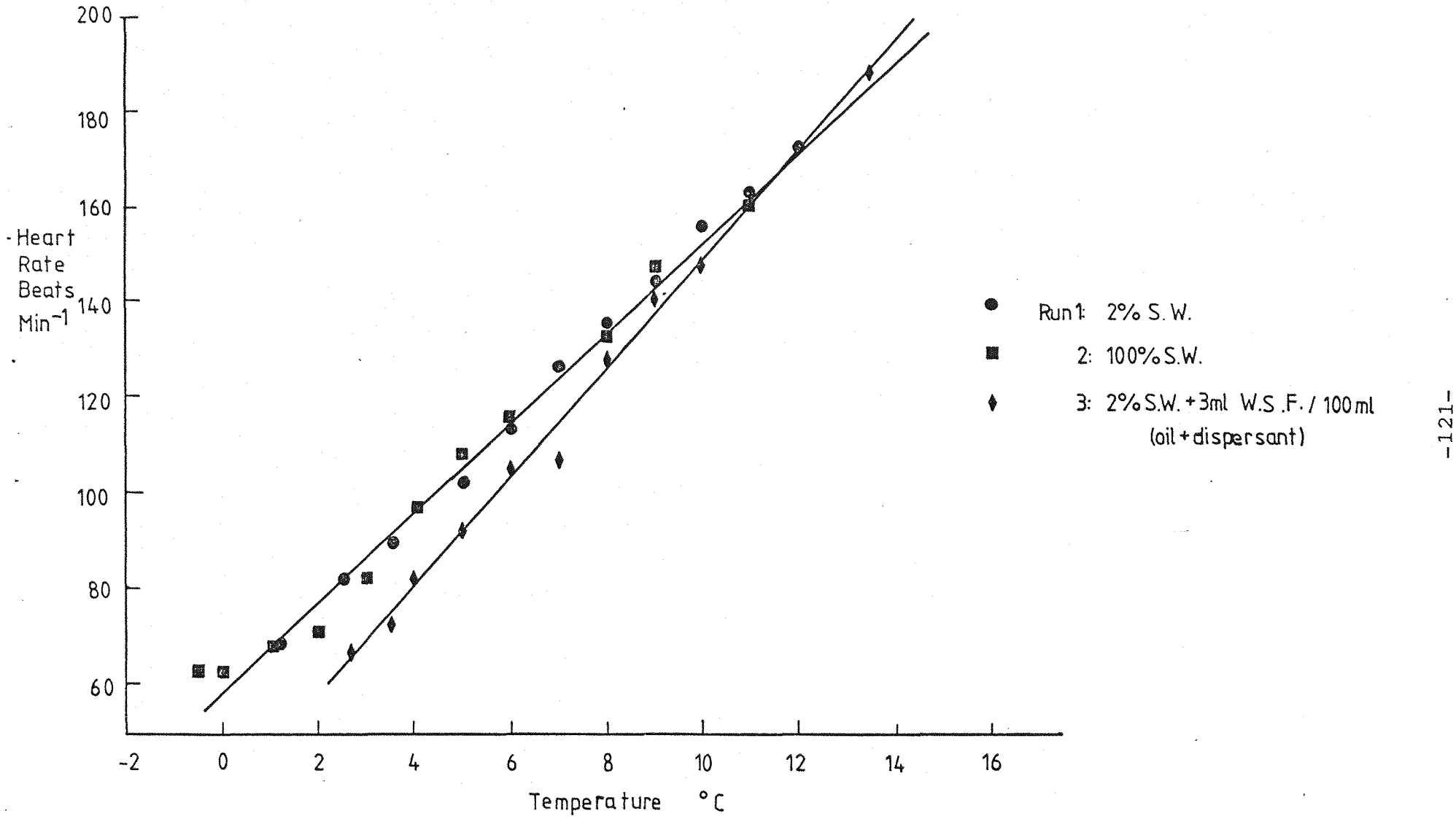
(iii) The addition of oil and dispersed oil Water Soluble Fraction .

The addition of varying concentrations of W.S.F. did not effect the heart rate of G. setosus.

These results show that the heart rate of G. setosus is positively correlated to temperature, while being

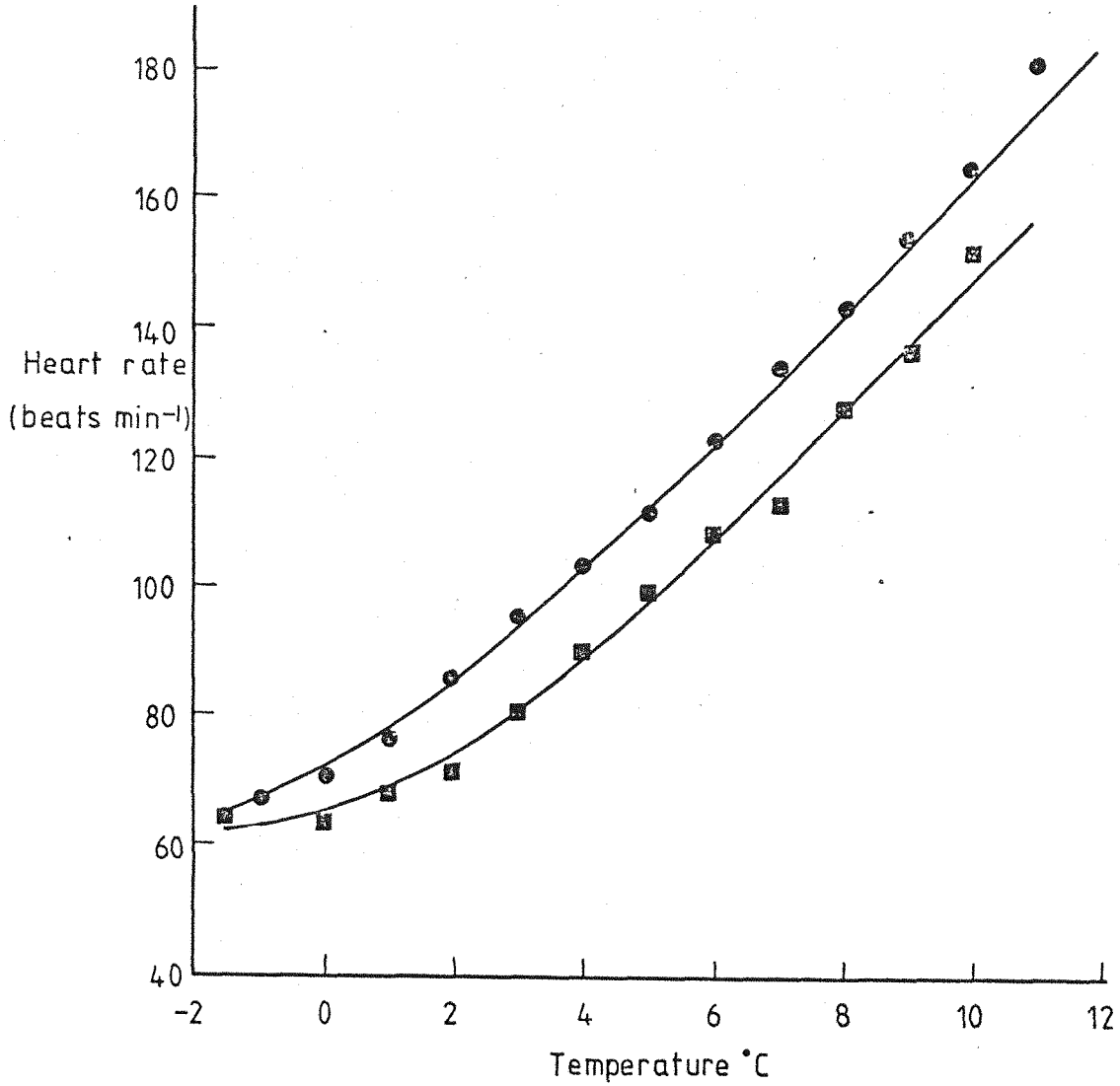
Heart rate against temperature for a single G. setosus.

Fig 5·18



Heart rate against temperature for a single G.setosus.

Fig 5·19



unaffected either by changes in external medium concentration or by the addition of oil and dispersed oil W.S.F.

DISCUSSION

The amphipods O. litoralis and G. setosus have been shown to live in an environment where, during the summer months, the salinity can vary from virtually fresh water to full strength sea water within a few centimetres. The ability of these animals to survive in a layer of fresh water in a marine environment confers an advantage as some local marine predators (e.g. the ctenophores) cannot penetrate into the fresh water. This affords the amphipods some protection while they graze, especially as the presence of the fresh water layer coincides with the late spring algal blooms on the underside of the ice. G. setosus is also found around the ice cracks in shallow tidal water during the summer, when the salinity is commonly less than 10‰. Thus these animals clearly demonstrate their ability to tolerate low salinities for short periods.

Comparison of the physiological responses of G. setosus with G. duebeni shows a remarkable difference in their responses to rapid changes in salinity. These dissimilarities reflect the differences in the habitat of the two species, G. duebeni often living for protracted periods in a very dilute medium, whereas G. setosus would normally have access to full strength sea water. Thus while G. duebeni must be able to achieve dynamic equilibrium with a dilute medium, G. setosus needs only to survive in fresh water for limited periods, being able to move into sea water at intervals.

The effect of oil and dispersed oil on G. setosus and O. litoralis has shown that these animals are particularly susceptable to contamination by untreated crude oil, especially as they are found on the underside of the ice where oil from an under ice oil spill would accumulate. They do not appear to be affected as much by dispersed oil. Thus it is concluded that any attempts at dispersing oil in an under ice oil spill would be advantageous to the epontic amphipod species.

GENERAL DISCUSSION

Osmoregulation in euryhaline crustaceans involves a variety of physiological adaptions to enable the concentration of extracellular fluid to be regulated, and the cells to compensate osmotic shock. Stenohaline crustacea which are unable to adapt to a dilute medium pay the penalty if exposed to such conditions. For example, Maia squinado and Galathea squamifera swell if subjected to reduced salinity, the latter dying after a weight increase of 12%, occurring as little as one hour after being transferred to 60% sea water. (Davenport 1972).

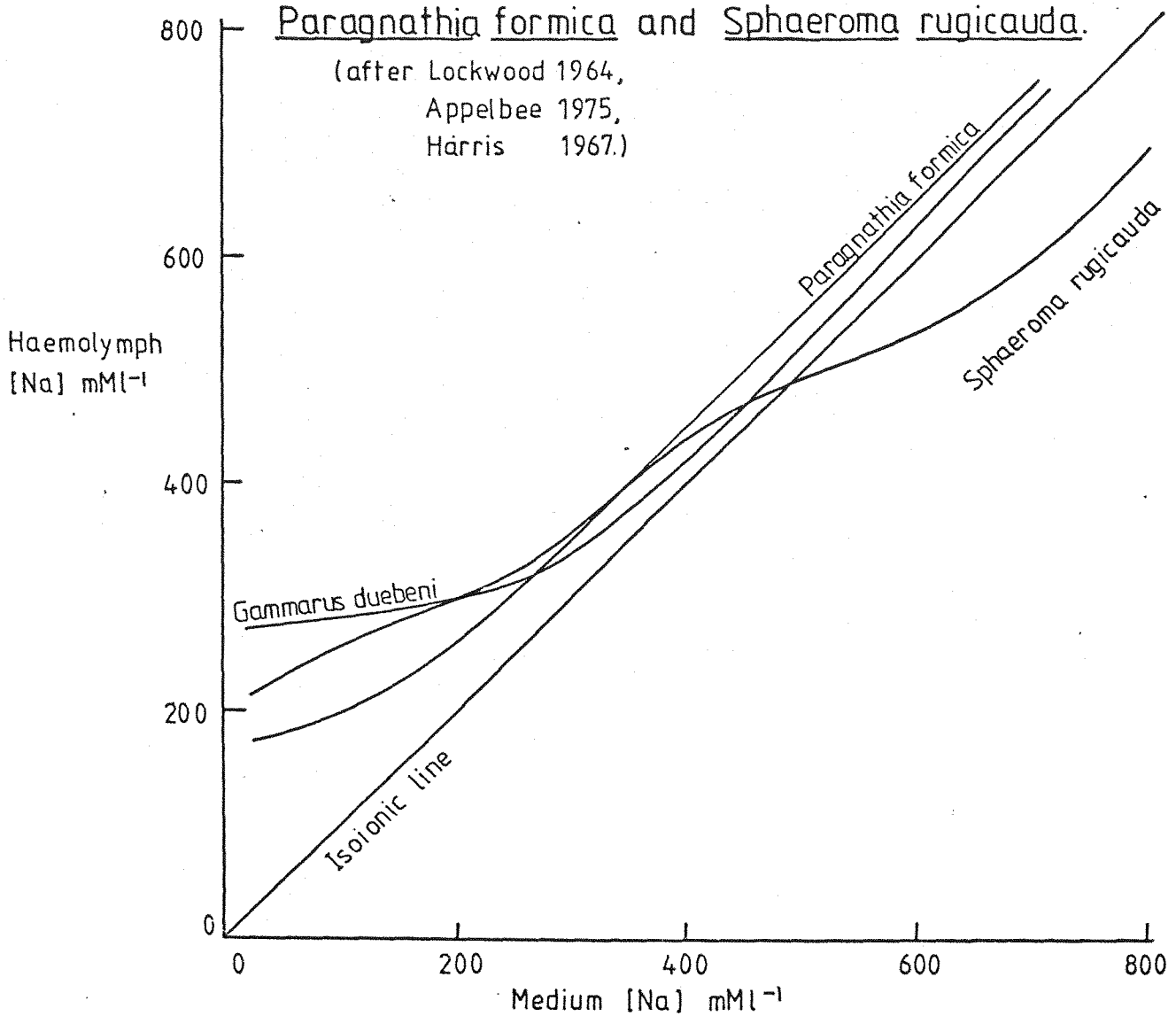
In order to survive, euryhaline crustacea must be effective at regulating their volume, matching the osmotic influx of water with urine output. Thus the crab Pachygraspus crassipes produces fifteen times as much urine in 50% sea water as in 100% s.w. (Gross and Marshall, 1960). The mechanism of increased urine flow has been demonstrated in Carcinus maenas (Norfolk, 1976) where at least three processes have been identified: variation in filtration rate, a change in pore dimensions at the filtration site, and a modification in the proportion of primary urine reabsorption.

While regulation of urine flow controls the overall value of the animal, the cells bathed in the extra cellular fluids are not protected from osmotic shock by this mechanism. Any such shock resulting from an altered haemolymph concentration would result in a cell volume variation causing physiological and circulatory problems.

To some degree this problem is lessened by adjusting the concentrations of the extracellular fluids so that the osmotic gradient between the cells and the extracellular fluids is minimised. This is achieved by reduced ion loss and increased active ion uptake. Thus euryhaline crustaceans are characterised by maintaining their haemolymph concentration hypertonic to the medium in conditions of low salinities. (Fig. D;1)

Although the osmotic gradient between the cells and the haemolymph is minimised it is still necessary for the cells to adjust their concentration to maintain an osmotic balance with

The relationship between haemolymph and medium sodium concentration in Gammarus duebeni, Paragnathia formica and Sphaeroma rugicauda. Fig. D·1



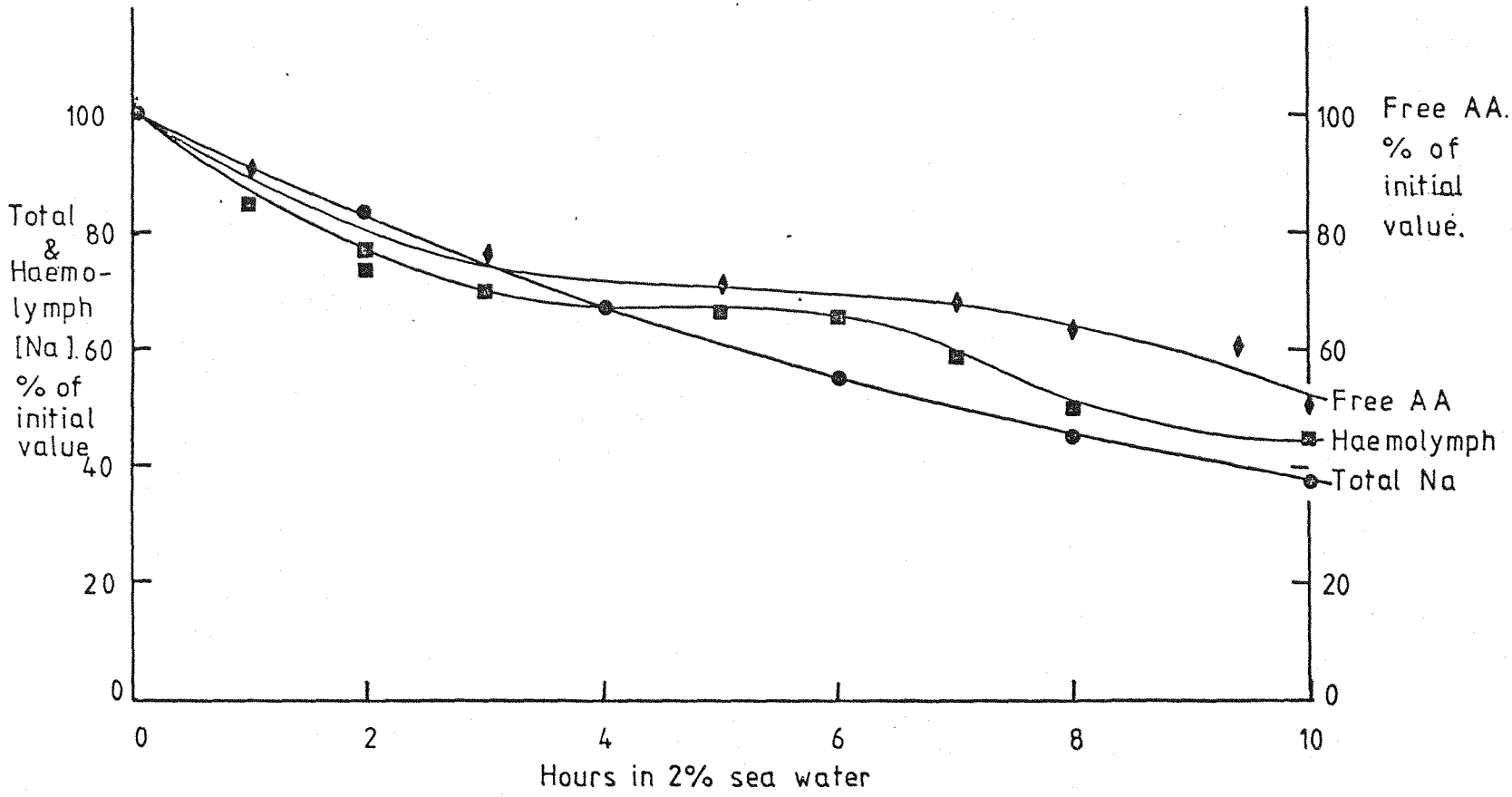
the surrounding fluids. Most crustacea have some potential for adjusting the level of free amino acids (F.A.A.) in the cells. This trait is well developed in euryhaline crustacea allowing adjustment of the cell osmotic concentration. This is demonstrated in the isopod Sphaeroma rugicauda where there is a decrease in F.A.A. following a dilution in external medium (Fig. D:2). (Harris, 1967).

The effectiveness of these methods in maintaining cell and body volumes is demonstrated in the amphipod Gammarus duebeni in which there is no detectable difference in the blood volume of animals in 100% and 2% sea water. (Lockwood and Inman, 1973). Similarly the water content in the muscles of Carcinus maenas increases by only 3.8% in animals in 40% sea water as compared to animals in 100% sea water (Shaw 1958).

A further mechanism by which some euryhaline crustacea minimise osmotic shock is to reduce their permeability to water and ions. Rhithropanopeus harrisi (Smith 1967), Carcinus maenas (Smith 1970) and Gammarus duebeni (Lockwood, Inman and Courtenay 1973) have all been shown to alter their apparent permeability to water. It is proposed that a changing permeability to water in some euryhaline amphipods is an important mechanism by which these amphipods successfully osmoregulate.

The physiological responses of the euryhaline amphipods have shown that, of the species studied in this thesis, only Gammarus duebeni, Gammarus setosus and Onisimus litoralis exhibit large changes in apparent water permeability. These amphipods are also the most tolerant to osmotic stress. An aim of this thesis is to demonstrate that the apparent water permeability changes found in G. duebeni are real, and not an artefact of experimental technique. In Chapters 1 and 2, various criticisms and possible errors of the technique of using THO as a marker to measure the permeability of the amphipods to water were discussed. From this study there are two main lines of evidence to suggest the permeability changes demonstrated in G. duebeni are real.

The relationship between cell free amino acid concentration, haemolymph sodium concentration & total sodium concentration in Sphaeroma rugicauda after transfer from sea water to 2% sea water. (after Harris 1967) Fig D·2



(i) There is a good correlation between change in water permeability found by using THO and calculated using urine clearance rates.

(ii) Chaetogammarus marinus and Gammarus locusta exposed to varying salinities do not demonstrate the water permeability changes found in G. duebeni under the same conditions. If the change in water permeability in G. duebeni were an artefact of the technique used, then it is likely that C. marinus and G. locusta would also exhibit the same pattern of permeability change.

It is felt that while either of these two arguments alone would not prove conclusive, taken in conjunction they strongly support the hypothesis that the change in water permeability in G. duebeni is indeed real This statement must be qualified as there is no indication that the values of $t_{\frac{1}{2}}$ in minutes are absolute. These results show a pattern of change and this limitation must be realised if any further calculations are based on the measured $t_{\frac{1}{2}}$ values.

Having demonstrated the value of using THO as a marker to indicate water permeability change, this technique was used on the arctic amphipods G. setosus and O. litoralis. However, it is felt that while the conclusions reached on the technique of using THO on G. duebeni would hold true for other amphipod species, the technique would need further justification if used in other phyla such as the Chordata where the problems are entirely different.

Although attempts were made to investigate possible mechanisms for the change in water permeability in Gammarus duebeni (Chapter 3), there is little evidence to suggest any specific mechanism. Further research should attempt to discover how such rapid and large changes in water permeability are brought about. Some work has been carried out using electron microscopy (Lockwood and Inman 1973), by photographing the micro structure of the gills of G. duebeni in 100% and 2% sea water, and there is some indication of increased mitrochondrial activity at 2% and a more convoluted endoplasmic reticulum. However, further research is needed with the animals in various salinity regimes

to confirm or contradict these observations.

In order to investigate further the effect of the potential difference on the permeability of the membranes, elegant use of micro-electrodes is necessary, ideally on a single excised gill preparation. However, it would be technically difficult to measure the water permeability of a single amphipod gill, and a larger experimental animal is indicated. Unfortunately, the larger crustacea have a relatively stable body fluid concentration due to the increased volume, and do not exhibit the changes in water permeability shown by G. duebeni.

Comparative work on various amphipod species has demonstrated a spectrum of physiological responses to osmotic stress. It is possible to relate these variations in responses to the ecology of the individual species. Amphipods occupy widely varying habitats, from the successful scavenger Hirondella gigas of the abyssal depths (Hessler et al, 1978) to Gammarus pulex in fresh water streams (Hynes, 1950). A significant number of species have evolved a tolerance to a fluctuating external salinity such as found in estuarine conditions. Each of these species has a different ecological niche, so that for the euryhaline amphipods each species would be expected to exhibit varying degrees of tolerance.

Of the British species, Gammarus duebeni is the most euryhaline. The population studies live in small pools on a salt marsh where they have little choice of environmental conditions, which may vary from fresh water to full strength sea-water. However, they have been observed to leave pools which are in danger of drying up and migrate to other pools overland, surviving for several hours out of water. (Segerstrale, 1946). Gammarus duebeni are also found in fresh water streams in coastal areas in North West Europe (Hynes, 1954) and in full strength sea water in coastal rock pools (Kinne, 1959).

Other British euryhaline amphipods include Gammarus locusta (Spooner, 1946), Chaetogammarus marinus (Spooner, 1946) (formally Marinogammarus marinus) and Gammarus zaddachi (Sergerstrale, 1947). C. marinus and G. locusta live in muddy intertidal zones and, although subject to fresh water run off and precipitation

at low tide, are not exposed to long periods of low salinity, being covered every high tide with sea water. While G. zaddachi are found in estuarine and salt marsh conditions, they do not compete with G. duebeni as the former occur in the more saline deeper drainage channels.

The circumpolar arctic amphipod Gammarus setosus is the most abundant species of Gammarus in the arctic. (Steele and Steele, 1974). This species is found in full strength sea water for eight months of the year and for the short Spring and Summer it is often associated with low salinities produced by melting snow and ice. The fresh water, present as a layer above the sea water allows the amphipods easy access to the salt water below. Thus while thay are found in salinities as low as 1‰, they are always able to return to the salt water. This contrasts with Gammarus duebeni which is often in fresh water for long periods of time. Onisimus litoralis is another euryhaline arctic amphipod which often co-exists with G. setosus in the fresh water layer under the ice in Spring and Summer. O. litoralis show very similar physiological responses to that found in G. setosus. This demonstrates the similarity of responses of two different genera with similar environmental conditions.

Comparison of the physiological responses of G. duebeni and G. setosus confirms the difference found in their environmental conditions. Both species are able to tolerate large and sudden changes in salinity, but the physiological responses to such salinity changes differ significantly. Both species show a high permeability to water if acclimated to high salinities. However, if the animals are transferred from a low salinity to a high salinity, the responses of the two species are different. G. deubeni maintains a low permeability to water for 16 hours after the transfer when there is a sudden increase in water permeability, while G. setosus becomes more permeable immediately after the change.

Related to this difference in water permeability is the time taken for the body fluids to reach equilibrium with the external medium following the increase in external medium

concentration. In G. duebeni the mean time is 16 hours, approximately corresponding to the period when the animals are still impermeable after the salinity shock. By contrast, the more permeable G. setosus takes only 3-4 hours to reach equilibrium.

Relating these differences to the ambient conditions for these animals suggests the following hypothesis: G. setosus is only found in fresh water in Spring and Summer, and even then has access to salt water. They are thus in a position to travel into the salt water to take up ions if the body fluids become depleted. It is therefore to their advantage to reach equilibrium with their surroundings rapidly after travelling into sea water, and to lose ions slowly in fresh water. Their high permeability to water after sudden increases in salinity allow these conditions to be met. Conversely, G. duebeni used in the present study are found in enclosed pools and have no "choice" of salinity, it is therefore more useful for the animals to be able to guard against sudden osmotic shock by remaining impermeable after a sudden increase of external medium so that the osmotic water flux out of the animal can be minimised.

Thus while these animals both tolerate extremes of salinity, the different environmental conditions have dictated different physiological responses to the problems of osmotic stress.

Chaetogammarus marinus and Gammarus locusta also show physiological responses which relate to their environmental conditions. Neither species is able to survive in salinities below 10% for extended periods, and this is reflected in a lack of significant permeability change when the animal is placed in varying salinities. These animals are unable to osmoregulate effectively when exposed to extreme osmotic stress. However, they are able to survive short periods at salinities down to 1‰ as demonstrated by their long term survival in the experimental cycling salinity system, where the salinity varies from 1‰ to 31‰ in a 12 hour 25 min. cycle.

Comparing the three British species of amphipod studied in the cycling salinity regime demonstrates the relative degrees

of stability of the blood concentration to such changes in salinity. G. duebeni maintains the haemolymph concentration relatively constant while G. locusta and C. marinus are unable to do so (c.f. Fig. 1:14). If these results are taken in conjunction with the permeability to water during the salinity cycle (c.f. Fig. 1:18) and the net fluxes calculated (c.f. Fig. 1:24), then the effect of the changes in permeability to water in G. duebeni can be seen. G. duebeni appears to be able to minimise net osmotic water loss and gain by being relatively impermeable during osmotically stressful periods, i.e. when there is a large haemolymph to external medium gradient, and the osmotic net fluxes would be large.

Thus it can be hypothesised that the changing permeability to water demonstrated in G. duebeni and G. setosus is an important facet of osmoregulation which allows these animals to successfully survive in habitats which exclude species which would otherwise compete. For example, G. duebeni is able to colonise the salt marsh conditions with no competition from Gammarus pulex found in a nearby fresh water stream, or from Gammarus zaddachi located in the deeper drainage channels of the marsh. Similarly the arctic epontic amphipods are able to graze freely on the underice algal blooms within a fresh water layer, safe from the ctenophore predators of mid-water which cannot penetrate the fresh water.

The isopod Sphaeroma rugicauda is found co-existing with Gammarus duebeni in the Salt Marsh pools at Totton, Southampton. (Harris, 1967). These animals are of a similar size and experience identical osmotic conditions. Under experimental conditions, S. rugicauda shows an extremely large individual variation of water permeability due to the animals behavioural response of rolling up into a tight ball when disturbed (Bolt unpublished). This could be overcome by measuring outflux of THO over a longer period of time when the animals were not disturbed. It would be interesting to note if a crustacean of the order isopoda living in the same environmental condition as G. duebeni would have evolved a similar response to osmotic stress.

In conclusion, this thesis has demonstrated that the permeability to water of some euryhaline species of amphipod alters when the external medium is changed. Furthermore, it appears that this ability is related to the animals capacity to survive in osmotically stressful conditions, and it is likely that changes in permeability constitute a significant mechanism in osmoregulation.

---oOo---

REFERENCES

APPLEBEE, J.F. (1975). An investigation into some aspects of the osmoregulation of Paragnathia formica (Hesse). M.Sc. dissertation, University of Southampton.

BEADLE, L.C. and CRAGG, J.B. (1940). Studies on adaption to salinity in Gammarus species. 1. Regulation of blood and tissue and the problem of adaption to fresh-water. J. exp. Biol. 17, 153-163.

BUCHANAN, R.A., CROSS, W.E., & THOMSON, D.H. (1977). Survey of the marine environment of Bridport Inlet, Melville Island. L.G.L. Ltd., environmental research associates, 414, 44 Eglinton Ave, West Toronto, Ontario, Canada.

CORNELL, J.C. (1973). A reduction in water permeability in response to a dilute medium in the stenohaline crab Libinia emarginata. (Brachyura, Majidae). Biol. Bull. mar. biol. Woods Hole 145, 430-431.

DAINTY, J & HOUSE, C.R. (1966). Unstirred layers in frog skin. J. Physiol., Lond. 182, 66-78.

DAVENPORT, J. (1972). Volume changes shown by some littoral anomuran crustacea. J. mar. biol. Assoc. U.K. 52, 863-877.

DAVENPORT, J. (1979). Cold resistance in Gammarus duebeni Lilljeborg. Astarte. 12, 21-26.

DAWSON, M.D. (1982). Aspects of osmoregulation in the amphipod Gammarus duebeni; the effects of changing salinity and some potential pollutants.

DAYTON, P.K. and MARTIN, S. (1971). Observations of ice stalactites in McMurdo Sound, Antarctica. J. Geophys. Res. 76: 1595-1599.

FETTIPLACE, R. (1978). The influence of the lipid on the water permeability of artificial membranes Biochem. biophys. acta. 513 (1) 1-10.

GERDENITSCH, Waltraud (1980). The influence of isoptin (verapamil) on the water permeability of the inner epidermal cells of Allium cepa. Phyton. Horn. 20 (3/4) 235-250.

GRAZIANI, Y. and LIVNE, A. (1972). Water permeability of Bilayer Lipid Membranes Sterol-Lipid Interaction. J. Membrane biol. 7, 275-284.

GREENAWAY, P. (1981). The fate of glomerular filtration markers injected into the haemolymph of the amphibious crab Holthuisana transversa. J. exp. biol. 91, 339-347.

GROSS, W.J. and MARSHALL, L.A. (1960). The influence of salinity on magnesium and water fluxes on a crab. Biol. Bull. mar.biol. Lab. Woods Hole, 119, 440-453.

HARRISON and LUNT, (1980). Biological Membranes. 2nd ed. Blackie & Son Ltd, Glasgow.

HARRIS, R.R. (1967). Aspects of ionic and osmotic regulation in two species of Sphaeroma (Isopoda). Ph.D thesis, University of Southampton.

HAYWOOD, G.P. (1970). A study of the osmotic changes in the blood and urine of three species of gammarid exposed to varying external salinities. M.Sc. dissertation, The University, Southampton.

HESSLER, Robert R; Camilla L. INGRAM; A. Aristides YAYANOS and Bryan R. BURNETT (1978). Scavenging amphipods from the floor of the Philippine Trench. Deep-Sea Res. 25 (11): 1029-1048.

HYNES, H.B.N. (1950). Preliminary report on fresh water species of Gammarus in the Isle of Man. Rep. Mar. Biol. Sta. Pt. Erin 62, 24-25.

HYNES, H.B.N. (1954). The ecology of Gammarus duebeni Lilljeborg and its occurrence in fresh water in Western Britain. J. anim. Ecol. 23, 38-84.

JØRGENSEN, C.B. and DALES, R.P. (1957). The regulation in some neried polychaetes. Physiol. Comp et Oecol 4 : 357-374.

KINNE, O. (1959) Ecological Data on the amphipod Gammarus duebeni. A monograph Veroff. Inst. Meeresforsch, Bremerhaven.

L.G.L. Ltd. (1980). A proposal for Baffin Island Oil Spill project, Biological component. (Benthic and epontic studies).
L.G.L. Ltd, environmental research associates, 414, 44 Eglinton Ave. West Toronto, Ontario, Canada

LOCKWOOD, A.P.M. (1964). Activation of the sodium uptake system at high blood concentrations in the amphipod Gammarus duebeni. J. exp. Biol. 41, 447-458.

LOCKWOOD, A.P.M. and INMAN, C.B.E. (1973). Water uptake and loss in relation to the salinity of the medium in the amphipod crustacean Gammarus duebeni. J. exp. Biol. 58, 149-163.

LOCKWOOD A.P.M. and INMAN, C.B.E. (1979). Ecophysiological responses of Gammarus duebeni to salinity fluctuations. Ecological processes in Coastal Environments. Edited by Jeffries R.L. and Davy, A.J.

LOCKWOOD, A.P.M., INMAN, C.B.E. and COURTENAY, T.H. (1973) The influence of environmental salinity on the water fluxes of the amphipod crustacean Gammarus duebeni. J. exp. Biol. 58, 137-48.

LOCKWOOD, A.P.M., JENKINSON, N.W., BOLT, S.R.L., DAWSON, M.E. and JENSEN, A.C. (1981). Microprocessor systems as an aid to estuarine studies in the laboratory. (Accepted for publication by Estuarine, Coastal & Shelf Science).

MONTAL, M. and MUELLER, P. (1972). Formation of Bimolecular Membranes from Lipid monolayers and a study of their electrical properties. Proc. nat. Acad. Sci. U.S. 69, 3561-3566.

MOTAIS, R., ISAIA, J., RANKIN, J.L. and MAETZ, J. (1969). Adaptive changes of water permeability of the teleostean gill epithelium in relation to external salinity. J. exp. Biol. 51, 529-46.

NERNST, W. (1904). Theorie der Reaktionsgerschwindigkeit. Heterogemen Sytemen 7 phys. chem. 47, 52-55.

NORFOLK, J.R.W. (1976). The control of urine production in Carcinus maenas. Ph.D. thesis, University of Southampton.

PAIGE, R.A. (1970). Stalactite growth beneath sea ice. Science 167, 171-172.

RAMSEY, J.A. and BROWN, R.H.J. (1955). Simplified apparatus and procedure for freezing point determinations upon small volumes of fluid. J. scient. Instrum. 32, 372.

RAMSEY, J.A., BROWN, R.H.J. and CROGHAN, P.C. (1955). Electrometric titration of chloride in small volumes. Vol. 32, No.4 pp 822-829. J. exp. Biol.

RUDY, P.P. (1967). Water permeability in selected decapod crustacea. Comp. Biochem. Physiol. 22, 581-9.

SEGERSTRÅLE, S.G. (1946). On the occurrence of the amphipod Gammarus duebeni. Lillj. in Finland, with notes on related species. Societas Scientiarum Fennica Conmentiores Biologicae IX 18.

SEGERSTRÅLE, S.G. (1947). New observations on the distribution and morphology of the amphipod Gammarus zaddachi (sexton) with notes on related species. Journal of the marine biological association of the United Kingdom. Vol. XXVII pp 219-244.

SHAW (1958). Osmoregulation in the muscle fibres of Carcinus maenas. J. Exp. Biol. 35, 920-929.

SINGER, S.J. and NICOLSON, G.L. (1972). The Fluid Mosaic Model of the Structure of Cell Membranes. Science 175, 720-731.

SMITH, R.I. (1967). Osmotic regulation and adaptive reduction of water permeability in a brackish water crab, Rhithropanopeus harrisi (Brachyura, Xanthidae). Biol. Bull. mar. biol. lab. Woods Hole. 144, 643-58.

SMITH, R.I. (1970) The apparent water permeability of Carcinus maenas (Crustacean, Brachyura, Portunidae) as a function of salinity. Biol. Bull. mar. biol. Lab. Woods Hole. 139, 351-62.

SMITH, R.I. (1976). Apparent water permeability variation and water exchange in crustaceans and annelids. Perspectives in Experimental Biology Vol.1. Zoology. Edited by Spencer Davies, P. Permagon Press, Oxford.

SPOONER, G.M. (1947). The distribution of Gammarus spp. in estuaries. Part 1. J. mar. biol. assoc. U.K. 27, 1-52.

STEELE, D.H. and STEELE, V.J. (1974). The biology of Gammarus (Crustacea, Amphipoda) in the northwest atlantic. VIII Geographic distribution of the northern species. Can. J. Zool. 52. 1115-1120.

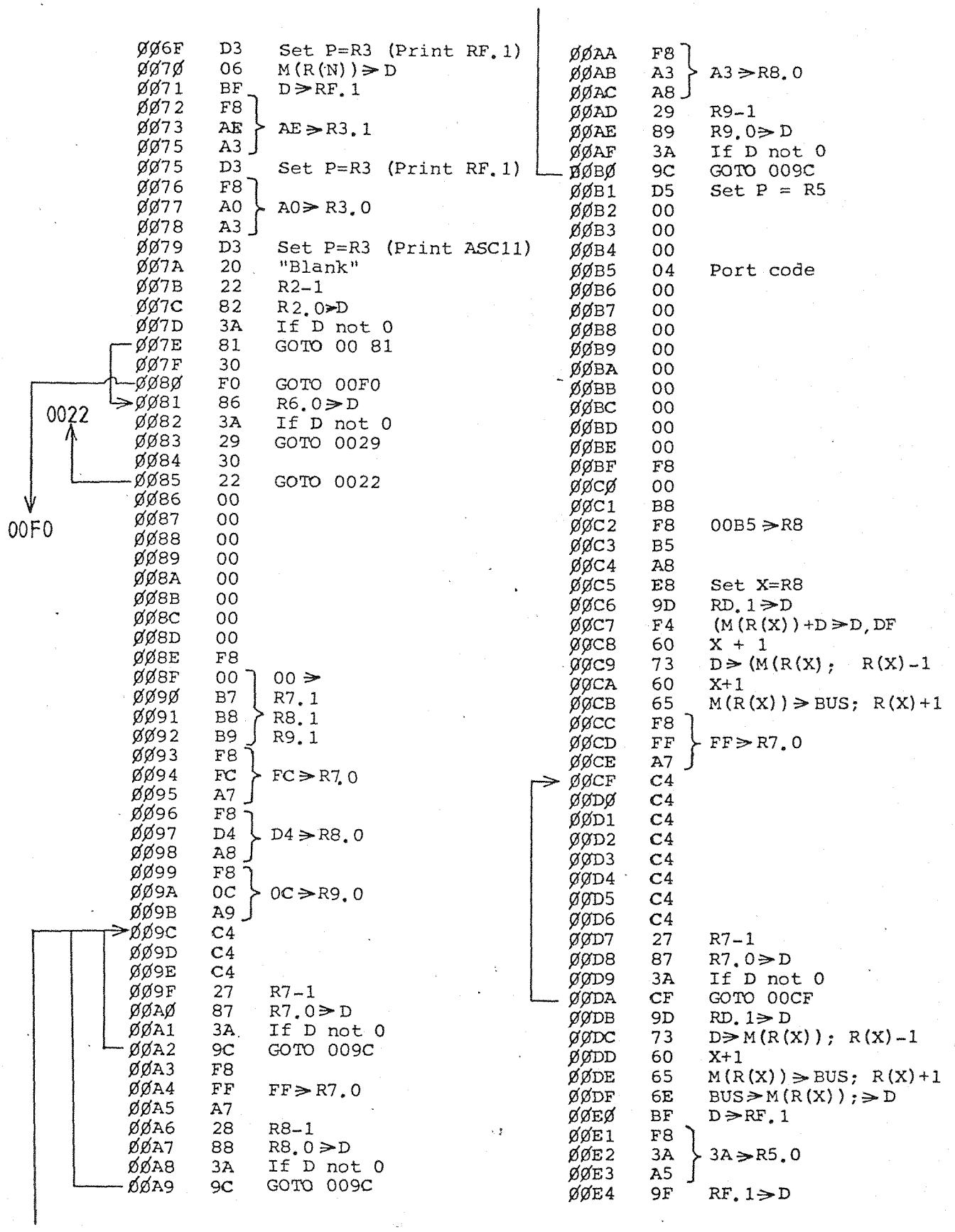
---oOo---

APPENDIX

1. Annotated microprocessor program for the salinity cycle

```
ØØØØ  C4
ØØØ1  F8
ØØØ2  00
ØØØ3  B5   Sets R5
ØØØ4  F8   = P
ØØØ5  12
ØØØ6  A5
ØØØ7  D5
ØØØ8  00
ØØØ9  00
ØØØA  00
ØØØB  00
ØØØC  00
ØØØD  00
ØØØE  00
ØØØF  00
ØØ1Ø  00
ØØ11  00
ØØ12  F8
ØØ13  02
ØØ14  B3.  Sets R3
ØØ15  F8   = P at 0250
ØØ16  50
ØØ17  A3   (subroutine)
ØØ18  D3
ØØ19  C4
ØØ1A  C4
ØØ1B  C4
ØØ1C  F8
ØØ1D  00
ØØ1E  B2   0008
ØØ1F  F8
ØØ2Ø  08   into R2
ØØ21  A2
ØØ22  F8
ØØ23  02
ØØ24  B6   0200
ØØ25  F8
ØØ26  00   into R6
ØØ27  A6
ØØ28  C4
ØØ29  26   R6-1
ØØ2A  F8
ØØ2B  00   00 ⩾ RA.1
ØØ2C  BA
ØØ2D  C4
ØØ2E  F8
ØØ2F  06   06 ⩾ RA.0
ØØ3Ø  AA
ØØ31  F8
ØØ32  00
ØØ33  B3   Sets R3
ØØ34  F8   = P at 00BF
ØØ35  BF
ØØ36  A3   (subroutine :
ØØ37  D3   call salinity)
ØØ38  00
ØØ39  02   Port code
ØØ3A  E6   Set X = R6
ØØ3B  F5   M(R(X))-D ⩾ DF,D
ØØ3C  BB   D  RB 1
ØØ3D  33   If DF=1,
ØØ3E  4D   GOTO 004D
ØØ3F  F8
ØØ4Ø  02   02 ⩾ RD.1
ØØ41  BD
ØØ42  F8
ØØ43  00
ØØ44  B9   0039 ⩾ R9
ØØ45  F8
ØØ46  39
ØØ47  A9
ØØ48  E9   Set X=R9
ØØ49  65   M(R(X)) ⩾ Bus MRX+1
ØØ4A  30
ØØ4B  5A   GOTO 005A
ØØ4C  01   Port code
ØØ4D  30
ØØ4E  4F   GOTO 004F
ØØ4F  F8
ØØ5Ø  01   01 ⩾ RD.1
ØØ51  BD
ØØ52  F8
ØØ53  00
ØØ54  B9   004C ⩾ R9
ØØ55  F8
ØØ56  4C
ØØ57  A9
ØØ58  E9   Set X = R9
ØØ59  65   M(R(X)) ⩾ Bus : MRX+1
ØØ5A  F8
ØØ5B  8E   8E - R3.0
ØØ5C  A3
ØØ5D  D3   Set P = R3
ØØ5E  2A   RA-1
ØØ5F  8A   RA.0 ⩾ D
ØØ6Ø  3A   If D not 0
ØØ61  31   GOTO 0031
ØØ62  C4
ØØ63  F8
ØØ64  81   Sets R3 = P
ØØ65  B3   at 81AE
ØØ66  F8   (Print RF.1
ØØ67  AE    subroutine)
ØØ68  A3
ØØ69  D3
ØØ6A  9B   RB.1 ⩾ D
ØØ6B  BF   D ⩾ RF.1
ØØ6C  F8
ØØ6D  AE   AE ⩾ R3.1
ØØ6E  A3
```

0031


ØØ6F D3 Set P=R3 (Print RF.1)
ØØ7Ø 06 M(R(N))⩾D
ØØ71 BF D⩾RF.1
ØØ72 F8
ØØ73 AE AE⩾R3.1
ØØ75 A3
ØØ75 D3 Set P=R3 (Print RF.1)
ØØ76 F8
ØØ77 A0 A0⩾R3.0
ØØ78 A3
ØØ79 D3 Set P=R3 (Print ASC11)
ØØ7A 20 "Blank"
ØØ7B 22 R2-1
ØØ7C 82 R2.0⩾D
ØØ7D 3A If D not 0
ØØ7E 81 GOTO 00 81
ØØ7F 30
ØØ8Ø F0 GOTO 00F0
ØØ81 86 R6.0⩾D
ØØ82 3A If D not 0
ØØ83 29 GOTO 0029
ØØ84 30
ØØ85 22 GOTO 0022
ØØ86 00
ØØ87 00
ØØ88 00
ØØ89 00
ØØ8A 00
ØØ8B 00
ØØ8C 00
ØØ8D 00
ØØ8E F8
ØØ8F 00 00⩾
ØØ9Ø B7 R7.1
ØØ91 B8 R8.1
ØØ92 B9 R9.1
ØØ93 F8
ØØ94 FC FC⩾R7.0
ØØ95 A7
ØØ96 F8
ØØ97 D4 D4⩾R8.0
ØØ98 A8
ØØ99 F8
ØØ9A 0C 0C⩾R9.0
ØØ9B A9
ØØ9C C4
ØØ9D C4
ØØ9E C4
ØØ9F 27 R7-1
ØØAØ 87 R7.0⩾D
ØØA1 3A If D not 0
ØØA2 9C GOTO 009C
ØØA3 F8
ØØA4 FF FF⩾R7.0
ØØA5 A7
ØØA6 28 R8-1
ØØA7 88 R8.0⩾D
ØØA8 3A If D not 0
ØØA9 9C GOTO 009C
0022
00F0
ØØAA F8
ØØAB A3 A3⩾R8.0
ØØAC A8
ØØAD 29 R9-1
ØØAE 89 R9.0⩾D
ØØAF 3A If D not 0
ØØBØ 9C GOTO 009C
ØØB1 D5 Set P = R5
ØØB2 00
ØØB3 00
ØØB4 00
ØØB5 04 Port code
ØØB6 00
ØØB7 00
ØØB8 00
ØØB9 00
ØØBA 00
ØØBB 00
ØØBC 00
ØØBD 00
ØØBE 00
ØØBF F8
ØØCØ 00
ØØC1 B8
ØØC2 F8 00B5⩾R8
ØØC3 B5
ØØC4 A8
ØØC5 E8 Set X=R8
ØØC6 9D RD.1⩾D
ØØC7 F4 (M(R(X))+D⩾D,DF
ØØC8 60 X + 1
ØØC9 73 D⩾(M(R(X); R(X)-1
ØØCA 60 X+1
ØØCB 65 M(R(X))⩾BUS; R(X)+1
ØØCC F8
ØØCD FF FF⩾R7.0
ØØCE A7
ØØCF C4
ØØDØ C4
ØØD1 C4
ØØD2 C4
ØØD3 C4
ØØD4 C4
ØØD5 C4
ØØD6 C4
ØØD7 27 R7-1
ØØD8 87 R7.0⩾D
ØØD9 3A If D not 0
ØØDA CF GOTO 00CF
ØØDB 9D RD.1⩾D
ØØDC 73 D⩾M(R(X)); R(X)-1
ØØDD 60 X+1
ØØDE 65 M(R(X))⩾BUS; R(X)+1
ØØDF 6E BUS⩾M(R(X));⩾D
ØØEØ BF D⩾RF.1
ØØE1 F8
ØØE2 3A 3A⩾R5.0
ØØE3 A5
ØØE4 9F RF.1⩾D

```
00E5  D5   Set P = R5
00E6  00
00E7  00
00E8  00
00E9  00
00EA  00
00EB  00
00EC  00
00ED  00
00EE  00
00EF  00
00F0  F8
00F1  08   08>R2.0
00F2  A2
00F3  F8
00F4  81
00F5  B3   Set P = R3
00F6  F8   at 81A0
00F7  A0   (Print ASC11
00F8  A3   subroutine)
00F9  D3
00FA  0A   Carriage Return
00FB  D3   Set P=R3
00FC  0D   Line Feed
00FD  30   GO TO
00FE  81   00 81
00FF  00
0200  F8
0201  02
0202  B5
0203  F8   0207>R5
0204  07
0205  A5
0206  D5   Set P = R5
0207  F8
0208  6E   6E>RE.1
0209  BE   (Set Baud rate=110)
020A  F8
020B  00
020C  B2   0008>R2
020D  F8   (counter)          0028
020E  08
020F  A2
0210  F8
0211  81
0212  B3   813B>R3
0213  F8   (READAH
0214  3B    subroutine)
0215  A3
0216  D3   Set P = R3
0217  8D   RD.0>D
0218  FE
0219  FE
021A  FE   Shift left
021B  FE
021C  F6
021D  F6   Shift right        026C
021E  F6
021F  F6
```

```
0220  B6   D>R6.1
0221  D3   Set P=R3
0222  8D   RD.0>D
0223  FE
0224  FE   Shift left
0225  FE
0226  FE
0227  A6   D>R6.0
0228  F8
0229  02
022A  B7   0240>R7
022B  F8
022C  40
022D  A7
022E  C4
022F  D3   Set P=R3
0230  8D   RD.0>D
0231  E7   Set X=R7
0232  FE
0233  FE
0234  FE   Shift left
0235  FE
0236  C4
0237  C4
0238  C4
0239  F6
023A  F6   Shift right
023B  F6
023C  F6
023D  73   D>M (R(X)); R(X)-1
023E  86   R6.0>D
023F  FC   M(R(P))+D>D,DF
0240  00
0241  A6   D>R.6.0
0242  F8
0243  02   Set P=R3
0244  B3   at
0245  F8   02 50
0246  50
0247  A3   (subroutine to
0248  D3   get Baud rate)
0249  C0
024A  00   GO TO
024B  28   00 28
024C  00
024D  00
024E  00
024F  00
0250  9E   RE.1>D
0251  FD   M(R(P))-D>D,DF
0252  30
0253  3B   If DF = 0
0254  6C   GO TO 026C
0255  F8
0256  02
0257  B7   0280>R7
0258  F8
0259  80
025A  A7
```

```
Ø25B  F8 ┐
Ø25C  00 │  0063
Ø25D  A8 ┘  ⩾ R8
Ø25E  F8 ┐
Ø25F  63 │
Ø26Ø  A8 ┘
Ø261  07    M(R(7))⩾D
Ø262  58    D⩾M(R(8))
Ø263  17    R7 + 1
Ø264  18    R8 +1
Ø265  07    M(R(7))⩾D
Ø266  58    D⩾M(R(8))
Ø267  17    R7 + 1
Ø268  18    R8 + 1
Ø269  07    M(R(7))⩾D
Ø26A  58    D⩾M(R(8))
Ø26B  D5    Set P=R5
Ø26C  F8 ┐
Ø26D  02 │
Ø26E  B7 ┘  0284⩾R7
Ø26F  F8 ┐
Ø27Ø  84 │
Ø271  A7 ┘
Ø272  F8 ┐
Ø273  63 ┘  63⩾R8.0
Ø274  A8 ┘
Ø275  07    M(R(7))⩾D
Ø276  58    D⩾M(R(8))
Ø277  17    R7 + 1
Ø278  18    R8 + 1
Ø279  07    M(R(7))⩾ D
Ø27A  58    D⩾M(R(8))
Ø27B  17    R7 + 1
Ø27C  18    R8 + 1
Ø27D  07    M(R(7))⩾D
Ø27E  58    D⩾M(R(8))
Ø27F  D5    Set P = R5
Ø28Ø  C0    GO TO
Ø281  02    0290
Ø282  90
Ø283  00
Ø284  F8 ┐
Ø285  81 ┘  81⩾R3.1
Ø286  B3 ┘
Ø287  00
Ø288  00
Ø289  00
Ø28A  00
Ø28B  00
Ø28C  00
Ø28D  00
Ø28E  00
Ø28F  00
Ø29Ø  F8 ┐
Ø291  81 │
Ø292  B3 │  Set P=R3
Ø293  F8 │  at 81AO
Ø294  AO │  (subroutine for
Ø295  A3 │  Print ASC11)
Ø296  D3 ┘
Ø297  41    "A"
Ø298  D3    Set P=R3
Ø299  43    "C"
Ø29A  D3    Set P=R3
Ø29B  54    "T"
Ø29C  D3    Set P = R3
Ø29D  3D    "="
Ø29E  F8 ┐
Ø29F  AE ┘  AE⩾R3.0
Ø2AØ  A3 ┘  (Subroutine to print
Ø2A1  D3    Set P=R3      RF.1)
Ø2A2  F8    AO⩾ R3.0
Ø2A3  AO    (Subroutine to print
Ø2A4  A3                  ASC11)
Ø2A5  D3    Set P  = R3
Ø2A6  20    Blank
Ø2A7  D3    Set P=R3
Ø2A8  44    "D"
Ø2A9  D3    Set P=R3
Ø2AA  49    "1"
Ø2AB  D3    Set P=R3
Ø2AC  46    "F"
Ø2AD  D3    Set P=R3
Ø2AE  3D    "="
Ø2AF  F8 ┐  AE⩾R3.0
Ø2BØ  AE ┘  (Subroutine to point
Ø2B1  A3 ┘                RF.1)
Ø2B2  9B    RB.1⩾D
Ø2B3  BF    D⩾RF.1
Ø2B4  D3    Set P=R3
Ø2B5  F8 ┐  AO⩾R3.0
Ø2B6  AO ┘  (subroutine to print
Ø2B7  A3 ┘                ASC11)
Ø2B8  D3    Set P=R3
Ø2B9  20    Blank
Ø2BA  D3    Set P=R3
Ø2BB  53    "S"
Ø2BC  D3    Set P=R3
Ø2BD  54    "T"
Ø2BE  D3    Set P=R3
Ø2BF  3D    "="
Ø2CØ  F8 ┐  AE ⩾ R3.0
Ø2C1  AE ┘  (Subroutine to print
Ø2C2  A3 ┘  RF.1
Ø2C3  06    M(R(6))⩾D
Ø2C4  BF    D⩾RF.1
Ø2C5  D3    Set P=R3
Ø2C6  F8 ┐  AO⩾R3.1
Ø2C7  AO ┘  (Subroutine to print
Ø2C8  A3 ┘  ASC11)
Ø2C9  D3    Set P=R3
Ø2CA  0D    Carriage  return
Ø2CB  D3    Set P=R3
Ø2CC  0A    Line feed
Ø2CD  C0    GO TO
Ø2CE  00    0028
Ø2CF  28
```

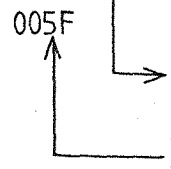
0028

2. Program for Voltage Clamp Apparatus

```
0000  F8 ┐
0001  00 │
0002  B5 │
0003  F8 ├ Set P=R5
0004  07 │ at 0007
0005  A5 │
0006  D5 ┘
0007  F8 ┐
0008  00 ├ 0000⇒RB
0009  AB │
000A  BB ┘
000B  C4
000C  C4
000D  C4
000E  C4
000F  C4
0010  F8 ┐
0011  00 │
0012  B8 ├ 00F5⇒R8
0013  F8 │
0014  F5 │
0015  AB ┘
0016  E8   Set X=R8
0017  61   M(R(X))⇒Bus;R(X)+1
0018  C4
0019  C4
001A  C4
001B  C4
001C  C4
001D  C4
001E  C4
001F  C4
0020  C4 ←┐
0021  3C   IF EF2=1
0022  20   GOTO 0020 ┘
0023  F8 ┐
0024  F0 │
0025  AE │
0026  F8 │
0027  7F ├ 00F0⇒R6
0028  AD ├ 009F⇒ RD
0029  F8 │
002A  00 │
002B  BD │
002C  BE ┘
002D  C4
002E  C4
002F  C4
0030  F8 ┐
0031  00 │
0032  B8 ├ Set X=R8
0033  F8 ├ at 00BE
0034  BE │
0035  A8 │
0036  E8 ┘
0037  65   M(R(X))⇒Bus;R(X)+1
0038  C4
```

```
0039  3E   IF EF3=0
003A  38   GOTO 0038
003B  65   M(R(X))⇒Bus;R(X)+1
003C  F8 ┐
003D  00 │
003E  B3 │
003F  F8 ├ 0081⇒R3
0040  81 │
0041  A3 ┘
0042  Bus  M(R(X))⇒D
0043  53   D⇒R3.0
0044  C4
0045  C4
0046  C4
0047  C4
0048  C4
0049  C4
004A  C4
004B  C4
004C  C4
004D  C4
004E  C4
004F  C4
0050  F8 ┐
0051  FF │
0052  B7 ├ FFFF⇒R7
0053  F8 │
0054  FF │
0055  A7 ┘
0056  C4 ←┐
0057  C4  │
0058  C4  │
0059  C4  │
005A  27   R7-1
005B  97   R7.1⇒D
005C  3A   IF D NOT 0
005D  56   GOTO 0056 ┘
005E  C4
005F  C4
0060  F8 ┐
0061  00 │
0062  BA ├ 00B5⇒RA
0063  F8 │
0064  B5 │
0065  AA ┘
0066  EA   Set X=RA
0067  F0   M(R(X))⇒D
0068  FC   M(R(P))+D⇒DF,D
0069  08
006A  73   D⇒M(R(X));R(X)-1
006B  60   R(X)+1
006C  65   M(R(X))⇒Bus;R(X)+1
006D  C4 ←┐
006E  3E   IF EF3=0
006F  6D   GOTO 006D ┘
0070  2A   RA-1
0071  F0   M(R(X))⇒D
```

```
                                                    005F
                                                     ^
ØØ72  FF   D-M(R(P))⇒DF,D                           |
ØØ73  08                                           |
ØØ74  73   D⇒M(R(X));R(X)-1                         |
ØØ75  60   R(X)+1                                   |
ØØ76  65   M(R(X))⇒Bus;R(X)+1                       |
ØØ77  6E   Bus M(R(X))⇒D                            |
ØØ78  C4                                            |
ØØ79  C4                                            |
ØØ7A  C4                                            |
ØØ7B  C4                                            |
ØØ7C  C4                                            |
ØØ7D  C4                                            |
ØØ7E  C4                                            |
ØØ7F  C4                                            |
ØØ8Ø  FC   M(R(P))-D⇒D,DF                           |
ØØ81  00                                            |
ØØ82  33   IF DF=1                                  |
ØØ83  90   GOTO 0090                                |
ØØ84  8E   RE.0⇒ D                                  |
ØØ85  FC   M(R(P))+D⇒ D,DF                          |
ØØ86  10                                            |
ØØ87  AE   D⇒ RE.0                                  |
ØØ88  C6   LONG SKIP IF D NOT 0                     |
ØØ89  1D   RD+1                                     |
ØØ8A  C4                                            |
ØØ8B  C4                                    005F    |
ØØ8C  C4                                     ^      |
ØØ8D  C4                                     |      |
ØØ8E  30   goto                              |      |
ØØ8F  99   0099                              |      |
ØØ9Ø  8E   RE.0⇒ D                           |      |
ØØ91  C6   LONG SKIP IF D NOT 0              |      |
ØØ92  2D   RD-1                              |      |
ØØ93  C4                                     |      |
ØØ94  C4                                     |      |
ØØ95  8E   RE.0⇒D                            |      |
ØØ96  FF D-M(R(P))⇒D,DF                      |      |
ØØ97  10                                     |      |
ØØ98  AE   D⇒RE.0                            |      |
ØØ99  F8 ┐                                   |      |
ØØ9A  B5 ├ B5⇒RA.0                           |      |
ØØ9B  AA ┘                                   |      |
ØØ9C  EA   Set X=RA                          |      |
ØØ9D  8E   RE.0⇒D                            |      |
ØØ9E  73   D⇒M(R(X));R(X)+1                  |      |
ØØ9F  8D   RD.0⇒D                            |      |
ØØAØ  73   D⇒M(R(X));(R(X)+1                 |      |
ØØA1  60   R(X)+1                            |      |
ØØA2  61   M(R(X))⇒Bus;R(X)+1                |      |
ØØA3  65   M(R(X))⇒Bus;R(X)+1                |      |
ØØA4  C4                                     |      |
ØØA5  C4                                     |      |
ØØA6  F8 ┐                                   |      |
ØØA7  00 |                                   |      |
ØØA8  B7 ├ 0010⇒ R7                          |      |
ØØA9  F8 |                                   |      |
ØØAA  10 |                                   |      |
ØØAB  A7 ┘                                   |      |
                                             |      |
ØØAC  C4                                     |      |
ØØAD  27   R7-1                              |      |
ØØAE  97   R7.1⇒D                            |      |
ØØAF  3A   IF D NOT 0                        |      |
ØØBØ  AC   GOTO 00AC                         |      |
ØØB1  30   GOTO                              |      |
ØØB2  5F   005F                              |      |
ØØB3  00                                     |      |
ØØB4  00                                     |      |
ØØB5  00   Port Code                         |      |
ØØB6  00                                     |      |
ØØB7  00                                     |      |
ØØB8  00                                     |      |
ØØB9  00                                     |      |
ØØBA  00                                     |      |
ØØBB  00                                     |      |
ØØBC  00                                     |      |
ØØBD  00                                     |      |
ØØBE  00                                     |      |
ØØBF  00                                     |      |
ØØCØ  00                                     |      |
ØØC1  00                                     |      |
ØØC2  00                                     |      |
ØØC3  00                                     |      |
ØØC4  00                                     |      |
ØØC5  35   IF IF2=1                          |      |
ØØC6  CD   GOTO 00CD                         |      |
ØØC7  30   GOTO                              |      |
ØØC8  5F   005F                              |      |
ØØC9  00                                     |      |
ØØCA  00                                     |      |
ØØCB  00                                     |      |
ØØCC  00                                     |      |
ØØCD  9B   RB.1⇒D                            |      |
ØØCE  3A   IF D NOT 0                        |      |
ØØCF  E3   GOTO 00E3                         |      |
ØØDØ  8B   RB.0⇒ D                           |      |
ØØD1  3A   IF D NOT 0                        |      |
ØØD2  E3   GOTO 00E3                         |      |
ØØD3  F8 ┐                                   |      |
ØØD4  00 |                                   |      |
ØØD5  B3 ├ 0081⇒R3                           |      |
ØØD6  F8 |                                   |      |
ØØD7  81 |                                   |      |
ØØD8  A3 ┘                                   |      |
ØØD9  03   M(R(3))⇒D                         |      |
ØØDA  FC   or FF M(R(P))±                    |      |
                 D⇒D,DF                      |      |
ØØDB  01                                     |      |
ØØDC  53   D⇒M(R(3))                         |      |
ØØDD  F8 ┐                                   |      |
ØØDE  02 |                                   |      |
ØØDF  BB ├ 0290⇒RB                           |      |
ØØEØ  F8 |                                   |      |
ØØE1  90 |                                   |      |
ØØE2  AB ┘                                   |      |
ØØE3  2B   RB-1                              |      |
```



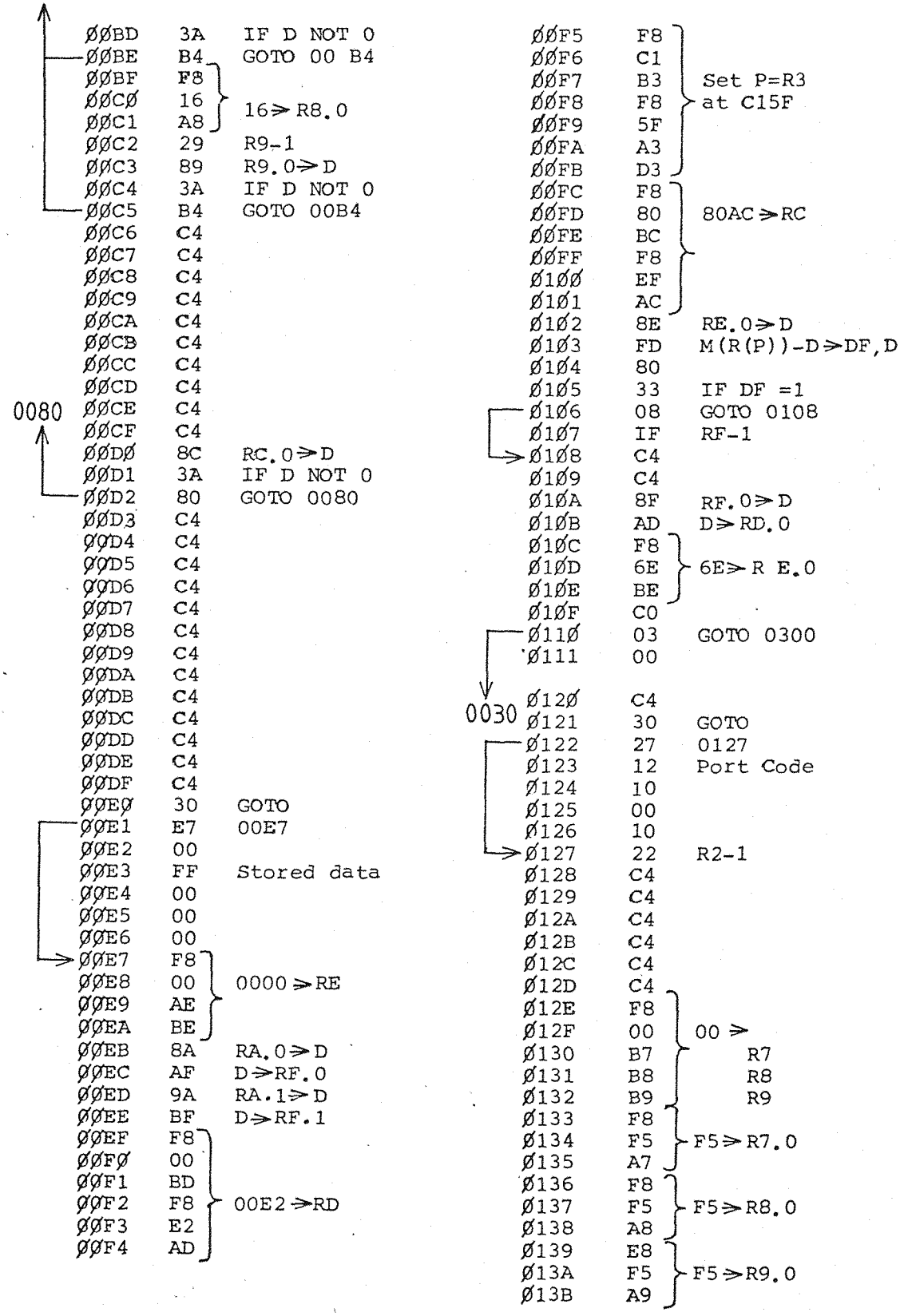

```
ØØE4   F8 ┐
ØØE5   00 │
ØØE6   B9 ├ 00⇒R9.1
ØØE7   BF │     RF.1
ØØE8   B3 ┘     R3.1
ØØE9   F8 ┐
ØØEA   FC ├ FC⇒R9.0
ØØEB   A9 ┘
ØØEC   F8 ┐
ØØED   FD ├ FD⇒RF.D
ØØEE   AF ┘
ØØEF   F8 ┐
ØØFØ   DA ├ DA⇒R3.0
ØØF1   A3 ┘
ØØF2   3C   IF EF1=0
ØØF3   F8   GOTO 00F8
ØØF4   09   M(R(9))⇒D
ØØF5   53   D  M(R(3))
ØØF6   30   GOTO
ØØF7   5F   005F
ØØF8   09   M(R(9))⇒D
ØØF9   53   D  M(R(3))
ØØFA   30   GOTO
ØØFB   5F   00 5F
ØØFC   FF   STORED DATA
ØØFD   FC
ØØFE
ØØFF
Ø1ØØ
Ø1Ø1
Ø1Ø2
Ø1Ø3
Ø1Ø4
Ø1Ø5
Ø1Ø6
Ø1Ø7
Ø1Ø8
Ø1Ø9
Ø1ØA
Ø1ØB
Ø1ØC
Ø1ØD
Ø1ØE
Ø1ØF
Ø11Ø
Ø111
Ø112
Ø113
Ø114
Ø115
Ø116
Ø117
Ø118
Ø119
Ø11A
Ø11B
Ø11C
Ø11D
Ø11E
Ø11F
```

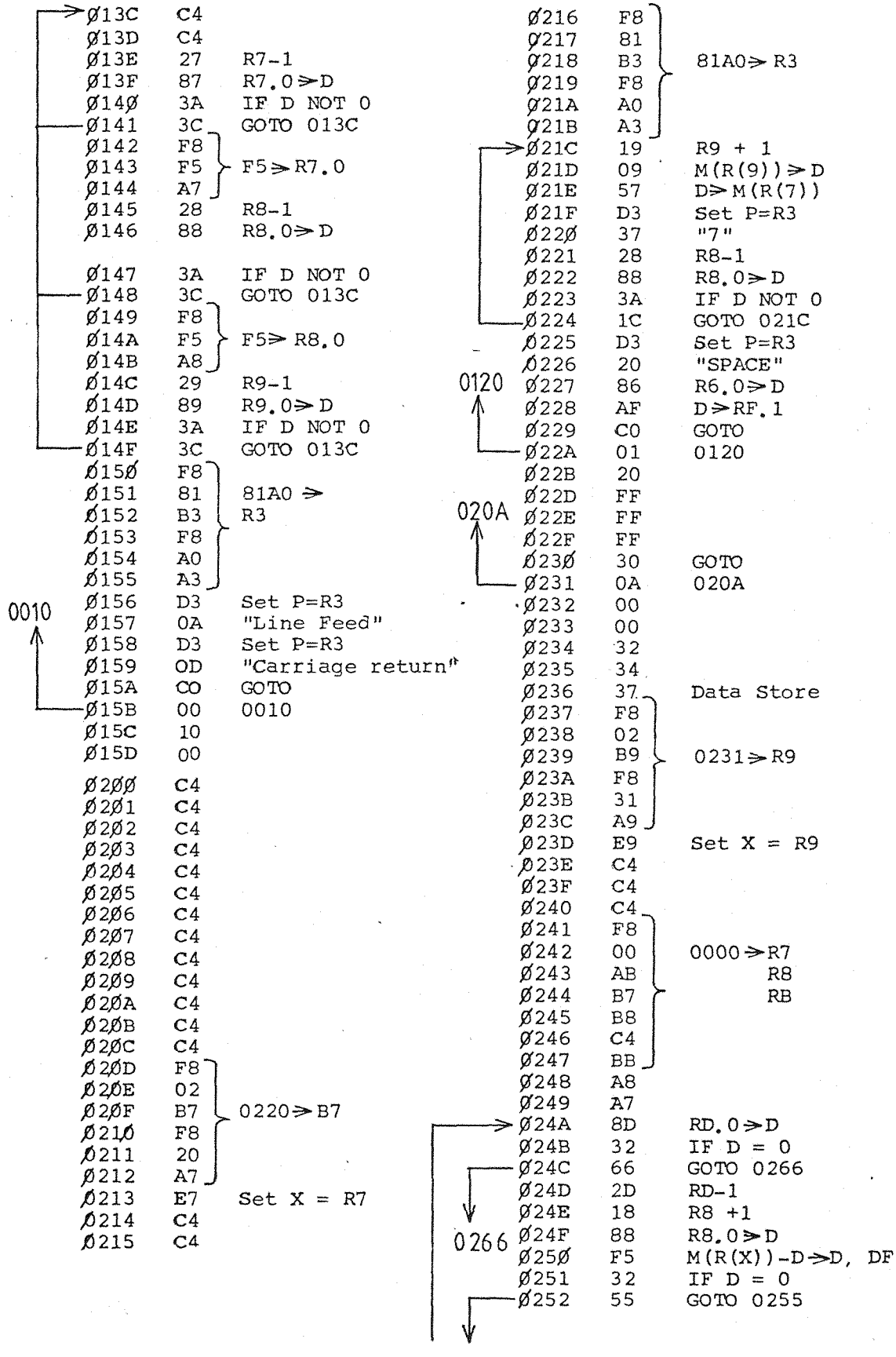
```
Ø121
Ø122
Ø123
Ø124
Ø125
Ø126
Ø127
Ø128
Ø129
Ø12A
Ø12B
Ø12C
Ø12D
Ø12E
Ø12F
Ø13Ø
Ø131
Ø132
Ø133
Ø134
Ø135
Ø136
Ø137
Ø138
Ø139
Ø13A
Ø13B
Ø13C
Ø13D
Ø13E
Ø13F
Ø14Ø
Ø141
Ø142
Ø143
Ø144
Ø145
Ø146
Ø147
Ø148
Ø149
Ø14A
Ø14B
Ø14C
Ø14D
Ø14E
Ø14F
Ø15Ø
Ø151
Ø152
Ø153
Ø154
Ø155
Ø156
Ø157
Ø158
Ø159
Ø15A
Ø15B
Ø15C
Ø15D
Ø15E
Ø15F
```

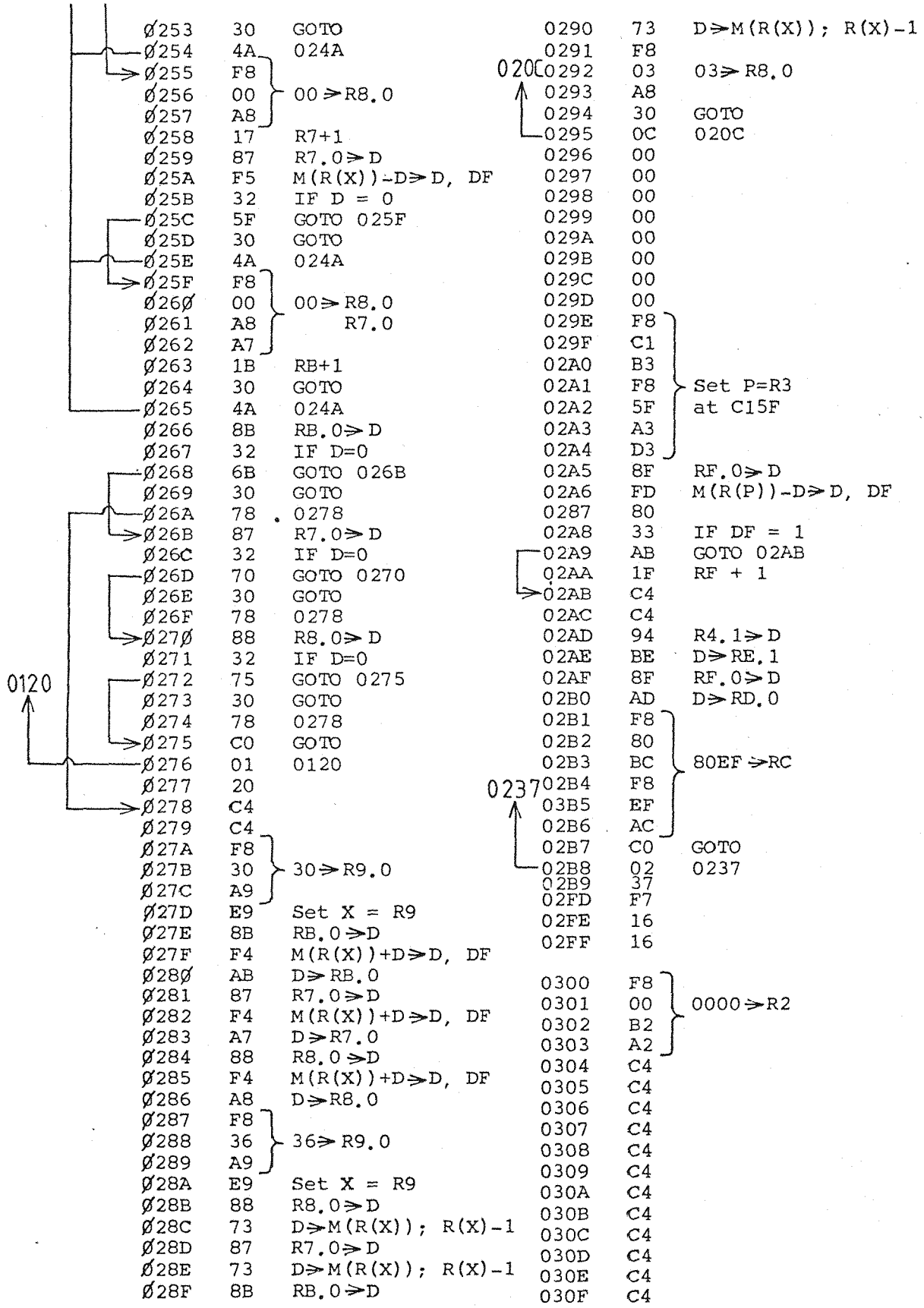
3. Microprocessor program for data processing urine flow rate data

```
0000  F8
0001  00
0002  B5
0003  F8   Set P=R5
0004  07   at 0007
0005  A5
0006  D5
0007  F8
0008  00   0000 ⇒R2
0009  A2
000A  B2
000B  C4
000C  C4
000D  C4
000E  C4
000F  C4
0010  F8
0011  00   00FF ⇒RC
0012  BC
0013  F8
0014  FF
0015  AC
0016  F8
0017  00
0018  A7
0019  A8
001A  A9   00⇒ R7
001B  AA        R8
001C  B7        R9
001D  B8        RA
001E  B9
001F  BA
0020  30   GOTO
0021  70   0070

0070  30   GOTO
0071  80   0080
0072  C4
0073  C4
0074  C4
0075  C4
0076  08   Port code
0077  00
0078  88   Stored data
0079  00
007A  C4
007B  C4
007C  C4
007D  C4
007E  C4
007F  C4
0080  F8
0081  00
0082  B8   0076
0083  F8    ⇒ R8
0084  76
0085  A8
```

```
0086  E8   Set X=R8
0087  65   M(R(X))⇒ Bus; R(X)+1
0088  C4
0089  3E   IF EF 3 = 0
008A  88   GO TO 88(wait
           for ADC)
008B  65   M(R(X))⇒ Bus; R(X+1)
008C  6E   Bus⇒M(R(X))⇒D
008D  BF   D⇒RF. 1
008E  C4
008F  C4
0090  2C   RC-1
0091  F8
0092  00   009B
0093  B9    ⇒ R9
0094  F8
0095  9B
0096  A9
0097  8A   RA. 0⇒ D
0098  59   D⇒M(R(9))
0099  9F   RF. 1⇒D
009A  FC   M(R(5))+D⇒DF, D
009B  58   Data
009C  AA   D⇒RA. 0
009D  3B   IF D=0
009E  A3   GOTO 00A3
009F  9A   RA. 1⇒D
00A0  FC   M(R(5))+D⇒DF, D
00A1  01   Data
00A2  BA   D⇒RA. 1
00A3  C4
00A4  C4
00A5  C4
00A6  F8
00A7  00   00 ⇒
00A8  B7   R7 1
00A9  B8   R8. 1
00AA  B9   R9. 1
00AB  F8
00AC  16   16⇒R9. 0
00AD  A9
00AE  F8
00AF  16   16⇒R8. 0
00B0  A8
00B1  F8
00B2  16   16⇒R7. 0
00B3  A7
00B4  27   R7 - 1
00B5  87   R7. 0⇒D
00B6  3A   IF D NOT 0
00B7  B4   GOTO 00B4
00B8  F8
00B9  16   16⇒R7. 0
00BA  A7
00BB  28   R8-1
00BC  88   R8. 0⇒D
```


ØØBD 3A IF D NOT 0
ØØBE B4 GOTO 00 B4
ØØBF F8
ØØCØ 16 16⇒R8.0
ØØC1 A8
ØØC2 29 R9-1
ØØC3 89 R9.0⇒D
ØØC4 3A IF D NOT 0
ØØC5 B4 GOTO 00B4
ØØC6 C4
ØØC7 C4
ØØC8 C4
ØØC9 C4
ØØCA C4
ØØCB C4
ØØCC C4
ØØCD C4
0080 ØØCE C4
ØØCF C4
ØØDØ 8C RC.0⇒D
ØØD1 3A IF D NOT 0
ØØD2 80 GOTO 0080
ØØD3 C4
ØØD4 C4
ØØD5 C4
ØØD6 C4
ØØD7 C4
ØØD8 C4
ØØD9 C4
ØØDA C4
ØØDB C4
ØØDC C4
ØØDD C4
ØØDE C4
ØØDF C4
ØØEØ 30 GOTO
ØØE1 E7 00E7
ØØE2 00
ØØE3 FF Stored data
ØØE4 00
ØØE5 00
ØØE6 00
ØØE7 F8
ØØE8 00 0000⇒RE
ØØE9 AE
ØØEA BE
ØØEB 8A RA.0⇒D
ØØEC AF D⇒RF.0
ØØED 9A RA.1⇒D
ØØEE BF D⇒RF.1
ØØEF F8
ØØFØ 00
ØØF1 BD
ØØF2 F8 00E2⇒RD
ØØF3 E2
ØØF4 AD
ØØF5 F8
ØØF6 C1
ØØF7 B3 Set P=R3
ØØF8 F8 at C15F
ØØF9 5F
ØØFA A3
ØØFB D3
ØØFC F8
ØØFD 80 80AC⇒RC
ØØFE BC
ØØFF F8
Ø1ØØ EF
Ø1Ø1 AC
Ø1Ø2 8E RE.0⇒D
Ø1Ø3 FD M(R(P))-D⇒DF,D
Ø1Ø4 80
Ø1Ø5 33 IF DF =1
Ø1Ø6 08 GOTO 0108
Ø1Ø7 IF RF-1
Ø1Ø8 C4
Ø1Ø9 C4
Ø1ØA 8F RF.0⇒D
Ø1ØB AD D⇒RD.0
Ø1ØC F8
Ø1ØD 6E 6E⇒R E.0
Ø1ØE BE
Ø1ØF C0
Ø11Ø 03 GOTO 0300
Ø111 00
0030 Ø12Ø C4
Ø121 30 GOTO
Ø122 27 0127
Ø123 12 Port Code
Ø124 10
Ø125 00
Ø126 10
Ø127 22 R2-1
Ø128 C4
Ø129 C4
Ø12A C4
Ø12B C4
Ø12C C4
Ø12D C4
Ø12E F8
Ø12F 00 00⇒
Ø130 B7 R7
Ø131 B8 R8
Ø132 B9 R9
Ø133 F8
Ø134 F5 F5⇒R7.0
Ø135 A7
Ø136 F8
Ø137 F5 F5⇒R8.0
Ø138 A8
Ø139 E8
Ø13A F5 F5⇒R9.0
Ø13B A9


Ø13C C4
Ø13D C4
Ø13E 27 R7-1
Ø13F 87 R7.0➔D
Ø14Ø 3A IF D NOT 0
Ø141 3C GOTO 013C
Ø142 F8
Ø143 F5 F5➔R7.0
Ø144 A7
Ø145 28 R8-1
Ø146 88 R8.0➔D
Ø147 3A IF D NOT 0
Ø148 3C GOTO 013C
Ø149 F8
Ø14A F5 F5➔R8.0
Ø14B A8
Ø14C 29 R9-1
Ø14D 89 R9.0➔D
Ø14E 3A IF D NOT 0
Ø14F 3C GOTO 013C
Ø15Ø F8
Ø151 81 81A0 ➔
Ø152 B3 R3
Ø153 F8
Ø154 A0
Ø155 A3
0010
Ø156 D3 Set P=R3
Ø157 0A "Line Feed"
Ø158 D3 Set P=R3
Ø159 OD "Carriage return"
Ø15A CO GOTO
Ø15B 00 0010
Ø15C 10
Ø15D 00
Ø2ØØ C4
Ø2Ø1 C4
Ø2Ø2 C4
Ø2Ø3 C4
Ø2Ø4 C4
Ø2Ø5 C4
Ø2Ø6 C4
Ø2Ø7 C4
Ø2Ø8 C4
Ø2Ø9 C4
Ø2ØA C4
Ø2ØB C4
Ø2ØC C4
Ø2ØD F8
Ø2ØE 02
Ø2ØF B7 0220➔B7
Ø21Ø F8
Ø211 20
Ø212 A7
Ø213 E7 Set X = R7
Ø214 C4
Ø215 C4
Ø216 F8
Ø217 81
Ø218 B3 81A0➔R3
Ø219 F8
Ø21A A0
Ø21B A3
Ø21C 19 R9 + 1
Ø21D 09 M(R(9))➔D
Ø21E 57 D➔M(R(7))
Ø21F D3 Set P=R3
Ø22Ø 37 "7"
Ø221 28 R8-1
Ø222 88 R8.0➔D
Ø223 3A IF D NOT 0
Ø224 1C GOTO 021C
Ø225 D3 Set P=R3
Ø226 20 "SPACE"
0120
Ø227 86 R6.0➔D
Ø228 AF D➔RF.1
Ø229 C0 GOTO
Ø22A 01 0120
Ø22B 20
Ø22D FF
020A
Ø22E FF
Ø22F FF
Ø23Ø 30 GOTO
Ø231 0A 020A
Ø232 00
Ø233 00
Ø234 32
Ø235 34
Ø236 37 Data Store
Ø237 F8
Ø238 02
Ø239 B9 0231➔R9
Ø23A F8
Ø23B 31
Ø23C A9
Ø23D E9 Set X = R9
Ø23E C4
Ø23F C4
Ø240 C4
Ø241 F8
Ø242 00 0000➔R7
Ø243 AB R8
Ø244 B7 RB
Ø245 B8
Ø246 C4
Ø247 BB
Ø248 A8
Ø249 A7
Ø24A 8D RD.0➔D
Ø24B 32 IF D = 0
Ø24C 66 GOTO 0266
Ø24D 2D RD-1
Ø24E 18 R8 +1
0266
Ø24F 88 R8.0➔D
Ø25Ø F5 M(R(X))-D➔D, DF
Ø251 32 IF D = 0
Ø252 55 GOTO 0255


Ø253 30 GOTO
Ø254 4A 024A
Ø255 F8
Ø256 00 00⇒R8.0
Ø257 A8
Ø258 17 R7+1
Ø259 87 R7.0⇒D
Ø25A F5 M(R(X))-D⇒D, DF
Ø25B 32 IF D = 0
Ø25C 5F GOTO 025F
Ø25D 30 GOTO
Ø25E 4A 024A
Ø25F F8
Ø26Ø 00 00⇒R8.0
Ø261 A8 R7.0
Ø262 A7
Ø263 1B RB+1
Ø264 30 GOTO
Ø265 4A 024A
Ø266 8B RB.0⇒D
Ø267 32 IF D=0
Ø268 6B GOTO 026B
Ø269 30 GOTO
Ø26A 78 0278
Ø26B 87 R7.0⇒D
Ø26C 32 IF D=0
Ø26D 70 GOTO 0270
Ø26E 30 GOTO
Ø26F 78 0278
Ø27Ø 88 R8.0⇒D
Ø271 32 IF D=0
0120
Ø272 75 GOTO 0275
Ø273 30 GOTO
Ø274 78 0278
Ø275 C0 GOTO
Ø276 01 0120
Ø277 20
Ø278 C4
Ø279 C4
Ø27A F8
Ø27B 30 30⇒R9.0
Ø27C A9
Ø27D E9 Set X = R9
Ø27E 8B RB.0⇒D
Ø27F F4 M(R(X))+D⇒D, DF
Ø28Ø AB D⇒RB.0
Ø281 87 R7.0⇒D
Ø282 F4 M(R(X))+D⇒D, DF
Ø283 A7 D⇒R7.0
Ø284 88 R8.0⇒D
Ø285 F4 M(R(X))+D⇒D, DF
Ø286 A8 D⇒R8.0
Ø287 F8
Ø288 36 36⇒R9.0
Ø289 A9
Ø28A E9 Set X = R9
Ø28B 88 R8.0⇒D
Ø28C 73 D⇒M(R(X)); R(X)-1
Ø28D 87 R7.0⇒D
Ø28E 73 D⇒M(R(X)); R(X)-1
Ø28F 8B RB.0⇒D
0290 73 D⇒M(R(X)); R(X)-1
0291 F8
020C 0292 03 03⇒R8.0
0293 A8
0294 30 GOTO
0295 0C 020C
0296 00
0297 00
0298 00
0299 00
029A 00
029B 00
029C 00
029D 00
029E F8
029F C1
02A0 B3
02A1 F8 Set P=R3
02A2 5F at C15F
02A3 A3
02A4 D3
02A5 8F RF.0⇒D
02A6 FD M(R(P))-D⇒D, DF
0287 80
02A8 33 IF DF = 1
02A9 AB GOTO 02AB
02AA 1F RF + 1
02AB C4
02AC C4
02AD 94 R4.1⇒D
02AE BE D⇒RE.1
02AF 8F RF.0⇒D
02B0 AD D⇒RD.0
02B1 F8
02B2 80
02B3 BC 80EF⇒RC
0237 02B4 F8
03B5 EF
02B6 AC
02B7 C0 GOTO
02B8 02 0237
02B9 37
02FD F7
02FE 16
02FF 16
0300 F8
0301 00 0000⇒R2
0302 B2
0303 A2
0304 C4
0305 C4
0306 C4
0307 C4
0308 C4
0309 C4
030A C4
030B C4
030C C4
030D C4
030E C4
030F C4

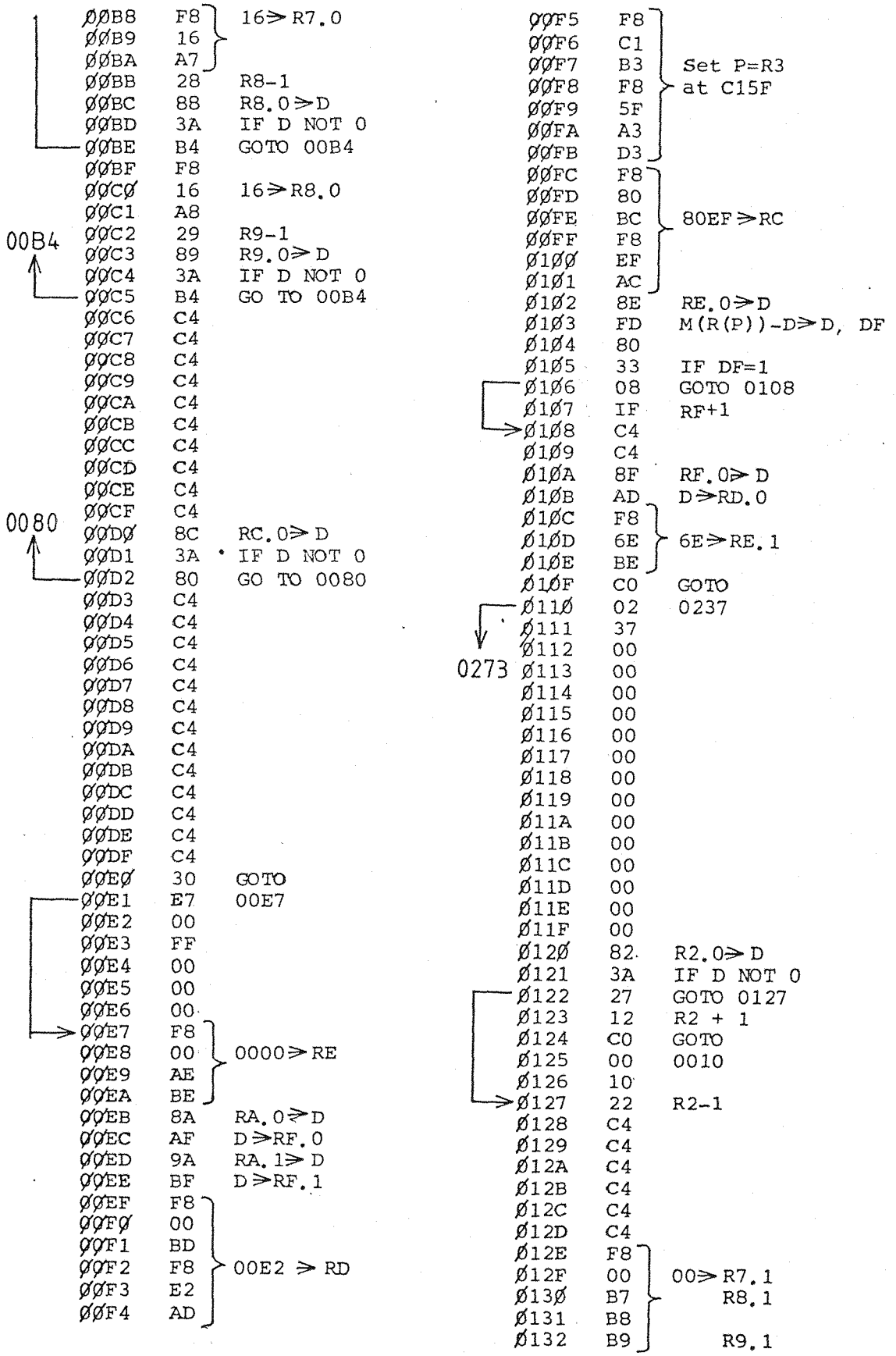
```
Ø31Ø  F8 ┐
Ø311  02 │ 02FE
Ø312  B9 │ ⇒
Ø313  F8 │ R9
Ø314  FE │
Ø315  A9 ┘
Ø316  E9    Set X=R9
Ø317  72    M(R(X))⇒D; R(X)+1
Ø318  73    D⇒M(R(X)); R(X)-1
Ø319  29    R9-1
Ø31A  72    M(R(X))⇒D; R(X)+1
Ø31B  73    D⇒M(R(X)); R(X)-1
Ø31C  8F    RF0⇒D
Ø31D  73    D⇒M(R(X)); R(X)-1
Ø31E  60    R(X)+1
Ø31F  60    R(X)+1
Ø32Ø  F8 ┐
Ø321  03 │
Ø322  B8 │ 032B⇒R8
Ø323  F8 │
Ø324  2B │
Ø325  A8 ┘
Ø326  F0    M(R(X))⇒D
Ø327  58    D⇒M(R(8))
Ø328  29    R9-1
Ø329  F0    M(R(X))⇒D
Ø32A  FD    M(R(P))-D⇒D, DF
Ø32B  16    R6+1
Ø32C  BF    RF.1⇒D
Ø32D  3B    IF DF = 0
Ø32E  A0    GOTO 03A0
Ø32F  C4
Ø33Ø  F8 ┐
Ø331  37 │ 37⇒R8.0
03A0 Ø332  A8 ┘
Ø333  72    M(R(X))⇒D; R(X)+1
Ø334  58    D⇒M(R(8))
Ø335  F0    M(R(X))⇒D
Ø336  FC    M(R(P))+D⇒D, DF
Ø337  F7    D-M(R(X))⇒DF,D
Ø338  A2    D⇒R2.0
Ø339  3B    IF DF=0
Ø33A  3E    GOTO 033E
Ø33B  F8 ┐
Ø33C  01 │ 01⇒R2.1
Ø33D  B2 ┘
Ø33E  30    GOTO
Ø33F  50    0350
Ø34Ø  C4
Ø341  C4
Ø342  C4
0350 Ø343  C4
Ø344  C4
Ø345  C4
Ø346  C4
Ø347  C4
Ø348  C4
Ø349  C4
Ø34A  C4
Ø34B  C4
Ø34C  C4

Ø34D  00
Ø34E  01
Ø34F  0D    Stored data
Ø35Ø  F8 ┐
Ø351  4F │
Ø352  AD │
Ø353  F8 │ 4F03⇒RD
Ø354  03 │
Ø355  BD ┘
Ø356  82    R2.0⇒D
Ø357  5D    D⇒M(R(D))
Ø358  2D    RD - 1
Ø359  92    R2.1⇒D
Ø35A  5D    D⇒M(R(D))
Ø35B  C4
Ø35C  C4
Ø35D  C4
Ø35E  C4
Ø35F  C4
Ø36Ø  F8 ┐
Ø361  00 │ 00⇒RE.1
Ø362  B6 │     RF.0
Ø363  AF ┘
Ø364  9F    RF.1⇒D
Ø365  AE    D⇒RE.0
Ø366  F8 ┐
Ø367  00 │ 00⇒RF.1
Ø368  BF ┘
Ø369  F8 ┐
Ø36A  C1 │ C1⇒RC.1
Ø36B  BC ┘
Ø36C  F8 ┐
Ø36D  5F │ 5F⇒R3.0
Ø36E  A3 ┘
Ø36F  D3    Set P=R3
Ø37Ø  2D    RD-1
Ø371  9F    RF.1⇒RF.1
Ø372  5D    D⇒M(R(D))
Ø373  ED    Set X=RD
Ø374  61    M(R(X))⇒Bus R(X)+1
Ø375  C4
Ø376  C4

Ø377  C4
Ø378  C4
Ø379  C4
Ø37A  C4
Ø37B  C4
Ø37C  C4
Ø37D  C4
Ø37E  C4
Ø37F  C4
Ø38Ø  F8 ┐
Ø381  00 │ 00⇒RD 1
Ø382  BD │    RF 1
Ø383  BF ┘
```

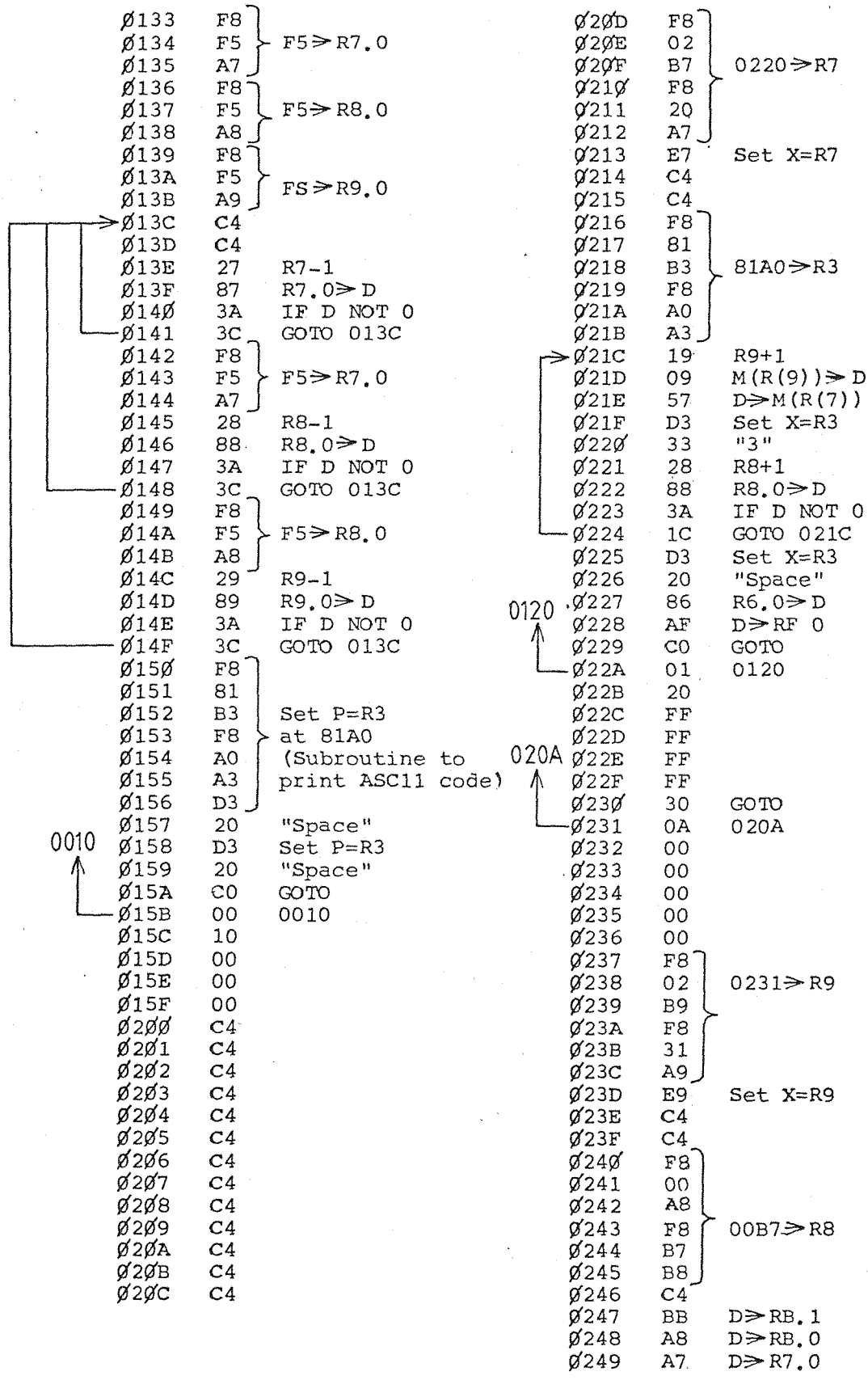
```
        Ø384    29      R9-1
        Ø385    09      M(R(9))  D
        Ø386    AD      D⇒RD.0
        Ø387    AF      D⇒RF.0
        Ø388    F8   ┐
        Ø389    80   │
        Ø38A    BC   │
        Ø38B    F8   ├  80 EF⇒RC
        Ø38C    EF   │
        Ø38D    AC   ┘
        Ø38E    C4
0237    Ø38F    C4
        Ø39Ø    F8   ┐
        Ø391    CE   ├  CE⇒RE.1
        Ø392    BF   ┘
        Ø393    C0      GOTO
        Ø394    02      0237
        Ø395    37
```

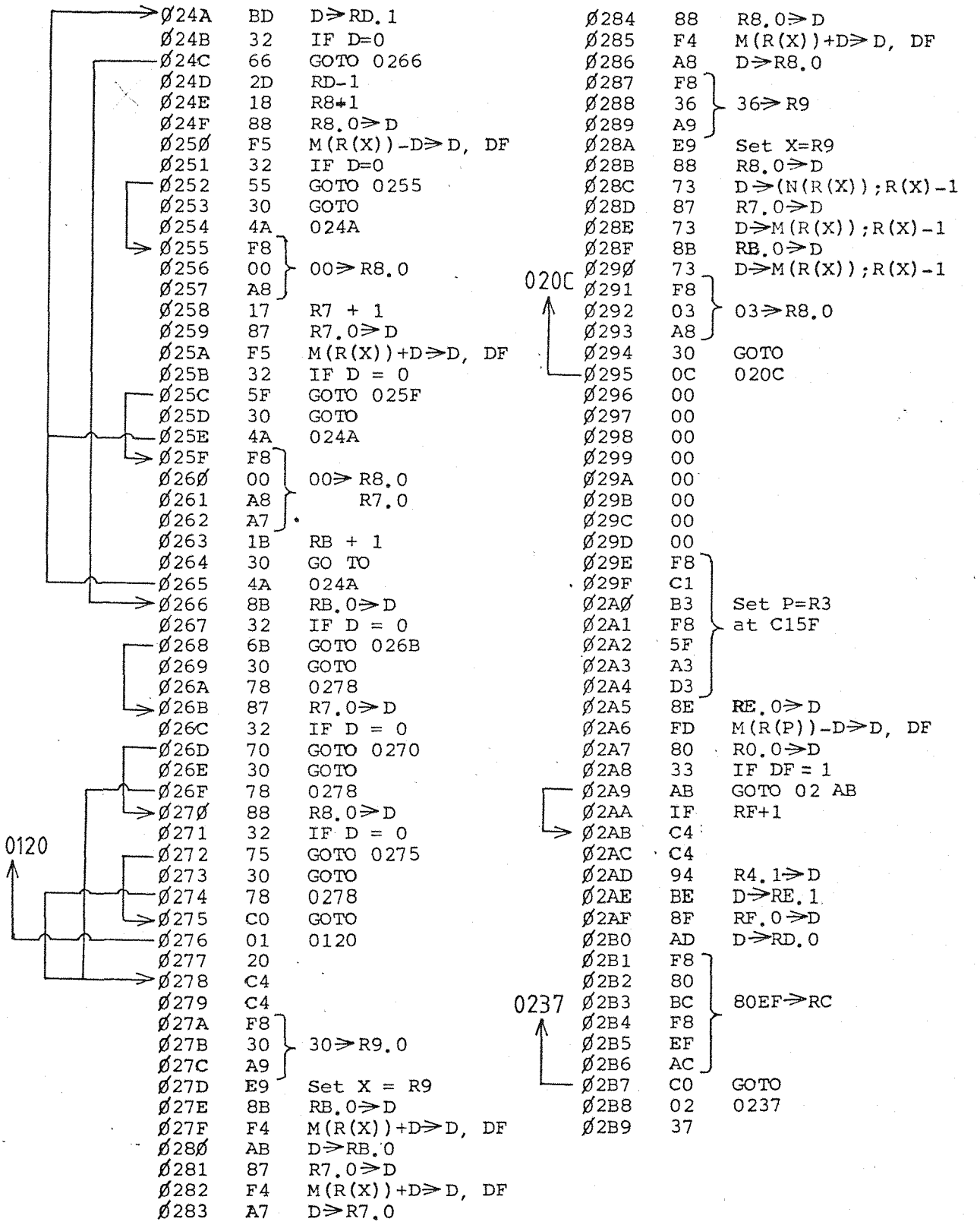
4. Microprocessor Program to aid Spectrophotometry

```
0000  F8
0001  00
0002  B5   Set P=R5
0003  F8   at 0007
0004  07
0005  A5
0006  D5
0007  F8
0008  00   0000≥R2
0009  A2
000A  B2
000B  C4
000D  C4
000E  C4
000F  C4
0010  F8
0011  00
0012  BC   00FF≥RC
0013  F8
0014  FF
0015  AC
0016  F8
0017  00
0018  A7
0019  A8
001A  A9   0000≥R7, 8, 9, A
001B  AA
001C  B7
001D  B8
001E  B9
001F  BA
0020  C4
0021  C4

0022  C4
0024  C4   through to 0070
0025  C4


0070  30   GO TO 0080
0071  80
0072  C4
0073  C4
0074  C4
0075  C4
0076  F8   Port code
0077  00
0078  21
0079  00
007A  C4
007B  C4
007C  C4
007D  C4
007E  C4
007F  C4
```

```
0080  F8
0081  00
0082  B8
0083  F8   Set X = R8
0084  76   at 0076
0085  A8
0086  E8
0087  65   M(R(X))≥Bus;R(X)+1
0088  C4
0089  3E   IF EF3 = 0
008A  88   GOTO 0088 (wait
                    for DAC)
008B  65   M(R(X))≥Bus;R(X)+1
008C  6E   Bus≥M(R(X))  D
008D  BF   D≥RF.1
008E  C4
008F  C4
0090  2C   RC-1
0091  F8
0092  00
0093  B9   009B≥R9
0094  F8
0095  9B
0096  A9
0097  8A   RA.0≥D
0098  59   D≥M(R(9))
0099  9F   RF.1≥D
009A  FC   M(R(P))+D≥D, DF
009B  46
009C  AA   D≥RA.0
009D  3B   IF DF=0
009E  A3   GOTO 00A3
009F  9A   RA.1≥D
00A0  FC   M(R(P))+D≥D, DF
00A1  01
00A2  BA   D≥RA.1
00A3  C4
00A4  C4
00A5  C4
00A6  F8
00A7  00
00A8  B7   00≥R7.1
00A9  B8        R8.1
00AA  B9        R9.1
00AB  F8
00AC  16   16≥R9.0
00AD  A9
00AE  F8
00AF  16   16≥R8.0
00B0  A8
00B1  F8
00B2  16   16≥R8.0
00B3  A7
00B4  27   R7-1
00B5  87   R7.0≥D
00B6  3A   IF D NOT 0
00B7  B4   GOTO 00B4
```


ØØB8 F8 16≥R7.0
ØØB9 16
ØØBA A7
ØØBB 28 R8-1
ØØBC 88 R8.0≥D
ØØBD 3A IF D NOT 0
ØØBE B4 GOTO 00B4
ØØBF F8
ØØCØ 16 16≥R8.0
ØØC1 A8
00B4
ØØC2 29 R9-1
ØØC3 89 R9.0≥D
ØØC4 3A IF D NOT 0
ØØC5 B4 GO TO 00B4
ØØC6 C4
ØØC7 C4
ØØC8 C4
ØØC9 C4
ØØCA C4
ØØCB C4
ØØCC C4
ØØCD C4
ØØCE C4
ØØCF C4
0080
ØØDØ 8C RC.0≥D
ØØD1 3A IF D NOT 0
ØØD2 80 GO TO 0080
ØØD3 C4
ØØD4 C4
ØØD5 C4
ØØD6 C4
ØØD7 C4
ØØD8 C4
ØØD9 C4
ØØDA C4
ØØDB C4
ØØDC C4
ØØDD C4
ØØDE C4
ØØDF C4
ØØEØ 30 GOTO
ØØE1 E7 00E7
ØØE2 00
ØØE3 FF
ØØE4 00
ØØE5 00
ØØE6 00
ØØE7 F8
ØØE8 00 0000≥RE
ØØE9 AE
ØØEA BE
ØØEB 8A RA.0≥D
ØØEC AF D≥RF.0
ØØED 9A RA.1≥D
ØØEE BF D≥RF.1
ØØEF F8
ØØFØ 00
ØØF1 BD
ØØF2 F8 00E2 ≥ RD
ØØF3 E2
ØØF4 AD
ØØF5 F8
ØØF6 C1
ØØF7 B3 Set P=R3
ØØF8 F8 at C15F
ØØF9 5F
ØØFA A3
ØØFB D3
ØØFC F8
ØØFD 80
ØØFE BC 80EF≥RC
ØØFF F8
Ø1ØØ EF
Ø1Ø1 AC
Ø1Ø2 8E RE.0≥D
Ø1Ø3 FD M(R(P))-D≥D, DF
Ø1Ø4 80
Ø1Ø5 33 IF DF=1
Ø1Ø6 08 GOTO 0108
Ø1Ø7 IF RF+1
Ø1Ø8 C4
Ø1Ø9 C4
Ø1ØA 8F RF.0≥D
Ø1ØB AD D≥RD.0
Ø1ØC F8
Ø1ØD 6E 6E≥RE.1
Ø1ØE BE
Ø1ØF C0 GOTO
Ø11Ø 02 0237
Ø111 37
Ø112 00
0273 Ø113 00
Ø114 00
Ø115 00
Ø116 00
Ø117 00
Ø118 00
Ø119 00
Ø11A 00
Ø11B 00
Ø11C 00
Ø11D 00
Ø11E 00
Ø11F 00
Ø12Ø 82 R2.0≥D
Ø121 3A IF D NOT 0
Ø122 27 GOTO 0127
Ø123 12 R2 + 1
Ø124 C0 GOTO
Ø125 00 0010
Ø126 10
Ø127 22 R2-1
Ø128 C4
Ø129 C4
Ø12A C4
Ø12B C4
Ø12C C4
Ø12D C4
Ø12E F8
Ø12F 00 00≥R7.1
Ø13Ø B7 R8.1
Ø131 B8
Ø132 B9 R9.1


Ø133 F8
Ø134 F5 F5⩾R7.0
Ø135 A7
Ø136 F8
Ø137 F5 F5⩾R8.0
Ø138 A8
Ø139 F8
Ø13A F5 FS⩾R9.0
Ø13B A9
Ø13C C4
Ø13D C4
Ø13E 27 R7-1
Ø13F 87 R7.0⩾D
Ø14Ø 3A IF D NOT 0
Ø141 3C GOTO 013C
Ø142 F8
Ø143 F5 F5⩾R7.0
Ø144 A7
Ø145 28 R8-1
Ø146 88 R8.0⩾D
Ø147 3A IF D NOT 0
Ø148 3C GOTO 013C
Ø149 F8
Ø14A F5 F5⩾R8.0
Ø14B A8
Ø14C 29 R9-1
Ø14D 89 R9.0⩾D
Ø14E 3A IF D NOT 0
Ø14F 3C GOTO 013C
Ø15Ø F8
Ø151 81
Ø152 B3 Set P=R3
Ø153 F8 at 81A0
Ø154 A0 (Subroutine to
Ø155 A3 print ASC11 code)
Ø156 D3
Ø157 20 "Space"
0010
Ø158 D3 Set P=R3
Ø159 20 "Space"
Ø15A C0 GOTO
Ø15B 00 0010
Ø15C 10
Ø15D 00
Ø15E 00
Ø15F 00
Ø2ØØ C4
Ø2Ø1 C4
Ø2Ø2 C4
Ø2Ø3 C4
Ø2Ø4 C4
Ø2Ø5 C4
Ø2Ø6 C4
Ø2Ø7 C4
Ø2Ø8 C4
Ø2Ø9 C4
Ø2ØA C4
Ø2ØB C4
Ø2ØC C4
Ø2ØD F8
Ø2ØE 02
Ø2ØF B7 0220⩾R7
Ø21Ø F8
Ø211 20
Ø212 A7
Ø213 E7 Set X=R7
Ø214 C4
Ø215 C4
Ø216 F8
Ø217 81
Ø218 B3 81A0⩾R3
Ø219 F8
Ø21A A0
Ø21B A3
Ø21C 19 R9+1
Ø21D 09 M(R(9))⩾D
Ø21E 57 D⩾M(R(7))
Ø21F D3 Set X=R3
Ø22Ø 33 "3"
Ø221 28 R8+1
Ø222 88 R8.0⩾D
Ø223 3A IF D NOT 0
Ø224 1C GOTO 021C
Ø225 D3 Set X=R3
Ø226 20 "Space"
0120
Ø227 86 R6.0⩾D
Ø228 AF D⩾RF 0
Ø229 C0 GOTO
Ø22A 01 0120
Ø22B 20
Ø22C FF
Ø22D FF
020A
Ø22E FF
Ø22F FF
Ø23Ø 30 GOTO
Ø231 0A 020A
Ø232 00
Ø233 00
Ø234 00
Ø235 00
Ø236 00
Ø237 F8
Ø238 02 0231⩾R9
Ø239 B9
Ø23A F8
Ø23B 31
Ø23C A9
Ø23D E9 Set X=R9
Ø23E C4
Ø23F C4
Ø24Ø F8
Ø241 00
Ø242 A8
Ø243 F8 00B7⩾R8
Ø244 B7
Ø245 B8
Ø246 C4
Ø247 BB D⩾RB.1
Ø248 A8 D⩾RB.0
Ø249 A7 D⩾R7.0


Ø24A BD D⇒RD.1
Ø24B 32 IF D=0
Ø24C 66 GOTO 0266
Ø24D 2D RD-1
Ø24E 18 R8+1
Ø24F 88 R8.0⇒D
Ø25Ø F5 M(R(X))-D⇒D, DF
Ø251 32 IF D=0
Ø252 55 GOTO 0255
Ø253 30 GOTO
Ø254 4A 024A
Ø255 F8
Ø256 00 00⇒R8.0
Ø257 A8
Ø258 17 R7 + 1
Ø259 87 R7.0⇒D
Ø25A F5 M(R(X))+D⇒D, DF
Ø25B 32 IF D = 0
Ø25C 5F GOTO 025F
Ø25D 30 GOTO
Ø25E 4A 024A
Ø25F F8
Ø26Ø 00 00⇒R8.0
Ø261 A8 R7.0
Ø262 A7
Ø263 1B RB + 1
Ø264 30 GO TO
Ø265 4A 024A
Ø266 8B RB.0⇒D
Ø267 32 IF D = 0
Ø268 6B GOTO 026B
Ø269 30 GOTO
Ø26A 78 0278
Ø26B 87 R7.0⇒D
Ø26C 32 IF D = 0
Ø26D 70 GOTO 0270
Ø26E 30 GOTO
Ø26F 78 0278
Ø27Ø 88 R8.0⇒D
Ø271 32 IF D = 0
Ø272 75 GOTO 0275
Ø273 30 GOTO
Ø274 78 0278
Ø275 C0 GOTO
Ø276 01 0120
Ø277 20
Ø278 C4
Ø279 C4
Ø27A F8
Ø27B 30 30⇒R9.0
Ø27C A9
Ø27D E9 Set X = R9
Ø27E 8B RB.0⇒D
Ø27F F4 M(R(X))+D⇒D, DF
Ø28Ø AB D⇒RB.0
Ø281 87 R7.0⇒D
Ø282 F4 M(R(X))+D⇒D, DF
Ø283 A7 D⇒R7.0
0120
Ø284 88 R8.0⇒D
Ø285 F4 M(R(X))+D⇒D, DF
Ø286 A8 D⇒R8.0
Ø287 F8
Ø288 36 36⇒R9
Ø289 A9
Ø28A E9 Set X=R9
Ø28B 88 R8.0⇒D
Ø28C 73 D⇒(N(R(X));R(X)-1
Ø28D 87 R7.0⇒D
Ø28E 73 D⇒M(R(X));R(X)-1
Ø28F 8B RB.0⇒D
Ø29Ø 73 D⇒M(R(X));R(X)-1
020C
Ø291 F8
Ø292 03 03⇒R8.0
Ø293 A8
Ø294 30 GOTO
Ø295 0C 020C
Ø296 00
Ø297 00
Ø298 00
Ø299 00
Ø29A 00
Ø29B 00
Ø29C 00
Ø29D 00
Ø29E F8
Ø29F C1
Ø2AØ B3 Set P=R3
Ø2A1 F8 at C15F
Ø2A2 5F
Ø2A3 A3
Ø2A4 D3
Ø2A5 8E RE.0⇒D
Ø2A6 FD M(R(P))-D⇒D, DF
Ø2A7 80 R0.0⇒D
Ø2A8 33 IF DF = 1
Ø2A9 AB GOTO 02 AB
Ø2AA IF RF+1
Ø2AB C4
Ø2AC C4
Ø2AD 94 R4.1⇒D
Ø2AE BE D⇒RE.1
Ø2AF 8F RF.0⇒D
Ø2B0 AD D⇒RD.0
Ø2B1 F8
Ø2B2 80
0237 Ø2B3 BC 80EF⇒RC
Ø2B4 F8
Ø2B5 EF
Ø2B6 AC
Ø2B7 C0 GOTO
Ø2B8 02 0237
Ø2B9 37
0237