J. Exp. Biol. (1963), 40, 613–623
With 2 text-figures
Printed in Great Britain

ANALYSIS OF THE HAEMOLYMPH OF *ARION ATER* L. (GASTROPODA: PULMONATA)

By D. K. ROACH
Department of Zoology, University College of South Wales and Monmouthshire, Cardiff

(*Received* 29 *April* 1963)

INTRODUCTION

Pulmonate smooth muscle structures are well suited for physiological investigation, since they are normally bathed directly by haemolymph and many of them are rhythmically contractile. Among the muscular organs investigated have been the heart (Cardot, 1921; Hogben, 1925; Arvanitaki & Cardot, 1931; Jullien *et al.* 1955; Krijgsman & Divaris, 1955; Jaeger, 1961), the buccal retractor (Bozler, 1930; Ramsay, 1940; Graham & Heathcote, 1952), the flagellum (Bachrach & Cardot, 1921), penis retractor (Jaeger, 1962) and alimentary tract (Wells, 1928; Graham & Heathcote, 1952). The buccal mass, crop and stomach are particularly suitable for kymograph studies and preparatory to an investigation of the motor activity of the alimentary tract of *Arion ater* it was realized that a blood analysis was necessary to form a basis for the preparation of a physiological saline for this animal. A number of pulmonate salines have been prepared (Krijgsman & Divaris, 1955; Lockwood, 1961), mostly for various *Helix* species, though also for *Lymnaea* (Carriker, 1946) and *Strophocheilos* (Jaeger, 1961). Very few salines, however, have been based on blood analyses. Salines for *Helix* species, based on limited analyses, have been produced by Bernard & Bonnet (1930), Arvanitaki & Cardot (1931) and Jullien *et al.* (1955), and further analyses of pulmonate blood have been made by Bernard (1931), Holtz & von Brand (1940), Saxena (1957) and Michon & Alaphilippe (1958). It is known that there is a considerable variation in water content from day to day in terrestrial pulmonates (Arvanitaki & Cardot, 1931; Kamada, 1933; Howes & Wells, 1934; Martin, Harrison, Huston & Stewart, 1958) and it might be supposed that an exact knowledge of the blood composition is not necessary to prepare a suitable saline. However, interchanging of salines between pulmonate species has not proved successful, and in some instances (Bozler, 1930; Ramsay, 1940) pooled haemolymph has had to be employed. Furthermore, it has been pointed out repeatedly (Cardot, 1921; Wells, 1928; Bernard & Bonnet, 1930; Ramsay, 1940; Hughes & Kerkut, 1956; Kerkut & Taylor, 1956) that the normal functioning of the muscular and nervous tissues of molluscs is considerably affected by variation both of the concentration of individual ions and of the overall osmotic concentration, making a knowledge of blood composition very desirable. Experimental methods are now available for a full analysis of very small volumes of blood and have been used here to find the haemolymph concentrations of sodium, potassium, calcium, magnesium, chloride, sulphate, phosphate, bicarbonate, protein and total electrolytes, as well as freezing-point depression (Δ) and pH, for the slug *Arion ater*.

MATERIALS AND METHODS

The animals, large garden slugs of the species *Arion ater* L. (subspecies *rufus*), were collected locally from gardens and maintained in wide, shallow terraria fitted with wire-mesh lids, with a plentiful supply of food and water. Cabbage and lettuce were provided, as well as supplementary artificial diets (BOCM 'Chick mash' and Oxoid 'Diet 41'). Haemolymph was collected by inserting the needle of a hypodermic syringe through the pneumostome into the mantle cavity, then through the postero-lateral wall of the cavity into the visceral haemocoele. Between 0·2 and 0·5 ml. of the opalescent, blue body fluid could then be withdrawn, uncontaminated by body-wall mucus, and without apparent harm to the animal. After 3 or 4 days a further sample of fluid could be taken from the same slug.

After withdrawal, the haemolymph sample was placed in a small Pyrex glass tube ($1\frac{1}{2} \times \frac{1}{2}$ in.) and centrifuged at 1500 *g* for 20 min. to remove debris and cells. The clear supernatant was then pipetted off, using a waxed pipette, into a clean tube which was finally sealed with a Polythene cap secured by a rubber ring. When the fluid samples were not used immediately they were stored by being frozen solid and kept at $-5°$ C.

Haemolymph samples were not pooled for the analyses. Determinations were made separately on samples from many slugs and, in the cases of chloride and bi-carbonate measurements, were repeated on individual samples. 'Analar' grade chemicals were used throughout the investigation.

ANALYSIS OF CATIONS

Sodium, potassium and calcium were determined by flame photometry. A preliminary analysis was made to estimate the approximate values for the three ions, and then two standard solutions were made up. The first contained 0·05 mM/l. NaCl, and was used for sodium estimations. The other contained 0·75 mM/l. NaCl, 0·05 mM/l. KCl and 0·05 mM/l. $CaCl_2$, and was the standard solution for potassium and calcium estimations, the added sodium chloride eliminating interference errors. 0·1 ml. of the haemolymph sample was diluted to 10 ml. (dilution × 100) and was used, with the appropriate filters, in an EEL flame photometer for analysis of potassium and calcium. 1 ml. of the diluted sample was then diluted further to 25 ml. (total dilution × 2500) and employed for sodium analysis. The two separate dilutions were made to match the highest sensitivity range of the photometer for the different ions.

Since flame photometry gives the total ion concentration, and some at least of the sodium, potassium and particularly calcium might exist in a bound form, the analyses were repeated on blood samples freed of protein by precipitation, either with 10% trichloroacetic acid or with a zinc-alkali mixture (Somogyi, 1945). In either case, the precipitate was centrifuged down and 0·2 ml. of the supernatant was pipetted off and diluted as above.

Magnesium was estimated colorimetrically, by the formation and measurement of the alkaline Titan yellow complex (method of Orange & Rhein, as modified by Croghan, 1958). Trichloroacetic acid-precipitated haemolymph was used, 0·1 ml. being added to 1·5 ml. of very dilute Titan yellow solution and 1·5 ml. of N-NaOH added. After mixing, the extinction was read at 520 mμ in a Unicam SP 600 spectrophoto-

meter. Magnesium concentrations were read off a calibration curve constructed from standard solutions of 1–10 mM/l. $MgCl_2$, the relationship being linear from 3 to 9 mM/l.

Results

An attempt has been made in each case to evaluate the variation to be expected from the experimental methods. These are expressed in Table 1 as average standard deviations and as average coefficients of variation, related to the mean haemolymph concentration of the ion in question. In the case of magnesium the average of the standard deviations (S.D.) of the values for the standard solutions from the expected (and corrected) straight line has been used. For the flame photometry results three

Table 1. *Variation resulting from analytical technique*

Ion measured	Average S.D. due to error of method (mM)	Average coefficient of variation relative to mean haemolymph concentration (%)
Sodium	±0·96	1·6
Potassium	±0·06	2·2
Calcium	±0·17	6·0
Magnesium	±0·27	4·5

Table 2. *Results of cation analysis*

Ion measured	No. of samples	Concentration (mM)	S.D.	Coefficient of variation (%)
Sodium				
Untreated haemolymph	27	61·49	±5·72	9·3
Trichloroacetic precipitated	8	65·06	±8·17	12·5
Zinc-alkali precipitated	8	61·92	±8·79	14·0
Potassium				
Untreated haemolymph	27	2·68	±0·80	29·9
Trichloroacetic precipitated	11	2·55	±0·78	30·5
Zinc-alkali precipitated	8	2·69	±0·98	36·5
Calcium				
Untreated haemolymph	28	2·81	±0·48	17·1
Trichloroacetic precipitated	12	2·97	±0·66	22·2
Zinc-alkali precipitated	8	2·30	±0·53	23·0
Magnesium				
Trichloroacetic precipitated	20	5·68	±0·87	15·3

readings had been taken for each diluted sample, alternating with measurement of the standard solution, so that it was possible to use the variations occurring between repeat measurements on individual samples as a measure of this 'reproducibility' value. Thus, by comparing the S.D. and coefficients of variation columns in Table 2 with those in Table 1, it is possible to estimate the extent to which the variation found in the results is due to error of the method.

Statistical analysis of the results shows that there is no significant difference ($P > 0·5$) between the untreated and protein-free bloods as far as sodium and

potassium are concerned—i.e. all the potassium and sodium present is in simple solution. For the calcium, however, it is quite clear that about 20% exists in bound form ($P < 0.02$) and the amount actually in solution is only 2·30 mM/l.

ANALYSIS OF ANIONS

The method described by Wigglesworth (1938) was used as the basis for the chloride analysis, i.e. addition of silver nitrate and back-titration with sodium thiocyanate with iron alum as indicator. 0·05 ml. of protein-free haemolymph (zinc-alkali precipitated) was pipetted on to a small Polythene dish, an excess of 50% nitric acid was added,

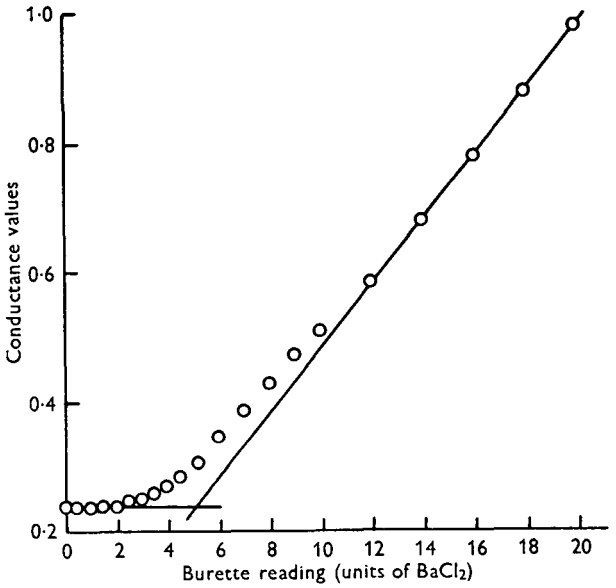

Fig. 1. Conductimetric titration of 0·1 ml. of 2 mM/l. Na_2SO_4 with 10 mM/l. $BaCl_2$.

followed by 0·2 ml. of 0·5% silver nitrate and a drop of saturated ferric alum. The mixture was then titrated with sodium thiocyanate until a permanent red flush appeared, the fluid drop being continually agitated by a jet of air. For titration a microburette was constructed after the Shaw pattern (1954) from thick-walled capillary tubing of 0·8 mm. internal diameter, so that it had a capacity of 0·1 ml. over a 20 cm. graduated length. As the bore of the burette was larger than that of Shaw's design, it was necessary to fit a delivery-speed control, consisting of a screw-driven, spring-loaded copper bellows. The burette units were arbitrary, since it was calibrated in terms of standard NaCl solution at regular intervals during the determinations.

For sulphate, a conductimetric method was employed, modified slightly from that described by Paulson (1953). 1 ml. of de-ionized water, 0·1 ml. of untreated haemolymph and 0·1 ml. of barium sulphate seeding agent (washed suspension of $BaSO_4$ in 0·2% acetic acid) were pipetted into an inverted 2 ml. capacity 'dip' conductivity cell (the side apertures being sealed by a wide rubber band) and thoroughly mixed with a suitably shaped glass stirrer. The initial conductance was measured with a Mullard E 7566 conductivity bridge, the balance being detected by an electron-beam

indicator ('magic eye'). Known volumes of 10 mM/l. $BaCl_2$ standard solution were then run in from the microburette described above and the conductance was re-measured after each addition, again after thorough stirring. The end-point was determined graphically (Fig. 1) by plotting conductance values against units of added titrant and extrapolating the two straight lines of initial and subsequent conductance values to their intersection. The microburette was calibrated by repeating the titration on standard 2 mM/l. Na_2SO_4 and K_2SO_4 solutions and the system was also tested by estimating the sulphate content of a mixture after successive additions of known volumes of standard sulphate solutions (sulphate recovery test), when full recovery was demonstrated.

Phosphate was estimated colorimetrically, as the molybdenum blue complex. Stannous chloride proved inconsistent as a reducing agent, but a method modified from Courgill & Pardee (1957) and Croghan (1958) worked consistently at the very low concentrations of phosphate encountered. 12·5 ml. of a filtered stock solution of reducing agent (0·5 g. of 1-amino-2-naphthol-4-sulphonic acid in 244 ml. of 15% sodium bisulphite and 6 ml. of 20% sodium sulphite) and 25 ml. of stock molybdic acid solution (5 g. ammonium molybdate in 222 ml. water and 28 ml. sulphuric acid) were mixed and diluted to 250 ml. immediately before use. 5 ml. of this mixture were pipetted into each of a series of tubes and 0·2 ml. of trichloroacetic acid-precipitated haemolymph was added to each tube. 10 min. after mixing, the extinction was read at 775 mμ and the phosphate concentration was read off a calibration curve constructed from standard 0·2–1·0 mM/l. Na_2HPO_4 solutions.

For the purposes of devising a physiological saline it was assumed that the total alkali reserve of the blood was made up of bicarbonate and phosphate ions, and, since the concentration of the latter was very low, the titratable alkalinity was taken as a measure of the bicarbonate concentration. 0·05 ml. of untreated (fresh) haemolymph was pipetted on to a waxed, white Perspex square and 0·05 N-HCl was titrated against it, using the microburette. For convenience and consistency of results the acid titrant was made up to contain 10 ml. of B.D.H. '4·5' indicator per 100 ml. The square was gently rotated by hand to mix the solutions, and the end-point was clearly given by the sharp colour change from blue, through grey to the first orange flush. The system was calibrated against 25 mM/l. $NaHCO_3$ solution. Stored, well-sealed frozen samples gave results very similar to those obtained with fresh haemolymph. The phosphate concentration (as m-equiv./l.) was subtracted from the alkalinity value to give the 'bicarbonate' concentration.

Results

As for the cation analytical methods, a measure of the reproducibility of the method has been calculated. In the case of phosphate analysis, average standard deviations of the readings for standard solutions from the corrected calibration curve were used, while variations between repeat determinations of the standard solutions were employed for chloride, sulphate and bicarbonate (see Tables 3 and 4).

FURTHER ANALYSES

Protein was estimated by the method of Folin (1922), modified by Danielson (1953). 0·05 ml. samples were diluted to 1 ml. and 5·5 ml. of mixed reagents added. The

extinction was read at 750 mμ and the concentration (expressed as mg./ml.) was read off a calibration curve constructed from 0·08–0·10 mg./ml. crystallized egg-albumin standard solutions.

Table 3. *Variation resulting from techniques of anion analysis*

Ion measured	Average S.D. due to error of method (mM)	Average coefficient of variation relative to mean blood concentration (%)
Chloride	±0·26	0·5
Sulphate	±0·06	2·9
Phosphate	±0·048	30·5
'Bicarbonate'	±0·24	0·97

Table 4. *Results of anion analysis*

Ion measured	No. of samples	No. of determinations	Concentration (mM)	S.D.	Coefficient of variation (%)
Chloride	20	62	52·45	±1·52	2·9
Sulphate	20	20	2·21	±0·24	10·8
Phosphate	20	20	0·157	±0·055	35·0
'Bicarbonate'	16	36	24·81	±2·28	9·2

Total electrolytes were estimated by a conductimetric method. 0·1 ml. of haemolymph was diluted with 10 ml. of de-ionized water in a boiling tube, held in a constant-temperature water bath, and its conductance was measured by inserting the 'dip' cell in the usual way. From the conductance value the total electrolytes, expressed as NaCl, were read off a calibration curve constructed from standard 0·02–0·11 M/l. NaCl solutions.

Δ was measured by the improved micro-cryoscopic method of Ramsay & Brown (1955), using both fresh and stored haemolymph samples. The results were checked against standard NaCl solutions, and also expressed in terms of NaCl concentrations.

The pH of the haemolymph was measured with a micro-electrode assembly connected to a Pye pH meter. It was possible to measure the pH of as little as 0·2 ml. of haemolymph by using a very small cup, into which the glass electrode just fitted. The reference electrode was lowered to make contact with the surface of the liquid in the cup. This arrangement was consistently accurate, provided frequent temperature checks and adjustments were made.

Results

Again, experimental error has been assessed (Table 5), from the calibration curve in the cases of protein analysis and conductance measurements, and from the average standard deviations of repeat determinations in the case of the Δ measurements.

The results of the anion and cation analyses can now be summarized, in terms of m-equiv./l. (Fig. 2 and Table 7). It can be seen that the anion and cation totals balance quite well, and that the total concentrations of anions and cations, expressed in m-equiv./l., are very near to the total electrolyte concentration (expressed as the

sum of the Na^+ and Cl^- concentrations in m-equiv./l.) as measured by the conductance of the haemolymph. These two facts lend support to the validity of the analytical results.

Table 5. *Variation resulting from techniques for additional analyses*

Determination	Average S.D. due to error of method	Average coefficient of variation relative to mean blood value (%)
Conductance	±1·33 mM (NaCl)	2·1
Δ	±1·38 mM (NaCl)	1·3
Protein analysis	±0·31 mg./ml.	4·4

Table 6. *Results of additional analyses*

Analysis	No. of samples	No. of determinations	Concentration	S.D.	Coefficient of variation (%)
Total electrolytes (as NaCl)	29	29	77·02 mM	±8·19	10·6
Protein (as albumin)	20	20	10·55 mg./ml.	±3·02	28·6
pH	26	26	8·83	±0·30	3·3
Δ (as NaCl)	27	83	107·6 mM	±23·6	22·0

$\Delta = 0{\cdot}3711° \pm 0{\cdot}0836°$ C.

A PHYSIOLOGICAL SALINE FOR *ARION*

From the measurements made on haemolymph as described above two salines have been devised, using the rules recommended by Lockwood (1961). They have of necessity been made with low phosphate and bicarbonate concentrations, since increasing these levels causes precipitation of the calcium at the very alkaline pH. The salines consisted of the following salts, which were anhydrous unless otherwise stated:

	Saline *A* (g./l.)	Saline *B* (g./l.)
NaCl	2·52	2·52
KCl	0·26	0·26
$CaCl_2.6H_2O$	0·66	0·66
$MgSO_4.7H_2O$	1·00	1·00
Na_2HPO_4	0·03	0·03
$NaHCO_3$	0·76	0·34
NaOH	0·064	0·068
Na_2SO_4	0·53	0·88
Glucose	11·50	11·50
Glycine		0·30

Saline *B* has the advantage of slightly better buffering capacity. For ease of preparation, stock solutions can be prepared:

Stock 1. 10 × NaCl, KCl, $CaCl_2$ and $MgSO_4$.

Stock 2. 10 × Na_2SO_4, $NaHCO_3$, Na_2HPO_4 and NaOH (+glycine in *B*).

Stock 3. 10 × glucose.

These stock solutions are stored separately in the refrigerator and mixed and diluted immediately before use.

Both salines have been tested and are capable of maintaining the motor activity of the gut and the heart for up to 30 hours and ciliary activity for over 48 hr. at about 5° C.

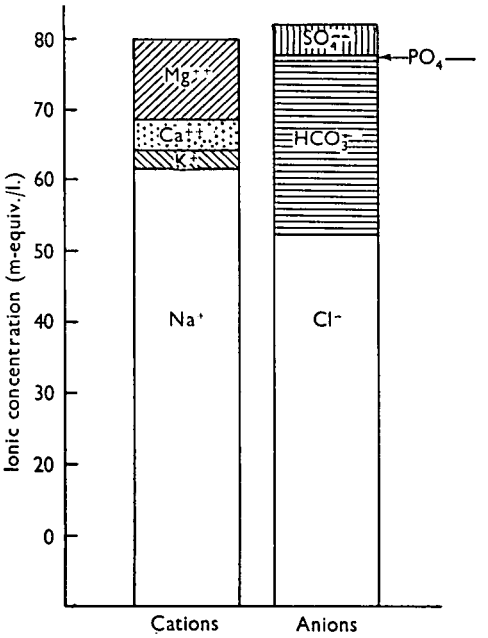

Fig. 2. Electrolyte balance in the haemolymph of *Arion ater*.

Table 7. *Summary of ionic concentrations of* Arion *haemolymph, expressed in m-equiv./l.*

Cations	
Na^+	61·49
K^+	2·68
Ca^{++}	4·60
Mg^{++}	11·36
Total	80·13
Anions	
Cl^-	52·45
SO_4^{--}	4·42
PO_4^{---}	0·47
$[HCO_3^-]$	24·81
Total	82·15
Total ions	162·28
Total ions (as measured by conductance)	154·04

DISCUSSION

Comparison of the figures for haemolymph concentrations with the estimates of analytical error shows that in nearly all cases the variations of the blood values are considerably larger than the variations due to the procedures adopted. Some of the methods admittedly introduce appreciable errors, compared with those developed by Robertson & Webb (1939), but the present methods gain over the gravimetric

procedures in speed and convenience of use, as well as only requiring 0·1 ml. of haemolymph instead of 1 ml. The phosphate figures are least reliable, but here the concentrations encountered are extremely low (some 5 parts of phosphorus per million).

Even when account is taken of the errors introduced by the analytical methods used, it can be seen that there is considerable variation in the haemolymph of *Arion ater*. This is particularly noticeable in the case of the Δ, which shows a coefficient of variation of 22%; the actual values ranged from 0·18° to 0·43° C. *Agriolimax* haemolymph has been shown to vary from 0·2° to 0·8° C. (calculated from Hughes & Kerkut, 1956), while Duval (1930) found variation in *Helix* from 0·37° to 0·43° C. in hibernating animals and 0·30° to 0·40° C. in active ones, the two ranges overlapping considerably. Kamada (1933), for the same animal, found 0·41° C. for 'hibernating' blood and 0·30° C. for 'active' blood. These figures for haemolymph variation agree well with observations on weight fluctuations by Howes & Wells (1934) and Dainton (1954), and also with the figures of Martin *et al.* (1958) for variation of the haemolymph volume of *Arion* from about 25 to 47% of the wet weight of the animal, while Arvanitaki & Cardot (1931) for *Helix pomatia* showed that the haemolymph volume was 10·7% of the total weight in hibernating animals and 16·2% in active animals. In view of this extreme variability it is somewhat surprising to find that the activity of the nervous system is considerably affected by osmotic pressure changes (Hughes & Kerkut, 1956; Kerkut & Taylor, 1956; Duncan, 1961) and so is the activity of the heart (Cardot, 1921).

The results seem to indicate that the greater part of this osmotic fluctuation is due to changes in concentration of the non-electrolytes in the blood, rather than of the electrolytes. The smaller variation of the latter (shown by the conductance measurements, supported by individual ion analyses) indicates that they are gained or lost with the water, whereas the non-electrolytes (as demonstrated by Δ and protein analysis) tend to remain in the haemolymph. This is of interest as many investigators have reported the disturbances of function in a number of nervous and muscular tissues when electrolyte ratios and concentrations have been altered (Bachrach & Cardot, 1921; Hogben, 1925; Wells, 1928; Bernard & Bonnet, 1930; Arvanitaki & Cardot, 1931; Ramsay, 1940; Krijgsman & Divaris, 1955; Duncan, 1961; Jaeger, 1961). In view of these findings, the relatively large variations of Ca^{++} and K^{+} levels in the haemolymph are of some significance. Dainton (1954) and Howes & Wells (1934) have suggested that the activity of slugs, or at least the degree of responsiveness to stimuli, is regulated by the degree of hydration of the animal. As the gain and loss of water apparently results in a change of concentration of Ca^{++} and K^{+}, and as both these ions have profound effects on nervous and muscular irritability, this is a possible mechanism causing the change of activity of the animal, in addition to the direct osmotic effect.

Because of the wide variations encountered in the haemolymph of *Arion*, it is probable that cycles of activity occur in the individual organs and tissues of the body, as well as in the behaviour of the animal as a whole. The physiological salines, then, are to be considered as maintaining, at best, an 'average' activity of the isolated organs, rather than a 'normal' one. This is inevitably an artificial condition, which is unable to illustrate the cycles of activity evident in the intact animal.

The constancy of the pH is all the more remarkable in contrast with the variation

of most other aspects of the haemolymph, though Arvanitaki & Blanc (1934) have described the profound effects of pH changes on the heart of *Helix*. It is, too, a remarkably alkaline pH, far more so than for almost any other animal studied in this respect. Most animals have a blood pH just on the alkaline side of neutrality, but values of 7·8–8·08 have been recorded for *Anodonta* and 8·4 for *Helix* (Lockwood, 1961), although 7·6–7·9 is generally quoted for the haemolymph of *Helix pomatia*; Michon & Alaphilippe (1958) give pH 7·8 for *Achatina*, and Saxena (1957) gives 7·86 for *Pila globosa*. The alkaline haemolymph of *Arion* has been the cause of difficulty in making up the salines, as the calcium tends to precipitate very readily at this pH, and it has meant that the phosphate concentration has had to be kept low, with consequent reduction of buffering power.

The most constant of the haemolymph constituents, then, are chloride, sodium and alkali reserve. This is at least suggestive of a mechanism for regulating these factors in the haemolymph when water is gained or lost by the animal, lending support to the concept of ionic regulation expressed by Holtz & von Brand (1940), who found evidence of this in the haemolymph of *Helix pomatia*. Saxena (1957) has recorded that chloride was the most stable constituent of the haemolymph of *Pila*. More information on this possible regulatory mechanism should be obtainable by following the changes in composition and volume of the haemolymph of individual slugs through their cycles of loss and gain of weight.

SUMMARY

1. Methods are described for the collection of slug haemolymph and its analysis for cations, anions, total electrolytes, protein, Δ and pH.

2. Of the cations measured, potassium and calcium show the greatest variation, being two to three times as variable as sodium, the most constant. About 20% of the blood calcium exists in a bound form.

3. Of the anions measured, chloride is the most concentrated and least variable. Sulphate and alkalinity are about equally variable, three times the chloride fluctuation. The phosphate concentration is very low.

4. The variation of total electrolytes is only half that of the total solutes, as measured by Δ, while protein is very variable.

5. The pH is remarkably alkaline but varies only within narrow limits.

6. Formulae are given for two physiological salines, capable of maintaining both muscular and ciliary activity.

My sincere thanks are due to Dr W. A. L. Evans for much advice and for reading the manuscript. The investigation was carried out under a D.S.I.R. Research Studentship.

REFERENCES

ARVANITAKI, A. & BLANC, H. (1934). Action des ions H et des ions OH sur la survie, la rythmicité, le tonus, le potential d'activité du myocarde de l'Escargot. *J. Physiol. Path. gén.* **32**, 775–91.

ARVANITAKI, A. & CARDOT, H. (1931). Solutions equilibrées pour le cœur des *Helix*, en rapport avec la composition de l'hémolymphe. *C.R. Soc. Biol., Paris*, **106**, 185–7.

BACHRACH, E. & CARDOT, H. (1921). Contractilité et excitabilité du flagelle de l'Escargot. *C.R. Soc. Biol., Paris*, **85**, 170–2.

BERNARD, A. (1931). Sur la composition minérale de l'hémolymphe de differents *Helix*. *C.R. Soc. Biol., Paris*, **106**, 183–4.

BERNARD, A. & BONNET, V. (1930). Composition minérale de l'hémolymphe et étude d'une solution physiologique pour l'Escargot. *C.R. Soc. Biol., Paris*, **103**, 1119–20.
BOZLER, E. (1930). Untersuchungen zur Physiologie der Tonusmuskeln. *Z. vergl. Physiol.* **12**, 579–602.
CARDOT, H. (1921). Action des solutions de Ringer hypertoniques sur le cœur isolé d'*Helix pomatia*. *C.R. Soc. Biol., Paris*, **85**, 813–16.
CARRIKER, M. R. (1946). Observations on the functioning of the alimentary system of the snail *Lymnaea stagnalis appressa* Say. *Biol. Bull., Woods Hole*, **91**, 88–111.
COURGILL, R. W. & PARDEE, A. B. (1957). In *Experiments in Biochemical Research Techniques*, p. 177. New York: Wiley and Sons.
CROGHAN, P. C. (1958). The osmotic and ionic regulation of *Artemia salina* L. *J. Exp. Biol.* **35**, 219–33.
DAINTON, B. H. (1954). The activity of slugs. I. *J. Exp. Biol.* **31**, 165–87.
DANIELSON, I. S. (1953). Amino-nitrogen in blood and its determination. *J. Biol. Chem.* **101**, 505–22.
DUNCAN, C. J. (1961). Spontaneous activity in the isolated nerves of pulmonate molluscs. *Comp. Biochem. Physiol.* **3**, 42–51.
DUVAL, M. (1930). Quoted in KAMADA (1933).
FOLIN, O. (1922). A system of blood analysis. III. A new colorimetric method for the determination of amino-nitrogen in blood. *J. Biol. Chem.* **51**, 377–91.
GRAHAM, J. D. P. & HEATHCOTE, R. St A. (1952). Pharmacological investigation of the nature of the innervation of the muscles of *Helix aspersa*. *J. Exp. Biol.* **29**, 192–5.
HOGBEN, L. T. (1925). Studies on the comparative physiology of contractile tissues. I. The action of electrolytes on invertebrate muscle. *Quart. J. Exp. Physiol.* **15**, 263–312.
HOLTZ, F. & VON BRAND, T. (1940). Quantitative studies upon some blood constituents of *Helix pomatia*. *Biol. Bull., Woods Hole*, **79**, 423–31.
HOWES, N. H. & WELLS, G. P. (1934). Water relations of snails and slugs. *J. Exp. Biol.* **11**, 327–51.
HUGHES, G. M. & KERKUT, G. A. (1956). Electrical activity in a slug ganglion in relation to the concentration of Locke solution. *J. Exp. Biol.* **33**, 282–94.
JAEGER, C. P. (1961). Physiology of Mollusca. I. Action of acetylcholine on the heart of *Strophocheilos oblongus*. *Comp. Biochem. Physiol.* **4**, 30–2.
JAEGER, C. P. (1962). Physiology of Mollusca. III. Action of acetylcholine on the penis retractor muscle of *Strophocheilos oblongus*. *Comp. Biochem. Physiol.* **7**, 63–9.
JULLIEN, A., ACOLAT, L., RIPPLINGER, J., JOLY, M. & VIELLE-CESSAY, C. (1955). La teneur en ions Na, K et Ca de l'hémolymphe déterminée au photomètre à flamme et ses rapports avec la composition de solutions artificielles aptes à assurer une activité de longue durée au cœur isolé chez les Hélicides. *C.R. Soc. Biol., Paris*, **149**, 723–5.
KAMADA, T. (1933). The vapour pressure of the blood of the edible snail. *J. Exp. Biol.* **10**, 75–8.
KERKUT, G. A. & TAYLOR, B. J. R. (1956). The sensitivity of the pedal ganglion of the slug to osmotic pressure changes. *J. Exp. Biol.* **33**, 493–501.
KRIJGSMAN, B. J. & DIVARIS, G. A. (1955). Contractile and pacemaker mechanisms in the heart of molluscs. *Biol. Rev.* **30**, 1–36.
LOCKWOOD, A. P. M. (1961). 'Ringer' solutions and some notes on the physiological basis of their ionic composition. *Comp. Biochem. Physiol.* **2**, 241–89.
MARTIN, A. W., HARRISON, F. M., HUSTON, M. J. & STEWART, D. M. (1958). The blood volumes of some representative molluscs. *J. Exp. Biol.* **35**, 260–79.
MICHON, J. & ALAPHILIPPE, F. (1958). Comparison de quelques constantes biologiques de l'hémolymphe d'individus normaux de *Achatina marginata* (Gastéropode Pulmone) et d'individus présentant une anomalie de croissance. *C.R. Soc. Biol., Paris*, **152**, 1349–52.
PAULSON, S. (1953). Biophysical and physiological investigations on cartilage and other mesenchymal tissues. IV. A semimicro method for conductimetric determination of sulfur. *Acta chem. scand.* **7**, 325–8.
RAMSAY, J. A. (1940). A nerve-muscle preparation from the snail. *J. Exp. Biol.* **17**, 96–115.
RAMSAY, J. A. & BROWN, R. H. J. (1955). Simplified apparatus and procedure for freezing-point determinations upon small volumes of fluid. *J. Sci. Instrum.* **32**, 372–5.
ROBERTSON, J. D. & WEBB, D. A. (1939). The microestimation of sodium, potassium, calcium, magnesium, chloride and sulphate in sea-water and the body fluids of marine animals. *J. Exp. Biol.* **16**, 155–77.
SAXENA, B. B. (1957). Inorganic ions in the blood of *Pila globosa* (Swainson). *Physiol. Zool.* **30**, 161–4.
SHAW, J. (1954). A simple procedure for the study of ionic regulation in small animals. *J. Exp. Biol.* **32**, 321–9.
SOMOGYI, M. (1945). Determination of blood sugar. *J. Biol. Chem.* **160**, 69–73.
WELLS, G. P. (1928). The action of potassium on muscle preparations from invertebrates. *J. Exp. Biol.* **5**, 258–82.
WIGGLESWORTH, V. B. (1938). A simple method of volumetric analysis for small quantities of fluid: estimation of chloride in 0·3 μl. of tissue fluid. *Biochem. J.* **31**, 1719–22.