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SODIUM ACTIVITY OF INSECT BLOOD: PHYSIOLOGICAL SIGNIFICANCE AND RELEVANCE TO THE DESIGN OF PHYSIOLOGICAL SALINE

BY J. E. TREHERNE, P. B. BUCHAN AND R. R. BENNETT*

A.R.C. Unit of Invertebrate Chemistry and Physiology, University of Cambridge, Department of Zoology, Downing Street, Cambridge, England

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SUMMARY

The apparent activity coefficients for sodium (γ'_{Na}) in the blood of six insect species have been calculated from measurements made with sodium-selective electrodes and a flame photometer. In every case γ'_{Na} was significantly lower than that for this cation in free solution (γ_{Na}). In *Periplaneta americana* γ'_{Na} varied considerably, during a period of 90 days, so that a relatively constant sodium activity (a_{Na}) was maintained in the blood in the face of large variations in the total sodium content measured by flame photometry. Despite the relative constancy of a_{Na} (of around 0·088 M) appreciable fluctuations were observed in the sodium and potassium content of nervous connectives over a period of 140 days. The values of a_{Na} and a_K were used to devise a satisfactory cockroach saline for use in experiments with isolated nerve cords.

INTRODUCTION

Many cellular functions are directly or indirectly dependent upon the activity of the inorganic ions in the body fluids, generally depending upon a high activity of sodium relative to that of potassium and upon critical and relatively low levels of divalent cations. In most physiological investigations the chemically determined concentrations are used as approximate estimates of the activities of inorganic ions in body fluids. In many cases this approximation appears to be justified, but would obviously be invalid if the activity coefficients of these ions differed markedly from those in free, aqueous, solutions. The present investigation was initiated to test the validity of this approximation, for insect blood, by measuring its sodium activity and relating the measurements to those obtained by flame photometry. The results of this investigation have also been used in an attempt to devise a satisfactory saline for use in physiological experiments with cockroach excitable tissues.

* Department of Biology, University of Massachusetts – Boston, Dorchester, Massachusetts 02125, U.S.A.

METHODS AND MATERIALS

(*a*) *Measurement of sodium activity*

Sodium-selective glass electrodes were constructed from Corning NAS 11–18 glass tubing (1·0 mm outside diameter). The tubing was drawn out to a diameter of approximately 0·25 mm and sealed using a microtorch with a gas–oxygen mixture. A thin-walled bulb, of 0·5 mm diameter, was then blown from the sealed glass tip. The shank of the electrode was coated with resin–wax mixture, leaving only the glass bulb uncoated. The glass tubing and the bulb was filled by capillarity using fine glass fibres, thrust down the inside of the tubing, and a fine glass micropipette. The fluid within the electrode was a solution containing 100 mM-NaCl and 100 mM tris base and was connected, via an Ag–Cl wire, to a Keithley 602 electrometer and a Servoscribe pen recorder. The indifferent electrode was constructed from a conventional glass microelectrode from which the tip had been broken before filling with 3·0 M-KCl in Agar.

The sodium electrode was calibrated with solutions of pure sodium chloride, the sodium activity corresponding to each concentration being calculated from data given by Robinson & Stokes (1959). A 59 mV slope was obtained for decade change in activity over the entire range from 0·001 to 0·15 M, indicating a satisfactory degree of sodium-selectivity.

Measurements in physiological saline, however, showed some departures from the predicted activities of low concentrations. Fig. 1 illustrates readings from the cockroach saline devised by Evans (1975), which has the following composition: 156·3 mM-Na^+, 10 mM-K^+, 1·8 mM-Ca^{2+}, 163·6 mM-Cl^-, 1·8 mM-HPO_4^{2-}, 0·2 mM-H_2PO_4, 2·5 mM-HCO_3^-. Lower sodium concentrations were achieved by replacement with tris. The graph shows sodium activity as measured by the calibrated electrode, plotted against concentration. At high concentrations the straight line indicates a constant activity-coefficient, but below about 0·01 M the activity is apparently greater than expected. This deviation is unlikely to be real, as the ionic strength of the saline was maintained approximately constant; it is probably due to a small contribution from other ions to the electrode potential. To avoid the effects of such interference, all measurements on blood were made in the range of concentrations where the graph is linear.

(*b*) *Flame photometry*

Measurement of sodium and potassium concentrations was achieved using the integrating flame photometer, built by J. A. Ramsay according to principles described by Ramsay (1953) and Öberg, Ulfendahl & Wallin (1967). The procedure employed was as described by Bennett, Buchan & Treherne (1975).

(*c*) *Preparation of blood samples and nervous connectives*

Penultimate connectives, taken from adult male cockroaches, were prepared for flame photometric determination of Na^+ as previously described (Bennett *et al.* 1975). Total sodium and potassium concentrations of whole blood were measured after appropriate dilution in ion-free water. Blood samples were obtained from most insects by puncturing previously waxed areas of abdominal cuticle with a steel

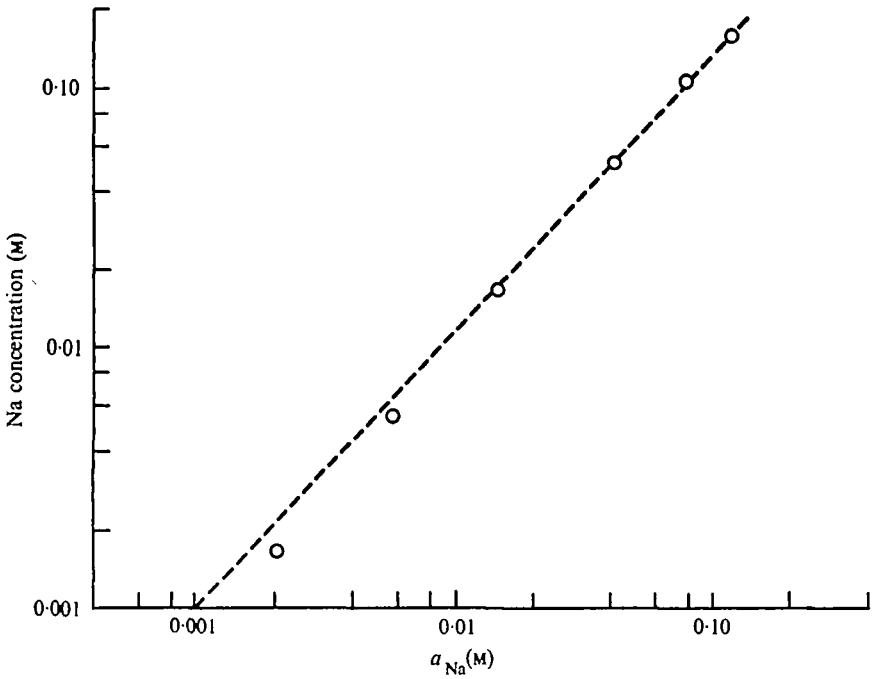

Fig. 1. The relation between sodium activity (a_{Na}) and concentration. The line shows the theoretical relation calculated for pure NaCl solutions from the data of Robinson & Stokes (1959). The points indicate the values of a_{Na} measured with the sodium-selective electrode in physiological saline.

needle and applying gentle pressure to the body. In cockroaches blood was also obtained, with a 1 μl micropipette, from the surfaces of the surgically exposed mushroom bodies. Samples of *Rhodnius* blood were obtained from the cut leg joints by application of gentle abdominal pressure.

The sodium activity in blood was measured by placing 5–10 μl drops beneath liquid paraffin and advancing the bulb of the sodium selective electrode and the tip of indifferent electrode into the drop. With cockroaches measurements were also made by gently inserting the bulb of the ion-selective electrode into the reservoir of blood associated with the accessory gland. Closely similar results were obtained for the sodium activities measured using both of the above methods.

(*d*) *Insect culture conditions*

The *Periplaneta americana* culture was maintained in large tanks, under normal daylight conditions, at a room temperature of 27–29 °C. Water was freely available. The cockroaches were fed on a diet of the following composition: 350 g rolled oats, 350 g wheat feed, 50 g linseed cake, 120 g grass meal, 35 g casein, 25 g dried powdered yeast, 25 g sucrose, 50 ml linseed oil, 50 ml arachis oil and 11 g W. salt mixture.

Tenebrio molitor was reared at approximately 20 °C, under natural daylight conditions, and were fed a diet consisting of 350 g rolled oats, 350 g wheat and 3·5 g dried yeast. *Rhodnius prolixus* were fed on rabbits, once every 4 weeks, and were maintained in darkness in incubators at 27·5–28·5 °C. *Carausius morosus* were fed on freshly gathered privet leaves, at a temperature of 18–20 °C in natural daylight

Table 1. *The sodium concentration (measured by flame photometry), the activity (a_{Na}) (determined by sodium-selective electrode measurements, see Fig. 1) and the effective activity coefficient (γ'_{Na}) calculated from these values for individuals from six insect species*

(The values are related to the activity coefficient of sodium ions in pure NaCl solutions (γ_{Na}) (calculated from the data of Robinson & Stokes, 1959). The experimentally determined values show the mean ± S.E.)

Species	Stage	Date	n	Na^+ concentration (M)	a_{Na}	γ'_{Na}	γ_{Na}	Ratio $\frac{\gamma'_{Na}}{\gamma_{Na}}$
Periplaneta americana	Adult	24. ii. 74	9	0·179 ± 0·010	0·085 ± 0·003	0·481 ± 0·024	0·752	0·640
Schistocerca gregaria	Adult	13. iii. 74	10	0·057 ± 0·003	0·0412 ± 0·002	0·734 ± 0·033	0·818	0·897
Carausius morosus	Adult	8. iii. 74	10	0·019 ± 0·002	0·0113 ± 0·001	0·642 ± 0·073	0·872	0·736
Rhodnius prolixus	5th instar	12. iii. 74	10	0·193 ± 0·004	0·114 ± 0·007	0·562 ± 0·021	0·748	0·751
Tenebrio molitor	5th instar	18. iii. 74	10	0·104 ± 0·007	0·059 ± 0·002	0·579 ± 0·029	0·777	0·745
Pieris brassicae	5th instar	14. iii. 74	10	0·013 ± 0·002	0·008 ± 0·001	0·746 ± 0·092	0·890	0·838

conditions. The *Schistocerca gregaria* culture was maintained on a 12 h day at 35 °C and 12 h night at 25 °C and was fed on potted wheat and bran. The larvae of *Pieris brassicae* were maintained with 16–18 h daylight and were fed on cabbage plants.

RESULTS

(*a*) *Measurement of total sodium content and sodium activity of insect blood*

Table 1 summarizes the results obtained with six insect species. The sodium concentrations, measured by flame photometry, approximate to those obtained by previous investigators (cf. Florkin & Jeuniaux, 1964). The value of 57 mM for *S. gregaria*, however, differs from the average value of 81·3 mM obtained by Duchâteau, Florkin & Leclercq (1953) and that of 180 mM measured by Phillips (1964).

The results show that for all of the species the activity coefficient of the blood sodium was significantly lower than that for this cation in free aqueous solution at equivalent concentrations. This is particularly evident with the blood of *P. americana* which showed the lowest effective activity coefficient for sodium ions.

(*b*) *Change in concentration and activity of sodium ions in cockroach blood*

Blood samples taken from batches of ten cockroaches for a period of 90 days showed substantial fluctuations in sodium content (Fig. 2). These changes in total sodium level did not appear to be paralleled by equivalent proportional changes in the gross potassium content of the blood, neither were they accompanied by any equivalent changes in the sodium activity of the blood which remained at relatively stable levels for the entire period.

The activity coefficient for blood sodium, calculated from the data illustrated in Fig. 2, showed large and significant fluctuations with time (Fig. 3). At the commencement of the period of observation the activity coefficient was relatively low ($\gamma'_{Na} = 0{\cdot}481 \pm 0{\cdot}024$), but increased after a period of 28 days ($\gamma'_{Na} = 0{\cdot}755 \pm 0{\cdot}027$)

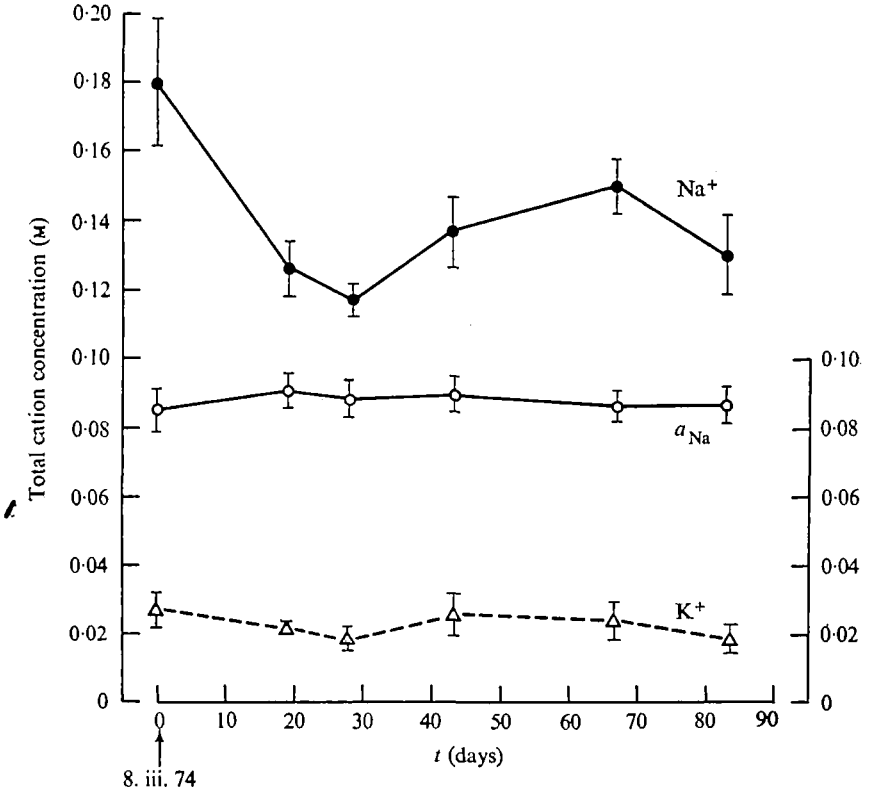

Fig. 2. The concentrations of sodium and potassium ions measured by flame photometry, and the sodium activity of cockroach blood over a period of 90 days (from 8. iii. 74 to 6. vi. 74). Each point represents the mean of measurements made on ten individuals, the vertical lines indicating the extent of twice the standard error of the mean.

to approach the theoretical value calculated for sodium chloride in free solution (γ_{Na} = 0·769). At this time, therefore, the blood sodium approached a fully ionized condition. Subsequently, a significant decline in the apparent activity coefficient was observed.

(*c*) *Changes in cation content of cockroach nerve cords*

Despite the relative constancy of the sodium activity of the blood appreciable individual variation and apparent fluctuations were observed in the sodium content per unit weight of nervous connectives. Fig. 4 illustrates the sodium contents measured in penultimate abdominal connectives from successive batches of cockroaches taken from the laboratory culture over a period of 140 days. These data show an apparent decline in sodium concentration with time, the values at 120 and 140 days being only half those measured initially. This decline was, however, associated at least in part with an increase in weight of the connectives, so that the total amount of sodium present remained approximately constant.

Apart from high initial values (24. ii. 74 and 16. iii. 74) the potassium concentration of connectives showed no significant variation during the subsequent period of observation (Fig. 5). In this case the terminal increase in weight was not associated with a decline in the gross concentration of this cation in the connectives.

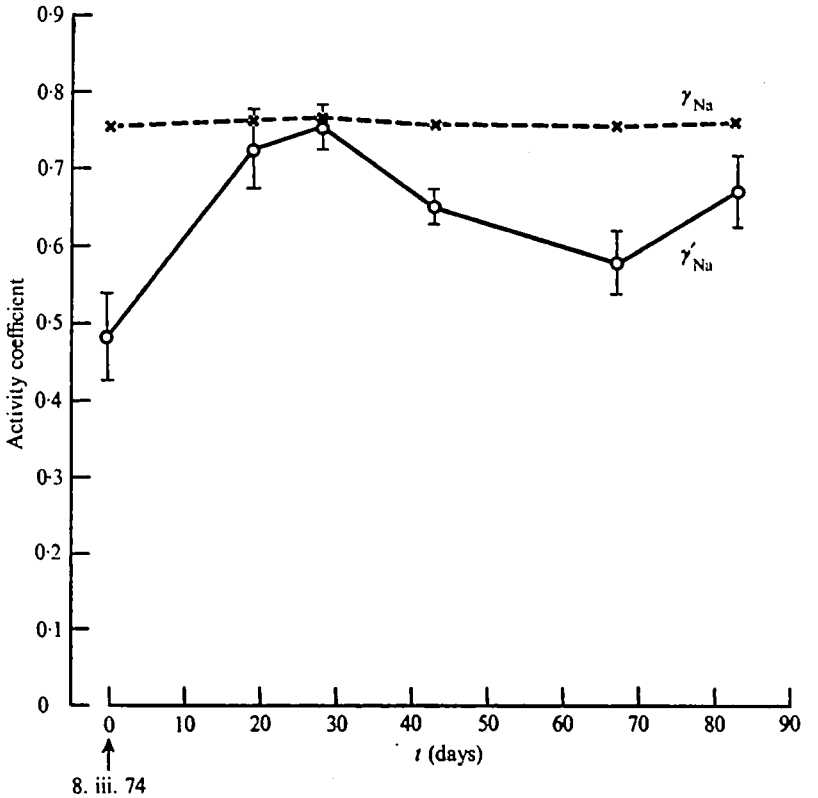

Fig. 3. The apparent activity coefficient (γ'_{Na}) of cockroach blood (●) over a period of 90 days (from 8. iii. 74 to 6. vi. 74) calculated from a_{Na} and the sodium concentrations determined by flame photometry shown in Fig. 2. These values are compared with the activity coefficient for sodium (γ_{Na}) calculated, from the data of Robinson and Stokes, for NaCl in free solution. The vertical lines show the extent of twice the standard error of the mean.

(*d*) *Sodium and potassium content of isolated cockroach connectives in physiological salines*

The measurements with sodium-selective electrodes indicate that sodium may not be present in a fully ionized state in the blood of some insect species, an observation which has important implications in the design of appropriate physiological salines. A variety of physiological salines have been used to maintain isolated organs and tissues of the cockroach, that of Yamasaki & Narahashi (1959) being frequently employed in electrophysiological experiments on cockroach nerve cords. It has recently been shown by Evans (1975), however, that the high osmotic concentration, largely resulting from the sodium content of this saline (210 mM), causes an appreciable weight-loss in intact, isolated, nerve cords. To prevent such a loss in weight the latter author devised a saline, containing 337·3 mOsmols, with lower sodium (156·3 mM) and higher potassium concentration (10 mM) (see p. 722) then in the saline of Yamasaki & Narahashi.

Experiments with isolated, ligatured, connectives confirmed the efficiency of the Evans' saline in reducing weight loss. It was found, however, that connectives gained sodium (Fig. 6) and lost potassium (Fig. 7) in this saline.

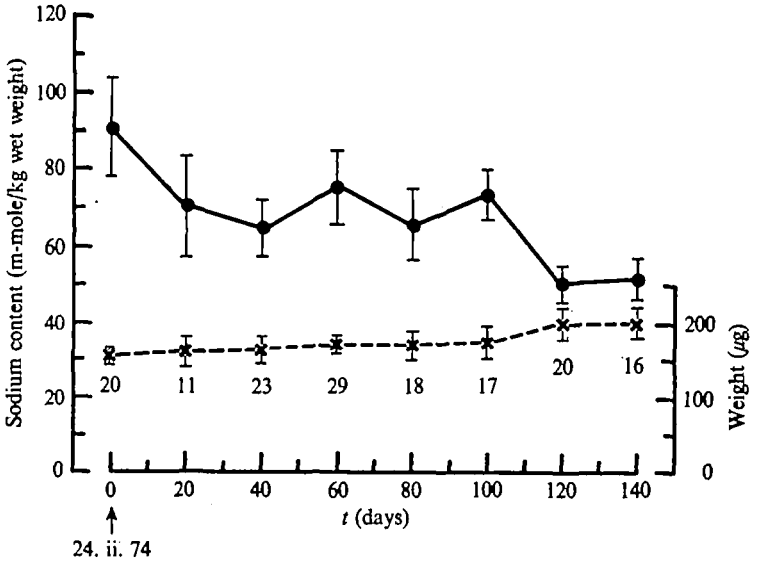

Fig. 4. Variations in the sodium content (●) and weight (×) of penultimate abdominal connectives taken from batches of cockroaches over a period of 140 days (from 24. ii. 74 to 15. vii. 74). The vertical lines indicate the extent of twice the standard error of the mean, the number of individuals in each batch being also indicated.

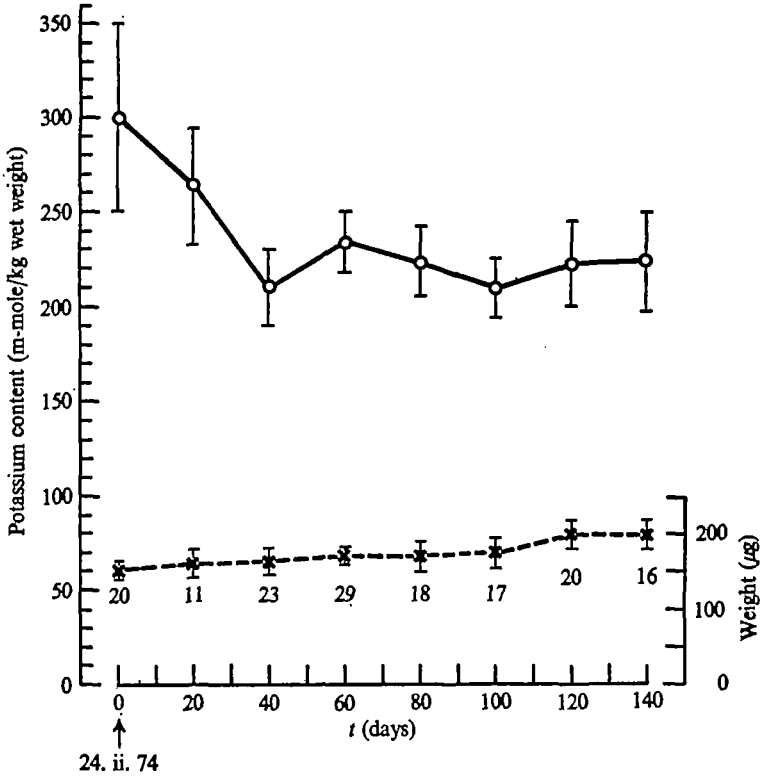

Fig. 5. Potassium content (○) and weight (×) of penultimate abdominal connectives taken from batches of cockroaches over a period of 140 days (from 24. ii. 74 to 15. vii. 74). The vertical lines indicate the extent of twice the standard error of the mean, the number of individuals in each batch being also indicated.

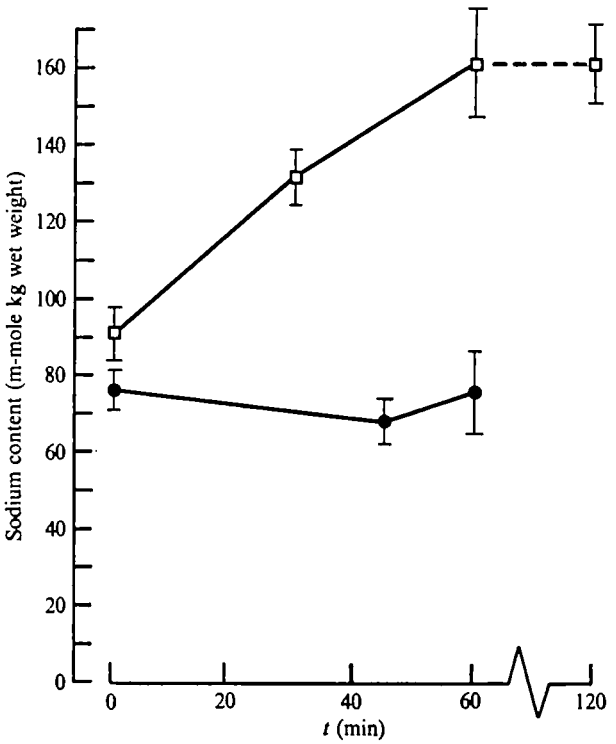

Fig. 6. Sodium content of isolated, ligatured, penultimate abdominal connectives, from *P. americana*, maintained in two different physiological salines. The saline devised by Evans (1975) (□) was based on sodium and potassium concentrations determined by flame photometry (156·3 mM-Na^+ and 10 mM-K^+); the composition of the present saline (●) was based on measurements made with ion-selective electrodes in this investigation (120 mM-Na^+ and 25 mM-K^+). The experiments with Evans saline were performed between 26. ii. 74 and 1. iii. 74 and those with the present saline between 25. iv. 74 and 1. v. 74. The vertical lines indicate the extent of twice the standard error of the mean (n = 10).

To avoid changes in the sodium and potassium content of isolated connectives a saline of appropriate sodium and potassium activity was devised. Our tests with sodium selective electrodes showed that the blood of cockroaches from the Cambridge colony maintained a sodium activity equivalent to a concentration of 120 mM over a period of 90 days, despite marked fluctuations in total sodium content (Fig. 2). Measurements with a potassium selective electrode, based on the design of Khuri, Agulian & Wise (1971) showed that the activity of potassium ions was equivalent to a concentration of around 25 mM.

As preliminary experiments indicated that relatively constant potassium concentrations were maintained in isolated connectives in saline containing 25 mM-K^+ a physiological saline of the following composition was devised: 120 mM-Na^+, 25 mM-K^+, 2·0 mM-Ca^{2+}, 2·0 mM-Mg^{2+}, 163·7 mM-Cl^-, 0·2 mM-PO_4^-, 1·8 mM-HPO_4^{2-}, 2·5 mM-HCO_3^-, 19·02 mM $tris^-$.

Nervous connectives maintained in the above saline showed no appreciable rise in sodium content (Fig. 6) and a proportionally smaller change in potassium content as compared with Evans saline over a period of 1 h (Fig. 7).

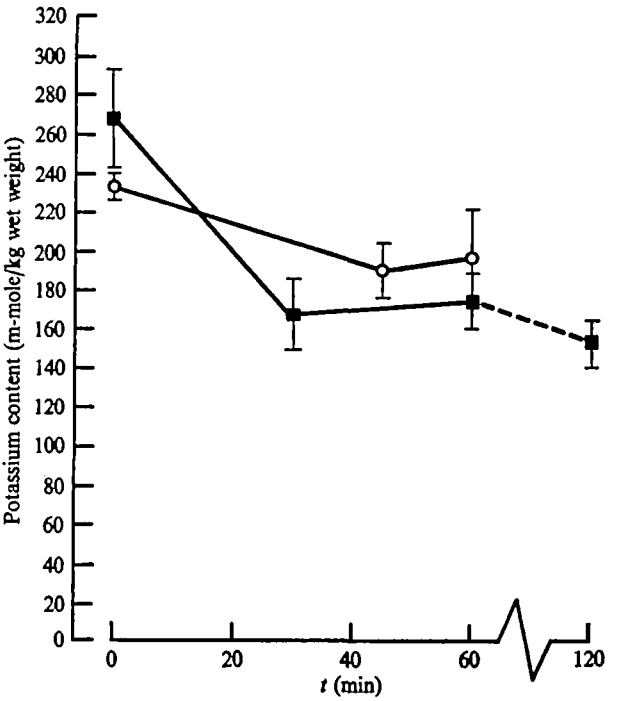

Fig. 7. The potassium content of isolated, ligatured, cockroach connectives maintained in Evans (■) and the present saline (○). The experiments with the former saline were performed between 26. ii. 74 and 1. iii. 74, those with the present saline between 25. iv. 74 and 1. v. 74. The vertical lines indicate the extent of twice the standard error of the mean (n = 10).

DISCUSSION

Insect blood appears to be characterized by extreme variability in the concentrations of the major inorganic cations. This is well exemplified by cockroach blood, in which the individual values for sodium concentration can differ by almost a factor of 3 (cf. Asperen & Esch, 1956; Pichon, 1970). The present observations confirm this variability, showing relatively large fluctuations in total sodium concentration of the blood.

Such extreme variations present obvious difficulties in the interpretation of cellular excitability. This is particularly evident with insect muscle fibres in which there is no evidence of any 'protection' from blood ions such as is provided for central neurones by the blood–brain barrier system (cf. Treherne, 1974). These difficulties are, however, at least partially resolved by the present observations. The observation that the sodium activity of cockroach blood remains relatively constant, despite large fluctuations in total sodium content, is particularly relevant for it indicates that the effective sodium concentration is regulated within relatively close limits. This appears to be a novel form of ionic regulation, at least for insect species.

The data indicate that discrepancies exist between the sodium concentrations, measured by flame photometry, and the values obtained using the sodium-selective electrode for six insect species (Table 1). This discrepancy was particularly evident in the case of *P. americana* in which the mean activity coefficient (γ'_{Na}) could be as

low as 0·481 as compared with the value of 0·752 (γ_{Na}) predicted for sodium ions in free solutions at the total concentration of 0·179 M recorded in these insects. This implies that only 64% of the sodium ions were effectively 'free' in the blood of this insect (on 24. ii. 74).

We have, as yet, little understanding of the factors causing a reduction of γ'_{Na} in the blood of these insect species. It is, however, difficult to attribute this effect to a sequestration of as much as 36% of the sodium within the blood cells. From the data of Brady (1967) it is possible to calculate that the haemocytes occupy an average of 2·31% of the volume of cockroach blood. To accommodate 36% of the blood sodium (0·0644 M) in such a volume would imply an improbable intracellular sodium concentration of 2·79 M. It is also difficult to attribute the progressive changes in γ'_{Na} to massive alterations in blood cell numbers, for these changes are not accompanied by significant changes in the potassium content of the blood (Fig. 2) which would certainly be predicted on the basis of a calculated intracellular potassium concentration of 0·116 M for cockroach haemocytes (Brady, 1967).

It seems most likely, therefore, that the low values of γ'_{Na} resulted from the interaction of the sodium ions with plasma constituents: such reduced values resulting from sodium 'binding' (such as might occur as a result of the formation of covalent linkages with large molecules), from electrical interactions with relatively large organic anions (such as amino acids, polypeptides and/or proteins) or from a combination of both of these factors. According to this interpretation the changes in γ'_{Na} with time (Fig. 3) would result from the alterations in the relative concentrations or the physico-chemical properties of unspecified plasma constituents.

Despite the relative constancy of the sodium activity of cockroach blood it was shown that significant changes could be measured in the sodium content of nervous connectives taken from batches of insects over an extended period. These changes were surprisingly large, involving a significant decline from $91{\cdot}0 \pm 6{\cdot}7$ (on 24. ii. 74) to $64{\cdot}8 \pm 3{\cdot}3$ mmoles/kg (on 5. iv. 74) and, subsequently, from $73{\cdot}2 \pm 3{\cdot}4$ (on 5. vi. 74) to $50{\cdot}6 \pm 2{\cdot}4$ (on 25. vi. 74) and $51{\cdot}9 \pm 2{\cdot}6$ mmoles/kg (on 15. vii. 74). The latter decline was associated with, and presumably resulted from, a significant increase in the weight of the connectives. A significant change in the gross concentration of potassium ions was also observed: from $299{\cdot}0 \pm 29{\cdot}9$ to $210{\cdot}2 \pm 10{\cdot}2$ mmoles/kg (between 24. ii. 74 and 5. iv. 74).

The evidence provides little insight as to the causes or the physiological significance of such fluctuations in the gross cation content of the central nervous tissues. The cockroaches used in this investigation were fed on a standard diet and maintained at a relatively constant temperature (27–29 °C). The culture was, however, exposed to natural daylight conditions. It is conceivable, therefore, that the fluctuations in cation levels could represent seasonal changes, perhaps associated with a changing diurnal photoperiod. The possibility also cannot be eliminated that, if these are non-random events, they could result from factors such as different degrees of crowding (this factor was not controlled) or undiagnosed metabolic disorders.

It would obviously be unprofitable, at this stage, to speculate at length as to the possible localization of the cation fractions which exhibit fluctuations within the nervous connectives. It may be worth noting, however, that recent experiments have shown that relatively large changes in the sodium content of cockroach connectives

can occur, on exposure to sodium-deficient saline, in the absence of significant changes in axonal function (Bennett *et al.* 1975). This sodium fraction has been provisionally identified with the glial elements. It is conceivable, therefore, that the present fluctuations may be, in part at least, associated with changes in glial sodium concentration, resulting from changes in sodium content or glial volume.

The important factor which emerges from these perplexing observations is that the fluctuations in the cation contents of the connectives cannot be directly related to changes in the sodium activity or the potassium content of the blood. It is possible, therefore, that the central nervous tissues were responding to some other, unspecified, physiological signals.

A previous investigation from this laboratory has shown that substantial changes in weight can be produced in isolated cockroach nerve cords by the use of physiological salines of inappropriate cation composition (Evans, 1975). Such weight changes were prevented by using a sodium concentration approximating to that measured by flame photometry (156·3 mM-Na^+). The results of the present investigation confirm Evans's observations, but show, in addition, that a substantial increase in sodium content occurs in the latter saline. Substitution of a saline containing sodium and potassium at concentrations equivalent to their activities in cockroach blood resulted in the maintenance of relatively stable concentrations of both of these cations in isolated connectives. It appears, therefore, that the saline devised in this investigation represents a realistic approximation to the effective blood concentrations of these cations.

The use of this novel saline has also been found to be most effective in reducing the extent of apparent ultrastructural artifacts which have, for example, been found to occur in nerve cords exposed to the high sodium, low potassium, saline of Yamasaki & Narahashi (1959). With the latter saline intact preparations incubated before fixation displayed lacunae between perineurial cells and also exhibited occasional cellular damage (Treherne, Schofield & Lane, 1973). Recent observations in this laboratory (Treherne & Lane, unpublished observations) have shown that such artifacts are reduced in the saline of Evans (1975), but are effectively eliminated using the present cockroach saline. With this saline the ultrastructural appearance of the perineurial elements is similar to that observed in preparations which were fixed directly *in situ*. It would appear, therefore, that the present saline may be of value not only in physiological investigations, but also in ultrastructural studies which involve the exposure of nerve cords, and perhaps other organs, to physiological saline.

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