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THE EFFECT OF IONS UPON NEUROMUSCULAR TRANSMISSION IN A HERBIVOROUS INSECT

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The ionic composition of the haemolymph of most insects is closely related to their diet (Boné, 1944; Clark & Craig, 1953; Duchateau, Florkin & Leclerq, 1953). In herbivorous insects the blood sodium and potassium levels are similar to those of the food plant, but magnesium is higher and calcium lower than in the food plant (Duchateau *et al.* 1953). The sodium:potassium and calcium:magnesium ratios of the haemolymph are both less than unity. In carnivorous and omnivorous insects, however, the proportions of the mineral constituents of the haemolymph are more comparable to those of vertebrate blood: there is a high sodium:potassium ratio, and the calcium:magnesium ratio approaches or exceeds unity.

In various Acrididae (Hagiwara & Watanabe, 1954; Hoyle, 1953, 1955*a*, *b*) and in the cockroach (Wilson, 1954; Hoyle, 1955*a*), an increase in the external potassium concentration results in a depolarization of the resting membrane of the muscle fibres. Hoyle (1955*a*) found that in the locust an increase in magnesium ions reduced the size of the end-plate potential and lowered the excitability of the muscle-fibre membrane. Calcium ions antagonized the action of magnesium on the end-plate potential and their presence was necessary for its development. No detailed study of the effect of sodium ions upon neuromuscular transmission in insects has yet been made. In vertebrates the presence of sodium is necessary for the full development of the end-plate potential (Fatt & Katz, 1952) and is essential for the production of the propagated action potential (see Hodgkin, 1951).

There is no evidence that propagated action potentials occur in insect muscle fibres. The distributed end-plate potentials excite the muscle to contract even when they are very small (Hoyle, 1955*a*). It might be expected that in herbivorous insects there will be a low resting potential due to high blood potassium, and a small action potential due to a combination of low resting potential and low blood sodium. Since in arthropod muscle the size of the

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mechanical response is directly related to the size of the muscle action potential, the muscles of herbivorous insects might contract less vigorously than those of carnivorous or omnivorous insects. At present there is no information about the relative performances of insect muscles. Hoyle (1955*a*) found that a concentration of 10 mM-Mg reduced the end-plate potential to a very small size and led to the disappearance of the active membrane of 'spike' response of locust and cockroach muscle. This value is only 0·5–0·2 of the values for the blood Mg of many herbivorous insects (Clark & Craig, 1953; Duchateau *et al.* 1953). If all the magnesium found in the blood of these insects is present in the form of unbound ions, there must be some difference in magnesium tolerance between the muscles of herbivorous and other insects.

The ionic composition of cockroach and locust bloods is typical of carnivorous and omnivorous insects. It seemed desirable, therefore, to examine the effects of the four common cations, sodium, potassium, calcium and magnesium, on neuromuscular transmission in a typical herbivorous insect. The present paper describes the results of such a study on the stick insect, *Carausius morosus* Br.

METHODS

Blood composition

Blood analyses were carried out upon small samples obtained by pricking the articular membrane between two of the thoracic segments, and taking up the resulting drop of blood in a haemocytometer pipette. A measured quantity of the blood was then pipetted into a known small quantity of distilled water. Sodium, potassium, and calcium were estimated on the samples by means of an 'E el' flame photometer. Before magnesium and phosphate estimations, protein was precipitated with trichloroacetic acid, and centrifuged off. Magnesium was determined by the titan yellow method of Heagy (1948), and phosphate by the method of Snell & Snell (1949). For chloride and bicarbonate Conway microdiffusion methods were used; the original method for bicarbonate (Conway, 1950), and a modified colorimetric method for chloride (Gordon, 1952).

The results of these analyses are given in Table 1. They show good agreement with the results of previous workers, although the magnesium value obtained was not quite so high as that of Duchateau *et al.* (1953). Nevertheless, the value of 53 mM-Mg obtained is 20–30 times that found in vertebrate blood or the haemolymph of a carnivorous insect.

A saline was prepared for experimental purposes utilizing the figures obtained by analysis, so as to approximate closely to the ionic composition of the haemolymph. Some allowance was made

TABLE 1. The ionic composition of *Carausius* haemolymph

Figures are given in mM as the mean, to the nearest whole number, of a number of analyses (in brackets), ± its S.E. The results of previous workers are included in the table.

	Na	K	Ca	Mg	Cl	Phosphate, as $H_2PO_4^-$	HCO_3^-
Author's analyses:							
Mean	15	18	7·5	53	101	16	5
S.E.	±3	±3	±1	±6	±11	±3	±2
No. of analyses	(17)	(17)	(12)	(13)	(17)	(7)	(3)
Boné, 1944	21	25	—	—	—	—	—
Duchateau *et al.* 1953	9	28	8	72·5	—	—	—
Ramsay, 1953, 1955	14	17	—	—	—	40*	—
'Serum': Ramsay, 1955	11	18	3·5	54	87	13*	

* as PO_4^{3-}

for probable binding of some of the magnesium by the blood amino acids (Albert, 1950). The composition of the saline was as follows: Na 15; K 18; Mg 50; Ca 7·5; H_2PO_4 6; HPO_4 4·5; Cl 133 (all mM). This saline has a pH of about 6·6, which was similar to the pH value obtained from fresh blood using a Pye pH meter with a glass electrode micro-assembly. Experimental salines were prepared by addition or subtraction of the chloride of the ion under investigation. To the normal saline was added 63·3 g of sucrose/l., and the tonicity of the other saline mixtures was adjusted to that of the normal saline by a suitable alteration of the sucrose content.

For a sodium-free mixture, potassium phosphates were substituted for sodium phosphates and the amount of potassium chloride adjusted to preserve the potassium level of the normal saline. It was found that the use of phosphate buffers resulted in the formation of precipitates, presumably of calcium phosphate, at low magnesium concentrations. Phosphate–bicarbonate buffering (12 mM-$H_2PO_4^-$; 8 mM-HCO_3^-) was accordingly used for zero and 25 mM–Mg salines. Some experiments were carried out in which all the magnesium salines were buffered in this way, and the results were not found to differ from experiments in which phosphate-buffered salines were used.

Even in the normal saline a precipitate formed on standing. No saline was used in this condition.

Tetraethylammonium, tetramethylammonium, and tetra-*n*-butylammonium iodides were converted to chlorides by dissolving them in distilled water and passing the solution through a column containing Permutit ion-exchange resin 'de-acidite FF' in chloride form, until the liquid passing through showed no trace of iodide. Their strength was adjusted to a suitable level for incorporation in the salines after a chromate titration for chloride.

Experimental procedure

The animal was placed ventral surface uppermost, upon a bed of 'Plasticene' contained in a Petri dish, and fastened to this bed by small strips of 'Plasticene' passing over the femora just behind the femoro-tibial joints in such a way that the tibiae were free to move. The particular leg to be examined was surrounded by 'Plasticene' to keep it supported, and a well was formed around it by a wall, also of 'Plasticene', which passed over the coxa and kept it fixed in position. The ventral cuticle was then removed from most of the femur, exposing the flexor tibialis muscle. This is the largest femoral muscle, and consists of pinnately arranged fibres running from the lateral cuticle to their insertion on the median apodeme, the fibres being grouped into bundles of two vertical rows of five fibres each. The muscle is innervated by the crural nerve, which contains a 'fast' axon sending branches to all the fibres of the muscle. In these experiments the crural nerve was stimulated in the thorax by transformer-differentiated square pulse stimuli applied to the nerve through polythene-insulated, tapered silver-wire electrodes. A single stimulus produced a 'twitch' type contraction in all the fibres of the muscle.

The electrical responses associated with this contraction were studied by means of glass capillary intracellular micro-electrodes filled with 3M-KCl (Ling & Gerard, 1949; Nastuk & Hodgkin, 1950), which were used to impale single muscle fibres. A cathode-follower input stage modified from Bishop (1949) and a d.c. pre-amplifier (Copeland, 1952) were used to feed the signal to a Cossor 1049 oscillograph. The effects of salines of different composition upon the muscle response were studied by changing the fluid in the 'Plasticene' well. The routine procedure was to impale about ten fibres selected at random over the surface of the muscle in each saline, after a replacement time of 30 min. The electrode was left in the last fibre impaled in each saline, so that the effect of the next saline could be seen on the same fibre at regular intervals up to the standard replacement time. In addition, the effect of a series of salines was sometimes examined with the electrode left in the same fibre for the whole time. Only superficial fibres were used for sampling, but the effects described were checked on deeper fibres from time to time, and found to be similar to those seen in superficial fibres.

RESULTS

If the experimental preparations are completed before the removal of the cuticle from the femur, it is possible to impale fibres of the flexor tibialis muscle within two minutes of covering the muscle with normal saline. The shortest

time within which a change in the ionic composition of the bathing fluid was seen to produce an effect upon a superficial fibre was about 1 min in high-potassium saline. In most superficial fibres it was a little longer than this. It should therefore be possible, if deeper fibres are impaled immediately after they have been covered in saline, to record the electrical responses of these fibres while they are still bathed in their normal medium. Two records obtained from deeper fibres in this way are shown in Fig. 1. They illustrate the variation in resting and action potentials between individual fibres. In Fig. 1*A* the resting potential is 37 mV and the action potential is 40 mV. Corresponding values for Fig. 1*B* are 42 and 30 mV respectively. Mean values obtained from six such deeper fibres in the same muscle were 41 mV ± S.E. 1·5 for the resting potential and 39 mV ± 2·2 for the action potential. Fig. 1*A* was recorded on a slower time base than Fig. 1*B*, to show the time course of the rising phase

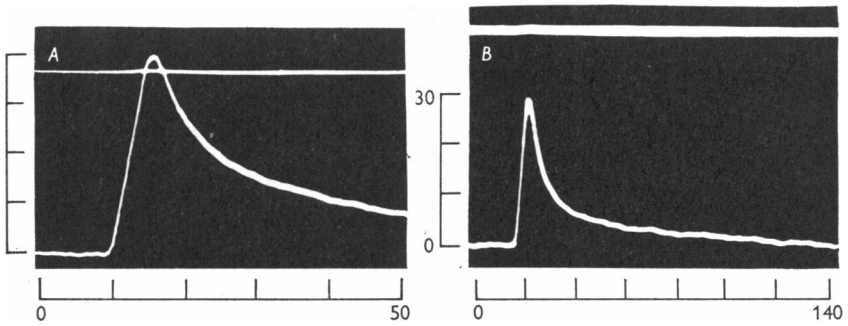

Fig. 1. Two examples, on different time bases, of the normal electrical response of *Carausius* muscle to single shock stimulation of the crural nerve. Calibration in all Figs. (except Fig. 12): mV and msec.

more clearly. The rising phase occupies about 6 msec and is followed by a long decay phase of about 100–150 msec (Fig. 1*B*). Small overshoots of zero potential, similar to that seen in Fig. 1*A*, were observed more often in young adults than in old adults. The former were used as far as possible in these experiments, the cuticle, which is harder in older animals, serving as a rough guide in their selection.

The long decay time observed was not a result of the appearance of a contraction artifact. The time course did not vary when small vertical movements of the electrode were made. Furthermore, the initial part of the recovery phase occurred more quickly in high-sodium saline, although the mechanical response was then greater. In low-calcium solution the recovery phase was slower, but the tension developed by the muscle was much smaller. Contraction artifacts were seen in some cases: they could always be distinguished from the time course of the action potential.

At first, no attempt was made to see that the normal saline was isotonic

with the muscle fibres. Roeder & Weiant (1950) and Wilson (1954) added a small quantity of sucrose to the saline they used for the cockroach, but Hoyle (1953) and Hagiwara (1953) added none to the saline they used for various Acrididae. The osmotic pressure of insect blood is high, owing to the presence of large quantities of free amino acids and reducing sugars. Figures for the osmotic pressure of the haemolymph of most of the insects studied by these authors are not available, but it is probable that some at least of the salines used by them were hypotonic to the muscle fibres they examined. However, no adverse effects were reported as a result of their use. There was some swelling of locust muscle fibres in the saline used by Hoyle (1953), but this had no effect upon their electrical and mechanical properties (Hoyle, personal communication).

This was not the case in *Carausius*. In the normal saline without added sucrose the magnitude of the resting and action potentials showed a substantial decline (Fig. 2), but recovered when sucrose was added to the saline. Within limits, depending upon the normal action of the salt concerned, recovery could be induced by the addition of almost any salt to the saline. For

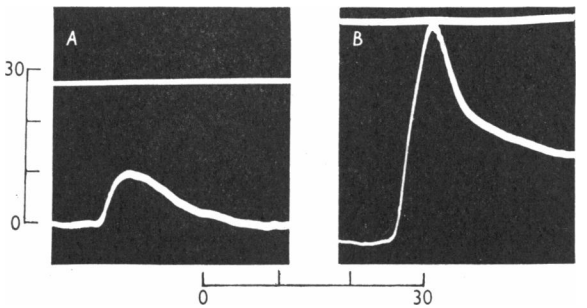

Fig. 2. *A*, normal response without addition of sucrose to saline; *B*, with sucrose (see text).

example, the addition of a moderate quantity of potassium ions resulted in a small increase in the size of the action potential, although the resting potential declined further. Following these observations the normal saline was made up with sucrose, as stated. The amount of sucrose to be added was determined by trial and error until resting and action potentials were obtained similar in size to those recorded from deeper fibres bathed in the insect's own body fluids.

The effect of sodium ions

Changes in the external sodium concentration affected the magnitudes of both the resting and action potentials. Fig. 3 is a graph of the results from a typical experiment. Below about 100 mM-Na the curves for the resting and action potentials follow an almost parallel course. The similar shape of the two curves may mean that at these sodium concentrations the change in size of the action potential is at least partly a result of the corresponding change in the

resting potential: del Castillo, Hoyle & Machne (1953) found that the size of the end-plate potential of locust muscle fibres was directly proportional to that of the resting potential.

At sodium concentrations above 100 mM both curves rise more slowly, but the curve for the action potential maintains a higher rate of rise than the curve for the resting potential. The differences are not statistically significant, but the same trend occurred in all experiments. Some factor other than the magnitude of the resting potential may be involved. In both resting and action potential the change in magnitude between 0 and 200 mM-Na is relatively small: about 6 mV in the resting potential, and 10 mV in the action potential.

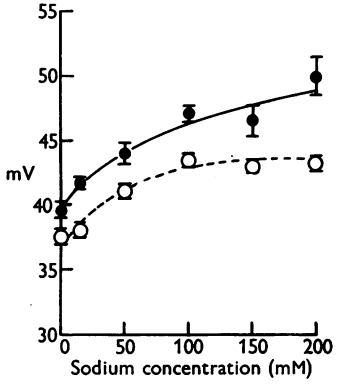

Fig. 3

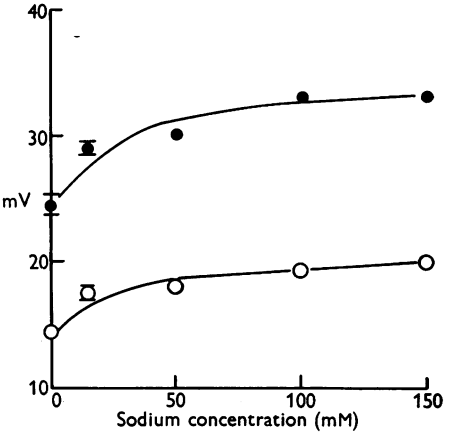

Fig. 4

Fig. 3. Relation between external sodium concentration and size of resting potential, ○, and action potential, ●. Limits of S.E. of mean shown by bars where they exceed radius of circle, and similarly throughout this paper.

Fig. 4. Relation between size of junctional potential, ○, active membrane response, ●, and external sodium concentration. Different experiment from Fig. 3.

By employing a suitably fast time base on the oscillograph, it is possible to determine the relative sizes of the end-plate component, which will hereafter be termed the junctional potential, and of the active membrane response. The junction between the two components then appears in some records as a slight bend in the rising phase of the action potential (the validity of this criterion is discussed on p. 131). Analysis of figures obtained from such selected fibres shows that the curves for the two components follow a parallel course which is similar in shape to that of the action potential as a whole (Fig. 4).

In Fig. 5 records are shown which illustrate the effect of lowering the sodium in the bathing fluid from normal to zero, or raising it from normal to 200 mM. The record in zero sodium was taken 3 hr after transference of the fibre to this

saline from normal saline: it was no different from the response recorded after 30 min. It is evident from these records that an alteration in external sodium concentration results in a corresponding alteration in the rate of rise of the action potential. The rate of rise of the junctional potential and active membrane response are affected in a similar manner by changes in sodium concentration (see Fig. 7), and it follows that the start-to-peak rate of rise of the action potential is a rough measure of the rate of rise of each of its two components. In three separate experiments the increase in the rate of rise of the action potential between zero and 200 mM-Na was 5·8, 6·1 and 7·9 V/sec, giving a mean value of 6·9 V/sec. The corresponding increase in the size of the action potential was 12, 10, and 12·5 mV respectively, giving a mean of 11·5 mV.

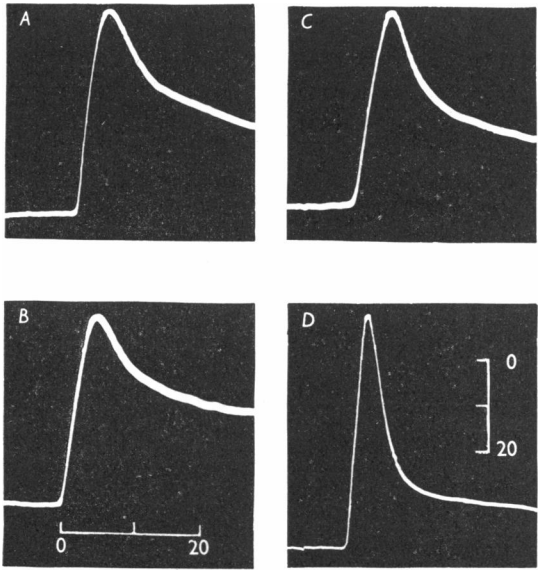

Fig. 5. Records from a fibre transferred from normal saline (*A*) to zero Na (*B*); and from another transferred from normal saline (*C*) to 200 mM-Na (*D*).

Quaternary ammonium ions

In an attempt to throw more light upon the role of sodium in the production of the action potential in *Carausius*, experiments were performed in which the sodium content of the salines was replaced by an equivalent quantity of a quaternary ammonium ion. The muscle became inexcitable after being bathed for 30 min in concentrations of tetrabutylammonium ions as low as 15 mM. It is not known whether this was due to a direct effect upon the muscle fibres or to some action upon the nerve endings. Conduction along the crural nerve was not affected. Excitability did not return when the saline was replaced with normal saline containing sodium.

Tetraethylammonium (TEA) ions tended to produce an irreversible fall in the value of the resting potential, the effect being quicker the higher the concentration. The fall in resting potential was accompanied by a fall in the magnitude of the action potential. Nevertheless, the rate of rise of the action potential increased considerably by comparison with sodium salines.

TEA salines also caused the muscle fibres to become abnormally excitable. The slightest mechanical stimulus to any part of the muscle, or a slight movement of the tibia, resulted in a vigorous and prolonged contraction of the whole muscle. A similar contraction followed single shock stimulation of the crural nerve. In sodium salines a single nerve stimulus produces only a small movement of the tibia, and the increase in size of the twitch in increased sodium concentrations is light. In concentrations of TEA as low as 15 mM there ensued complete and prolonged flexion of the tibia as a result of a single nerve stimulus. The vigorous and prolonged nature of the contraction in TEA salines appeared to be associated with tetanic trains of action potentials following each applied single stimulus.

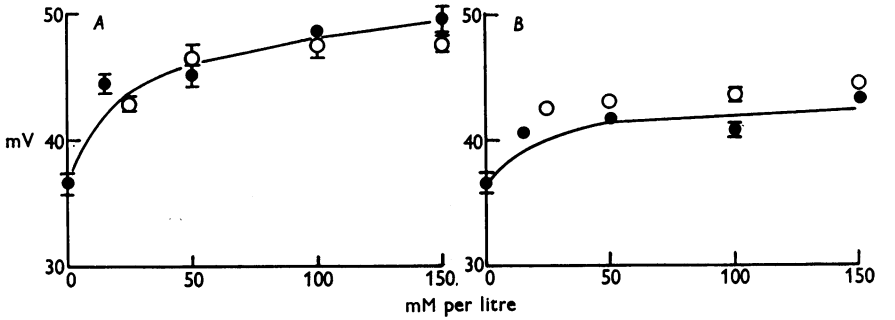

Fig. 6. Relation between size of *A*, resting potential, *B*, action potential and external sodium, ●, and tetramethylammonium, ○, concentrations.

When tetramethylammonium (TMA) ions were substituted for sodium ions, the values of the resting and action potentials were very similar to those obtained in the equivalent sodium salines (Fig. 6). However, although TMA ions proved a suitable substitute for sodium in this respect, their use resulted in a marked decrease in the rate of rise of both the junctional potential and the active membrane response by comparison with sodium ions (Figs. 7, 8). This effect could be reversed by bathing in normal saline containing sodium.

The effect of potassium ions

Potassium ions exerted a depolarizing action upon the muscle fibre membrane similar to that observed in other excitable tissues (Hodgkin, 1951; Fatt & Katz, 1953; Hoyle, 1953). The largest resting potential occurred in zero potassium: as the external potassium concentration was raised, the magnitude of the resting potential fell (Fig. 9).

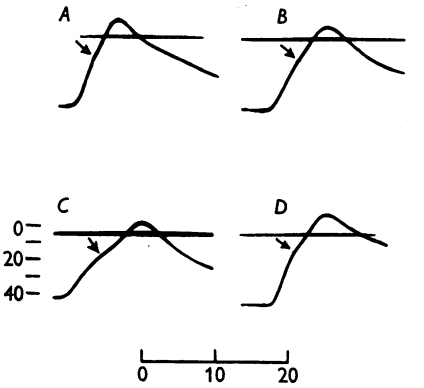

Fig. 7. Records from different fibres. *A*, in 50 mM-Na; *B*, in 15 Na; *C*, in 100 TMA; *D*, in 50 TMA. The arrows mark the inflexion in the rising phase.

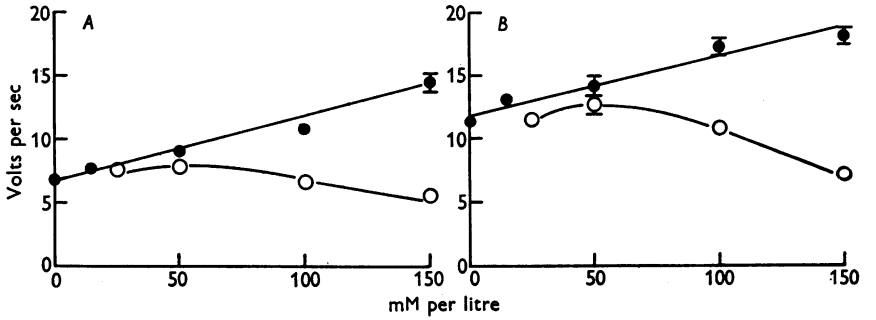

Fig. 8. Relation between rate of rise of *A*, junctional potential, *B*, active membrane response and external sodium, ●, and tetramethylammonium, ○, concentrations.

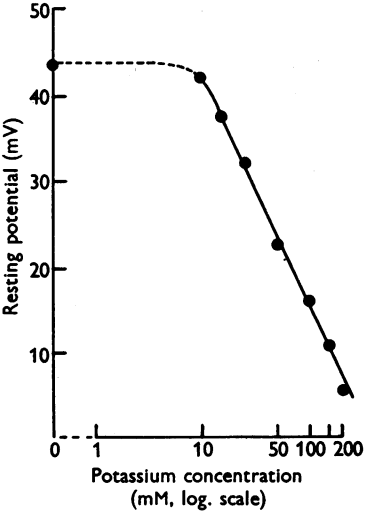

Fig. 9. Relation between size of resting potential and external potassium concentration (logarithmic). Equilibration time of 30 min.

The action potential was also affected by alterations in the potassium level. It declined in magnitude as the potassium concentration was raised (Fig. 10). In a typical case the action potential fell, after 30 min in 100 mM-K, to about 20 mV, compared with a mean value of $44 \pm 1{\cdot}2$ mV from fibres of the same muscle in zero potassium saline. Records in which the junctional potential (j.p.) and active membrane response (a.m.r.) could be distinguished appeared to agree with the findings of Hoyle (1955*a*) in locust and cockroach muscle fibres. He found that in raised potassium the a.m.r. showed a progressive decline which was correlated with a decline in the j.p. In fibres of *Carausius* which show an overshoot of zero potential in normal saline, the overshoot persists when the potassium concentration is raised up to about 100 mM, though above this figure it tends to disappear.

A replacement time of 30 min is not sufficient for the complete development of the effect of a change in potassium concentration in superficial fibres. The decline in size of the resting and action potentials in raised potassium begins

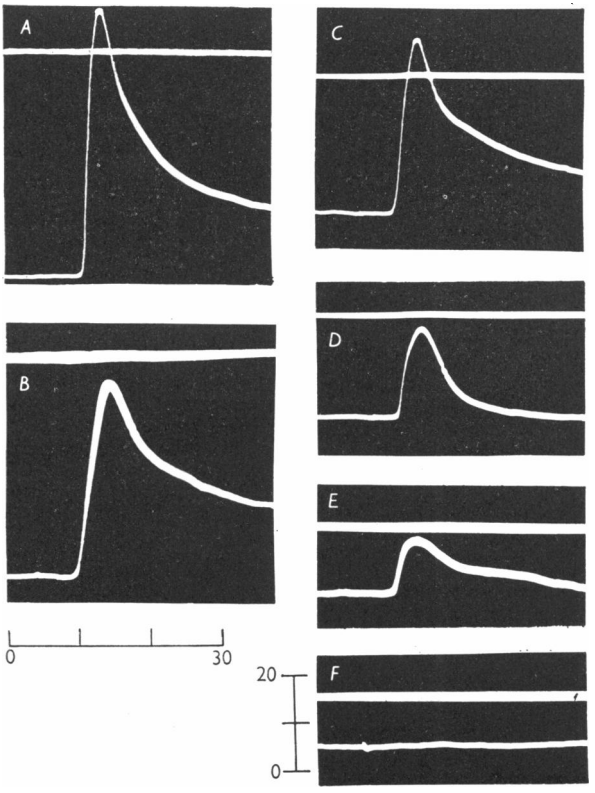

Fig. 10. Sample action potentials from a muscle at different potassium concentrations. *A*, zero; *B*, 18; *C*, 50; *D*, 100; *E*, 150; *F*, 200 mM. Note the overshoot in *C*, and the presence of both j.p. and a.m.r. in *D*.

almost immediately the saline has been changed and is rapid initially, but becomes slower, and the final equilibrium levels may not be reached for an hour or more. Thus, with a longer replacement time, the curve relating resting potential to potassium concentration (Fig. 9) slopes more steeply to cross the base line at about 150 mM-K, at which concentration, if the muscle is allowed to soak for a sufficient period, the action potential is abolished. If it is soaked in the saline long enough for all the fibres of the muscle to be affected in this way, the muscle fails to contract in response to either single or repetitive stimulation of its nerve.

The effect of calcium ions

A rise in the external calcium concentration is accompanied by an increase in the magnitude of the resting and action potentials (Fig. 11). The increase in size of the resting potential from zero to 10 mM-Ca was about 5 mV: that of the action potential was much greater, amounting to about 36 mV. The change

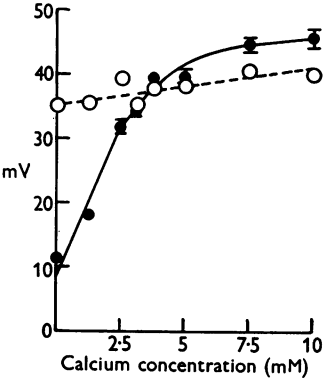

Fig. 11. Relation between size of resting potential, ○, and action potential, ●, at different calcium concentrations.

in size of the action potential was greatest between zero and 5 mM-Ca, the part of the curve between these values being approximately linear. Above about 5 mM an increase in calcium concentration has a progressively smaller effect upon the action potential, and it will be seen from Fig. 10 that the slope of the curve relating to the action potential is more comparable to that of the resting potential. In a few cases the muscle was bathed in 25 mM-Ca, but this resulted in an increase in the action potential of only a few mV, indicating that the curve is almost horizontal at concentrations above 10 mM.

When the muscle was transferred from normal saline to zero calcium, there was a rapid alteration in the shape of the action potential (Fig. 12). The a.m.r. came off the j.p. progressively later, and the time course of the whole response became slower. As the lack of calcium took full effect both components declined in size, but the decline was more rapid in the case of the a.m.r., and it

disappeared while the j.p. itself was still quite large. The j.p. itself did not fall below about 8 mV by the end of the standard replacement time of 30 min.

The separation of the j.p. from the a.m.r. in reduced calcium allowed the magnitudes and rates of rise of the two components to be examined separately. As in the case of the results obtained in sodium salines described earlier, the

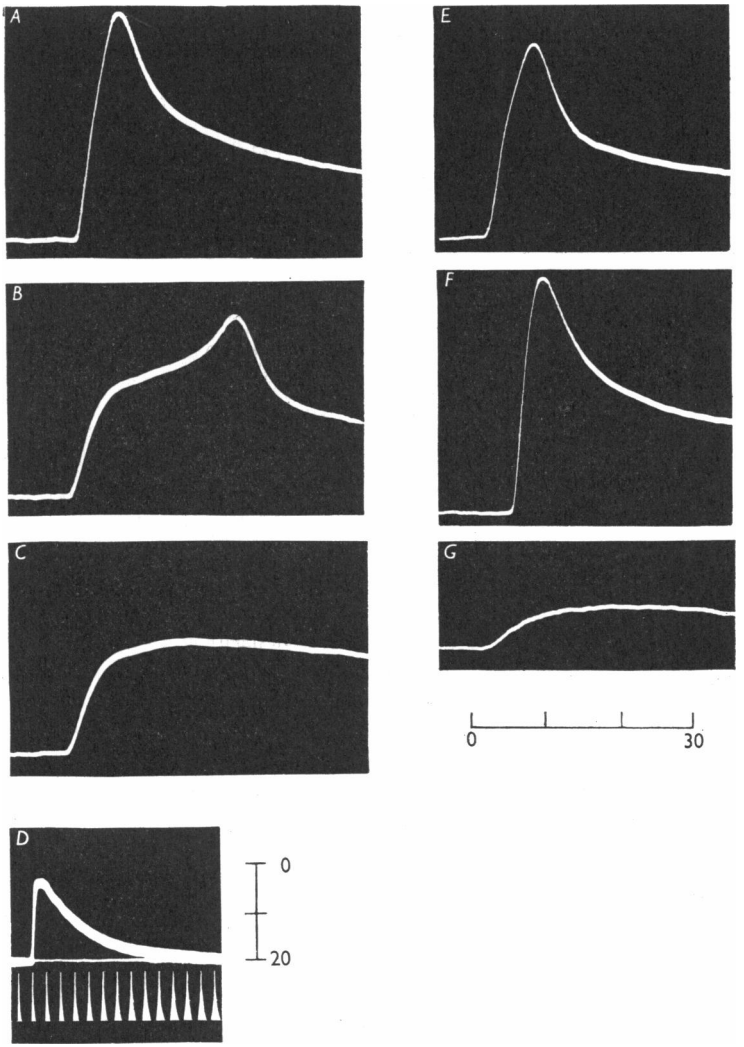

Fig. 12. The effect of calcium. *A–D*, records from the same fibre: *A*, in normal saline; *B*, 3 min after transference to zero calcium; *C*, after a further 3 min; *D*, a record taken a few minutes after *C*, on a faster time base to show time course. *E–G*, records from another fibre. *E*, in normal saline; *F*, in 10 mM-Ca; *G*, in 0 Ca (after 30 min). Calibration: 50 c/s in *D*, otherwise msec and mV.

bend in the rising phase of the action potential was taken as the junction between the two. To investigate the validity of this criterion, the muscle was transferred from normal to zero-calcium saline, and when the supposed a.m.r. had just disappeared, a pair of suitably spaced stimuli were applied to the crural nerve. Each stimulus of the pair produced a separate response of the muscle fibre membrane, and the two responses tended to summate, when they were followed by a further membrane response, similar in size and shape to the supposed a.m.r. which had previously disappeared (Fig. 13). The two responses were not followed by a further response when the paired stimuli were sufficiently far apart to prevent summation; nor when further soaking in zero calcium had reduced the size of the two summating responses below a certain level. The summating responses must apparently reach a certain critical height if they are to give rise to the further membrane response. It is evident from these results that the hump in the rising phase seen in reduced calcium marks the junction of two distinct components, and it is assumed that the similar,

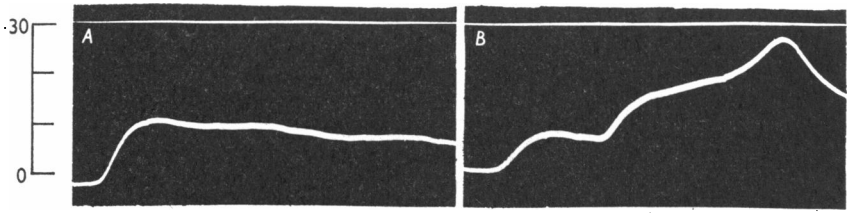

Fig. 13. *A*, in zero Ca, junctional potential only. *B*, same fibre a few minutes later: paired stimuli elicited an active membrane response.

but slighter, hump noted in other salines is comparable. It appears reasonable to suppose, by analogy with the muscle fibres of other animals, that the two components involved are the end-plate or junctional potential and the active membrane response.

Fig. 14 shows curves which relate the magnitudes of the two components to the calcium concentration. Between about 2·5 and 5 mM-Ca the relationship between the calcium concentration and the a.m.r. is substantially linear. An a.m.r. is not present below 2–2·5 mM-Ca: above this level it shows only about a 3 mV variation up to 10 mM-Ca. A similar degree of variation occurs in the resting potential in the same calcium concentrations (Fig. 11). It is possible that the size of the a.m.r. in different calcium concentrations is related to the value of the resting potential.

The rates of rise of the j.p. and a.m.r. are affected by alterations in the calcium concentration (Fig. 15). Between zero and 7·5 mM-Ca the rate of rise of the j.p. is directly proportional to the external calcium concentration. After an initially steep rise the curve for the a.m.r. continues upwards at a

slower rate. The rate of rise of the a.m.r. increases by about 5 V/sec when the calcium is raised from 2·5 to 7·5 mM.

The sensitivity of the junctional potential to changes in the calcium level suggests that in *Carausius*, as in the locust and cockroach (Hoyle, 1955*a*), calcium exerts an effect upon the release of some neuromuscular transmitter, similar to that seen in vertebrates (del Castillo & Stark, 1952). Hoyle (1955*a*)

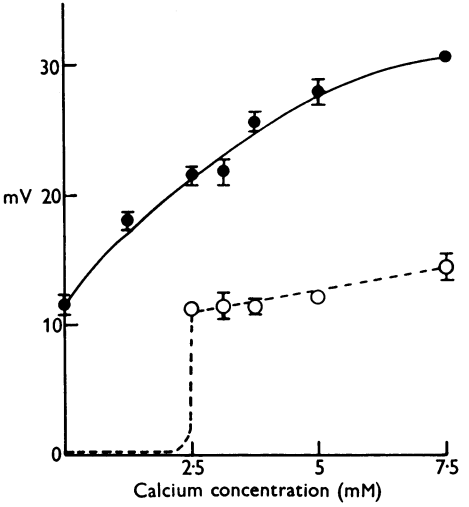

Fig. 14. Relation between size of junctional potential, ●, active membrane response, ○, and external calcium concentration.

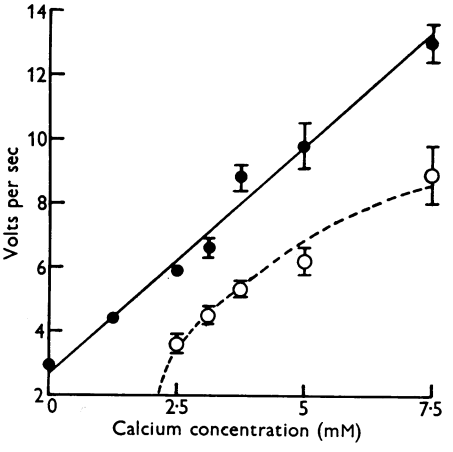

Fig. 15

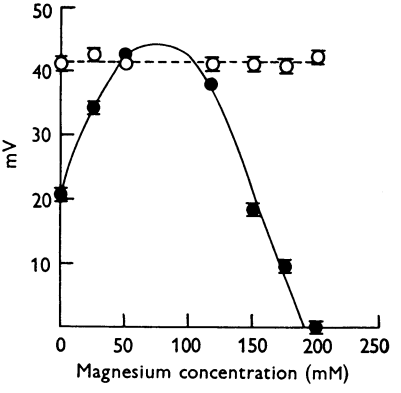

Fig. 16

Fig. 15. Relation between rate of rise of junctional potential, ●, active membrane response, ○, and external calcium concentration.

Fig. 16. Relation between size of resting potential, ○, action potential, ●, and external magnesium concentration.

pointed out that, nevertheless, calcium does not alter the time course of the vertebrate end-plate potential. He found that in the locust and cockroach, as in *Carausius*, the time course of the junctional potential became slower in low calcium.

The effect of magnesium ions

The relationship between the magnitudes of the resting and action potentials and the external magnesium concentration is shown in Fig. 16. The resting potential does not alter significantly in size between zero and 200 mM-Mg. The action potential, however, decreased in size above and below a magnesium level of about 50–75 mM-Mg. In the locust and cockroach (Hoyle, 1955*a*) and in vertebrates (del Castillo & Engbaek, 1954) magnesium inhibits the release of the neuromuscular transmitter, and a decrease in magnesium concentration is accompanied by an increase in the height of the end-plate or junctional potential. In *Carausius* it appears to be the action potential as a whole which declines, as in high potassium.

Above about 75 mM-Mg the action potential falls in size as the magnesium level is raised. The change in the action potential is similar in some respects to the neuromuscular block which occurs in other muscle fibres subjected to magnesium concentrations several times greater than normal (del Castillo &

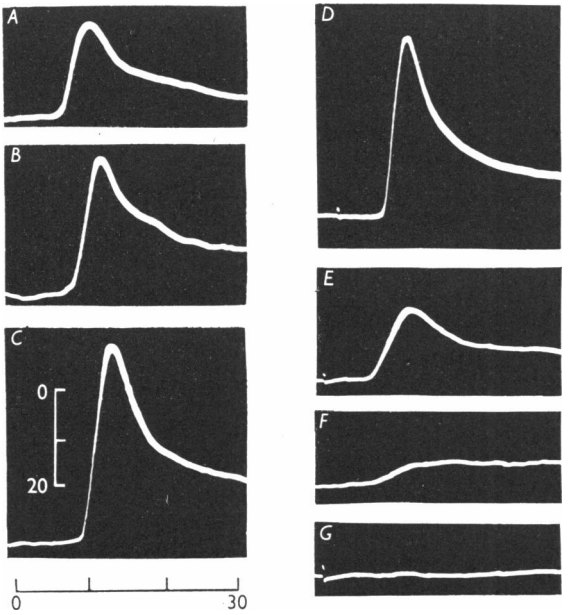

Fig. 17. Effect of low and high magnesium on action potential. *A–C*, records from the same fibre: *A*, in zero-Mg; *B*, in 25 mM-Mg; *C*, in 50 mM-Mg. *D–F*, records from another fibre: *D*, in 50 mM-Mg; *E–G*, progressive deterioration in 200 mM-Mg; records at 5 min intervals after transference to this saline.

Engbaek, 1954; Hoyle, 1955*a*). As the magnesium level is raised, the decline in the action potential increases, and the a.m.r. eventually disappears, the decrease in size of the j.p. being more gradual (Fig. 17). The general effect is therefore similar to that seen in low calcium; and in the locust and cockroach (Hoyle, 1955*a*) and in vertebrates (del Castillo & Engbaek, 1954) calcium antagonizes the neuromuscular block produced by magnesium. However, the changes produced in these animals by high magnesium are not entirely comparable to those which occur in *Carausius*, where the a.m.r. and j.p. did not become separated in time, as they did in low calcium; and the effect of high magnesium was not antagonized by calcium ions. The addition to the 200 mM-Mg saline of sufficient calcium to restore the calcium: magnesium ratio to that of the normal saline neither abolished nor arrested the progressive decline of the action potential. The hypertonicity of the 200 mM-Mg saline would not be expected to cause a decline of this magnitude, if at all.

In all cases where the action potential was reduced in size, the twitch tension of the muscle fell. When the muscle was soaked in 200 mM-Mg long enough for all the muscle fibres to be affected, the twitch tension fell to zero and no contraction could be elicited either by single shocks or repetitive stimulation. The effect could be reversed by prolonged washing in normal saline.

The action of hyaluronidase

In order to test the possibility that some kind of barrier surrounds the muscle fibres in *Carausius*, experiments were carried out in which 'Hyalase', a commercial preparation of hyaluronidase kindly supplied by Messrs Bengers, was added to certain salines. It was hoped that any connective tissue component of such a barrier would be attacked by the enzyme, resulting in a significant alteration in the action of the salines. The hyalase was added to the normal saline at a strength of 1000 u./100 ml. and the muscle was soaked in this saline for 3 hr. It was then transferred to zero or 200 mM-Na salines, also containing hyalase. No change was observed in the electrical response of the muscle fibres as a result of soaking in any of these salines.

DISCUSSION

The nerves of the locust (Hoyle, 1953), and possibly of all insects (Hughes, 1953), are surrounded by a sheath which probably acts as a selective barrier and which provides a more favourable ionic environment for the nerve than the haemolymph. In the locust this sheath ends at the motor nerve ending (Hoyle, 1955*b*). Several of the facts reported here might be considered to indicate the presence of a similar kind of selective barrier around the muscle fibres of *Carausius*. These are: (i) the relatively small effect of sodium ions upon the size of the action potential; (ii) the high magnesium concentrations required to produce neuromuscular block; (iii) the rather long replacement

times necessary for changes in the external ionic concentration to exert their full action upon the muscle fibres; and (iv) the considerable depression of neuromuscular transmission which takes place in hypotonic salines. The latter could be due to the osmotic passage of water through the barrier, the absence of a compensating passage of ions resulting in dilution of the fluid contained between the barrier and the muscle fibre.

There is much evidence against the possibility that any barrier in *Carausius* is actively selective. The absence of any change in the action of salines after the application of hyaluronidase is interesting but not at all conclusive. However, there are also a number of facts which suggest that the various ions are, in fact, reaching the muscle fibres and acting directly upon them, but that in some cases their action upon the fibres is different from that expected from a comparison with the muscle fibres of other animals.

Thus, a selective barrier of the kind under discussion might be expected largely to exclude magnesium and possibly potassium ions, but to concentrate sodium ions around the muscle fibres. In this case it seems probable that after transference to a low magnesium saline the barrier would either tend, at least for a time, to withdraw even more magnesium from the fluid surrounding the fibre; or, if slow acting, maintain the Mg level at its normal value for the duration of the experiment. From results obtained with the muscle fibres of other animals (del Castillo & Engbaek, 1954; Hoyle, 1955*a*) this should result in an enhancement of neuromuscular transmission or in no effect at all. Even if the barrier adjusted so rapidly that its action was missed, a depression of neuromuscular transmission in low magnesium salines would not be expected. The fact that such a depression is observed in *Carausius* suggests that the reduced magnesium concentration is acting directly upon the muscle fibres, but the effect is different from that seen in other animals so far studied.

The long replacement times are adequately explained by the presence of tracheolated connective tissue around the groups of muscle fibres, and around individual fibres. Hoyle (1953) found that the tracheolated membrane around locust muscle acted as a partial diffusion barrier to potassium ions. A comparable tracheolated membrane is present in *Carausius*, and would be expected to exert a similar delaying effect.

The size of the action potential in different sodium salines varies less than might have been expected in view of the results obtained from vertebrate muscles (Fatt & Katz, 1952). However, similar variations are observed when sodium ions are replaced by TMA ions. It is therefore possible that any 'carrier' mechanism which may be involved in the inward current of the action potential (Hodgkin, 1951) is not specific for sodium ions.

Sodium and TMA ions also influence the rate of rise of the action potential, but here their actions are different. Increased sodium concentrations result in an increased rate of rise, whereas TMA salines have the reverse effect. If

sodium ions are not, in fact, specific carriers of the inward current, it could be argued that their major effect is upon the way in which charge is transported across the membrane. If TMA ions cannot exert a similar influence, the reason for the different rates of rise in the two salines is explicable. It is necessary to remember, however, that the reason might equally lie in the relative ability of a 'carrier' to deal with the two different ions.

It is thus evident that a selective barrier is not the only possible explanation of the alterations in the action potential in sodium and TMA salines. It is interesting to note that Fatt & Katz (1953) replaced sodium ions by quaternary ammonium ions in the fluids bathing the muscle fibres of certain crustaceans, and found that they continued to function as well as, or better than, in comparable sodium salines. They suggested that the results they obtained with TMA salines could be explained on the assumption that the action potential in these animals involves an influx of calcium or magnesium ions, or an outflux of some internal anion; and that sodium plays only an indirect role during the production of the action potential, 'conditioning' the excitatory reaction of the membrane without being a carrier of the current. A similar explanation could be applied to *Carausius*.

It must be admitted that such an explanation does not account for the fact that impulses passing down the motor nerve were able to excite the muscle fibres in zero sodium. Histologically, the visible sheath which is regarded as the ion barrier which protects the nerve from adverse environmental conditions (Hoyle, 1953; Twarog & Roeder, 1956) appears to end at the point where the motor nerve ending branches out into the terminal claw, or end-plate. The end-plate is therefore exposed to solutions bathing the muscle fibres. If the observed facts were due merely to the presence of a passive barrier, then a steady but slow decline in both nerve and muscle response would be expected with prolonged soaking, since some effect is observable within a few minutes of changing the experimental saline. In these experiments, soaking in zero sodium extended over 3 hr, and no change occurred in the muscle response after the expiry of the experimental time of 30 min; and the nerve was still conducting after 3 hr. If, on the other hand, a selective barrier is operating, it must be operating only for sodium ions, since calcium and potassium act in a comparable way in *Carausius* and in vertebrates; and a selective barrier in respect to magnesium ions has already been rejected. On balance, therefore, the presence of motor nerve activity in zero sodium is best left as a fact which at present cannot be explained.

The question arises whether calcium or magnesium, or both, contribute to the inward current involved in the production of the action potential. While it seems highly probable that calcium ions affect the size and rate of rise of the junctional potential, through their influence upon the release of a neuromuscular transmitter, the fact that the active membrane response varies by

only 3 mV between 2·5 and 10 mM-Ca suggests that any contribution of this kind by calcium ions is negligible.

If magnesium contributes to the inward current, an explanation of the decline in size of the action potential at low magnesium concentrations becomes apparent. The tolerance of the muscle fibres of *Carausius* to high magnesium concentrations is so striking, however, by contrast with other muscle fibres (del Castillo & Engbaek, 1954; Hoyle, 1955*a*) that it is possible that the decline in low concentrations is bound up with the mechanism involved in this tolerance. There is no evidence to show what this mechanism may be.

The suggestion of Fatt & Katz (1952) that in certain crustacean muscle fibres an outward flux of some internal anion might be responsible for the action current must also be considered in regard to *Carausius*. Direct evidence is lacking, but it is just possible that hypotonic salines depress the muscle response by producing an osmotic inflow of water into the fibre which results in the dilution of such an anion within the muscle fibre. Although both the resting and action potentials are altered in size by hypotonic salines, the reduction in the action potential is greater than the corresponding reduction in the resting potential.

There is nothing to show how sodium and TMA ions alter the magnitude of the resting potential. It might be supposed that changes in the resting potential, when the concentrations of these ions are altered, merely reflect the accompanying change in chloride concentration. However, there is no evidence that the muscle fibre membranes of insects are freely permeable to chloride ions. In *Carausius* variation in the magnesium chloride content of the bathing fluid over a wide range of concentrations had no effect upon the magnitude of the resting potential.

The effect of calcium upon the resting potential may be connected with the shift it produces in the curves relating sodium and potassium permeability to the membrane potential of the squid giant axon (Frankenhaeuser & Hodgkin, 1955).

The action of potassium ions upon the resting potential appears to be similar to that observed in other muscle fibres. The time necessary for replacement of these ions may be due to tracheolated tissue surrounding the muscle fibre membrane; or to a slight impermeability to potassium ions. However, replacement does eventually occur, and the normal resting potential is rather low compared with, for example, that found in the locust (Hoyle, 1953) but not so low that the functioning of the muscle fibres is seriously impaired. It may be that in other herbivorous insects with a much higher blood potassium level (e.g. see Duchateau *et al.* 1953) mechanisms may be discovered which counteract the effect of such potassium concentrations. Such mechanisms are not needed in *Carausius*.

SUMMARY

1. Analyses of the blood ionic composition of the stick insect, *Carausius morosus*, have confirmed that this animal is a typical herbivorous insect, with low sodium and high magnesium concentrations in the blood, resulting in sodium:potassium and calcium:magnesium ratios of less than unity.

2. The effects of changing the sodium, potassium, calcium, and magnesium concentrations of the bathing fluid upon the 'fast' type electrical response of the flexor tibialis muscle of *Carausius* have been studied with glass capillary intracellular micro-electrodes.

3. Lowering the sodium concentration results in a decrease in the magnitudes of the resting and action potentials, but an active membrane response is still present in zero sodium. The rate of rise of the action potential increases as the sodium concentration is raised.

4. When tetramethylammonium ions are substituted for sodium ions in the bathing fluid, the magnitudes of the resting and action potentials vary in different concentrations to a similar extent to the corresponding sodium salines; but the rate of rise decreases as the TMA level is raised.

5. It is suggested that sodium ions may not be specific carriers of the action current, but that they may affect the excitability of the muscle fibre membrane.

6. Calcium ions are necessary for the development of the junctional potential. It is suggested that they influence the liberation of a neuromuscular transmitter substance.

7. When the magnesium concentration is raised above about 75 mM the action potential declines in size, until neuromuscular block resembling that observed in low calcium salines results. This effect is not antagonized by calcium ions.

8. The action potential declines in size when the magnesium concentration is lowered below about 50 mM.

9. A rise in the external potassium concentration results in a fall in the magnitudes of the resting and action potentials, but a long replacement time is necessary for full development of this effect.

10. The possibility that a selective ion barrier is present around the muscle fibres of *Carausius* is discussed, and rejected, with reservations in the case of sodium ions.

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