Biochemical and electrophysiological studies on muscle fibres from Periplaneta Americana L. and Sphinx Ligustri L

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BIOCHEMICAL AND ELECTROPHYSIOLOGICAL STUDIES ON MUSCLE FIBRES FROM *PERIPLANETA AMERICANA* L. AND *SPHINX LIGUSTRI* L.

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ABSTRACT

The permeability properties of skeletal muscle from Periplaneta americana and Sphinx ligustri have been studied by several methods and in each the effects of pharmacological agents and of temperature have been investigated.

The properties of the microsomal preparation of skeletal muscle were characterized. Compared with preparations from many other types of excitable cell, although probably not muscle, the microsomal preparation was atypical in lacking an enzyme synergistically stimulated by monovalent cations and in being insensitive to strophanthin G.

Normal trans-membrane inorganic ion distributions of skeletal muscle were determined. The ratios were similar in the two animals and not markedly dissimilar from those of vertebrate muscle / blood ratios. The results obtained with Sphinx muscle differed from those of previous workers. Based on the results obtained a new saline was developed for bathing the muscles of Periplaneta and Sphinx which proved to be more suitable than salines used by other workers.

Intracellular microelectrode recordings were made from resting and active skeletal muscle of Periplaneta. The relative contributions to the resting membrane potential of actively and passively distributed ions were determined. A considerable hyperpolarizing contribution to the resting membrane potential was made by a metabolically dependent constant current generator. Similar results were obtained using Sphinx skeletal muscle. An electrical model was derived to account for the phenomena observed. The individual contributions of inorganic ions to depolarizing electrogenesis were determined. Sodium was found to make a major contribution. The possible role of electrogenic pumps in the development of end-plate potentials was discussed. The excitatory postsynaptic potential of Periplaneta

skeletal muscle was compared with the end-plate potential of vertebrate skeletal muscle and the possible nature of the biphasic response of Periplaneta muscle to excitation discussed. Possible mechanisms of excitatory and inhibitory electrogenesis were related to the model developed to describe the resting membrane potential.

CHAPTER 1 GENERAL INTRODUCTION.

The literature contains several very good reviews of the current state of knowledge concerning insect muscle neurophysiology (Hoyle, 1962; Aidley, 1967; Usherwood, 1967a & 1969). These reviews have been concerned with neuromuscular transmission, the excitatory mechanisms of muscle and the effect of inorganic ion distributions on membrane potentials. These papers aim to draw together information concerning many different insect species. One point which is evident from a study of the literature is that, although the mechanisms underlying the production and maintenance of bioelectric potentials are different from those in vertebrate muscle, they are not clearly understood. The purpose of this work therefore, was, by making a detailed study of one species, to make a contribution to the understanding of bioelectric phenomena in insect muscle.

The anatomy and histology of Periplaneta americana has been well documented (Guthrie & Tindall, 1968). The muscles used in the present study were the skeletal muscles of the coxae of the cockroach. The fibres of these muscles are striated and normally contract and relax quickly, usually one twitch lasting less than 100 msec (Usherwood, 1962b). Histologically the muscles are very similar to those of vertebrates. Periplaneta muscle fibres are about 100 μm in diameter. The contents of the fibres are bound by the plasma membrane or sarcolemma and include mitochondria, sarcoplasmic reticuli, sarcoplasm, nuclei and fibrils which contain the two contractile elements. The fibrils consist of interdigitating thick (myosin) and thin (actin) filaments, characteristic of vertebrate skeletal muscle. They are regularly striated with A- and I-bands and divided transversely into sarcomeres by dense Z-lines.

The plasma membrane of the muscle fibres is normally about 7.5 nm thick with the characteristic "unit membrane" structure. It is invaginated to form the transverse tubular system with two morphologically

distinct elements, tubules and sarcolemmal invaginations (Cochrane & Elder, 1967). The sarcolemmal invaginations are circular in cross section but the tubules are flattened (Hoyle, 1965). Sarcolemmal invaginations enter at the Z-lines and give off vertical and horizontal branches. The tubules enter opposite the overlap regions of actin and myosin filaments. Branches of the transverse tubular system make contact with the sarcoplasmic reticuli where they form diadic junctions. There are two bioelectric components to the plasma membrane, an electrically excitable, nonsynaptic part, and an electrically inexcitable, but chemically excitable, synaptic region. These regions are structurally indistinct (Usherwood, 1967a).

The plasma membrane of muscle cells is selectively permeable. It has been proposed that the membrane contains aqueous channels, possibly with positive and negative fixed charge sites controlling the passage of ions (Shanes, 1958). This has been called the passive control of permeability. Analyses of the permeability properties of the plasma membrane could lead to forecasts of the electrical properties of excitable cells. An unequal distribution of permeable ions across the plasma membrane will result in the development of a potential difference predictable by the distributions of the ions involved according to the Nernst relationship. Electrogenic pumps (the active movement of ions across the membrane so that there is a net current flow in one direction) may also contribute to the potential difference established. In insects it is also possible that the unequal distribution, and perhaps the active pumping, of organic ions may also contribute to the potential developed (Usherwood, 1969). All these factors may contribute to the potential difference established across an excitable cell membrane at rest i.e. the resting membrane potential.

Two examples of the resting potential of an excitable cell being

dependent upon the distribution of potassium across the cell membrane are found in squid axon (Curtis & Cole, 1942) and in frog muscle (Hodgkin & Horowicz, 1959). In the former case the membrane is thought not to be freely permeable to chloride ions and it has been suggested that an active inward movement of chloride occurs (Keynes, 1963). It has not been shown if this inward anion movement contributes to the resting membrane potential. In frog muscle on the other hand, chloride ions are freely permeable and are therefore distributed according to the potential across the membrane (Hodgkin & Horowicz, 1959). Kernan (1968) however has proposed that under certain conditions an electrogenic contribution to the resting potential of frog muscle may be observed. Kernan has suggested that the membrane potential of frog muscle is determined by a diffusion potential and a parallel active depolarizing process. The depolarizing process is suggested to be a potassium pump, activated by decamethonium and dependent upon metabolic energy for maintenance. The contribution of potassium and chloride ions to the resting potential of cockroach skeletal muscle fibres is not fully understood. The muscle membrane is permeable to potassium ions and the resting potential is thought to be determined by their distribution (Hoyle, 1953; Wood, 1957 & 1963). The membrane has been suggested to be only slightly permeable (Wood, 1965), and fully permeable (Usherwood, 1967a & 1967b), to chloride ions. There has been no investigation made as to the possibility of a contribution to the resting potential by an electrogenic pump.

Frequently, the observations of bioelectric potentials have failed to agree with values predicted by the Nernst or Goldman equations based upon inorganic ion distributions (Huddart, 1966a; Ashhurst, 1967; Carpenter & Alving, 1968). In members of lower insect orders such as Periplaneta the inorganic ion concentrations of the haemolymph approach

those of vertebrate extracellular muscle fluid. However in some phytophagous primitive insects (e.g. Cheleutoptera) and in some higher insect orders the ionic content of the haemolymph is markedly different from that of vertebrates (Usherwood, 1969). For example the haemolymph of some Lepidoptera may contain high potassium, high magnesium and low sodium concentrations (Huddart, 1966a). These are interesting conditions since the muscles of these animals function in an environment in which a vertebrate muscle would become inexcitable.

Insect haemolymph also contains metabolites, particularly a high concentration of amino acids. In Lepidoptera trehalose is found in very high concentrations. The main organic acids in insect haemolymph are the substrates of the tricarboxylic acid cycle (Usherwood, 1969). Changes in amino acid content of the haemolymph are thought to compensate for fluctuations in inorganic ion concentrations (Florkin & Jeuniaux, 1964), although some ionic regulation does occur (Stobbart & Shaw, 1964).

During excitation of nerve and muscle it has usually been found that some cation species, usually sodium, enters the cell. Where this is the case some system must be present to remove those ions which have entered. Enzyme systems thought to be responsible for the active transport of cations have been found in the plasma membrane of many tissues (Skou, 1964). No equivalent study of insect muscle has been attempted, but knowledge of the enzymic properties of the cockroach muscle membrane might be of value in establishing the nature of the ionic current developed during excitation. Cockroach skeletal muscle fibres are different from vertebrate fibres in that they are frequently polyneurally innervated i.e. they receive endings from more than one axon (Usherwood, 1967a). Furthermore, they may be innervated by both excitatory and inhibitory nerves (Usherwood & Grundfest, 1964 & 1965). One of the

most striking characteristics of insect muscles, first described by Foettinger in 1880 (from Usherwood, 1967a), is multiterminal innervation of the fibres. Nerve endings are distributed at 30-60 μm intervals along the length of the fibres (Edwards, 1959). As a result of this form of innervation the propagation of an impulse along muscle fibres, as occurs in vertebrates, is unnecessary and decremental propagation results. Furthermore, the electrical response to excitation is graded and depends upon stimulus strength. The nature of the ionic current during excitation of muscle is not understood in insects. Usherwood (1969) has suggested that sodium ions are involved to some extent in locust and stick insect excitatory responses, whereas Wood (1963) suggested that cockroach muscle fibres have only a limited permeability to sodium ions during activity. In insects (such as Sphinx ligustri), where, in comparison with vertebrate muscle, the sodium gradient is reversed across the muscle membrane, it is less likely that sodium could be involved. Inhibitory responses have also been recorded from cockroach muscle fibres (Usherwood, 1967a). The inhibitory potential developed is thought to be due to an increase in permeability to chloride ions (Usherwood & Grundfest, 1965).

The aim of this project was to study the muscle physiology of one animal (Periplaneta americana) in detail and then to refer to an animal with an apparently different physiology (Sphinx ligustri), for comparison. It was the intention to approach the problem from several angles and to combine in one study the biochemistry of, the electrophysiological properties of, and ionic movements and distributions across, the skeletal muscle membrane. In such a way it was hoped that it would be possible to erect a hypothesis to account for the production and maintenance of bioelectrical potentials in one insect species. The wider application of this hypothesis could then be determined using the data

obtained from Sphinx ligustri and published data for other species.

CHAPTER 2 GENERAL METHODS AND MATERIALS.

1. Animal culture.

a. Periplaneta americana. Periplaneta is a relatively easy animal to rear. There are 9-13 stadia to maturity (Willis, Riser & Roth, 1958) and the duration of this nymphal period is 4-5 months at 25-30°C (Biellmann, 1960). Adults range from 29-40 mm in body length and at 25-30°C will live for up to three years. Both male and female adults were used in this study. Three cultures were maintained so that a ready supply of adults was available. They were kept in fish tanks at 28°C in a 12 hour light and 12 hour dark regime. Their staple diet was bran, supplemented occasionally with slices of carrot and potato. Animals were caught and prepared for experimentation without any anaesthesia.

b. Sphinx ligustri. Sphinx, otherwise known as the Privet Hawk Moth, is one of the largest native Hawk Moths of the British Isles. It has a wing span of up to one decimetre and a body length of 4 centimetres. The moth is beautifully marked in shades of brown and purple. The fully fed caterpillar is also very striking in appearance and may exceed 8 centimetres in length. It is bright apple-green with seven sloping white stripes, edged in front with violet, and behind with yellow. Pupae were kept in the dark at 4°C during the winter. In March they were exposed to room temperature and natural day and night rhythms. Usually within one month the adult moth emerged and was kept at room temperature. In the adult stage the animals were not fed since experimental animals were used within a few days of emergence. A few moths were kept alive for longer and allowed to breed. Fertilised eggs were collected and placed on damp filter paper. Caterpillars typically hatched after 10-14 days when they were fed on chopped privet leaves. When larger the caterpillars were allowed to browse on branches of privet. Just prior to pupation the caterpillars developed a brown

streak dorsally. At this stage they were placed on sand into which they burrowed and then pupated. Two to three weeks later it was possible to dig up the sand and recover the pupae which were transferred to 4°C for storage. Since no reliable method was found to break the pupal diapause, experiments with these moths were restricted by the times of emergence. Experimental animals were caught and prepared without anaesthesia.

2. Glassware.

Glassware used for measuring purposes was E-MIL gold line standard. All glassware was washed six times in hot water, four times in distilled water and rinsed in deionized water. Deionized water was obtained by passing distilled water through an Elgastat deionizer column.

3. Chemicals.

Inorganic salts were AnalaR grade where possible and supplied by British Drug Houses, Poole, as were DNP, EDTA, TCA, DOCA and Folin & Ciocalteau's phenol reagent. Bovine serum albumen (fraction V), Tris, histidine, Fiske and Subbarow reducing reagent, guanidine, strophanthin G, sodium ∝ glycerophosphate, cytochrome c and the sodium salts of ATP, ITP, UTP and CTP were obtained from Sigma Chemical Company, St Louis, USA. ATP, ITP, CTP, UTP and BSA serum albumen were stored desiccated at -20°C.

4. Radioactive isotope.

^{22}Na was obtained as sodium chloride solution. Both ^{22}Na and tritiated inulin were supplied by the Radiochemical Centre, Amersham. I am indebted to Professor Boulter of the Botany Department of Durham University for the loan of radioactive counting equipment and for the use of his liquid scintillation counter.

Class : INSECTA

Sub-Class : PTERYGOTA

Section : POLYNEOPTERA

Super-Order : BLATTOPTEROIDEA

Order : DICTYOPTERA

Sub-Order : BLATTODEA

Series : NEOBLATTARIA

Super-Family : BLATTOIDEA

Family : BLATTIDAE

Species : PERIPLANETA AMERICANA Linnaeus.

CHAPTER 3 THE BIOCHEMISTRY OF THE MUSCLE MEMBRANE.

INTRODUCTION

Reports of studies of membrane enzyme systems using ATP as a substrate are numerous in the literature. These ATPases (E.C. 3.6.1.3.) have been described for many varied tissues, including brain (Bowler & Duncan, 1968a), kidney (Skou, 1962), and muscle (Duggan, 1965). These enzymes require magnesium ions and hydrolyse ATP to ADP plus inorganic phosphate. This basic activity is stimulated in the presence of sodium and potassium ions, which are described as acting synergistically. The classical membrane ATPase system is composed of two fractions. The first is a $Na^+ + K^+$ stimulated Mg^{2+}-dependent ATPase, inhibited by cardiac glycosides and associated with the active transport of cations (Skou, 1965). The second is a Mg^{2+}-dependent ATPase, unaffected by cardiac glycosides and which has been suggested as being responsible for the control of passive permeability of cells (Duncan, 1967).

From studies of such enzyme systems it has been concluded that the monovalent cation stimulated ATPase represents the enzyme located in the plasma membrane which exchanges intracellular sodium ions for extracellular potassium ions (Cochrane & Elder, 1967). In this way cells characteristically maintain a high internal potassium and low sodium content. Consequently a study of the characteristics of the ATPases of muscle membranes could lead to an understanding of the permeability characteristics of those membranes. The membrane ATPases of vertebrate striated muscle have been characterised by Samaha & Gergely (1965) for man, and by Duggan (1965) for frog. Schwartz (1962) has also studied the membrane ATPases of mammalian cardiac muscle. No such study has been made on insect muscle membranes. Recently it has been reported that the ventral nerve cord of Periplaneta does contain the classical $Na^+ + K^+$ stimulated Mg^{2+}-dependent ATPase (Grasso, 1967). However the nerves of Periplaneta are bathed in a fluid of different composition

from the haemolymph by their enclosure in a nerve sheath (Treherne & Smith, 1963). Hence the muscles are exposed to different conditions from the nerves and need not necessarily be expected to exhibit similar membrane characteristics.

METHODS

1. Preparation of microsomal fractions.

Coxal muscles were taken from three adult cockroaches, yielding about one gram of tissue. All the cuticle was removed and the muscles placed in ice-cold 0.25M sucrose buffered to pH 7.2 with 50 mM Tris-HCl and containing 10 mM EDTA. Homogenisation was carried out using a Potter-Elvehjem homogeniser with a teflon pestle (clearance 0.1-0.15 mm). The time taken for homogenisation was not longer than two minutes during which time the preparation was always surrounded by ice.

The resultant homogenate was centrifuged for 10 minutes at 2,000 *g* at 0°C and the pellet discarded. Mitochondria were removed at 0°C with two successive spins of 20 minutes at 12,500 *g* (250,000 *g* min.) using a M.S.E. Automatic Superspeed centrifuge, head No. 2409. The mitochondrial pellets from each spin were discarded. The supernatant was spun at 0°C in the Superspeed 40, head No. 2409, at 107,000 *g* for 60 minutes ($6.42 \times 10^6 g$ min.) to give the microsomal pellet. The supernatant was carefully withdrawn with a Pasteur pipette. Any remaining liquid was drawn off from around the pellet with slivers of filter paper. The microsomal pellet was suspended in the required volume of ice-cold 50 mM Tris-HCl or other stated buffer at pH 7.2 to give the enzyme preparation.

To determine the effect of deoxycholate on the preparation 0.75% deoxycholate was added to the extraction medium. No deoxycholate was added to the reaction medium.

2. Estimation of enzyme activity.

The incubation technique depended upon the conditions of the experiment. However the standard reaction media were as follows : either 4 mM $MgCl_2$, or 4 mM $MgCl_2$ + 50 mM NaCl + 50 mM KCl, made up in 50 mM histidine buffered to pH 7.2 with HCl and containing 2 mM ATP (Tris

salt made from Na salt by ion exchange). All concentrations were calculated to give the stated values in the final volume of reaction medium. 1.8 ml of the reaction medium was thermoequilibrated and the reaction started by the addition of 0.2 ml of enzyme preparation. The reaction was carried out usually at 25°C, and in all cases the temperature was controlled to $\pm$ 0,1°C. After a stated time, usually 20 minutes, the reaction was stopped by the addition of 2 ml of ice-cold 15% trichloracetic acid (TCA) and the mixture transferred to ice.

3. Estimation of inorganic phosphate.

The cold reaction mixture was centrifuged at 4°C to remove the precipitated protein and the assay of inorganic phosphate liberated by the enzyme reaction followed the method of Fiske and Subbarow (1925). The absorbancy of the solutions was determined at 660 nanometres using a Hilger Watts spectrophotometer. Two 1 ml aliquots of the supernatant were assayed and their inorganic phosphate content averaged.

4. Standard curve for inorganic phosphate determination.

A range of phosphate concentrations were made up and assayed for phosphate as above. Their absorbancy values at 660 nanometres were plotted against phosphate concentrations to give the standard curve shown in figure 1.

5. Estimation of microsomal protein.

The protein content of the enzyme preparation was determined by the method of Lowry, Rosebrough, Farr and Randall (1951), using bovine serum albumen (BSA) fraction V as standard.

Reagents: Solution 1 2% (w/v) sodium carbonate in 0.1 N sodium hydroxide,

Solution 2 0.5% copper sulphate.

Solution 3 1% sodium potassium tartrate.

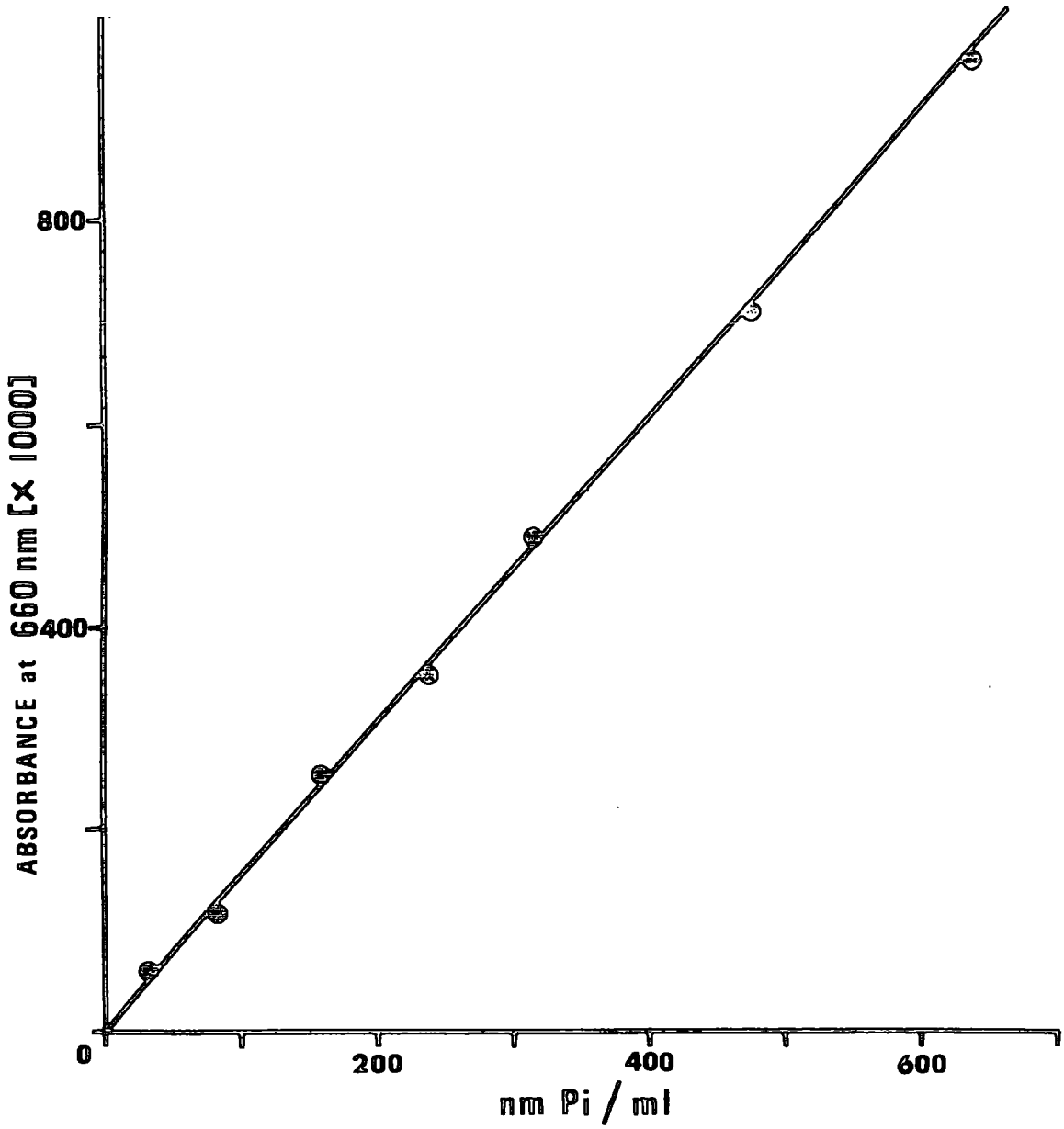
Folin's reagent (Solution A) was prepared by diluting 4 volumes of Folin & Ciocalteau's phenol reagent with 6 volumes of distilled water. Solution

FIGURE 1. STANDARD PHOSPHATE CURVE.

Phosphate concentrations were determined according to the method of Fiske and Subbarow (1925).

Ordinate: Absorbance at 660 nanometres.

Abscissa: Nanomoles inorganic phosphate.


ABSORBANCE at 660 nm [x 1000]
800
400
0
200
400
600
nm Pi / ml

B was made by mixing equal volumes of solutions 2 and 3 and adding 50 volumes of solution 1 to each volume of the mixture.

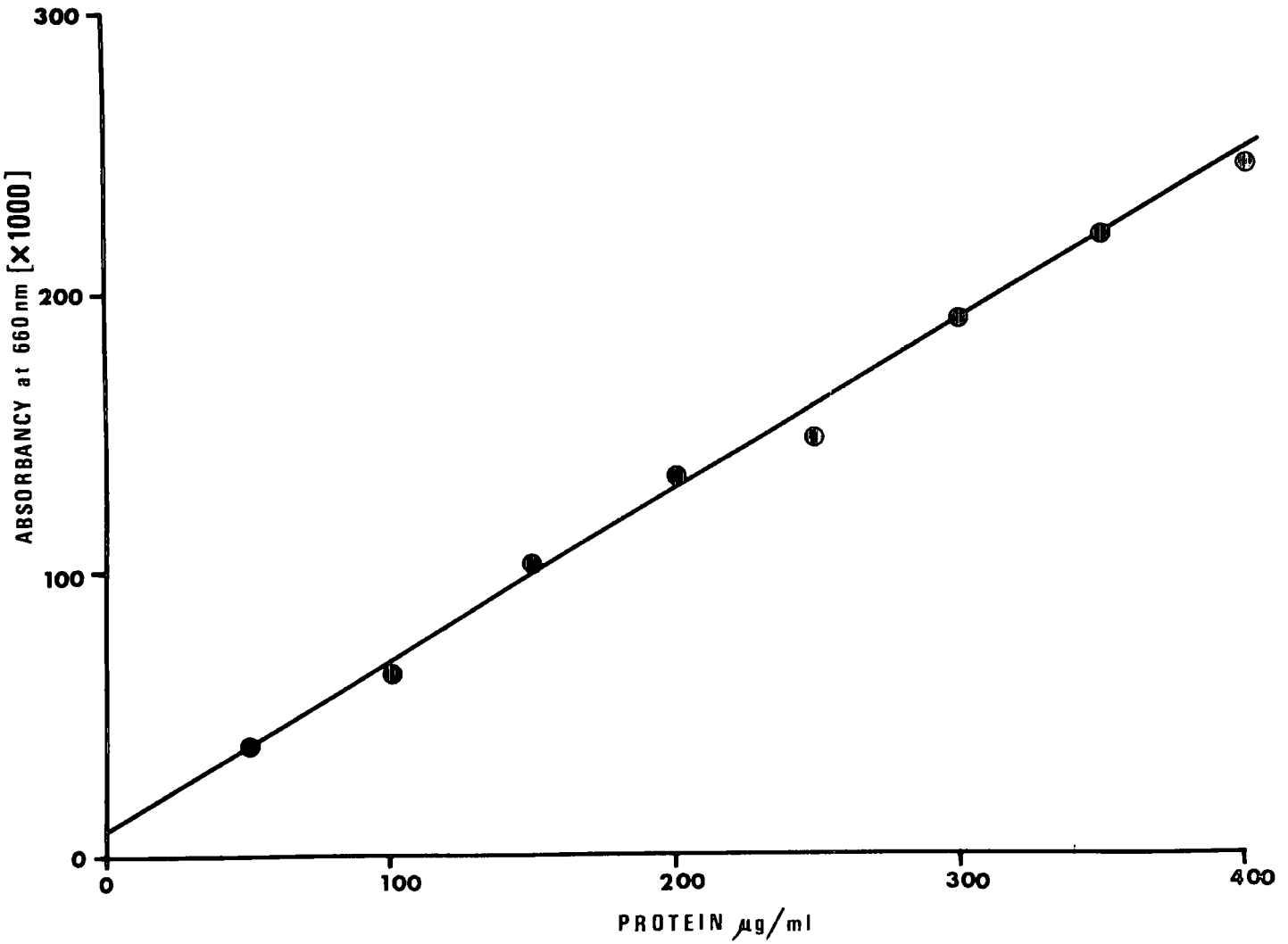
Standard protein solutions were made up ranging from 0 to 10^{-3} g BSA/ml. The microsomal preparation was diluted if necessary to give protein levels within this range. 0.2 ml of protein solution was added to 3 ml of solution B and left for 30 minutes at room temperature. 0.3 ml of solution A was then added and the mixture left for 60 minutes at room temperature. After this time the mixture was poured into silica cuvettes (1 cm light path) and their optical density measured at 500 nanometres in a Hilger-Watt spectrophotometer. From the standard protein solutions a calibration curve was plotted and the unknowns determined from this curve. The standard curve is shown in figure 2.

FIGURE 2. STANDARD PROTEIN CURVE.

Protein standards were prepared from bovine serum albumen (BSA) fraction V. Protein concentrations were determined according to the method of Lowry et al.(1951).

Ordinate: Absorbance at 660 nanometres.

Abscissa: Protein concentration (μg / ml).


300
200
100
0
ABSORBANCY at 660nm [×1000]
0
100
200
300
400
PROTEIN μg/ml

RESULTS

1. Mg^{2+}-dependent ATPase.

The effect of Mg^{2+} on the ATPase activity of the muscle membrane microsomal fraction of Periplaneta coxal muscle is shown in figure 3. Maximal activity occurred between 2 mM and 6 mM Mg^{2+}. Above 7 mM Mg^{2+} enzyme activity fell off, very little activity was found at Mg^{2+} concentrations below 0.25 mM. Indeed, as can be seen from table 1 (expts. 7-14), very little activity was found in the absence of Mg^{2+}. Substitution of Mg^{2+} with Ca^{2+} gave an ATPase activity only about 10% of that with 4 mM Mg^{2+}. The enzyme activity was not stimulated by the presence of the monovalent cations Na^{+} and K^{+} in the absence of Mg^{2+}.

In all subsequent experiments a concentration of 4 mM Mg^{2+} was used, the reaction being linear up to 20 minutes in these conditions, see figure 4.

2. Monovalent cation stimulation of the Mg^{2+}-dependent ATPase.

In table 1 the effect of the addition of alkali metal ions is shown as the additional enzyme activity found above that in the presence of 4 mM Mg^{2+} alone (expt. 9 or 1a.). No correction is made for the small activity found in the presence of sodium or potassium ions alone (expts. 11-14). Enzyme activity sensitive to strophanthin G is calculated as [a - b - (1a - 1b)]. All experiments were carried out at 25°C.

Addition of either sodium or potassium ions in the presence of 4 mM Mg^{2+} increased the ATPase activity although the Mg^{2+}-dependent ATPase activity constituted the majority of the total activity (table 1, expts. 15-18). Potassium ions always caused greater activation than sodium ions (table 1, expts. 17 and 18). When sodium and potassium ions were added together very little, if any, synergistic activity was found (table 1, expts. 3-6 and 19-21).

Strophanthin G classically inhibits the synergistic stimulation by

FIGURE 3. The effect of increasing Mg^{2+} concentrations on the activity of the Mg^{2+}-dependent ATPase of a microsomal preparation obtained from cockroach coxal muscle.

Reaction medium: 2 mM ATP, 50 mM Tris (pH 7.2) and increasing concentrations of Mg^{2+}.

Incubation: 20 minutes at 25°C.

Ordinate: ATPase activity expressed as nanomoles of inorganic phosphate liberated per mg of protein per minute.

Abscissa: Mg^{2+} concentration (mM).

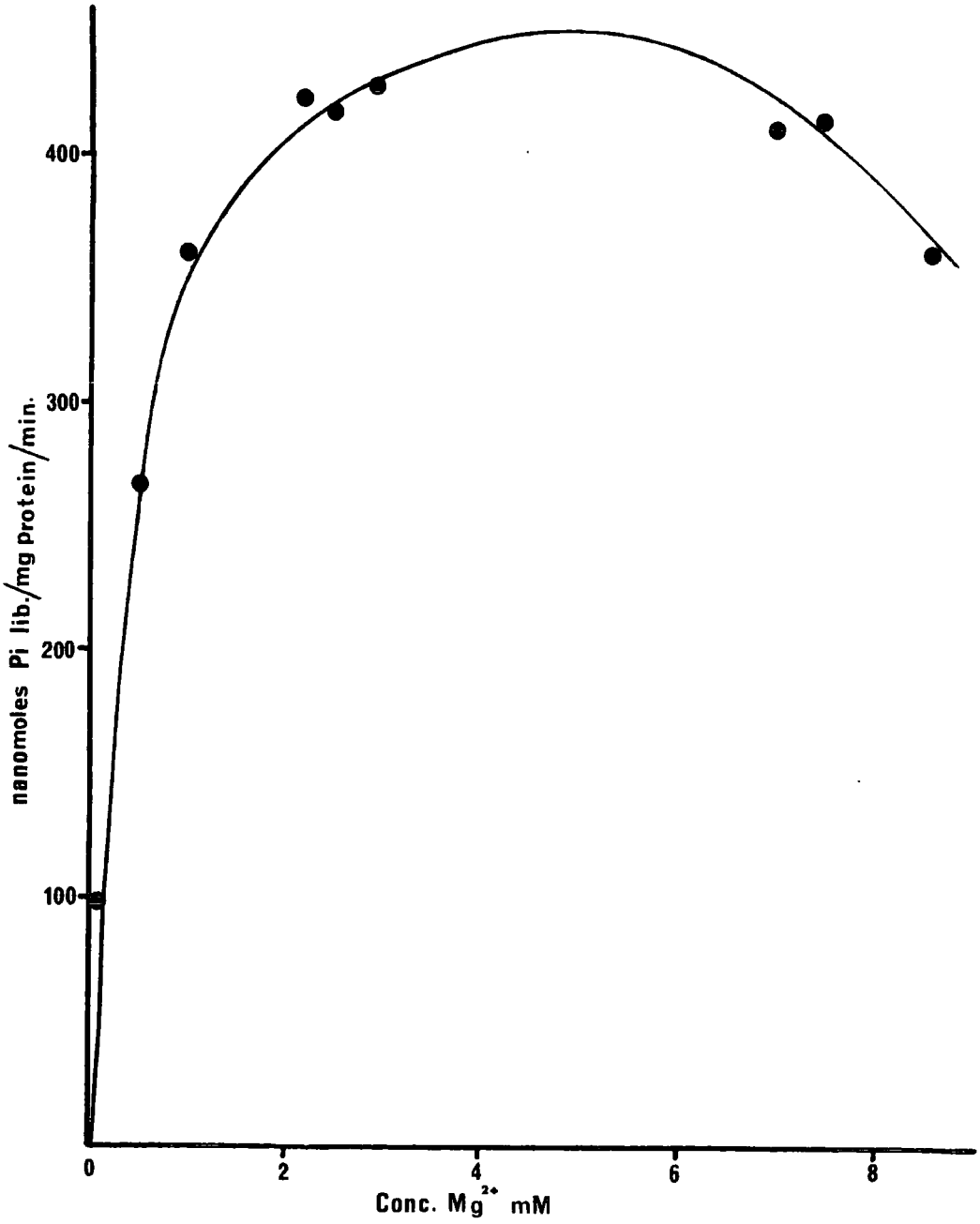

400
300
200
100
nanomoles Pi lib./mg protein/min.
0
2
4
6
8
Conc. Mg2+ mM

TABLE 1. The effect of alkali metal ions and strophanthin G on the ATPase activity of a microsomal preparation obtained from cockroach coxal muscle.

Conditions: All experiments were carried out at 25°C in the presence of 2 mM ATP and 50 mM Tris (pH 7.2). ATPase activity is expressed as nanomoles of inorganic phosphate liberated per mg protein per minute.

Expt. No.	Cation Conc. (mM) Mg^{2+}	Ca^{2+}	Na^{+}	K^{+}	Strophanthin G 10^{-4}M	ATPase Activity	Activity due to Monovalent Cations	Strophanthin G Sensitive Activity
1a	4	0	0	0	-	507		
b	4	0	0	0	+	474		3
2a	4	0	0	20	-	828	321	
b	4	0	0	20	+	764		31
3a	4	0	10	20	-	843	336	
b	4	0	10	20	+	729		81
4a	4	0	20	20	-	876	369	
b	4	0	20	20	+	710		133
5a	4	0	50	20	-	790	283	
b	4	0	50	20	+	716		41
6a	4	0	100	20	-	760	253	
b	4	0	100	20	+	657		70
7	0	1	0	0	-	59		
8	0	10	0	0	-	69		
9	4	0	0	0	-	523		
10	8	0	0	0	-	598		
11	0	0	0	10	-	18	18	
12	0	0	0	50	-	17	17	
13	0	0	10	0	-	23	23	
14	0	0	50	0	-	25	25	
15	4	0	0	10	-	714	191	
16	4	0	0	50	-	762	239	
17	4	0	10	0	-	607	84	
18	4	0	50	0	-	707	184	
19	4	0	10	10	-	740	217	
20	4	0	50	10	-	730	207	
21	4	0	10	50	-	813	290	

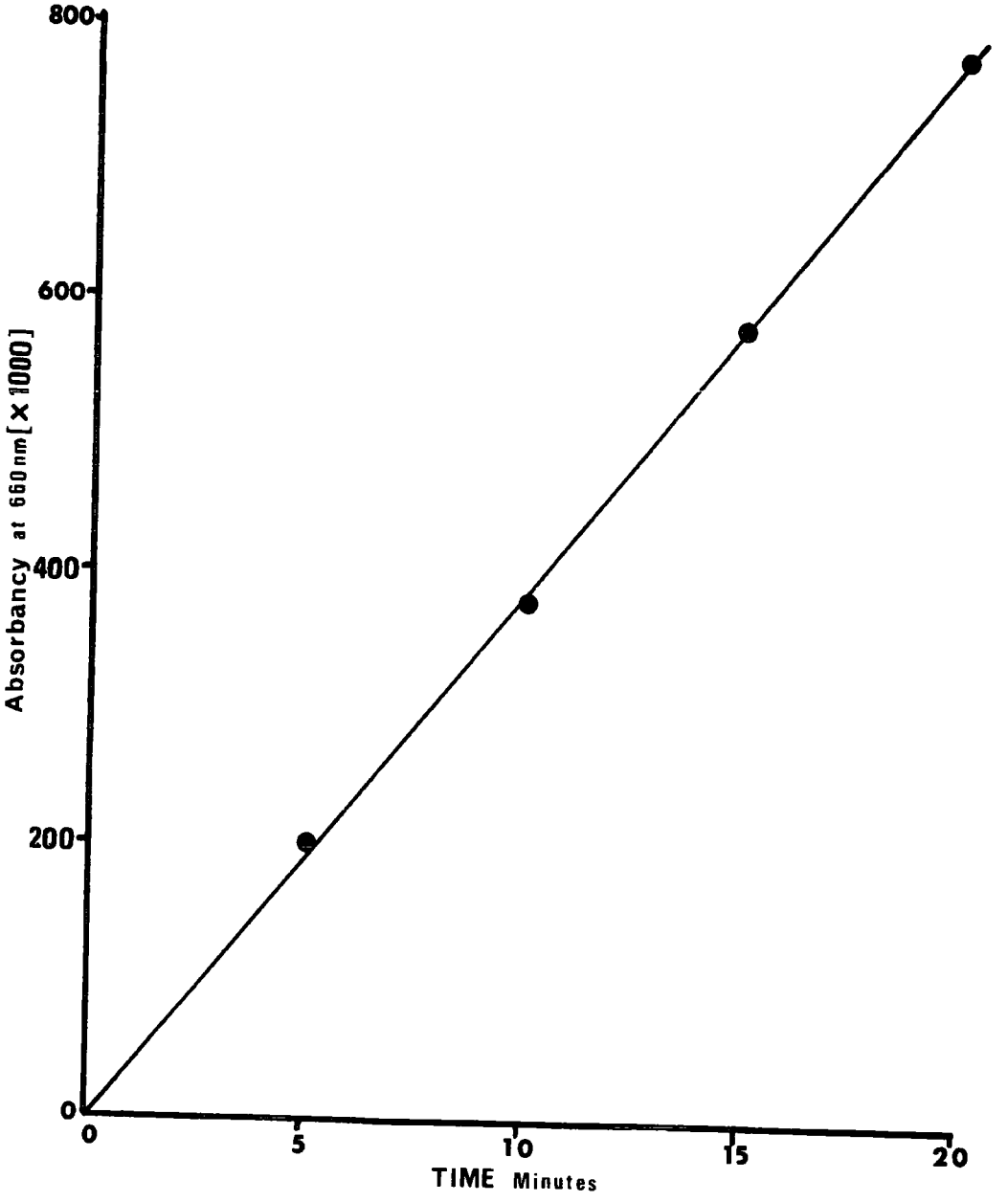
FIGURE 4. The effect of increasing incubation time on the activity of the Mg^{2+}-dependent ATPase of a microsomal preparation obtained from cockroach coxal muscle.

Reaction medium: 4 mM Mg^{2+}, 2 mM ATP, 50 mM Tris (pH 7.2).

Incubation: Increasing time at 25 °C.

Ordinate: ATPase activity expressed as absorbancy at 660 nanometres x 10^3.

Abscissa: Time of incubation in minutes.


800
600
400
200
0
Absorbancy at 660 nm [X 1000]
0
5
10
15
20
TIME Minutes

sodium and potassium of the Mg^{2+}-dependent APTase (Skou, 1965; Dunham & Glynn, 1961). In this preparation high concentrations of strophanthin G (10^{-4}M) had a small inhibitory effect on the monovalent cation stimulation of the Mg^{2+}-dependent ATPase activity (table 1, expts. 1-6). This latter result, taken with the lack of synergistic activation by sodium and potassium, is good evidence that in this preparation there is little genuine $Na^+ + K^+$ activated Mg^{2+}-dependent ATPase with an obligatory requirement for both sodium and potassium ions as stated by Skou (1964).

3. Treatment with deoxycholate.

In many studies on vertebrate membrane preparations it has been possible to increase the ratio of the $Na^+ + K^+$ activated Mg^{2+}-dependent ATPase to Mg^{2+}-dependent ATPase activity by having deoxycholate present during homogenisation (Skou, 1962 & 1965; Schwartz, 1962). Table 2 shows the activity of the Periplaneta muscle membrane preparation resulting from extraction in the presence of 0.75% deoxycholate. As can be seen this treatment results in an overall decrease in activity of the preparation and furthermore the stimulation of the Mg^{2+}-dependent ATPase by monovalent cations has been lost.

4. Substrate specificity.

Active transport of ions in red blood cells has been shown to require ATP, other nucleoside triphosphates being of less effect as substrates. The substrate specificity of the muscle membrane ATPase from cockroach skeletal muscle was determined using ATP, ITP, UTP and GTP. The nucleotide triphosphate was added to the reaction medium to give a final concentration of 2 mM. The ionic concentration of the reaction medium was either 4 mM Mg^{2+} or 4 mM Mg^{2+} plus 50 mM K^+ and 10 mM Na^+, which were the conditions giving maximal stimulation (see table 1). The results are shown in table 3, and it is clear that monovalent

TABLE 2. The effect of the presence of 0.75 % deoxycholate in the extraction medium on the ATPase activity of a microsomal preparation obtained from cockroach coxal muscle.

Conditions: Incubated at 25°C for 15 minutes in the presence of 2 mM ATP. The concentration of cations in the reaction media was as shown in the table. All the reaction media comtained 50 mM Tris (pH 7.2). Enzyme activity is expressed as nanomoles of inorganic phosphate liberated per mg protein per minute.

Cation Concentration mM			ATPase Activity
4 Mg^{2+}			362
	25 K^+		17
4 Mg^{2+}	25 K^+		355
		25 Na^+	9
4 Mg^{2+}		25 Na^+	359
4 Mg^{2+}	25 K^+	25 Na^+	359

TABLE 3. The substrate specificity of a microsomal preparation obtained from cockroach coxal muscle.

Conditions: Either a reaction medium containing 4 mM Mg^{2+} (Mg^{2+} ATPase), or 4 mM Mg^{2+} + 10 mM Na^{+} + 50 mM K^{+} (total activity). Both media contained 50 mM Tris (pH 7.2). The Na^{+} - K^{+} ATPase activity is calculated from the difference between total activity and that in the presence of Mg^{2+} alone. All nucleotides were added to give a final concentration of 2 mM. Incubated at 25°C for 15 minutes. ATPase activity is expressed as nanomoles of inorganic phosphate liberated per mg protein per minute.

Substrate	Mg^{2+} ATPase	Total Activity	Na^{+} - K^{+} ATPase
ATP	904	1443	539
ITP	728	829	101
UTP	222	208	0
CTP	92	79	0

cation stimulation is found only with ATP and ITP, although the former substrate is much more effective.

5. Metabolic inhibitors.

a. Effect of 2 - 4 dinitrophenol.

The effect of DNP upon the microsomal ATPase preparation depended whether the preparation was preincubated with DNP (i.e. before addition of substrate) or whether DNP was present in the reaction mixture only. The results are shown in figure 5. In general when DNP is present in the reaction medium only, the higher the concentration of DNP the greater the inhibition, although no inhibition was found at concentrations below 0.5 mM DNP. Indeed low concentrations of DNP (c 0.5 mM) in the reaction medium caused a small stimulation of Mg^{2+} ATPase of about 20% (see table 4 and figure 5).

A 15 minute preincubation with 1 mM DNP reduced the Mg^{2+} ATPase activity from 606 to 468 nanomoles Pi / mg protein / minute. The subsequent presence of DNP in the reaction medium caused relatively little further inhibition. For example with 5 mM DNP the enzyme activity was still about 400 nanomoles Pi / mg protein / minute. Again low concentrations of DNP (c 0.5 mM) in the reaction medium caused a small stimulation of the Mg^{2+} ATPase.

The addition of 0.25 mM DNP to the incubation medium had no effect on the activity of the monovalent cation stimulated activity (see expt. A, table 4).

In an experiment where the whole muscle was exposed (as described in chapter 4) and bathed in saline for two hours before extraction and homogenisation, insignificant activation (4.9%) of the Mg^{2+}-dependent ATPase was found to occur in the muscles bathed in 0.5 mM DNP as compared with the control (table 4B). In neither case was there any stimulation of the activity on the addition of sodium or potassium ions.

FIGURE 5. The effect of DNP on the Mg^{2+}-dependent ATPase activity of a microsomal preparation obtained from cockroach coxal muscle.

The lower curve represents the result of preincubating the preparation for 15 minutes with DNP, the upper curve the result of including DNP in the reaction medium only.

Reaction media: 4 mM Mg^{2+}, 2 mM ATP, increasing concentrations of DNP.

Incubation: The lower curve shows the results of preincubation with DNP for 15 minutes and subsequent incubation at 25°C. The upper curve shows the results from preparations which were not preincubated but were incubated at 25°C. The results are a combination of three experiments and have been presented to show the percentage change of activity due to the presence of DNP.

Ordinate: Percentage activity in relation to that activity in the absence of DNP.

Abscissa: Concentration of DNP (mM) in the reaction medium.

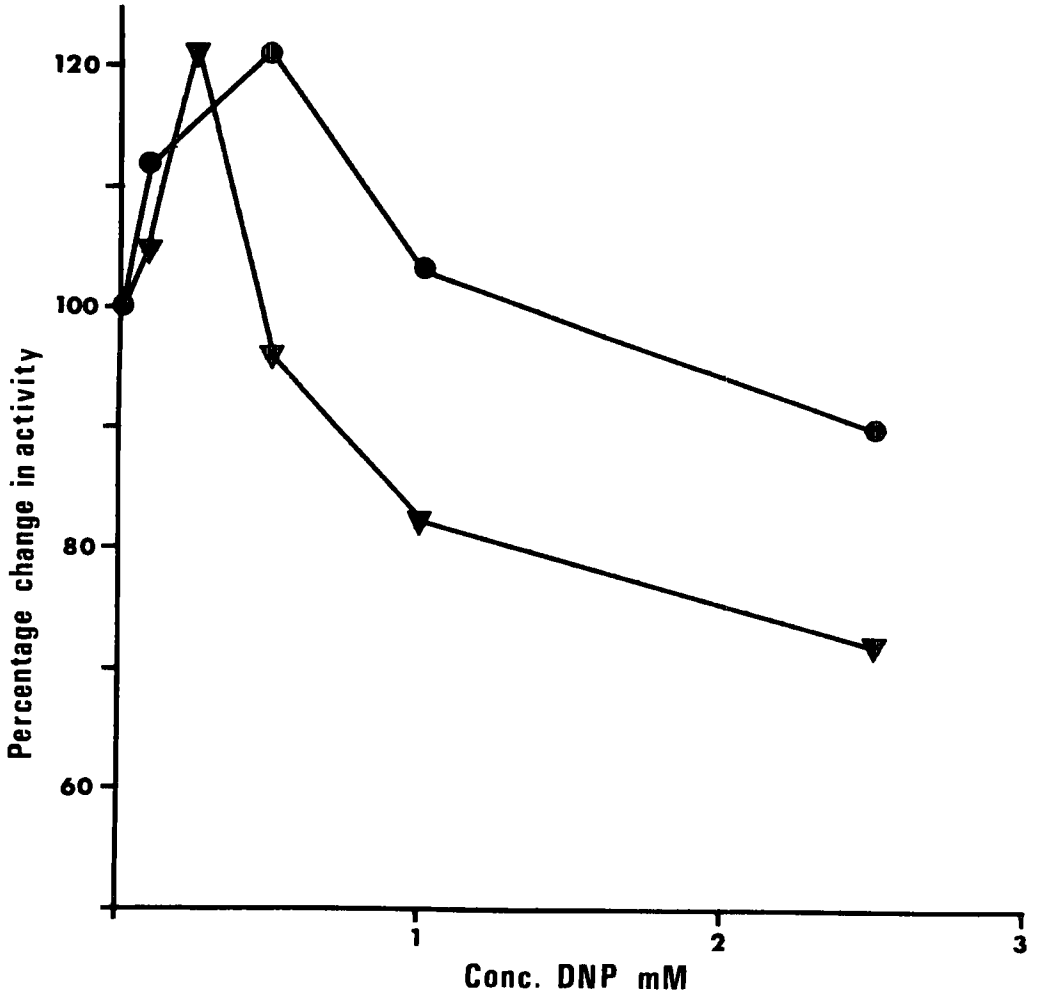

120
100
80
60
Percentage change in activity
1
2
3
Conc. DNP mM

TABLE 4. The effect of Dinitrophenol on ATPase activity of a microsomal preparation obtained from cockroach coxal muscle.

A. Effect of DNP in the incubation medium.

B. Effect of preincubating whole muscle in either saline or saline containing DNP on the subsequent ATPase activity of the microsomal preparation.

Conditions: A. Incubated at 25°C for 15 minutes in the presence of 2 mM ATP. The reaction medium contained 4 mM Mg^{2+} ± 0.25 mM DNP, or 4 mM Mg^{2+} + 50 mM Na^{+} + 50 mM K^{+} ± 0.25 mM DNP. Both media contained 50 mM Tris (pH 7.2). ATPase activity is expressed as units of absorbancy at 660 nanometres.

B. Whole muscle was preincubated in saline ± 0.5 mM DNP for two hours at room temperature before preparation of microsomes. The microsomal preparation was incubated for 15 minutes at 25°C in the presence of 2 mM ATP. The reaction medium contained 4 mM Mg^{2+} ± 0.25 mM DNP, or 4 mM Mg^{2+} + 50 mM Na^{+} + 50 mM K^{+}. Both media contained 50 mM Tris (pH 7.2). ATPase activity is expressed as nanomoles of inorganic phosphate liberated per mg protein per minute.

	Pre-treatment of Muscle	Cations in Reaction Medium (mM)	DNP in Reaction Medium (mM)	ATPase Activity
A.	none	4 Mg^{2+}	0	0.203
	none	4 Mg^{2+}	0.25	0.256
	none	4 Mg^{2+} 50 Na^{+} 50 K^{+}	0	0.336
	none	4 Mg^{2+} 50 Na^{+} 50 K^{+}	0.25	0.333
B.	saline	4 Mg^{2+}	0	141
	saline + DNP	4 Mg^{2+}	0	148
	saline	4 Mg^{2+}	0.25	179
	saline + DNP	4 Mg^{2+}	0.25	199
	saline	4 Mg^{2+} 50 Na^{+} 50 K^{+}	0	124
	saline + DNP	4 Mg^{2+} 50 Na^{+} 50 K^{+}	0	139

It appeared that bathing the whole muscle in saline affected the extraction of the muscle membrane ATPases because even the control Mg^{2+}-dependent ATPase activity was reduced when compared with that of a freshly isolated preparation (compare table 1 with table 4 B), although the muscle did function normally after two hours exposure to saline as can be seen in later chapters.

b. Effect of Cyanide.

When the muscle microsomal preparation was incubated with sodium cyanide the Mg^{2+}-dependent ATPase activity was stimulated, greater stimulation being found as the cyanide concentration was raised (table 5). This was not strictly a Mg^{2+}-dependent ATPase activity since cyanide was employed as the sodium salt, although the concentration of Na^{+} present would be small, and certainly not large enough to cause a stimulation of the magnitude found in the presence of sodium cyanide (see tables 1 and 5). When the preparation was preincubated with cyanide before adding ATP the activation was even greater. Duggan (1965) found that the Mg^{2+}-dependent ATPase of frog muscle was similarly sensitive to cyanide. However at higher cyanide concentrations Duggan found inhibition to occur and he suggested that the initial activation seen at low cyanide concentrations was due to the removal of a heavy metal inhibitor.

6. Oxygen consumption of homogenates.

Since both DNP and cyanide are metabolic inhibitors, and DNP stimulates mitochondrial ATPase activity, and as they both had an effect on this enzyme preparation, it was considered worthwhile to ensure that there was no mitochondrial contamination of the microsomal preparation. A pellet from each mitochondrial spin and from the microsomal spin were suspended in 0.1 M potassium phosphate buffer at

TABLE 5. The effect of cyanide on the Mg^{2+} ATPase activity of a microsomal preparation obtained from cockroach coxal muscle.

Conditions: Incubated for 15 minutes at 25°C in the presence of 2 mM ATP. The reaction media contained 4 mM Mg^{2+}, 50 mM Tris and increasing concentrations of sodium cyanide. Where stated the preparation was preincubated for 15 minutes at 25°C in the reaction medium, but in the absence of ATP. ATPase activity is expressed as nanomoles of inorganic phosphate liberated per mg protein per minute. % activation relates to the increase in Mg^{2+} ATPase activity induced by cyanide.

Preincubation Time (min)	Ions in Reaction Medium (mM)	Total Activity	% Activation
none	4 Mg^{2+}	219	none
none	4 Mg^{2+} + 0.5 NaCN	334	52.9
none	4 Mg^{2+} + 2.0 NaCN	352	60.8
none	4 Mg^{2+} + 5.0 NaCN	395	80.4
15	4 Mg^{2+}	204	none
15	4 Mg^{2+} + 0.5 NaCN	265	29.9
15	4 Mg^{2+} + 2.0 NaCN	459	125.0
15	4 Mg^{2+} + 5.0 NaCN	501	145.6

pH 7.4 containing:-

250 mM ∝glycerophosphate

1 x 10^{-4} M cyctochrome c

4 x 10^{-3} M $CaCl_2$

4 x 10^{-3} M Al_2 Cl_3

10 ml Warburg flasks were set up to record oxygen consumption at 25°C. The protein content of each resuspended pellet was estimated, as described in the methods, and the results expressed as nanomoles O_2 consumed / mg protein / hour. The oxygen consumption of the first mitochondrial pellet was 10.52 nanomoles O_2 / mg protein / hour. The second mitochondrial pellet consumed only 2.49 nanomoles O_2 / mg protein / hour. The microsomal pellet did not consume a detectable amount of oxygen and it was therefore considered that any mitochondrial contamination of the microsomal preparation was insignificant.

7. Guanidine-HCl.

Guanidine is an agent which is known to inhibit the Mg^{2+}-dependent ATPase to a greater degree than the $Na^+ + K^+$ activated Mg^{2+}-dependent ATPase of red cell ghosts (Wins & Schoffeniels, 1966). It is therefore possible to increase the ratio of the $Na^+ + K^+$ activated Mg^{2+}-dependent to Mg^{2+}-dependent ATPase activity by treating the preparation with guanidine, although relatively high concentrations are required to do this. In the experiment shown in table 6 no protein values were obtained but the ratio of $Na^+ + K^+$ activated Mg^{2+}-dependent ATPase to Mg^{2+}-dependent ATPase activity is given. Guanidine at a concentration of 75-100 mM did increase this ratio in this preparation. The effect was similar to that found in red cell ghosts by Wins and Schoffeniels (1966), although the ratio of the monovalent cation stimulated ATPase activity to Mg^{2+}-dependent ATPase activity was always greater than one in their preparation.

TABLE 6. The effect of guanidine-HCl on the ATPase activity of a microsomal preparation obtained from cockroach coxal muscle.

Conditions: Either 4 mM Mg^{2+} (Mg^{2+} ATPase) or 4 mM Mg^{2+} + 50 mM Na^{+} + 50 mM K^{+} (total activity), incubated at 25°C for 15 minutes in the presence of 2 mM ATP. Na^{+} - K^{+} ATPase activity is obtained by subtracting the activity in the presence of Mg^{2+} alone from the total activity. ATPase activity is expressed as absorbancy units at 660 nanometres.

Guanidine Conc. (mM)	Mg^{2+} ATPase Activity	Total Activity	Na^{+} - K^{+} ATPase Activity	Mg^{2+}/ Na^{+} K^{+} Ratio
0	0.680	0.898	0.218	1 : 0.32
25	0.407	0.440	0.033	1 : 0.08
50	0.281	0.369	0.088	1 : 0.31
75	0.150	0.307	0.157	1 : 1
100	0.119	0.194	0.075	1 : 0.63

8. pH sensitivity.

The effect of pH on the activity at 25°C of the ATPase of the microsomal preparation of Periplaneta muscle membranes, in the presence of 4 mM Mg^{2+} or 4 mM Mg^{2+} + 50 mM Na^{+} + 50 mM K^{+}, is shown in figure 6. Solutions between pH 6 and 7 were buffered with 50 mM histidine-HCl. Solutions of higher pH were buffered with 50 mM Tris-HCl. From the results it is seen that the Mg^{2+}-dependent ATPase activity had a pH optimum between 7 and 8 whilst the Na^{+} + K^{+} activated Mg^{2+}-dependent ATPase activity was relatively unaffected by pH except at pH 9.2. This suggests that there may only be a single ATPase present in this preparation whose activity is dependent upon magnesium ions and is slightly enhanced in the presence of monovalent cations. Usually it has been found that each enzyme has a separate and quite distinct pH optimum. For example Duggan (1965) found that for frog muscle membrane the Mg^{2+}-dependent ATPase activity had an optimum at pH 8.5 and the K^{+} activated Mg^{2+}-dependent ATPase activity had an optimum at pH 7.5.

9. Thermal sensitivity.

a. Effect of Preincubation at 20-45°C.

In experiments involving incubations at several different temperatures 50 mM histidine-HCl was used as a buffer. The preparation was preincubated at either 20°, 25°, 30°, 35°, 40°or 45°C for 15 minutes in 24 mm test tubes in the presence of 4 mM Mg^{2+} or 4 mM Mg^{2+} + 50 mM K^{+} and then stored on ice. Na^{+} was omitted since addition of Na^{+} to the medium gave little increase in activity (table 1). Each tube was then transferred to 25°C and, after 5 minutes equilibration, the incubation was started by adding ATP to give a final concentration of 2 mM. Preincubation greatly increased enzyme activity up to 35°C, above which temperature inactivation occurred. From figure 7 it can be seen that the peak of activation for the Mg^{2+}-dependent ATPase activity was about

FIGURE 6. The effect of pH on the ATPase activity of a microsomal preparation obtained from cockroach coxal muscle.

The upper curve represents Mg^{2+} ATPase activity and the lower line represents the activity in the presence of 4 mM Mg^{2+} subtracted from the activity in the presence of 4 mM Mg^{2+} + 50 mM Na^{+} + 50 mM K^{+}.

Reaction media: 4 mM Mg^{2+}, and 4 mM Mg^{2+} + 50 mM Na^{+} + 50 mM K^{+}. Both media contained 2 mM ATP and were buffered with 50 mM histidine (for pH 6-7) and 50 mM Tris (for pH 7-9).

Incubation: 25°C.

Ordinate: ATPase activity expressed as μmoles inorganic phosphate liberated per mg protein per minute.

Abscissa: pH of incubation medium.

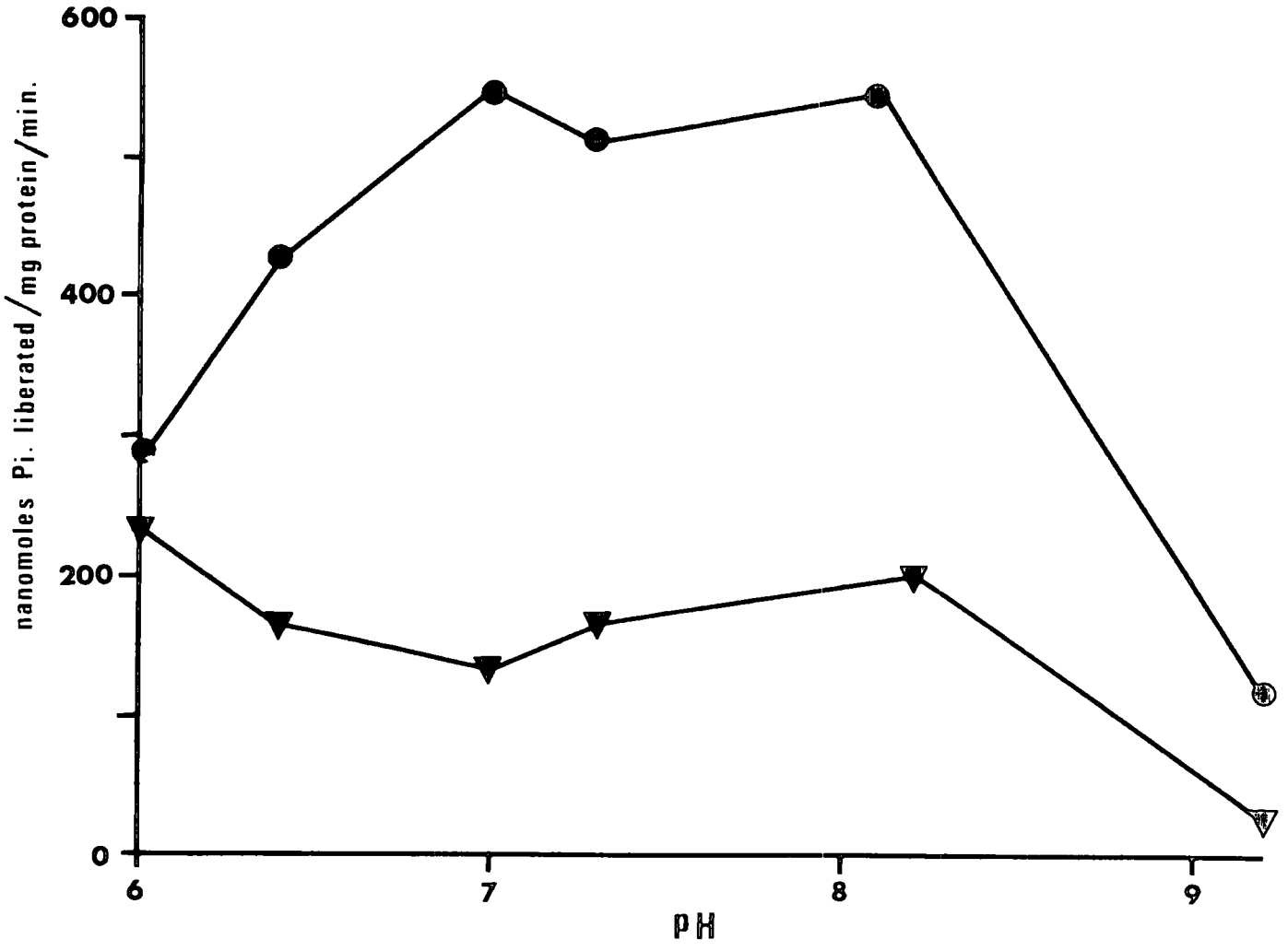

600
400
200
0
nanomoles Pi. liberated/mg protein/min.
6
7
8
9
pH

FIGURE 7. The effect of preincubation on the ability to hydrolyse ATP of a microsomal preparation obtained from cockroach coxal muscle.

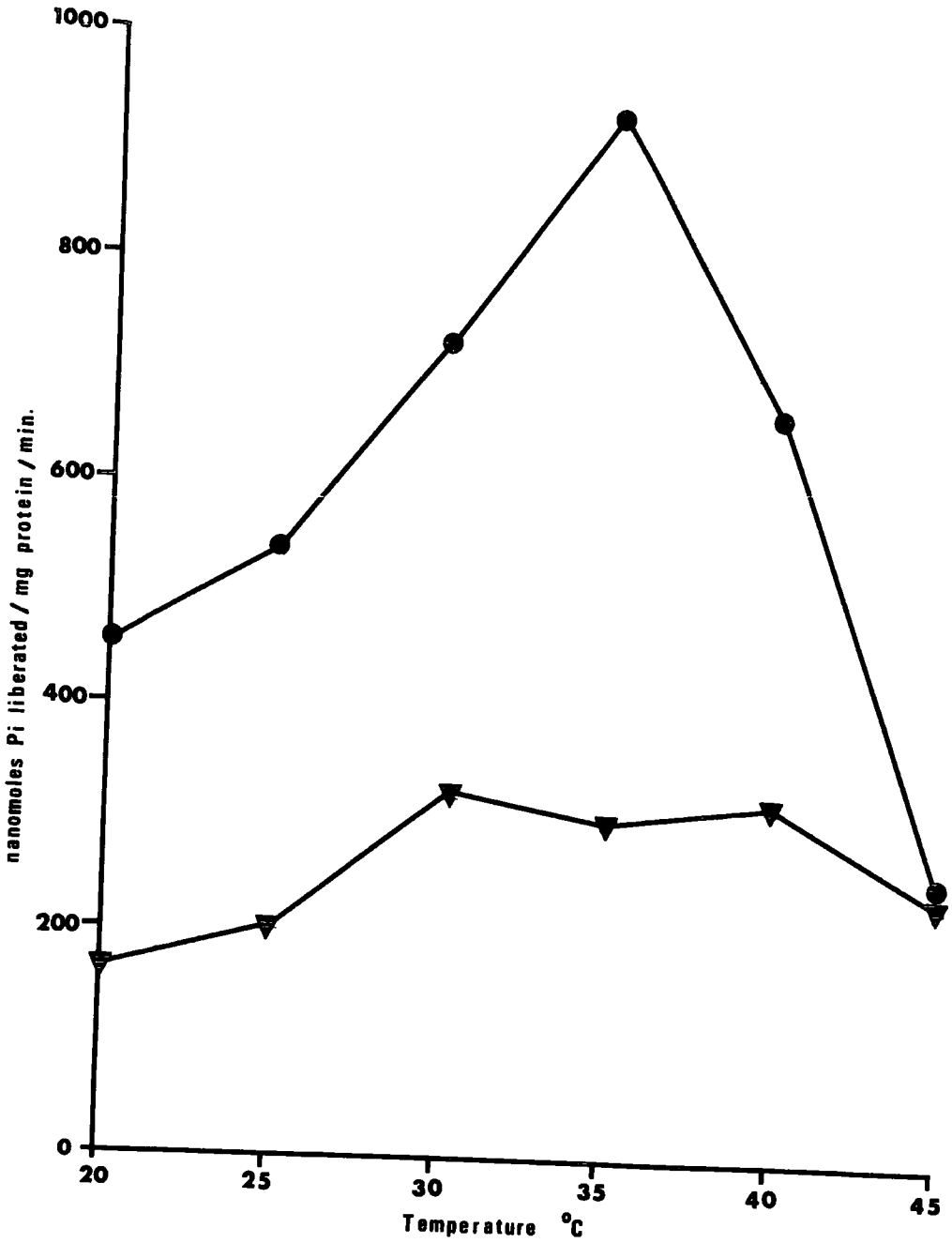
The upper curve represents Mg^{2+} ATPase activity and the lower curve the activity in the presence of 4 mM Mg^{2+} subtracted from the activity in the presence of 4 mM Mg^{2+} + 50 mM K^{+}.

Reaction media: 4 mM Mg^{2+}, and 4 mM Mg^{2+} + 50 mM K^{+}. Both media contained 2 mM ATP and 50 mM histidine (pH 7.2)

Incubation: The enzyme preparation was preincubated at increasing temperatures and its subsequent activity assayed at 25°C.

Ordinate: ATPase activity expressed as nanomoles of inorganic phosphate liberated per mg protein per minute.

Abscissa: Incubation temperature.


1000
800
600
400
200
0
nanomoles Pi liberated / mg protein / min.
20
25
30
35
40
45
Temperature °C

35°C although the enzyme was still activated even after preincubation at 40°C, but after preincubation at 45°C the enzyme was about 50% inhibited. The K^+ activated Mg^{2+}-dependent ATPase was affected differently by preincubation, being stimulated by all preincubation temperatures. Maximal stimulation was found by preincubation at 30-40°C.

In a further experiment the preparation was preincubated for varying times at 30°C and then incubated for 5 minutes at 30°C. The effect of preincubation time at this temperature was to increase the activity of both the Mg^{2+}-dependent ATPase and the monovalent cation activated ATPase (figure 8).

b. Arrhenius plot.

The preparation was incubated at temperatures ranging from 0.5°C to 40°C in the presence of 4 mM Mg^{2+} and 4 mM Mg^{2+} plus 50 mM K^+. The results were expressed as a μplot in figure 9. The effect of DNP in the reaction medium on the μplot for the Mg^{2+}-dependent ATPase is shown in figure 10.

The energy of activation for the Mg^{2+}-dependent enzyme was 18.39 K cal $mole^{-1}$. In the presence of DNP the energy of activation of the Mg^{2+}-dependent ATPase was unaltered. The energy of activation for the K^+ activated Mg^{2+}-dependent ATPase was 19.59 K cal $mole^{-1}$. The μplots for the activity in the presence of 4 mM Mg^{2+} and the increased activity due to potassium were parallel and therefore possessed the same sensitivity to temperature.

FIGURE 8. The effect of preincubation time at 30°C on the ability to hydrolyse ATP at 30°C of a microsomal preparation obtained from cockroach coxal muscle.

The upper curve represents Mg^{2+} ATPase and the lower curve represents the activity in the presence of 4 mM Mg^{2+} subtracted from the activity in the presence of 4 mM Mg^{2+} + 50 mM K^{+}.

Reaction media: 4 mM Mg^{2+}, and 4 mM Mg^{2+} + 50 mM K^{+}. Both media contained 2 mM ATP and 50 mM histidine (pH 7.2).

Incubation: The preparation was preincubated for increasing times at 30°C and the subsequent activity assayed at 30°C.

Ordinate: ATPase activity expressed as nanomoles of inorganic phosphate liberated per mg protein per minute.

Abscissa: Time of preincubation at 30°C.

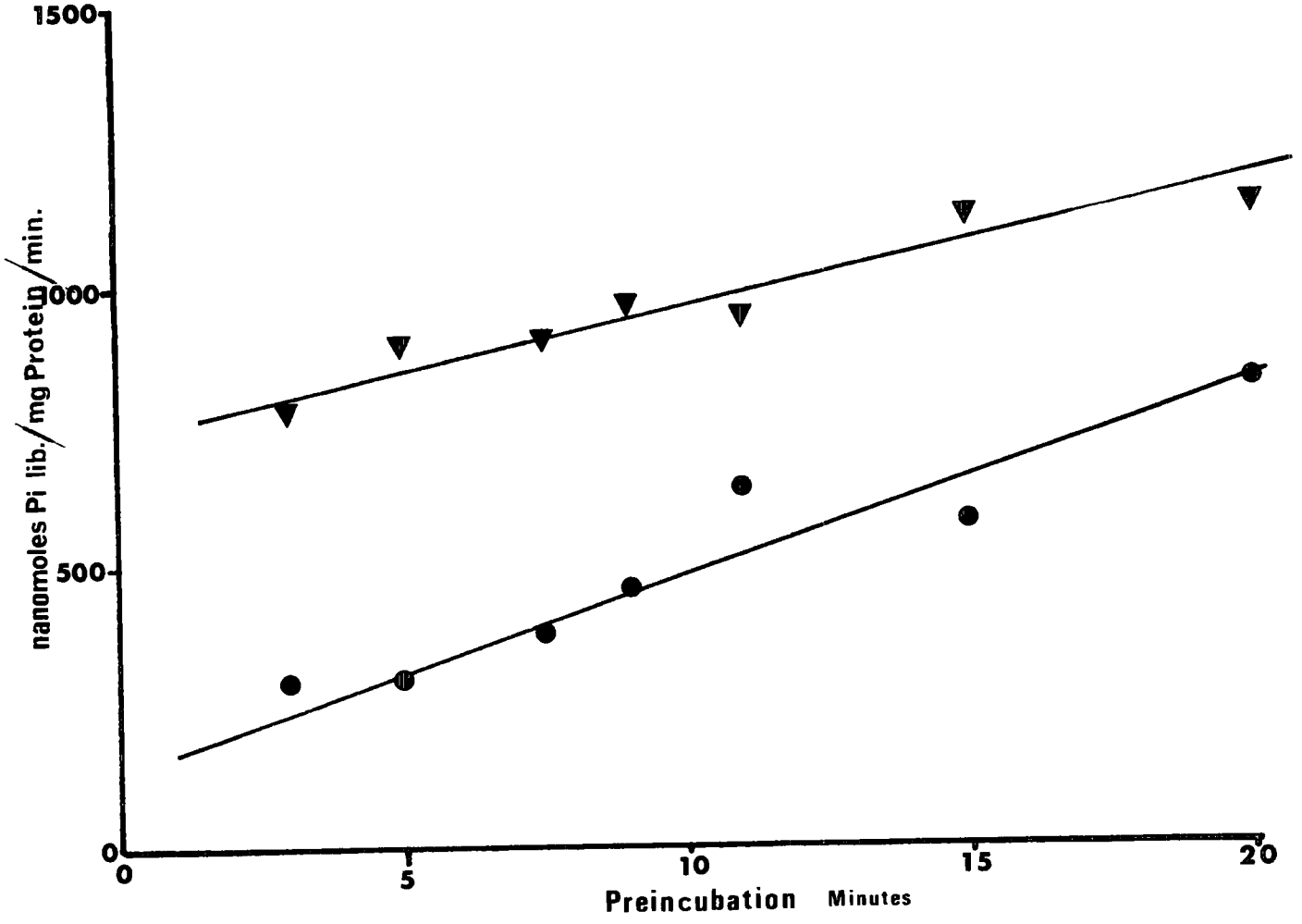

1500
1000
500
0
nanomoles Pi lib./mg Protein/min.
0
5
10
15
20
Preincubation Minutes

FIGURE 9. Arrhenius plots for the Mg^{2+} ATPase (upper curve) and the K^+ stimulation of the Mg^{2+}-dependent ATPase (lower curve) of a microsomal preparation obtained from cockroach coxal muscle.

Reaction media: Upper curve 4 mM Mg^{2+}. The lower curve was obtained as the difference between the activity in the presence of 50 mM K^+ + 4 mM Mg^{2+} and in the presence of 4 mM Mg^{2+} alone. Both media contained 2 mM ATP and 50 mM histidine (pH 7.2).

Ordinate: ATPase activity expressed as nanomoles of inorganic phosphate liberated per mg protein per minute.

Abscissa: $\frac{1}{T^\circ A} \times 10^3$.

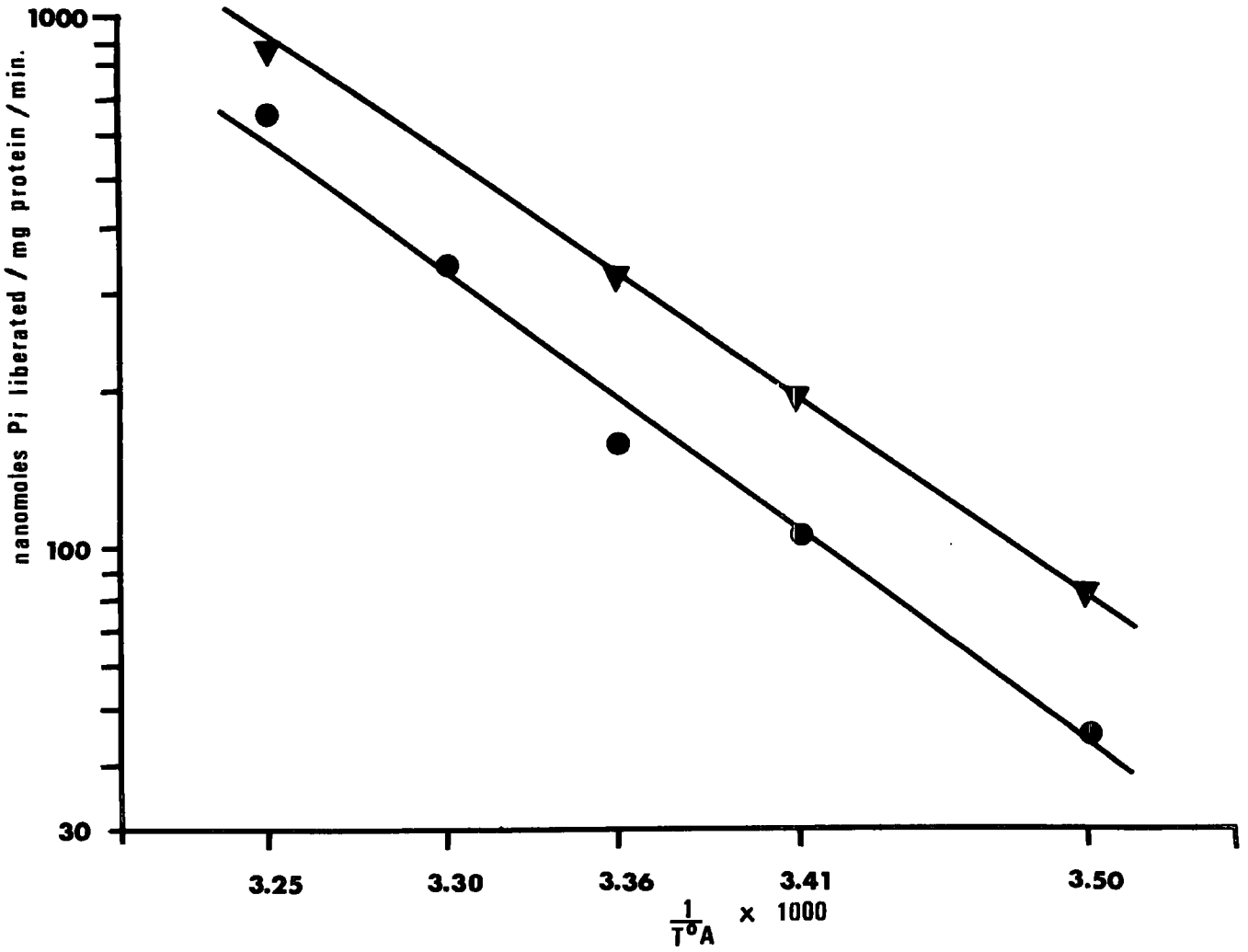
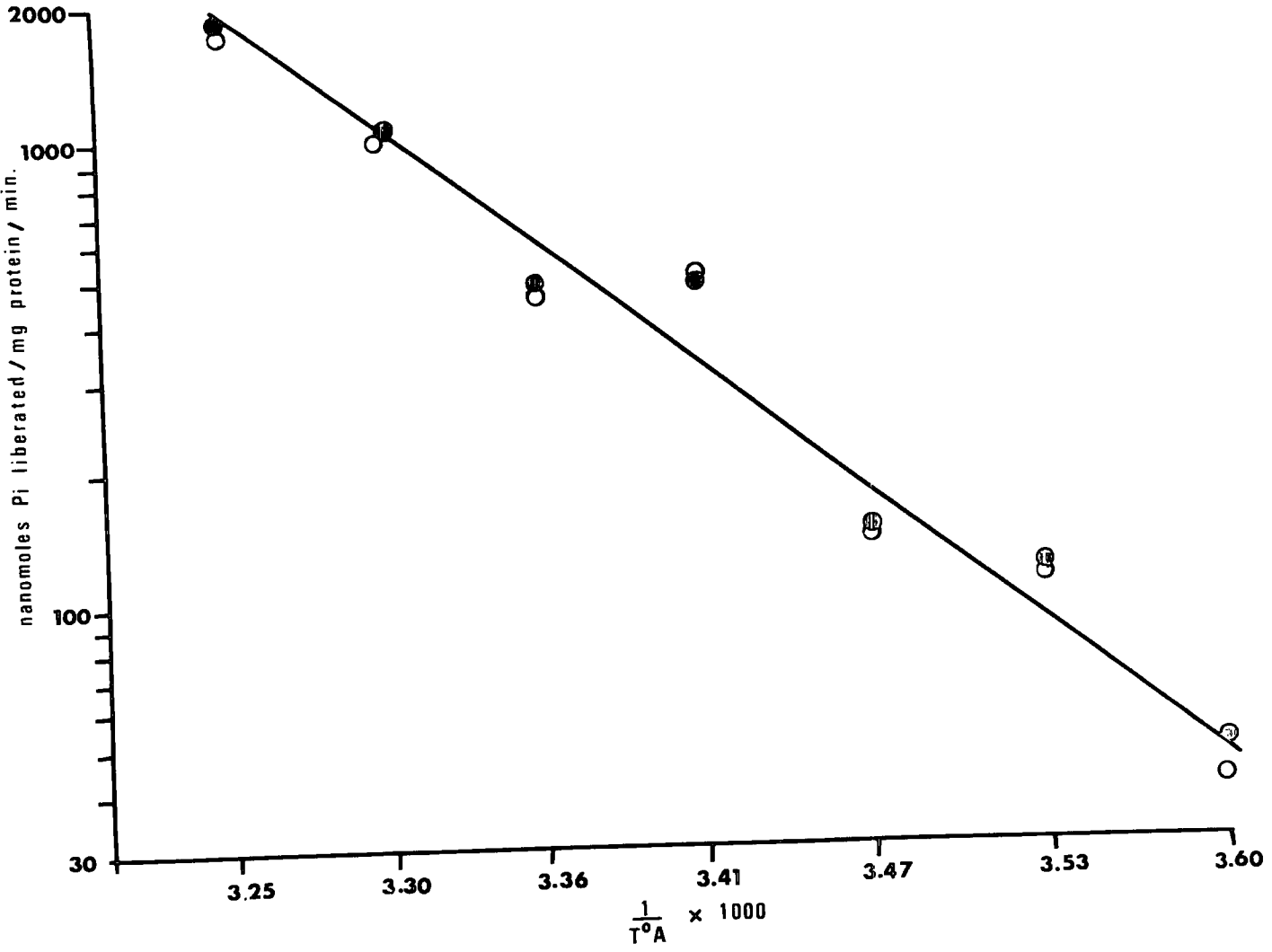

1000
100
30
nanomoles Pi liberated / mg protein / min.
3.25
3.30
3.36
3.41
3.50
1/T°A x 1000

FIGURE 10. Arrhenius plot for the Mg^{2+} ATPase in the presence (O) and absence (Θ) of 0.5 mM DNP of a microsomal preparation obtained from cockroach coxal muscle.

Reaction Media: 4 mM Mg^{2+} (Θ) and 4 mM Mg^{2+} + 0.5 mM DNP (O). Both media contained 2 mM ATP and 50 mM Tris (pH 7.2).

Ordinate: ATPase activity expressed as nanomoles of inorganic phosphate liberated per mg protein per minute.

Abscissa: $\frac{1}{T^{\circ}A} \times 10^{3}$.


2000
1000
100
30
nanomoles Pi liberated / mg protein / min.
3.25
3.30
3.36
3.41
3.47
3.53
3.60
1/T°A x 1000

DISCUSSION

Since 1957 when Skou described an enzyme system hydrolysing ATP in crab nerve membranes similar enzyme systems have been identified in preparations of other nerve cells (Abood & Gerard, 1964; Bonting, Caravaggio & Canady, 1964), brain tissues (Järnefelt, 1960; Schwartz, Bachelard & Mollwain, 1962; Skou, 1962), and cardiac muscle (Schwartz, 1962; Lee & Yu, 1963). The ATPase activity of all these membrane preparations has two fractions representing either two ATPases with different metal ion requirements, or one ATPase with two different sites of activity (Schwartz, 1962).

Typically one component of this ATPase activity is stimulated by magnesium ions alone (the Mg^{2+}-dependent ATPase) and it has been suggested that this is the enzyme responsible for the control of passive permeability of cells (Duncan, 1967). The other component of the ATPase activity, dependent upon the monovalent cations for activation, is thought to represent the cation pump of excitable cell membranes (Skou, 1965). The two components typically differ in their properties (Skou, 1957, 1960, 1962; Post, Merritt, Kinsolving & Albright, 1960; Dunham & Glynn, 1961) in certain distinct ways.

The Mg^{2+}-dependent ATPase is not inhibited by cardiac glycosides; its activity is increased by small amounts of calcium in the presence of magnesium; its activity is not dependent on the presence of sodium and potassium ions; it can utilize both ITP (Skou, 1960) and ADP (Herbert, 1956) as well as ATP. Skou (1965) listed the requirements which the other component must fulfil to be considered as a sodium transporting system. It should :

a. Be located in the cell membrane.

b. Have an affinity for sodium that is higher than for potassium at a site located on the inside of the cell membrane.

c. Have an affinity for potassium that is higher than for sodium at a site located on the outside of the cell membrane.

d. Contain an enzyme system that can catalyse the hydrolysis of ATP and thus convert the energy from ATP into a movement of cations.

e. Be capable of hydrolysing ATP at a rate dependent on the concentration of sodium inside the cell and on the concentration of potassium outside the cell.

f. Be found in all cells in which an active, linked transport of sodium and potassium occurs.

g. A close correlation must be found between the effects of cardiac glycosides on the cation transport in the intact cell and their effect on the isolated system.

Clearly the membrane preparation from Periplaneta coxal muscle does not comply with all those conditions which have been tested. The system is located in the cell membrane and does contain an enzyme system that can catalyse the hydrolyses of ATP. However, it was virtually unaffected by strophanthin G (although in Chapters 4 & 5 it will be shown that the intact cell is affected by strophanthin G). It cannot be considered as being involved in linked sodium and potassium transport since, although the ATPase activity was stimulated by potassium, there was no further stimulation caused by the addition of sodium i.e. there was no synergistic stimulation by sodium and potassium. It was shown that the monovalent cation stimulation of the Mg^{2+}-dependent ATPase only occurred in the presence of ATP and ITP, UTP and CTP being ineffective as substrates. This is in agreement with Hoffman (1960) who found that active transport of ions in the red cell required ATP as substrate and with ITP as an energy source there is reduced sodium transport and with GTP and UTP none at all. Duggan (1965) also found that the monovalent cation stimulated ATPase of frog muscle membrane showed a distinct preference

for ATP over other nucleotide triphosphates. However the inability of this Periplaneta muscle membrane preparation to fulfil many of the requirements laid down by Skou suggests that it does not contain an ATPase associated with linked sodium and potassium transport.

It is possible that there is only one ATPase in this preparation. This view, which has been suggested before (Wareham, Duncan & Bowler, 1968), is supported by the following observations. The high level of Mg^{2+}-dependent ATPase activity was not due to mitochondrial contamination since the extraction procedure (in the absence of DOCA) was such that the mitochondria should not be disrupted and would have been sedimented out of the homogenate in the second and third spins. The oxygen consumption of the second mitochondrial pellet was very low and that of the microsomal pellet not detectable. Furthermore the pH optimum of the Mg^{2+}-dependent ATPase activity was not sharply defined but lay between pH 7 and 8, whereas Kielley and Kielley (1953) showed that mitochondrial ATPase (from rat liver) had a well defined optimum at pH 8.5. The Arrhenius μ plot for the Mg^{2+}-dependent ATPase was linear and the energy of activation (18.4 K cal $mole^{-1}$) was similar to that determined for this enzyme from other tissues (Bowler & Duncan, 1968a, 1968b). The value for the activation energy indicates that this enzyme preparation is different from myosin ATPase which has an activation energy of 9-12 K cal $mole^{-1}$ (Ouellet, Laidler & Morales, 1952; Levy, Sharon & Koshland, 1959). The μ plot does differ from that obtained for a frog brain preparation in the higher temperature range (Bowler & Duncan, 1968a) where a line was obtained to which a definite break could be fitted at about 15°C. Significantly the Arrhenius μ plot for the monovalent cation stimulation of Periplaneta muscle membrane ATPase activity was also linear and parallel to the Arrhenius μ plot for the Mg^{2+}-dependent ATPase. Furthermore the energies of activation for the two enzymes

were also similar, 18.4 for the Mg^{2+}-dependent ATPase and 19.6 for the monovalent cation stimulation. This is in marked contrast to the results of Bowler and Duncan (1968a, 1968b, 1969) who found that the two enzymes in frog, rat and hedgehog brain preparations had distinctly different energies of activation.

Skou (1964) found that deoxycholate increased the $Na^+ + K^+$ activated ATPase to Mg^{2+}-dependent ATPase ratio. In this preparation monovalent cation stimulation was completely inhibited when 0.75% DOCA was incorporated in the extraction medium. It was not possible to increase the degree of monovalent cation stimulation by any treatment described other than by high concentrations of guanidine.

In association with the findings that there is no synergistic monovalent ion activation or strophanthin G sensitivity these results could be taken to show the lack of a sodium transport system in Periplaneta muscle membranes. The ATPase activity present could be attributed to a single enzyme with two different sites of activity, one stimulated by Mg^{2+} alone and one further stimulated by monovalent cations.

This does not necessarily mean that insect muscle is unusual in its biochemical properties. Work on rat skeletal muscle (M.A. Radcliffe, personal communication) has not demonstrated an ATPase system synergistically stimulated by sodium and potassium. Furthermore Duggan (1965), was unable to demonstrate synergistic stimulation by sodium and potassium, or inhibition by strophanthin G, in frog muscle microsomes. Samaha and Gergely (1965), working with human striated muscle microsomes, were able to demonstrate a degree of synergistic stimulation by sodium and potassium which was inhibited by strophanthin G, but to do so required deoxycholate extraction, the presence of sodium azide and even ageing of the preparation. Even then they were unable to decide whether there

were two separate enzymes or whether the changed ATPase activity was merely an artifact. It is clear that it is extremely difficult to demonstrate the presence in muscle membrane of a $Na^+ + K^+$ activated Mg^{2+}-dependent ATPase activity. Whether this is a result of employing the wrong extraction methods or whether there is no such enzyme in muscle membranes it is not possible to say. It is however certain that there is active extrusion of sodium ions from vertebrate muscle cells (Keynes & Maisel, 1954; Conway, Kernan & Zadunaisky, 1961; Dee & Kernan, 1963). It is by no means so certain that there is such a sodium extrusion system in Periplaneta muscle membranes (Wood, 1963). Hence at the present stage no conclusion may be drawn as to the presence or absence of a sodium pump in Periplaneta skeletal muscle membranes.

It is however pointed out that the characteristics of Periplaneta muscle microsomes do show a considerable similarity to the microsomes of frog muscle which does actively extrude sodium (Duggan, 1965). The ATPase activity found in frog muscle microsomal preparations is not synergistically stimulated by sodium and potassium, is not inhibited by strophanthin G and has a similar pH sensitivity, substrate specificity and magnesium requirements to cockroach muscle microsomes. The monovalent cation stimulation is inhibited by treatment with DOCA and the Mg^{2+}-dependent activity may be increased in the presence of cyanide.

To examine further the permeability properties of Periplaneta muscle membranes and to try and relate the properties of the microsomal ATPase activity to the physiology of the intact muscle it was considered necessary to investigate the passage of ions across the cell membrane and the electrical phenomena inevitably involved. Cyanide and DNP are two agents which have been used in studying bioelectric properties of muscles (Huddart & Wood, 1966) and their effect has usually been inter-

preted as being via cell respiration. However it has been shown that both agents are able to increase the Mg^{2+}-dependent ATPase activity, cyanide by 100% and DNP by 25% in this preparation. How these effects may be interpreted in terms of bioelectric potentials is not clear but it is suggested that cyanide and DNP also have a direct effect on the muscle membrane (see Chapters 4 & 5). By studying the movements of ions and changes in membrane potentials it was possible to determine the effect of agents such as strophanthin G on the intact cell. Similarly the permeability of the cell membrane to inorganic ions was studied in the intact cell. In such a way it was hoped to relate the properties of the ATPase activity of the membrane preparation to the physiology of the intact cell.

CHAPTER 4. IONIC DISTRIBUTIONS BETWEEN INTRACELLULAR AND EXTRACELLULAR PHASES.

INTRODUCTION

The distribution of permeable ions across the cell membrane is involved in the production of transmembrane bioelectric potentials. The relationship can be described by the Nernst equation:

$$E_m = \frac{RT}{nF} \times \log_e \frac{[K^+]_i}{[K^+]_o}$$

A more detailed account of the relationship between ionic gradients, permeability and membrane potentials is given in chapter 5. However it can be seen that one of the factors in the establishment of membrane potentials is the concentration of the permeable ions in the extracellular and intracellular ionic phases.

Much of the literature concerning intracellular and extracellular ionic concentrations in insects consists only of a list of values; a useful summary is given by Florkin and Jeuniaux (1964) and by Stobbart and Shaw (1964). The interpretation of these data with regard to the generation of bioelectric potentials in insect nerve and muscle is made difficult for the following reasons.

Firstly, some of the intracellular ions may be bound to proteins, as calcium almost certainly is, (Ebashi, Ebashi & Kodama, 1967). Secondly, in the muscle fibre ions may be isolated into compartments (Dunham & Gainer, 1968). Thirdly, it is not known to which ions insect muscle membranes are permeable. The reported values of insect haemolymph inorganic ion concentrations vary considerably. For example some reported values for the haemolymph ionic concentrations of a few representative insects are given in table 7. The variations reported may be due to diurnal fluctuations in ion levels (Jones, 1964) or to the presence of haemocytes (Brady, 1967b). The presence of cells in insect haemolymph would be expected to cause errors in the prediction of membrane potentials if the cells played an ion regulatory role (Brady, 1967a & 1967b).

TABLE 7. Some published values for insect haemolymph ion concentrations.

Example	Haemolymph conc. mmoles/litre Na^+	K^+	Mg^{2+}	Ca^{2+}	Author
Dictyoptera	110.6	13.3	-	-	Hoyle (1955)
Periplaneta americana	107.0	17.0	1.7	-	Tobias (1948)
	157.0	7.6	5.4	4,2	Van Asperen and Esch (1956)
			22.9	8.5	Clark and Craig (1953)
			13.5	17.8	Van Asperen and Esch (1954)
Cheleutoptera	21.0	25.0	-	-	Boné (1944)
Carausius morosus	14.0	16.0	-	-	Ramsay (1953)
	11.0	18.0	108.5	7.0	Ramsay (1955)
	8.7	27.5	145.0	16.2	Duchâteau, Florkin and Leclerq (1953)
	15.0	18.0	106.0	15.0	Wood (1957)
Orthoptera	67.4	9.0	27.0	15.2	Duchâteau et al. (1953)
Locusta migratoria migratorioides	75.0	20.0	-	-	Ramsay (1953)
	74.0	15.0	-	-	Ramsay (1953)
	102.0	22.0	-	-	Ramsay (1953)
Lepidoptera	2.6	52.8	49.2	16.4	Duchâteau et al. (1953)
Sphinx ligustri (pupae)	3.0	54.1	50.0	40.9	Duchâteau et al. (1953)
	4.3	48.4	-	15.0	Drilhon (1934)

The determination of myoplasm ion content of cockroach coxal muscles is also made difficult because they are composed of many fibres; indeed it has been argued by Usherwood (1969) that as the extracellular space around the innermost fibres may not be easily equilibrated with the bathing saline they are not suitable tissues for permeability studies. The ideal preparation, as Usherwood points out, would be a single fibre one, but, as even the most careful and delicate dissection would damage both the tracheal supply and innervation, which are essential for normal muscle function, results from such damaged preparations would be of questionable value. Related to this is the necessity to determine extracellular space volume of the muscle. It is shown below, however, that the extracellular space equilibrates with the bathing saline in about one hour and, consequently, Usherwood's suggestion that the coxal muscles are unsuitable for such studies may be unduly pessimistic.

In some insects the haemolymph has been found to contain a high potassium and low sodium concentration in which no vertebrate muscle could function. For example the leg muscle of Carausius morosus has a $[Na^+]_i / [Na^+]_o$ ratio close to unity and magnesium is the major external cation (Wood, 1957). In Tenebrio molitor larvae (Belton & Grundfest, 1962) and the moth Telea polyphemus and Sphinx ligustri (Huddart, 1966a) the haemolymph potassium level is very high and $[Na^+]_i > [Na^+]_o$.

Apart from the inorganic ions sodium, potassium, magnesium, calcium, chloride and bicarbonate, organic metabolites which are normally retained in the cells of other animals, are abundant in the haemolymph of insects. High aminoacidoemia is characteristic of insects. The amino acid content may compensate for fluctuations in haemolymph inorganic ion levels. Also present in the haemolymph are urea, uric acid, proteins, lipids, phospholipids and carbohydrates. Trehalose is often the major carbohydrate, particularly in Lepidoptera. Usherwood has suggested (1969)

that osmotic control is exercised by changing the amino acid haemolymph levels and not by the alteration of inorganic ion levels as in vertebrates although some control of inorganic ion levels is also achieved in insect haemolymph.

The approach used in this work was to determine, as accurately as possible, the values for internal and external ionic concentrations of sodium, potassium and magnesium under various conditions. It was considered of little value to assay calcium levels since this ion is largely bound in the muscle. The technique was standardised as far as possible so that the values obtained were comparable with each other if not necessarily with those obtained by other workers. The results obtained were interpreted in relation to the enzymic properties of the muscle microsomes.

METHODS

1. Haemolymph analysis.

A cockroach was fixed on its back by strips of plasticene to expose the soft cuticle at the base of the coxae. A small cut was made and a drop of haemolymph allowed to collect. 10 μl of this were taken up in a Drummond "microcap" micropipette and transferred to a 10 ml volumetric flask containing deionised water acidified with a drop of AnalaR concentrated nitric acid. The solution was made up to 10 ml and well shaken. As long as the procedure was carried out quickly no coagulation was found to occur. Nitric acid was used to leach the ions out of any precipitate which might have occurred. The samples were assayed for inorganic ions using a Unicam SP90 spectrophotometer and expressed as mmoles / litre of haemolymph.

2. Myoplasm analysis.

In control analyses the cuticle was stripped from the coxae and the coxal muscles removed by dissection to cause as little damage to the integrity of the fibres as possible. The excised muscles were then vigorously washed with a fine stream of deionised water for 5 seconds and then dried on Whatmann ashless filter paper where any remaining cuticle was removed. The excised muscles were weighed to an accuracy of 0.1 mg to give their wet weight. They were dried to constant weight at 60°C in a vacuum oven and reweighed to give the dry weight of muscle. The wet weight less the dry weight gave the weight of muscle water. The muscles were then placed in a muffle furnace at 350°C for 24 hours when only a fine white ash remained. This was carefully dissolved in deionised water acidified with a drop of AnalaR concentrated nitric acid and made up to 10 ml in a volumetric flask. The ions were assayed on a Unicam SP90 atomic absorption spectrophotometer. The results were expressed as mmoles / kg fibre water.

The effect of exposing the muscles to experimental salines, pharmacological agents and temperature, on the ionic composition of the muscle fibres was determined as follows. A cockroach was fixed on its back and a plasticene muscle bath constructed around the coxae. As much cuticle as possible was removed from the coxal muscles under examination without damaging the fibres. A certain amount of damage due to disruption of the tracheal system of the surface fibres must inevitably occur. The experimental saline was put in the muscle bath for a specified time at room temperature ($22^{\circ}C \pm 2^{\circ}$) unless otherwise stated. At the end of the exposure the saline was removed and the coxal muscles severed close to the body to avoid cutting the fibres unnecessarily. Thereafter the muscles were treated as has been described for the control muscles.

Since the muscle bath was constructed of plasticene it was important to ensure that plasticene did not release ions into the saline. A piece of plasticene was soaked in deionized water for 2 hours with frequent agitation before assaying the solution for Na^{+}, K^{+} and Mg^{2+}. No such ions were found to be present.

3. Atomic absorption spectrophotometry.

The experimental samples were compared to a series of standards. Sodium and potassium standards were prepared by serial dilution of B.D.H. volumetric solutions with deionized water. The magnesium standards were made up using magnesium chloride ($MgCl_2.6H_2O$). Magnesium was read by absorption and sodium and potassium by emission on a Unicam SP90 atomic absorption spectrophotometer.

4. Tracer methods.

Of the three types of experiments in which the rate of entry of ^{22}Na was determined, the methods used in two were essentially the same as has been described earlier for the ionic analyses of myoplasm. In one case the muscles were soaked in saline containing 3.6 μcuries ^{22}Na / ml and

the increase in intracellular ^{22}Na level monitored. A muscle was excised after a stated time, briefly washed in isotope-free saline and dried on filter paper. It was weighed and then dissolved in concentrated AnalaR nitric acid on a stainless steel planchette. This was then dried under an infra-red lamp. The activity of the sample was read using a Mullard end-window Geiger Müller tube, the count being displayed on an I.D.L. Scaler 1805. The readings presented were always greater than five times the background count, which was about 40c.p.m. This value was subtracted from all readings.

In the other case the coxal muscles of the two opposite legs were exposed to saline containing 3.6 μcuries ^{22}Na / ml. One leg was stimulated to twitch at a stated rate whilst the other leg was not stimulated and acted as a control. The differences in the amount of ^{22}Na which entered the two legs was determined as described above.

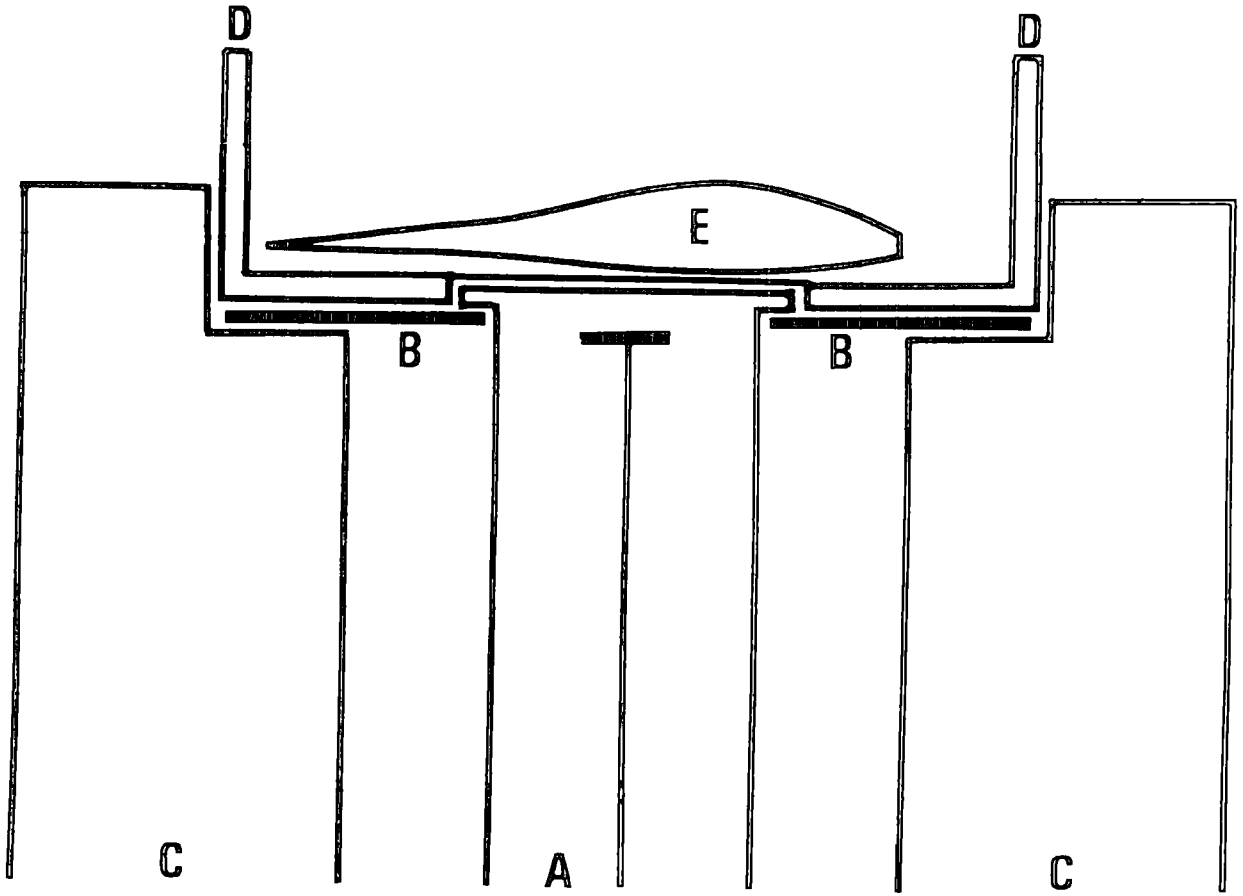
In the third type of experiment, an animal was injected with 0.1 ml of saline containing 0.4 μcuries of ^{22}Na / ml 24 hours previous to the experiment. The third thoracic segment was severed from the animal with its two legs. The exposed leg muscles were arranged as shown in figure 11. The saline bathing the muscles was replaced before each reading and agitated with a Pasteur pipette after each reading. In such a way the total activity of the muscles could be constantly monitored and the efflux of ^{22}Na followed without destroying the muscles. However it must be noted that the muscles were no longer attached to their tracheal systems or to their nervous systems as in other experiments. (The background count was subtracted from the readings)

5. Determination of muscle space volume.

Muscles, arranged in the same way as when determining the effect of experimental salines, were bathed in saline containing tritiated inulin with a specific activity of about 1.65×10^6 c.p.m. At the end of a set

FIGURE 11. Apparatus used to follow the efflux of ^{22}Na from cockroach coxal muscles injected 24 hours previously with 0.4 μcuries of ^{22}Na.

The Geiger Müller tube (A) is supported by an alloy disc (B) resting in a recess on the top of a one inch thick lead cylinder (C). A perspex muscle bath (D) sits on the top of the alloy disc. The end of the Geiger Müller tube fits a hole in the base of the muscle bath which has been sealed with a sheet of mica 20-30 μ thick. The muscles (E) were fixed across the mica window and the bath filled with saline.


D
D
E
B
B
C
A
C

time the muscles were washed rapidly with distilled water, blotted and weighed. They were dissolved in 0.2 ml of KOH and added to 12 ml of scintillation fluid. The scintillation fluid consisted of 30% methanol in toluene containing 6 g / litre of 2.5 diphenyl oxazole (PPO) and 0.1 g / litre of 2-phenylenebris (5-phenyl oxazole) (POPOP). The samples were read using a Beckmann 4S-200B liquid scintillation counter. A reading of the activity in the saline was obtained by taking 20 μl of the saline at the end of the experiment and adding it to 50 μl of normal saline. 10 μl of this was put into 12 ml of scintillation fluid. Such a dilution brought the level of activity down to that obtained from the muscle itself. The activity in the saline was calculated as follows:

Level in saline equals

$$\frac{\text{(count-background)}}{\text{sample volume } (\mu\text{l})} \times \text{dilution factor} \times 1000 \text{ counts / ml / minute.}$$

Similarly for the muscle:

$$\frac{\text{(count-background)}}{\text{wet weight (mg)}} \times 1000 \text{ counts / g wet weight / minute.}$$

Therefore the percentage extracellular space of the muscle equals

$$\frac{\text{activity in 1 g muscle}}{\text{activity in 1 ml saline}} \times 100.$$

This method is essentially the same as that used by Van der Kloot (1966) for determining crayfish muscle space.

6. Temperature.

Whole animals were exposed to 46°C by putting them in glass tubes lowered into a water bath at 46°C. A small hole was left in the top of the tubes for ventilation purposes. Saturated vapour pressure was maintained by adding a few drops of water to each tube.

To determine the effect of a temperature of 5°C upon exposed muscles

animals were arranged as described in section 2, and the experiments performed in a cold-room kept at 5° C.

RESULTS

1. The ionic balance between haemolymph and muscle.

The concentrations of inorganic ions resulting from the analyses of coxal muscle and haemolymph of Periplaneta are given in table 8. No allowance has been made for muscle space volume since it will be shown below to be small. In table 8 the results are compared with previous published values for Periplaneta. It can be seen that the haemolymph sodium content found in this work is similar to that found by other workers. The intracellular sodium content of 22.2 mmoles / kg fibre water is not significantly lower than the figure given by Hoyle (1955) although the value given by Tobias (1948) is far greater. The haemolymph potassium concentration found here is the same as that found by Tobias although higher than that found by Hoyle. There is a significant difference between the myoplasm potassium concentration of 86.7 mmoles / kg fibre water given here and the higher values given by Hoyle and Tobias. Since the potassium distribution depends to some extent upon diet this may explain some of the variation (Hoyle, 1954). The magnesium concentrations found in this work are considerably higher than those given by Tobias.

2. Extracellular muscle space.

Cell membranes are not permeable to inulin due to its large molecular size and consequently it can be used to determine the extracellular space of tissues (Van der Kloot, 1966). Wood (1963) estimated, using the inulin method of Roe, Epstein and Goldstein (1949), that the extracellular space of cockroach skeletal muscle never exceeded 4% of the total muscle volume. He considered this value to lie within his experimental error and therefore did not include it in the evaluation of his results.

Using tritiated inulin a value of less than 4% was found for the

TABLE 8. The concentration of Na^+, K^+, and Mg^{2+} in Periplaneta haemolymph and coxal muscle.

Standard errors of the mean have been given where available.

Ion	Haemolymph conc. mmoles / litre	Muscle conc. mmoles / kg fibre water	Author
Na^+	109.1 ± 10.6	22.2 ± 1.2	Present
Na^+	110.6 ± 7.7	26.9 ± 2.9	Hoyle (1955)
Na^+	107.0	46.0	Tobias (1948)
K^+	17.0 ± 1.1	86.7 ± 1.8	Present
K^+	13.3 ± 1.2	110.4 ± 7.3	Hoyle (1955)
K^+	17.0	112.0	Tobias (1948)
Mg^{2+}	6.6 ± 0.3	14.2 ± 0.3	Present
Mg^{2+}	1.7	7.4	Tobias (1948)

extracellular space for Periplaneta coxal muscle fibres (table 9). After soaking for 5 hours in tritiated inulin the value for the percentage space of the muscle volume was only 3.9% and this had not increased markedly from the value of 3.8 for a 1 hour soak. From the results for different exposure times to tritiated inulin (table 9), the time course of penetration into the muscle can be followed. In 30 minutes the inulin had only penetrated into half of the muscle space but within 1 hour complete equilibration had occurred. This is an important observation in view of criticisms concerning the length of time necessary for the complete equilibration of experimental salines around muscle fibres (Usherwood, 1969). More evidence to support an equilibration time of 1 hour will be given in chapter 5.

3. Saline composition.

On the basis of the haemolymph analyses, and with reference to the saline used by Wood (1965), a normal saline was constructed with the following composition:

Na^+ 140 mM : K^+ 16 mM : Mg^{2+} 6 mM : Ca^{2+} 10 mM : Cl^- 172 mM : HCO_3^- 16 mM.

The saline was brought to pH 7.2 by bubbling carbon dioxide through it. The justification for the use of this saline was that fibres bathed in it maintained their functional integrity for periods of more than 4 hours. As can be seen from table 11 significant redistribution of ions did not occur during a 2 hour exposure to normal saline. A comparison of the effects of buffers of various composition on the resting membrane potential is shown in chapter 5 where it is also shown that in this saline the muscle retains its bioelectrical properties for several hours.

4. Effect of pharmacological agents on ion movements.

a. 2.4 -Dinitrophenol.

Periplaneta coxal muscles which were exposed as described previously and soaked in saline containing 0.5 mM DNP at pH 7.0 for 2 hours showed some alteration in ion levels (table 10). The approximate values

TABLE 9. Determination of Periplaneta coxal muscle space volume.

The percentage muscle volume was calculated from the activity in the whole muscle after different times of exposure to tritiated inulin. In this respect the value for muscle space serves as an index of the rate of penetration of inulin around the muscle fibres. Note the increase in activity up to one hour exposure but the subsequent lack of increase after longer exposures.

Time of Exposure to Tritiated Inulin (hrs)	Calculated %age Extracellular Space in Muscle
0.5	2.7
1.0	3.8
1.5	3.3
3.0	4.0
5.0	3.9

TABLE 10. The effect of including 0.5 mM DNP in saline on the intracellular ion concentrations of cockroach coxal muscle.

The values attributed to Huddart and Wood were taken from Comparative Biochemistry and Physiology, Volume 18, page 684, figure 2, and therefore represent only approximate values.

Time of Exposure to 0.5 mM DNP (hrs)	% Change in Myoplasm Ion conc. Na (mM)	K (mM)	Mg (mM)	Author
1.0	+ 21.8	- 19.5	none	Present
1.0	+ 12.5	- 75.6	none	Huddart & Wood (1966)
1.5	+ 26.4	- 20.6	none	Present
1.5	+ 18.8	- 81.8	none	Huddart & Wood (1966)
2.0	+ 59.0	- 30.0	none	Present
2.0	+ 38.0	- 83.0	none	Huddart & Wood (1966)

TABLE 11. The effect on intracellular ion concentrations of Periplaneta coxal muscles due to exposure to normal saline and Strophanthin G.

Muscles were exposed for 2 hours at room temperature to saline only, or containing 5×10^{-4} M Strophanthin G.

Treatment for 2 hrs.	Sodium		Potassium		Magnesium	
	Conc. (mmoles / Kg muscle water)	% Change	Conc. (mmoles / Kg muscle water)	% Change	Conc. (mmoles / Kg muscle water)	% Change
Saline	22.5 ± 2.3	0	85.9 ± 2.9	0	14.8 ± 0.6	0
Strophanthin G	29.6 ± 1.0	+ 31.5	85.6 ± 4.9	- 0.9	13.4 ± 0.7	- 9.4

obtained for similar treatment of cockroach coxal muscle by Huddart and Wood (1966) are also shown. Clearly, the potassium loss by the present preparation was far less, and the sodium gain greater, than they found. After 2 hours Huddart and Wood found an 83% fall in $[K^+]_i$ and a 38% increase in $[Na^+]_i$. This compares with a 30% decrease in $[K^+]_i$ and a 59% increase in $[Na^+]_i$ in this preparation. No change was found in $[Mg^{2+}]_i$.

Hence it may be concluded that in 0.5 mM DNP at pH 7.0 there was a net outward movement of potassium ions and a net inward movement of sodium ions in these muscle fibres although the magnitude of the changes differs from that found by earlier workers.

b. Strophanthin G.

Exposed coxal muscles soaked for 2 hours in saline containing 5×10^{-4} M strophanthin G showed an increase of 31.5% in $[Na^+]_i$. Strophanthin G typically inhibits the linked Na K transport in a variety of cells, notably excitable cells. It is also known to inhibit glycolysis of erythrocytes (Murphy, 1963; Whittam & Ager, 1965). If a sodium pump is present in this preparation then the expected effect of strophanthin G would be to increase the level of intracellular sodium, because sodium entering along the electrochemical gradient could not be expelled from the cells. However, the classical theory of such a pump requires that intracellular sodium ions be expelled in exchange for potassium ions, so under the influence of strophanthin G it might be expected that an increase in $[Na^+]_i$ would be associated with a decrease in $[K^+]_i$. Table 11 shows that the potassium level in the muscle fibres remained constant in the presence of strophanthin G. The magnesium level showed a small fall of 9.4%. It would appear that the main effect of strophanthin G on Periplaneta muscle fibres is to increase intracellular sodium

concentration.

5. Effect of varying external pH.

The pH of the saline was altered by the addition of N - 2 - hydroxyethyl piperazine - N'- 2 - Ethanesulphonic acid buffer to give values between 6.15 and 7.85. No correction was made for the alteration in osmotic pressure of the salines since it was found that the percentage of water in the muscles remained constant during a 2 hour exposure at all pH values. The results are shown in table 12, together with the ionic content of the myoplasm. It can be seen that there was some fluctuation in the levels of all ions, but, overall it was clear that there was no real change in $[Na^+]_i$ or in $[K^+]_i$ except at pH 6.15 where $[K^+]_i$ was higher. It is apparent that such variations in external pH had little effect upon the intracellular concentrations of those ions assayed.

6. Effect of temperature.

a. On whole animal.

Animals were exposed to a temperature of 46°C for increasing times at saturated water vapour pressure. Ten minutes was allowed for the air temperature in the vials to equilibrate with the bath temperature before all exposure times. Haemolymph and muscle analyses were carried out as described for control animals. The analysis of the haemolymph ions (table 13) showed that the level of potassium rose sharply with time whilst the level of sodium dropped and the magnesium level rose slightly. Over one hour there was a 45% drop in $[Na^+]_o$, a 29.7% increase in $[K^+]_o$ and a 21.2% increase in $[Mg^{2+}]_o$. Ion analysis of muscle after treatment (table 13) showed that the fibres lost potassium and gained sodium during a one hour exposure. The magnesium level in the muscle was unchanged. The water content of the muscles remained more or less constant during the exposures.

TABLE 12. The effect of saline pH on myoplasm ion concentrations of cockroach coxal muscle.

Muscles were bathed for two hours at room temperature. Saline at pH 7.2 and 7.85 was produced with bicarbonate buffered saline alone and saline at pH 6.55 and 6.15 by the addition of N - 2 - hydroxyethyl piperazine - N'- 2 - Ethanesulphonic acid.

pH	Expts.	Na (mmoles / Kg tissue water)	Range	K (mmoles / Kg tissue water)	Range	Mg (mmoles / Kg tissue water)	Range	% Water in Wet Wt.
6.15	3	22.5	19.9-27.3	99.7	96.3-103.5	13.4	12.3-14.0	79.6
6.55	5	23.0	19.6-24.6	84.8	72.9- 99.6	12.1	11.8-12.7	78.7
7.20	9	22.2	15.8-36.7	85.9	72.7-100.0	14.2	12.9-19.0	80.1
7.85	4	21.7	16.3-25.3	83.2	79.3- 87.0	11.5	11.3-11.8	80.2

TABLE 13. Effect of exposure of the whole animal to 46°C on ionic distributions between the myoplasm and haemolymph of the cockroach.

Animals were exposed to 46°C at saturated vapour pressure for the times stated.

Concentrations of Na, K and Mg are expressed as mmoles / Kg muscle water in the case of myoplasm values and as mmoles / litre for haemolymph values.

Sample	Time of Exposure (min)	Sodium	Potassium	Magnesium	% water
Haemolymph	0	109.1	17.0	6.6	
Myoplasm	0	22,2	86.7	14.2	81.0
Haemolymph	15	107.5	21.0	5.8	
Myoplasm	15	16.8	70.0	15.2	81.3
Haemolymph	30	92.5	34.5	5.5	
Myoplasm	30	19.6	71.3	15.3	81.3
Haemolymph	45	55.0	71.0	8.8	
Myoplasm	45	20.4	71.6	16.9	78.6
Haemolymph	60	60.0	67.5	8.0	
Myoplasm	60	24.4	73.9	16.1	80.6

The time course of the change in haemolymph and muscle were different. In the haemolymph sodium was lost whilst potassium was gained so that after 45 minutes at 46°C $[Na^+]_o$ was reduced by 54.1 mM and $[K^+]_o$ was increased by 54 mM. This apparent one for one exchange was not reflected in the muscle where $[Na^+]_i$ did not show a consistent change during 60 minutes and $[K^+]_i$ was reduced by 16.7 mM within 15 minutes but did not show any further decrease.

The changes in haemolymph ion concentrations were more dramatic than those in muscle ion concentrations. It is predicted that this is mainly because haemolymph volume is small relative to tissue volume and so an exchange of a certain quantity of an ion between haemolymph and muscle would be reflected in a larger concentration change in the former tissue. It is also unlikely that the exchange of ions was restricted to haemolymph and muscle, and other tissues such as fat body and gut may also have been involved. It is concluded that exposure of the whole animal to 46°C causes an exchange of Na for K between haemolymph and tissues.

b. <u>On exposed muscle</u>.

To ensure that the pH of the normal saline did not alter over the temperature range employed the temperature / pH relationship was determined. It was found that for a 15°C fall in temperature from 20°C there was a reduction of 0.18 of a pH unit. The saline at 5°C had a pH of 7.02. From previous work on the effect of pH on ion movements (see result 5) it is unlikely that such a small fall in pH would have any significant effect on ionic distributions.

When the muscle fibres were soaked in saline for 2 hours at 5°C the following results were obtained upon assaying the fibres for their ion content. $[Na^+]_i$ = 19.3 mmoles / Kg fibre water, a 14.2% decrease ; $[K^+]_i$ = 95.5 mmoles / Kg fibre water, an 11.2% increase ; $[Mg^{2+}]_i$ =

13.2 mmoles / Kg fibre water, a 10% decrease. Hence lowering the temperature to 5°C caused only a small redistribution of the myoplasm ions. These results are of significance to the electrophysiology section on the effects of low temperature (chapter 5).

7. Movement of ^{22}Na.

a. Sodium exchange rate.

Exposed coxal muscles were soaked for varying times in saline containing 3.6 μcuries / ml of ^{22}Na. The muscles were monitored for ^{22}Na activity at the stated times (table 14). The results showed that there was a significant permeability to sodium since the ^{22}Na content of the fibres increased with exposure time. Some of the activity present was obviously due to contamination by ^{22}Na of the extracellular spaces resistant to the washing out process. The extracellular space volume of a 30 mg muscle is about 1 μl. It was determined that 1 μl of saline with an activity of 3.6 μcuries of ^{22}Na / ml would give a reading of about 1,600 c.p.m. It was estimated from the graph shown in figure 12 that the contamination was not greater than 17 c.p.m. / mg wet weight of muscle. Furthermore strophanthin G at 5×10^{-4} M, which has been shown to increase $[Na^+]_i$, raised the rate of ^{22}Na entry (table 14 and figure 12). Indeed the ^{22}Na level in the muscle after 60 minutes gave an activity of 66 c.p.m. / mg wet weight of tissue which rose to only 69 c.p.m. after another hour. This finding clearly shows that the results obtained in the control muscle were not solely due to ^{22}Na entering the extracellular spaces since strophanthin G would be expected to have little effect on such a diffusion process.

b. Excitation and ^{22}Na entry into muscle.

Exposed coxal muscles were soaked for 15 minutes in saline containing ^{22}Na, at a concentration of 3.6 μcuries / ml, and then stimulated at one pulse per second for 15, 25 or 30 minutes. The results

TABLE 14. The effect of Strophanthin G on the passive influx of ^{22}Na into resting muscle of the cockroach.

Muscles were soaked in saline containing 3.6 μ curies ^{22}Na / ml at room temperature. The activity of these muscles was compared to those in which strophanthin G at 5×10^{-4} M was included in the saline.

Time of Exposure to ^{22}Na (min)	Activity of Muscle c.p.m. / mg wet wt. of Muscle	Strophanthin G 5×10^{-4} M	Increase in Activity due to Strophanthin G
30	20.4	-	2.5
30	22.9	+	
60	23.0	-	11.8
60	34.8	+	
90	25.7	-	37.0
90	62.7	+	
150	32.9	-	36.6
150	69.5	+	
270	36.3	-	

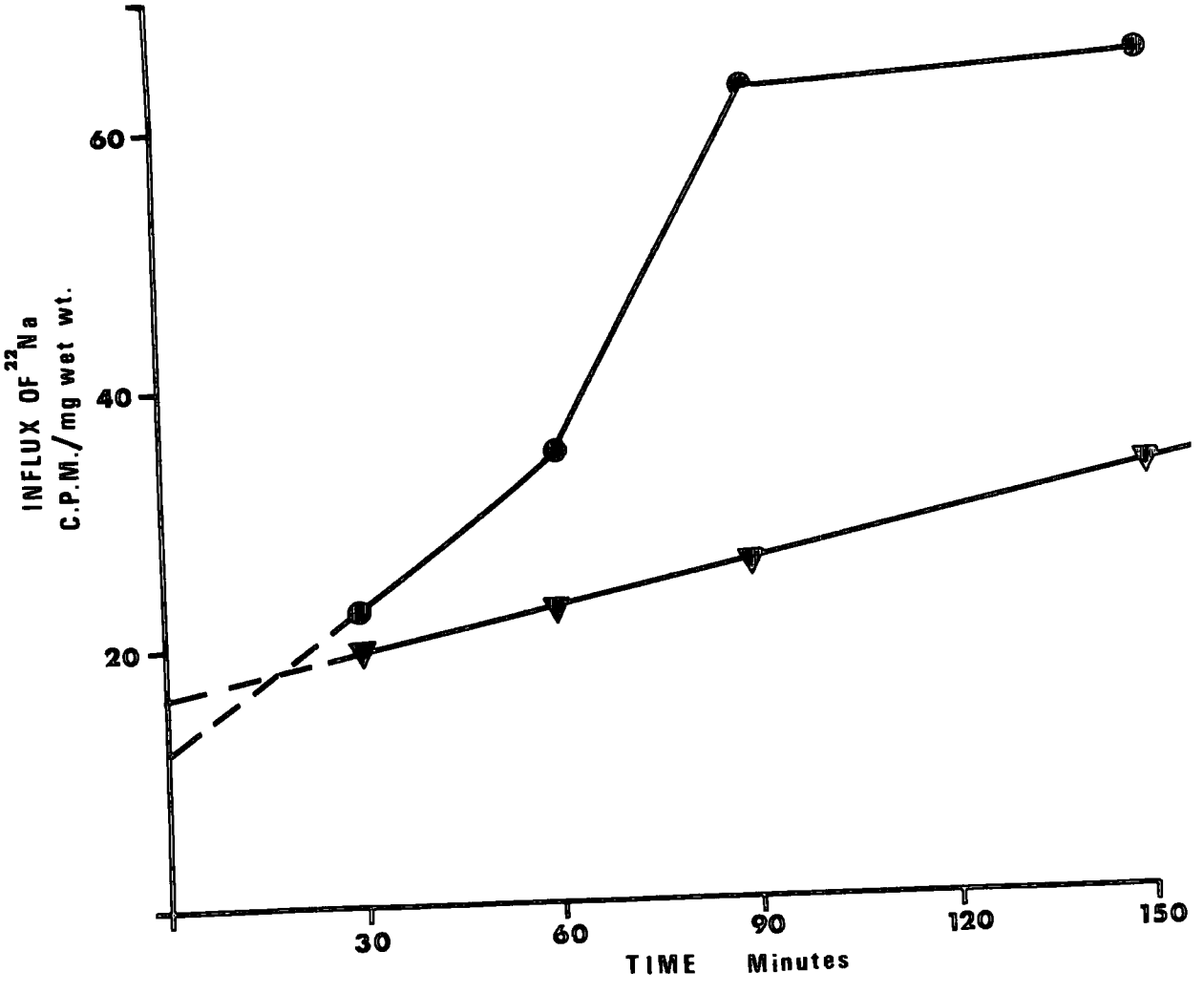
FIGURE 12. The effect of strophanthin G on the passive permeability to ^{22}Na of cockroach coxal muscle.

Experiments were conducted at room temperature in saline containing 3.6 μ curies per ml of ^{22}Na (▲) Strophanthin G was added to this saline at a concentration of 5×10^{-4} M (○).

Ordinate: Influx of ^{22}Na shown as the counts / minute / mg wet wt. of muscle.

Abscissa: Time (minutes) of exposure to saline containing 3.6 μ curies per ml of ^{22}Na.

Data obtained from Table 14.


INFLUX OF ^{22}Na
C.P.M./mg wet wt.
60
40
20
30
60
90
120
150
TIME Minutes

are shown in table 15. It was found that the ^{22}Na content of the stimulated muscle was higher than the control muscles which had not been stimulated, although there was some variation in the radio activities recorded. This may have been due to the difficulty in ensuring that all the fibres were responding to the stimuli. It is certainly possible that the increased ^{22}Na activity of the stimulated muscles was in part due to a faster penetration of the isotope into the extracellular spaces as a result of the contraction of these fibres. However, if sodium ions are involved in carryimg the inward current upon stimulation, then part or all of the increased activity could be due to the increased permeability to sodium during excitation.

c. Efflux of ^{22}Na from muscle.

Several experiments were attempted where an animal was injected with 0.1 ml saline containing 0.4 μ curies of ^{22}Na / ml and left overnight. The leg muscles were then exposed and arranged (figure 11) so that the ^{22}Na content could be monitored continuously. Table 16 gives the results obtained for the efflux of ^{22}Na into saline as the percentage loss of activity from the initial ^{22}Na activity of the whole muscle and the actual activities recorded. It can be seen that there was an initial high washout in the first few minutes followed by a very slow fall in the activity of the muscles. For safety reasons, it was not possible to load the animals with higher levels of ^{22}Na, and, at the levels used it was not possible to determine accurately the rate of efflux; it was therefore not feasible to determine the effects of pharmacological agents or temperature upon the efflux rate.

TABLE 15. The effect of repetitive stimulation on the influx of ^{22}Na into cockroach coxal muscle.

Muscles were stimulated at room temperature at the rate of one stimulus per second. The control muscles were simply bathed in the saline for an equivalent time. The saline contained 3.6 μ curies / ml ^{22}Na.

Total Time in ^{22}Na (min)	Time Stimulated (min)	Activity above Background c.p.m. / mg wet weight
30	15	50.8
30	none	15.8
40	25	36.8
40	none	23.0
45	30	41.0
45	none	15.8

TABLE 16. Efflux of ^{22}Na from resting coxal muscle of the cockroach.

Experiments were conducted at room temperature. Muscles were loaded with ^{22}Na by injecting the animal with 0.1 ml of saline containing 0.4 μ curies of ^{22}Na / ml. The actual activities recorded are given along with the percentage loss activity with time. The efflux was monitored using the apparatus shown in figure 11.

Time for Efflux (min)	c.p.m. of Whole Muscle	Percentage loss of activity by the Muscle in Saline
0	562.3	0
15	379.2	32.6
30	350.8	37.6
60	312.6	44.4
90	296.4	47.3
120	266.2	52.7

DISCUSSION

Although the results of this chapter are best discussed in the light of the results of the electrophysiological investigations, it is worthwhile considering briefly the significance of these results and to relate them to the findings of the previous chapter.

The results obtained for the intracellular and extracellular ionic distributions compare reasonably well with some other published values. From the figures obtained it is possible to forecast the expected membrane potential of the muscle fibres if it is assumed that they behaved according to the Nernst hypothesis. This states that for potassium ions

$$E_K = \frac{RT}{nF} \times \log_e \frac{[K^+]_i}{[K^+]_o} \text{ volts}$$

and for sodium ions

$$E_{Na} = \frac{RT}{nF} \times \log_e \frac{[Na^+]_o}{[Na^+]_i} \text{ volts}$$

where

R = the gas content,

T = absolute temperature,

F = Faraday's constant,

n = valency,

E_K = potassium equilibration potential,

E_{Na} = sodium equilibration potential.

From such calculations, using the values for $[K^+]$ and $[Na^+]$ obtained in this work, $E_K = -41.4$ mV, and $E_{Na} = +40.4$ mV. In the case of sodium, the Nernst equation describes the theoretical maximum to which the membrane potential should deviate from zero in a positive direction during excitation if the muscle were to become a pure Na battery (when $P_K = 0$). If the membrane were solely permeable to potassium ions in the resting state, and this permeability was of a passive nature, then

the potential developed (E_K) will be the result of a Donnan equilibrium and be an expression of the distribution of potassium ions across that membrane.

Before considering the effects of agents upon the permeability of the muscle membrane certain facts concerning the applicability of Periplaneta coxal muscles to this kind of study must be considered. Usherwood (1969) in criticizing the results of Wood (1963, 1965) obtained from work on cockroach coxal muscles stated that "..... I am convinced that the large leg muscles used by Wood are most unsuitable .. ... his (Wood) results reflect to some extent the difficulties of equilibrating these muscles with saline." These statements are supported by a personal communication (to Usherwood) from Belton who found that the retractor unguis muscle from (an anonymous) moth leg, which was composed of only a few fibres, still contracted three hours after immersion in distilled water. It is difficult to assess such a statement without experimental details. The muscle used was, by Belton's own admission, peculiar in being composed of only a few fibres and as such one cannot rule out the possibility of a structural barrier protecting them. Furthermore, no evidence was given of an intracellular examination to determine the distribution of ions in these muscles. A possible explanation of this phenomenon is given later. Certainly it would appear to be weak evidence to discontinue, as Usherwood has suggested, working with cockroach coxal muscles.

The evidence presented here, using tritiated inulin, is contrary to Usherwood's view. Complete penetration around the fibres had occurred within one hour of soaking the large coxal muscles of the cockroach in saline containing inulin. Evidence given in the sections on electrophysiology shows that agents which affect the bioelectric potentials of the central fibres do so within one hour of application.

Hence it is suggested that the problem which Usherwood has raised concerning the equilibration of these muscles with experimental salines is largely groundless.

The results presented here confirm the effect of DNP on ion movements found by Huddart and Wood but differ from their results in detail. They describe much greater decreases in $[K^+]_i$ and smaller increases in $[Na^+]_i$ than reported here. A point of contrast is that Huddart and Wood have used phosphate buffered saline in their work, which, as is shown in chapter 5, is unsuitable for the two hour exposure period. Thus, it is likely that soaking alone for this period in phosphate saline would cause some ion movements. Unfortunately Huddart and Wood did not include a control series to check this. It has been shown that exposure to bicarbonate buffered saline not only sustains normal resting membrane potentials (chapter 5) but also maintains internal sodium and potassium concentrations (table 11).

Huddart and Wood have tentatively suggested that the primary action of DNP is on oxidative phosphorylation so reducing the supply of ATP for active transport. However DNP has also been shown to affect membrane enzymes (see Chapter 3), and so it is also possible that this agent has a direct effect upon membrane permeability. DNP, acting as an uncoupling agent, has the ability to separate protons and electrons (Robertson, 1968) which might result in local changes in internal pH which could also affect permeability (Stephens, 1969). McCann (1967) has proposed the existence of a K-pump in moth heart muscle. This pump transports potassium inwards, with no linked sodium efflux. It is inhibited by DNP which causes an increased potassium efflux. Such a pump would contribute to the resting membrane potential unless chloride ions entered the fibres with potassium ions. Indeed, evidence for the existence of an electrogenic component to the resting membrane potential

in cockroach skeletal muscle fibres will be given in later chapters.

The effect of strophanthin G on this preparation is difficult to explain. It has been clearly demonstrated that strophanthin G does not affect the ATPase activity of the microsomal preparation of Periplaneta skeletal muscles, yet it does cause an increase in $[Na^+]_i$. If it is suggested that there is a classical $Na^+ + K^+$ activated Mg^{2+} dependent ATPase present in the muscle membrane, then either it is inactivated by the extraction procedure, or makes only a very small contribution to the total ATPase activity. The effect of strophanthin G on the sodium level in the muscle may reflect the blockage of a sodium pump via inhibition of a glycolytic energy supply (Murphy, 1963; Whittam & Ager, 1965). However one would expect that with a classical $Na^+ + K^+$ -exchange system strophanthin G would also affect $[K^+]_i$. In this preparation this is not the case and, as strophanthin G and potassium are thought to compete for a common enzyme site, if a sodium pump does exist it is probably not of the classical type.

No mention has yet been made of chloride distribution. The most recently published data for cockroach muscle is given by Wood (1963) where $[Cl^-]_i$ was found to be 10 mM and $[Cl^-]_o$ 95.7 mM. Chloride efflux probably occurs across the membranes of the transverse tubular system in the skeletal muscle of insects and crayfish (Brandt, Reuben, Girardier & Grundfest, 1965; Cochrane & Elder, 1967; Usherwood, 1967a). Wood (1965) suggests that a Donnan equilibrium involving potassium and chloride ions does not occur in locust and cockroach muscle. It has been correctly pointed out by Usherwood (1969) that Wood's analysis of $[Cl^-]_i$ could have included extracellular chloride ions held within the transverse tubular system of the muscle fibres. The possible contribution of chloride ions to the membrane potentials of cockroach muscle fibres is fully discussed in the next chapter.

It was shown in chapter 3 that the ATPase activity was reduced at low temperatures. Since such membrane enzymes have been implicated in the control of membrane permeability it was not surprising that exposure of the whole muscles to 5°C caused some redistribution of ions. It is suggested that the effect of low temperatures on these muscles is to reduce the ability of the muscle membrane to control the passage of ions across it, either passively or by active movement of ions. These results have considerable bearing on the effect of low temperatures on the resting membrane potential. The effect of high temperature on the whole animal was to cause drastic changes in haemolymph ion concentrations but since it was concluded that these changes were not fully reflected in myoplasm ion changes some other tissue or tissues may also be involved.

From the work using ^{22}Na it was shown that Periplaneta skeletal muscle fibres are permeable to sodium ions in the resting state and that sodium ions enter the fibres during excitation. Cells which become permeable to sodium ions during excitation typically possess a sodium pump to extrude those ions which enter. Significantly strophanthin G caused an increase in the ^{22}Na content of cockroach muscle fibres.

Previous workers have, from analyses of the ionic content of haemolymph and myoplasm, predicted that the ionic basis to the excitability of some insect muscles must be very different from that of vertebrate muscle. The present work has shown that in Periplaneta muscle $[K^+]_i > [K^+]_o$, that $[Na^+]_i < [Na^+]_o$ and that $[Mg^{2+}]_i > [Mg^{2+}]_o$ which is essentially an ionic distribution similar to that of vertebrates. However, the interpretation of these data in terms of bioelectric potentials is made difficult for several reasons. A major problem is the possible role of haemocytes in the control of ion balances.

Brady (1967a, 1967b, 1968) believes that haemocytes do contribute to the potassium level in the haemolymph, and as such, would lead to errors in predicting membrane potentials. Ion binding may occur in the extracellular space of muscle fibres. Carrington and Tenney (1959) conclude that 10% of the calcium and 20% of the magnesium in the haemolymph of Telea polyphemus (Lepidoptera) is bound to organic particles. For the same animal Weevers (1966) suggested a figure of 60% for calcium and magnesium binding. Since equations of the Nernst and Goldman type depend on values of ion activities rather than gross ion concentrations it is clear that the occurrence of any ion binding presents a real difficulty in predicting bioelectric potentials.

However bioelectric predictions from ionic distributions can be of some value especially when comparing the actual value of membrane potential developed with the ion distributions. The application of the data obtained in this chapter in determining the origin of bioelectric potentials may be seen in the following chapters on the electrophysiology of cockroach coxal muscles.

CHAPTER 5. THE RESTING MEMBRANE POTENTIAL.

INTRODUCTION

As a result of the unequal distributions of positive and negative ions across the cell membrane, muscle fibres in the resting state are in a polarized condition with the inside negative to the outside. This chapter involves an examination of the mechanism of production and maintenance of this resting membrane potential.

Theories for the origin of the resting potential of excitable cells originated in modern times from the work of Bernstein (1902 & 1912), who considered that the resting membrane was selectively permeable to potassium ions only. In such a situation the potential developed could be calculated according to the Nernst relationship for a potassium electrode:

$$E_m = \frac{RT}{nF} \times \log_e \frac{[K^+]_i}{[K^+]_o} \text{ volts}$$

where

n = valency,

F = Faraday's constant,

R = the gas constant,

T = absolute temperature,

E_m = resting membrane potential,

$[K^+]_o$ = extracellular potassium ion concentration,

$[K^+]_i$ = intracellular ion concentration.

Boyle and Conway (1941) demonstrated that frog sartorius muscle fibres at rest were permeable to both potassium and chloride ions and it was suggested that their distribution was in accordance with a Donnan equilibrium.

$$\frac{[K^+]_o}{[K^+]_i} = \frac{[Cl^-]_i}{[Cl^-]_o}$$

In this case the resting potential was forecast by the unequal dis-

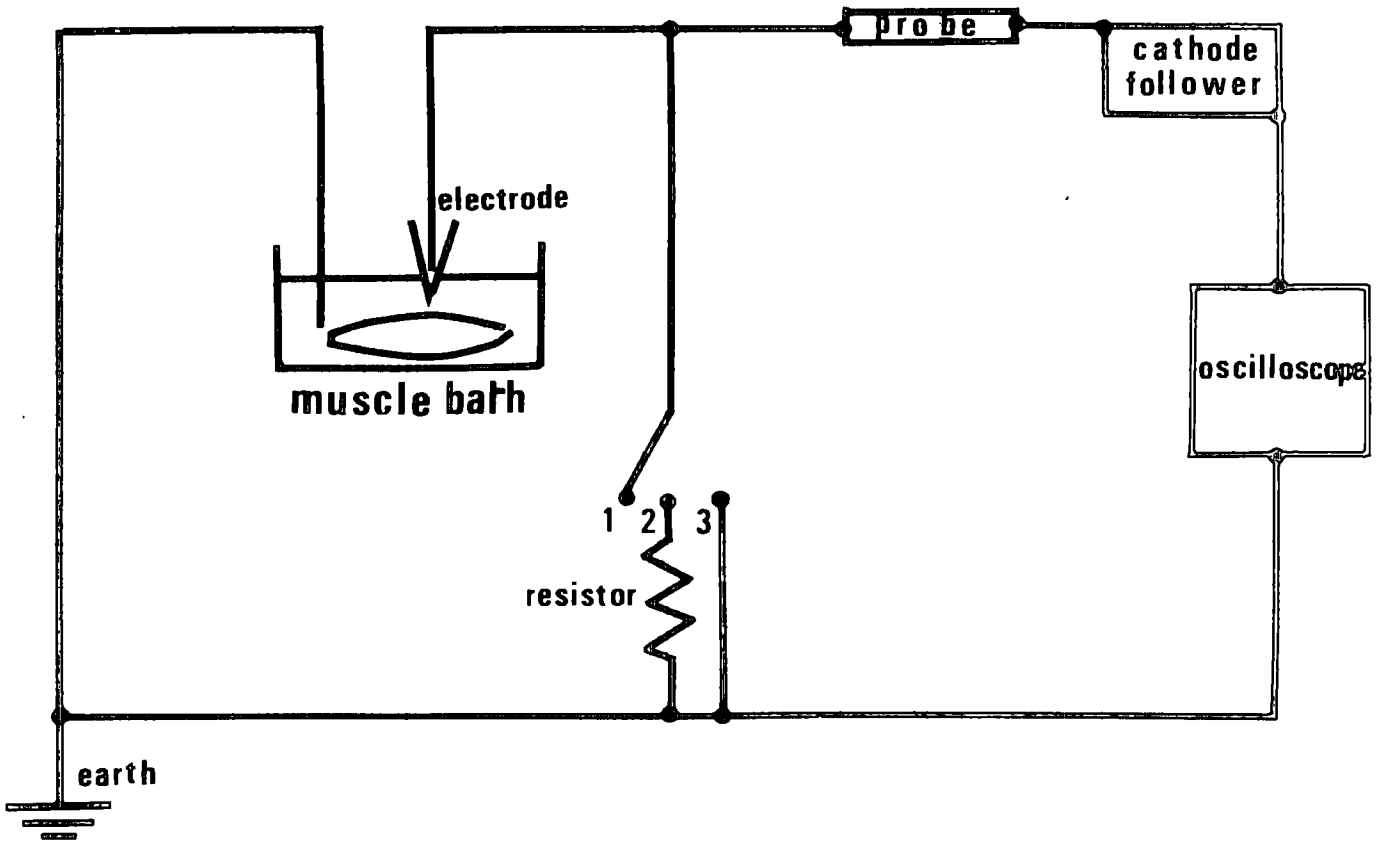
tribution of these permeable ions across the cell membrane. The distribution was held to be unequal due to the presence inside the cell of large, impermeable, negatively charged ions, and outside the cell of positively charged, impermeable sodium ions. Such a system with a fixed negative charge inside and a fixed positive charge outside constitutes the "double" Donnan equilibrium described above.

This theory had to be modified when it was found that sodium was not a totally impermeable ion in the resting state. In order to maintain the marked difference in intracellular sodium and potassium concentrations either sodium ions alone or both sodium and potassium ions had to be actively transported across the cell membrane. In frog skeletal muscle sodium is removed from the cell in exchange for potassium in an active, metabolically dependent, process working against the concentration gradient. Chloride ions in this case appear to be passively distributed depending on E_m and on the concentration of impermeant intracellular anions. The extrusion of sodium ions alone would be sufficient to maintain the unequal distribution of potassium and sodium but, since it would involve the unidirectional transport of a charged particle, it would result in the establishment of a potential i.e. it would constitute an electrogenic pump. Any potential due to an electrogenic pump would sum algebraically with the diffusion potentials of potassium, chloride and sodium ions and the most permeant ions, potassium and chloride, would become passively distributed according to the electromotive force of the cell. A pump exchanging potassium for sodium equally would be electrically neutral and then the EMF of the cell would be determined by the relative permeabilities of all the ions in the system. The Goldman Equation (1943) refers to such a steady state system, where the intracellular and extracellular activities of the ions are equal:

$$E_m = \frac{RT}{nF} \times \log_e \frac{P_K [K^+]_o + P_{Na} [Na^+]_o + P_{Cl} [Cl^-]_i}{P_K [K^+]_i + P_{Na} [Na^+]_i + P_{Cl} [Cl^-]_o}$$

where P_K, P_{Na}, P_{Cl} are the relative permeabilities for potassium, sodium and chloride ions. In frog muscle $P_K > P_{Na}$, E_m approaches E_K and chloride ions are thought to be distributed according to E_m. The applicability of these various equations to cockroach skeletal muscle will be discussed in the light of the results presented here and those of other workers.

An examination is made of the effects of metabolic inhibitors and temperature upon the resting potential in an attempt to determine the extent to which active processes underlie its production and maintenance. The extent to which electrogenic pumps may contribute to such a potential is discussed in relation to the findings on the characteristics of the membrane ATPase already described.


probe
cathode follower
electrode
muscle bath
oscilloscope
1
2
3
resistor
earth

electrodes were not stored but used when fresh, reduced the likelihood of anomalous electrode potentials developing.

6. Resting potential measurements.

A microelectrode was inserted into a specially constructed brass holder held in a Prior micromanipulator. The electrode was connected by a short piece of silver-silver chloride wire to a cathode follower probe. The cathode follower was supplied by Electrophysiological Instruments Ltd. The cathode follower was connected to a Tektronix 502A dual beam oscilloscope. The saline in the muscle bath was earthed via a piece of silver-silver chloride wire. The circuit employed is shown in figure 13. A resting potential was recorded by lowering an electrode into the muscle with the manipulator until a sudden change in potential was recorded indicating that the electrode had penetrated a muscle fibre. Usually at least thirty different fibres were penetrated and their mean resting potential held to constitute a single reading.

RESULTS

1. The effect of saline composition on the resting membrane potential.

The literature describes a great variety of different saline compositions for insect muscle preparations. It is essential that the saline employed should not alter the properties of the muscles studied. In deciding upon the composition of a suitable saline two criteria were used. Firstly, the resting potential should be maintained and the muscle fibres retain their excitability for at least four hours at room temperature. Secondly, the resting potential recorded from muscles bathed in saline should be the same as that recorded in haemolymph. Three different salines were tested and their composition is shown in table 17. Phosphate buffered saline was prepared according to Wood (1965) and phosphate / bicarbonate buffered saline according to Wood (1961). Bicarbonate buffered saline was based upon the haemolymph analyses previously described (table 8).

The effect of a four hour exposure to these salines on the resting potential of Periplaneta skeletal muscle is shown in figure 14. In phosphate buffered saline the resting potential was depolarized by -18 mV after four hours. Similarly in phosphate / bicarbonate buffered saline the resting potential depolarized with the time of exposure such that it was reduced by 13 mV after four hours. On the other hand, in the bicarbonate buffered saline there was a depolarization of only -2 mV after four hours. Furthermore, in bicarbonate buffered saline there was no apparent loss of excitability whereas in the other salines the response to stimulation was reduced, although no quantitative data was obtained. Other buffering agents such as Tris, HEPES and L-histidine were experimented with, but none proved as effective as bicarbonate in maintaining the resting potential over long periods of time. In bicarbonate saline the resting potential

TABLE 17. Saline composition.

The salines were made up in deionised water. Salines containing bicarbonate were brought to pH 7.2 by bubbling with CO_2. Bicarbonate was added as the potassium salt.

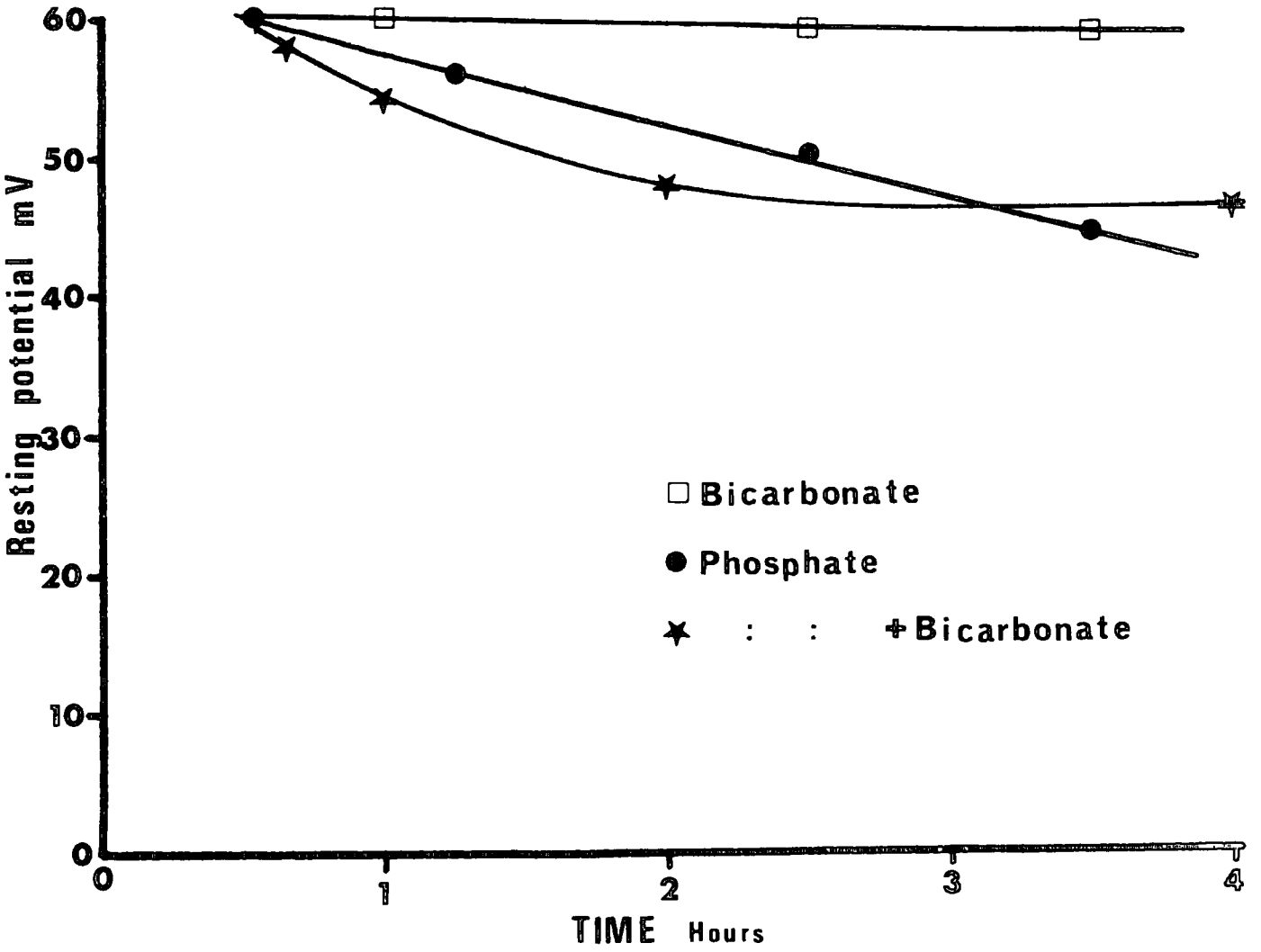
Saline	mM								pH
	Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-	HCO_3^-	HPO_4	H_2PO_4	
Phosphate	143	12	2	3	150	-	4.5	6	7.3
Phosphate / bicarb.	100	14	4	10	142	4	-	6	7.2
Bicarbonate	140	16	10	6	172	16	-	-	7.2

FIGURE 14. The effect of saline composition on the resting potential of cockroach coxal muscle.

Each point on the graph is the mean of at least 30 recordings from different muscle fibres. The standard errors are not great enough to be drawn in. The composition of the salines is shown in table 17. Muscles were soaked at room temperature.

Ordinate: Resting membrane potential (mV).

Abscissa: Time of exposure of the muscles (min.).


60
50
40
30
20
10
0
Resting potential mV
0
1
2
3
4
TIME Hours
□ Bicarbonate
● Phosphate
★ : : + Bicarbonate

recorded was very similar to that recorded in haemolymph (table 26, chapter 6). As a result of these findings it was decided that the bicarbonate buffered saline was a reasonably good physiological saline and consequently it was used as the normal saline for cockroach muscle.

2. The contribution of potassium and chloride to the resting membrane potential.

A series of experiments were undertaken to examine the dependency of the resting potential upon the distribution of potassium and chloride ions. The potassium concentration of the saline was altered in the absence of chloride ions, where proprionate salts were used, and in the presence of chloride, in such a way that the product of the potassium and chloride concentrations remained constant. In chloride free saline isosmocity with K 150 mM saline was maintained by the addition of sucrose to salines with lower potassium concentrations. Muscle fibres were equilibrated for two hours in each potassium concentration before recording potentials. Two hours has been shown to be adequate for equilibration around these muscle fibres (table 9). The effect of potassium concentration in the absence of chloride is shown in figure 15. The slope for a ten-fold change in potassium concentration was 41 mV. The effect of potassium concentration on the resting potential whilst keeping the product of the potassium and chloride concentrations constant is shown in figure 16. The slope for a ten-fold change in potassium concentration was 43 mV.

The effect on the resting potential of replacing chloride in the saline with an impermeant anion was tested. Chloride was replaced by an equivalent amount of sulphate, citrate, succinate or proprionate. An initial measurement of the resting potential was made in bicarbonate saline and then after bathing with chloride free saline for various times. The resting potential depolarized to a steady level after

FIGURE 15. The effect of potassium concentration in the absence of chloride on the resting potential of cockroach coxal muscle.

Muscles equilibrated with the salines for two hours before recording. Chloride was replaced by proprionate and the experiments were performed at room temperature. Standard errors were calculated for the readings but were not great enough to be shown on the graph.

Isosmocity with K 150 mM saline was maintained by the addition of sucrose to saline with lower K^+ concentrations.

Ordinate: Resting membrane potential (mV).

Abscissa: Concentration of potassium (mM) in bathing saline.

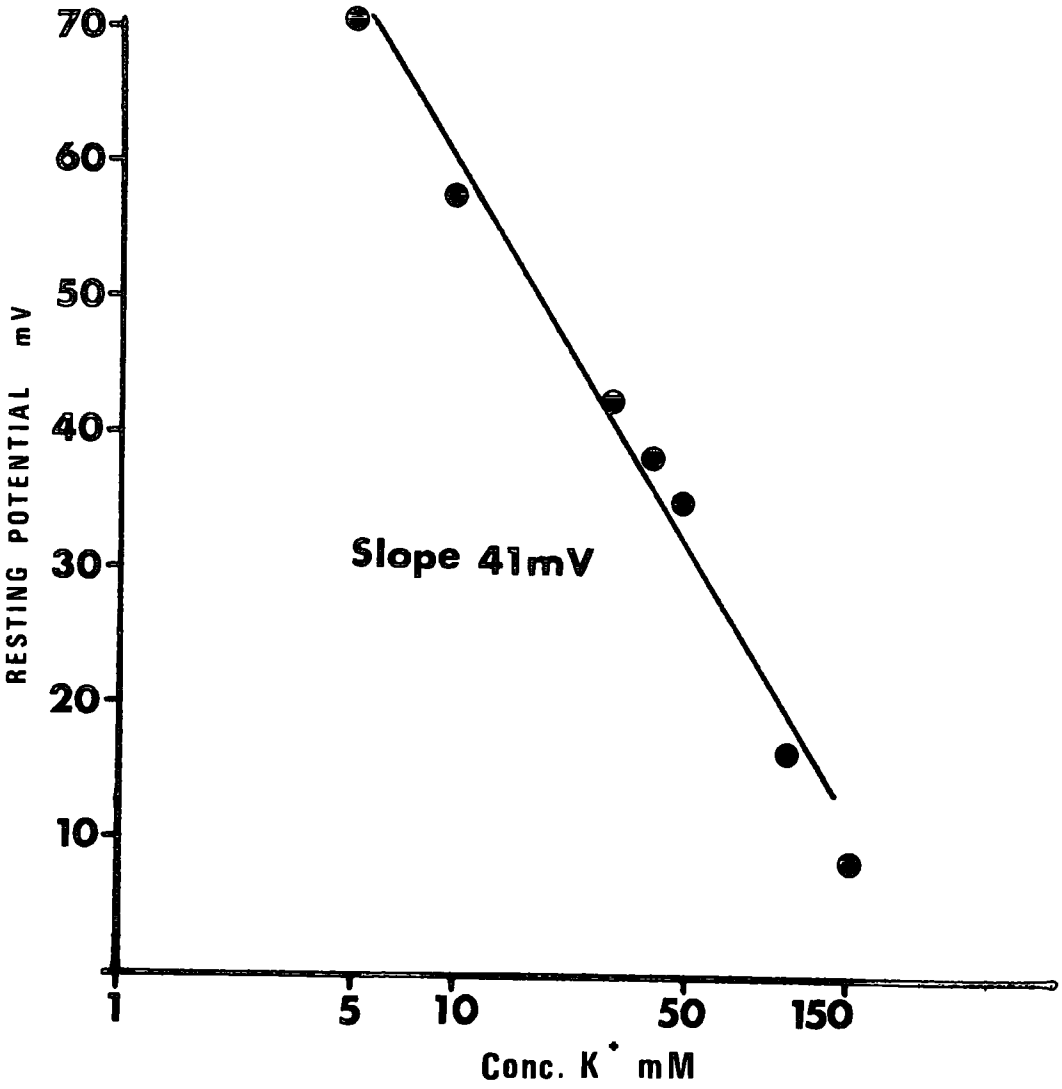
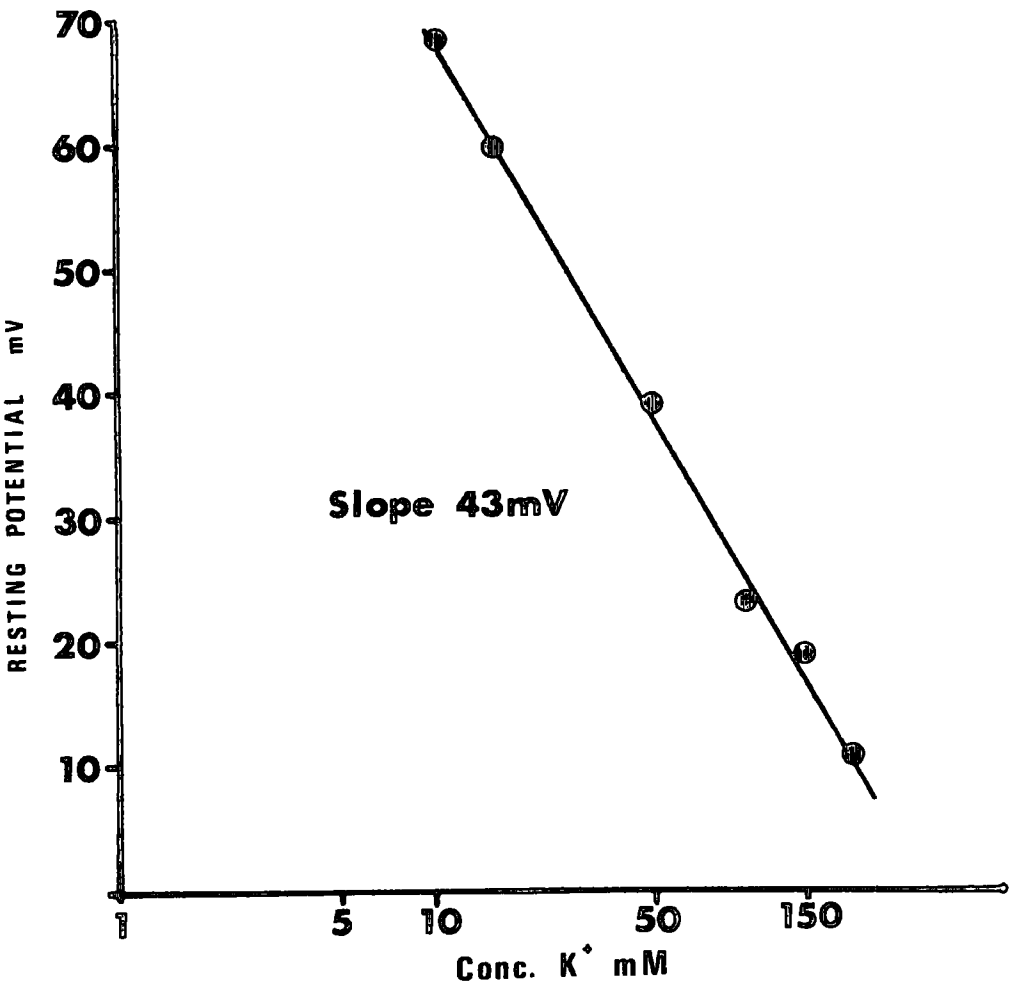

70
60
50
40
30
20
10
RESTING POTENTIAL mV
Slope 41mV
1
5
10
50
150
Conc. K+ mM

FIGURE 16. The effect of saline potassium concentration in the presence of chloride on the resting potential of cockroach coxal muscle.

Muscles were equilibrated with the salines for two hours before recording. Concentrations of potassium and chloride were altered so that their product remained a constant. The experiments were performed at room temperature. Standard errors were calculated for the readings but were not great enough to be shown on the graph.

Ordinate: Resting membrane potential (mV).

Abscissa: Concentration of potassium (mM) in the bathing saline.


70
60
50
40
30
20
10
RESTING POTENTIAL mV
Slope 43mV
1
5
10
50
150
Conc. K⁺ mM

45 minutes in proprionate saline (table 18, expt. 1). In succinate saline the membrane was depolarized but did not reach a steady level (table 18, expt. 2). Where chloride was replaced with citrate the membrane was depolarized and when replaced in normal saline it repolarized (table 18, expt. 3). A similar depolarization occurred when a muscle was bathed in sulphate saline but when 150 mM choline chloride was added to the saline the membrane repolarized (table 18, expt. 4). The time course for depolarization upon removal of chloride ions and repolarization upon replacement of chloride was similar.

3. The contribution of sodium to the resting membrane potential.

When choline chloride was substituted for sodium chloride in the saline bathing Periplaneta coxal muscles, no immediate change in resting potential was observed (table 19). After two hours there was no significant depolarization, but after three hours the membrane became depolarized. However it has been shown that an equilibration time of one hour is sufficient for permeation of saline around the muscle fibres and no effect on the resting potential was seen for exposures of up to two hours. The depolarization after two hours may well be due to choline entering the muscle cells.

4. The effect of temperature on the resting membrane potential.

It was found that the resting potential of Periplaneta skeletal muscles was very sensitive to low temperature. A series of experiments were carried out to measure the resting potential at a number of different temperatures between 20°C and 5°C. It was found that the potential depolarized with temperature to a far greater degree than predicted by the Nernst equation (figure 17). At 5°C the potential recorded approximated to E_K calculated from the potassium distribution at 5°C shown in chapter 4 (results section 6b). E_K at 5°C was calculated to be -42.5 mV and the recorded potential was -40.4 mV.

TABLE 18. The effect of chloride on the resting membrane potential of cockroach coxal muscle.

Muscles were bathed in saline at room temperature. Standard errors were calculated from the averaged resting potentials of at least 30 different muscle fibres. In the experiments chloride salts were replaced by proprionate, succinate, citrate and sulphate salts. In experiment 4 it was replaced by the addition of 150 mM choline chloride to the saline containing sulphate salts. All salines were buffered with 16 mM HCO^-_3 and CO_2 to pH 7.2.

Expt.	Saline	Time of Exposure (min.)	Resting Potential (mV)	S.E.
1	Bicarbonate	10	-59.9	0.5
	Proprionate (Cl-free)	30	-54.9	0.6
	Proprionate (Cl-free)	45	-44.9	0.7
	Proprionate (Cl-free)	75	-42.2	0.9
2	Bicarbonate	10	-59.8	0.7
	Succinate (Cl-free)	30	-41.6	0.6
	Succinate (Cl-free)	60	-21.3	1.5
3	Bicarbonate	15	-59.1	0.8
	Citrate (Cl-free)	30	-41.2	0.8
	Bicarbonate	40	-57.1	1.2
4	Bicarbonate	10	-60.5	0.9
	Sulphate (Cl-free)	30	-44.4	0.9
	Sulphate + 150 mM Choline Chloride	30	-59.0	0.6

TABLE 19. The effect of sodium-free saline on the resting membrane potential of cockroach coxal muscle.

Sodium chloride was replaced by choline chloride. Muscles were bathed in saline at room temperature. Standard errors were calculated from the resting potential of at least 30 different muscle fibres.

Saline	Time of Exposure (min.)	Resting Potential (mV)	S.E.
Bicarbonate	15	-57.7	1.0
Na-free Bicarb.	30	-59.0	0.6
Na-free Bicarb.	60	-59.3	0.6
Na-free Bicarb.	75	-59.2	0.6
Na-free Bicarb.	120	-55.8	1.2
Na-free Bicarb.	180	-49.7	0.7
Na-free Bicarb.	300	-48.0	0.9

FIGURE 17. The effect of decreasing temperature upon the resting membrane potential of cockroach coxal muscle.

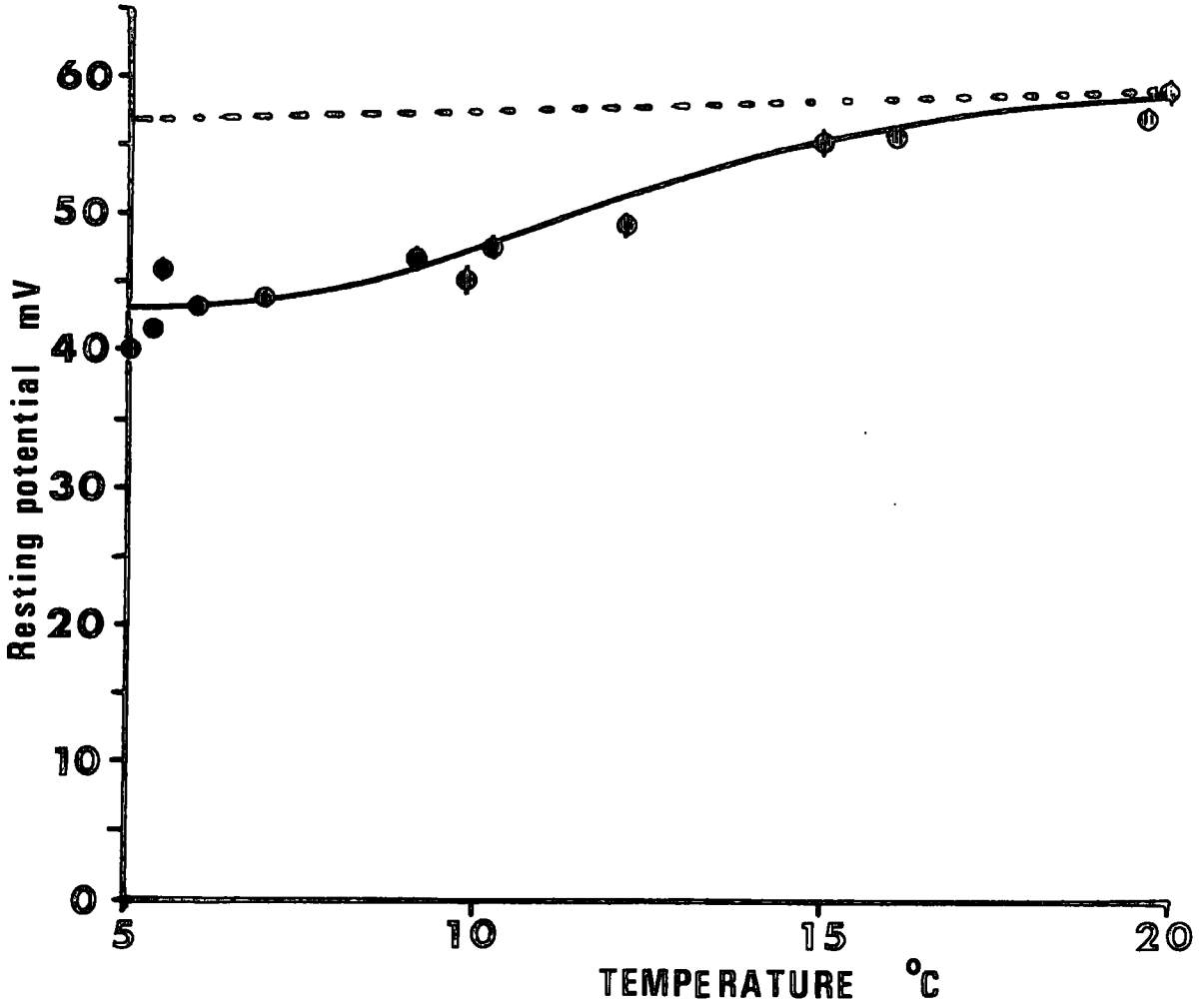
——— = recorded value.

- - - = predicted value from the Nernst equation.

The muscles were equilibrated to each temperature for two hours. The muscle bath was surrounded by water, cooled, using the Peltier effect described in Methods, section 2, and the temperature in the muscle bath monitored by means of a thermocouple. Standard errors are shown where large enough.

Ordinate: Resting potential (mV) recorded.

Abscissa: Temperature of the saline (°C).


60
50
40
30
20
10
0
Resting potential mV
5
10
15
20
TEMPERATURE °C

To determine the contribution of extracellular potassium and chloride to the temperature sensitive component of the resting potential, muscles were cooled in potassium-free and chloride-free salines. The results are shown in table 20. It was found that potassium ions did not contribute to the temperature sensitivity but chloride did. In chloride-free saline the resting potential did not recover after being depolarized by low temperature (table 20, expt.3), whereas the lack of potassium did not affect the recovery (table 20, expt.2).

At this stage it appeared that the resting membrane potential was composed of two parts, one dependent upon a temperature sensitive process involving chloride ions, and the other a temperature insensitive component, which approximated in magnitude to E_K. To test the possibility that this latter component was a potassium electrode the effect of potassium concentration on the resting potential at 5° C was determined. Two series of experiments were performed, one in which potassium concentration was altered in the absence of chloride ions (figure 18), and one where potassium concentration was altered whilst keeping the product of the potassium and chloride concentrations constant (figure 19). Under both conditions over the range 0-50 mM potassium in the saline, at 5° C after two hours, no change in the resting potential was recorded. Further at higher levels of potassium the resting potential approximated to the slopes obtained at room temperature, (figures 15 and 16).

5. Action of pharmacological agents on the resting membrane potential.

a. Strophanthin G.

Strophanthin G has been shown to have little effect upon the membrane ATPase activity of Periplaneta skeletal muscle, whilst it did cause an increase in $[Na^+]_i$ in whole muscles of the same preparation. Muscles were exposed for one hour to salines containing 10^{-5} M, 5×10^{-5} M

TABLE 20. The effect of temperature on the resting potential of cockroach coxal muscle as affected by the presence and absence of K^+ and Cl^-.

In chloride-free saline sulphate was substituted for chloride. Standard errors were calculated for the averaged resting potentials from at least 30 different fibres. Muscles were equilibrated for 60 minutes at each temperature. Chloride was replaced by an equivalent amount of sulphate and potassium was replaced by an equivalent amount of choline chloride.

Expt.	Saline	Temperature °C	Resting Potential (mV)	S.E.
1	Bicarbonate	17.1	-59.9	0.7
	Bicarbonate	5.8	-42.1	0.9
	Bicarbonate	17.1	-55.7	0.5
2	Bicarbonate	26.0	-58.3	0.7
	K-free Bicarbonate	6.0	-41.3	0.5
	K-free Bicarbonate	20.0	-60.2	0.8
3	Bicarbonate	20.7	-60.5	0.9
	Cl-free Bicarbonate	6.9	-35.4	0.8
	Cl-free Bicarbonate	24.3	-43.6	0.4

FIGURE 18. The effect of saline potassium concentration at 5°C in the absence of chloride on the resting potential of cockroach coxal muscle.

Each point represents the average of the resting potentials of at least 30 different fibres. The standard errors are not great enough to be shown. Muscles were equilibrated to 5°C for two hours. The muscle bath was surrounded by cold water and the temperature of the saline in the muscle bath monitored with a thermocouple. The dotted line represents the behaviour of Em at 20°C under the same conditions (figure 15).

Ordinate: Resting potential recorded (mV).

Abscissa: Concentration of potassium (mM) in the bathing saline.

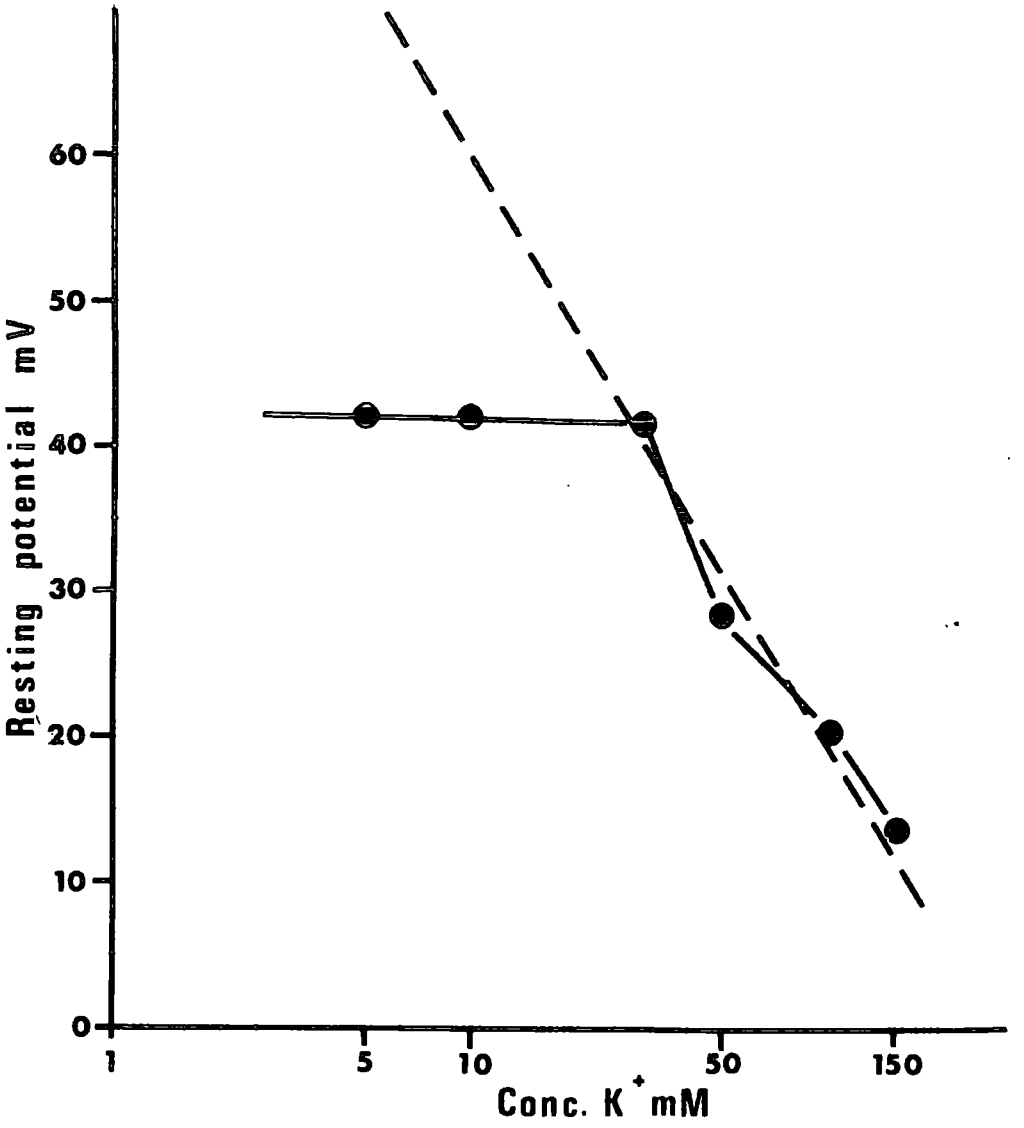
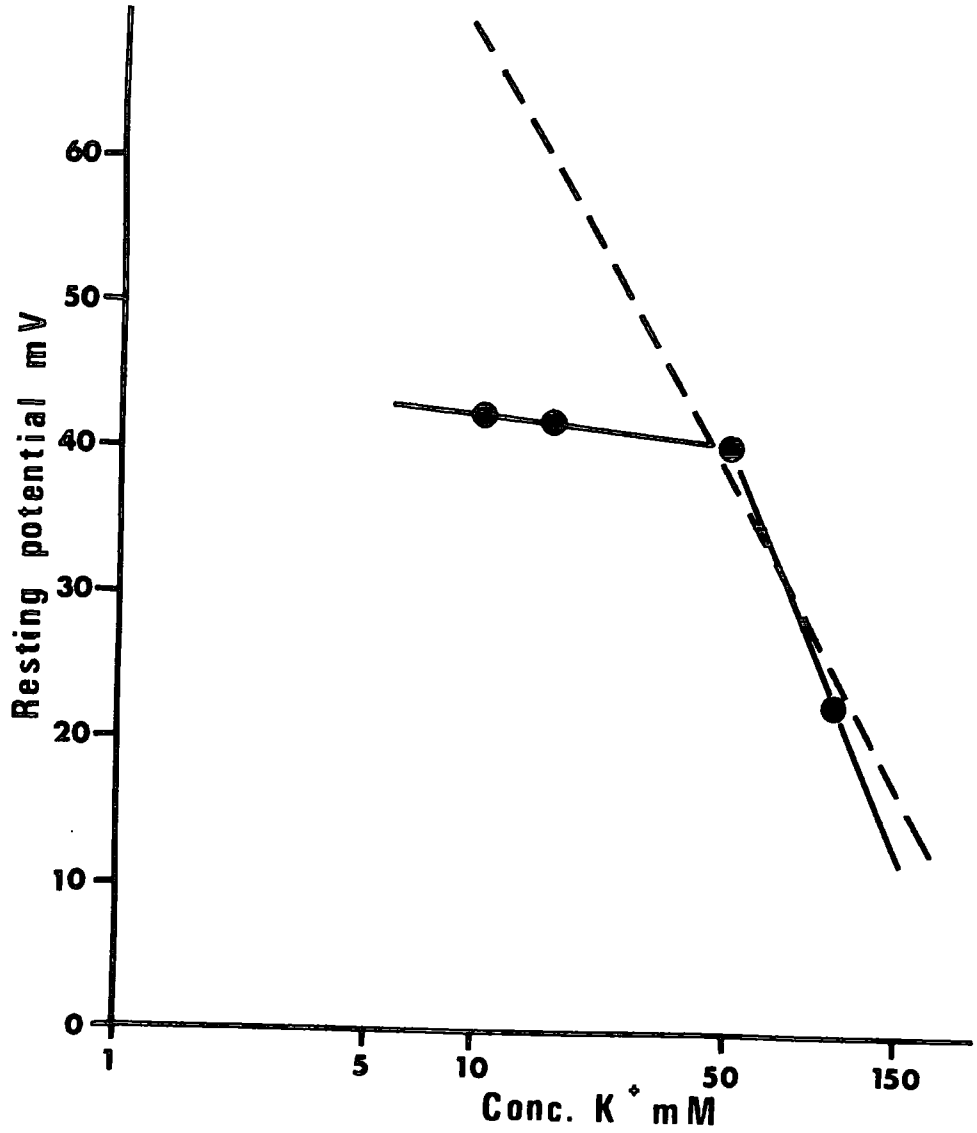

Resting potential mV
60
50
40
30
20
10
0
1
5
10
50
150
Conc. K+ mM

FIGURE 19. The effect of saline potassium concentration at 5°C in the presence of chloride on the resting potential of cockroach coxal muscle.

Potassium concentration was altered in such a way that the product of the potassium and chloride concentrations remained constant. Each point represents the average of the resting potentials of at least 30 different fibres. The standard errors are not great enough to be shown. Muscles were equilibrated to 5°C for two hours. The muscle bath was surrounded by cold water and the temperature of the saline in the muscle bath monitored with a thermocouple. The dotted line represents the behaviour of Em at 20°C under the same conditions (figure 16.).

Ordinate: Resting potential recorded (mV).

Abscissa: Concentration of potassium (mM) in the bathing saline.


Resting potential mV
60
50
40
30
20
10
0
1
5
10
50
150
Conc. K+ mM

and 5 x 10^{-4} M strophanthin G and the resting potentials recorded (table 21). Muscles were also exposed to 5 x 10^{-4}M strophanthin G at low temperatures. At a concentration of 5 x 10^{-5}M strophanthin G at room temperature, the resting potential was reduced almost to E_K being -42.4 mV. At low temperatures, strophanthin G at 5 x 10^{-4} M had very little further effect upon the resting potential. In the absence of $[Cl^-]_o$ strophanthin G at 5 x 10^{-4} M had no significant effect at room temperature. It appears that strophanthin G removes the temperature sensitive, chloride-dependent, component of the resting membrane potential. It is unlikely that the depolarization due to the presence of strophanthin G was caused by the observed increase in $[Na^+]_i$ (see table 11) since strophanthin G had a similar effect on the resting potential in sodium-free saline (table 21).

b. 2.4 -Dinitrophenol.

McCann (1967) has shown that moth heart muscle is sensitive to DNP and to sodium azide; the resting potential falls rapidly and potassium ions leave the muscle fibres. He considers this good evidence for the existence of an electrogenic potassium pump contributing to the resting potential. Huddart and Wood (1966) found that inclusion of 0.5 mM DNP in the saline bathing skeletal muscle of Sphinx ligustri and Periplaneta americana resulted in a triphasic decline in the resting potential. They concluded that the resting potential depended for its maintenance upom oxidative metabolism. It has been shown (chapter 4, Result section 4a) that exposure of cockroach skeletal muscle fibre to 0.5 mM DNP caused an increase in $[Na^+]_i$ and a decrease in $[K^+]_i$. Figure 20 shows the effect of 0.1 mM DNP at pH 7.1 on the resting potential of this preparation. There was a fairly rapid decline (over 120 minutes) in the resting potential which reached a steady level around -40 mV.

TABLE 21. The effect of strophanthin G on the resting potential of cockroach coxal muscle at low temperatures and in the absence of $[Na^+]_o$ and $[Cl^-]_o$.

Standard errors were calculated from the measurement of the resting potential of at least 30 different fibres. Muscles were equilibrated for one hour to each concentration of strophanthin G, Na-free saline, Cl-free saline and to each temperature. Sodium was replaced by an equivalent amount of proprionate in experiment 2 and chloride by an equivalent amount of sulphate in experiment 4.

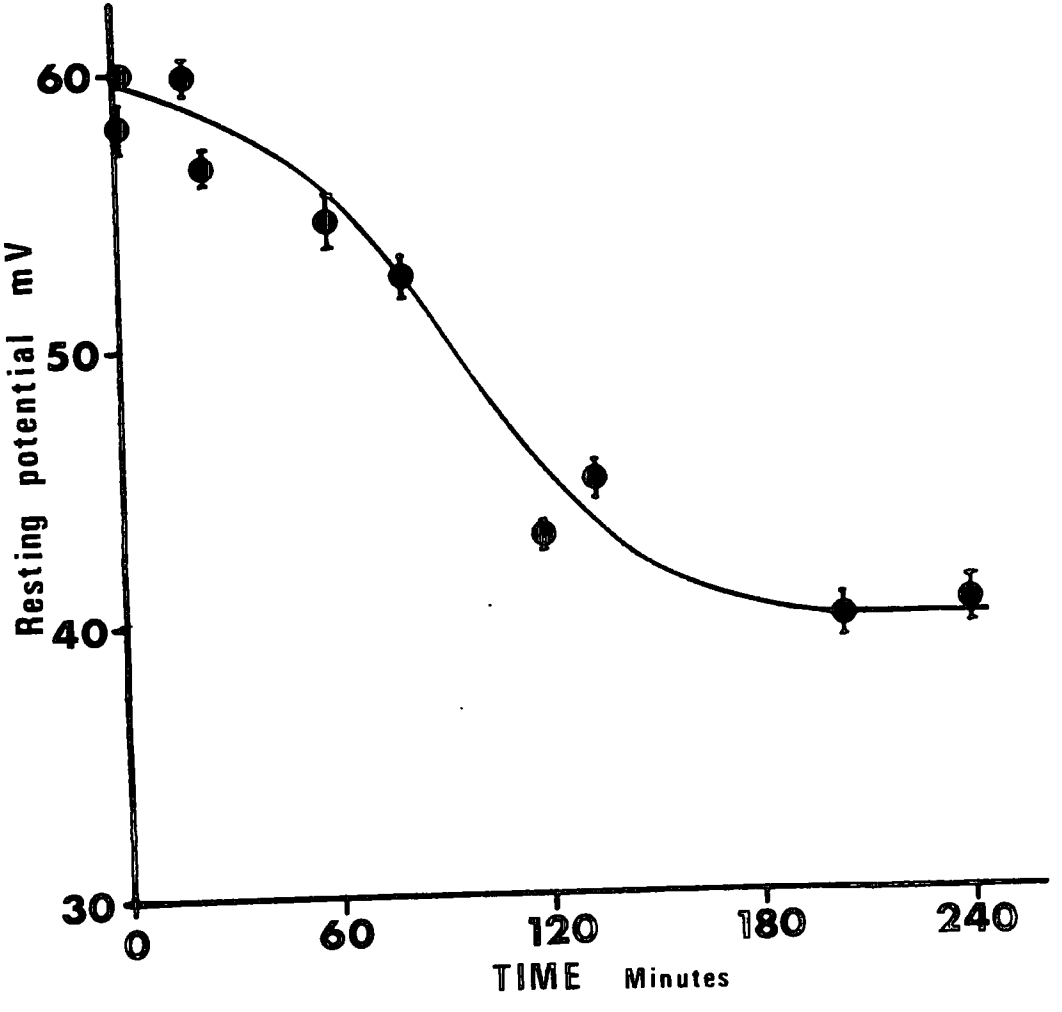
Expt.	Saline	Temperature °C	Resting Potential (mV)	S.E.
1	Bicarbonate + 10^{-5} M strophanthin G	c 20.0	-52.4	0.5
	Bicarbonate + 5×10^{-5} M strophanthin G	c 20.0	-41.4	0.7
	Bicarbonate + 5×10^{-4} M strophanthin G	c 20.0	-42.4	0.6
2	Bicarbonate	c 20.0	-59.9	0.8
	Na-free	c 20.0	-61.1	0.6
	Na-free + 5×10^{-4} M strophanthin G	c 20.0	-43.1	0.5
3	Bicarbonate	21.8	-61.0	0.7
	Bicarbonate	5.1	-46.1	0.7
	Bicarbonate + 5×10^{-4} M strophanthin G	6.6	-42.7	0.6
	Bicarbonate + 5×10^{-4} M strophanthin G	20.0	-42.4	0.6
	Bicarbonate + 5×10^{-4} M strophanthin G	7.4	-38.3	0.8
4	Cl-free	24.3	-43.6	0.4
	Cl-free + 5×10^{-4} M strophanthin G	24.3	-41.9	0.6

FIGURE 20. The effect of 10^{-4} M Dinitrophenol on the resting membrane potential of cockroach coxal muscle.

Muscles were exposed to bicarbonate saline containing DNP at pH 7.1 at room temperature. The standard errors shown are the results of at least 30 separate measurements.

Ordinate: Resting potential (mV) recorded.

Abscissa: Time (minutes) of exposure to saline containing 10^{-4} M DNP.


60
50
40
30
Resting potential mV
0
60
120
180
240
TIME Minutes

c. Cyanide.

Sodium cyanide at a concentration of 10^{-3} M in the saline bathing the muscles caused a similar depolarization of the resting potential as did DNP (figure 21). Under the influence of 10^{-3} M cyanide the resting membrane potential was found to be no longer sensitive to low temperature (table 22), giving further evidence that the membrane potential is partly supported by a metabolic process. It was found that when the cyanide was washed out of the preparation the resting potential recovered to some extent (table 22). An experiment was performed to determine the effect of varying $[K^+]_o$ whilst keeping the product $[K^+]_o \times [Cl^-]_o$ constant in the presence of 1 mM sodium cyanide for a 60 minute exposure of the muscle fibres at room temperature (table 23). The result was virtually identical with the same experiment in normal saline at 5°C (figure 19).

d. Acetazolamide (Diamox).

Owing to the apparent requirement by the muscles of the cockroach for bicarbonate ions it was of interest to determine the effect of acetazolamide, an inhibitor of carbonic anhydrase, on this preparation. The enzyme carbonic anhydrase is associated with the transport of H^+ and Cl^- ions across frog gastric mucosa (Imamura, 1970) and plays some role in the K^+ -Na^+ or H^+ -Na^+ exchange mechanism in renal tubules (Suzuki & Ogawa, 1968). When acetazolamide at a concentration of 5 mM was added to the saline bathing the coxal muscle preparation the resting potential was depolarized after two hours to a similar extent as caused by cyanide, strophanthin G and DNP (figure 22). It is possible that carbonic anhydrase activity is necessary for the maintenance of the resting potential by controlling intracellular pH. If H^+ ions were to be distributed in a Donnan equilibrium with potassium or chloride ions then any change in internal pH will alter the resting potential.

FIGURE 21. The effect of 10^{-3} M sodium cyanide on the resting membrane potential of cockroach coxal muscle.

Muscles were exposed to bicarbonate saline containing sodium cyanide (pH 7.2) at room temperature. The standard errors shown are the result of at least 30 separate measurements.

Ordinate: Resting potential (mV) recorded.

Abscissa: Time (minutes) of exposure of the muscles to 10^{-3} M sodium cyanide.

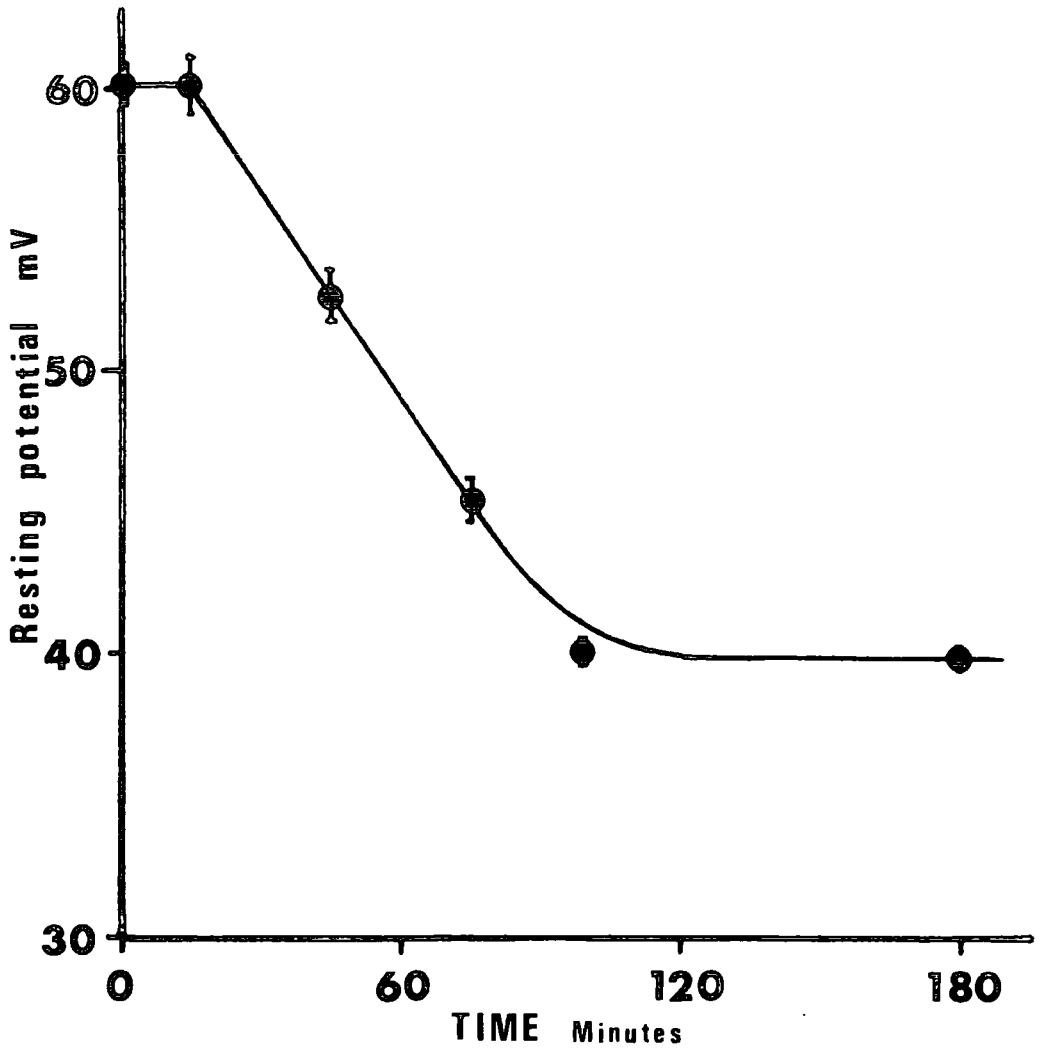

60
50
40
30
Resting potential mV
0
60
120
180
TIME Minutes

TABLE 22. The effect of sodium cyanide on the temperature sensitive component of the resting membrane potential of cockroach coxal muscle.

The muscles were exposed at room temperature and at 5°C. Standard errors were calculated from measurements of the resting potentials of at least 30 different fibres.

Saline	Time of Exposure (min.)	Temperature °C	Resting Potential (mV)	S.E.
Bicarbonate	10	c 20	-60.2	0.7
Bicarbonate + 10^{-3} M CN	100	c 20	-40.0	0.5
Bicarbonate + 10^{-3} M CN	60	5	-42.4	0.6
Bicarbonate	45	c 20	-54.4	0.7

TABLE 23. The effect of saline potassium concentration, in the presence of chloride and 10^{-3} M sodium cyanide, on the resting membrane potential of cockroach coxal muscle.

The potassium concentration was altered so that the product of the potassium and chloride concentrations remained constant. Muscles were equilibrated for 60 minutes, at room temperature, to saline containing 10^{-3} M sodium cyanide. Standard errors were calculated from measurements of at least 30 different fibres.

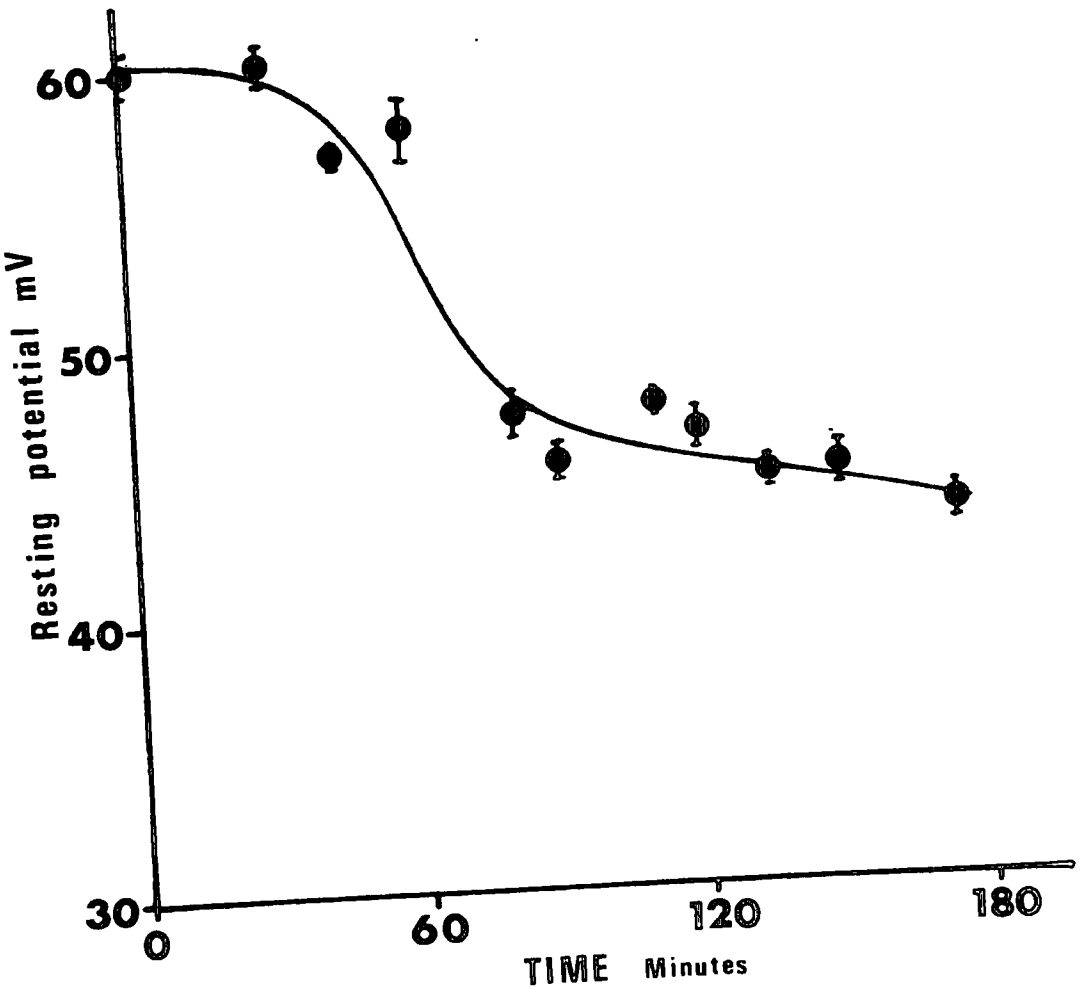
Potassium Conc. (mM)	Resting Potential (mV)	S.E.
10	-44,8	1.0
16	-42.5	0.6
20	-40.4	0.7
50	-42.6	0.7
150	-14.8	0.8

FIGURE 22. The effect of 5×10^{-3} M Acetazolamide on the resting membrane potential of cockroach coxal muscle.

Muscles were equilibrated with saline containing acetazolamide (Diamox) for various times at room temperature. The standard errors shown are the results of at least 30 separate measurements.

Ordinate: Resting potential (mV) recorded.

Abscissa: Time (minutes) of exposure to 5×10^{-3} M Acetazolamide.


60
50
40
30
Resting potential mV
0
60
120
180
TIME Minutes

However it must be noted that a relatively high concentration of acetazolamide was used, since lower concentrations were ineffective in reducing the resting potential, and might in this case be having another effect beside the inhibition of carbonic anhydrase activity.

6. The effect of external pH upon the resting membrane potential.

The pH of the saline was made acidic by the addition of HEPES and alkaline by the addition of sodium bicarbonate to the normal saline. No adjustments were made for the alteration of osmotic pressure of the salines. The results of a two hour exposure on the resting potential at each pH are shown in table 24. It can be seen that there was a significant depolarization at pH 6.5, but no further depolarization at pH 6.1. The depolarization seen at pH values below 7 may simply be due to the low concentration of bicarbonate ions. (At pH 7.2 and 25°C there is 10 mM HCO_3^- but at pH 6.4 there is only 1 mM.) It is not possible to determine the direct effect of altering pH if the membrane is sensitive to levels of bicarbonate ions.

7. The effect of divalent cations on the resting membrane potential.

Any direct contribution by divalent cations to the resting potential seems unlikely due to their low concentrations both inside and outside the muscle fibres. It has been shown in chapter 4 that under a great variety of conditions internal magnesium levels remain constant, suggesting either that the membrane is not permeable to them or that they are bound inside the fibres. A series of experiments were performed where muscles were bathed for one hour in saline buffered with 10 mM Tris (to avoid precipitation) and containing increasing calcium concentrations. Concentrations of calcium up to 50 mM did not significantly alter the resting potential (table 25). In another series of experiments the magnesium concentration was raised in the presence of 20 mM calcium. Again there was no effect on the resting potential (table 25).

TABLE 24. The effect of pH of the bathing saline on the resting membrane potential of cockroach coxal muscle.

pH 6,1 and 6.5 were obtained by the addition of HEPES to the saline and pH 7.8 by the addition of sodium bicarbonate. The muscles were equilibrated for two hours at room temperature. The standard errors were determined from the resting potential of at least 30 different fibres.

pH	Resting Potential (mV)	S.E.
7.8	-59.2	0.6
7.2	-60.4	0.9
6.5	-51.0	0.5
6.1	-50.6	0.6

TABLE 25. The effect of divalent cations on the resting membrane potential of cockroach coxal muscle.

Muscles were equilibrated with each saline for two hours. Salines were buffered with Tris and did not contain any bicarbonate since this would precipitate at high calcium concentrations. The experiments were performed at room temperature. The standard error represents the resting potential from at least 30 different fibres.

Calcium (mM)	Magnesium (mM)	Resting Potential (mV)	S.E.
10	6	-60.6	0.5
15	6	-59.2	0.5
20	6	-59.3	0.6
30	6	-59.4	0.4
50	6	-60.3	0.5
20	20	-60.0	0.4
20	40	-60.4	0.4

DISCUSSION

The experimental evidence which has been presented indicates that the generation and control of the resting membrane potential of Periplaneta skeletal muscle fibres are complex processes. It is, however, apparent that the resting membrane potential has two distinct components, which, although probably separate processes, are probably also inter-related.

The first is an "active," electrogenic system which is inhibited at low temperatures and is dependent upon the presence of HCO_3^- and Cl^- in the bathing media. The contribution of this component is approximately 20 mV when the muscle is bathed in a suitable saline or by its own haemolymph and when it is maintained at about 22°C. Strophanthin G, CN^-, DNP and acetazolamide all reduce the normal resting potential by approximately 20 mV, although the time-courses of these inhibitions do vary. Exposure to 5°C produces a characteristic graph when the resting potential is plotted against $\log [K^+]_o$ (see figure 19) and a similar plot is obtained in the presence of CN^-, suggesting that this anion and low temperature have the same action.

The second component superficially resembles a mixed ion electrode, although, as will be shown later, there are a number of marked differences with the ionic electrodes typically found in excitable cells. It contributes approximately 40 mV to the total resting potential. When the muscle is maintained in an appropriate saline at about 22°C, the resting potential varies with the logarithm of $[K^+]_o$, but has a slope of 43 mV for a ten-fold change in $[K^+]_o$ (see figure 16), instead of the 58 mV slope expected (Boyle & Conway, 1941).

When a membrane is freely permeable to only K^+ then the equilibrium

potential for that ion may be described by the Nernst relationship:

$$E_K = \frac{RT}{nF} \times \log_e \frac{[K^+]_i}{[K^+]_o} \text{ volts}$$

At room temperature this may be simplified when:

$$E_K = 58 \times \log_{10} \frac{[K^+]_i}{[K^+]_o} \text{ mV}$$

Hence for a ten-fold change in $[K^+]_o$ there should be a 58 mV change in potential and the slope of the graph of resting potential against log $[K^+]_o$ will be 58 mV. Although Wood (1963) concluded that Periplaneta muscle fibres were relatively impermeable to chloride ions, Usherwood (1967a) has suggested that they are permeable to both potassium and chloride ions. When a membrane is freely permeable to both K^+ and Cl^- then the distribution of ions across that membrane is described by the Donnan equilibrium:

$$\frac{[K^+]_i}{[K^+]_o} = \frac{[Cl^-]_o}{[Cl^-]_i} \quad \text{or} \quad [K^+]_i \times [Cl^-]_i = [K^+]_o \times [Cl^-]_o$$

Hence alteration of $[K^+]_o$ will cause an alteration in this balance of ions and a slope of 58 mV would not be seen for a ten-fold change in $[K^+]_o$. A slope of 58 mV will be seen, however, if $[K^+]_o$ and $[Cl^-]_o$ are varied in such a way that their product remains constant. Since, under neither condition, when the product $[K^+]_o \times [Cl^-]_o$ was kept constant or in the absence of $[Cl^-]_o$, did the results obtained give even an approximation to a 58 mV slope for a ten-fold change in $[K^+]_o$, it may be concluded that the resting membrane of Periplaneta skeletal muscle fibres is not freely permeable to potassium and chloride ions.

The suggestion that the membrane potential is the sum of ionic and metabolic components has been advanced for the giant neurones of

the mollusc Anisodoris (Gorman&Marmor, 1970a & 1970b). The two components are also readily separable by exposure to temperature below 5°C, although in this preparation the active component is an electrogenic $Na^+ - K^+$ pump and the behaviour of the ionic component can be predicted by a constant field type of equation. These findings on Anisodoris extend the previous studies of Carpenter and Alving (1968) on Aplysia neurones.

Two further points may be made:

(a) Calculated values for E_K, using either values obtained in this study, or those of previous workers (Tobias, 1948; Hoyle, 1953; Wood, 1963), lie below the observed E_m in contrast with the typical situation (e.g. frog muscle, Hodgkin & Horowicz, 1959) where $E_K > E_m$, the resting potential being reduced by a small permeability to Na^+.

(b) The abnormal situation apparent in insect muscle may well be related to the need to have an efficient system controlling both E_m and excitatory events in the face of fluctuations in haemolymph $[K^+]$ diurnally (Jones, 1964) and after feeding (Hoyle, 1953).

The ionic electrode.

Several previous studies on insect muscle (Belton, 1958; Belton & Grundfest, 1962; Wood, 1965; Huddart, 1966a & 1966b) have drawn attention to the fact that the resting potential obtained from muscle of many species, and its behaviour when $[K]_o$ is varied, is not readily explained by a constant field type of equation. The anomalies described have been:

(i) a resting potential considerably greater than E_K,

(ii) a markedly non-linear graph when E_m is plotted against $\log [K]_o$, especially at low values for $[K]_o$, and

(iii) an insensitivity to changes in $[K]_o$.

Usherwood (1969) has emphasized the difficulties when working with

large and complex muscles and the probable sources of error in determining $[K]_i$ and $[Cl]_i$ and the activities of these ions and has suggested that it is important to maintain the product $[K]_o \times [Cl]_o$ constant in experiments involving alterations of $[K]_o$.

In the presence of HCO_3^- and Cl^- and at a temperature of 20°C quite a good linear plot was obtained for E_m vs log $[K]_o$, when $[K]_o \times [Cl]_o$ was maintained constant (figure 16), although the slope of the line was 43 mV for a ten-fold change in $[K]_o$, instead of the value of 58 mV obtained with other excitable cells. Under these conditions, with normal $[K]_o$ and $[Cl]_o$, E_m appears to lie much closer to E_{Cl} than to E_K. Usherwood (1967b) reports that the cockroach skeletal muscle behaves as a good potassium electrode. However figure 16 was obtained when the active component of E_m was functional; when the experiment was repeated at 5°C an atypical relationship between $[K]_o$ and E_m was found (figure 19). Above about 50 mM $[K]_o$ the graph was comparable with that at 20°C but, below 50 mM $[K]_o$, E_m was apparently almost independent of changes in $[K]_o$. Figure 19 therefore probably represents the true properties of the ionic electrode, since it was obtained when the active component was inhibited. The unusual features of the electrode may be summarized:

(i) Over the range of $[K]_o$ where the muscle membrane apparently resembles a K electrode (K >50 mM), E_m is proportional to $[K]_o$, the slope of the line (figures 18 & 19) is about 43 mV for a ten-fold change in $[K]_o$.

(ii) E_m is apparently insensitive to changes in $[K]_o$ in the range below 50 mM, E_m remaining at approximately-42 mV. This part of the curve may be comparable with results obtained by Huddart (1966b) with Lepidopteran muscle (E_m little altered when $[K] < 50$ mM) and by Belton and

Grundfest (1962) with Tenebrio larvae (E_m unaffected when $[K]_o < 100$ mM, but at higher concentrations the relationship is logarithmic, with a slope of about 58 mV).

(iii) If the muscle is cooled to 5°C with a normal $[K]_o$ of 16 mM, E_m falls to 42 mV which is probably close to E_K (i.e. when E_m is apparently independent of $[K]_o$, in the range 5-50 mM, it is "clamped" at a value close to E_K for a muscle in its own haemolymph). In spite of the difficulties in estimating the activity of K^+ inside the muscle, it is probably true that if $[K]_o$ is raised, with the active component inhibited, E_m recorded exceeds the theoretical E_K. This is, of course, the reverse of the situation in typical excitable cells where $E_K > E_m$, the theoretical potential being reduced by a small permeability to Na^+ (Hodgkin & Horowicz, 1959). Of course, when the active component is contributing to E_m of cockroach muscle, the resting potential almost certainly exceeds E_K.

The following suggestions can be made regarding the properties of this ionic electrode:

1. The departure of figure 19 from linearity is reminiscent of the same plot found with frog muscle (Hodgkin & Horowicz, 1959). In this preparation, however, this behaviour is readily explained as being due to the significant effect of P_{Na} at low $[K]_o$ values. P_{Na} / P_K in frog muscle at rest is 0.01. This non-linearity is only found in the range of <10 mM $[K]_o$ and similar logarithmic plots, curvilinear in the range 0-10 mM, have been described for Carausius (Wood, 1957) and Schistocerca (Hoyle, 1953). It could be suggested that the exaggerated non-linearity obtained with cockroach muscle at 5°C (figures 18 & 19) is due to a much higher Na permeability (or

conceivably a permeability to divalent cations) with a consequent involvement of these ions in the control of E_m . This hypothesis is rejected on the grounds that:

a. E_m proved to be little affected by variation in $[Na]_o$.

b. E_m was not markedly affected by variation in the external concentration of divalent cations, and an elevated $[Ca]_o$ in Carausius can cause a hyperpolarization (Wood, 1957). In any event concentration ratios for divalent ions are probably low (although there are even greater difficulties in determining the internal concentrations of free ions) and, being divalent, their contribution to an ionic electrode is proportionally less.

c. Substitution of a variety of values for P_{Cl} , P_K and P_{Na} will not simulate the marked independence of E_m at $[K]_o$ values of 5-50 mM. It is, therefore, this "clamping" of the ionic electrode in the absence of the active component that appears to be the most specialized and atypical property of the resting potential of cockroach muscle.

2. Strickholm and Wallin (1967), working with crayfish giant axons, have pointed out that plots of E_m versus log $[K]_o$ are not necessarily very meaningful; they find that both P_{Cl} and P_K are dependent on the membrane resting potential (P_{Na} is, of course, known to be dependent on E_m in most excitable cells). That such an explanation is also possible in cockroach muscle is borne out by an examination of figures 16 and 19. E_m approximates to E_{Cl} at very low values of E_m (high $[K]_o$), but approximates to E_K as E_m rises from 0 to about -40 mV. This transition from a predominantly Cl^- electrode to a predominantly K^+ electrode as $[K]_o$ is decreased would account for the deviation from the 58 mV slope in the range when $[K] > 50$ mM. In vivo, therefore,

but with the active component inhibited by low temperature, $P_K > P_{Cl}$. When the active component contributes to E_m in vivo, the transmembrane potential changes from about -42 mV to about -60 mV and presumably the P_K / P_{Cl} ratio would rise still further. This suggestion differs from Usherwood's (1969) conclusion from studies on rates of KCl efflux in grasshopper muscle that P_K and P_{Cl} are of similar magnitude. It must be emphasized, however, that Usherwood (1967b, 1969) has suggested that a marked deviation from a slope of 58 mV is due to faulty equilibration. However, Hoyle (1954) has measured E_m in locust muscle when the insects have been fed previously on diets which cause marked changes in the K^+ concentration of the haemolymph. E_m plotted against $[K]_o$ produced a straight line plot (5-150 mM $[K]_o$), but inspection suggests that the slope is about 50 mV; equilibration is clearly established in these experiments and since they were conducted at room temperature in the insects' own haemolymph the active component (if present in locust muscle) would have been operating. The experiments with tritiated inulin also suggest that equilibration in experimental salines is completed within 60 minutes.

3. The above suggests that:

   (i) Cl^- is implicated in the control of E_m and

   (ii) this ion is not in equilibrium, in contrast with the situation in frog muscle where it is passively distributed in obedience to E_m (Hodgkin & Horowicz, 1959).

Such a suggestion would be in accord with experiments which indicate that the hyperpolarization of the IPSP of insect muscle is generated by an increase in P_{Cl} (Usherwood & Grundfest, 1965; Usherwood, 1968; locust and grasshopper) and others in which an increased Cl^- permeability is described when ibotenic acid is applied to locust muscle (Lee & Usherwood, 1970). All these experiments, however, appear to

be at variance with those in which a transient variation in E_m of grasshopper muscle is reported in response to a rapid alteration in $[Cl]_o$ (Usherwood & Grundfest, 1965; Usherwood, 1967b), corresponding with experiments described in frog muscle (Hodgkin & Horowicz, 1959).

4. Usherwood (1967a, 1969), using grasshopper, locust and cockroach muscle, has shown, from measurements of swelling and of E_m when $[K]_o$ is changed, that P_K is high in an outward direction, but lower in the opposite direction. Similar results were observed when measuring E_m in the present study, and this rectification is similar to that reported in frog muscle (Hodgkin & Horowicz, 1959).

These properties of the ionic electrode are summarized in figure 23; in short, its properties include:

a. anomalous rectification (Usherwood, 1969).

b. inward rectification of P_K.

c. departure from a 58 mV slope.

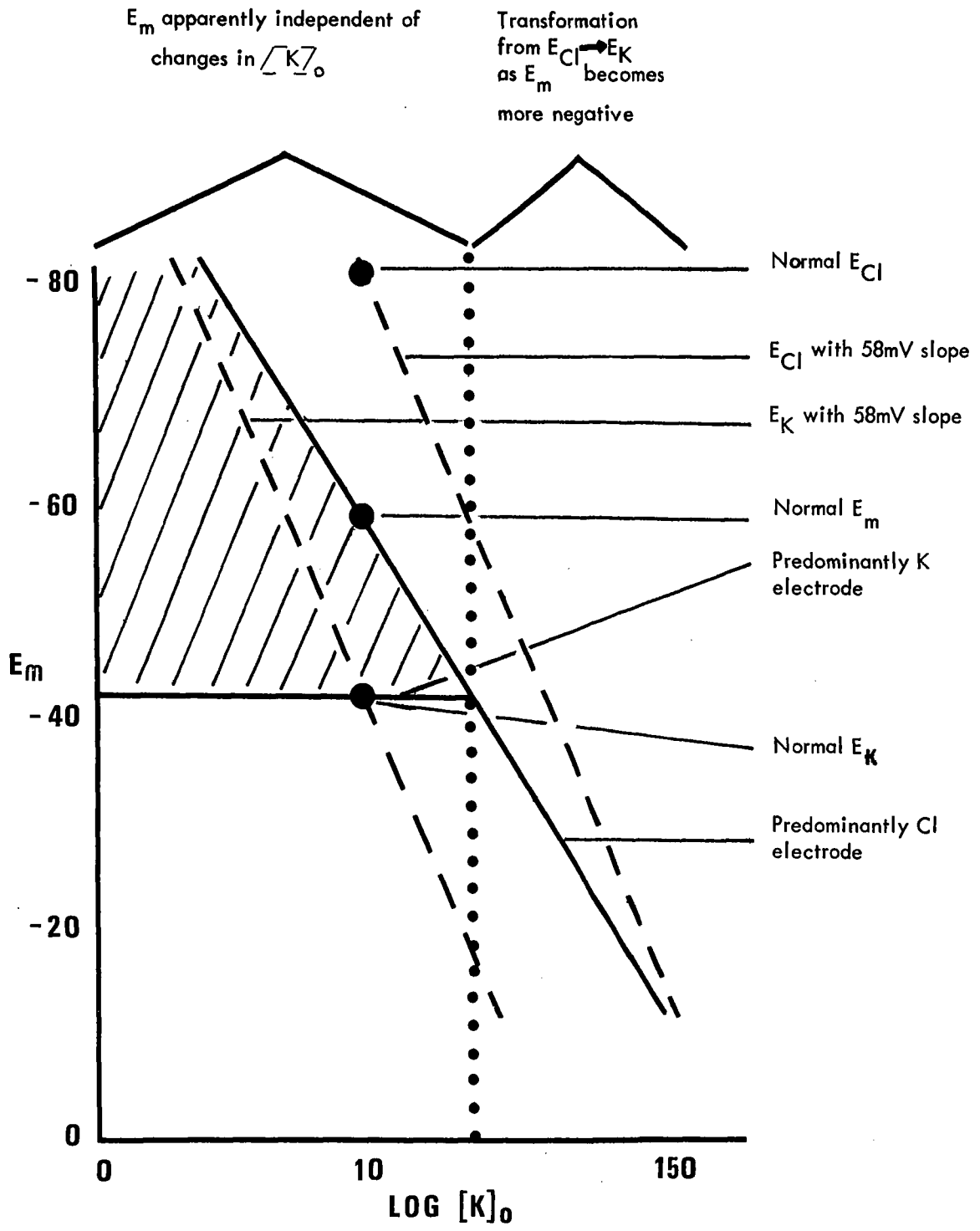
d. an independence of variation of $[K]_o$ at concentrations below 50 mM.

The active component.

An electrogenic component to the resting potential has been described in other excitable cells, including frog muscle (Cross, Keynes & Rybova, 1965; Adrian & Slayman, 1966; Corrie & Bonting, 1966), and lobster axon (Senft, 1967), and it has been suggested that such a system could be involved in the genesis of excitatory and inhibitory potentials at synaptic membranes (Kernan, 1967, 1968; Kerkut, Brown & Walker, 1969; Pinsker & Kandell, 1969; Sokolove & Cooke, 1971) although this has been questioned (Kehoe & Ascher, 1970). An electrogenic sodium pump has been shown to contribute to the membrane potential of Aplysia neurons (Carpenter & Alving, 1968; Carpenter, 1970), and has been implicated in the generation of the receptor potential

FIGURE 23. Summary of the properties of the ionic electrode present in the resting potential of cockroach skeletal muscle.

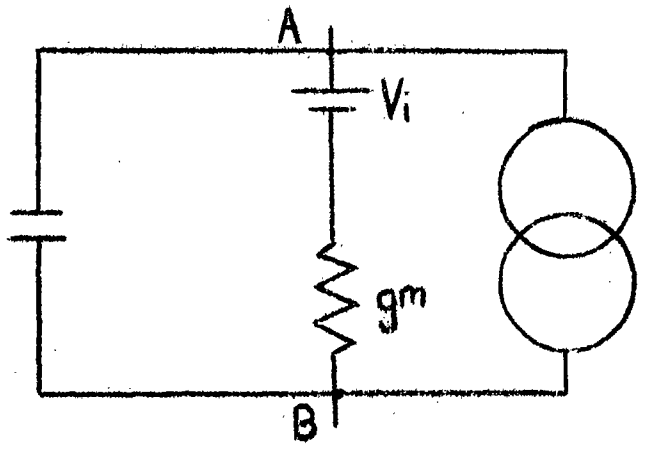
The diagram illustrates the effect of variation in $[K^+]_o$ on E_K and E_{Cl} if they were to behave according to the Nernst equation. The solid line represents observed E_m. The shaded portion of the figure is meant to represent the contribution to E_m by an electrogenic pump.


E_m apparently independent of changes in $[K]_o$
Transformation from $E_{Cl} \rightarrow E_K$ as E_m becomes more negative
Normal E_{Cl}
E_{Cl} with 58mV slope
E_K with 58mV slope
Normal E_m
Predominantly K electrode
Normal E_K
Predominantly Cl electrode
E_m
-80
-60
-40
-20
0
0
10
150
LOG $[K]_o$

of the photoreceptor cells in Limulus (Smith & Stell, 1968a & 1968b). Kerkut and Ridge (1961) obtained a Q_{10} value for Periplaneta muscle fibres when at rest of 1.296 ± 0.046. This value was significantly greater than the value of 1.035 expected if the membrane were to behave as a passive system. They concluded that the resting potential was dependent upon metabolic processes.

The active component of Periplaneta muscle membrane potential is hyperpolarizing and is therefore due to a net movement of cations outwards or anions inwards. In vivo amino acids and other charged organic molecules, which are present in the haemolymph (Florkin & Jeuniaux, 1964), could be implicated. It seems unlikely that this could be so since the saline developed in the present study appears to be comparable with the haemolymph in the maintenance of bioelectric potentials.

The equivalent circuit of the membrane can be represented as follows:



where Vi is the battery formed from the combined ionic electrodes, and gm is the membrane conductance. The membrane potential between A and B would be Vi + I / gm.

The measured E_m is not, however, the simple sum of the addition of a 20 mV active component to the ionic electrode. The shaded portion of figure 23 shows that the magnitude of the contribution

of the active component is dependent on E_m (or $[K]_o$) and is zero when $[K]_o > 50$ mM. A very similar analysis is presented for E_m and the contribution of the electrogenic Na pump in Anisodoris giant neurones; the contribution of the pump vanishes when $[K]_o$ is about 60 mM and $E_m \simeq 25$ mV (Gorman & Marmor, 1970a).

It has been shown that subjecting the muscle to any of the following treatments produced a fall in E_m of approximately 20 mV:

(i) a temperature of 5°C

(ii) the absence of HCO_3^- in the saline

(iii) the absence of Cl^- in the saline

(iv) 5×10^{-3} M acetazolamide

(v) 10^{-3} M CN

(vi) 10^{-4} M DNP

(vii) 5×10^{-4} M strophanthin G.

Although there are differences in the time courses of these changes in E_m with the different treatments, it seems reasonable to suggest that the same active component is inhibited in each case and that the differences are related to differential rates of penetration of drugs. Since similar plots of E_m versus log $[K]_o$ are obtained either at low temperature or in the presence of CN^-, it seems probable that all these treatments have the same ultimate action on E_m.

Candidate ions that could be implicated in the active component are: cations K^+, Na^+ and H^+, and anions Cl^- and HCO_3^-. It has not been possible to determine which of these ions is associated with electrogenic activity in cockroach muscle; considerable uncertainty persists in other preparations which are more accessible, e.g. toad bladder (Gonzalez, Shamoo & Brodsky, 1969; Gonzalez, 1969; Gonzalez & Schilb, 1969; Green, Steinmetz & Frazier, 1970) and Nitella plasmalemma (Hope, 1965; Kitasato, 1968; Walker & Hope, 1969; Spanswick, 1970).

(1) Sodium ions.

Since strophanthin G and agents that suppress the oxidative production of ATP (Caldwell, 1960; Glynn, 1964) inhibit the active component, it is tempting to suggest that an electrogenic Na pump is present. This is the typical system generating a contribution to E_m in muscles and opisthobranch neurones, and the marked temperature sensitivity would also be in accord with the known inhibition of the mammalian Na transport system at about 10°C (Bowler & Duncan, 1968a; Wieth, 1970). It has been shown that a $Na^+ - K^+ - Mg^{2+}$ ATPase which is typically associated with the active Na extrusion (Skou, 1965) is absent in microsomal preparations of muscle from Periplaneta. However, subsequent comparative studies with both vertebrate and invertebrate muscles (Bowler & Duncan, unpublished) suggest that the presence of an ATPase that is synergistically activated by Na^+ and K^+ may not be universally demonstrable in muscle microsomal preparations as was previously supposed. Bittar (1966) has suggested that the Na^+ pump of Maia muscle has two components, one the $Na^+ - K^+ - Mg^{2+}$ ATPase and the other an electrogenic mechanism driven by respiration and possibly by direct coupling of glycolytic energy. Na^+ extrusion in frog and mammalian muscle is favoured by the presence of HCO_3^- in the saline (Creese, 1949, 1952; Carey & Conway, 1954) and a comparable situation might explain the dependence of the cockroach preparation on this ion.

On the other hand, sensitivity to strophanthin G is not conclusive evidence of a system for Na^+ extrusion since this cardiac glycoside also inhibits glycolysis in skeletal muscle (Kypson, Triner & Nahas, 1968). The experiments with ^{22}Na efflux indicated that there was a very slow exchange of Na^+ across the muscle membrane. Finally it is not clear why such a Na^+ transport system should be sensitive to $[Cl]_o$ and $[HCO_3]_o$, although there is evidence that HCO_3^- is

associated with some Na^+ extrusion systems, as in vertebrate muscle (see above) and corneal epithelium (Hodson, 1971).

(2) Potassium ions.

Since E_m exceeds E_K *in vivo*, when the active component is functional, K^+ is presumably transported outwards, reducing $[K]_i$. If this transport is not coupled, it would generate a hyperpolarizing potential. A potassium pump has been suggested in moth heart muscle although, in this preparation, DNP produced a 60% reduction in E_m, a treatment which also caused an associated outward K^+ movement (McCann, 1967). The situation reported in Periplaneta skeletal muscle is also puzzling (Huddart & Wood, 1966); E_m falls in the presence of DNP to -40 mV over two hours but this is preceded during the first hour by a rapid K^+ efflux. The influx of Na^+ is much slower. One might infer that metabolic inhibitors stop active transport, causing a readjustment and a consequent fall in E_m. However, if $E_m > E_K$ it is not clear why K^+ should move outwards. If, on the other hand, DNP were to inhibit an electrogenic component, one would expect to see K^+ efflux follow only when E_m had fallen below E_K.

(3) Chloride and Bicarbonate ions.

Active chloride transport has been established in rabbit cornea (Green, 1965), the intestine of marine Teleosts (House & Green, 1965), the gall bladder of several species (Diamond, 1962), goldfish gills (Krogh, 1937), turtle bladder (Brodsky & Schilb, 1966), and many plant cells (Robertson, 1968).

An hyperpolarizing electrogenic pump involving chloride ions in Periplaneta muscle, necessitates a net inward movement of ions down their chemical gradient, but against their electrical gradient. The evidence favouring an inwardly directed chloride movement is considerable. The resting membrane potential was depolarized to a steady

level in the absence of extracellular chloride ions. Significantly, in such a condition the resting potential was not further altered by metabolic inhibitors, strophanthin G, or low temperature. All these factors caused a depolarization in the presence of extracellular chloride ions. Hence this is direct evidence that the active component of the resting potential is dependent upon the presence of extracellular chloride ions. Further evidence of active chloride transport is provided by the effect of bicarbonate ions. The maintenance of the resting potential was shown to be dependent upon the presence of extracellular bicarbonate ions, a feature demonstrated in other chloride transporting systems, such as the frog gastric mucosa (Imamura, 1970) and turtle bladder (Gonzalez et al., 1969). Finally the resting potential was depolarized in the presence of acetazolamide, the carbonic anhydrase inhibitor, although at high concentrations. Transport of chloride and bicarbonate ions across the turtle bladder is inhibited in the presence of acetazolamide, despite the fact that homogenates of turtle bladder do not possess carbonic anhydrase activity (Gonzalez, 1969).

Hence it is concluded that the electrogenic component of the resting potential of Periplaneta muscle fibres is intimately connected with chloride ions, most probably resulting in their inward transport. Further, the electrogenic component requires the presence of extracellular bicarbonate ions, possibly for active transport as in frog gastric mucosa (Imamura, 1970) and in turtle bladder (Gonzalez et al., 1969), or possibly for its buffering action (Spanswick, 1970).

(4) Protons.

There is no evidence implicating protons in the generation of the active component of the resting potential of Periplaneta muscle fibres, other than the circumstantial evidence that the system is sensitive

to the presence of external bicarbonate ions. It would be of interest to determine the effect of pH on the system but this is hampered by the effect of pH on bicarbonate concentration. Hutter and Warner (1967a & 1967b) have shown that permeability of frog muscle membranes to chloride ions was markedly affected by the pH of the bathing saline.

It is clear that much less is known about the role of protons than about other ions involved in membrane potentials. Stephens (1969) has emphasized the probable importance of protons in electrically excitable membranes. Kitasato (1968) has suggested that proton conductance has a major contribution to the total conductance of the plasmalemma of Nitella, a view which has been challenged by Walker & Hope (1969) who consider that the flux of protons is low and that the major contributor to plasmalemma conductance is potassium. Hope (1965) considers that the existence of an inwardly directed electrogenic bicarbonate pump is the most likely explanation for the hyperpolarization observed in cells of Chara in the presence of extracellular bicarbonate ions. Spanswick (1970) believes, however, that bicarbonate acts primarily as a buffer. Hence the plasmalemma potential of single cells of the Characeae is another preparation where the ionic basis is not clearly established.

On the evidence available it is difficult to suggest the nature of the ionic basis to the active component of Periplaneta muscle resting potential. The situation is reminiscent of that of the turtle bladder where, although short-circuit current is readily measureable in that preparation, the role of each ion is uncertain. The isolated bladder actively transports sodium, chloride, and bicarbonate ions from the mucosal to the serosal bathing fluid. The active transport of bicarbonate rather than of protons in the opposite direction, has been established by Schilb and Brodsky (1966), although Steinmetz (1967)

has invoked proton transport from the serosal to the mucosal surface to account for the observed acidification of bicarbonate-free mucosal fluid. Although active transport occurs in bicarbonate-free saline, the isohydric addition of bicarbonate to the serosal fluid of short-circuited turtle bladder produces an approximate doubling in the rate of sodium transport. A quantitatively similar but smaller effect was obtained from the addition of chloride ions to the serosal fluid (Gonzalez, 1969; Gonzalez & Schilb, 1969; Gonzalez et al., 1969). Acetazolamide (0.1 mM) added to the serosal fluid inhibits active transport of chloride ions and bicarbonate ions, although this cannot be related to carbonic anhydrase activity (Gonzalez & Schilb, 1969; Maren, 1967).

It is possible that the basis to the active component of Periplaneta muscle resting potential is a similar, complex, interacting system, resulting in a net outward flow of ionic current. The total membrane potential of Periplaneta skeletal muscle fibres may be summarized in a conventional diagram (Hubbard, Llinas & Quastels, 1969) as shown in figure 24. It is emphasized that this is only a tentative attempt to explain all the phenomena observed in a single model. Furthermore, such a model in no way is meant to describe structural properties of the muscle membrane. The active component to the resting potential has been represented as an outward current generator and is thought to be the result of a net inward movement of chloride ions.

In such a system, changes in the permeability of the membrane to one or more ions (due, for example, to the action of excitatory and inhibitory transmitters) could alter the voltage produced by the sum of the ionic batteries (Vi) or membrane conductance (gm), thereby altering the contribution of the constant current generator to membrane potential (E_m) as shown in equation 2. Thus if Vi approximated to E_K,

FIGURE 24. Tentative model to account for the electrical behaviour of Periplaneta muscle membranes.

Where:

$$gm = gK + gCl + gNa + gL \quad \ldots\ldots\ldots(1)$$

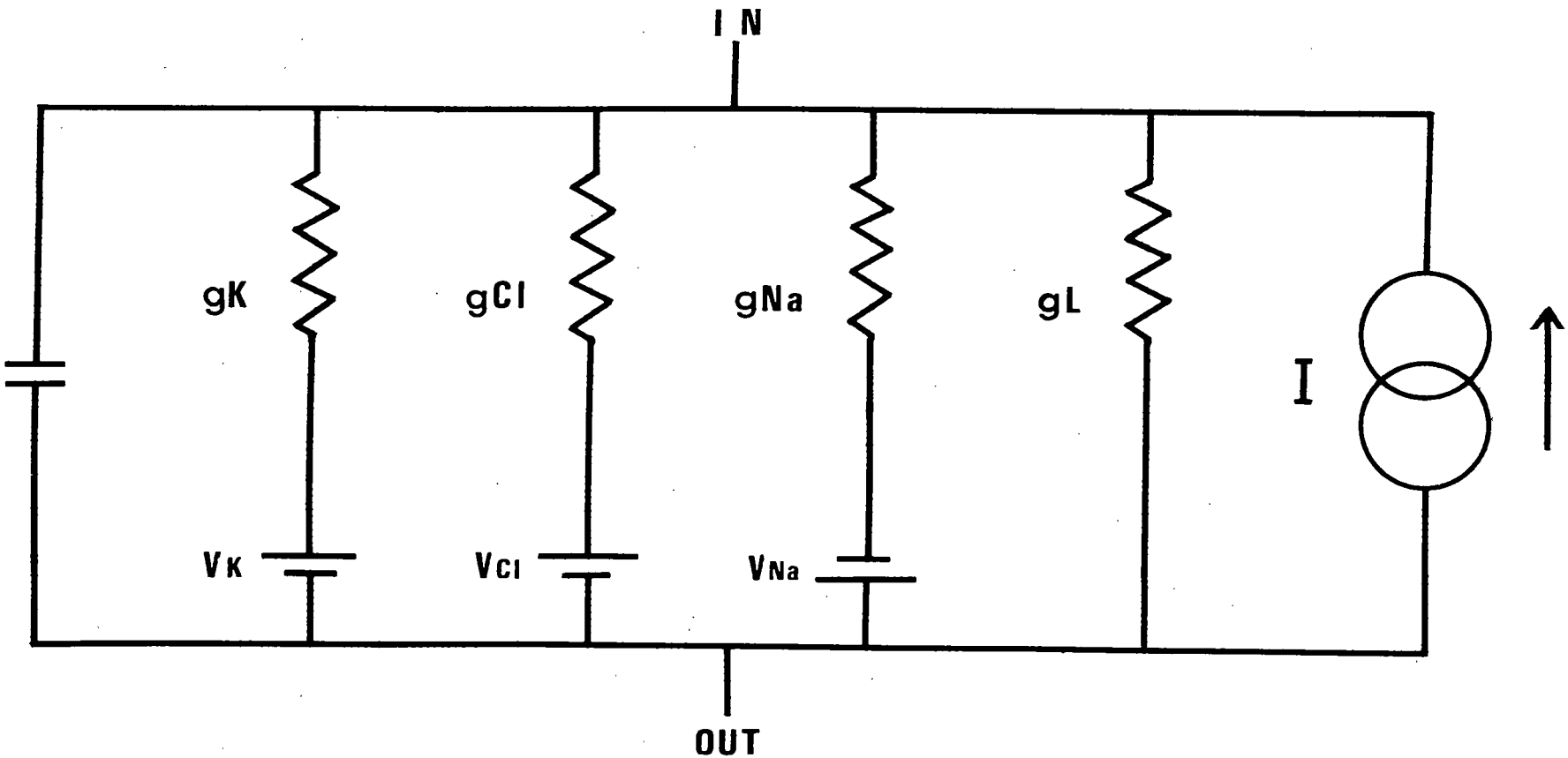
$P_K > P_{Cl}$ *in vivo*

V_K = c -40 mV

V_{Cl} = c -60 mV

The sum of the ionic batteries (V_i) = c -40 mV

$$E_m = V_i + \frac{I}{gm} \quad \ldots\ldots\ldots\ldots\ldots\ldots(2)$$


I N
gK
gCl
gNa
gL
I
VK
VCl
VNa
OUT

whilst an increase in conductance to potassium (gk) will have little or no effect on Vi, the value of I / gm will decrease and the net effect could be depolarizing. Conversely, an increase in conductance to chloride ions (gCl) will tend to have an hyperpolarizing action on Vi (since E_m will approach E_{Cl}), but I / gm will again decrease giving a compensatory depolarization. It is possible that the system postulated can produce an hyperpolarizing IPSP by the activation of the active hyperpolarizing part of the resting potential. Such an IPSP initiation would still be dependent upon the extracellular presence of chloride ions (Usherwood, 1968).

It is interesting that Usherwood (1963) has shown that 20 days after motor nerve section the resting potential of locust muscle eventually falls by 25%. He suggests (Usherwood, 1969) that this could be due to a failure of a metabolic pump to maintain the normal distribution of ions across the muscle fibre membrane. It could also be explained more directly as the failure of the electrogenic contribution of such a pump. Figure 23 reveals that the contribution of the electrogenic component is dependent on E_m; as the membrane is depolarized its contribution is reduced. A similar situation is found in Anisodoris. There is, therefore, an intimate association between the two components of E_m. It is clear that the inclusion of an electrogenic component in any excitable cell can cause a number of difficulties when an analysis of E_m is attempted. Switching on the pump will produce change in P_K, ion fluxes and E_K. A solution of a system of equations involved is given by Frumento (1965). The consequences of such a system on excitatory and inhibitory electrogenesis is considered in the next chapter.

In this model the membrane potential has been treated as being composed of two separate, but clearly inter-related, components.

However, it is conceivable that the situation may be even more involved. Figure 16 (the effect of potassium concentration on the resting potential) may represent the response of a single complex system controlling the passive ion permeabilities, in which metabolic energy is required for changes in permeability to occur. In the absence of such "metabolic priming" changes in permeability are difficult and the membrane becomes inexcitable.

CHAPTER 6. THE ELECTRICAL RESPONSE TO EXCITATION.

INTRODUCTION

The postsynaptic muscle membrane of the cockroach is typically inexcitable by electrical stimulation (Grundfest, 1956 & 1957) and responds only to the chemical transmitter, thought to be glutamic acid (Usherwood, 1969). Since innervation of these muscles is multiterminal a considerable area of the muscle surface is electrically inexcitable, resulting in the production of non-propagating potentials.

The literature on insect muscle physiology has been confused by the use of vague terminology. In particular, the use of the term action potential to describe the response of the muscle to stimulation is unwise since it implies a similarity with vertebrate propagated, all-or-none potentials. The excitation potential of insect muscle is graded, not all-or-none and, typically, does not overshoot the zero potential. The terminology employed here will be that used by Usherwood, (1969). There are three responses to stimulation. Two are junctional potentials, the excitatory postsynaptic potential (EPSP) and the inhibitory postsynaptic potential (IPSP), and the third is an extrajunctional potential termed the electrically excited response (EER). There is some similarity between the EPSP and a vertebrate end-plate potential since it shows refractoriness (Cerf, Grundfest, Hoyle & McCann, 1959) and can be converted into an all-or-none action potential under certain conditions (Werman, McCann & Grundfest, 1961; Hoyle, 1962; Usherwood, 1962a). The EPSP and IPSP are responses to chemical stimulation i.e. the release of neuromuscular transmitter, whereas the EER is a response to the electrical stimulus of the EPSP.

The vertebrate muscle end-plate potential is thought to represent a local depolarization with a rapid rising phase and a slower decay phase. Upon release of transmitter it is believed that the postsynaptic membrane is depolarized by an inward flow of ionic current,

thought to involve sodium and potassium ions only (Takeuchi & Takeuchi, 1960). The postsynaptic potential (EPP) is always excitatory in frog muscle and reaches a peak in 1 msec and lasts for 20 msec. The transmitter (acetylcholine) action lasts for only 5 msec. Takeuchi and Takeuchi (1960) have also shown that there is a linear relationship between the resting membrane potential and the postsynaptic potential and between the resting potential and the current which flows during the postsynaptic potential. These relationships appear to establish that the synaptic current in vertebrate skeletal muscle is a result of ions moving down their concentration gradients (Eccles, 1964).

In contrast to this conclusion Kernan (1967) has suggested that the end-plate potential of rat skeletal muscle might be generated by acetylcholine activating an electrogenic pump involving the inward movement of potassium ions. In rat skeletal muscle fibres the spontaneous miniature end-plate potentials are suppressed by hypoxia (Hubbard & Loyning, 1966) suggesting an active contribution to membrane depolarization. Synaptic activation of an electrogenic sodium pump due to the presence of acetylcholine has been suggested by Pinsker and Kandel (1969) to result in a prolonged IPSP in molluscan interneurons. Their conclusions have, however, been questioned by Kehoe and Ascher (1970) who claim that the prolonged IPSP is due to a change in potassium conductance. In insect and crustacean muscle it has been shown that the inhibitory current is carried by chloride ions (Boistel & Fatt, 1958; Grundfest, 1959; Usherwood, 1969), where the IPSP has a faster rise and slower falling phase than the EPSP.

The propagated action potential of vertebrate muscle is due primarily to a specific increase in permeability to sodium ions. In response to a depolarization sodium conductance increases until at a threshold level the net current direction is inward rather than outward.

Then the net inward current causes still further depolarization and greater increase in sodium conductance. Sodium conductance increases in theory to reach the equilibrium potential for sodium. It does not in fact do so since inactivation of the sodium current occurs and potassium conductance increases as a result of depolarization. These two factors result in repolarization so terminating the action potential.

Although transmembrane ionic flux is involved in the action potential, the action potential does not depend on an appreciable ionic concentration change across the membrane. Some ionic movement does occur (Hodgkin & Keynes, 1955) however and as a consequence of this ionic redistribution a pump must be present to restore the ionic balances in order to maintain excitability.

Electrically excitable membranes are termed rectifying since membrane conductance can alter as a result of altering the transmembrane potential. They cannot be said to be rectifying when the conductance change causes regenerative activity as does the sodium conductance system. They are rectifying when potassium conductance increases in response to depolarization. This action is however termed delayed rectification since potassium conductance increases more slowly than the depolarization. Delayed rectification results in the membrane potential being displaced temporarily towards the potassium equilibrium potential after an action potential. Anomalous rectification, which occurs in frog skeletal muscle fibres (Katz, 1949; Nakajima, Iwasaki & Obata, 1962; Adrian, 1964), is rectification in the opposite direction. Then potassium conductance is reduced upon depolarization and increased upon hyperpolarization.

These concepts of the generation of the action potential have evolved mainly from studies on the squid giant axon and frog skeletal muscle. Electrophysiological studies of other excitable cells have

disclosed electrogenic phenomena of a different nature. Insect muscle fibres have often been shown to exist in an ionic environment very different from squid axons and frog muscle and to exhibit qualitatively different electrical responses.

The all-or-none action potential typical of nerves and vertebrate muscle fibres does not occur in insect muscle fibres. The electrical response of insect fibres to nerve stimulation is graded and spike electrogenesis is decrementally propagated. The response may however be converted into an all-or-none action potential under certain conditions (Werman et al., 1961; Hoyle, 1962; Usherwood, 1962a). The graded response of insect muscle is a function of the electrically excitable membrane. The EPSP is by definition, graded to the quanta of transmitter released. Werman et al. (1961) have suggested that the depolarizing current due to inward cation movement is damped by an increase in potassium conductance to produce graded responses. In other words, they suggest that the potassium rectifying current is not as delayed in insect muscle as in vertebrate muscle. If this be so, then the peak depolarization of the EER will bear little relation to the equilibrium potential for the ion or ions involved in the depolarizing current. In fact, to overshoot the zero potential, a very large EPSP must first be generated. Multiterminal innervation ensures that the whole fibre is depolarized at the same time, so avoiding the necessity for propagation of impulses. One practical consideration that evolves from these properties is that insect muscle fibres cannot be directly stimulated electrically. To elicit a muscle contraction it is necessary to stimulate via the appropriate nerve and consequently the properties of the presynaptic membrane must be taken into account when determining the effect of an agent upon the excitation of the

muscle fibres.

The muscles studied were the extensores trochanteris of the coxae, muscles 136 + 137 according to the nomenclature of Carbonell (1947).

METHODS

The animal was arranged as described for measurements of resting membrane potentials. The same recording circuit was also used (figure 13). The resting membrane potential was recorded when the microelectrode was in a single fibre and the oscilloscope beam was set on single sweep triggered by the output of a square wave stimulator. The single sweep upon stimulation was recorded on Ilford 5G91 recording film using a Cossor oscillograph camera.

A muscle was stimulated by placing a pair of silver electrodes connected to a Palmer square wave stimulator on the corresponding ventral ganglion. Single pulses of 1 msec duration were fed through a radio frequency coupling device to the electrodes. This isolator effectively removed the stimulus artefact from the recordings. The signal generator voltage served as the source of power for a midget radiofrequency oscillator which converted the signal to amplitude-modulated carrier energy. The radiofrequency energy was transmitted across a small air gap to a receiving circuit where it was demodulated and filtered. The voltage thus recovered was a faithful reproduction of the original signal but was isolated from the input circuit as well as from ground and so avoided the recording of a stimulus artefact. The radiofrequency coupler used was constructed according to the details given by Schmitt and Dubbert (1949).

The result of a nerve stimulation was a single twitch of the muscle fibres. The mechanical deformation caused by fibre contraction did not alter the properties of the electrode. It was apparent, however, that after a few contractions the seal between the implanted electrode and the cell membrane was broken and the potentials recorded diminished. Therefore it was not usually possible to follow the effect of agents upon the excitability of a single fibre but several different fibres had to be sampled and the results meaned.

RESULTS

1. Measurement of the excitatory postsynaptic potential and the electrically excited response.

Intracellular recordings were taken of the excitation of muscle fibres whilst bathed in their haemolymph, and after equilibration for two hours with the normal saline described previously. The parameters of the EPSP and the EER were measured from filmed records. Usually at least 30 different responses were measured; the mean values are given in table 26. A photograph of the typical excitatory response in haemolymph and in saline is shown in figure 25. It has been shown that the fast fibres in cockroach muscle fatigue rapidly in both their electrical and mechanical responses to repetitive stimulation (Smit, Becht & Beemakkers, 1967). Care was taken in this series of experiments not to overstimulate the fibres. No substrate was added to the saline since it was found that the addition of α glycerophosphate, trehalose or glucose did not improve the survival of the fibres or affect their electrical responses.

Since the various parameters for the electrical responses to excitation are very similar in both haemolymph and saline (table 26) it is suggested that the effective composition of the normal saline is very close, in respect of its inorganic ion concentrations, to that of the haemolymph. From table 26 it can be seen that the rate of rise of the EPSP is greater than that of the EER. Furthermore the depolarization of the EPSP is 21.4 mV greater than that of the EER and there is typically no overshoot of the zero potential. These parameters may be compared to those of a vertebrate action potential. The rate of rise of the end-plate potential of frog muscle fibres is 220 volts / second and of the action potential is 670 volts / second (Nastuk, 1953), and the action potential typically does overshoot the zero potential.

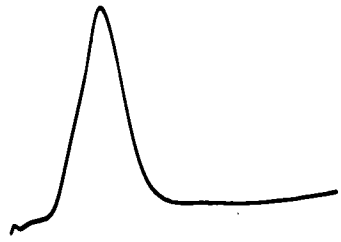
TABLE 26. The parameters of the response to excitation of Periplaneta coxal muscle in haemolymph and saline.

Experiments were conducted at room temperature. Standard errors are calculated from measurements from at least 30 different muscle fibres. Before recording from fibres bathed in saline the muscles were equilibrated for two hours at room temperature.

Condition	EPSP Size mV	EPSP Rate of Rise Volts / sec.	EER Size mV	EER Rate of Rise Volts / sec.	Resting Potential mV
Haemolymph	38.5 ± 0.4	52.0	17.1	29.4	59.9 ± 0.3
Saline	38.2 ± 0.9	55.4	15.9	31.1	60.6 ± 0.5

FIGURE 25. The electrical responses to excitation of cockroach coxal muscle recorded in haemolymph and saline.

Trace A was obtained after the muscles had been allowed to equilibrate with the bicarbonate saline described in chapter 5. Trace B was obtained from muscle bathed in haemolymph. Both are intracellular records. The coxal muscles used were those of the third thoracic segment and maximally stimulated by square wave pulses applied to the ventral ganglion of the third thoracic segment. The scale is shown alongside the records. The resting potential of the muscles in haemolymph and saline were not significantly different.



A. SALINE

20mV

5ms

B. HAEMOLYMPH

The rate of rise of the EPSP and the EER of cockroach muscle fibres is approximately 4 and 22 times slower respectively than the rate for the EPP and AP of frog muscle fibres. The lack of similarity between the responses to excitation in the two animals suggests that the mechanisms underlying spike electrogenesis in them may be very different.

2. The effect of divalent cations.

a. Calcium.

To determine the contribution of calcium to the electrical current during excitation $[Ca^{2+}]_o$ was raised and the responses to excitation filmed and measured. The muscles were equilibrated for one hour to each concentration of calcium and the responses of at least 30 different fibres recorded. The responses in saline containing 10-50 mM calcium are given in table 27 and typical film records shown in figure 26. The high calcium salines were buffered with 10 mM Tris to avoid precipitation of calcium carbonate. The omission of bicarbonate ions from the saline did not effect the resting potentials during the first hour of exposure.

As $[Ca^{2+}]_o$ was increased from 10-50 mM the resting potential was not significantly altered. The absolute size of the EPSP did not change either. There was, however, an increase of 45.3% in the size of the EER. The second effect of high $[Ca^{2+}]$ was to slow the rate of rise of the EPSP whilst accelerating the rate of rise of the EER. At no concentration of calcium was there a significant overshoot of the zero potential. It is thought that calcium enhances the release of chemical transmitter at the nerve muscle junctions of insects (Usherwood, 1963), but does not contribute to the ionic current, since synaptic potentials can be elicited from locust muscle fibres by the addition of glutamate in the absence of external calcium

TABLE 27. The effect of Calcium on the EER and EPSP of cockroach coxal muscle.

Salines were buffered with 10 mM Tris. The responses of at least 30 different fibres were measured at each concentration of $[Ca^{2+}]_o$ after equilibration for one hour. $[Mg^{2+}]_o$ was maintained at 6 mM. Experiments were conducted at room temperature. Standard errors are calculated from measurements of at least 30 different muscle fibres.

$[Ca^{2+}]$ mM	EPSP mV	Rate of rise Volts / sec.	EER mV	Rate of Rise Volts / sec.	R.P. mV
10	38.2 ± 0.9	55.4	15.9	31.1	60.6 ± 0.5
15	36.2 ± 1.1	52.4	17.9	35.1	59.2 ± 0.5
20	40.5 ± 0.7	46.0	20.8	40.0	59.3 ± 0.6
30	37.8 ± 0.8	42.0	18.6	31.0	59.4 ± 0.4
50	38.0 ± 0.4	42.2	23.1	40.0	60.3 ± 0.5

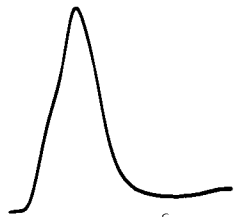
FIGURE 26. The effect of altering the concentration of Mg^{2+} and Ca^{2+} in the bathing saline on the electrical responses to stimulation of cockroach coxal muscle.

Records C and D show the effect of saline buffered to pH 7.2 with 10 mM Tris, containing 10 mM and 50 mM Ca^{2+} in the presence of 6 mM Mg^{2+}. Muscles were equilibrated to each saline for one hour.

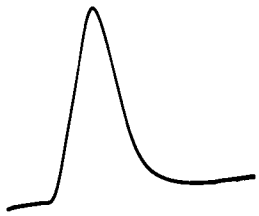
Records E, F and G show the effect of raising $[Mg^{2+}]_o$ in the presence of 20 mM Ca^{2+} on the excitation of muscle equilibrated for one hour.

All traces are intracellular records. The coxal muscles recorded from were those of the third thoracic segment which were maximally stimulated by applying square wave pulses to the ventral ganglion of that segment. The resting potentials of the muscles were not significantly different (see tables 27 & 28).

C. Mg^{2+} 6 mM
Ca^{2+} 10mM

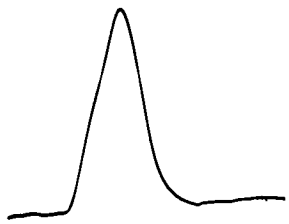


D. Mg^{2+} 6mM
Ca^{2+} 50mM

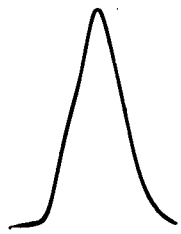


20mV
5ms

E. Mg^{2+} 6mM
Ca^{2+} 20mM



F. Mg^{2+} 20mM
Ca^{2+} 20mM



G. Mg^{2+} 40mM
Ca^{2+} 20mM

ions (Usherwood, 1969). In the absence of calcium ions nerve-muscle transmission is blocked. Whilst it is unlikely that calcium contributes to synaptic current in this preparation, since increasing $[Ca^{2+}]$ had little effect upon the magnitude of the EPSP, it is possible that it is able to contribute to the ionic current of the EER.

b. Magnesium.

In the presence of 20 mM Ca^{2+}, the magnesium concentration of the saline (buffered with 10 mM Tris) was raised to 20 and 40 mM. The responses to stimulation of muscles which had been equilibrated for one hour with these salines are analysed in table 28, and typical film records are shown in figure 26. Increasing $[Mg^{2+}]_o$ resulted in a slight decrease in the rate of rise of both the EPSP (11.5%) and the EER (17.0%). Furthermore, whilst the absolute magnitude of the EPSP was not affected by high external $[Mg^{2+}]$ there was an increase of 14.9% in the magnitude of the EER in 40 mM Mg^{2+}, which resulted in a small (4.5 mV), but significant, overshoot of the zero potential.

3. The effect of Sodium Ions.

Muscle fibres were equilibrated with salines of different sodium composition for one hour. Sodium was replaced with choline chloride. It has already been shown that after one hour in saline containing no sodium the resting potential was not altered (see table 19). In saline containing a low concentration of sodium the absolute sizes of the EPSP and the EER were significantly reduced (table 29). Film records of the effect of altering $[Na^{+}]_o$ are shown in figure 27. The depolarization of the EPSP increased linearly with increasing $[Na^{+}]_o$ up to 100 mM sodium which appeared to produce the maximum response (figure 28). The depolarization of the EER also increased with the sodium concentration but not in a linear manner. When the

TABLE 28. The effect of Magnesium on the EER and EPSP of cockroach coxal muscle.

The salines were buffered with 10 mM Tris. The responses of at least 30 different fibres were measured at each concentration of Mg^{2+} after equilibration for one hour. Experiments were conducted at room temperature.

$[Ca^{2+}]_o$ mM	$[Mg^{2+}]_o$ mM	EPSP Size mV	EPSP Rate of Rise Volts / sec.	EER Size mV	EER Rate of Rise Volts / sec.	R.P. mV
20	6	40.5 ± 0.7	46.0	20.8	40.0	59.3 ± 0.6
20	20	37.8 ± 0.1	43.5	22.4	31.5	60.0 ± 0.4
20	40	41.9 ± 0.5	40.7	23.9	33.2	60.4 ± 0.4

TABLE 29. The effect of $[Na^+]_o$ on the size and rate of rise of the EPSP and the EER of cockroach coxal muscle.

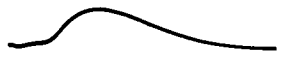
Experiments were conducted at room temperature. The responses of at least 30 different fibres were measured after equilibration for one hour to each concentration of Na^+. In salines containing less than 140 mM sodium chloride choline chloride was added to maintain isosmocity. Saline containing 240 mM Na^+ was obtained by the addition of 100 mM NaPr to the normal saline.

$[Na^+]_o$ mM	EPSP mV	EPSP Rate of Rise Volts / sec.	EER mV	EER Rate of Rise Volts / sec.	TOTAL RESPONSE mV
20	6.94 ±3.2	5.06	0	0	6.94 ±3.2
40	14.96 ±1.0	12.07	7.00 ±0.8	8.33	21.96 ±1.7
60	24.43 ±1.1	21.11	15.22 ±0.8	21.87	39.2 ±2.1
80	33.33 ±1.0	32.53	20.33 ±0.5	32.12	53.11 ±1.3
100	34.96 ±0.7	31.30	22.33 ±0.4	37.20	56.88 ±0.8
140	35.00 ±0.6	47.30	19.80 ±0.3	37.57	54.80 ±0.7
240	40.65 ±0.7	47.21	20.17 ±0.6	35.39	60.83 ±1.1

FIGURE 27. The effect of $[Na^+]_o$, and of replacing $[Na]_o$ with Li^+, on the electrical responses to stimulation of cockroach coxal muscle.

Records H to L show the effect of increasing concentrations of $[Na]_o$ on the intracellular records of the response to excitation after an equilibration time of one hour. Isosmocity with saline containing 140 mM NaCl was maintained by the addition of choline chloride.

Record M shows the effect of replacing $[NaCl]_o$ with LiCl on the intracellular record of the response to excitation after equilibration for two hours.



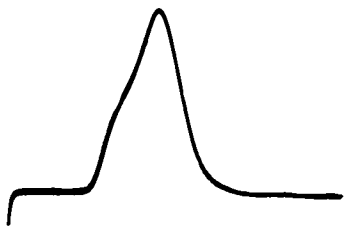
H. Na^+ 20mM

I. Na^+ 40mM

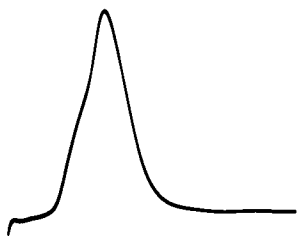
J. Na^+ 60mM

20mV

5ms



K. Na^+ 80mM



L. Na^+ 100mM

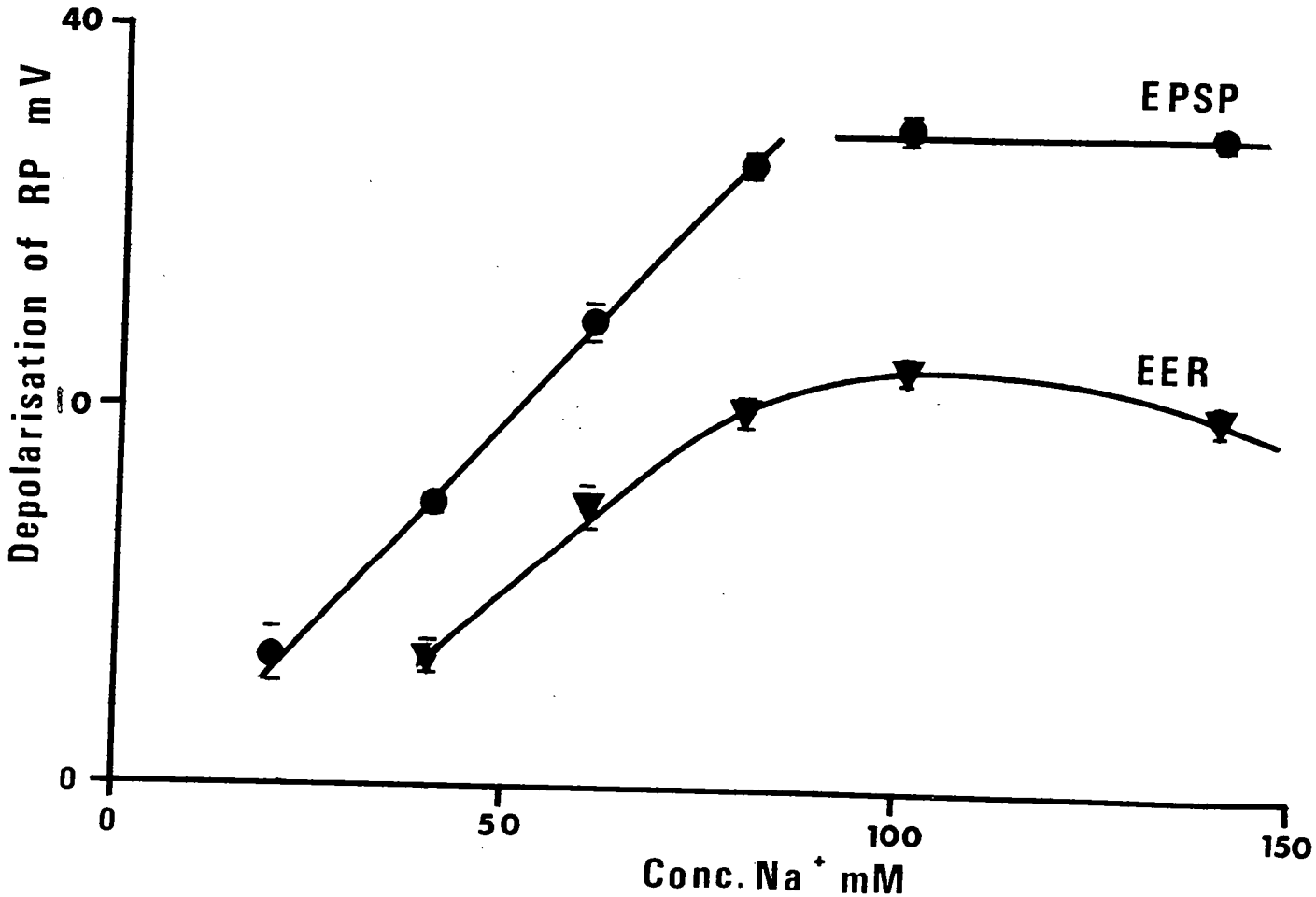
M. Li^+ 100mM

FIGURE 28. The effect of increasing $[Na^+]_o$ on the magnitude of the EPSP and the EER of cockroach coxal muscle.

Experiments were conducted at room temperature. In salines containing less than 140 mM NaCl, choline chloride was added to maintain isosmocity. The responses of at least 30 different fibres were measured after one hour equilibration to each concentration of Na^+. Standard errors are shown where large enough to be greater than the diameter of the points.

Ordinate: Membrane potential (mV) recorded.

Abscissa: Concentration of sodium in the saline (mM).


40
0
Depolarisation of RP mV
EPSP
EER
0
50
100
150
Conc. Na+ mM

rate of rise of the EPSP was plotted against increasing $[Na^+]_o$ it was seen that this rate increased linearly in relation to the sodium concentration up to a level of 140 mM (figure 29). The rate of rise of the EER increased to a greater extent than that of the EPSP in response to increased $[Na^+]_o$. The rate of rise of the EPSP is typically faster than that of the EER in normal saline, but it can be seen from figure 29 that at sodium levels between 60-120 mM the rate of rise of the EER is greater than that of the EPSP.

When the mean size of the EPSP was plotted against the mean size of the EER at increasing $[Na^+]_o$ a linear relationship resulted (figure 30). This suggests that the absolute size of the EER is dependent upon the size of the EPSP. However, when the rate of rise of the EPSP was plotted against the rate of rise of the EER at varying $[Na^+]_o$ the relationship obtained was not linear (figure 31). This indicates that the rate of rise of the EER is dependent upon other factors than the rate of rise of the EPSP.

Because of the complex relationship between the EPSP and the EER it cannot be claimed that sodium ions have been shown to carry all the inward current during excitation. It is however suggested that sodium ions are necessary for the production of the EPSP and that the rate of rise and the magnitude of the EPSP is related to the external sodium concentration.

4. The effect of lithium ions.

Lithium ions have been found to substitute effectively for sodium in the action potentials of the giant axon of Loligo (Hodgkin & Katz, 1949), and myelinated nerve fibres of the frog (Huxley & Stampfli, 1951). That lithium could substitute for sodium in the action potential of frog muscle fibres was first demonstrated by Overton (1902). When lithium (100 mM) replaced sodium in the saline bathing cockroach

FIGURE 29. The relationship of the rates of rise of the EPSP and the EER of cockroach coxal muscle to increasing $[Na^+]_o$.

The values of the rates of rise of the EPSP and EER were obtained from table 29. No standard errors are shown.

Ordinate: Rate of rise (V sec).

Abscissa: Concentration of sodium in the bathing saline (mM).

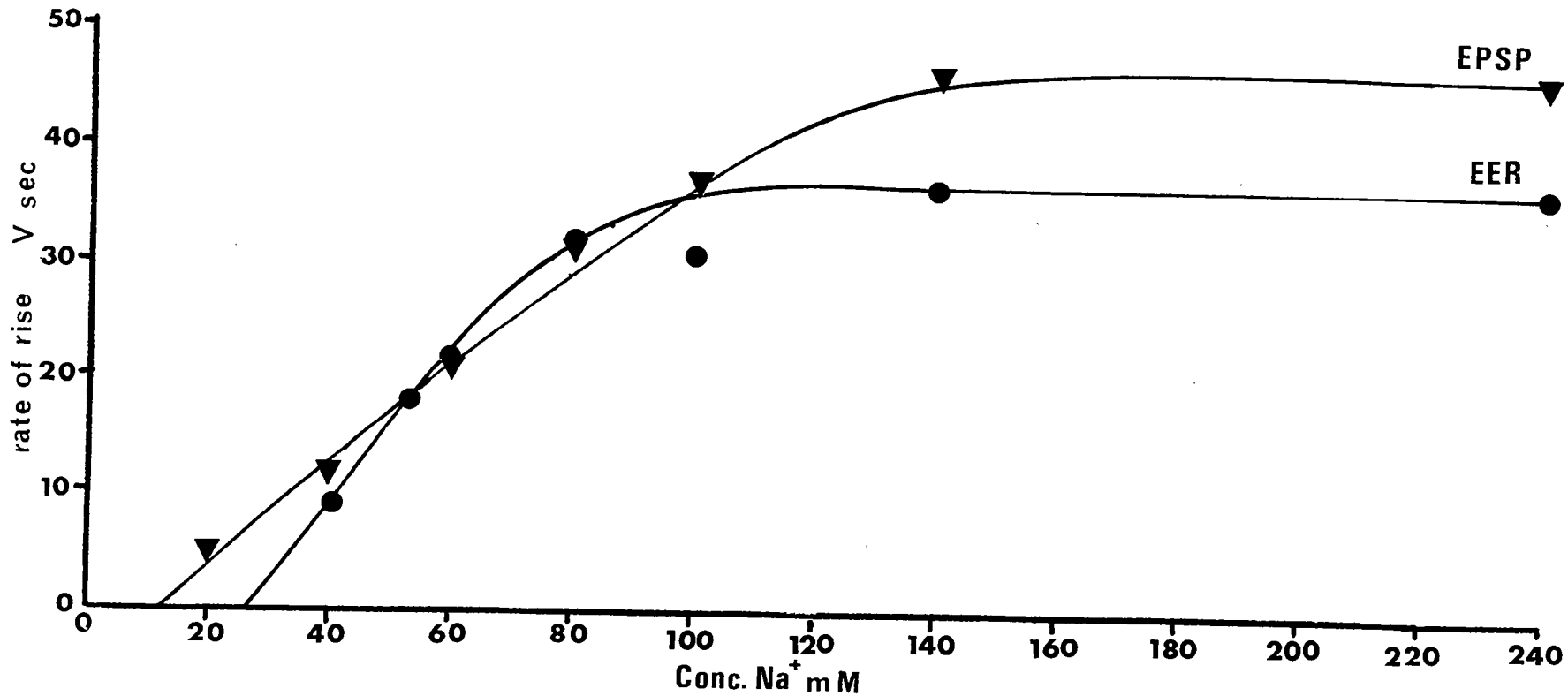

50
40
30
20
10
0
rate of rise V sec
0
20
40
60
80
100
120
140
160
180
200
220
240
Conc. Na+ m M
EPSP
EER

FIGURE 30. The relationship of the mean size of the EPSP to the mean size of the EER of cockroach coxal muscle at varying $[Na^+]_o$.

The size of the EPSP and the EER were obtained from table 29. No standard errors are shown.

Ordinate: Magnitude of the EER (mV).

Abscissa: Magnitude of the EPSP(mV).

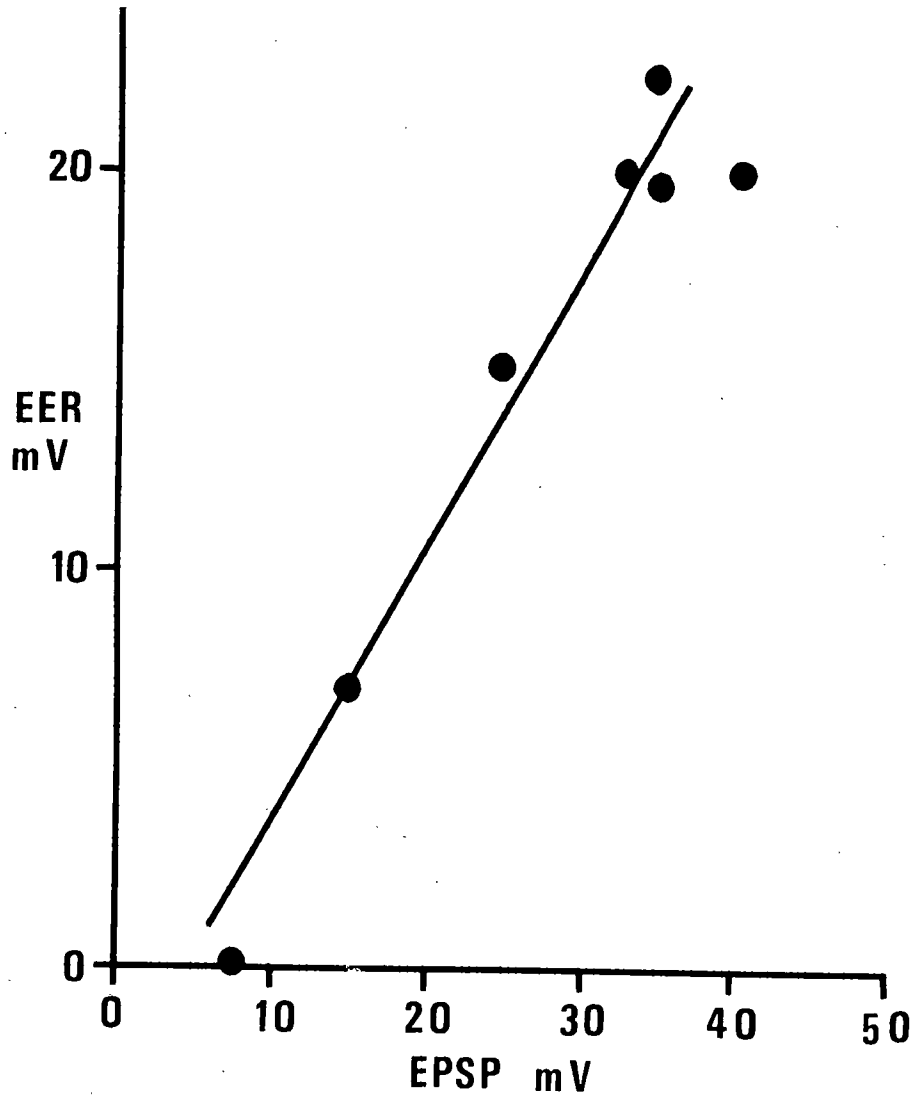
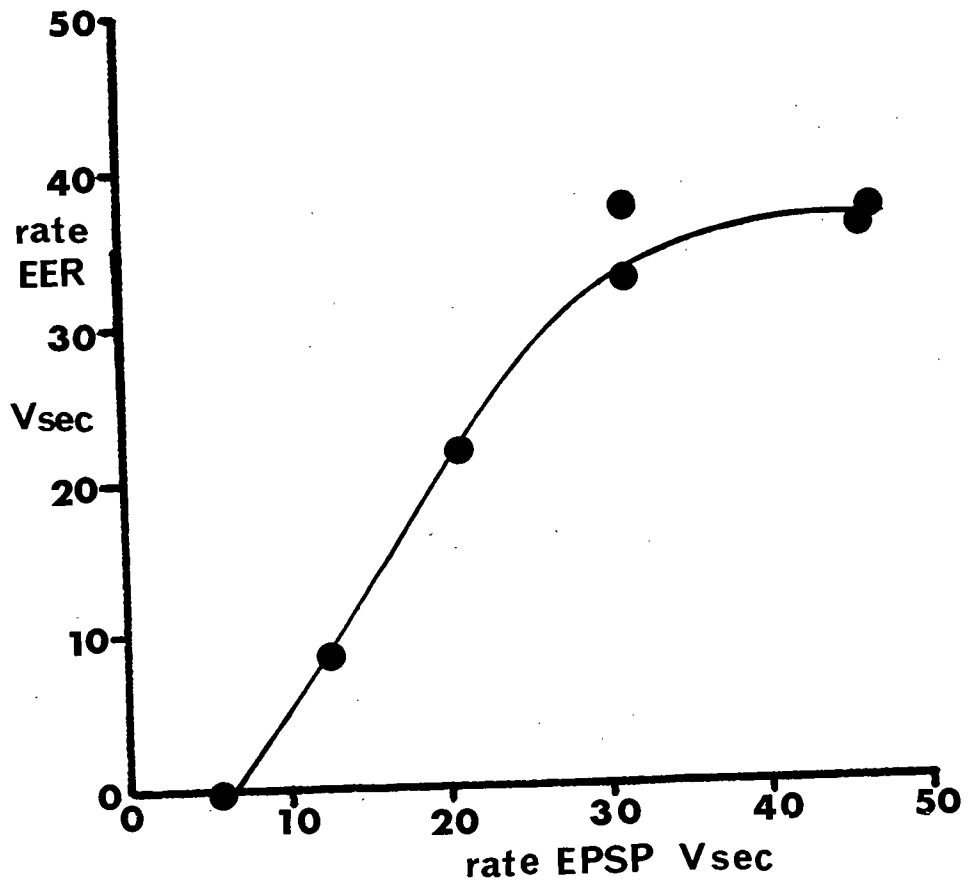

20
10
0
EER
mV
0
10
20
30
40
50
EPSP mV

FIGURE 31. The relationship of the mean rates of rise of the EPSP and the EER of cockroach coxal muscle at different $[Na^+]_o$.

The values of rates of rise of the EPSP and EER were obtained from table 29. No standard errors are shown.

Ordinate: Rate of rise of the EER (Volts / sec.).

Abscissa: Rate of rise of the EPSP (Volts / sec.).


50
40
rate
EER
30
Vsec
20
10
0
0
10
20
30
40
50
rate EPSP Vsec

skeletal muscle fibres, electrical activity and contractions could still be elicited after two hours equilibration, but both the EPSP and the EER were reduced (figure 27). The EER was reduced by about 50% and the EPSP by about 30% of their values in 100 mM sodium. Similarly the rate of rise of the EER was reduced by c. 60% whilst the rate of rise of the EPSP was reduced by c. 30%. Hence lithium was able to substitute only in part for sodium.

5. The effect of Potassium.

After a one hour equilibration with saline containing 50 mM potassium the resting potential was -35.3 mV ± 0.3. In this condition the muscles were still excitable, although the magnitude of the response was greatly reduced, no overshoot of the zero potential occurred and repolarization was delayed (figure 32). The EPSP was only 9.8 mV ± 0.4 and the EER was only 4.9 mV ± 0.5. The rate of rise of the EPSP was 15.7 V / sec. and that of the EER was 6.9 V / sec. Since del Castillo et al. (1953) have shown that the size of the EPSP is linearly related to the degree of polarization of the resting potential, in 50 mM K^+ a reduced response was expected.

6. The effect of Chloride Ions.

The absence of chloride from the saline bathing cockroach muscle fibres has been shown to result in a depolarization of the resting potential. After three hours equilibration with sulphate-substituted saline the muscle fibres were still excitable (figure 32) although the resting potential was -42 mV. However the magnitude of the depolarization was reduced, probably due to the relation of the EPSP with the size of the resting potential, and it was not possible to distinguish between the EPSP and the EER. The rate of rise of the observed depolarization was reduced to c. 30 V / sec. (cf. table 26). The rate of repolarization was markedly reduced, suggesting that

FIGURE 32. The effect of chloride-free saline and saline containing 50 mM $[K^+]_o$ on the response to excitation of cockroach coxal muscle.

Experiments were conducted at room temperature.

Record N shows the intracellular response to excitation of muscle equilibrated for three hours with Cl^--free saline. The resting potential was depolarized to-42 mV. Cl^- was replaced by an equivalent amount of sulphate.

Record O shows the intracellular response to excitation of muscles equilibrated for one hour to saline containing 50 mM K^+. The resting potential was - 35.3 mV.

N. Cl^- - FREE SALINE

O. K^+ 50mM

chloride conductance might be involved in the process of repolarization.

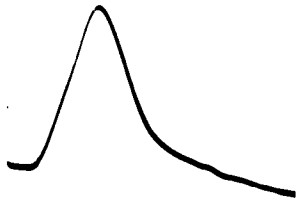
7. The effect of temperature.

Exposure of the muscle fibres of the cockroach to a temperature of 5°C has been shown to result in a depolarization of the resting potential to c.-40 mV. Despite this depolarization it was found that after equilibration to 5°C the muscle fibres were inexcitable. Low temperature would be expected to reduce the rate of release of neuromuscular transmitter. To test the possibility that a synaptic block occurred at 5°C the spontaneous activity of the ventral nerve cord of an animal was recorded. An animal was fixed on its back and a median incision made to expose the large ventral nerve cord. A single silver wire was hooked around the nerve and connected to a preamplifier. The recording was displayed on an oscilloscope and the record filmed. The rate of firing at 20°C was 55 / sec. When the animal was cooled to 5°C spontaneous activity still occurred with a firing rate of 43 / sec. Hence chemical transmission and electrical transmission were occurring in the central nervous system at 5°C. Therefore it may be tentatively suggested that the effect of 5°C in inactivating cockroach muscle is directly on the muscle membrane itself.

Figure 33 shows the effect of temperatures between 10°C and 20°C on the response of these muscles to excitation. The muscles were equilibrated to different temperatures for 30 minutes and their responses filmed. Since the resting potential becomes depolarized at low temperatures, and since the EPSP size is related to the resting potential, the responses were expected to be small. As the temperature was lowered the depolarization became less and the rate of rise slowed. However it is clear from the records in figure 33 that the most dramatic effect of low temperature was to slow the rate of repolarization.

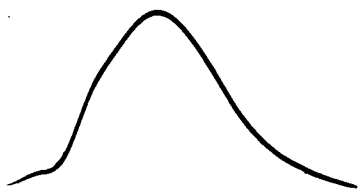
FIGURE 33. The effect of lowering the temperature on the response to excitation of cockroach coxal muscle.

Records P to S show the effect of lowering the saline temperature on the intracellular responses to stimulation. Muscles were equilibrated to each temperature for 30 minutes and the temperature was recorded by means of a thermocouple placed alongside the muscle.

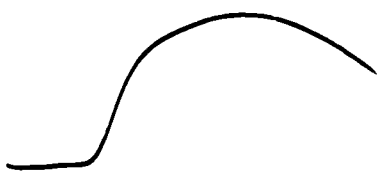


P. 18.0 °C

Q. 14.2 °C



R. 12.9 °C



S. 11.6 °C

Since the EER is dependent upon the depolarization of the EPSP, and this in turn is dependent upon the polarization of the resting potential, it may be that the loss of excitability at low temperatures is simply a reflection of resting membrane depolarization. However in 50 mM K^+, when the resting potential was only -35.3 mV, the fibres were still excitable.

8. Excitation and metabolic inhibitors.

It has been demonstrated that metabolic inhibitors inhibit the temperature-sensitive component of the resting potential of Periplaneta skeletal muscle fibres. After a one hour equilibration with saline containing either 0.5 mM DNP, 0.5 mM strophanthin G or 1.0 mM cyanide, the muscles were also found to be inexcitable. It could be suggested that this loss of excitability is due to the removal by the inhibitors of the temperature-sensitive component of the resting potential since in high $[K^+]_o$, although the resting potential was depolarized to a similar extent, the muscles were still excitable. However, when $[Cl]_o$ was reduced to zero the muscles were still found to be excitable.

DISCUSSION

Interpretation of the experimental results presented above is complicated by the nature of the preparation studied. Periplaneta skeletal muscle fibres are multiterminally innervated and it is not possible to stimulate them directly. The following factors must therefore be taken into consideration because of the necessity of stimulating the muscle fibres via their nerves.

a) Any treatment apparently altering the electrical responses of the muscle fibres, such as changes in ionic environment, or topical administration of drugs, could be having a presynaptic effect. Although the nerves are protected from the haemolymph or saline by a sheath, the nerve terminals are not fully protected, although some glial cells are present.

b) Since an in vivo preparation was used there is a possibility that the recorded responses to stimulation were modified by neural activity in motor nerves other than those stimulated. Inhibitory postsynaptic potentials have been recorded from muscle of locust, a grasshopper, a bee and a beetle (Usherwood & Grundfest, 1964, 1965; Ikeda & Boettiger, 1965a & 1965b; Hoyle, 1966a & 1966b). Excitation of inhibitory axons results in the attenuation of depolarizing EPSP's and reduction in the force of the mechanical responses. In locust and grasshopper jumping muscles only fibres innervated by the "slow" excitor axons receive inhibitory innervation, and the main effect of peripheral inhibition is thought to be on the slow responses (Usherwood & Grundfest, 1965). Hence inhibition is unlikely to have modified the "fast" responses recorded in this present work from Periplaneta coxal muscles. It has been suggested that it does occur in Blaberus giganteus and Blaberus craniifer (Usherwood & Grundfest, 1965), although no experimental evidence has been published.

c) Certain treatments affecting the responses of the muscle fibres could act at the postsynaptic terminals. Any agent affecting the release or the postsynaptic action of the neuromuscular transmitter, thought to be L-glutamic acid (Usherwood & Machili, 1968; Usherwood, Machili & Leaf, 1968; Beranek & Miller, 1968a & 1968b), will lead to a reduction in the size of the EPSP and consequent loss of excitability.
d) Production of the EER is dependent upon the magnitude and rate of rise of the EPSP. Consequently any treatment altering the EPSP will also affect the EER. The magnitude of the EPSP is linearly related to degree of polarization of the resting potential (del Castillo et al., 1953), and any agent causing the resting membrane potential to depolarize will result in a reduction in the sizes of the EPSP and the EER.

In view of the above considerations it is clear that the results presented must be interpreted with care and that conclusions may only be drawn with certain reservations. However the above criticisms also apply to many previous studies and it will be assumed that the major effect of changes in ionic content of the bathing saline is on the muscle membrane directly.

The results showed that the electrical responses of Periplaneta coxal muscle fibres are similar whether the muscles are immersed in haemolymph or in normal saline. The parameters of the responses compare well with the values given by Hoyle (1955) for the same preparation, who found that ".....the EPSP was the largest and fastest part of the insect muscle action potential in response to nerve stimulation." He gave values for the rate of rise of the EPSP (38 V / sec.) and the EER (36 V / sec.) which are similar to those found here.

The divalent cations magnesium and calcium have been implicated

in synaptic events in cockroach muscle. It is thought that calcium is necessary for the release of chemical transmitter, a process inhibited by high concentrations of magnesium (Hoyle, 1955). The results presented here indicate that divalent cations may contribute to some extent to spike electrogenesis. In high calcium saline (50 mM Ca^{2+} + 6 mM Mg^{2+}) there was a significant increase in the size of the EER (45.3%). It has been suggested that the muscle membrane has a low permeability to magnesium since a variety of treatments, resulting in the drastic redistribution of potassium and sodium, had little effect upon the magnesium distribution. Nevertheless, increasing the magnesium concentration from 6 to 40 mM, in the presence of 20 mM calcium, resulted in a small (14.7%) increase in the size of the EER. In neither case can a synaptic transmission effect be invoked since, not only was the absolute size of the EPSP unaffected under these conditions, its rate of rise was in fact slightly reduced. Hence it is concluded that under these experimental conditions the divalent cations magnesium and calcium are able to play a part in the electrogenesis of the EER but not of the EPSP.

The contribution of potassium and chloride ions to depolarizing electrogenesis is difficult to evaluate due to their contribution to the resting membrane potential. A significant effect in the absence of chloride was the slower rate of repolarization, a similar effect being produced in high potassium saline. Usherwood (1969) suggested that potassium and possibly chloride ions were responsible for repolarizing electrogenesis in insect muscle. It has previously been suggested that conductance to potassium and chloride ions is very temperature sensitive. Significantly at low temperatures the rate of repolarization was considerably slowed. Hence both potassium and chloride ions are implicated in the process of repolarizing

electrogenesis.

There was a progressive loss of excitability as the temperature of the muscles was lowered and at 5°C the muscle fibres were inexcitable. Metabolic inhibitors also rendered the muscle fibres of this preparation inexcitable. Under both these conditions the resting membrane potential was depolarized prior to stimulation. This is an apparent anomaly in that depolarization has brought about a loss of excitability, and is in direct contrast to vertebrate muscle where depolarization of the resting membrane potential towards the threshold of the propagated spike potential results in increased excitability.

If in fact the loss of excitability in this preparation is due to depolarization of the resting potential then any treatment causing the resting potential to depolarize to about -40 mV should abolish excitability. Clearly this is not the case since in 50 mM potassium saline the muscle fibres were still excitable, despite the fact that the resting potential was only -35.5 mV. The treatments which abolished excitability, low temperatures, DNP, cyanide and strophanthin G, were the treatments which were found to inhibit the temperature-sensitive component of the resting membrane potential. It may be suggested that the presence of all or part of this actively maintained component is necessary for excitability. Against this conclusion may be placed the fact that, whilst chloride ions have been shown to be involved in the production of the active component of the resting membrane potential, in chloride-free saline the muscle fibres did retain some degree of excitability. The value of this result is difficult to assess. Inspection of figure 32 reveals that the response is abnormal in that there is no apparent division into EPSP and EER and the rate of rise is very fast, and the rate of repolarization very slow. Hence this result is difficult to interpret and may be seen as an objection

to the explanation of the loss of excitability due to the influence of agents affecting the active component of the resting potential.

A number of experiments were carried out in an attempt to elucidate the possible contribution of sodium ions to spike electrogenesis. Although sodium is the major cation present in the haemolymph of Periplaneta it has been suggested that the muscle membrane is relatively impermeable to sodium ions during activity (Wood, 1963). In chapter 5 it was shown that sodium ions do not contribute to the resting membrane potential of Periplaneta muscle fibres. Substitution of sodium chloride with choline chloride in the bathing saline had little effect on the resting potential over two hours. The contribution of sodium ions to excitation was examined by several different approaches.

Figures 28 and 29 show that variation of $[Na]_o$ has an effect on the amplitude and rate of rise of both the EPSP and the EER at concentrations from 0 to 100 mM. Increasing $[Na]_o$ above 100 mM had little further effect. However when $[Na]_o < 100$ mM there was an approximately linear relationship between $[Na]_o$ and the amplitude of both the EPSP and the EER. Similar results have been obtained with Carausius (Wood, 1957), locust, cockroach (Wood, 1961) and Bombyx (Huddart, 1966b). In all examples a small excitatory depolarization persists when $[Na]_o = 0$, so that the overall effect of variation of $[Na]_o$ does not appear to be very marked. In most excitable cells, of course, the amplitude of the action potential is linearly related to log $[Na]_o$ (e.g. squid axon, Hodgkin & Katz, 1949; frog myelinated nerve, Huxley & Stampfli, 1951; frog muscle, Nastuk & Hodgkin, 1950) where the genesis of the action potential is associated with a very great increase in P_{Na} and the membrane transiently transforms almost completely into a Na^+ electrode. In

cockroach muscle the increase in ionic permeability must be much smaller, relative to P_K. This greater effect of variation of $[Na]_o$ could be explained in two ways:

(i) $[Na]_o$ will determine the size of action potential in the presynaptic nerve terminals, which in turn will modify the release of transmitter and so alter the EPSP. $[Na]_o$ variation would, therefore, have a dual action on excitatory potentials.

(ii) The increase in Na^+ permeability in the muscle membranes is a modest one when compared with K^+ permeability.

As explained above, it is assumed, in the first instance, that this is the true explanation and that the changes in ionic concentrations are not having a marked presynaptic effect.

The results are similar to those obtained with other input components, namely the perfused Pacinian corpuscle (Diamond, Gray & Inman, 1958) and the end-plate of frog muscle (Fatt, 1950; Fatt & Katz, 1952; Nastuk, 1953). Difficulties were encountered with both preparations; the Pacinian corpuscle did not recover well from perfusion with Na^+-free saline and the frog muscle had to be depolarized by applied acetylcholine, since variation in $[Na]_o$ had a marked effect presynaptically. However, both preparations reveal the following features in common with excitatory electrogenesis of cockroach muscle:

(i) The amplitude of the potential recorded is progressively reduced as $[Na]_o$ is reduced.

(ii) The relationship between potential amplitude and $[Na]_o$ is markedly less than a 58 mV change for a ten-fold change in $[Na]_o$.

(iii) A potential persists in low or zero $[Na]_o$; the frog EPP is about 50% of normal when $[Na]_o$ is reduced to 12.5% (Fatt & Katz, 1952).

Thus, the properties of the potentials of excitatory electrogenesis in cockroach muscle have several features in common with those recorded at other input components.

In cockroach (although not in many other insects) the major cation of the haemolymph is Na^+ and the peak depolarization recorded during excitation is a small "undershoot," although small "overshoots" have been occasionally recorded. It is, therefore, possible that during excitation there is a change in gm such that gNa approximates to gK. In frog muscle, the EPP is generated by an increase in conductance to both Na^+ and K^+ (with the ratio Δ gNa /ΔgK constant) but not to Cl^- (Takeuchi & Takeuchi, 1960). A situation such that the ratio gNa / gK approximates to 1 (and the possibility of the membrane producing a graded response) could be achieved in insect muscle in one of two ways:

(i) By a large increase in Na^+ permeability comparable to that seen in a typical, electrically excitable axon. However, if the resting P_K is sufficiently high, or if the increase in P_K triggered by electrical stimulation occurred early enough, the depolarizing regenerative response initiated by a large increase in P_{Na} would be highly damped (Werman et al., 1961; Usherwood, 1969).

(ii) The increase in P_{Na} is small, when compared with that seen in electrically excitable components. On this view the change in cation permeability recorded in insect muscle in response to the excitatory transmitter (and probably in other input components) is both quantitatively and qualitatively different from that of an electrically excitable axon.

It seems probable (but by no means certain) that in situation (i) Li^+ would substitute completely for Na^+, as in the electrically excitable axon (Hodgkin & Katz, 1949; Huxley & Stämpfli, 1951),

whereas in situation (ii), where a limited increase in P_{Na} is postulated, this would not necessarily be so. The hydrated ionic radii are Li^+ = 4.5 Å, Na^+ = 3.4 Å, Ca^{2+} = 4.5 Å (Shanes, 1958). The records of figure 27 show that Li^+ can substitute for Na^+ in excitatory electrogenesis of cockroach muscle, although it is less effective. Usherwood (1969) suggests that Li^+ interferes presynaptically with synaptic transmission, since locust retractor unguis muscle also responds to application of glutamate (but not to neural stimulation) when the bathing saline contains Li^+ instead of Na^+. Again, this situation is parallel in other input components; the EPSP of cat superior cervical ganglion neurones (Pappano & Volle, 1967), the amphibian electroretinogram (Furukawa & Hanawa, 1955; Hamasaki, 1963) and the EPSP of crayfish muscle fibres (Ozeki & Grundfest, 1967) are not produced when Li^+ is substituted for Na^+. The generator potentials of Limulus photosensitive neurones (Obara & Grundfest, 1967, 1968) are reduced in the presence of Li^+, suggesting that the receptor membrane is less permeable to Li^+ than it is to Na^+ (Obara & Grundfest, 1967, 1968). However, the clearest parallels are found in the genesis of the frog EPP, where the substitution of Li^+ for Na^+ produces an EPP which is 85% of normal amplitude and also causes a presynaptic blocking action (Odenara & Yamakawa, 1966). At a variety of input components, therefore, Li^+ is either unable, or only partially able, to replace Na^+.

It is therefore suggested that a modest increase in cation permeability, rather than a damping effect by an increase in P_K, is the cause of the graded non-propagated, excitatory depolarization in cockroach muscle. It may well be that this is the typical permeability change that occurs in the membrane of many electrically-inexcitable input components.

This modest increase in membrane permeability in cockroach muscle is probably rather unselective since under experimental conditions Ca^{2+} can apparently be partially effective in producing a depolarization. Werman et al., (1961) also report that Ba^{2+} and Sr^{2+} can substitute for Na^{+} during the EPSP in the grasshopper Romalea. Two factors will determine the efficacy of any one ion:

(i) Hydrated ionic radius, since the larger ions will probably have a smaller permeability relative to P_K .

(ii) Valency, since divalent ions will have a smaller effect in electrogenesis.

Thus, Usherwood (1969) concludes that Ca^{2+} contributes very little to synaptic current in locust muscle fibres in vivo, since glutamate potentials can still be evoked at synapses when neuro-muscular transmission has been blocked by the removal of this divalent ion. Although there are considerable difficulties in determining values for $[Ca]_i$ and $[Mg]_i$, it is probable that their ionic gradients are low and that these divalent ions have little effect in vivo.

In figure 24 E_m was specified as Vi + I / gm.

This may be expanded thus:-

$$E_m = \left[\frac{I}{gNa + gK + gCl} \right] + \left[\frac{(gCl \cdot VCl) + (gK \cdot VK) + (gNa \cdot VNa)}{gNa + gK + gCl} \right]$$

If it is assumed that at 20°C the appropriate values for E_K, E_{Cl} and E_{Na} are -40 mV, -60 mV and +40 mV respectively, suitable values for conductance of the resting membrane to give a contribution of the active component equivalent to -20 mV and a contribution of the ionic electrode of -40 mV are gCl / gK = 0.2 and gNa / gK = 0.05. These values are only estimates, but are not unreasonable in the light of the evidence presented here and in chapter 5. If one now assumes that during excitation in vivo in cockroach, when there is

an increase in cation permeability, the current is carried almost completely by Na^+ (the major cation in the haemolymph) figure 34 shows the effect of changes in gNa on the contribution of the two components.. The total depolarizing potential would reach the zero line when gNa = 2.0 x gK. If gNa = 1.5 x gK, E_m would be approximately -6 mV. The majority of normal recorded potentials do in fact undershoot by about 5 or 6 mV (table 26) suggesting that the change in gNa does lie at about 1.5 x gK and that it has a depolarizing effect on both components of the resting potential. The schematic illustration in figure 34 is an over-simplification since it assumes that only gNa increases during excitation. Clearly if gK also increases the relative contribution of both VCl and the active component will be reduced.

The effect of variation of $[Na]_o$ on depolarizing electrogenesis in such a situation, where gNa = 1.5 x gK, is shown in figure 35; it is not dissimilar to the experimental plots obtained (figure 28) and by Wood (1963). When $[Na]_o$ is very low a small depolarization will probably still persist on excitation since the I / gm contribution will be reduced (figure 34). This may explain the report cited by Usherwood (1969) of small retractor muscles from moth leg being observed to contract for up to three hours in distilled water. Even in preparations where there is no second, electrogenic component present, inspection of equation 3 shows that a small depolarization could still be expected when $[Na]_o = [Na]_i$ following an increase in gNa consequent upon excitation. Again a parallel is found in the mammalian Pacinian corpuscle (Diamond et al., 1958) and the EPP of frog muscle produced by the application of acetylcholine (Fatt, 1950; Nastuk, 1953) where a small potential persists in Na^+-free solutions.

FIGURE 34. The theoretical effect of changes in gNa on membrane potential of cockroach coxal muscle.

The contribution of the two components of the resting potential are calculated from the equations:

$$\frac{I}{gm} = \frac{I}{gNa + gK + gCl}$$

$$Vi = \frac{(gCl \cdot VCl) + (gK \cdot VK) + (gNa \cdot VNa)}{gNa + gK + gCl} \quad \ldots\ldots\ldots (3)$$

assuming that $gCl / gK = 0.2$ and that $gNa / gK = 0.05$.

The effect of gNa on E_m is also shown as the sum of the two components.

Ordinate: Membrane potential (mV).

Abscissa: Ratio of gNa to gK.

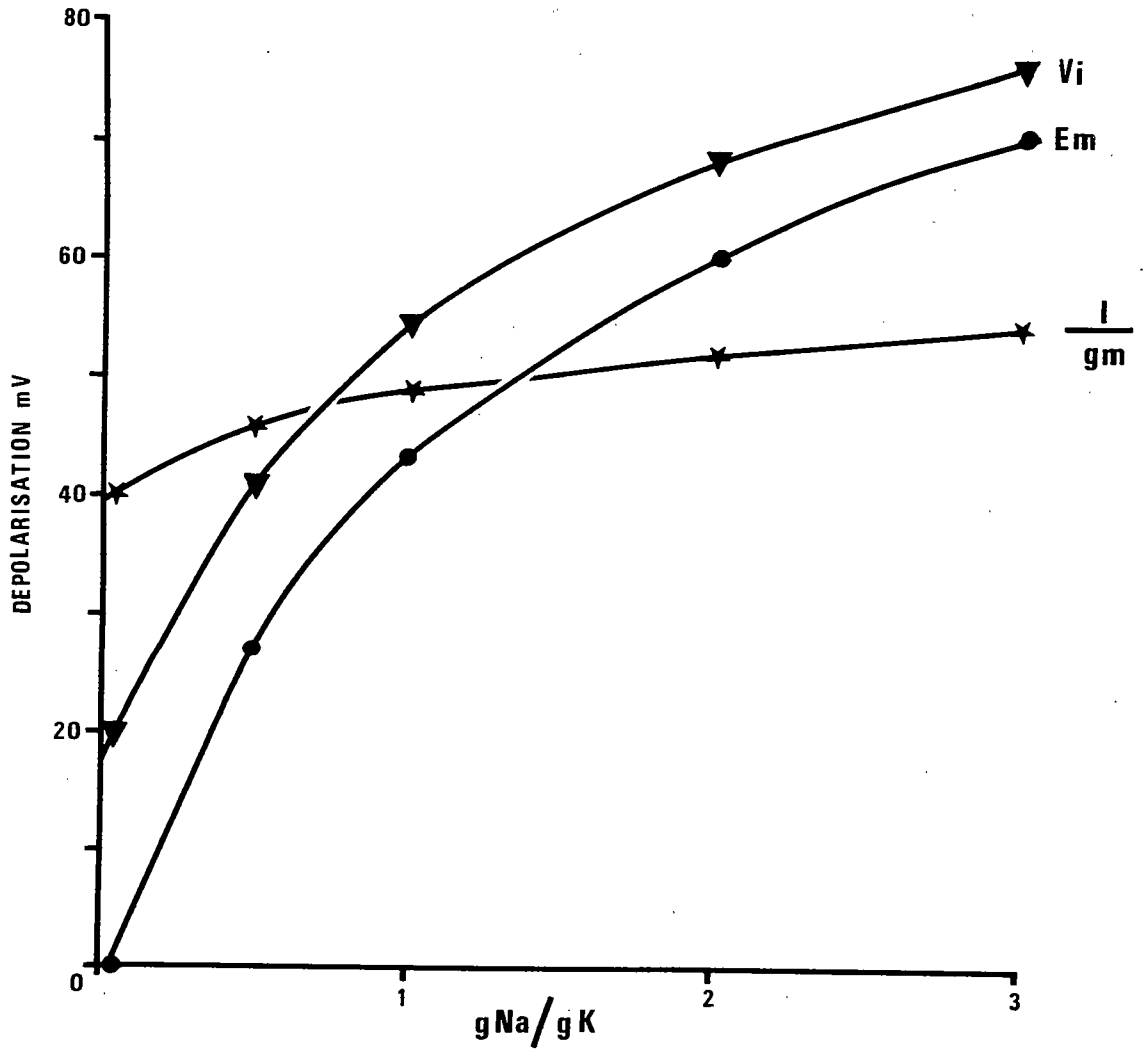

80
60
40
20
0
DEPOLARISATION mV
Vi
Em
I
gm
1
2
3
gNa/gK

FIGURE 35. The theoretical effect of variations in $[Na]_o$ on depolarizing electrogenesis where gNa = 1.5 x gK.

$[Na]_i$ is taken to be 20 mM.

VNa at the peak of depolarization is calculated from the equation:

$$VNa = 58 \times \log \frac{[Na]_o}{[Na]_i}$$

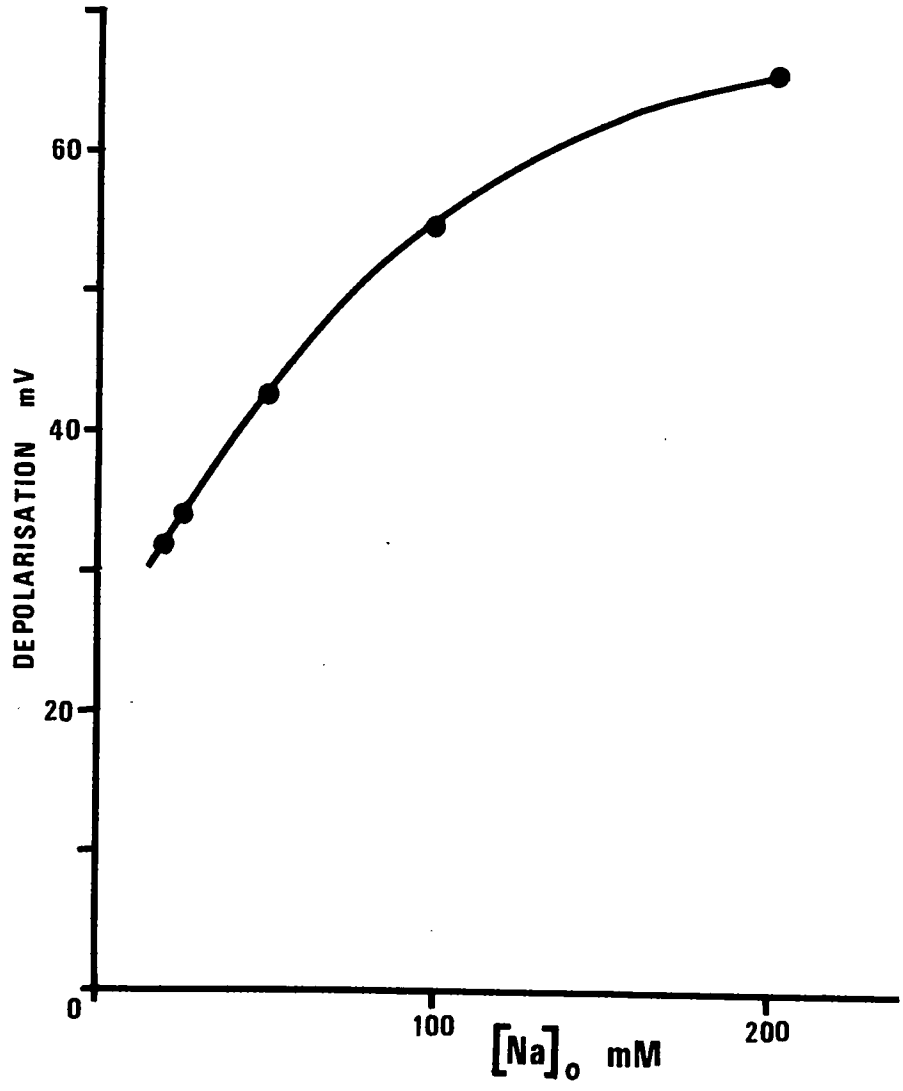
This value has then been used in the equation:

$$E_m = \left[\frac{I}{gNa + gK + gCl} \right] + \left[\frac{(gK \cdot VK) + (gCl \cdot VCl) + (gNa \cdot VNa)}{gNa + gK + gCl} \right]$$

where gCl / gK = 0.2 and gNa / gK = 1.5.

Ordinate: Membrane potential (mV).

Abscissa: External sodium concentration (mM).


DEPOLARISATION mV
60
40
20
0
100
200
[Na]o mM

The nature of excitatory potentials.

The separation of the response to excitatory transmitters into EPSP and EER could be explained as:

a. Being due to two separate phenomena, as in the EPP and action potential of the vertebrate neuromuscular junction in striated muscle.

b. Excitatory electrogenesis is a single phenomenon, but has a complex time course.

The general assumption implicit in the literature on insect muscle is that the EPSP and EER are essentially two different types of potential, by analogy with the situation in vertebrates. There is good evidence in support of such a view, in particular the production of all-or-none, electrically-excited action potentials which propagate without decrement in orthopteran muscle fibres by treating them with Ba^{2+} (Werman et al., 1961). It might be suggested that one potential represents the cessation of the active component and the other the general increase in cation permeability. However, the following evidence suggests that the EPSP and the EER could be two manifestations of the same phenomenon:

(i) The EER is not the equivalent of the action potential of vertebrate muscle; it normally has the properties of an EPSP, being graded and non-propagated.

(ii) The size of the EER is proportional to the size of the EPSP, although the rate of rise of the EER is not proportional to the rate of rise of the EPSP.

(iii) Variation of $[Na]_o$ has a very similar effect on the EER and the EPSP, suggesting that the same cation-permeability change is implicated in the genesis of both potentials.

The following points may be noted in connexion with the above:

a. It has been shown in the previous chapter that the magnitude of the contribution of the active component is reduced as the membrane is depolarized, vanishing when E_m approximates to -40 mV. A similar situation is found with the electrogenic Na^+ pump of Anisodoris neurones (Gorman & Marmor, 1967a & 1967b). This might be explained as an increase in gm with depolarization and hence a progressive reduction in the contribution of I / gm. Possibly the EPSP represents the depolarization associated with the reduction of both components of E_m, whilst the EER (when $E_m <$ approximately-40 mV) is the change in Vi alone, consequent upon the changes of membrane conductance. Experiments by Cerf et al. (1959) described by Usherwood (1969, p.246) on Romalea muscle fibres show that the size of the EPSP is augmented by hyperpolarization whereas, although the EER is at first augmented, it is finally blocked by hyperpolarization.

b. The EPP recorded in vertebrate muscle does not always show a smooth rise, sometimes the rising phase reveals a "step" and this effect is particularly apparent when the preparation is cooled to 2-6°C (del Castillo, 1955; Katz & Miledi, 1965) and is believed to be due to an asynchronism in the liberation of transmitter. The EPP's recorded in diaphragm muscle from rats at birth were also complex and resulted from the summation of 2-4 units. These potentials became simpler as the rat grew older and, at 10 days, potentials similar to the cockroach EPSP and EER were recorded; when 16 days old, the EPP's resembled those recorded in adult rat muscle (Redfern, 1970).

The nature of inhibitory potentials.

Usherwood (1968) has shown that the IPSP in locust and grasshopper muscle is produced by an increase in chloride permeability; the IPSP reverses when the muscle is bathed in chloride-free saline

(Usherwood & Grundfest, 1965). Figure 36 illustrates the probable consequences on E_m when the chloride conductance is increased in the system suggested here for cockroach muscle membranes. The ionic electrode hyperpolarizes by shifting towards E_{Cl}, whilst the contribution of the active component is reduced due to an increase in gm. The net effect (equation 3 and figure 36) is that E_m remains constant. Nor can hyperpolarizing, inhibitory synaptic potentials be readily explained in such a situation as an increase in gK (figure 37) which would have little effect on the ionic electrode, whilst the contribution of the active component would be markedly reduced. The net effect would be a depolarization of E_m. In such a situation, the simplest way of explaining the generation of a hyperpolarizing potential would be the stimulation of the active component under the influence of the inhibitory transmitter.

There is a further anomaly concerning the excitation of insect muscle. If frog muscle is transferred to saline where $[Cl]_o = 0$ and then cooled from 22 to 2°C it depolarizes, excitability rises and spontaneous firing may begin (Erlij & Van der Kloot, 1967). This is not found in insect muscle; cooling to 5°C produces the 20 mV depolarization associated with the cessation of the active component, but excitability does not increase.

Such considerations as these suggest that E_m might not be so clearly compartmentalised but that the "active component" might represent a fraction of E_m that is sensitive to temperature and metabolic inhibitors as a consequence of an unusual system controlling K^+ and Cl^- permeability, as suggested at the end of chapter 5.

FIGURE 36. The theoretical effect of an increase in gCl / gK ratio on the two components of the resting potential as described by equation 3. for cockroach coxal muscle.

The effect of increasing the ratio of gCl / gK on the two components of equation 3, Vi and $\frac{I}{gm}$ is shown.

Ordinate: Membrane potential (mV).

Abscissa: Ratio of gCl to gK.

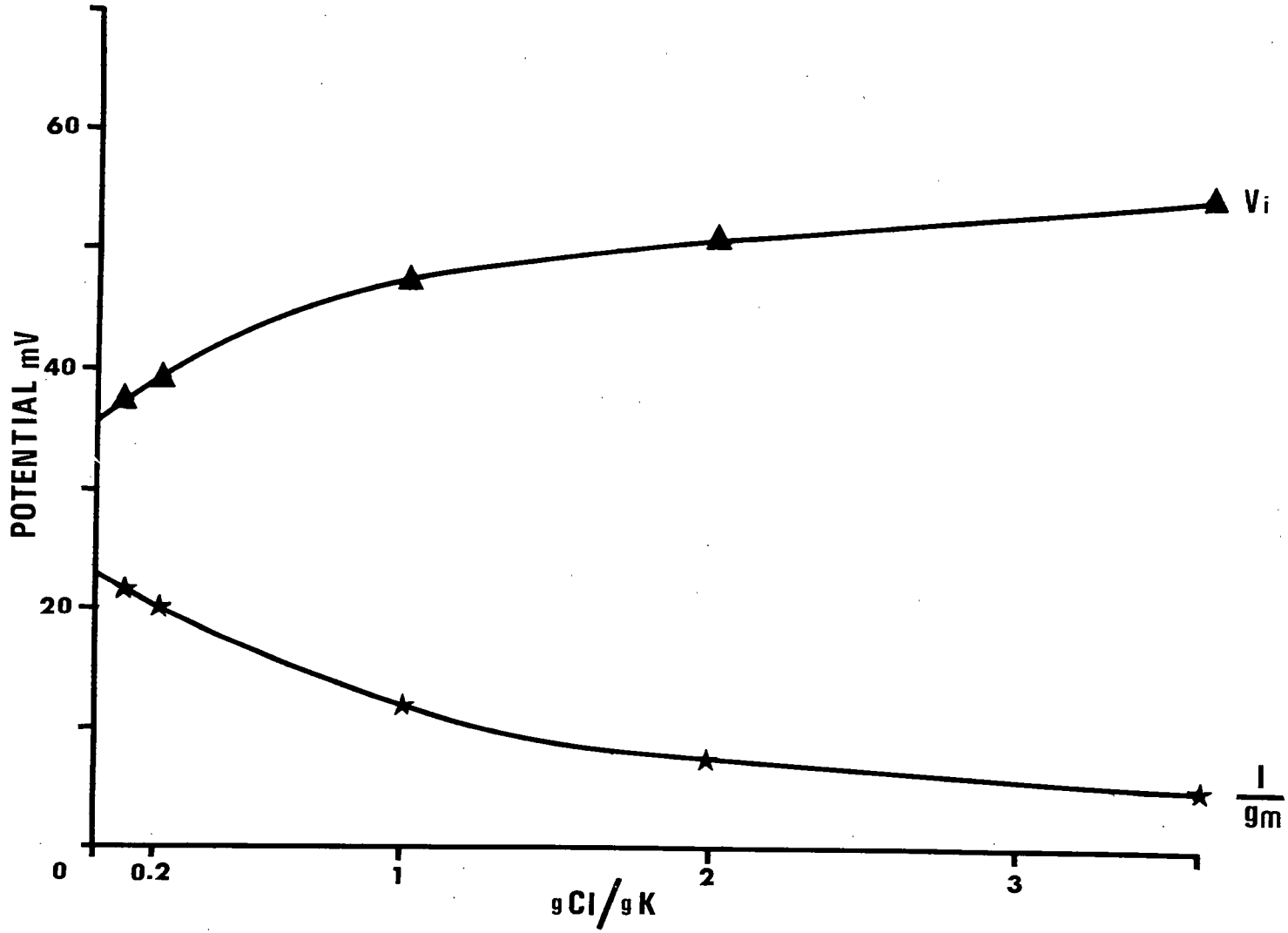
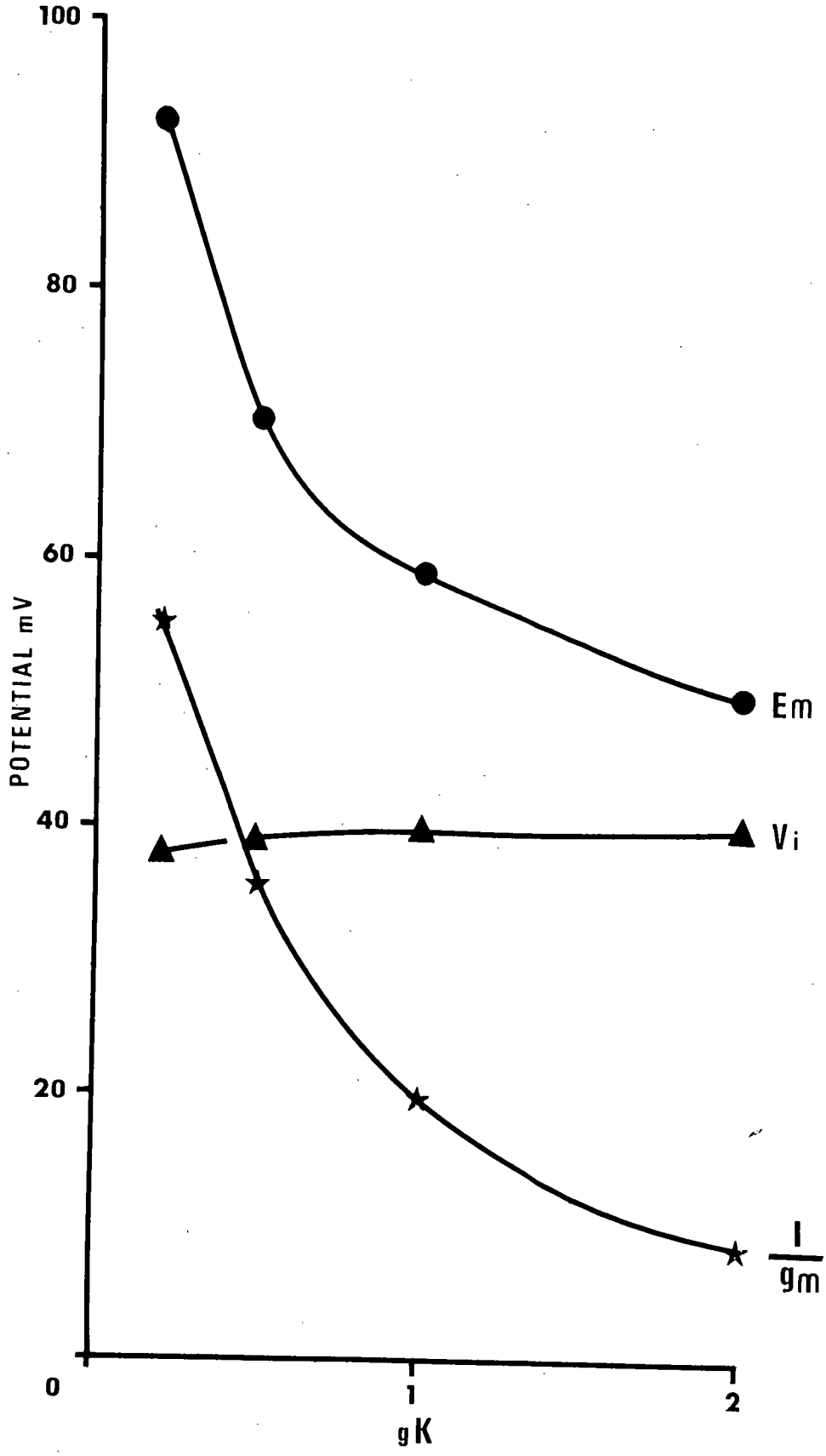

60
40
20
0
POTENTIAL mV
Vi
I/gm
0.2
1
2
3
gCl/gK

FIGURE 37. The theoretical effect of altering gK on the two components of the resting potential as described by equation 3 for cockroach coxal muscle.

The effect of gK on the two components of equation 3, Vi and $\frac{I}{gm}$ is shown.

Ordinate: Membrane potential (mV).

Abscissa: gK


100
80
60
40
20
0
POTENTIAL mV
Em
Vi
1
gm
1
2
gK

CHAPTER 7. A COMPARISON WITH SPHINX LIGUSTRI.

Class : INSECTA

Sub-Class : PTERYGOTA

Super-Order : MECOPTEROIDES

Order : LEPIDOPTERA

Sub-Order : HOMONEURA

Super-Family : SPHINGOIDEA

Family : SPHINGIDAE

Species : SPHINX LIGUSTRI Linnaeus.

INTRODUCTION

A comparative study of the physiology of Sphinx muscle was carried out to determine to what extent the basic features of Periplaneta muscle physiology applied to an insect with allegedly atypical features. Sphinx has been reported to possess a very unusual ionic distribution between its myoplasm and haemolymph (Huddart, 1966a). The potassium concentration in the haemolymph was found to be unusually high and the sodium content unusually low, less in fact than in the myoplasm. Further it has been reported that the resting membrane potential of Sphinx muscle fibres is not related to transmembrane potassium distributions (Huddart, 1966a), and that the transmembrane distribution ratios K_i / K_o and Cl_o / Cl_i are not reciprocal (Huddart, 1967). Huddart has suggested that the resting membrane potential is dependent upon cell metabolism for its maintenance. This is of significance in view of the results already presented for Periplaneta muscle fibres.

Little information is available concerning the ionic basis to spike electrogenesis, although it has been suggested that divalent cations might be involved (Huddart, 1966b). No study has been made of the muscle membrane ATPase activity in Sphinx. In chapter 3 it was shown that Periplaneta muscle membranes did not possess a classical $Na^+ + K^+$ -dependent ATPase characteristic of excitable cell membranes. In view of the reports that ionic distributions across Sphinx muscle were unusual it was of interest to see if this was reflected in different permeability properties of the muscle membranes.

METHODS

1. Membrane Preparation.

It was found impractible to use coxal muscle from Sphinx due to their small size. Instead flight muscle was used for enzyme studies. Due to the limited material available the experiments were designed simply to determine the degree of monovalent cation stimulation of the preparation and the level of Mg^{2+}-dependent ATPase activity. The microsomal preparation was obtained as described for Periplaneta in chapter 3. Four different incubation media were employed, all buffered at pH 7.2 with 50 mM Tris-HCl.

a. 4 mM $MgCl_2$

b. 4 mM $MgCl_2$ + 50 mM NaCl

c. 4 mM $MgCl_2$ + 50 mM KCl

d. 4 mM $MgCl_2$ + 50 mM KCl + 50 mM NaCl

The preparation was incubated at 25°C for 20 minutes with ATP added to give a final concentration of 2 mM. The protein content was assayed as before and the activity expressed as nanomoles Pi liberated / mg protein / minute. The effect of 0.25 mM DNP on the activity of the Mg^{2+}-dependent ATPase activity was also determined.

2. Ionic Distributions.

The muscles used were the coxal muscles of the second thoracic segment. The coxae were exposed by cutting the animal transversely between the second and third segments and pinning the body out for dissection of the coxal muscles. Analysis of the Na^+, K^+ and Mg^{2+} content of the myoplasm was carried out as previously described for Periplaneta. The results were expressed as m moles / kg fibre water. Analyses were carried out on animals 2-3 days and 14 days after emergence from the pupa.

Haemolymph samples were obtained by pinning the whole animal

down with strips of plasticene and making a small incision at the base of the second coxae. A drop of haemolymph was allowed to collect and was taken up in a 10 μl "Drummond Microcap." The sample was analysed for Na^+, K^+ and Mg^{2+} as previously described. The results were expressed as m moles/ litre of haemolymph. Haemolymph samples were taken from animals 2-3 days after emergence from the pupa.

3. Electrophysiology.

An animal was cut transversely on either side of the second thoracic segment, so leaving two legs attached to the severed body section. The body segment was laid out and a plasticene bath constructed around the coxae. The cuticle was stripped from part of one of the coxae and the exposed muscles bathed in saline. The normal saline was constructed on the basis of the ionic analyses of the haemolymph and contained 20 mM K^+; 20 mM Na^+; 16 mM Mg^{2+}; 10mM Ca^{2+}; 10 mM HCO_3^-; 82 mM Cl^-; 100 mM sucrose; made up in deionized water and brought to pH 7.2 by bubbling with carbon dioxide.

Resting membrane potentials were recorded with intracellular glass microelectrodes as previously described for Periplaneta. The effect of alteration of $[Cl^-]_o$, addition of strophanthin G and DNP, and of low temperature, on the maintenance of the resting membrane potential was determined.

RESULTS

1. ATPase activity.

The ATPase activity in the presence of 4 mM Mg^{2+} alone was low. This activity was increased in the presence of DNP (table 30). The addition of either Na^+ or K^+ to the reaction medium did not result in any stimulation of ATPase activity. In fact it was apparent that monovalent cations caused the Mg^{2+}-dependent ATPase activity to be inhibited by about 20% (table 30).

2. Ionic distributions.

The results of the analyses of haemolymph and muscle of Sphinx 2-3 days and 14 days after emergence are given in table 31. Also given are the values found by Huddart (1966a) for this preparation. It can be seen that there is a marked divergence between the results. In the present work the ratio Na_o / Na_i = 21.6 / 3.8 (for 2-3 day old animals). However Huddart describes this as being 3.6 / 20.7. He also found a higher value for K_i and a lower value for K_o than in this work. The standard errors shown for the present values are small. The analyses of muscle ionic content from animals 2-3 days old did not differ significantly from those from 14 day old animals.

3. Electrophysiology.

Resting membrane potentials were measured whilst the muscle fibres were bathed in haemolymph and after they had equilibrated with normal saline for one hour (table 32, experiment A). There was no significant difference between the resting potentials recorded under the two conditions.

When the chloride in the saline was replaced with proprionate the resting potential became depolarized (table 32, experiment B). After 75 minutes in chloride free saline the resting potential had fallen to -41.6 mV from its initial value of -60.3 mV. Upon replacing the

TABLE 30. The ATPase activity of the microsomal preparation of Sphinx flight muscle.

The preparation was incubated at 25°C for 20 minutes in the presence of 2 mM ATP.

Conc. ions in reaction medium (mM)				m moles Pi liberated /
Mg^{2+}	Na^{+}	K^{+}	DNP	mg protein / minute
4	0	0	0	207
4	0	0	0.25	287
4	50	0	0	168
4	0	50	0	160
4	50	50	0	153

TABLE 31. Ionic analyses of haemolymph and myoplasm of Sphinx ligustri.

Values attributed to Huddart are taken from Arch. Internat. Physiol. Biochem. 74, 603-613 (1966).

The haemolymph analyses presented here represent samples taken in March, 1969, and June, 1970. There was no difference between the two groups.

Sample	Age (days)	Na (mM)	K (mM)	Mg (mM)	Author
Haemolymph	2-3	21.6 ± 2.2	22.4 ± 0.9	16.2 ± 0.6	Present
Haemolymph	-	3.6 -	49.8 -	36.0 -	Huddart
Muscle	2-3	3.8 ± 0.4	111.4 ± 2.6	16.5 ± 0.3	Present
Muscle	14	4.7 ± 0.6	109.1 ± 3.3	17.4 ± 0.6	Present
Muscle	-	20.7 -	84.4 -	- -	Huddart

TABLE 32. Factors which depolarize the resting potential of coxal muscle of Sphinx ligustri.

The saline used was buffered with bicarbonate / Co_2 to pH 7.2.

In experiment B the chloride in the saline was replaced with proprionate. Where the chloride was replaced with choline chloride no allowance was made for the alteration in osmolarity of the saline.

Standard errors were calculated from the mean of at least 30 different resting potentials measured.

Expt.	Conditions	Exposure Time (min)	Saline Temp. °C	Resting Potential (mV)	± S.E.
A	Haemolymph	-	20	-59.8	0.9
	Saline	60	20	-60.7	1.1
B	Saline	30	20	-60.3	1.1
	Proprionate	75	20	-41.6	0.9
	Proprionate + 62 mM choline chloride	75	20	-60.3	1.5
	Proprionate	75	20	-42.9	0.8
C	Saline	60	20	-60.7	1.1
	Saline + 5×10^{-4}M strophanthin G	30	20	-58.0	0.8
	Saline + 5×10^{-4}M strophanthin G	60	20	-48.9	0.6
	Saline + 5×10^{-4}M strophanthin G	90	20	-43.1	0.4
	Saline + 5×10^{-4}M strophanthin G	150	20	-41.5	0.5
D	Saline	30	20	-61.6	1.1
	Saline	30	5	-44.7	0.9

chloride by adding 62 mM choline chloride to the saline the resting potential repolarized within 75 minutes.

When 5×10^{-4} M strophanthin G was included in the saline the resting potential became depolarized (table 32, experiment C). After 90 minutes equilibration the resting potential was depolarized by almost one third of its normal value. Longer exposure had only a small additional effect.

Reducing the temperature of the normal saline to 5°C again reduced the resting potential by about one third of the value recorded at room temperature (table 32, experiment D).

DISCUSSION

It was stated in the general introduction that having established a hypothesis for the production and maintenance of bioelectric potentials in cockroach skeletal muscle it was intended that a comparison would be made with muscle of an insect with reportedly unusual features concerning its electrophysiology.

The number of results presented above are not as many as would have been liked. This was partly because the interference of pupal diapause led to a restricted availability of adult moths but also because it was shown that the myoplasm and haemolymph ion concentrations were not as dissimilar from those of the cockroach as was previously believed (see below). However, the experimental results may be compared with the results of similar experiments with Periplaneta muscles.

The specific activity of the Mg^{2+}-dependent ATPase was much lower than of the Periplaneta preparation, although the activity was similarly stimulated by DNP. The inhibitory effect of monovalent cations on the ATPase was unusual. Whereas with studies on the Periplaneta preparation it was concluded that there was little evidence for the existence of a classical $Na^{+} + K^{+}$ activated Mg^{2+}-dependent ATPase, in this preparation there was no suggestion of such an enzyme. However, it must be remembered that although such an enzyme system was not demonstrated in the Periplaneta muscle microsomal preparation, electrophysiological evidence pointed strongly to the conclusion that there was in fact active pumping of ions in the intact muscle. Therefore it may only be concluded that in this Sphinx muscle microsomal preparation no evidence was obtained to indicate the existence of a cation pump.

The ionic analyses shown in table 31 were shown to differ markedly

from other published values for Sphinx skeletal muscle. Haemolymph sodium and magnesium concentrations were higher and potassium concentrations lower than found by Huddart (1966a). The myoplasm ion concentrations are also at variance with the values published by Huddart in that a lower sodium and higher potassium concentration was found in this work. It is very disturbing that the divergences are so great. The analyses given by Huddart bear a closer resemblance to the analyses of Sphinx pupal haemolymph by Duchateau et al. (1953) and Drilhon (1934), shown in table 7, than it does with the present results.

It is very difficult to suggest a reason for these divergences. In the present study the results were carefully scrutinized for possible errors. Haemolymph samples from animals emerging in March, 1969, and June, 1970, gave identical results. Myoplasm analyses of animals which were 2-3 days old and 14 days old gave the same results. Hence time of emergence, or ageing, cannot explain the differences between the two sets of results. The animals used in this study were not fed at all as adults. The caterpillars were fed exclusively on green privet leaves. It is not possible to explain the disagreement with the results of other workers, although the results presented above are considered to represent real values for the experimental animals used. These results represent a disappointment since they obviate the major reason for including Sphinx muscle in this study. It was reported that the ionic distribution of sodium between haemolymph and muscle was the reverse of the situation in Periplaneta and that the potassium ratio was much lower. In fact this analysis has shown that the ratio Na_o / Na_i = 5.4 and the ratio K_i / K_o = 6.9. An essentially similar situation occurs in Periplaneta where Na_o / Na_i = 6.9 and K_i / K_o = 5.6. Hence the intention of

comparing an insect with unusual muscle permeability characteristics to Periplaneta is lost.

Evidence that the haemolymph ionic analyses were correct was given by the finding that the resting membrane potential recorded whilst the muscles were bathed in haemolymph was the same as when they had equilibrated with the normal saline, which was constructed upon the findings of these analyses. The fact that the resting membrane potential recorded at 5°C was reduced by one third of its value at room temperature suggests that there is a metabolic component to this potential. This was the conclusion arrived at by Huddart and Wood (1966), who found that DNP caused a reduction (depolarization) of the resting membrane potential of Sphinx skeletal muscle fibres. Hence these muscles have similar properties to Periplaneta muscles. Significantly chloride is implicated in the active component of Sphinx muscle since the omission of chloride from the saline resulted in a depolarization of about one third of the resting potential. Strophanthin G also caused a depolarization, as in Periplaneta muscles, similar in extent to the effect of low temperature and chloride free saline. Under all these depolarizing treatments it was significant that the resting potential closely approached the equilibrium potential for potassium of -41.1 mV, calculated from the normal potassium distribution. Hence the resting membrane potential of Sphinx muscle also appears to be composed of two components, one actively maintained and contributing about -20 mV, and the other passively produced and contributing about -40 mV.

From the literature it would not be expected that the permeability characteristics of skeletal muscle from Periplaneta and Sphinx would be similar. It was nevertheless shown that in fact they are similar in respect of their lack of a membrane $Na^+ + K^+$ activated Mg^{2+}-depen-

dent ATPase, their distribution ratios of inorganic ions across the muscle membranes, and in their electrophysiological properties.

It is concluded that the skeletal muscle membranes of Periplaneta and Sphinx have very similar properties and that it would be valid to postulate a single model for their control of permeability.

CHAPTER 8. DISCUSSION AND CONCLUSIONS.

The aim of this study was to make a useful contribution to the understanding of the production and maintenance of bioelectric potentials in insect muscle cells. To do this, three aspects of the permeability properties of skeletal muscle membranes from Periplaneta americana were studied, namely, possible biochemical control mechanisms, ionic distributions, and electrophysiological characteristics. Having erected a hypothesis for the production and maintenance of bioelectric potentials in Periplaneta muscle it was intended to make a detailed comparison with data obtained from Sphinx ligustri skeletal muscle, where inorganic ion distributions were believed to be very different from the situation found in Periplaneta muscle (Huddart, 1966a & 1967). However, since it was found in this study that the inorganic ionic distribution ratios are not so very different in the two animals it is perhaps not surprising that the permeability properties of the muscles from the two insects appear to be very similar.

The results of the studies on the microsomal preparations of muscle from both Periplaneta and Sphinx did not indicate the presence of synergistic stimulation by monovalent cations of ATPase activity indicative of the cation pump found in many excitable cells (Skou, 1965). There was, however, a component of the ATPase activity, stimulated by the presence of magnesium ions, similar to the enzyme thought to be responsible for the control of passive permeability of cell membranes (Duncan, 1967). It was not possible to alter the activity of the preparation in any way to demonstrate synergistic stimulation by sodium and potassium ions i.e. to "uncover" a pump. The effect of including deoxycholate in the extraction medium was inhibitory, and strophanthin G always had little inhibitory effect. Samaha and Gergely (1965) were able to demonstrate synergistic

stimulation of human striated muscle microsomes by sodium and potassium, an activity which was inhibited by strophanthin G. However to do this required the presence of deoxycholate in the extraction medium, sodium azide treatment and even ageing of the preparation. As a result of the need for such drastic extraction techniques they were unable to decide whether their preparation contained two types of ATPase or a single ATPase whose activity was moderated by a variety of factors.

The presence of a monovalent cation stimulated ATPase activity of the kind typically found in nerve cell microsomal preparations (Jarnfelt, 1960; Schwartz et al., 1962; Skou, 1962; Abood & Gerard, 1964; Bonting et al., 1964) and cardiac muscle microsomal preparations (Schwartz, 1962; Lee & Yu, 1963) is not typically found in similar preparations of skeletal muscle cells. Duggan (1965) was unable to demonstrate such a system in frog muscle microsomes. Similarly such a system was not found in rat skeletal muscle (M.A. Radcliffe, personal communication). Hence it appears that the properties of the microsomal preparation studied here are probably not atypical when compared with vertebrate skeletal muscle microsomal preparations.

However the significance of such studies is in considerable doubt. Wood (1963) suggested that there was no active extrusion mechanism for sodium ions in Periplaneta muscle cells. Hence as a result of the present studies on the muscle microsomal preparation of Periplaneta it would have appeared reasonable to conclude that this statement was supported (Wareham et al., 1968). However, the conclusion drawn from studies on vertebrate skeletal muscle microsomal preparations would be that there too no sodium extrusion mechanism existed. The evidence from studies of sodium permeability suggests strongly that there is an active extrusion of sodium ions

from vertebrate muscle cells (Keynes & Maisel, 1954; Conway et al., 1961; Dee & Kernan, 1963), despite the fact that the biochemical nature of this extrusion cannot be demonstrated with certainty. In the present work it has been shown that strophanthin G, which typically inhibits the cation stimulated ATPase of excitable cells, increased the intracellular sodium concentration when applied to whole muscles whilst having no effect on the microsomal ATPase activity. Further, evidence has been presented showing that sodium ions are clearly involved in the electrical responses to excitation. If sodium ions do enter the muscle fibres during excitation then a sodium extrusion mechanism must be present. Hence it appears that Periplaneta muscle may be compared to vertebrate muscle in respect of its apparent lack of membrane enzyme activity responsible for cation movements, whilst presenting obvious requirements for such a sodium extrusion mechanism.

The above leads to two possible conclusions. Either the extraction procedure and subsequent treatment of the microsomal muscle preparation is inadequate, resulting in the loss or alteration of some or all of the enzyme activity, or the sodium extrusion mechanism of skeletal muscle is different from that of nerve cells. Studies with both vertebrate and invertebrate muscles (Bowler & Duncan, unpublished) have suggested that the presence of an ATPase synergistically activated by Na^+ and K^+ may not be as universally demonstrable as was believed in muscle microsomal preparations.

The resting potential of Periplaneta was shown to have complex origins. The resting potential of excitable cells is the result of unequal distributions of permeable ions across the cell membrane. The sign and size of the potential is determined by the relative permeabilities of these ions in association with the activity of ionic pumps where present. In purely passive systems the potential developed

is found to be equal to the equilibrium potential of the permeable ions, usually K^+ or Cl^- (whichever is the greater). The resting potential of Periplaneta skeletal muscle could not be described from the distribution of K^+ or Cl^- across the cell membranes. This suggested that some form of actively maintained potential was present in the system. This has been termed a constant current generator in the present work and serves to maintain the resting potential about 20 mV negative to the equilibrium potential for K^+ under normal conditions. The presence of an electrogenic contribution to the resting potential of excitable cells has been postulated for other systems, such as frog muscle (Cross et al., 1965; Adrian & Slayman, 1966; Corrie & Bonting, 1966), crab muscle (Bittar, 1966), guinea-pig's taenia-coli (Casteels, 1969), lobster axon (Senft, 1967), snail neurones (Moreton, 1969) and Aplysia neurones (Carpenter & Alving, 1968; Carpenter, 1970).

That there was an electrogenic contribution to the resting potential of cockroach skeletal muscle was demonstrated in many ways. A depolarization of about 20 mV resulted from treatment with metabolic inhibitors, strophanthin G and by cooling the preparation. However, although all these agents were thought to be affecting the same energy-dependent process, the exact nature of the constant current has not been determined. It has not been possible to decide whether this component of the resting potential is the result of a unidirectional movement of one species of ion or the result of the unequal movements of several ions, or indeed, whether the resting potential represents a single complex system controlling passive ion permeabilities and where metabolic energy is required for changes in permeability to occur. The evidence for or against any of these possibilities have been discussed in detail in chapter 5, but certain

facts are worthy of emphasis.

The active component appeared to be dependent on the presence of extracellular Cl^- since the treatments which inhibited the constant current generator in the presence of Cl_o^- had no effect in the absence of Cl_o^- . If an electrogenic Cl^- pump were present it would necessarily have to move Cl^- into the cells to have the required hyperpolarizing effect on E_m . In such a situation it is conceivable that the hyperpolarizing potential (IPSP) observed by Usherwood in the locust (1968) could be the result of increased pump activity under the influence of the inhibitory transmitter. Active chloride transport has been established in a variety of different preparations (Krough, 1937; Diamond, 1962; Green, 1965; House & Green, 1965; Brodsky & Schilb, 1966). However the apparent dependence of the resting potential of Periplaneta muscle on the presence of extracellular HCO_3^- suggests a more complicated situation. Several Cl^- transporting systems are dependent upon $[HCO_3^-]_o$, such as frog gastric mucosa (Imamura, 1970) and turtle bladder (Gonzalez, 1969), where Cl^- transport is also inhibited by acetazolamide, despite the absence of carbonic anhydrase activity. The isolated turtle bladder actively transports Na^+, Cl^- and HCO_3^- from the mucosal to the serosal bathing fluid (Gonzalez, 1969; Gonzalez & Schilb, 1969; Gonzalez et al., 1969). It is considered possible that the basis to the active component of the resting potential of Periplaneta skeletal muscle is a similar complex, interacting system, resulting in a net outward flow of ionic current.

The part of the resting potential remaining in the absence of the constant current generator is thought to represent the passive distribution of the permeable ions. Clearly the characteristics of this component will be related to the activity of the active component.

An attempt to describe the permeability properties of the muscle membrane has been made in the formulation of the circuit diagram in figure 24. The behaviour of the membrane may be forecast from equation 3. This equation has been discussed in chapters 5 and 6. The behaviour of the membrane at rest is thought to result in the net inward movement of Cl^-. The evidence obtained from Sphinx muscle suggests that a similar situation pertains there. There was a temperature-sensitive component to the resting potential which showed a dependence on the presence of extracellular Cl^-. Significantly, the resting potential only equalled E_K when the active component was inhibited.

In the situation described by equation 3, hyperpolarizing IPSPs can only occur from an increase in the contribution of the constant current generator since simple changes in membrane permeability would have no real effect on E_m (see figure 36). Although equation 3 explains the apparent insensitivity of E_m to changes in $[K^+]_o$ and the fact that E_m is considerably greater than E_K it does not readily explain the markedly non-linear graph when E_m is plotted against log $[K^+]_o$ in the absence of the constant current generator. This atypical behaviour of E_m was discussed in chapter 5 and the properties of the ionic electrode have been summarised in figure 23.

In order to determine the nature of permeability changes the electrogenesis of Periplaneta skeletal muscle was studied. Apart from a small contribution by Ca^{2+} and Mg^{2+} to depolarizing electrogenesis under experimental conditions it was shown that a part of the depolarizing current was carried by Na^+. This was demonstrated in a variety of ways including the increase in sodium flux in stimulated muscles and by the effect of $[Na^+]_o$ on the absolute size and rate of rise of the depolarization. In the absence of the constant current generator the

muscles became inexcitable, suggesting that either this component of the resting potential is involved in the production of the EPSP (e.g. the excitatory transmitter could switch off the pump), or that the activity of the component is necessary to allow changes in membrane conductances to occur.

The EPSP of cockroach muscle shows several features in common with other input components such as the Pacinian corpuscle (Diamond et al., 1958) and the frog end-plate (Fatt, 1950; Fatt & Katz, 1952; Nastuk, 1953) in that:

(i) the amplitude of the potential recorded is progressively reduced as $[Na^+]_o$ is reduced,

(ii) the relationship between potential amplitude and $[Na^+]_o$ is markedly less than a 58 mV change for a 10-fold change in $[Na^+]_o$ and

(iii) a potential persists in low $[Na^+]_o$.

It was concluded that the increase in P_{Na} at peak depolarization in Periplaneta muscle is small when compared with electrically excitable components, a conclusion previously arrived at by Wood (1963). An attempt was made to reproduce the experimental results of the effect of $[Na^+]_o$ on electrogenesis by using equation 3. It was concluded that an increase in gNa such that gNa = 1.5 x gK at peak depolarization was sufficient to account for the observed results. This limited increase in gNa could explain the typical lack of positive overshoot in Periplaneta muscle.

By analogy with the situation in vertebrates there is an assumption in the literature that the EPSP and EER of insect muscle are essentially two different types of potential. However it has already been pointed out that, although the EPSP is analagous to the vertebrate EPP, the EER is not analogous to the action potential of

vertebrates since it is graded and non-propagated. Since the size of the EER is proportional to the size of the EPSP and since variation in $[Na^+]_o$ had a similar effect on both the EPSP and the EER the same cation permeability change is implicated in both potentials. The possibility that the EPSP and EER may represent an end-plate potential with a complex time-course is suggested by two pieces of evidence obtained from vertebrate studies. The EPP's of rat diaphragm muscle at 10 days after birth show a biphasic rise similar to that seen in insects (Redfern, 1970). The EPP of vertebrate muscle at 2-6°C often shows a "step" in the rising phase, thought to be due to an asynchronous release of acetylcholine (del Castillo, 1955; Katz & Miledi, 1965). Although there is insufficient evidence to reach a definite conclusion it is suggested that the biphasic release, or effect, of neuromuscular transmitter could produce the EPSP and EER typically seen in insect muscle, and that the permeability changes occurring are the same in the two phases of the potential. It is concluded that electrogenesis in Periplaneta muscle bears a considerable resemblance to the EPP of vertebrate muscle.

The fact that electrogenesis of Periplaneta muscle is similar to that of the vertebrate EPP is not necessarily surprising, since Na^+ is the most favourably distributed cation to carry a depolarizing current in both cases. A similar situation may well exist in Sphinx where E_{Na} could theoretically reach +37.8 mV (calculated from table 31). On the other hand the potassium concentration of insect haemolymph fluctuates considerably whereas the blood potassium of vertebrates does not and it is not surprising that mechanisms for the maintenance of the resting potential in insect muscle is so very different from that of vertebrates. It is interesting to note that $[Na^+]$ measured in haemolymph from Periplaneta kept under a variety of conditions

rarely falls below 100 mM (100 mM Na^+_o gives the maximal depolarizing response). Pichon (1970) found that $[Na^+]_o$ lay between 102 mM and 180 mM when animals were kept on diets of tap water, lettuce leaves or milk powder. Under the same conditions $[K^+]_o$ varied between 9.1 mM and 37.4 mM. Hence, whilst electrogenesis based on an increase in gNa could work at maximum efficiency, the resting potential based on passive permeability to K^+ would be unacceptably variable under these conditions. Therefore the significance of the apparently complex basis to the resting membrane potential of insect muscle may well be due to their inability to maintain their haemolymph inorganic ion balances constant.

SUMMARY

1. Coxal muscles of the cockroach Periplaneta americana were studied in a variety of ways in an attempt to understand the basis to the production and maintenance of bioelectric potentials in insects.

2. A biochemical study was carried out on muscle microsomes prepared by differential centrifugation of homogenates of Periplaneta coxal muscles.

3. A considerable percentage of the total adenosine triphosphatase activity was Mg^{2+}-dependent. This activity was stimulated by DNP. The properties of this enzyme appeared to be comparable to the Mg^{2+}-dependent ATPase found in other excitable cell preparations.

4. Addition of monovalent cations did not produce any synergistic activation of the Mg^{2+}-dependent ATPase activity. It was not possible to demonstrate any $Na^{+}K^{+}$ ATPase activity, even by treatment with DOCA, characteristic of excitable cell membrane preparations.

5. Although no enzyme responsible for Na^{+} transport could be isolated from this preparation it was pointed out that similar isolation techniques have been unsuccessful in demonstrating such an enzyme in some other excitable tissues, despite strong evidence indicating the presence of active Na^{+} transport in the intact cells.

6. Na^{+}, K^{+} and Mg^{2+} concentrations in haemolymph and myoplasm were determined. The values compared well with other published figures.

7. Muscle extracellular space was determined using tritiated inulin and found to account for less than 4% of the total muscle volume. The time taken for complete diffusion of inulin into the muscles indicated that a fairly rapid equilibration with saline could be expected.

8. A new saline containing 16 mM HCO_3^- was developed for bathing Periplaneta muscles. In this saline muscles remained excitable and the resting potential was maintained for several hours. It was demonstrated that some salines described in the literature were unsuitable for bathing muscles over long periods of time.

9. Exposure of muscles in vivo to strophanthin G caused an increase in $[Na]_i$. DNP also caused an increase in $[Na]_i$ but also caused a reduction of $[K]_i$.

10. Exposure of muscles in vivo to low temperature did not alter myoplasm ion concentrations. However, exposure of whole animals to high temperatures caused an apparent exchange of Na^+ and K^+ between myoplasm and haemolymph, although it was considered that the major exchanges were taking place in some other tissue than muscle.

11. Repeated electrical stimulation of muscles in vivo raised the rate of ^{22}Na influx.

12. The resting potential, measured when muscles had equilibrated with normal saline and when in haemolymph, were the same.

13. $[Mg]_o$ and $[Ca]_o$ did not contribute to the resting potential.

Similarly $[Na]_o$ did not appear to affect the resting potential, although the results were complicated by the apparent permeability of the muscles to choline chloride during prolonged exposures to Na-free saline.

14. Recorded E_m was c 15-20 mV hyperpolarized with respect to E_K. The effect of $[K]_o$ was considerably less than predicted by the Nernst equation.

15. In Cl-free saline there was a maintained depolarization of c 15-20 mV. Metabolic inhibitors and low temperature resulted in a similar depolarization in normal saline.

16. Alteration of $[K]_o$ at 5°C, and in the presence of cyanide, produced a biphasic plot of E_m vs $[K]_o$ with an apparent insensitivity of E_m to $[K]_o$ less than 50 mM.

17. It was concluded that the resting potential was maintained by the relative distribution of K^+ and by the action of a metabolically dependent ion pump operating to hyperpolarize the membrane. This constant current generator required the presence of extracellular chloride and bicarbonate ions to maintain E_m negative to E_K.

18. An electrical model was proposed to account for the results obtained.

19. The parameters of the EPSP and EER were the same measured when the muscles had equilibrated with normal saline and when in haemolymph.

20. Divalent cations Ca^{2+} and Mg^{2+} were found to make no contribution to depolarizing electrogenesis at physiological levels.

21. The major cation involved in depolarizing electrogenesis was sodium. $[Na]_o$ affected both the size and rate of rise of the EPSP and the EER. Lithium ions were not able, however, to substitute *in toto* for Na^+.

22. The excitability of the muscle membrane was apparently dependent upon the activity of the constant current generator since at low temperatures, or in the presence of metabolic inhibitors, the muscles were rendered inexcitable.

23. The results were discussed in relation to the model developed to describe the resting potential. A comparison was drawn with the properties of the vertebrate end-plate potential.

24. A comparison was made with muscle from *Sphinx ligustri*. Flight muscle microsomes contained no Na^+K^+ ATPase activity. Unexpectedly, the trans-membrane ionic ratios of *Sphinx* coxal muscle were similar to those found using cockroach coxal muscle. The resting potential of *Sphinx* coxal muscle had similar properties to that of *Periplaneta*.

25. It was concluded that whilst depolarizing electrogenesis in insect skeletal muscle could be compared with the end-plate potential in vertebrate skeletal muscle, the resting potential has different properties concomittant with existence in an environment subject to fluctuating potassium concentrations.

ABBREVIATIONS

ADP	adenosine diphosphate
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
[ ]	concentration
c.p.m.	counts per minute
CTP	creatine triphosphate
DNP	2·4 dinitrophenol
DOCA	deoxycholate
E_{Cl}	chloride equilibrium potential
EDTA	ethylenediaminetetra-acetic acid
EER	electrically excitable response
E_K	potassium equilibrium potential
E_m	membrane potential
EMF	electromotive force
E_{Na}	sodium equilibrium potential
EPP	end-plate potential
EPSP	excitatory postsynaptic potential
F	"Faraday"
gCl	chloride conductance
gK	potassium conductance
gL	leak conductance
gm	membrane conductance
gNa	sodium conductance
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid
I	current
IPSP	inhibitory postsynaptic potential
ITP	inosine triphosphate
n	valency

nm	nanometres
P_{Cl}	chloride permeability
P_K	potassium permeability
P_{Na}	sodium permeability
R	gas constant
Rel	electrode resistance
T	absolute temperature
TCA	trichloroacetic acid
Tris	Tris(hydroxymethyl) methylamine
UTP	uridine triphosphate
Vi	sum of ionic batteries

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