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A CRITICAL STUDY OF THE EVIDENCE FOR PERIPHERAL INHIBITORY AXONS IN INSECTS

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INTRODUCTION

Usherwood & Grundfest (1964, 1965) and Ikeda & Boettiger (1965*a*, *b*) have proposed that some insect muscles are controlled in part by peripheral inhibitory axons. For example, some fibres of the metathoracic extensor tibiae muscle of locusts and grasshoppers are innervated by an inhibitory axon, and the excitatory electrical responses and mechanical responses of these fibres are attenuated during stimulation of this axon (Usherwood & Grundfest, 1964, 1965). The extensor tibiae muscle is innervated by two excitatory axons, designated 'fast' and 'slow' (Hoyle, 1955*a*; Cerf, Grundfest, Hoyle & McCann, 1959). Only fibres innervated by the 'slow' excitatory axon receive endings from the inhibitory axon. Peripheral inhibition of the 'fast' response is therefore minimal and the main effect of inhibitory activity is on the 'slow' responses of the extensor tibiae muscle (Usherwood & Grundfest, 1965). The electrical response of the extensor tibiae muscle fibre to stimulation of the inhibitory axon consists of an inhibitory postsynaptic potential (IPSP), which is usually hyperpolarizing. Hyperpolarizing IPSPs have been recorded from other muscle fibres of locusts and grasshoppers (Usherwood & Grundfest, 1965), from cockroach muscle fibres (Usherwood & Grundfest, 1965; Guthrie, personal communication), from bee muscle fibres (Ikeda & Boettiger, 1965*a*) and from beetle muscle fibres (Ikeda & Boettiger, 1965*b*). In orthopteran muscles the IPSP results from a transient increase in the chloride permeability of the muscle fibre, or more precisely the inhibitory postsynaptic component of the muscle fibre membrane (Usherwood & Grundfest, 1965). Therefore during the IPSP the membrane potential of the muscle fibre moves toward the chloride equilibrium potential (E_{Cl}) which is also the inhibitory equilibrium potential (E_{IPSP}). γ-Aminobutyric acid (GABA) mimics the transmitter at orthopteran inhibitory neuromuscular synapses while picrotoxin blocks activation of the inhibitory postsynaptic membrane both by GABA and the inhibitory transmitter (Usherwood & Grundfest, 1964, 1965).

On the basis of these observations the occurrence of peripheral inhibition in insects seemed firmly established although the functional significance of the inhibitory axons was not established. Nevertheless two recent publications by Hoyle (1966*a*, *b*) in which the properties of the metathoracic anterior coxal adductor (a.c.a.) muscle in locusts and grasshoppers are described, are somewhat critical of this conclusion. The a.c.a. muscle is innervated by two axons, one of which is clearly excitatory. The properties of the other axon are apparently less clearly defined since it sometimes enhances (excites) and at other times attenuates (inhibits) the contractions of the a.c.a. muscle.

In view of its apparent diverse properties Hoyle (1966*a*, *b*) has called this axon inhibitory-conditioning (I-C) rather than inhibitory. The electrical responses recorded intracellularly from fibres of the a.c.a. muscle during I-C stimulation ranged from large depolarizing postsynaptic potentials to large hyperpolarizing postsynaptic potentials. In some muscles the I-C responses were exclusively hyperpolarizing and inhibitory while in other preparations, where depolarizing I-C responses predominated, a slight enhancement of the contractions of the a.c.a. muscle in response to stimulation of the excitatory axon was recorded during I-C stimulation. In some preparations the electrical responses to I-C stimulation changed during the course of the experiment from hyperpolarizing to depolarizing potentials. Hoyle (1955*b*) has previously used the term 'conditioning' to describe one of the axons which innervates the locust metathoracic extensor tibiae muscle. Although this axon is clearly identical with the inhibitory axon described by Usherwood & Grundfest (1964, 1965), Hoyle (1955*b*) failed to demonstrate its inhibitory properties. Nevertheless he did record hyperpolarizing postsynaptic potentials from fibres of the extensor tibiae muscle during stimulation of his 'conditioning' axon although these did not apparently attenuate the depolarizing excitatory postsynaptic potentials (EPSPs). In fact a slight enhancement of the 'fast' and 'slow' contractions of the extensor tibiae muscle was observed during stimulation of the 'conditioning' axon. Hoyle (1966*b*) suggested that this axon might be an evolutionary relic which has lost its primeval function of peripheral inhibition. However, it is difficult to understand how stimulation of this axon could have much effect on the 'fast' response of the extensor tibiae muscle since it does not innervate most of the fibres which receive endings from the 'fast' excitatory axon (Usherwood & Grundfest, 1965). In fact the evidence favouring an inhibitory function for *this* axon is so overwhelming that there seems no reason to continue using the term 'conditioning'. However, the suggestion that the a.c.a. muscle in locusts and grasshoppers is innervated by an 'inhibitory-conditioning' axon rather than an inhibitory axon cannot be rejected quite so easily, and although much of the evidence for this is inconclusive the suggestion does warrant further investigation. In view of this the properties of the metathoracic a.c.a. and extensor tibiae nerve-muscle preparations have been re-examined in locusts and grasshoppers. The results of these studies indicate conclusively that the a.c.a. muscle and extensor tibiae muscle are both innervated by inhibitory axons of the type described by Usherwood & Grundfest (1964, 1965). It is possible that all the diverse responses obtained by Hoyle (1966*a*, *b*) from his a.c.a. nerve-muscle preparations can be accounted for partly by variations in the haemolymph–potassium levels in locusts and grasshoppers (Hoyle, 1953) and partly by an increase in the chloride content of the a.c.a. muscle fibres as a result of KCl leakage from muscles removed during dissection of the a.c.a. muscle.

MATERIAL AND METHODS

In situ and isolated preparations of the metathoracic a.c.a. nerve-muscle (Hoyle, 1966*a*, *b*) and *in situ* preparations of the metathoracic extensor tibiae nerve-muscle (Usherwood & Grundfest, 1965) were examined in the locusts *Schistocerca gregaria* and *Locusta migratoria* and in the grasshopper *Romalea microptera*. Intracellular recordings from a.c.a. and extensor tibiae muscle fibres were made with glass micro-

electrodes filled with either 3 M potassium chloride or potassium propionate. The recording and display equipment was standard (e.g. Katz, Webb & Sorem, 1964). Mechanical responses of the muscles were obtained using a Grass Strain Gauge (Usherwood & Grundfest, 1965).

Most preparations were bathed for periods in locust saline (10 K saline) containing 10 mM-KCl, 140 mM-NaCl, 2 mM-$CaCl_2$, 6 mM-NaH_2PO_4, 4 mM-$NaHCO_3$. This medium is similar to Hoyle's (1953) locust saline except that $MgCl_2$ was omitted. In some experiments the potassium content of the saline was increased. Salines containing an increased potassium concentration were prepared in one of two different ways: (1) potassium was substituted for sodium whilst maintaining the chloride concentration constant (154 m-equiv./l.); this type of saline will be referred to as high-K saline; (2) potassium was substituted for sodium whilst maintaining the product of the potassium and chloride concentration constant, i.e. an increase in the potassium concentration was compensated by a decrease in the chloride concentration. The impermeant anion propionate was substituted for chloride. This type of saline will be referred to as high-K (constant product) saline. All the salines were effectively isotonic and buffered at pH 6·8. The preparations were perfused continuously throughout the experiments with a constant stream of saline.

RESULTS

The main object of these studies was to determine whether the diverse inhibitory-conditioning (hereafter called inhibitory) responses obtained by Hoyle (1966*a*, *b*) from his preparations of the a.c.a. nerve-muscle of locusts and grasshoppers represent the normal responses of these muscles. As a starting-point the possibility was examined that the actual preparation of *in situ* and isolated a.c.a. muscles alters the responsiveness of these muscles to the inhibitory axon compared with the *in vivo* muscle. For example, significant changes in the ionic environment of the a.c.a. muscle could occur when the normal bathing medium of the muscle, haemolymph, is replaced by locust saline, and these changes might have implications for the response of the a.c.a. muscle fibre to inhibitory stimulation. Exposure and isolation of the a.c.a. muscle and associated nerves might also modify the inhibitory responses of the a.c.a. muscle fibres. To test these possibilities about twenty *in situ* a.c.a. muscles with their associated nerves were prepared and the inhibitory responses of the a.c.a. muscle fibres were examined at various stages during the dissections. The preparations were bathed in their own haemolymph at all times. Similar studies were made on six locust extensor tibiae nerve-muscle preparations.

The results obtained from these preparations were conclusive. The contractions of the a.c.a. muscles and the 'slow' contractions of the extensor tibiae muscles were always attenuated during inhibitory stimulation and the electrical responses to stimulation of the inhibitory axon were exclusively hyperpolarizing IPSPs (Fig. 1). Depolarizing IPSPs were never recorded from muscles bathed in haemolymph. Considerable resting tensions (*c.* 3 g. in the locust and *c.* 5 g. in the grasshopper) were often recorded from both a.c.a and extensor tibiae muscles when these muscles were bathed in haemolymph and stretched to maximal body length. The largest 'resting' tensions were recorded from muscles of insects fed on a diet (e.g. grass)

containing a high concentration of potassium. Hoyle (1954) found a relationship between the potassium content of the haemolymph and the diet of the locust and showed that the haemolymph–potassium concentration is elevated following a meal containing a high concentration of potassium. Usherwood & Little (to be published) have recently confirmed that the potassium content of locust and grasshopper haemolymph is dependent to some extent on diet although large variations in haemolymph–potassium levels have not been recorded. Muscle resting potentials are often significantly lower in grass-fed locusts than in starved locusts. A correlation

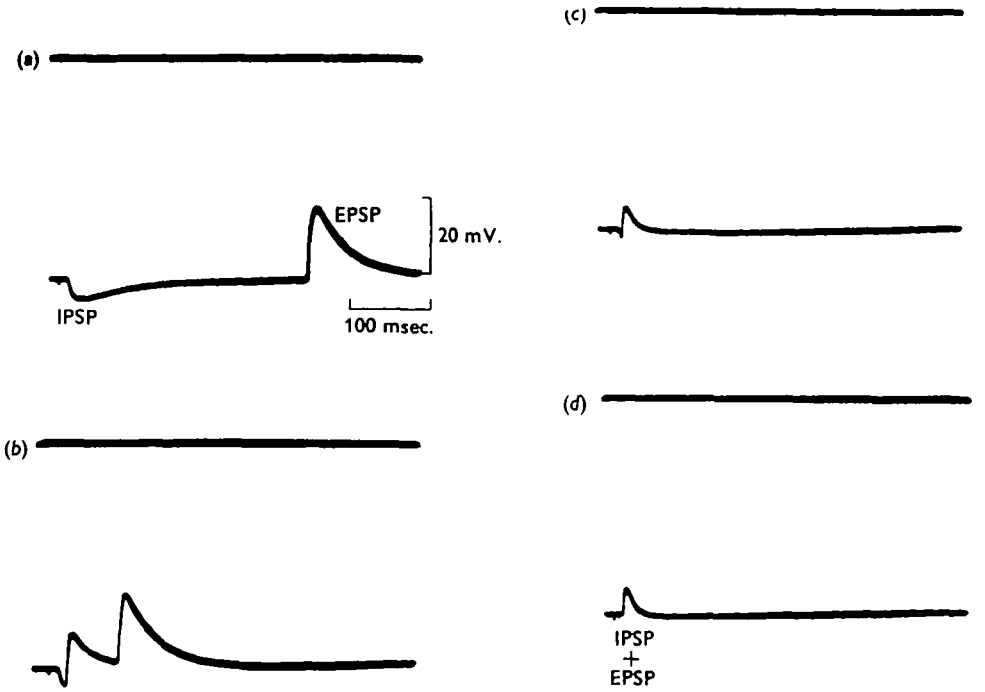

Fig. 1. Intracellular recordings from an a.c.a. muscle fibre of a starved locust showing interaction between EPSP and IPSP. Maximum attenuation of the EPSP occurred when the EPSP arose slightly before the peak of the IPSP (Usherwood & Grundfest, 1965). Similar responses were obtained from other dually innervated fibres of this a.c.a. muscle, when the muscle was bathed in haemolymph. Time and voltage calibrations were the same for *a–d*. Two EPSPs occurred during the IPSP in (*b*).

between haemolymph–potassium levels and muscle fibre membrane potentials is of course to be expected since locust muscle fibres are known to be reasonably good potassium electrodes for a wide range of potassium concentrations (Usherwood, 1967*a*, *b*). The extensor tibiae muscles in locusts and grasshoppers contain some tonic fibres (Usherwood, 1967*a*) and it seems reasonable to assume that the a.c.a. muscle also contains some tonic fibres since it gives a tonic contraction during potassium depolarization although in good preparations the tonic contraction is always preceded by a brief phasic response. In fact Hoyle (1966*b*) has reported the occurrence of at least two types of fibre in the a.c.a. muscle. Presumably the tonic fibres respond to potassium depolarization with a maintained contracture and this could account for the abnormally large 'resting' tensions recorded from muscles of locusts fed on a high-

potassium diet. Stimulation of the inhibitory axon to both a.c.a. (Fig. 2) and extensor tibiae muscles of grass-fed locusts transiently relaxes these muscles. Studies of free-walking locust preparations (Runion & Usherwood, 1966; Galloway, Runion & Usherwood, 1966) indicate that the inhibitory axons to both the a.c.a. and extensor tibiae muscles are active when these muscles are not being excited by the excitatory motor innervation (Usherwood & Runion, to be published). Inhibitory impulses could

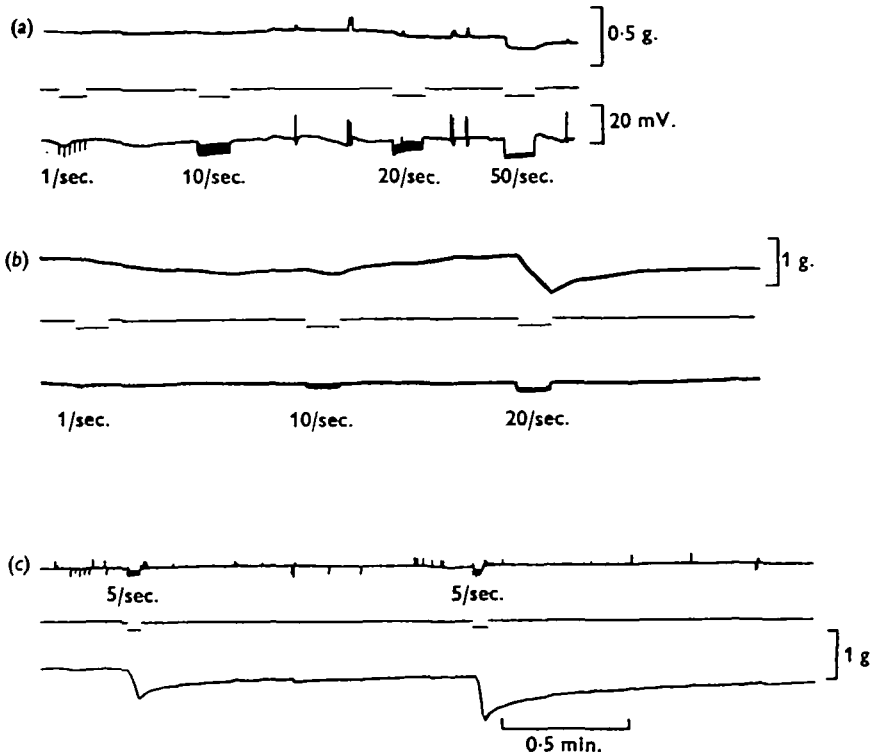

Fig. 2. Inhibition of locust a.c.a. muscle. All intracellular recordings (lower traces (*a*–*b*), upper trace (*c*)) were at the same amplification. The inhibitory neurone to the a.c.a. muscle was stimulated reflexly by exciting the left connective between the mesothoracic and metathoracic ganglia. The excitatory neurone to the a.c.a. muscle was spontaneously active in (*a*) and (*c*). The muscles were bathed in their own haemolymph. Stimulation of the inhibitory axon at frequencies shown below each intracellular recording relaxed the 'resting' muscles, especially in (*b*) and (*c*), where the haemolymph–potassium concentration was probably high as a result of the high potassium content of the diet (grass). In (*a*) the muscle was from a starved insect with presumably a lower haemolymph–potassium concentration. The tonic fibres of this muscle are therefore less contracted and the effect of the inhibitory axon is correspondingly reduced. The resting potentials of the muscle fibres from which the intracellular records were obtained could not be determined exactly because of the small quantity of fluid around the muscles and the respiratory movements of the thorax. However, they appeared to be quite low (*c.* 30–40 mV.) in (*b*) and (*c*), which is reflected in the small EPSPs and IPSPs recorded from these fibres. Approximate 'resting' tensions at maximal body length (*a*), 1 g.; (*b*) 3 g.; (*c*) 3 g. Time and voltage calibrations were the same for all records.

bring about small changes in the 'resting' tension of these muscles by virtue of their action on the tonic fibres especially if the haemolymph–potassium concentration is high, since the IPSPs are always hyperpolarizing and inhibitory regardless of the level of potassium in the haemolymph. Enhancement of the excitatory responses

during inhibitory stimulation, like that seen by Hoyle (1966*a*, *b*) in his a.c.a. preparations, never occurred in the present experiments when the muscles were in haemolymph. It seems reasonable therefore to assume that *in vivo* the inhibitory axons to a.c.a. muscles and extensor tibiae muscles in locusts and grasshoppers have similar properties. The term 'conditioning' as defined by Hoyle (1966*a*, *b*), cannot be used to describe these axons *in vivo* since they never 'condition' the muscle to produce a larger response to an excitatory stimulus.

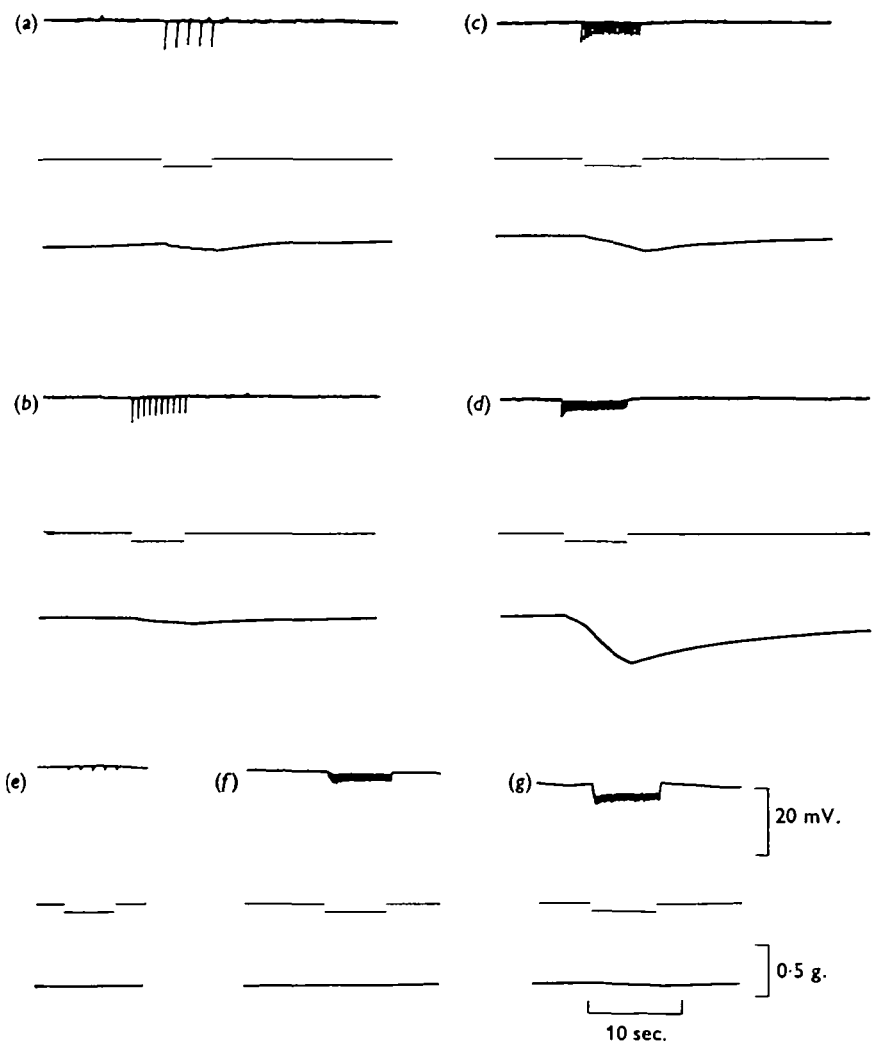

Fig. 3. Electrical responses (upper traces) of a single a.c.a. muscle fibre and mechanical responses (lower traces) of the *in situ* whole muscle from a grass-fed locust during inhibitory stimulation. (*a–d*) Muscle in haemolymph. Note transient relaxations of muscle during 'reflex' stimulation of the inhibitory axon to the a.c.a. muscle at (*a*), 1/sec.; (*b*), 2/sec.; (*c*) 5/sec. and (*d*), 10/sec. Resting tension of muscle at maximal body length was 5 g. (*e–g*) After 4 hr. in 10 K saline, hyperpolarizing IPSPs (upper traces) and relaxations (lower traces) to inhibitory stimulation ((*e*), 1/sec.; (*f*), 10/sec.; (*g*), 20/sec.) were considerably reduced and the resting tension of the muscle was less than 1 g. Time, tension and voltage calibrations were the same for all records.

The effects of locust saline on the properties of the a.c.a. and extensor tibiae muscles were examined next. The electrical and mechanical responses of over 100 a.c.a. nerve-muscle preparations and about 10 extensor tibiae nerve-muscle preparations from locusts and grasshoppers were studied first in haemolymph and then more critically in locust saline (10 K saline). In haemolymph the electrical responses during inhibitory stimulation were, as before, exclusively hyperpolarizing IPSPs and were often accompanied by transient relaxations of the muscles (Fig. 3). However, when the

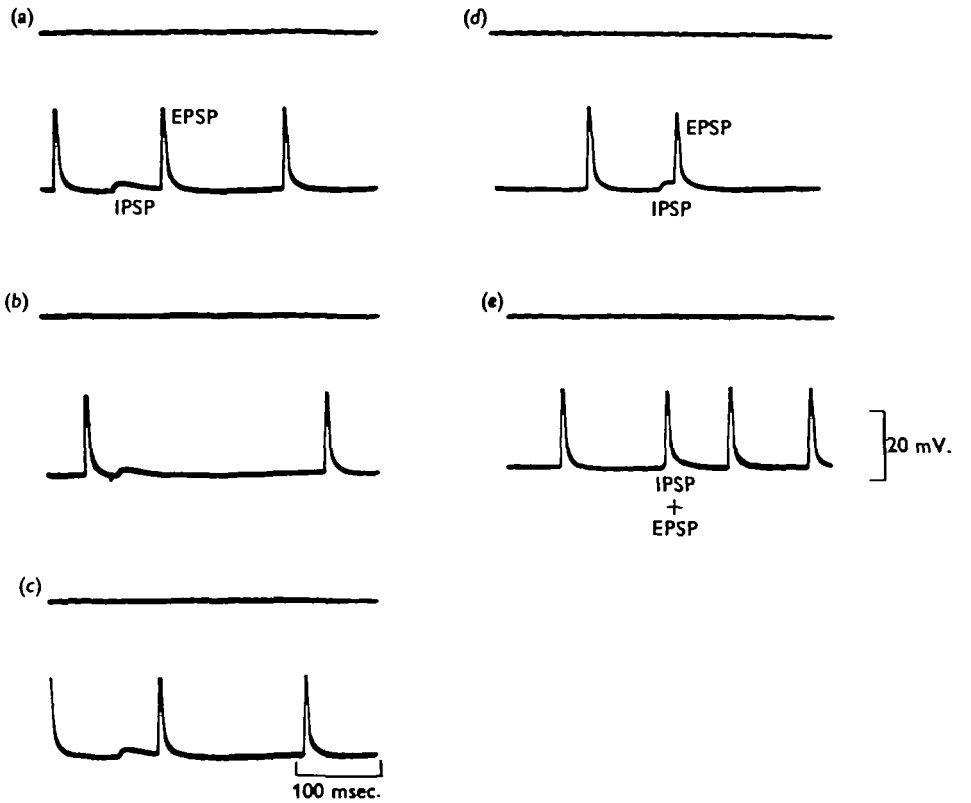

Fig. 4. Interaction between EPSPs and depolarizing IPSPs of a locust a.c.a. muscle fibre. Intracellular recording. Inhibitory neurone stimulated reflexly: excitatory neurone spontaneously active. *In situ* muscle preparation from a grass-fed locust. Muscle soaked in 10 K saline for 60 min. Note that although the IPSP is depolarizing it does not sum with the EPSP to give a larger combined depolarization, when both responses occur together (*d–e*). Instead, the EPSP is attenuated when it occurs during the IPSP. Therefore contractions of this muscle fibre in response to excitatory stimulation would be inhibited to some extent during the IPSP despite the fact that the inhibitory response is itself a depolarizing potential possibly capable of evoking a mechanical response (contraction) from the fibre. Time and voltage calibrations were the same for (*a–e*).

haemolymph was replaced with 10 K saline changes in the responses to the inhibitory axon often occurred. In some preparations the IPSPs became slightly smaller and the relaxations of the muscles in response to inhibitory stimulation slowly diminished (Fig. 3). At the same time a slight fall in muscle 'resting' tension was recorded. These results were most frequently obtained from muscles of starved insects with low

haemolymph–potassium concentrations (*c.* 7 m-equiv./l.). In most preparations from animals fed on a high-potassium diet, reversal of the muscle IPSPs from hyperpolarizing to depolarizing responses occurred in many fibres when the preparation was exposed to 10 K saline (Fig. 4). Reversal of the IPSPs also occurred in a few preparations from starved locusts. With the appearance of depolarizing IPSPs small contractions were frequently recorded from the muscles during inhibitory stimulation

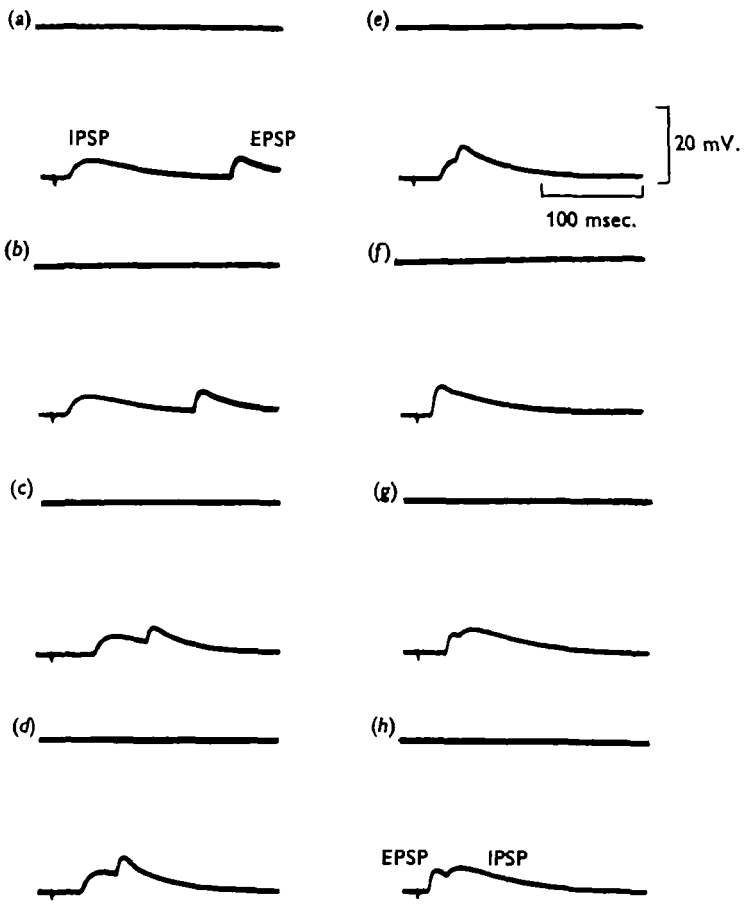

Fig. 5. Summation of depolarizing IPSPs and EPSPs. Intracellular recordings from another fibre of a.c.a. muscle preparation described in Fig. 4. When the EPSP occurs during the IPSP the resultant depolarization of the muscle fibre is greater than the depolarization during the EPSP alone, and the mechanical response of this muscle fibre might appear enhanced at this time. Stimulation of the inhibitory neurone to this muscle evoked a small contraction even in the absence of EPSPs. Time and voltage calibrations were the same for all records.

although contractions did not invariably occur. Interaction between depolarizing IPSPs and EPSPs was complex (Figs. 4, 5). On occasions the EPSP and IPSP summated to give what might appear to be an enhanced excitatory response (Fig. 5). At other times, provided the EPSP was large, the EPSP was attenuated by the depolarizing IPSP (Fig. 4). The effects of interaction between the EPSP and IPSP on the mechanical responses of the a.c.a. and extensor tibiae muscles were unpredictable

since these muscles contain many fibres innervated by both excitatory and inhibitory axons and the EPSPs and IPSPs recorded from the different fibres are never identical. The ionic basis for interactions between the EPSPs and IPSPs will be discussed later. Depolarizing IPSPs first appeared about 10 min. after the haemolymph was replaced by 10 K saline and were recorded from some preparations up to 6 hr. later. However, after the muscles had been soaked for about 8 hr. in 10 K saline the IPSPs were once again mainly hyperpolarizing. Stimulation of the inhibitory axon at this time usually failed to elicit any mechanical response from the muscles. Resting potentials of a.c.a. and extensor tibiae muscle fibres of grass-fed locusts increased progressively in 10 K saline but usually did not attain the predicted value of 60 mV. (Usherwood, 1967*a*, *b*) for some hours.

It appears from these results that 10 K saline can have a marked effect on the responses of muscle fibres of locusts and grasshoppers to inhibitory stimulation. This could undoubtedly explain why the inhibitory responses recorded by Hoyle (1966*a*, *b*) from his a.c.a. muscles were so diverse. Hoyle (1966*a*, *b*) reported that his a.c.a. preparations were examined in 'standard' locust saline which contains 10 m-equiv./l. potassium (Hoyle, 1953) and which he suggests should give similar results to average results obtained from muscles bathed in their own haemolymph. However, in view of the variable potassium content of locust haemolymph a significant fall in the potassium concentration of the external environment of the muscle could occur when the muscle is transferred from haemolymph to 10 K saline which could influence the magnitude and sign of the IPSP. This possibility was examined by observing the effect of controlled changes in the potassium concentration of the medium bathing a.c.a. or extensor tibiae nerve-muscle preparations on the inhibitory responses of these preparations.

About 50 a.c.a. and extensor tibiae nerve-muscle preparations from starved locusts were equilibrated for 1–2 hr. in 10 K saline. The haemolymph of starved locusts contains about 7 m-equiv./l. potassium and changes in the characteristics of the muscle IPSP occur less frequently when nerve-muscle preparations from these insects are exposed to 10 K saline. At the end of the equilibration period resting potentials of about 60 mV. were recorded from most superficial muscle fibres and the electrical responses to inhibitory stimulation were always hyperpolarizing IPSPs. Stimulation of the inhibitory axon of these 'equilibrated' preparations had little or no perceptible effect on muscle tension (Fig. 6). The 10 K saline was then replaced with a high-K saline containing 20–100 m-equiv./l. potassium and the preparations were perfused with this high-K saline for periods ranging from 10 min. to 2 hr. The superficial muscle fibres quickly depolarized in the high-K saline but with potassium concentrations less than 50 m-equiv./l. the excitatory and inhibitory axons were still capable of conducting impulses. The EPSPs were immediately reduced in magnitude but the IPSPs were initially enhanced (Fig. 7) although they also became smaller eventually (Fig. 8). Nevertheless the IPSPs were always hyperpolarizing in high-K saline. When the potassium concentration of the high-K saline exceeded 50 m-equiv./l. axonal conduction was blocked. After treatment with the high-K saline the preparations were returned to the control 10 K saline. Repolarization of the muscle fibres in 10 K saline was slow, the rate of repolarization being related to the concentration of potassium in the high-K saline and to the length of time in the high-K saline, i.e. repolarization of the muscle fibres was slowest following a 2 hr.

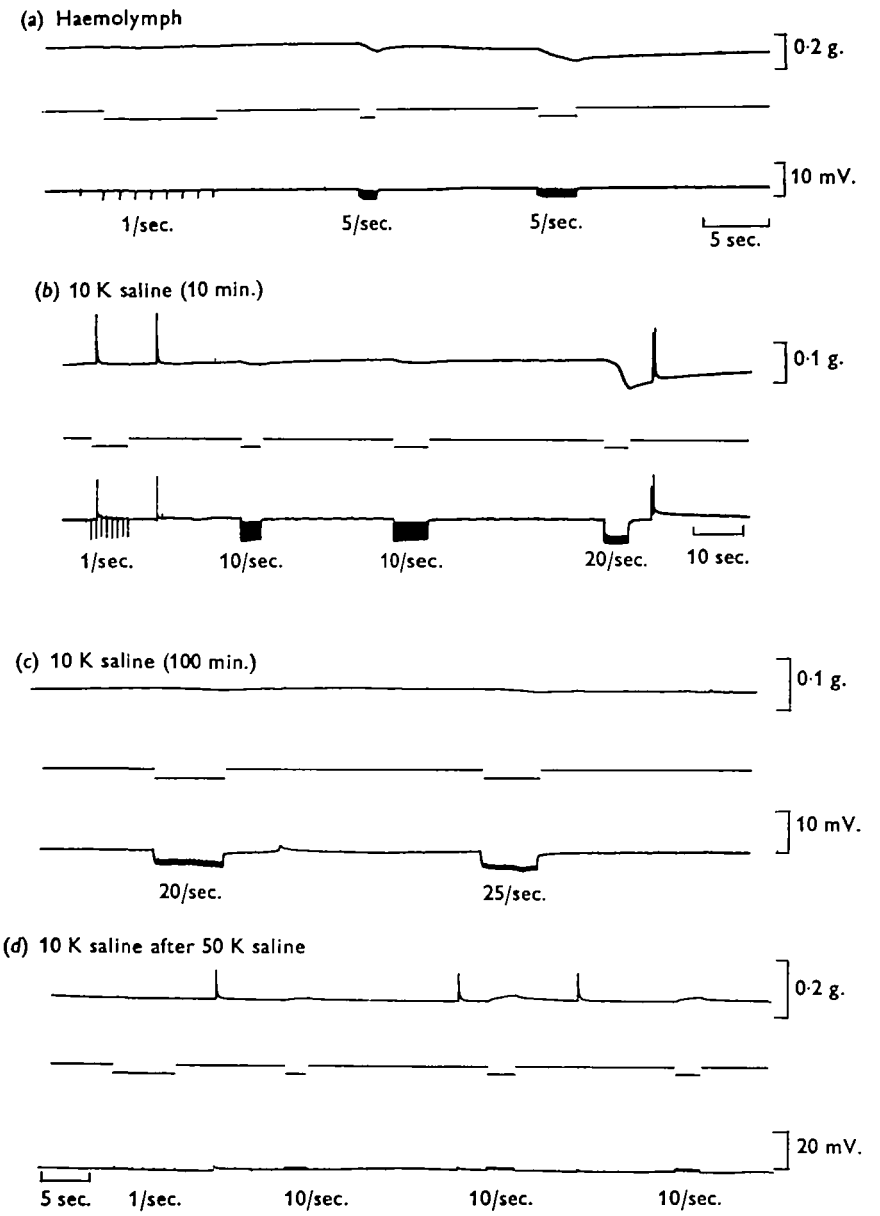

Fig. 6. Effect of changes in the potassium concentration of the medium bathing a grasshopper a.c.a. muscle. In haemolymph (*a*) the intracellular responses of the a.c.a. muscle fibres during inhibitory stimulation were exclusively hyperpolarizing IPSPs. A recording from a single a.c.a. muscle fibre (RP = 46 mV.) is shown in the lower trace of (*a*). The mechanical responses (upper trace) to inhibitory stimulation consisted of twitch-like relaxations at low frequencies and tonic-like relaxations at high frequencies. The muscle 'resting' tension in haemolymph was about 2·5 g. Electrical recordings from two other fibres of this muscle (lower traces) and mechanical recordings from the muscle (upper traces) at different times after transferring the muscle to 10 K saline are illustrated in (*b*) and (*c*). Hyperpolarizing IPSPs were recorded from the two fibres in (*b*) and (*c*) although depolarizing IPSPs were found in other fibres. The mechanical response (relaxation) to inhibitory stimulation gradually diminished but no contractions were recorded from this preparation during inhibitory stimulation after 100 min. in 10 K saline. (*d*) Mechanical response of a.c.a. muscle (upper trace) and intracellular recording from same fibre as (*c*) (lower trace) obtained after 10 min. in 10 K saline following 20 min. in 50 K saline. Note contractions and depolarizing IPSPs during inhibitory stimulation. Some spontaneous EPSPs accompanied by contractions are seen in (*b–d*). Voltage calibration same for (*a–b*). Time calibration same for (*c–d*).

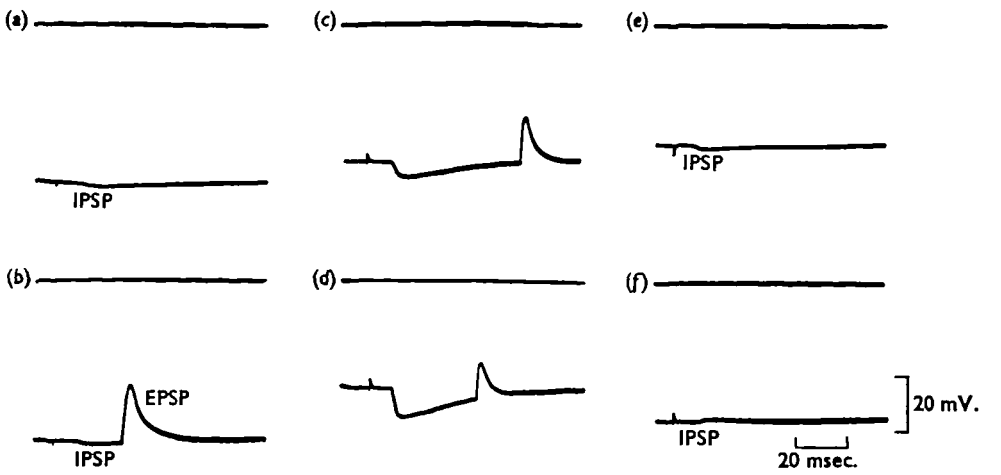

Fig. 7. Reversal of IPSP following treatment of locust a.c.a. muscle with high-K saline (40 m-equiv./l.). Reflexly evoked IPSPs (*a–f*) and spontaneous EPSPs (*b–d*) recorded intracellularly from a single superficial a.c.a. muscle fibre. A.c.a. muscle obtained from starved locust and soaked for 1 hr. in 10 K saline. Responses at end of equilibration shown in *a–b*. The IPSP initially increased in magnitude when 40 K saline was substituted for the 10 K saline (*c–d*) but the EPSP became smaller as the fibre depolarized. When the 10 K saline was reintroduced the IPSP became smaller as the muscle repolarized (*e*) and then reversed to a depolarization (*f*). Time and voltage calibrations were the same for all records.

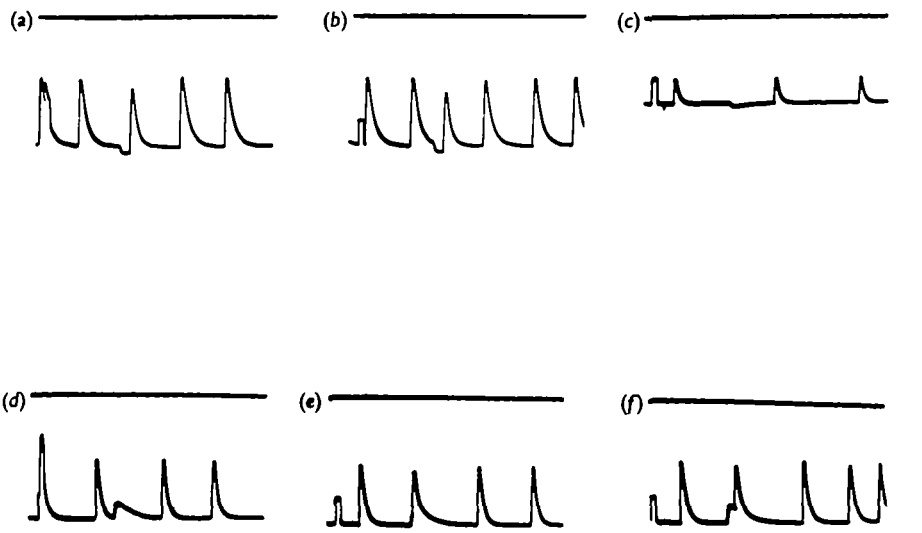

Fig. 8. Reversal of IPSPs of grasshopper a.c.a. muscle fibres. Inhibitory neurone stimulated reflexly. The excitatory neurone was spontaneously active. IPSPs were hyperpolarizing in 10 K saline (*a–b*) and the EPSP was attenuated when it occurred during the IPSP. The excitatory responses were much smaller in 40 K saline and the resting potential of the fibre was lower (depolarized) (*c*). The IPSP was initially enhanced, however, when the muscle was placed in the 40 K saline, although this change is not illustrated here. On returning the preparation to 10 K saline the IPSPs reversed to depolarizing responses (*d–f*). Nevertheless the EPSP was still attenuated to some extent when it occurred during the depolarizing IPSP. Calibration pulse at beginning of each record was 10 mV.–20 msec. (After Usherwood, 1967*a*.)

exposure to 100 K saline. The effects on the IPSP of exposing a preparation for 10 min. to 20 K saline and then returning it to 10 K saline were minimal. In fact the only noticeable difference seen before and after treatment with the 20 K saline was a slight transient reduction in the magnitude of the IPSP. After longer periods in high-K saline, particularly in saline containing more than 50 m-equiv./l. potassium, the polarity of the IPSP was altered when preparations were returned to 10 K saline

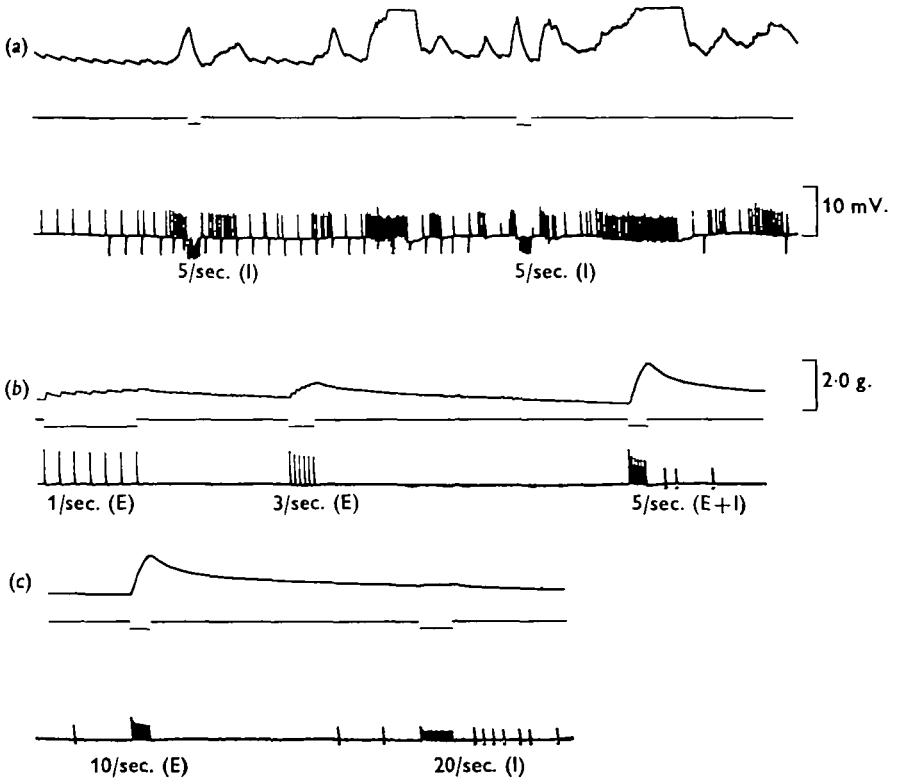

Fig. 9. An isolated locust a.c.a. nerve-muscle preparation. (*a*) Muscle soaked for 30 min. in 10 K saline. The excitatory and inhibitory neurones to this muscle were spontaneously active and the EPSPs were attenuated by the IPSPs (lower trace). The effect of the spontaneous inhibitory activity on the mechanical responses (upper trace) was not clear. However, in (*a*) when the inhibitory axon was stimulated reflexly (I) the inhibition of the a.c.a. muscle contraction was quite marked. GABA (10^{-4}, w/v) also reduces the responses of the a.c.a. muscle to excitatory stimulation (not shown in this Fig.). (*b–c*) Depolarizing IPSPs (I) and EPSPs (E) (lower traces) recorded intracellularly for one of the a.c.a. muscle fibres following exposure of the muscle for 30 min. to 50 K saline and then returning it to 10 K saline for 20 min. The depolarizing IPSPs in (*c*) are accompanied by small contractions (upper trace) of the muscle. Spontaneous IPSPs are indicated by dots under lower traces of (*b–c*). Note stimulation of inhibitory axon alone evokes a contraction from the muscle. Also inhibitory stimulation no longer attenuates the mechanical response of the a.c.a. muscle to the excitatory axon. Voltage calibration and tension calibration were the same for all records.

(Figs. 7, 8). Stimulation of the inhibitory axon now evoked a small contraction from the muscles (Figs. 6, 9, 10), whereas before high-K treatment either a small relaxation or no mechanical response at all was observed during inhibitory stimulation. In some experiments the preparation was treated with high-K saline and then the potassium concentration of the saline was lowered by 5 m-equiv./l. every 30 min. to a final value of

10 m-equiv./l. Under these conditions the response to the inhibitory axon was at all times a hyperpolarizing IPSP. Depolarizing IPSPs therefore only occur as a result of a large fast reduction in the potassium concentration of the bathing medium. Significantly, when depolarizing IPSPs appear they can be quickly reconverted to hyperpolarizing responses merely by increasing the potassium concentration of the bathing medium.

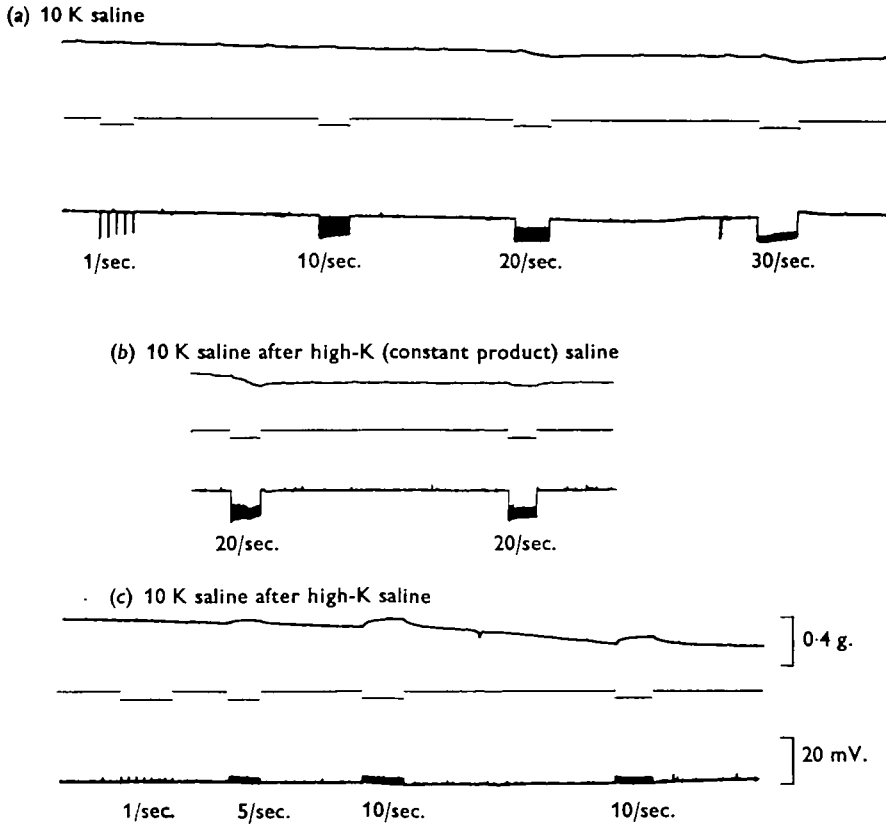

Fig. 10. Effect of different salines on the intracellular electrical responses (lower traces) and mechanical responses (upper traces) of an a.c.a. nerve-muscle preparation from a starved locust. The responses in 10 K saline to inhibitory stimulation were exclusively hyperpolarizing IPSPs accompanied by slight relaxations of the muscle. After treatment for 20 min. with 50 K (constant product) saline (*b*) the responses were unchanged when the muscle was returned to 10 K saline. However, following treatment for 10 min. with 50 K saline (*c*) the IPSPs became depolarizing and the mechanical responses to inhibitory stimulation were contractions rather than relaxations. Voltage and tension calibrations were the same for all records. Intracellular recordings were from the same fibre. RPs: (*a*), 58 mV., (*b*) 60 mV.; (*c*) 48 mV.

Recent studies of metathoracic retractor unguis muscles in locusts and grasshoppers have shown that an increase in the concentration of the potassium ions in the bathing medium results in swelling of these muscles as a result of influx of potassium chloride into the muscle fibres accompanied by an osmotic influx of water (Usherwood, 1967*a*, *b*). Changes in muscle volume occur only when the potassium concentration of the bathing medium is increased whilst maintaining the chloride

concentration constant. If the potassium concentration is increased and compensatory changes in the chloride concentration are made in order to maintain the product of the potassium and chloride concentrations constant there is no influx of potassium chloride and water, and the muscle fibres do not swell (Usherwood, 1967*a*, *b*). An increase in the internal chloride concentration $[Cl_i^-]$ of the muscle fibres would be expected therefore during treatment with high-K saline. This change in $[Cl_i^-]$ could account for the appearance of depolarizing IPSPs when the muscles are returned to 10 K saline since the IPSP is a chloride potential. If this is the case, then the inhibitory responses should not be altered after treatment with high-K (constant product) salines since this should not alter $[Cl_i^-]$. This was tested by treating a.c.a. and extensor tibiae nerve-muscle preparations, which had been equilibrated with 10 K saline for 2 hr., with high-K (constant product) salines containing potassium concentrations of 20–100 m-equiv./l. The muscle fibres were quickly depolarized by the high-K (constant product) salines and there was some reduction in the magnitudes of the IPSPs and EPSPs. The IPSPs were not enhanced initially in the high-K (constant product) salines as they were in the high-K salines. On returning the preparations to 10 K saline the muscle fibres quickly repolarized and the IPSPs and EPSPs returned to normal (Fig. 10). The IPSPs were always hyperpolarizing even following prolonged periods (up to 4 hr.) in 100 K (constant product) saline.

DISCUSSION

The IPSPs of the metathoracic a.c.a. and extensor tibiae muscles in locusts and grasshoppers can be converted from hyperpolarizing to depolarizing responses by soaking these muscles for a short period in saline containing a high concentration of potassium ions and then returning them to saline containing a low concentration of potassium ions, provided that $[Cl_o^-]$ is maintained constant. However, if the products of the potassium and chloride concentrations of these salines are equal (constant product salines) the IPSP is relatively unaffected. Entry of potassium chloride into the muscle fibres during treatment with saline containing a high concentration of potassium appears essential for reversal of the IPSP.

Most of the current during the IPSP is carried by chloride ions (Usherwood & Grundfest, 1965). When the chloride in the medium bathing one of these insect muscles is replaced by the impermeant anion propionate, the IPSPs reverse in sign to become depolarizing potentials (Usherwood & Grundfest, 1965) and the muscle contracts slightly during stimulation of the inhibitory innervation (Fig. 11). The equilibrium potential for the IPSP (E_{IPSP}) is normally more negative than the resting membrane potential (E_M) (Usherwood & Grundfest, 1965) and since the IPSP is a chloride potential (Fig. 12) the chloride equilibrium potential (E_{Cl}) must also be negative to E_M (Fig. 13). The resting muscle fibre in locusts and grasshoppers is permeable to both potassium and chloride ions (Usherwood, 1967*a*, *b*) so that some form of outward chloride pump could account for the discrepancy between E_M and E_{Cl}. Since these insect muscle fibres are relatively good potassium electrodes it is reasonable to assume that the potassium equilibrium potential (E_K) is close to E_M. When locust and grasshopper muscle fibres are exposed to high-K (constant product) salines the EMF of the potassium battery and the EMF of the chloride battery should change

simultaneously. E_K and E_{Cl} will become less negative and the muscle fibres will be depolarized (Fig. 13). On the assumption that E_K and E_{Cl} change to the same extent the magnitude of the IPSP should not change although the increased conductance of the depolarized muscle fibre could account for the reduced magnitude of the IPSP observed in the high-K (constant product) saline. The EPSP is always reduced in magnitude when the muscle is depolarized since E_M is then closer to the

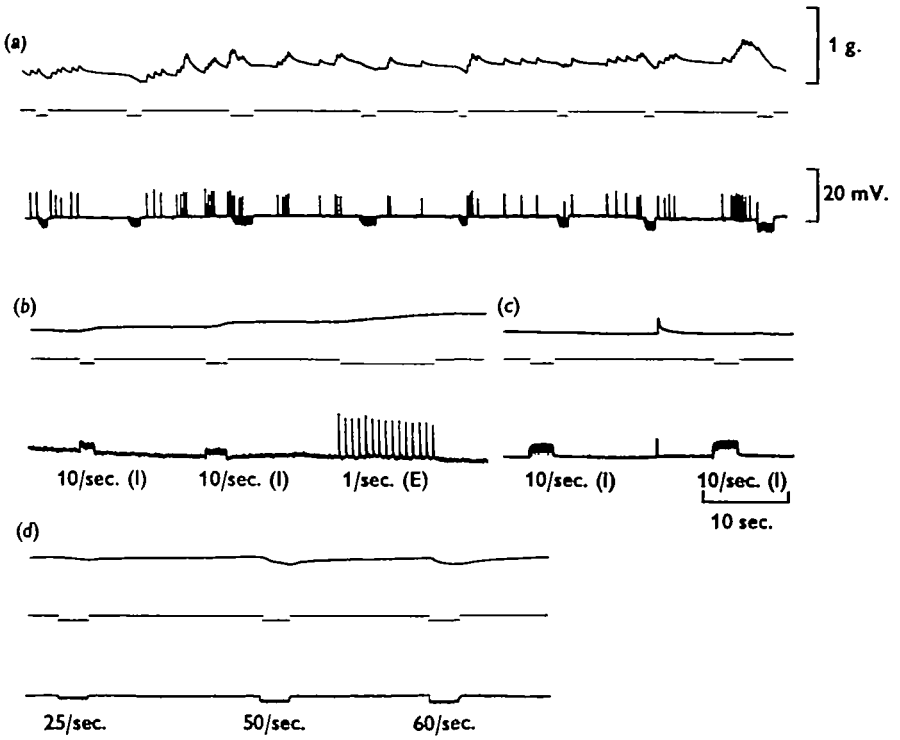

Fig. 11. Reversal of IPSPs of a locust a.c.a. muscle in a chloride-free (propionate) saline. Intracellular responses from a single a.c.a. muscle fibre (lower traces) and accompanying mechanical responses of the a.c.a. muscle (upper traces). In (*a*) the excitatory neurone was spontaneously active. The inhibitory neurone was excited reflexly at 10/sec. at times indicated on centre trace. Note attenuation of EPSPs and muscle tension during inhibitory stimulation. (*b*), The IPSPs (I) were reversed to depolarizations in 10 K chloride-free saline and the EPSPs and mechanical responses to excitatory stimulation (E) were initially reduced in magnitude. A transient depolarization of the muscle fibre accompanied by a contraction of the muscle was recorded when the muscle was first perfused with 10 K propionate saline. At the end of the 'chloride transient' the membrane potential of the muscle fibre returned to its initial value (*c.* 50 mV.). (*d*) On returning the muscle to the 10 K chloride-saline hyperpolarizing IPSPs reappeared and very slight transient relaxations of the muscle were recorded (upper trace) during inhibitory stimulation at frequencies indicated below intracellular trace. Time, voltage and tension calibrations were the same for all records.

equilibrium potential for the EPSP (E_{EPSP}) (Cerf *et al.* 1959) and the driving force on the ions which carry the current during the EPSP is reduced. Potassium chloride does not enter muscle fibres exposed to high-K (constant product) saline since the distribution of potassium chloride in the intracellular and extracellular environments is determined by the equilibrium $[K_i^+] \times [Cl_i^-] = [K_o^+] \times [Cl_o^-]$ (where $[K_i^+]$ and

$[K_o^+]$ are the internal and external potassium concentrations (activities) and $[Cl_i^+]$ and $[Cl_o^-]$ are the internal and external chloride concentrations (activities)) and replacement of 10 K saline with a high-K (constant product) saline does not upset this equilibrium. When muscles equilibrated in high-K (constant product) saline are returned to 10 K saline the superficial fibres repolarize very rapidly as E_K and E_{Cl} return to more negative levels simultaneously. Therefore E_{IPSP} is always more negative than E_M and the IPSP is at all times a hyperpolarizing response (Fig. 13).

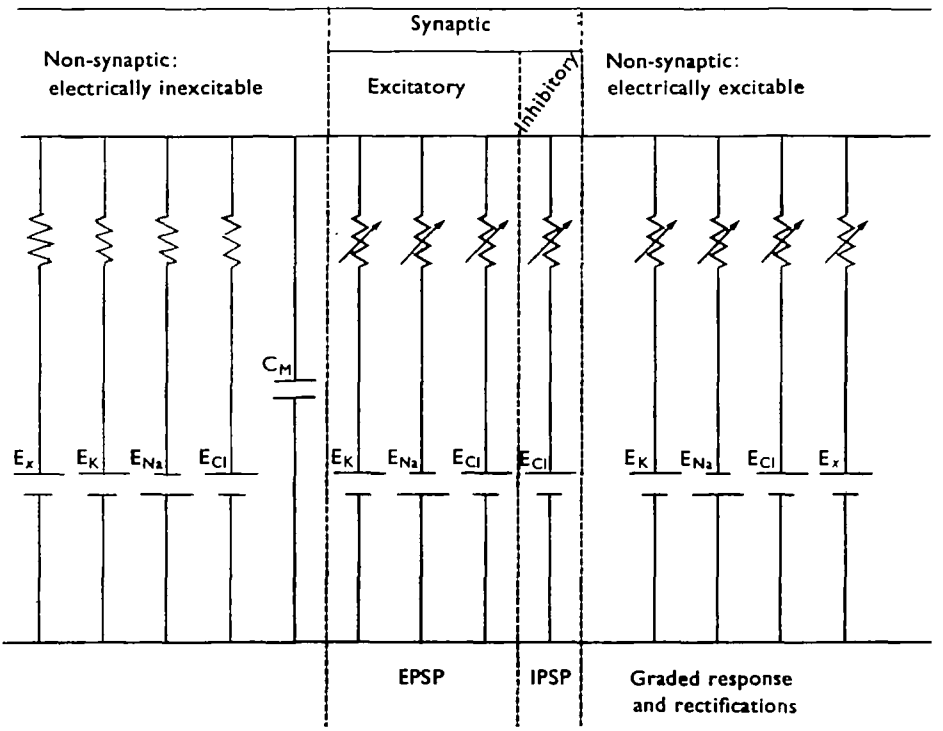

Fig. 12. Model of the electrical components of the membrane of the electrically excitable muscle fibre of locusts and grasshoppers. C_M is the membrane capacity. E_K, E_{Cl}, E_{Na} are the potassium, chloride and sodium batteries (EMF = equilibrium potential). Ions other than sodium and potassium for the excitatory synaptic membrane component and sodium, potassium and chloride for the non-synaptic membrane component possibly carry some of the current during activity of these membrane components. The battery for these ions which could be either anions or divalent cations is denoted by E_x. (Modified after Grundfest, 1964.)

When the a.c.a. or extensor tibiae muscle is exposed to a high-K saline with a normal chloride concentration (154 m-equiv./l.) the superficial muscle fibres are quickly depolarized, presumably as a result of the change in the EMF of the potassium battery across the muscle fibre membrane (Fig. 12). The chloride concentration of the bathing medium has not been altered and E_{Cl} does not therefore change immediately although it slowly becomes less negative as potassium chloride enters the muscle fibre and $[Cl_i^-]$ increases. Since E_{Cl} changes more slowly than E_M or E_K, the driving force on the chloride ions is temporarily increased and the IPSP is enhanced. The IPSP then slowly declines in magnitude as the equilibrium $[K_o^+] \times [Cl_o^-] = [K_i^+] \times [Cl_i^-]$

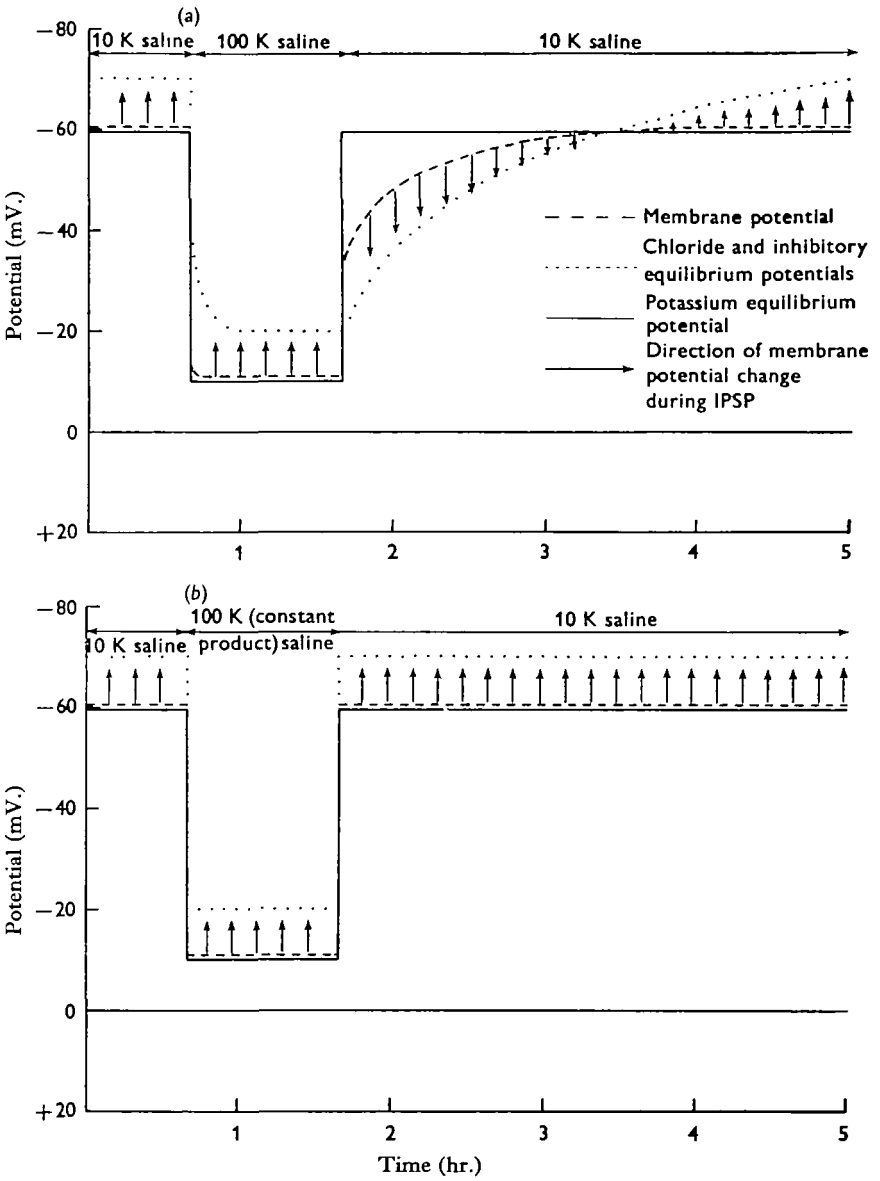

Fig. 13. Diagrammatic representation of effects on membrane potential (E_M), chloride equilibrium potential (E_{Cl}) and potassium equilibrium potential (E_K) of a grasshopper or locust muscle fibre of changes in the potassium concentration of the bathing medium $[K^+_o]$. (*a*) When $[K^+_o]$ is changed whilst maintaining the chloride concentration of the medium $[Cl^-_o]$ constant at 154 m-equiv./l., E_{Cl}, E_K and E_M change at different rates. When $[K^+_o]$ is reduced under these conditions, E_{Cl} becomes positive to E_M and the IPSP (a chloride potential) becomes depolarizing. However, when $[K^+_o]$ is changed whilst maintaining the product $[K^+_o] \times [Cl^-_o]$ constant (*b*), E_K, E_{Cl} and E_M change simultaneously and the IPSP is hyperpolarizing for both increasing and decreasing $[K^+_o]$. In a whole-muscle preparation considerable variation from fibre to fibre in the time course of changes in E_M, E_K and E_{Cl} will presumably occur.

is re-established. The extra potassium chloride and water which enters the muscle fibres during high-K treatment will be lost when the muscle is returned to the 10 K saline. However, efflux of potassium chloride from muscle fibres of locusts and grasshoppers is much slower than influx of potassium chloride (Usherwood, 1967*a*, *b*). When a muscle fibre loaded with potassium chloride is returned to 10 K saline the extra potassium chloride leaves the fibre only very slowly and E_M changes only very slowly (Usherwood, 1967*a*, *b*). Since $[K_i^+]$ has not altered significantly during treatment with high-K saline, E_K will immediately assume the value (60 mV.) appropriate for the new low external potassium concentration according to the Nernst equation $E_K = (RT/F) \log \frac{[K_o^+]}{[K_i^+]}$, where R, T and F have their usual meanings. $[Cl_i^-]$ increases during treatment with high-K saline and since potassium chloride efflux is slow E_{Cl} and therefore E_{IPSP} will not change very rapidly when a muscle fibre loaded with potassium chloride is returned to 10 K saline. E_M should assume a value somewhere intermediate between E_K and E_{Cl} and therefore for some time after the fibre is replaced in 10 K saline, E_{Cl} will be less negative than E_M and depolarizing IPSPs will appear during inhibitory stimulation. As potassium chloride diffuses out of the fibre E_{Cl} will become more negative but so will E_M and therefore the IPSP will remain depolarizing. However, when E_M, E_K and E_{Cl} become equal the IPSP will disappear and then as the rest of the extra potassium chloride diffuses or is pumped out of the fibre, E_{Cl} will become negative to E_M and E_K, and the IPSP will reappear as a hyperpolarizing response.

If at the end of high-K treatment the potassium concentration of the saline is reduced only slowly then the rate of potassium chloride efflux will be high enough to maintain E_{Cl} below E_K and the IPSP will remain hyperpolarizing throughout the period of potassium chloride efflux.

According to these arguments the diverse results obtained by Hoyle (1966*a*, *b*) from his a.c.a. and extensor tibiae nerve-muscle preparations could be accounted for by the different haemolymph-potassium concentrations of his insects. Hoyle (1954) has suggested that the potassium content of locust haemolymph varies between 5 and 40 m-equiv./l. according to the potassium content of the diet. Usherwood & Little (to be published) have failed to obtain such large variations in haemolymph–potassium levels by feeding locusts and grasshoppers on diets containing different potassium concentrations, although haemolymph from insects fed on grass (a high-potassium diet) usually contains about 4 m-equiv./l. more potassium than haemolymph from starved insects. However, fluctuations in the haemolymph–potassium levels are not apparently accompanied by changes in the chloride content of the haemolymph (Usherwood & Rees, unpublished). Therefore any variations in haemolymph–potassium concentration will be accompanied by changes in the chloride concentration of the muscle fibres. Since the haemolymph–potassium concentration presumably varies only very slowly E_{Cl} will always be more negative then E_M and the *in vivo* response to inhibitory stimulation will be at all times a hyperpolarizing IPSP.

Changes in the responses of these insect muscle fibres to inhibitory stimulation probably result mainly from leakage of KCl from muscles cut during dissection of the nerve-muscle preparations. A number of closely adjacent muscles must be removed to obtain a.c.a. nerve-muscle preparations in locusts and grasshoppers, and since these

muscles contain a high concentration of potassium it is possible that some of this potassium could leak out from the cut ends of the fibres during dissection to produce local increases in the potassium concentration of the medium bathing the a.c.a. muscle. Some fibres of the a.c.a. muscle could therefore be subjected for short periods to a high concentration of potassium with resultant increase of the chloride content of these fibres. Perhaps this would explain why in the present experiments depolarizing IPSPs were recorded more frequently from isolated a.c.a. nerve-muscle preparations which involve a considerable amount of dissection. It could also account for the varied inhibitory responses obtained from fibres of the same muscle preparation and also for the fact that depolarizing IPSPs were sometimes recorded also from isolated a.c.a. muscles from starved locusts despite the fact that the potassium concentration in the haemolymph of these insects should be similar to or less than that of 10 K saline. Reversal of the IPSPs to depolarizing responses as a result of potassium leakage from cut fibres could presumably occur even when the muscles were in haemolymph although this did not happen in the present experiments. Artifacts of this type are less likely to arise with the extensor tibiae muscle since this muscle is more easily exposed and in any event is protected to some extent, by the tracheolar system, from any potassium that might leak out during removal of the flexor tibiae and retractor unguis muscles during preparation.

Disregarding any possible alteration of $[Cl_i^-]$ during dissection the responses recorded from a.c.a. and extensor tibiae muscle fibres when the haemolymph is replaced by 'standard' locust saline (10 K) will depend on the haemolymph–potassium concentration or, more exactly, on the difference between the products of the potassium and chloride concentrations of the haemolymph and 10 K saline. The possibility that ionized amino acids play some part in determining the membrane potentials of insect muscle fibres cannot be ignored, but the role, if any, that these substances play is not yet understood. If replacement of haemolymph by 10 K saline reduces the external potassium concentration of the a.c.a. muscle and thereby alters the product of the external potassium and chloride concentrations then E_K will change faster than E_{Cl} (E_{IPSP}) and E_{Cl} could become less negative than E_M. The IPSP would change from a hyperpolarizing to a depolarizing response and contractions rather than relaxations could be recorded from the muscle during inhibitory stimulation. The nature of the interaction between an EPSP and a depolarizing IPSP depends to some extent on the magnitude of the excitatory response. If the EPSP is large then it is attenuated by the depolarizing IPSP. Presumably the peak of the EPSP in this instance is above (less negative than) E_{IPSP}. When the EPSP is small and the peak of the response is below (more negative than) E_{IPSP}, then summation of the inhibitory and excitatory response will occur. The magnitude of the EPSP varies from fibre to fibre in both the a.c.a. and extensor tibiae muscles, and since these muscles are large, considerable differences in E_{IPSP} and E_M of different a.c.a. muscle fibres might be expected for some time after exposure to 10 K saline. These differences could account to some extent for the many different inhibitory and excitatory potentials recorded by Hoyle (1966*a*, *b*) from different fibres of his a.c.a. nerve-muscle preparations.

Usherwood & Grundfest (1964, 1965) found that the IPSPs of the extensor tibiae muscle fibres in locusts and grasshoppers were always hyperpolarizing even in 10 K saline, although some differences in the magnitude of the resting potential, EPSP

and IPSP were seen in different fibres. This occurred despite the fact that the locust preparations used by Usherwood & Grundfest (1964, 1965) were obtained from locusts that had been starved and were always equilibrated for about 1 hr. in 10 K saline before any recordings were made from the muscle fibres. Any effect on $[Cl^-_i]$ of differences in the potassium concentrations of the haemolymph and 10 K saline should therefore have been minimized. The low resting potentials recorded from many a.c.a. and extensor tibiae muscle fibres (Hoyle, 1966*a*, *b*; Usherwood & Grundfest, 1964, 1965) were probably in many cases the result of inferior penetration of these fibres with the recording electrodes rather than to an abnormally high $[Cl^-_i]$. It is possible of course that tonic fibres have lower resting potentials than phasic fibres (Usherwood & Grundfest, 1965). The fact that Usherwood & Grundfest (1965) found that the reversal potential for the IPSP was the same in fibres with low and high resting potentials suggest that the chloride concentrations of these fibres were identical. In the present experiments considerable care was taken to select electrodes with relatively high resistances (*c.* 20 MΩ) and low tip potentials (*c.* 5 mV.). Using these electrodes the resting potentials of superficial fibres in any given muscle were relatively constant (± 2 mV.), provided of course that these muscle fibres had been completely equilibrated with the saline environment.

In view of the large size and complexity of the a.c.a. and extensor tibiae muscles, considerable errors in measurement of membrane potentials must arise, especially when these involve changes in the ionic environment of the muscles. Changes in membrane potential of the more deeply situated a.c.a. muscle fibres were much slower than for the superficial fibres of the muscle. This was especially true for decreasing $[K^+_o]$. Presumably the full effects of a reduction of $[K^+_o]$ resulting in potassium chloride efflux from the fibres are delayed to some extent by the slow rate of diffusion of potassium from the fibres and from the extracellular spaces between the fibres. Quantitative information on the permeabilities of a.c.a. and extensor tibiae muscle fibres cannot therefore be obtained easily from whole-muscle preparations. Obviously, isolated single-fibre preparations are the complete answer to this problem but preparations of this type are very difficult to obtain, at least in the grasshopper and locust. Usherwood (1967*a*, *b*) and Cochrane & Elder (1967) have investigated the potassium and chloride permeabilities of isolated retractor unguis muscles in locusts and grasshoppers. These preparations contain only seventeen fibres and are considerably better than the a.c.a. and extensor tibiae muscles for investigation of ion fluxes. Unfortunately the retractor unguis muscle is not innervated by an inhibitory axon and the muscle does not contain any tonic fibres.

The main object of this publication was to substantiate claims by Usherwood & Grundfest (1964, 1965) that some orthopteran muscles are innervated by peripheral inhibitory axons similar to those found in some crustaceans. The experiments described herein were not done with a view to arriving at a definition of the role of peripheral inhibitory innervation in insects. This aspect will be considered at some length in a later publication.

SUMMARY

1. The metathoracic anterior coxal adductor (a.c.a.) muscle of the locust and the grasshopper is innervated by a peripheral inhibitory axon similar to the inhibitory axon which innervates the metathoracic extensor tibiae muscles of these insects. No evidence was found to justify calling this axon an inhibitory-conditioning axon.

2. Hyperpolarizing inhibitory postsynaptic potentials (IPSPs) are normally recorded from a.c.a. muscle fibres during stimulation of this axon, and if the bathing medium contains a high concentration of potassium ions the tonic fibres of the a.c.a. muscle relax slightly during inhibitory stimulation.

3. The IPSPs are chloride potentials and can be converted to depolarizing responses by changing either the external or internal chloride concentration of the a.c.a. muscle fibres. Depolarizing IPSPs are frequently accompanied by small contractions of a.c.a. muscle fibres innervated by the inhibitory axon.

4. The a.c.a. muscle fibres are permeable to potassium and chloride ions but influx of potassium chloride is much faster than efflux. Therefore when a.c.a. muscle fibres are loaded with chloride by exposing them to high-K saline (20–100 m-equiv. potassium/l.) and are then returned to normal (10 m-equiv. potassium/l.) saline the internal chloride concentration remains elevated for some time and during this period the equilibrium potential for the inhibitory response is less negative than the resting potential and the IPSPs are depolarizing.

5. Depolarizing IPSPs are usually recorded from a.c.a. muscle fibres of locusts and grasshoppers when these fibres are transferred from their normal bathing medium, haemolymph, to 10 K saline. Probably the main reason for this reversal of the IPSPs is the entry of KCl into the muscle fibres during dissection of the nerve-muscle preparations. Large quantities of KCl would be released into the environment surrounding these preparations from muscle fibres cut and removed during dissection.

6. Depolarizing IPSPs were more frequently recorded from muscle fibres of grassfed locusts than from fibres of starved locusts. The potassium concentration of haemolymph of grass fed locusts is higher than that of locust saline (10 m-equiv./l.).

7. The potassium concentration of locust haemolymph presumably fluctuates *in vivo* but these fluctuations are too slow to affect the sign of the IPSP. The IPSPs are therefore always hyperpolarizing *in vivo*.

8. The effect of changes in the potassium concentration of the bathing medium on the magnitude and polarity of the IPSP could account for the diverse responses recorded previously from a.c.a. muscle fibres of locusts and grasshoppers.

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