J. Exp. Biol. (1974), 60, 673–705
With 4 plates and 10 text-figures
Printed in Great Britain

STUDIES ON LOCUST NEUROMUSCULAR PHYSIOLOGY IN RELATION TO GLUTAMIC ACID

BY A. N. CLEMENTS AND TERRY E. MAY
Woodstock Laboratory, Shell Research Limited, Sittingbourne, Kent

(*Received* 11 *October* 1973)

INTRODUCTION

It is well known that application of L-glutamic acid to insect excitatory neuromuscular synapses leads to the production of depolarizing postsynaptic potentials, and this, with other evidence, has led to the belief that L-glutamic acid may be the chemical transmitter at these synapses (Pitman, 1971). It is also known that the haemolymph that bathes insect muscles contains L-glutamate at concentrations which would block neuromuscular transmission in experimental preparations, and it has been uncertain how the neuromuscular system of the living insect functions under such conditions. The work described here was undertaken to learn more about the action of glutamate on insect excitatory neuromuscular systems, and to try to explain how they function in the presence of haemolymph glutamate. Observations were made predominantly upon the mechanical responses of the locust retractor unguis muscle. Compared with the use of microelectrodes this approach told little about cellular mechanisms, but it did provide valuable information which could not be obtained with the other methods. For example, it revealed that certain experimental conditions could cause abnormal muscle contractions.

The experimental results were sometimes difficult to interpret, partly because the physiological salines differed so much in chemical composition from locust haemolymph that it was difficult to extrapolate from the experimental situation to the living insect, and partly because the experimental conditions could generate abnormal responses. The study was extended to investigate some of these additional problems, and this gave us a better understanding of the physiology of locust muscle under natural and experimental conditions. We consider also that our study has thrown some light on the mechanisms which protect locust neuromuscular synapses from haemolymph glutamate.

MATERIALS AND METHODS

Insects

All work was done with the desert locust, *Schistocerca gregaria* Forskål, a culture of which was maintained as described by Hunter-Jones (1966). The locusts were given fresh green food daily, supplemented with stabilized wheat germ. Male locusts which had fledged 2–4 weeks earlier were selected for experiments.

Nerve–muscle preparations

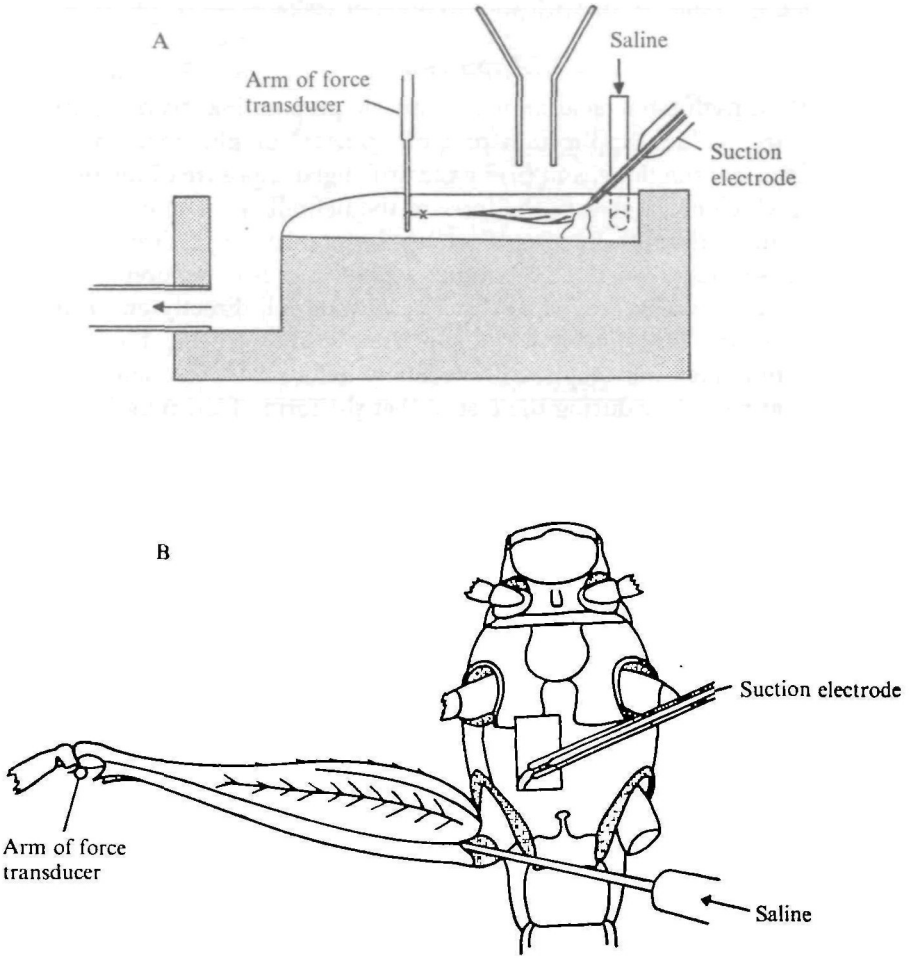
The retractor unguis muscle in the hind leg of the locust consists of two bundles of muscle fibres in the femur and a number of smaller units in the tibia, all attached to the same apodeme (Albrecht, 1953). The two bundles of fibres in the femur, which alone were used in this work, are innervated by two neurones whose axons run in nerve $5b_2$, which is a branch of the crural nerve. Two side branches of nerve $5b_2$ supply the retractor unguis muscle in the femur, and each branch contains processes from the two excitatory axons (Hoyle, 1955*a*; Rees & Usherwood, 1972). The axons ramify within each fibre bundle to produce a multiterminal innervation, and the many nerve endings lie within the connective tissue layer which separates the individual fibres. Stimulation of the crural nerve at low frequencies produces twitch contractions, and at high frequencies produces tetanic contractions in the retractor unguis. The muscle responds to potassium depolarization in a phasic manner, giving a brief contracture (Usherwood & Machili, 1968). Experiments were carried out on two different preparations which were called 'isolated preparations' and 'perfused-femur preparations'.

Isolated preparations

The isolated preparations were made much as described by Hoyle (1953) and Usherwood & Machili (1968). A hind leg was removed and placed in a sloping channel, 6 mm wide and 6 mm deep at the mid-point, cut in a block of Perspex. The basal segments of the leg were set in wax, and the leg was dissected under flowing saline to leave the retractor unguis muscle, still attached at its origin to cuticle, and its nerve. The apodeme of the retractor unguis muscle was tied to the insulated arm of a force transducer (Grass Instrument Co., FT.03C) with a fine Terylene fibre, and the muscle was adjusted to its natural rest length which had previously been noted (Text-fig. 1A). The volume of saline surrounding the preparation was 0·9 to 1·0 ml, and saline was pumped past the preparation by a peristaltic pump (Watson-Marlow Ltd., MHRE.72) at from 0·4 to 1·4 ml per min. Preparations were stimulated electrically, either by a suction electrode connected to the crural nerve or by pin electrodes placed in the saline. Preparations were stimulated every 10 sec with pulses of 1 msec duration, and of about 4 V with the suction electrode and about 12 V with the pin electrodes. The deflexions of the force transducer caused by muscle contractions were recorded by pen recorder (Devices Instruments Ltd., M4). For most recordings the output from the force transducer was damped with a low-pass filter in the pre-amplifier to attenuate the high-frequency oscillations of the transducer. When the time course of the muscle contractions was to be studied, the pre-amplifier was connected to an oscilloscope, and the output from the transducer was not damped.

Perfused-femur preparations

The thorax of a locust was isolated by removal of head, abdomen and alimentary canal, and the openings were sealed with soft wax. A small area of thoracic sternite was removed on one side of the midline, the crural nerve was cut near the meta-thoracic ganglion, and the free end of the crural nerve was drawn into a suction



Text-fig. 1. Locust retractor unguis preparations. (A) Isolated preparation. (B) Perfused-femur preparation.

electrode. This opening of the thorax was then sealed with a mixture of wax and liquid paraffin. The soft membrane at the femoral–tibial joint was cut, the apodeme of the retractor unguis muscle was tied to the arm of a force transducer, and the apodeme was cut beyond this connexion (Text-fig. 1 B). A 25-gauge hypodermic needle was inserted through the base of the femur into the blood space in which the retractor unguis muscle lay, and saline was pumped at 0·1 ml/min through the femur to emerge through the cut membrane at the femoral–tibial joint. When dye was added to the perfusion fluid it was seen to pass quickly through the blood spaces of the leg, and back flow into the thorax was usually negligible or absent. When it was required to measure hydrostatic pressure within a perfused femur, the perfusion line was connected through a side arm to a pressure transducer (Consolidated Electrodynamics, Type 4-327-L221), which was further connected to the pen recorder. In such experiments the needle piercing the femur was of a larger diameter

(i.d. = 0·61 mm), to prevent the formation of pressure differences across the needle, and was sealed into the femur with wax to prevent leakage.

Experiments

The actions of L-glutamic acid on nerve–muscle preparations were studied in two ways: (i) by the sudden application of 2 ml 'pulses' of glutamate, which could stimulate muscle contractions, and (ii) by the prolonged exposure of the preparations to glutamate, which could reduce the force of the neurally evoked contractions, and which could cause other effects also. To study its agonist properties, glutamate was applied in 2 ml saline through a small funnel held over an isolated preparation (Text-fig. 1 A). The funnel was arranged so that the solution fell directly on to the muscle but without causing any mechanical disturbance visible on the force transducer record. The stimulator was stopped immediately before each test, and the recorder chart was run at 5 mm/sec during the test so that the form of the muscle contraction was recorded. To study the effects of glutamate on neurally evoked contractions, isolated and perfused-femur preparations were exposed to glutamate in saline at the normal flow rate, and the force of the neurally evoked contractions was measured after appropriate periods of exposure. When required, the chart speed was increased so that the form of the muscle contractions could be distinguished. Unless otherwise stated, all references to solutions of glutamic acid or of glutamate imply solutions of sodium L-glutamate (B.D.H.) in the standard saline. The pH of test solutions was always measured, and when necessary was adjusted to 6·8.

Salines

Several salines were used, and all were based on the salines described for use with locust preparations by Hoyle (1953) and Usherwood & Grundfest (1965). It was considered important that the salines should have osmolalities close to that of haemolymph, and so measurements were made with a Knauer Electronic Semi-Micro Osmometer, over a period of 2 years, of the osmolalities of samples of haemolymph from individual locusts, including both larvae and adults which had fledged two to four weeks previously. The following results were obtained:

Vth instar	366 mOsm	(S.D. = 22; n = 15)
Adult males	428 mOsm	(S.D. = 16; n = 20)
Adult females	407 mOsm	(S.D. = 18; n = 15)

Sucrose was added to the salines to bring their osmolalities close to that of the haemolymph of adult male locusts.

Standard saline

NaCl 140, KCl 10, NaH_2PO_4 4, Na_2HPO_4 6, $CaCl_2$ 2, sucrose 90 mmol/l. When necessary the pH was adjusted to 6·8 with NaOH or HCl. The osmolality of this saline is 410 mOsm, and without the sucrose it is 310 mOsm. This saline contains calcium and phosphate ions at near the solubility limit of calcium phosphate, and to prevent precipitation it is necessary to add the calcium chloride in solution after the other constituents have been dissolved in near maximum volume. When prepared carefully it can be kept quite satisfactorily at 5 °C for up to a week.

Tris saline

Tris saline was used when the actions of divalent metal ions were to be studied, and they could be added to the basic recipe given here. NaCl 140, KCl 10, Trizma base (Sigma) 10, $CaCl_2$ 2, sucrose 90 mmol/l. The pH was adjusted to 6·8 with HCl. The osmolality of this saline is 435 mOsm.

Chloride-free saline

CH_3SO_4Na 140, CH_3SO_4K 10, NaH_2PO_4 4, Na_2HPO_4 6, $CaSO_4$ 2, sucrose 90 mmol/l. The pH was adjusted to 6·8 when necessary, with H_2SO_4 or NaOH. The osmolality of this saline is 410 mOsm.

High-potassium saline

To prevent the rapid uptake of KCl and water by the muscles, which is liable to occur in high concentrations of potassium ions, the saline used for producing potassium contractures was designed so that the product of $[K^+] \times [Cl^-]$ was equal to that obtaining in the standard saline (Usherwood, 1967*a*, *b*). K_2SO_4 24, Na_2SO_4 55, NaCl 28, NaH_2PO_4 4, Na_2HPO_4 6, $CaCl_2$ 2, sucrose 90 mmol/l. The pH was adjusted to 6·8 with H_2SO_4 or NaOH when necessary. The osmolality of this saline is 350 mOsm. Application of a 2 ml pulse of this saline produced maximal potassium contractures. To obtain submaximal contractures it was mixed with the standard saline to give potassium concentrations of 25 m-equiv./l or less.

The extraction of haemolymph and assay of certain constituents

Haemolymph was obtained from adult male locusts by cutting off a mesothoracic leg at the base, applying slight pressure to the abdomen, and collecting the drops of haemolymph which fell from the small wound. When large volumes of haemolymph were required for experimental purposes Vth-instar locusts were used as the source.

The concentrations of sodium, potassium, calcium and magnesium in whole haemolymph were measured separately by atomic absorption spectrometry on samples of haemolymph taken from individual adult male locusts. The concentration of L-glutamic acid in whole haemolymph from individual adult male locusts was measured enzymically with L-glutamic dehydrogenase, using the procedure described by Bergmeyer (1965). Samples of haemolymph which were used for assay of glutamate were made to drop directly into 2·5% trichloroacetic acid, and sample volumes were calculated from the increase in weight of the collecting vessel (specific gravity of locust haemolymph = 1·06). Attempts to measure the glutamate concentration of haemolymph samples from individual locusts were frustrated at first because of the dense pigment which forms at the high pH of the enzymic assay. This problem was overcome by using a very stable spectrophotometer, the Zeiss PMQ II, to measure NADH formation, and backing off the absorption due to extraneous pigments. Measurements were also made of the concentrations of all non-peptide amino acids in pooled samples of locust haemolymph, using the Technicon NC-1 Automatic Amino Acid Analyser with Type A resin and gradient elution.

Preparation for electron microscopy

Tissues were fixed at room temperature with 3% glutaraldehyde in a dilute saline which consisted of 1 part standard saline and 2 parts water. The osmolality of the fixative was 544 mOsm, and its pH was adjusted to 6·8. Fixative was applied to the preparations in the same manner as saline, i.e. by irrigation in the case of isolated preparations, and by perfusion with the perfused-femur preparations. The isolated preparations and some perfused-femur preparations were exposed to saline for a period before fixation, but some perfused-femur preparations were fixed without prior perfusion with saline. All perfused-femur preparations were dissected in fixative after perfusion with fixative for 1 h or longer, and were then exposed to fixative for a further 0·5 h. The tissues were washed in buffered saline at room temperature, postfixed in 1% osmium tetroxide in buffer, and washed in buffer. The tissues were dehydrated with ethanol, and then infiltrated and embedded with Spurr's resin (Spurr, 1969). Thin sections were stained with uranyl acetate and lead citrate.

THE CHARACTERISTICS OF THE MUSCLE CONTRACTIONS

The different forms of muscle contraction, and the responses to glutamate

Retractor unguis muscles can contract in rather different ways under various experimental conditions, and it proved necessary to distinguish these different forms of contraction before attempting to interpret the results of different treatments. Studies were made on both isolated and perfused-femur preparations, and the following types of contraction were observed.

(1) *Neurally evoked contractions.* Twitch contractions caused by stimulating the crural nerve.

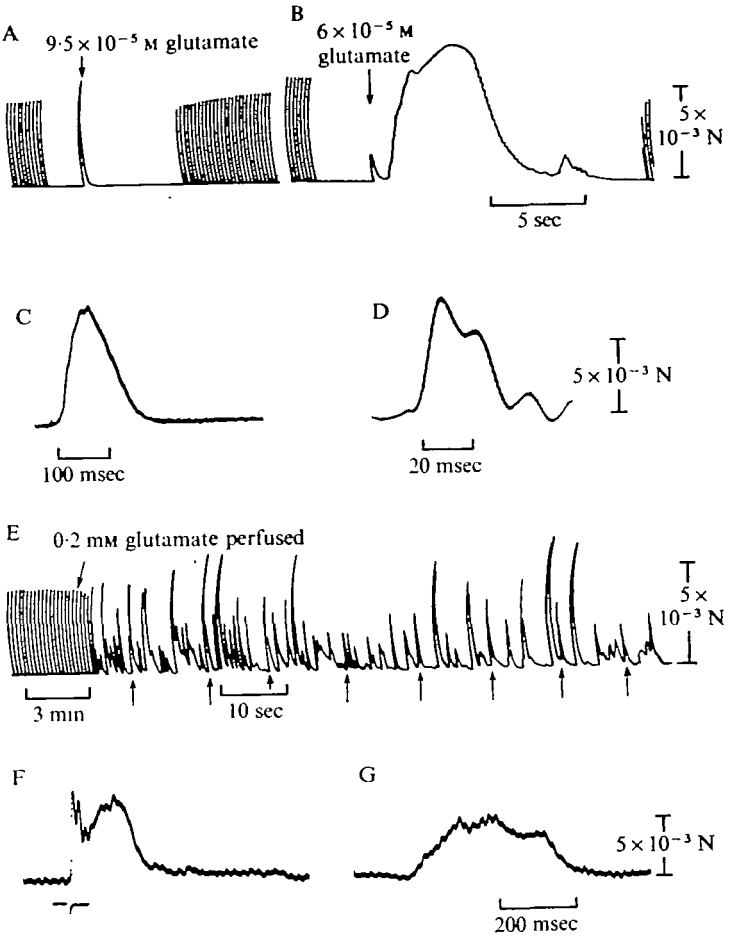
(2) *Repetitive contractions.* Contractions which occurred, under certain experimental conditions, immediately after the neurally evoked contractions.

(3) *Spontaneous contractions.* Contractions which sometimes occurred under the conditions which caused repetitive contractions, but which were separated in time from the neurally evoked and repetitive contractions. Spontaneous contractions could also occur in the absence of neurally evoked contractions.

(4) *Glutamate contractions.* Contractions caused by the sudden application of glutamate solutions, of sufficient concentration, to isolated preparations.

Neurally evoked contractions

The neurally evoked contractions, which were elicited by electrically stimulating the crural nerve, were similar in the isolated and perfused-femur preparations, and had the form of a twitch with a rise time, when measured on an oscilloscope, of 11 msec. When the stimulating voltage was slowly varied it was always possible to distinguish two twitch heights, which probably resulted from the firing of either one or both of the two axons that supply the muscle. At certain intermediate voltages, i.e. between 5 and 10 V but not above, the muscle contractions of the perfused-femur preparations would usually show a further substantial increase in amplitude, again



Text-fig. 2. Muscle contractions given by retractor unguis preparations. (A), (B) and (C) are glutamate contractions given by isolated preparations challenged with 2 ml pulses of glutamate solution. (A) is a twitch contraction, and (B) is a contraction with twitch and contracture components; in both cases the transducer output was damped to attenuate high-frequency oscillations. (C) is a glutamate contraction, undamped and photographed from an oscilloscope. (D) is a neurally evoked contraction obtained from the same preparation as (C) and recorded in the same way. (E) shows the repetitive and spontaneous contractions of a perfused-femur preparation. The start of the trace shows neurally evoked contractions recorded while the retractor unguis muscle was still bathed in haemolymph. On perfusion of the femur with 0·2 mM glutamate the neurally evoked contractions continued but, even at the faster chart speed, they were masked by repetitive and spontaneous contractions. The times of stimulation of the crural nerve, during the period of perfusion with glutamate, are indicated by arrows below the trace. (The slightly irregular spacing of the arrows is due to irregularity of chart movement.) (F) shows a neurally evoked contraction followed by repetitive contractions, undamped and photographed from an oscilloscope. (G) is a spontaneous contraction of the same preparation, recorded in the same way.

in two steps, whether the muscles were perfused with haemolymph or saline, but the cause of this further increase is not known. When isolated preparations were dissected in fast-flowing saline the amplitude of the neurally evoked contractions would stabilize after about 30 min. When perfused-femur preparations were first exposed to saline the twitch height would generally increase by about 30% and remain high for

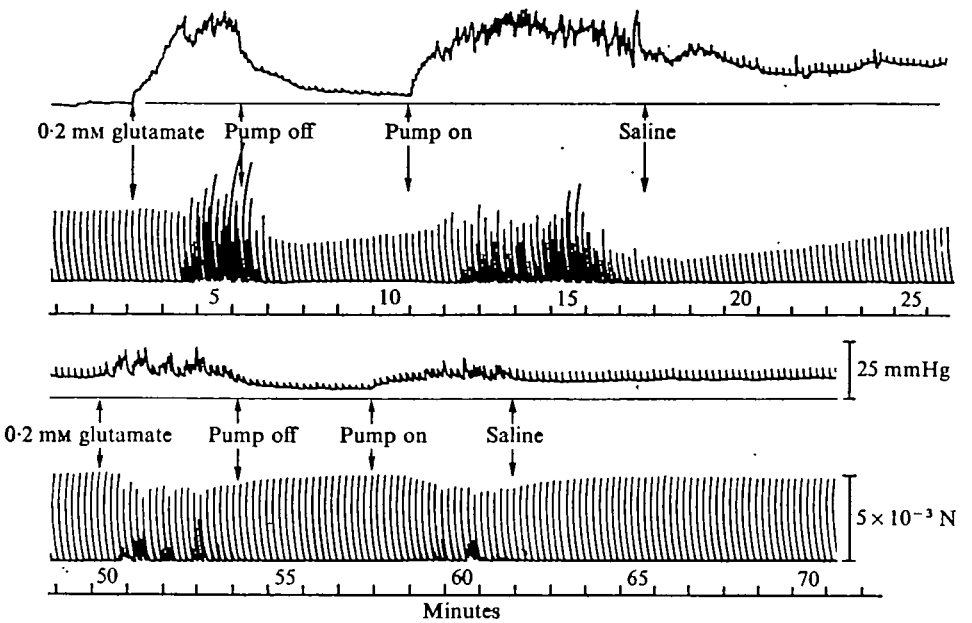
some minutes, but would later fall slightly and stabilize at an amplitude slightly higher than that originally recorded while the femur still contained its haemolymph. Both types of preparation were routinely stimulated at voltages which were low but sufficient to fire both axons, and under these conditions the force of the neurally evoked contractions was about 8×10^{-3} newtons (N), although damping of the transducer output normally reduced the measured force to about 5×10^{-3} N.

When L-glutamate was added to the saline used to irrigate isolated preparations, at concentrations greater than 10^{-5} M, the force of the neurally evoked contractions would fall gradually for a short time and then remain steady at a level which was dependent on the concentration. In many measurements, a linear relationship was always found between the extent of the depression of neurally evoked contractions and the log molar concentration of glutamate (Clements & May, 1974). The mean concentration of glutamate causing a 50% reduction in the twitch height of isolated preparations after 20 min irrigation was $6{\cdot}4 \times 10^{-5}$ M (S.D. = $1{\cdot}8 \times 10^{-5}$ M). Glutamate concentrations of 4×10^{-4} M or higher would generally block neuromuscular transmission in isolated preparations almost completely. Contrary to the observations of Usherwood & Machili (1968), we obtained no potentiation of neurally evoked contractions on irrigating isolated preparations with very low concentrations of glutamate (10^{-11} to 10^{-6} M). The amplitude of the neurally evoked contractions of perfused-femur preparations was reduced on perfusion with glutamate in saline but, as will be described in detail later, perfused-femur preparations were much less sensitive to glutamate, in this respect, than were isolated preparations.

Repetitive and spontaneous contractions

In the presence of glutamate both the isolated and perfused-femur preparations would sometimes give muscle contractions which were additional to the neurally evoked contractions. These additional contractions often occurred immediately after a neurally evoked contraction, in which case they were called 'repetitive contractions', but they also occurred well separated in time from the neurally evoked contractions, in which case they were called 'spontaneous contractions' (Text-fig. 2 E–G). The repetitive contractions most often consisted of one additional contraction which occurred from a few ms to 100 ms after the start of the neurally evoked contraction, but sometimes two or more additional contractions closely followed a neurally evoked contraction. The rise times of the repetitive contractions ranged from 60 to 200 ms, compared to the 11 ms rise time of the neurally evoked contractions. The rise times of the spontaneous contractions ranged from 160 to 240 msec. In the case of the isolated preparations, the repetitive and spontaneous contractions were normally not very pronounced and would stop within a minute or two after first exposure to glutamate, but with the perfused-femur preparations the abnormal contractions were stronger and more frequent, and might continue for 20 min or more.

It was observed that the effects of glutamate on perfused-femur preparations were greater at faster perfusion rates. A faster perfusion rate might cause flow through additional channels within the femur or it might cause an increase in hydrostatic pressure within the femur, and the second possibility was investigated by connecting a pressure transducer to the perfusion line. It was found that increases in pump speed led to increases in hydrostatic pressure in the perfusion line and, presumably,



Text-fig. 3. Record of the muscle contractions and hydrostatic pressure of a perfused-femur preparation at two stages in an experiment. The start of the trace shows the neurally evoked contractions recorded when the preparation had just been set up and while the retractor unguis muscle was still bathed in haemolymph. The hydrostatic pressure was zero at that time. After 3 min, perfusion with 0·2 mM glutamate in saline was started. The hydrostatic pressure rose sharply when the femur was first perfused, and fell when the pump was switched off. Abnormal muscle contractions occurred only when both glutamate was present and the pressure was high. After 50 min the pressure was low and the response to glutamate was slight.

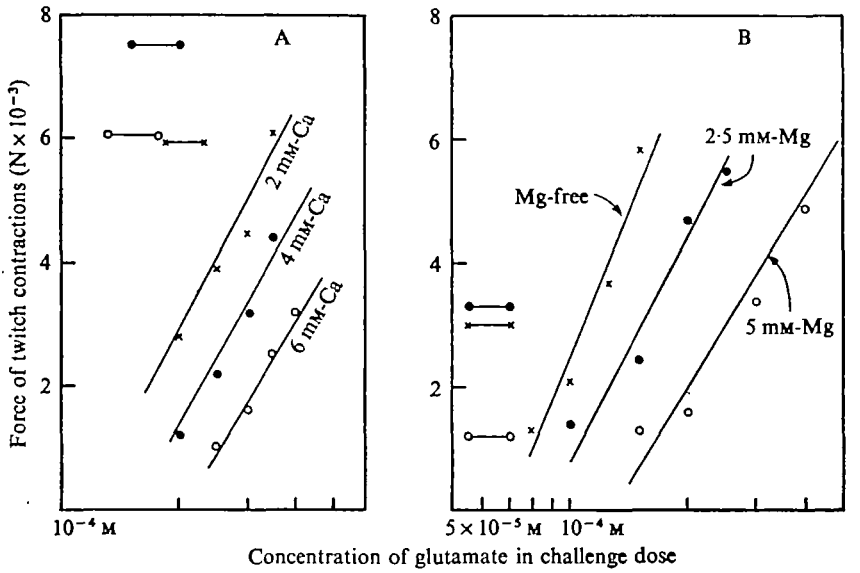
also within the femur itself, at least at its proximal end where the retractor unguis muscle is situated. Even at the normal low perfusion rate of 0·1 ml/min considerable hydrostatic pressure would build up when the standard saline was first perfused through the femur, although the pressure would always fall during the later stages of an experiment. During the first minutes of perfusion with standard saline, when the hydrostatic pressure was generally raised, the neurally evoked contractions of the retractor unguis muscle showed no change in form or amplitude which could be associated with the raised pressure, even up to 65 mmHg. However, if during this early phase of an experiment saline containing glutamate was perfused, the retractor unguis muscles gave strong repetitive and spontaneous contractions and the force of the neurally evoked contractions fell. In some such experiments neuromuscular transmission was blocked by glutamate concentrations as low as 0·2 mmol/l. If, during perfusion with glutamate, the perfusion pump was switched off for a few minutes, the hydrostatic pressure would fall almost to zero, the repetitive and spontaneous contractions would stop, and the force of the neurally evoked contractions would start to increase. Observations suggested that the perfusing saline did not drain out of the femur while the pump was switched off. When the pump was switched on again there was a rapid rise in hydrostatic pressure, the repetitive and spontaneous contractions quickly returned, and the twitch height started to fall

again (Text-fig. 3). Considerable variation was observed in the hydrostatic pressures which developed on perfusion with saline. In a series of 11 experiments the average pressure on first exposure to saline was 21 mmHg, and this rose to 27 mmHg when saline containing 0·2 mmol/l glutamate was perfused. The increased pressure recorded on perfusing glutamate possibly resulted from the frequent and strong muscle contractions which occurred at that time. After perfusion with saline for an hour practically all the preparations had lower hydrostatic pressures, the average being about 10 mmHg, and the preparations were little affected by 0·2 mmol/l glutamate at that time (Text-fig. 3). The osmolality of standard saline is slightly lower than that of the haemolymph of adult male locusts, and it seems probable that the tissues of the femur take up water and swell when first exposed to standard saline, but subsequently equilibrate with the saline. Preparations that maintained a high pressure for a long period showed a sensitivity to glutamate for a long period, but preparations in which the pressure fell after a few minutes perfusion with saline only showed sensitivity to glutamate for a few minutes. However, even during the later stages of an experiment, actions which led to a rise in hydrostatic pressure, such as changes in pump speed or in the osmolality of the saline, would also cause a return of sensitivity to glutamate.

Glutamate contractions

The sudden application of a 2 ml 'pulse' of glutamate solution to an isolated preparation would stimulate the retractor unguis muscle to contract. The characteristic form of a glutamate contraction was a twitch with a rise time of about 45–55 msec (Text-fig. 2A, C). When experimental conditions were carefully controlled, a linear relationship was always found between twitch force and log molar concentration of glutamate (Clements & May, 1974). In most cases the twitch threshold lay between 3 and 8×10^{-5} M. From dose/response curves obtained from 36 isolated preparations, the mean concentration of L-glutamic acid causing a twitch equal in force to the neurally evoked contractions was $2{\cdot}4 \times 10^{-4}$ M (S.D. = $1{\cdot}7 \times 10^{-4}$ M). Higher concentrations would stimulate twitch contractions of up to about twice the amplitude of the neurally evoked contractions. The force of the glutamate twitch contractions was depressed when isolated preparations were irrigated with $2{\cdot}5 \times 10^{-5}$ M or higher concentrations of glutamate, although lower concentrations of glutamate potentiated the contractions (see below).

Not infrequently the glutamate contractions given by isolated preparations would consist not only of a twitch but of a twitch followed by a brief contracture of 6 sec or more duration (Text-fig. 2B). Nothing certain was learned about the mechanism of the contractures, but the effects of different experimental conditions on the contractures was observed. The amplitude of the contractures bore no simple relationship to glutamate concentration, whether it was measured as maximum force or as the area within the curve of the contracture record. The contractures were observed more often during the first hours after a preparation had been set up than during the later hours. Contractures were given by preparations made from locusts denervated 5–7 days previously. Both twitches and contractures were obtained when isolated preparations were challenged with pulses of other excitatory amino acids including L-cysteic acid, DL-homocysteic acid, and kainic acid. Irrigation with 0·2 mM picro-



Text-fig. 4. The effects of irrigation with various concentrations of calcium and magnesium ions on the force of glutamate twitch contractions. (A) Upon irrigation successively with saline containing 2, 4, and 6 mM-$CaCl_2$. (B) Upon irrigation successively with Mg-free saline, 2·5, and 5 mM-$MgCl_2$ in saline (in each case with 2 mM-$CaCl_2$ also). The horizontal bars indicate the force of the neurally evoked contractions in each solution.

toxin potentiated both the twitch and contracture components of glutamate contractions, and would cause the appearance of glutamate-induced contractures in preparations which had previously not shown them. Irrigation with 1 mM 4-aminobutyric acid (GABA) caused a reduction in the force of glutamate-induced twitches and the disappearance of glutamate-induced contractures. The glutamate-induced contractures were not affected by a change in the irrigation saline from standard saline to chloride-free saline.

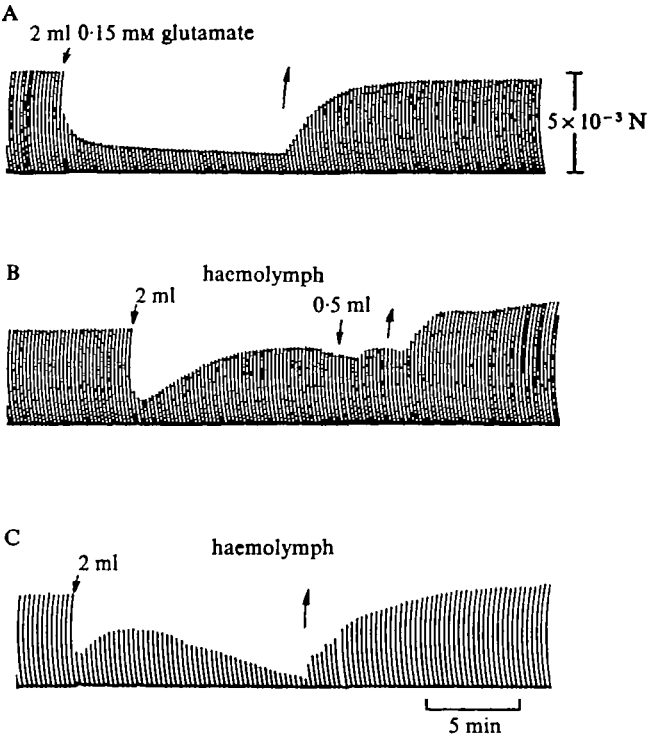
The effects of calcium and magnesium ions

Calcium and magnesium ions are known to have both pre- and postsynaptic effects on insect neuromuscular systems (Hoyle, 1955*b*). In the present study it was necessary to know the extent to which these ions affected muscle contractions in order to be able to interpret the behaviour of the retractor unguis preparations when exposed to haemolymph or to physiological salines. As the calcium concentration of the Tris saline was increased from 2 to 4 to 6 mM the force of the neurally evoked contractions increased progressively, but the force declined slightly at 8 mM (occasionally even at 6 mM) and declined further at 10 mM. When isolated preparations were irrigated successively with saline containing 2, 4 and 6 mM calcium chloride, and were challenged with pulses of glutamate, the dose/response curve shifted to the right at the higher calcium concentrations indicating that more glutamate was needed to stimulate muscle contraction (Text-fig. 4A). The repetitive and spontaneous contractions which often appeared when perfused-femur preparations were treated with

saline containing glutamate were progressively reduced when the calcium concentration was raised from 2 to 4 to 6 mM. Calculation of the extent of binding of glutamate and calcium ions from the stability constants cited by Lumb & Martell (1953), indicated that the effects of calcium ions on glutamate action were greater than could be accounted for by the binding of glutamate to calcium ions.

When 2·5 mM magnesium chloride was added to Tris saline, the force of the neurally evoked contractions given by isolated and perfused-femur preparations sometimes increased slightly, but on other occasions was reduced by up to 40%. Exposure to 5 mM magnesium chloride caused a marked reduction in the force of neurally evoked contractions. Treatment of isolated preparations with 2·5 mM magnesium chloride led to a marked reduction in the force of glutamate twitch contractions, and an even greater reduction followed treatment with 5 mM magnesium chloride (Text-fig. 4B). The repetitive and spontaneous contractions which often appeared when perfused-femur preparations were treated with saline containing glutamate were also reduced or eliminated by the presence of 3 mM magnesium. These effects of magnesium ions on glutamate actions were greater than could be explained by the binding between magnesium ions and glutamate, as calculated from the stability constants cited by Lumb & Martell (1953). Indeed, when 5 mM magnesium chloride was added to the challenge dose of glutamate, but not to the irrigated saline, the force of the glutamate contractions given by isolated preparations was only slightly reduced.

Attempts were made to find whether calcium and magnesium ions acted on the nerve–muscle preparations to alter the normal effect of prolonged exposure to glutamate, which was a depression of the neurally evoked contractions. The experimental results were not easy to interpret because the neurally evoked contractions increased in force as the calcium concentration was raised, and generally decreased in force in the presence of magnesium. However, these effects of the metal ions could generally be taken into account. The neurally evoked contractions of perfused-femur preparations were rather less depressed on prolonged exposure to 0·5 mM glutamate when the perfusing saline contained 4 to 6 mM calcium than when it contained 2 mM calcium. Similarly, the effects of 0·1 mM glutamate on isolated preparations were less in the presence of 4 or 6 mM calcium than in the presence of 2 mM calcium. These effects of calcium were greater than would be expected from the estimated binding of glutamate to calcium ions, and imply that calcium affects the preparations themselves in some way that reduces their sensitivity to glutamate. When isolated and perfused-femur preparations were treated alternately with salines containing magnesium chloride and glutamate it was found that both substances generally depressed the neurally evoked contractions, yet when magnesium (2·5–5 mM) and glutamate (0·1–0·5 mM) were applied simultaneously the depressant effects of the magnesium and glutamate were not additive. This result is difficult to explain. The estimated binding of glutamate and magnesium ions is not sufficient to explain the result, but the observation suggests that magnesium ions possibly acted on the preparations to reduce their sensitivity to glutamate. The extent to which the calcium, magnesium and glutamate in locust haemolymph affect neuromuscular systems in the living insect will depend upon the concentrations of these various ligands that are free, not bound, and this point is developed later in this paper.



Text-fig. 5. The effects of haemolymph on the neurally evoked contractions of two isolated preparations. Small volumes of cell-free haemolymph, or glutamate, were cautiously pipetted into the bath, with no subsequent flow of saline until the preparation was washed. (A) Response to 0·15 mM glutamate. (B) Response of the same preparation to haemolymph, shortly after the exposure to glutamate. (C) Response of another preparation to haemolymph.

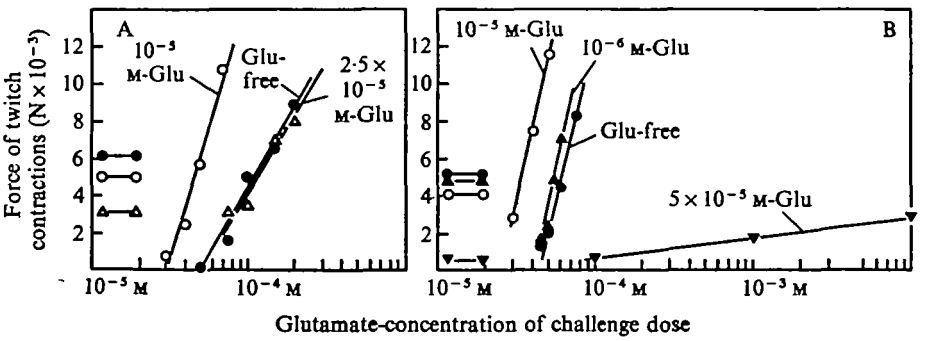
The responses to haemolymph

Pooled haemolymph from Vth-instar locusts was used for studies on the effects of haemolymph on retractor unguis preparations. It was found that the preparations responded in the same way to whole, sonicated, and cell-free haemolymph, and so cell-free haemolymph which could be most easily perfused was normally used. The effects on over 30 isolated preparations of a fairly prolonged exposure to haemolymph were examined by carefully pipetting 1–2 ml of haemolymph into the bath, so displacing the saline, and recording the neurally evoked contractions for 10–20 min, during which period there was no flow of saline. The characteristic response was an immediate fall in twitch height of about 50% followed by a partial recovery. Over half of the preparations then maintained the twitch height in the continued presence of haemolymph, and on washing with saline the twitch height not only returned to normal but was enhanced by up to 30% for the following one to two hours (Text-fig. 5). The other preparations showed a subsequent decline of twitch height in the continued presence of haemolymph, two preparations declining to near block, and although the twitch height was partially or wholly restored on washing it was not enhanced. The preparations were also exposed to 2 ml of 0·1 or 0·2 mM glutamate applied in the same manner as the haemolymph. 0·1 mM glutamate generally de-

pressed the twitch height by about the same amount as did haemolymph, but 0·2 mM glutamate caused a greater depression than haemolymph. The composition of haemolymph is so complex that it cannot be claimed that the depression of twitch height observed in the presence of haemolymph was due to its content of glutamic acid. When isolated preparations irrigated with saline were challenged by the sudden application of pulses of haemolymph, the retractor unguis muscles would contract. The form of the contraction was a twitch, sometimes followed by a brief contracture.

When perfused-femur preparations were perfused with haemolymph from Vth-instar larvae, without prior perfusion with saline, the hydrostatic pressure would often rise substantially; pressures up to 70 mmHg were recorded. This rise in hydrostatic pressure was not surprising because the mean osmolality of haemolymph samples from Vth-instar locusts was 366 mOsm. (S.D. = 22), whereas that of haemolymph samples from adult male locusts, aged 2–4 weeks after fledging, was 428 mOsm (S.D. = 16). Perfusion of an adult femur with larval haemolymph would be expected to cause water uptake by the tissues, with consequent swelling and reduction in flow. Simultaneous with the rise in hydrostatic pressure which accompanied perfusion with Vth-instar haemolymph could be observed the onset of repetitive and spontaneous contractions. If the perfusion pump was switched off briefly the hydrostatic pressure would fall and the abnormal contractions would stop, although the femur apparently remained full of haemolymph. When pumping of haemolymph was resumed the rise in hydrostatic pressure was accompanied by the reappearance of abnormal contractions. When perfused-femur preparations which had been perfused with saline for many minutes were perfused with haemolymph, the twitch height would drop to a level close to that which had been recorded at the start of the experiment when the femur still contained its own haemolymph. In most cases where haemolymph was perfused late in an experiment there was no rise in hydrostatic pressure, and the preparations showed little tendency to give repetitive and spontaneous contractions.

At raised hydrostatic pressures the perfused-femur preparations responded more severely to 0·2 mM glutamate in standard saline than to haemolymph, although at very high pressures both 0·2 mM glutamate and haemolymph could block neuromuscular transmission. Perfused-femur preparations generally responded in a rather similar manner to haemolymph and to saline containing both 0·2 mM glutamate and 3 mM magnesium. When *perfused-femur* preparations which had been perfused with saline for some time were perfused with haemolymph and then with haemolymph containing an additional 0·4 mmol/l glutamate, there was a fall in twitch height on the change from saline to haemolymph, but there was no further fall in twitch height when the haemolymph containing added glutamate was perfused, although a few preparations gave rather more repetitive and spontaneous contractions. In contrast, when *isolated* preparations which had been irrigated with saline were exposed to haemolymph and then to haemolymph containing an additional 0·2 or 0·4 mmol/l glutamate, the twitch height fell on first exposure to haemolymph and fell further when the haemolymph with added glutamate was applied. It appeared therefore that the isolated preparations showed a greater sensitivity to glutamate added to haemolymph than did the perfused-femur preparations.

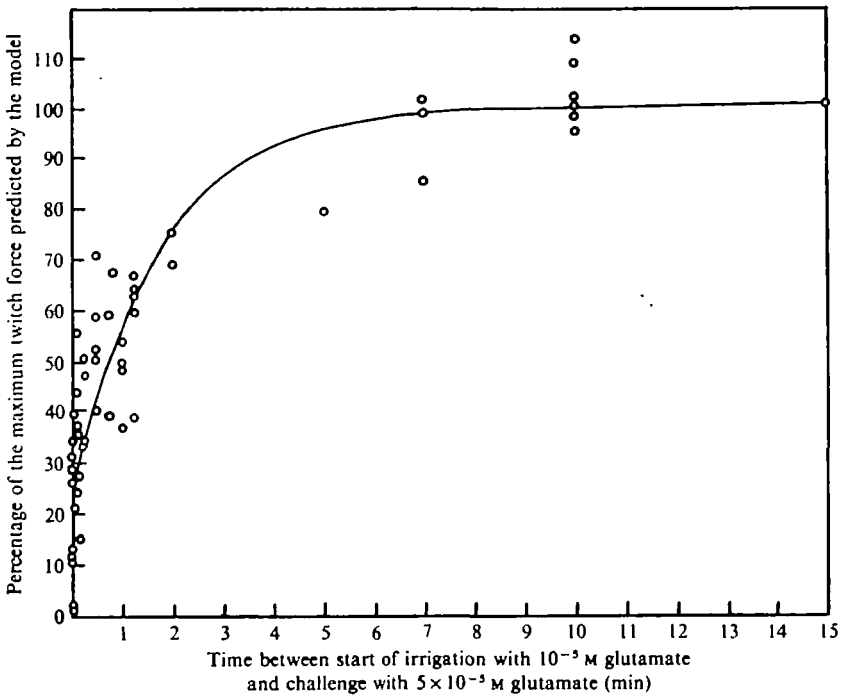


Text-fig. 6. Potentiation and depression of the glutamate twitch contractions given by isolated preparations on irrigation with low concentrations of glutamate. Retractor unguis muscles were stimulated to contract by application of 2 ml pulses of glutamate. The relationship between twitch force and concentration of glutamate in the challenge pulse is shown under conditions of irrigation with glutamate-free saline and with saline containing different concentrations of glutamate. The horizontal bars indicate the force of the neurally evoked contractions in each solution.

Potentiation of glutamate contractions by glutamate

The muscles of a living insect are bathed by haemolymph which contains glutamate, and so it was of interest to measure the responses of isolated retractor unguis preparations to pulses of glutamate while they were irrigated with saline containing glutamic acid. In a series of experiments, isolated preparations were allowed to equilibrate with glutamate-free saline, and were then irrigated with salines of increasing glutamate concentration for periods of 1–2 h at each concentration. After the neurally evoked contractions had settled at a constant height in each solution, the preparations were challenged with pulses of glutamate, and dose/response curves were obtained. Isolated preparations which were irrigated with low concentrations of glutamate showed an increased sensitivity to challenge with pulses of glutamate. During irrigation with 10^{-6} M glutamate the curve relating twitch force with concentration of glutamate in the challenge dose shifted slightly towards lower glutamate concentrations (Text-fig. 6B). During irrigation with 10^{-5} M glutamate the curve shifted strongly towards lower glutamate concentrations (Text-fig. 6A, B). Irrigation with 5×10^{-5} M glutamate caused a very marked decrease in sensitivity to pulses of glutamate (Text-fig. 6B), and irrigation with concentrations between 1 and 5×10^{-5} M produced variable results, in some preparations increasing the sensitivity to glutamate, but in others decreasing it. It is interesting to observe that a sudden increase in glutamate concentration from 1×10^{-5} M (irrigating concentration) to 4×10^{-5} M (challenge concentration) is sufficient to stimulate a strong twitch contraction (Text-figs. 6A, B). In contrast to the twitch component of the glutamate contraction, the contracture component was unchanged by irrigation of preparations with 10^{-5} M glutamate.

Preparations made with muscles denervated 5–7 days previously, which failed to respond to indirect electrical stimulation, would contract when challenged with L-glutamic acid, and the glutamate contractions became considerably stronger when the denervated preparations were irrigated with 10^{-5} M glutamate. This showed that the increase in sensitivity was not a presynaptic phenomenon involving increased



Text-fig. 7. Time course of the development of potentiation of glutamate contractions shown by seven isolated preparations following irrigation with 10^{-5} M glutamate. The preparations were stimulated to contract by challenge with 2 ml pulses of 5×10^{-5} M glutamate while irrigated with glutamate-free saline, and after irrigation for different periods with 10^{-5} M glutamate. The points marked are the experimental observations, and the curve shows the relationship obtained with the equation actual response $= A(100 - B')e^{-k(t-60)}$, in which A = maximum twitch force/100 for any preparation, and B' = percentage of maximum response obtained from zero time onwards.

release of transmitter. Irrigation with 10^{-5} M glutamate had no effect on the force of the submaximal potassium contractures which were produced on challenging preparations with 2 ml of saline containing 25 m-equiv. of potassium ion per litre.

Experiments were undertaken to measure the rate of development of the change of sensitivity which followed irrigation with 10^{-5} M glutamate. Seven isolated preparations were irrigated with glutamate-free saline and challenged with a 2 ml pulse of 5×10^{-5} M glutamate. These preparations were then irrigated with 10^{-5} M glutamate, the bath contents being changed rapidly, and were challenged seconds or minutes later with another pulse of 5×10^{-5} M glutamate. After each challenge the preparations were re-equilibrated with glutamate-free saline before further irrigation with 10^{-5} M glutamate and further challenge. The increased sensitivity was seen as potentiation of the response to 5×10^{-5} M glutamate, and it was found that full potentiation of the glutamate contraction took 7 min or more to develop (Text-fig. 7). Statistical analysis of the results from the seven preparations showed that after irrigation with 10^{-5} M glutamate for 60 sec the twitch height had reached, on average, only $56{\cdot}7 \pm 8{\cdot}6\%$ (95% confidence limits) of the maximum attained later by the same preparation. In contrast to its relatively slow development, the potentiation

of the glutamate contractions disappeared very rapidly when the irrigating saline was changed to glutamate-free. Indeed, two preparations which were challenged with glutamate 15 and 30 sec after the baths had been flushed with glutamate-free saline showed no potentiation.

The slight reduction in the force of the neurally evoked contractions observed during irrigation with 10^{-5} M glutamate can probably be explained as an action of glutamate on the postsynaptic membrane, since Usherwood (1967*b*) has shown that glutamate, at such concentrations, reduces the amplitude of miniature EPSPs, but no explanation is apparent for the simultaneous potentiation of glutamate contractions. Walther & Usherwood (1972) found that irrigation of a *Schistocerca* retractor unguis preparation with 9×10^{-6} M L-glutamate caused a maintained depolarization of about 4 mV, and they suggested that the potentiation of glutamate contractions which is described in the present paper could be explained by a summation of glutamate potentials with a maintained depolarization. This explanation accords with the relationship that is known to hold between membrane potential and tension in crustacean muscle fibres (Orkand, 1962) but two observations contradict it. First, potassium contractures were not potentiated by irrigation of the preparation with 10^{-5} M glutamate, and second, the maintained depolarization described by Walther & Usherwood (1972) reached a maximum within 30 sec, whereas potentiation of the glutamate contractions took 7 min or more to reach its maximum. The time course of the onset of potentiation also precludes the possibility that the glutamate receptor has an allosteric mechanism which is an important factor in the increase of sensitivity.

HAEMOLYMPH GLUTAMATE AND ITS RELATIONSHIP TO NEUROMUSCULAR TRANSMISSION

Insect nerve–muscle preparations are sensitive to L-glutamic acid, yet relatively high concentrations of L-glutamic acid are found in insect haemolymph. The resolution of this paradox must lie in a mechanism which protects the sensitive component, presumably synaptic receptors, from the haemolymph glutamate. The experimental approach reported here involved: (1) a re-analysis of locust haemolymph for L-glutamic acid and other constituents, (2) a search for a component of haemolymph that might bind glutamic acid, and (3) a search for a diffusion barrier.

The composition of locust haemolymph

Haemolymph was obtained from adult male *Schistocerca gregaria* which had fledged 2–4 weeks earlier, and the concentrations of sodium, potassium, calcium, magnesium, and L-glutamic acid were measured in separate haemolymph samples taken from individual locusts. Pooled haemolymph samples were used to assay non-peptide amino acids and to measure the total non-peptide amino acid concentration. The results of the assays are summarized in Table 1.

The mean concentration of L-glutamic acid, measured in individual haemolymph samples from 19 adult male locusts, was 0·22 mmol/l, and the range was 0·06–0·45 mmol/l. These results were confirmed by assays on pooled samples of haemolymph, measured enzymically and by automatic amino acid analysis. The haemolymph of Vth-instar locusts contained similar concentrations of L-glutamic acid.

Table 1. *The concentrations of certain constituents of the haemolymph of adult male* Schistocerca gregaria

Constituent	Mean concentration (mmol/l)	Standard deviation	No. of samples
Potassium	8·1	1·3	34
Sodium	121·2	9·6	34
Calcium	5·0	1·1	43
Magnesium	11·6	2·9	43
L-Glutamic acid	0·22	0·10	19
Total amino acids	45·0	—	4

The exposure of isolated retractor unguis preparations to such concentrations of L-glutamic acid in saline would severely depress the neurally evoked contractions and could even block neuromuscular transmission. Assays of all free amino acids in the haemolymph, made by automatic amino acid analysis, showed that the concentration of glutamic acid was not exceptionally high or low when compared with those of other amino acids, although it was well below those of glycine and proline which were sometimes present at 10 mmol/l or more. The total concentration of amino acids in haemolymph ranged from 34 to 54 mmol/l, with a mean of 45 mmol/l.

It is important to know whether binding between amino acids and divalent metal ions appreciably affects the availability of such components of haemolymph as glutamate and calcium and magnesium ions. As direct measurements of complex formation could not be made, the approximate extent was calculated from the published stability constants for amino acid–metal complexes. In solutions of pH 6·8 approximately 99% of glutamate molecules will exist as the monoanion with all three functional groups ionized, and the reaction of glutamate with the common divalent metal ions will be predominantly as follows:

$$\underset{a-x-y}{\text{Glutamate}^-} + \underset{b-x}{\text{Mg}^{2+}} \rightleftharpoons \underset{x}{\text{Mg-Glu}^+} \quad K_1 = \frac{x}{(a-x-y)(b-x)},$$

$$\underset{a-x-y}{\text{Glutamate}^-} + \underset{c-y}{\text{Ca}^{2+}} \rightleftharpoons \underset{y}{\text{Ca-Glu}^+} \quad K_2 = \frac{y}{(a-x-y)(c-y)},$$

where a, b and c are the initial concentrations of the glutamate, magnesium and calcium ions, and x and y those of the complexes. Lumb & Martell (1953) measured the stability constants of complexes between the glutamate dianion

$$^-OOC—CH_2—CH_2—CH(NH_2)COO^-$$

and magnesium and calcium ions, and obtained the values $K_1 = 80$ and $K_2 = 27$. For present purposes the assumption is made that the stability constants known for the dianion are applicable to the monoanion, and this appears reasonable since the stability constants of amino acids such as alanine are close to those of the glutamate dianion. The additional assumption is made that these same constants can be used to obtain a working approximation of the extent of binding between the magnesium and calcium ions and the total non-protein amino acids of locust haemolymph.

Table 2. *Concentrations of divalent metal ions and amino acids which are free and bound in locust haemolymph, calculated from the stability constants of glutamate-metal complexes*

	Approximate proportion bound	Total concn (mM)	Approximate concn bound (mM)	Approximate concn free (mM)
Calcium	48% (to amino acids)	5·0	2·4	2·6
Magnesium	73% (to amino acids)	11·6	8·5	3·1
Glutamic acid	5·3% (to calcium) 18·7% (to magnesium)	0·22	0·05	0·17
Total amino acids	5·3% (to calcium) 18·7% (to magnesium)	45	10·8	34·2

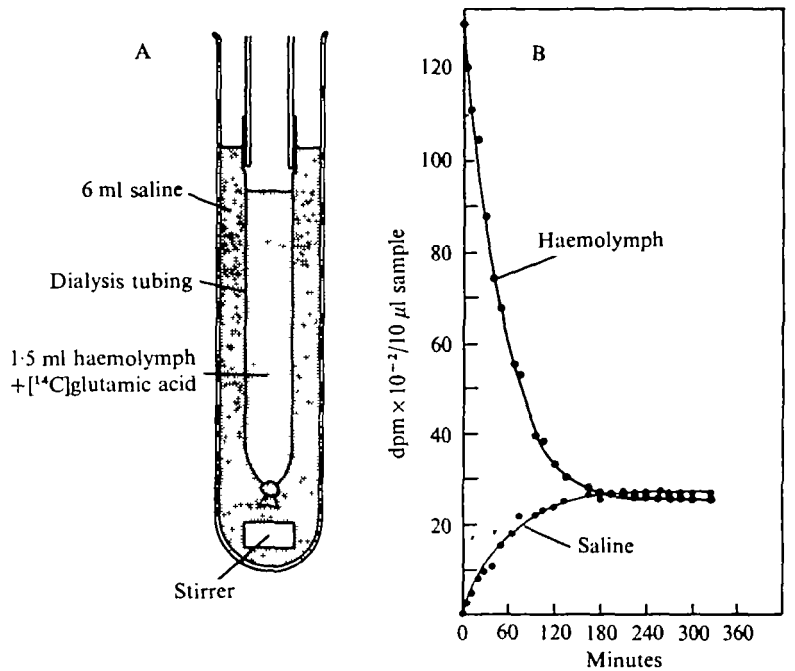
Table 2 lists the mean concentrations in locust haemolymph of these various ligands, and shows the proportions which are calculated to be bound and free at equilibrium. The calculations indicate that the concentration of free glutamate is reduced by about 25% through complex formation, whereas the concentration of calcium ions is reduced by about 50% and that of magnesium ions by about 75%. These values should be regarded as very approximate, since the stability constants of some amino acids are lower and those of other amino acids higher than those of glutamate.

Tests for the sequestration of glutamic acid by haemocytes and plasma proteins

Because calculation of the extent of binding between glutamate and divalent metal ions in locust haemolymph showed that only about 25% of the glutamate is bound in this way, it appeared that the formation of metal-amino acid complexes cannot protect neuromuscular synapses completely from haemolymph glutamate. However, other systems are known which sequester small molecules in blood. In the crab *Carcinus maenas* (L.) the blood amino acids are contained very largely within the haemocytes (Evans, 1972; Miller, Leaf & Usherwood, 1973), and the plasma albumin of some mammals and birds has a high affinity binding site for indoles, including L-tryptophan (McMenamy & Watson, 1968). The possibility was explored that the glutamate present in locust haemolymph is sequestered in one of these ways.

When haemolymph was taken from a number of locusts, pooled and centrifuged at a low speed so that the haemocytes sedimented without rupturing, the glutamate content of the plasma was found to be similar to that of whole haemolymph. It is not possible to say that when haemolymph was removed from locusts the haemocytes remained unchanged in their glutamate concentration, but there is no reason to believe the contrary. It can only be said that no evidence was found for any sequestration of glutamate by haemocytes. Miller *et al.* (1973) conducted similar experiments with *Schistocerca* haemolymph, and also concluded that there was no sequestration of glutamic acid by the haemocytes.

Equilibrium dialysis was used to test for the binding of glutamic acid by haemolymph proteins. Haemolymph taken from a number of adult or larval locusts was pooled and centrifuged gently to remove the haemocytes. A small amount of radioactive L-glutamic acid was added to the cell-free haemolymph, which was then left for 1 h. The haemolymph was then placed inside a length of dialysis tubing and dialysed against 4 vol. of Tris saline. 10 μl samples were taken for radioassay at 10 min



Text-fig. 8. Equilibrium dialysis of locust haemolymph. (A) Apparatus used, 1·5 ml cell-free haemolymph, containing an added 1·5 µl of [^{14}C]L-glutamic acid solution, was dialysed against 6 ml saline. (B) Radioactivity of samples of haemolymph and saline taken at 10 min intervals.

intervals. In a series of experiments it was found that after 2–3 h dialysis the levels of radioactivity were identical in the cell-free haemolymph and saline, indicating that there was no binding of glutamic acid by any non-diffusible material in the haemolymph. The design and results of a typical experiment are shown in Text-fig. 8.

Locusts were little affected by the injection of considerable quantities of glutamate into the haemocoele. For example, the injection of 20 µl 0·1 M sodium glutamate had no discernible effect on the walking or flight of an adult locust, although it should have temporarily raised the haemolymph glutamate concentration to 3 mM, on the assumption that adult male locusts have a haemolymph volume of 680 µl (Lee, 1961; Hill, Luntz & Steele, 1968). It appeared possible that the injected glutamate was sequestered by haemocytes or plasma proteins, and to test this possibility the injection experiment was repeated with locusts whose haemolymph had been replaced with saline. A piece of cuticle was cut out of the front of the head of each locust in the region where the aorta discharges, the locust was held in an inverted position so that its haemolymph drained out, and standard saline was pumped into the haemocoele through a hypodermic needle inserted into the abdomen. Over a period of 30 min the haemolymph was displaced by passing 2–3 ml of saline through the locust. Control locusts were operated on, but their haemolymph was not displaced. When perfusion was stopped the locusts remained inverted for 6–8 min to allow excess saline to drain out. They were then taken down, and both perfused and unperfused locusts were able to walk and fly strongly. One to two minutes after being taken down the locusts were injected with 20 µl 0·1 M sodium glutamate, and additional injections of 10 µl 0·1 M

Table 3. *Muscle contractions given by isolated retractor unguis preparations when challenged with drops of locust haemolymph*

Age of haemolymph	No. of challenges	Response (%)		
		No response	Weak + medium	Strong
Direct from locust	18	50	28	22
1–4 min	49	65	24	10
5–59 min	49	37	28	35
60 min +	48	19	54	27

sodium glutamate were made at 2 min intervals. After each injection walking and flight abilities were examined. With the three perfused and three unperfused locusts which were tested it required the injection of 40 μl or more of 0·1 M sodium glutamate to seriously reduce or stop walking and flight, and there was little difference of sensitivity between the perfused and unperfused individuals. This experiment confirmed that *in vivo* the locust muscles concerned with walking and flying are relatively insensitive to injected glutamate, and so suggested that haemolymph glutamate is not necessarily sequestered by haemocytes or haemolymph proteins.

Miller *et al.* (1973) removed haemolymph from *Schistocerca*, allowed it to age for varying periods, and then applied it as drops to isolated retractor unguis preparations. They found that haemolymph which had been aged could stimulate muscle contractions, but that haemolymph tested within 5 min of removal from the locust could not stimulate muscle contractions. They concluded that the contractions they observed were possibly glutamate contractions, and that the failure of the freshly drawn haemolymph to stimulate contractions suggested that the glutamate was bound in some way in the haemolymph. Our attempts to repeat these experiments did not lead to very similar findings (Table 3). Aged haemolymph stimulated muscle contractions somewhat more frequently than did freshly drawn haemolymph, but haemolymph dropped directly from wounded locusts stimulated muscle contractions in 50% of cases, suggesting that the stimulating agent must be free in normal haemolymph.

Search for a diffusion barrier

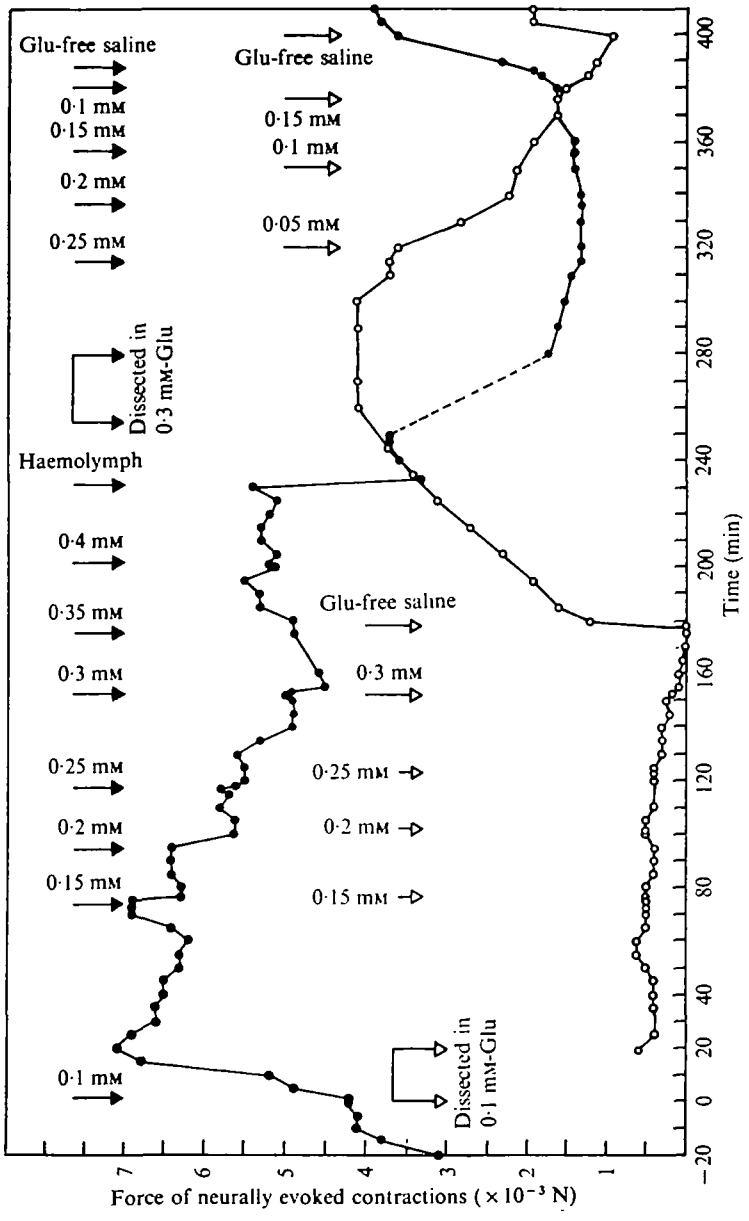
When the search for a sequestering mechanism in haemolymph proved negative, the possibility of a structural diffusion barrier was considered. To test the hypothesis that dissection of the isolated retractor unguis preparation damaged a diffusion barrier which in life protected the neuromuscular synapses from haemolymph glutamate, the perfused-femur preparation, described in the Methods section, was developed. With this preparation it was possible to observe the effects of glutamic acid on the mechanical responses of retractor unguis muscles which had suffered no mechanical disturbance from dissection, and it was possible to compare the actions of glutamic acid on isolated preparations and perfused-femur preparations derived from the same individual.

At the start of each experiment with a perfused-femur preparation, the crural nerve would be connected to a suction electrode, and the saline supply would be connected via a hypodermic needle inserted into the base of the femur. The retractor unguis muscle would then be stimulated via the crural nerve, but without perfusion of the

femur, and the contractions of the retractor unguis muscle would be observed, from the movement of the tarsus, for a period of an hour. After this time the soft membrane at the femoral–tibial joint would be cut, and the apodeme of the retractor unguis muscle tied to a strain gauge. The muscle contractions would be recorded directly for a period of about 5 min, while the muscle continued to be bathed in haemolymph, and perfusion with saline would then be started. It was known that the perfused saline did indeed bathe the retractor unguis muscle, because when the potassium concentration of the saline was raised or the calcium concentration was lowered a response was seen in the perfused preparation as quickly as in an isolated preparation run in parallel.

More than 100 experiments were carried out in which the responses of perfused-femur preparations were compared with those of isolated preparations made from the same individual. The characteristic behaviour of a freshly dissected isolated preparation was an initial brief decline in the force of the neurally evoked contractions from that recorded on first stimulation, and then a slow recovery over a period of 30–60 min, after which the force of the neurally evoked contractions stabilized at about 5×10^{-3} N. In contrast, the perfused-femur preparations normally gave neurally evoked contractions of about 3×10^{-3} N while bathed in their own haemolymph, but the twitch force rose sharply to 6 or 7×10^{-3} N when the haemolymph was displaced with saline, and later stabilized at about 5×10^{-3} N. It was soon found that perfused-femur preparations were much more tolerant of glutamate in saline than were isolated preparations, and not infrequently isolated preparations would be blocked or severely depressed by concentrations of glutamate which did not reduce the twitch height of perfused preparations to the levels previously recorded in haemolymph. Perfused preparations did however, show a much greater tendency than isolated preparations to give repetitive and spontaneous contractions on exposure to glutamate (Text-fig. 10A).

The different sensitivities of the isolated and perfused-femur preparations will be illustrated by one fairly typical experiment (Text-fig. 9). The right leg of a locust was set up ready for perfusion, and its retractor unguis muscle was stimulated indirectly for 20 min while bathed in haemolymph. The twitch height increased somewhat during the first 10 min and then became steady. At time 0 perfusion with 0·1 mM glutamate in saline was started and the twitch height increased strongly. At the same time dissection of the left leg was started in saline containing 0·1 mM glutamate, and after 18 min the weak contractions of the isolated preparation were first recorded. After 75 min the concentration of glutamate was progressively raised. The twitch height of the perfused-femur preparation fell slightly as the concentration rose to 0·4 mM, and when this was followed by perfusion with cell-free haemolymph the twitch height dropped to that recorded at the start of the experiment. The isolated preparation was strongly depressed by all concentrations of glutamate and contractions finally stopped in 0·3 mM glutamate. The isolated preparation recovered strongly when it was irrigated with glutamate-free saline, but declined again on exposure to glutamate. After 255 min the perfused-femur preparation was dissected in 0·3 mM glutamate and converted into a second isolated preparation. This also was fairly strongly depressed by glutamate but recovered in glutamate-free saline. From results such as these it was concluded that a retractor unguis muscle within an intact femur contains a diffusion barrier which is damaged or destroyed by the mechanical disturbance involved in dissecting out the isolated preparation.



Text-fig. 9. Comparison of the effects of glutamic acid on the neurally evoked contractions of isolated and perfused-femur preparations. The force of the neurally evoked contractions of retractor unguis muscles was recorded from an isolated preparation dissected from the left leg of a locust, and from a perfused-femur preparation from the right leg of the same locust. The closed symbols (● and ⬇) refer to the perfused-femur preparation, and the open symbols (○ and ⇩) refer to the isolated preparation. For the period −20 to 0 min the muscle within the right femur was bathed in haemolymph. At time 0 perfusion of the right femur with 0·1 mM glutamate was started and dissection of the left leg in 0·1 mM glutamate was also started.

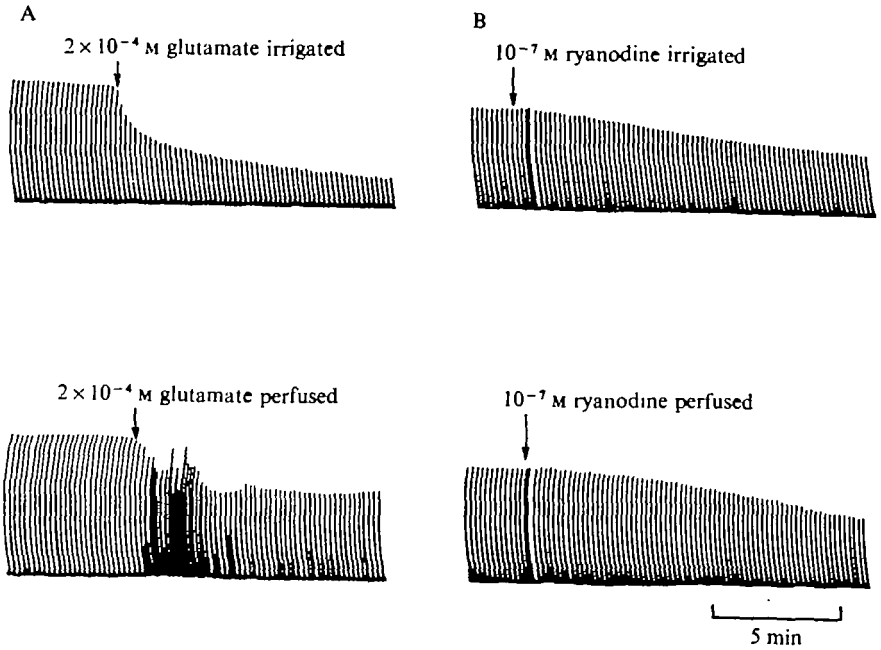
Because some perfused-femur preparations showed appreciable sensitivity to glutamate it was considered important to know the concentration of glutamate in the haemolymph of the individual locusts from which nerve-muscle preparations were derived. Samples of haemolymph were taken from seven locusts, which were shortly afterwards dissected to give isolated and perfused-femur preparations. The sensitivities of the preparations to irrigation or perfusion with glutamate solution were found while the L-glutamate concentrations of the haemolymph samples were measured enzymically. The haemolymph glutamate concentrations ranged from 0·075 to 0·40 mM. In most cases the neurally evoked contractions of the perfused-femur preparations were depressed about 50% by concentrations of glutamate in saline equal to or lower than the haemolymph glutamate concentration of the same individual. These preparations gave very marked repetitive and spontaneous contractions on perfusion with such concentrations of glutamate. In contrast, the isolated preparations showed few repetitive or spontaneous contractions on irrigation with glutamate, but their neurally evoked contractions were much more strongly depressed than those of the perfused-femur preparations. These results indicated that the diffusion barrier, which was postulated to be intact in the perfused-femur preparations, did not fully protect the neuromuscular system from concentrations of glutamate such as occur in haemolymph, at least under these experimental conditions. It was later found that, as has been described above, perfused-femur preparations were very sensitive to glutamate when the hydrostatic pressure was raised, as it often was on first perfusion with saline, and were relatively insensitive when the pressure was low. This effect of pressure almost certainly accounts for the responses of the seven perfused-femur preparations to glutamate at haemolymph concentrations, and the differences between the isolated and perfused-femur preparations in sensitivity to glutamate can still be taken as indirect evidence of a diffusion barrier.

Location of the diffusion barrier

Experimental evidence

If the postulated diffusion barrier protects only the nerve endings, then one would expect isolated preparations (with a damaged diffusion barrier) to be more sensitive to synaptic blocking agents than the perfused-femur preparations (with an intact diffusion barrier), but one would expect compounds acting on the non-synaptic membrane to affect both preparations equally. In contrast, if the diffusion barrier protects the whole muscle, then the (damaged) isolated preparations should show greater sensitivity than the (intact) perfused-femur preparations to both classes of compounds. Compounds that are believed to act predominantly on the non-synaptic muscle membrane include ryanodine (Usherwood, 1962), ibotenic acid (Lea & Usherwood, 1973*a*, *b*), and potassium chloride (Hoyle, 1953). The action of glutamic acid is predominantly at the neuromuscular synapse (Usherwood & Machili, 1968). Harmine blocks the response of the locust retractor unguis muscle to glutamate, and preliminary experiments suggest that it is acting at the postsynaptic membrane (Clements & May, 1974).

To test this hypothesis, isolated and perfused preparations were set up in pairs, each pair being derived from the same locust, and were exposed to one of the five compounds at various concentrations for periods up to 50 min. Ryanodine depressed



Text-fig. 10. Comparison of the sensitivities of isolated and perfused-femur preparations. (A) Effects of L-glutamic acid on the neurally evoked contractions of isolated and perfused-femur preparations from the same individual. (B) Effects of ryanodine on the neurally evoked contractions of isolated and perfused-femur preparations prepared from the same individual.

the neurally evoked contractions of the irrigated and perfused preparations to an almost identical extent (Text-fig. 10B). With potassium chloride there was on average less than 10% difference in effect on the isolated and perfused preparations. Ibotenic acid depressed the perfused-femur preparations on average about two-thirds as much as it depressed the isolated preparation. In general, therefore, the compounds believed to act predominantly on the non-synaptic membrane affected the isolated and perfused-femur preparations to a similar extent. In contrast, glutamic acid and harmine depressed the neurally evoked contractions of the isolated preparations very much more strongly than those of the perfused preparations (Text-fig. 10A). These results are consistent with the concept that the diffusion barrier protects the nerve endings rather than the whole muscle, and they further suggest that it is not specific for glutamic acid.

Structural evidence

The femoral component of the metathoracic retractor unguis muscle of *Schistocercea gregaria* is composed of two tightly packed bundles of fibres, one generally containing eight and the other nine fibres. The fine structure of this muscle has been described by Cochrane, Elder & Usherwood (1972) and by Rees & Usherwood (1972). An attempt was made to locate the postulated diffusion barrier by comparing the fine structure of retractor unguis muscles from isolated and perfused-femur preparations. Muscles were fixed under three different conditions: (i) isolated preparations which had been irrigated with saline for 30–90 min were irrigated with fixative for 2 h;

(ii) perfused-femur preparations which had been perfused with saline for 30–90 min were perfused with fixative for 1·5 h, then dissected in fixative and exposed to fixative for a further 0·5 h; and (iii) femora were prepared for perfusion in the normal way but the haemolymph was displaced by perfusion with fixative, which continued for 1 h before dissection in fixative and continued fixation for 0·5 h. The one distinct and regular difference that was found between the muscles from isolated and perfused-femur preparations was in the connective tissue that surrounds the fibres. A description will therefore first be given of the connective tissue of retractor unguis muscles from femora perfused with fixative without previous perfusion with saline, because these muscles will have been the least affected by experimental conditions.

Each muscle fibre is bounded by a sarcolemma consisting of a plasma membrane and an outer connective tissue sheath some 0·1–0·25 μm thick (Pls. 1 A, 4 A). The connective tissue sheath is generally very compact, and little structure can be distinguished within it, but sometimes regions which are less compact can be found when the connective tissue is seen to contain collagen fibrils within a matrix of amorphous material. Where two muscle fibres meet, the connective tissue continues over their apposed surfaces producing a sheath of double thickness (Pl. 1 A). At the levels of the Z bands there occur sarcolemmal invaginations, in which both the plasma membrane and the connective tissue invaginate into the cell (Pl. 4 A). These sarcolemmal invaginations can be traced for 10 μm or more in single sections, and they provide a route along which tracheoles penetrate into the depths of the fibres. Sarcolemmal invaginations arise both from the concealed surfaces of the fibres and from the surfaces which are exposed on the outside of the muscle. Many transverse tubules originate from the sarcolemmal invaginations, as well as from the general surface of the muscle fibres (Pl. 1 B).

No significant differences of fine structure were found between retractor unguis muscles from perfused-femur preparations which had been fixed with or without prior exposure to saline. However, striking differences were found between the muscles from isolated preparations, which had been dissected before fixation, and the muscles from perfused-femur preparations, which had been dissected after fixation. Transverse sections of the retractor unguis muscles of isolated preparations always showed a greater or lesser separation of the muscle fibres (Pl. 3). The extent of the separation could vary in different parts of the same muscle; sometimes the separation between adjacent fibres was slight, extending only some 10–20 μm into the muscle, but in other cases the clefts extended to the centre of the muscle. In contrast, the retractor unguis muscles from perfused-femur preparations always had tightly packed fibres, and transverse sections showed at most only slight separation of fibres (Pl. 2). Where, in isolated preparations, adjacent muscle fibres had been pulled apart, the connective tissue had a very dispersed appearance with a clear separation of collagen fibrils and matrix (Pls. 1 B, 3). The connective tissue over the muscle surface of isolated preparations also tended to show a dispersed structure, which gave it a stratified appearance, and the connective tissue had often largely disappeared from the sarcolemmal invaginations in these regions (Pls. 1 B, 4 B). These differences between the isolated and perfused-femur preparations were not absolute, for muscles from isolated preparations showed some tightly packed fibres, and muscles from perfused-femur preparations showed some stratification of the connective tissue sheath,

and occasional slight separation of fibres. However, the muscles from the isolated preparations consistently showed a much greater separation of fibres and opening up of connective tissue than did the muscles from the perfused-femur preparations.

The fine processes of the two excitatory axons that supply the retractor unguis muscle lie within the connective tissue between adjacent fibres. Most axon terminals are enclosed within the fibre bundles, but very occasionally one is seen on the outer surface of a fibre. The separation of muscle fibres which occurred in the isolated preparations exposed a number of axon terminals (Pl. 3). This exposure of axon terminals, together possibly with changes in the permeability of the connective tissue sheath, seems likely to account for some of the increased sensitivity of isolated preparations to glutamate, and the connective tissue sheath may be the diffusion barrier which has been postulated from the experimental results.

DISCUSSION

The actions of glutamate

The application of L-glutamic acid to locust nerve–muscle preparations can cause a variety of effects, depending upon the concentration of glutamate and the experimental conditions. Applied as a pulse it may stimulate muscle contractions of a twitch-like or of a brief contracture-like form. On longer exposure it may potentiate or depress the glutamate twitch contractions, depress neurally evoked contractions, and induce repetitive contractions and spontaneous contractions. It is impossible to do more than suggest the possible causes of these actions.

The relatively rapid rise time of the glutamate-stimulated twitch contractions suggests that they result from a transient depolarization of the postsynaptic membrane by the glutamate, such as occurs on nervous stimulation. However, the cause of the glutamate-stimulated contractures is quite uncertain. It is possible that they result from prolonged synaptic potentials, such as those observed with locust and crayfish muscle on the prolonged application of glutamate (Beranek & Miller, 1968; Evoy & Beranek, 1972). It is also possible that the contractures result from activation of non-synaptic receptors, for Cull-Candy & Usherwood (1974) have shown that glutamate activates non-synaptic receptors on locust muscle, causing a biphasic potential change. Passage of the ion involved in the depolarizing component of this potential change could possibly account for the glutamate-induced contractures.

When retractor unguis preparations, particularly the perfused-femur preparations, were exposed to glutamate, the muscles would give repetitive contractions after a single electrical stimulus to the nerve, and would also give spontaneous contractions. The muscles showed the greatest tendency to give repetitive and spontaneous contractions when glutamate was applied to perfused-femur preparations at high hydrostatic pressure. High pressure did not affect the responses of these preparations to harmine, ryanodine or KCl. The glutamate-induced repetitive and spontaneous contractions were diminished or disappeared when 3 mM magnesium chloride was added to the saline, and they were also reduced when the calcium concentration was raised. Repetitive contractions have been described from mammalian muscles after intravascular application of eserine, neostigmine, or acetylcholine (Brown, Dale & Feldberg, 1936; Masland & Wigton, 1940; Feng & Li, 1941). It is thought that a

raised acetylcholine concentration leads to activation of presynaptic receptors, causing backfiring of the motor nerves and release of transmitter, followed by muscle contraction (Katz, 1969).

The depression of neurally evoked contractions which is caused by the prolonged exposure of isolated and perfused-femur preparations to glutamate probably results mainly from the partial 'desensitization' of postsynaptic glutamate receptors, although the mechanism of desensitization is not known. Like McDonald, Farley & March (1972) but, unlike Usherwood & Machili (1968), we were unable to potentiate the neurally evoked contractions by irrigating with very low concentrations of glutamate. It is possible that differences in the composition and tonicity of salines are the cause of these conflicting observations.

The protection of neuromuscular synapses from haemolymph glutamate

Locust haemolymph contains glutamic acid at a concentration which can seriously affect neuromuscular function, particularly in dissected preparations. No evidence was found of sequestration of glutamate by haemocytes or of glutamate binding to haemolymph proteins, but binding to divalent metal ions probably reduces the concentration of free glutamate by about 25%.

It was consistently found that isolated preparations, in which the retractor unguis muscle was dissected out, were much more sensitive to glutamate than perfused-femur preparations, and it was concluded that dissection of the femur probably disrupted a diffusion barrier. Electron micrographs of retractor unguis muscles from the two types of preparation showed a consistent difference in the fine structure of the connective tissue sheath which surrounds the fibres of the retractor unguis muscle. Sections of muscles from isolated preparations, which had been dissected before fixation, showed that the muscle fibres had separated to a greater or lesser extent and that the connective tissue sheath had a rather dispersed structure. Sections of muscles from perfused-femur preparations, which had been dissected after fixation, showed that there had been very little separation of fibres, and that the connective tissue sheath had a relatively compact structure. In the perfused-femur preparations the great majority of axon terminals were concealed between the tightly packed muscle fibres, but in the isolated preparations a proportion of the axon terminals had become exposed through the separation of the muscle fibres. It seems possible that the connective tissue sheath acts as a diffusion barrier and reduces the rate at which glutamate molecules can diffuse from the haemolymph towards the nerve endings, the great majority of which lie within the connective tissue sheath between adjacent muscle fibres. Further protection may be afforded by the glial cells and tracheal sheath cells which surround the axon terminals and which take up glutamate (Faeder & Salpeter, 1970; Salpeter & Faeder, 1971). These cells may also take up glutamate, of presynaptic origin, which diffuses out of the synaptic clefts.

Calcium and magnesium ions and sensitivity to glutamate

Calcium and magnesium ions have marked effects on nerve–muscle preparations, but before the knowledge of such effects can be used to predict the actions of these ions in the normal animal it is necessary to know the extent to which they are bound

to other ligands in haemolymph and, in particular, to know the concentrations that remain free. Analysis of whole haemolymph from adult male *Schistocerca gregaria* gave mean concentrations of 5·0 (±0·17) mmol/l calcium, and 11·6 (±0·44) mmol/l magnesium, concentrations which are substantially lower than the frequently cited values of 9 mmol/l calcium and 17 mmol/l magnesium recorded by Duchâteau, Florkin & Leclercq (1953) from the haemolymph of Vth-instar *S. gregaria*. A proportion of the calcium and magnesium ions present in haemolymph will be bound to amino acids. Using the mean concentrations of metal ions and total amino acids measured in the haemolymph of adult male locusts in the present study, and the stability constants published for amino acid-metal complexes (Lumb & Martell, 1953), the best estimate of binding that can be made suggests that approximately 50% of the calcium ions and approximately 75% of the magnesium ions are bound to amino acids. Therefore, on average, some 2·6 mmol/l calcium and 3·1 mmol/l magnesium remain free (Table 2). It must be emphasized that the extent of binding has only been estimated, and not measured directly, and that there is considerable variation in ion concentrations in the haemolymph of different individuals. However, these estimates of the mean concentrations of free divalent metal ions are probably more useful for most physiological applications than is knowledge of the total concentrations, and it is interesting that the estimated free ion concentrations are close to those found experimentally, by Hoyle (1953), to be suitable for locust saline. The concentration of free glutamate, as of the other amino acids in locust haemolymph, is reduced about 25% by binding to the divalent metal ions.

The results described in this paper showed that both raising the calcium concentration of saline above 2 mmol/l and introducing magnesium ions made nerve–muscle preparations less sensitive to glutamate. This was apparent in the higher thresholds of the glutamate contractions (Text-fig. 4), in the reduction of the depression of neurally evoked contractions caused by glutamate, and in the prevention of glutamate-induced repetitive and spontaneous contractions in perfused-femur preparations. These effects were greater than could be accounted for by the binding of glutamate to calcium and magnesium ions, and so imply an action of the metal ions on the preparation itself. When the experimental conditions approached the average conditions believed to obtain in the intact insect, i.e. zero hydrostatic pressure, an intact diffusion barrier, a free calcium concentration of about 2 mmol/l, a free magnesium concentration of about 3 mmol/l, and a free glutamate concentration of about 0·2 mmol/l, the force of the neurally evoked contractions was generally very close to the force that was given when the femur contained its own haemolymph. Moreover, under these conditions the perfused-femur preparations did not give repetitive or spontaneous contractions. It is possible, therefore, but not proved, that when the postulated diffusion barrier is intact and not subjected to high hydrostatic pressure, the calcium and magnesium ions that are free in haemolymph, acting in whatever ways, are sufficient to complete the protection of neuromuscular synapses from haemolymph glutamate.

General conclusions on the problem of haemolymph glutamate

The concentration of glutamate in locust haemolymph appears not to rise much above 0·5 mmol/l. It is likely, therefore, that the concentration of glutamate is con-

trolled, as possibly are the concentrations of other amino acids in haemolymph. Murdock & Koidl (1972*a*, *b*) have shown that glutamate is extensively metabolized in the locust gut wall, and that little or no L-glutamate enters the haemolymph from the gut. They found that glutamate injected into the haemolymph was rapidly converted to glutamine.

In the case of the nerve supply to the retractor unguis muscle at least, the great majority of nerve endings are enclosed between the muscle fibres, and are separated from the haemolymph by a connective tissue sheath. Our experimental results suggest that a diffusion barrier partially isolates the nerve endings from the haemolymph, and that the diffusion barrier is damaged by dissection of nerve–muscle preparations. The postulated diffusion barrier also appears to fail under conditions of raised hydrostatic pressure. Electron micrographs of retractor unguis muscles fixed before and after dissection suggest that the connective tissue sheath that surrounds the muscle fibres possibly constitutes the diffusion barrier. Further protection of nerve endings is probably afforded by the glial cells and tracheal sheath cells that surround the nerve endings and which Faeder & Salpeter (1970) have shown take up glutamate.

Constituents of the haemolymph itself apparently play some part in the protection of neuromuscular synapses from glutamate, for the perfused-femur preparations were more sensitive to the standard saline containing glutamate than to haemolymph containing similar to higher concentrations of glutamate. Moreover, perfused-femur preparations were usually no more affected by haemolymph containing added glutamate than by normal haemolymph. There appears to be no sequestration of glutamate by haemocytes, or binding of glutamate to plasma proteins, but approximately 25% of haemolymph glutamate is probably bound to calcium and magnesium ions. These divalent metal ions have also been shown to reduce the sensitivity of nerve–muscle preparations to glutamate in saline to a greater extent than can be accounted for by their estimated binding of glutamate, and it is likely that the free calcium and magnesium ions of the haemolymph interact with the muscles and nerves in ways which reduce the sensitivity of the neuromuscular system to haemolymph glutamate.

SUMMARY

1. Two nerve–muscle preparations were used to investigate the physiology of the locust retractor unguis muscle in relation to L-glutamic acid. These were an 'isolated preparation', in which the muscle and its nerve were dissected out, and a 'perfused-femur preparation', in which the muscle suffered no mechanical disturbance.

2. Exposure of the nerve–muscle preparations to glutamate caused a variety of responses, some of which were shown to be abnormal and due to the experimental conditions.

3. When locust femora were perfused with saline or haemolymph the retractor unguis muscles were much more severely affected by glutamate if the hydrostatic pressure was slightly raised. At raised pressures the perfused-femur preparations were particularly prone to give repetitive and spontaneous contractions.

4. Analysis of haemolymph from adult male locusts showed that it contained, on average, 0·2 mmol/l L-glutamate, 45 mol/l total non-peptide amino acids, 5·0 mmol/l calcium, and 11·6 mmol/l magnesium. It was calculated that approximately 50% of

the calcium and 75% of the magnesium ions are bound to amino acids, and that approximately 25% of the glutamic acid is bound to divalent metal ions.

5. The isolated preparations were severely affected by glutamate at the concentration at which it occurs in haemolymph, and it was concluded that in the intact locust some mechanism must protect the neuromuscular synapses from haemolymph glutamate. No evidence could be obtained of the sequestration of glutamate by haemocytes, or of binding of glutamate to haemolymph proteins.

6. Calcium and magnesium ions reduced the sensitivity of nerve–muscle preparations to glutamate to a greater extent than could be accounted for by the formation of amino acid–metal complexes. This suggests that the protection afforded by calcium and magnesium involves an interaction of the metal ions with the neuromuscular system itself.

7. The retractor unguis muscle was much less sensitive to glutamate when it was contained within an undissected femur than in an isolated preparation. It was concluded that the muscle is normally protected from haemolymph glutamate by a diffusion barrier which is damaged on dissection.

8. Comparison of the fine structure of retractor unguis muscles, fixed either after dissection or while still contained within the femur, showed that dissection normally caused a partial separation of muscle fibres and damage to the connective tissue sheath, with the resultant exposure of some nerve endings. The connective tissue sheath may constitute the postulated diffusion barrier.

9. The excitatory synapses of the locust retractor unguis muscle are believed to be isolated from haemolymph glutamate by a diffusion barrier, which is tentatively identified with the connective tissue sheath that binds the muscle fibres together. Calcium and magnesium ions reduce the sensitivity of nerve–muscle preparations to glutamate, and may have such a role in the living insect.

We have benefited very greatly from the interest and advice which Dr P. N. R. Usherwood has given us. We are pleased to acknowledge also the help which we have received from Dr D. E. Ashhurst, Dr R. M. Scott and Dr R. J. Dowson during the course of the work, and from Dr J. E. Treherne who read the manuscript.

REFERENCES

ALBRECHT, F. O. (1953). *The Anatomy of the Migratory Locust*, xvi + 118 pp. London: Athlone Press.

BERANEK, R. & MILLER, P. L. (1968). The action of iontophoretically applied glutamate on insect muscle fibres. *J. exp. Biol.* **49**, 83–93.

BERGMEYER, H. U. (1965). *Methods of Enzymatic Analysis*, xxiii + 1064 pp. New York and London: Academic Press.

BROWN, G. L., DALE, H. H. & FELDBERG, W. (1936). Reactions of the normal mammalian muscle to acetylcholine and to eserine. *J. Physiol.* **87**, 394–424.

CLEMENTS, A. N. & MAY, T. E. (1974). Pharmacological studies on a locust neuromuscular preparation. *J. Exp. Biol.* **61**.

COCHRANE, D. G., ELDER, H. Y. & USHERWOOD, P. N. R. (1972). Physiology and ultrastructure of phasic and tonic skeletal muscle fibres in the locust, *Schistocerca gregaria*. *J. Cell Sci.* **10**, 419–41.

CULL-CANDY, S. G. & USHERWOOD, P. N. R. (1974). Two populations of L-glutamate receptors on locust muscle fibres. *New Biol.* **246**, 62–64.

DUCHÂTEAU, G., FLORKIN, M. & LECLERCQ, J. (1953). Concentrations des bases fixes et types de composition de la base totale de l'hémolymphe des insectes. *Arch. int. Physiol.* **41**, 518–49.

EVANS, P. D. (1972). The free amino acid pool of the haemocytes of *Carcinus maenas* (L.). *J. exp. Biol.* **56**, 501–7.

EVOY, W. H. & BERANEK, R. (1972). Pharmacological localization of excitatory and inhibitory synaptic regions in crayfish slow abdominal flexor muscle-fibres. *Comp. gen. Pharmac.* **3**, 178–86.

FAEDER, I. R. & SALPETER, M. M. (1970). Glutamate uptake by a stimulated insect nerve muscle preparation. *J. Cell Biol.* **46**, 300–7.

FENG, T. P. & LI, T. H. (1941). Studies on the neuromuscular junction. XXIII. A new aspect of the phenomena of eserine potentiation and post-tetanic facilitation in mammalian muscles. *Chinese J. Physiol.* **16**, 37–55.

HILL, L., LUNTZ, A. J. & STEELE, P. A. (1968). The relationships between somatic growth, ovarian growth, and feeding activity in the adult desert locust. *J. Insect Physiol.* **14**, 1–20.

HOYLE, G. (1953). Potassium ions and insect nerve muscle. *J. exp. Biol.* **30**, 121–35.

HOYLE, G. (1955*a*). The anatomy and innervation of locust skeletal muscle. *Proc. R. Soc.* B **143**, 281–92, 2 pls.

HOYLE, G. (1955*b*). The effects of some common cations on neuromuscular transmission in insects. *J. Physiol.* **127**, 90–103.

HUNTER-JONES, P. (1966). *Rearing and Breeding Locusts in the Laboratory*, 12 pp. London: Anti-Locust Research Centre.

KATZ, B. (1969). *The Release of Neural Transmitter Substances*, ix + 60 pp. Liverpool University Press.

LEA, T. J. & USHERWOOD, P. N. R. (1973*a*). Effect of ibotenic acid on chloride permeability of insect muscle fibres. *Comp. gen. Pharmac.* **4**, 351–363.

LEA, T. J. & USHERWOOD, P. N. R. (1973*b*). The site of action of ibotenic acid and the identification of two populations of glutamate receptors on insect muscle fibres. *Comp. gen. Pharmac.* **4**, 333–350.

LEE, R. M. (1961). The variation of blood volume with age in the desert locust (*Schistocerca gregaria* Forsk.). *J. Insect Physiol.* **6**, 36–51.

LUMB, R. F. & MARTELL, A. E. (1953). Metal chelating tendencies of glutamic and aspartic acids. *J. phys. Chem.* **57**, 690–3.

MASLAND, R. L. & WIGTON, R. S. (1940). Nerve activity accompanying fasciculation produced by prostigmin. *J. Neurophysiol.* **3**, 269–75.

MCDONALD, T. J., FARLEY, R. D. & MARCH, R. B. (1972). Pharmacological profile of the excitatory neuromuscular synapses of the insect retractor unguis muscle. *Comp. gen. Pharmac.* **3**, 327–38.

MCMENAMY, R. H. & WATSON, F. (1968). Indole-albumin association: a comparative study. *Comp. Biochem. Physiol.* **26**, 329–35.

MILLER, R., LEAF, G. & USHERWOOD, P. N. R. (1973). Blood glutamate in arthropods. *Comp. Biochem. Physiol.* **44**A, 991–6.

MURDOCK, L. L. & KOIDL, B. (1972*a*). Limited permeability and metabolism of L-glutamate in the locust gut wall. *J. exp. Biol.* **56**, 781–94.

MURDOCK, L. L. & KOIDL, B. (1972*b*). Blood metabolites after intestinal absorption of amino acids in locusts. *J. exp. Biol.* **56**, 795–808.

ORKAND, R. K. (1962). The relation between membrane potential and contraction in single crayfish muscle fibres. *J. Physiol., Lond.* **161**, 143–159.

PITMAN, R. M. (1971). Transmitter substances in insects: a review. *Comp. gen. Pharmac.* **2**, 347–71.

REES, D. & USHERWOOD, P. N. R. (1972). Fine structure of normal and degenerating motor axons and nerve-muscle synapses in the locust, *Schistocerca gregaria*. *Comp. Biochem. Physiol.* **43**A, 83–101, 5 pls.

SALPETER, M. M. & FAEDER, I. R. (1971). The role of sheath cells in glutamate uptake by insect nerve muscle preparations. *Progr. Brain Res.* **34**, 103–14.

SPURR, A. R. (1969). A low-viscosity epoxy resin embedding/medium for electron microscopy. *J. Ultrastruct. Res.* **26**, 31–43.

USHERWOOD, P. N. R. (1962). The action of the alkaloid ryanodine on insect skeletal muscle. *Comp. Biochem. Physiol.* **6**, 181–199, 12 pls.

USHERWOOD, P. N. R. (1967*a*). Permeability of insect muscle fibres to potassium and chloride ions. *J. Physiol.* **191**, 29*P*–30*P*.

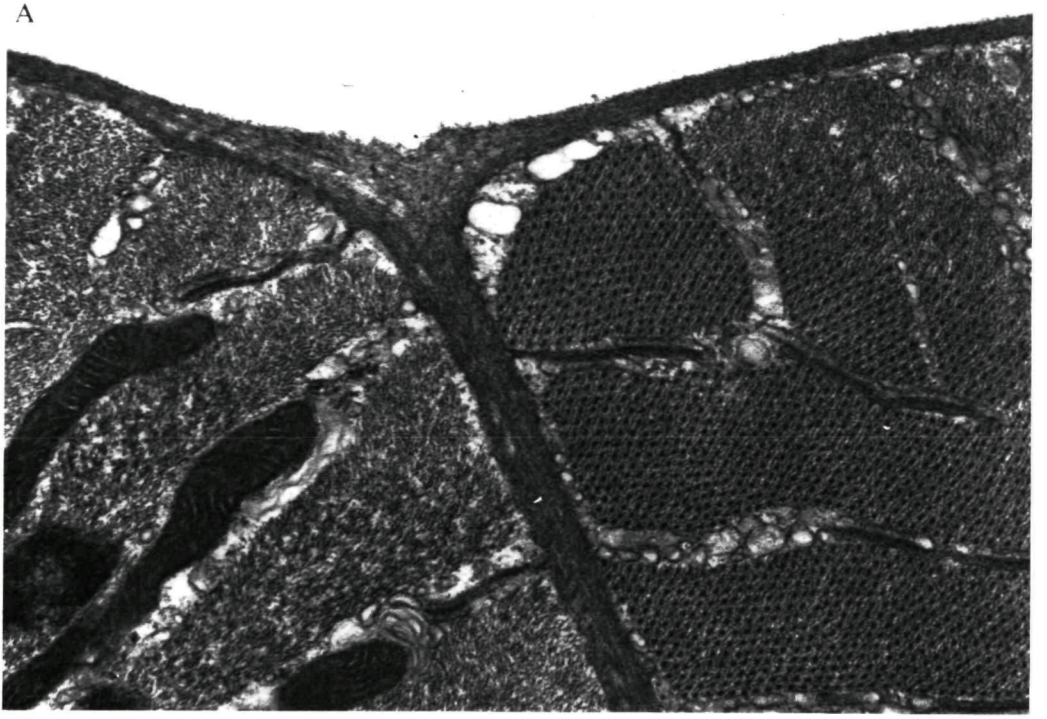
USHERWOOD, P. N. R. (1967*b*). Insect neuromuscular mechanisms. *Am. Zool.* **7**, 553–582.

USHERWOOD, P. N. R. & GRUNDFEST, H. (1965). Peripheral inhibition in skeletal muscle of insects. *J. Neurophysiol.* **28**, 497–518.

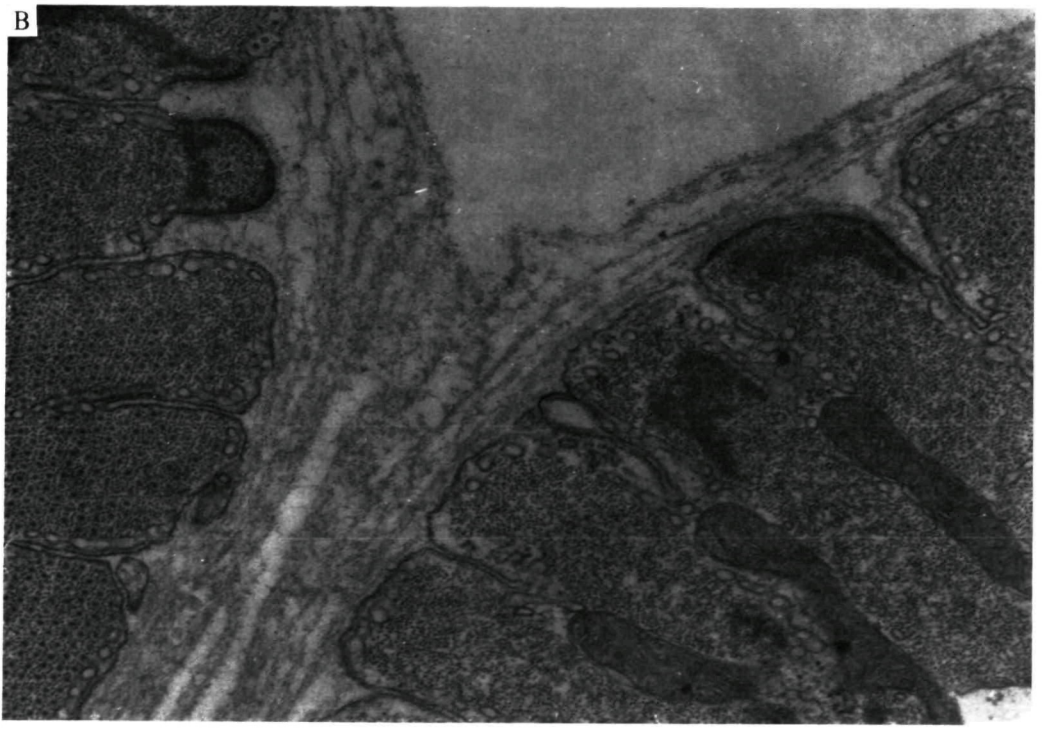
USHERWOOD, P. N. R. & MACHILI, P. (1968). Pharmacological properties of excitatory neuromuscular synapses in the locust. *J. exp. Biol.* **49**, 341–61.

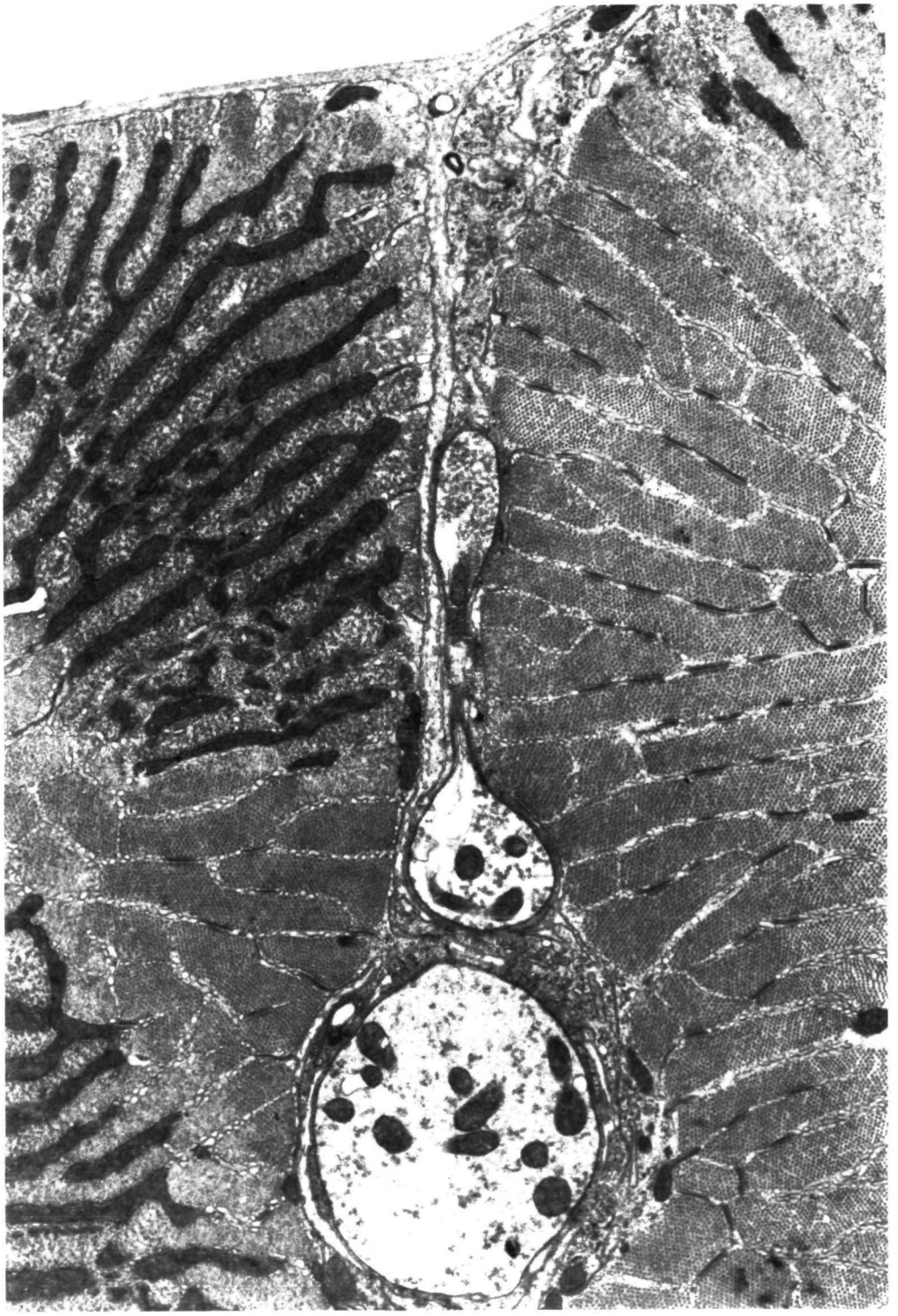
WALTHER, C. & USHERWOOD, P. N. R. (1972). Characterization of the glutamate receptors at the locust excitatory neuromuscular junction. *Verh. dt. zool. Ges.* **65**, 309–12.

A

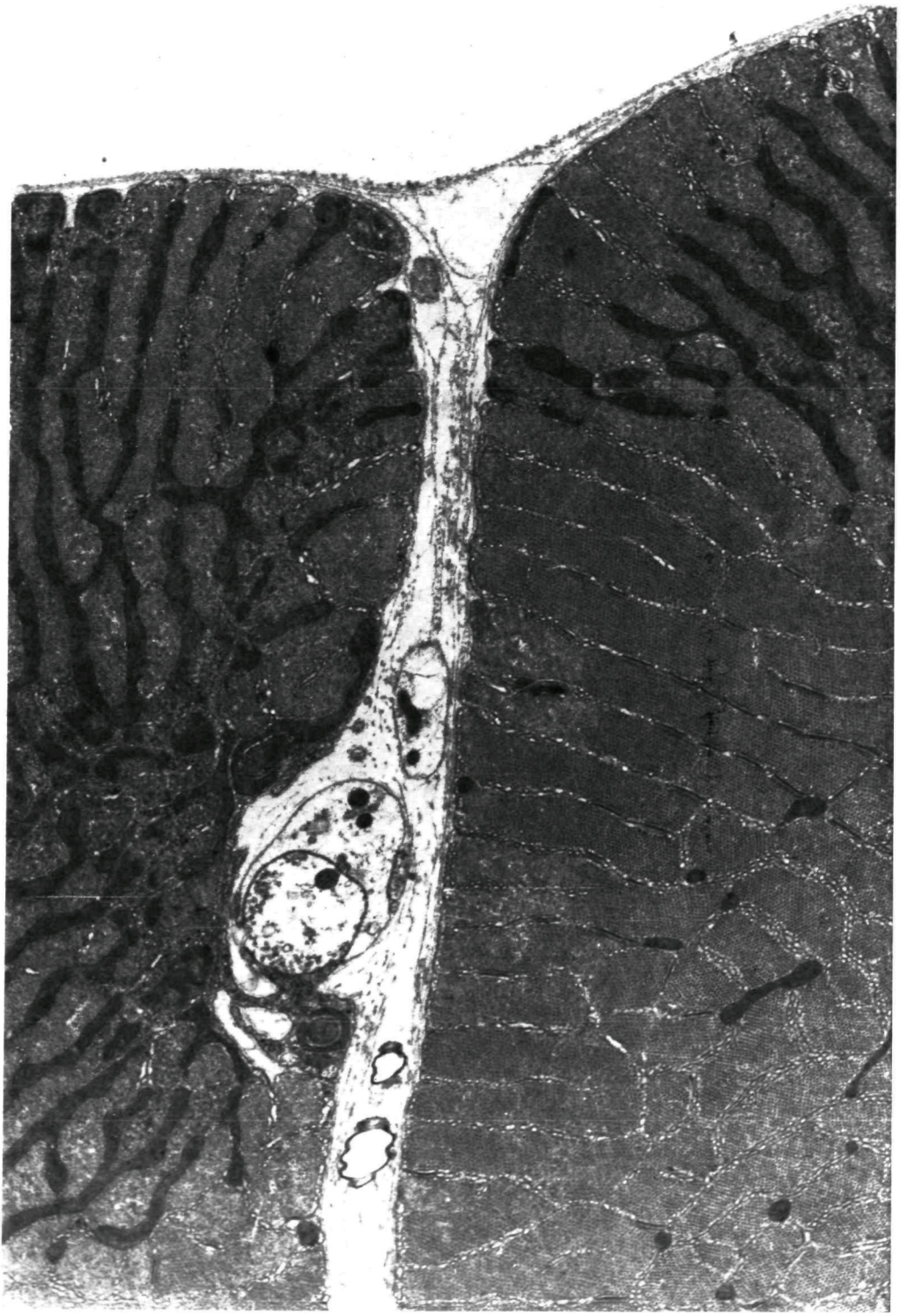


B



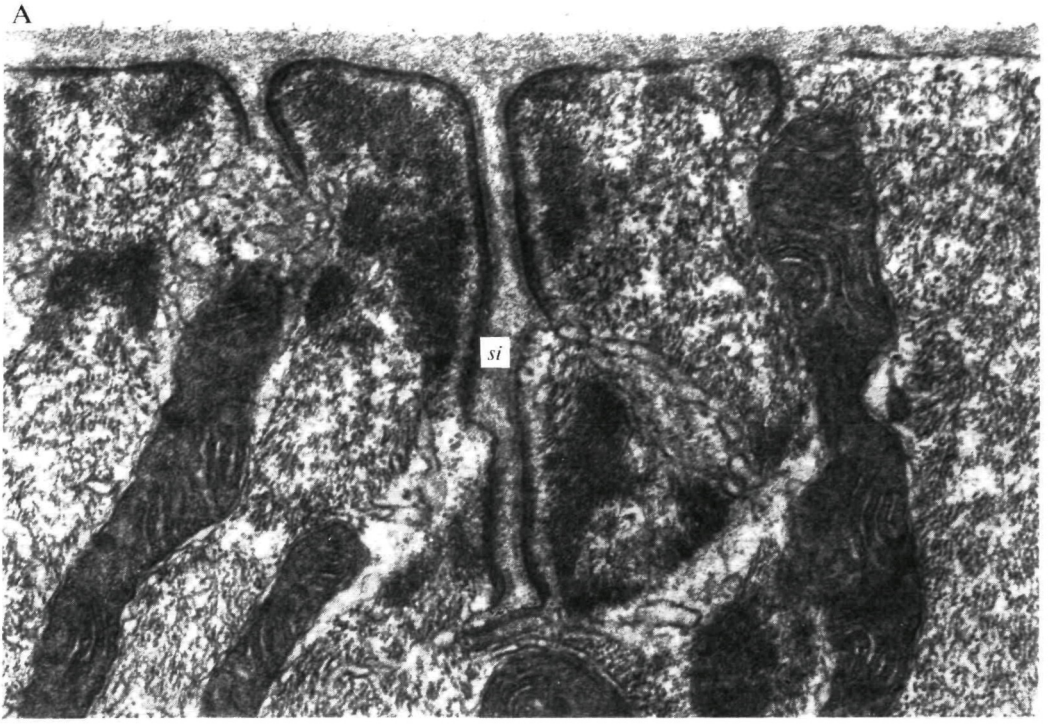


A. N. CLEMENTS AND T. E. MAY

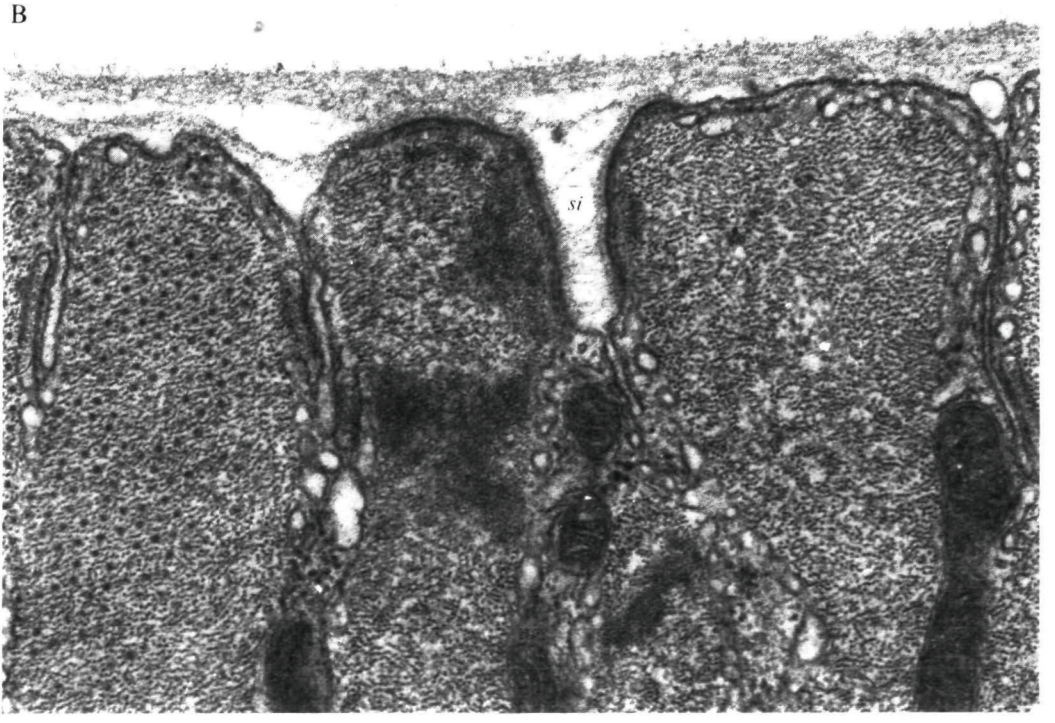


A. N. CLEMENTS AND T. E. MAY

A



B



A. N. CLEMENTS AND T. E. MAY

EXPLANATION OF PLATES

All plates show transverse sections of retractor unguis muscles from femora of the locust *Schistocerca gregaria*.

PLATE 1

Junction of two fibres at the surface of the muscle. A. From a perfused-femur preparation, fixed before dissection. The connective tissue sheath is compact. × 32 500. B. From an isolated preparation, fixed after dissection. The fibres have separated slightly, and the connective tissue sheath has an open appearance. × 32 500.

PLATE 2

Junction of two fibres from a perfused-femur preparation. An axon terminal is situated between the two fibres, but the connective tissue sheath isolates it from the outside of the muscle. × 13 000.

PLATE 3

Junction of two fibres from an isolated preparation. An axon terminal is situated between the two fibres, but owing to the separation of the fibres and the opening up of the connective tissue sheath, the axon terminal is effectively exposed. × 13 000.

PLATE 4

Transverse sections through the outer surface of the muscle. A. From a perfused-femur preparation. The connective tissue sheath has a compact appearance, and connective tissue fills the sarcolemmal invagination (*si*). × 51 000. B. From an isolated preparation. The connective tissue sheath has a stratified appearance, and the sarcolemmal invagination (*si*) appears empty. × 51 000.