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PHARMACOLOGICAL PROPERTIES OF EXCITATORY NEUROMUSCULAR SYNAPSES IN THE LOCUST

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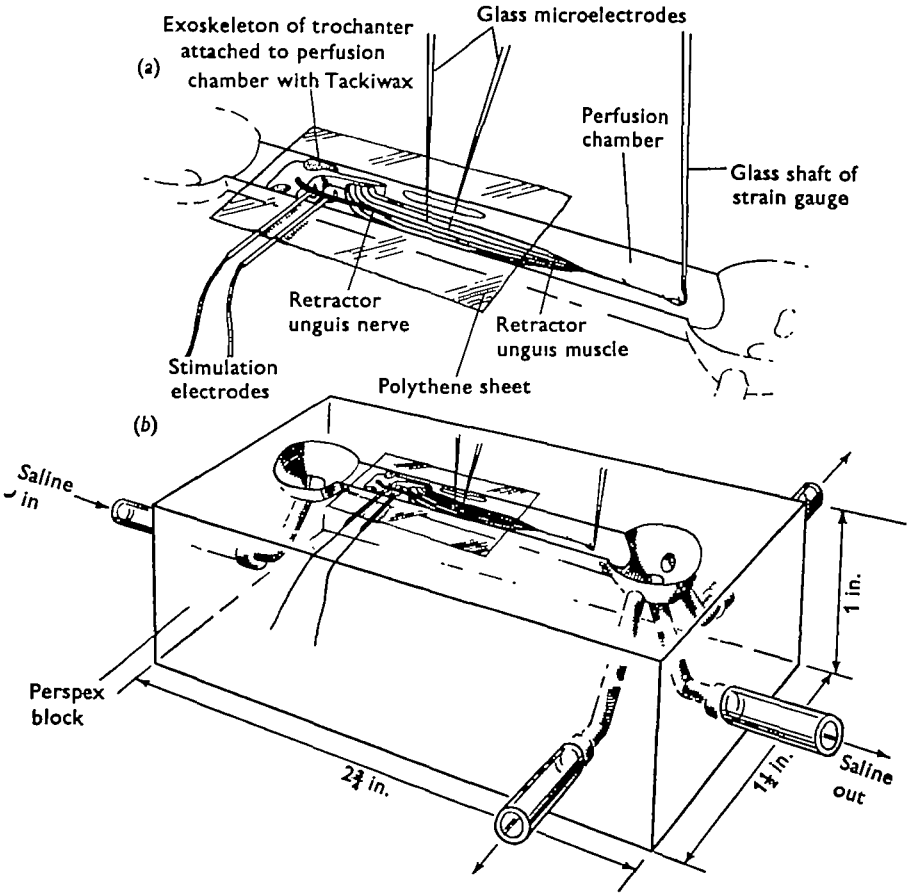
INTRODUCTION

Although insect nervous tissue is known to contain concentrations of acetylcholine far in excess of those found in the central nervous tissue of vertebrates (Long, 1961) and there is some evidence for cholinergic transmission at insect central synapses (Treherne & Smith, 1965), it is now generally accepted that chemical transmission at the insect neuromuscular junction is not cholinergic (e.g. Hill & Usherwood, 1961; Usherwood, 1963*a*; Usherwood & Grundfest, 1964, 1965). A large number of substances have been tested on insect nerve-muscle preparations in attempts to identify the chemical mediator or mediators involved in neuromuscular synaptic transmission in insects. Of these substances the most promising results have been obtained with certain amino acids, notably L-glutamic acid (e.g. Usherwood & Grundfest, 1965; Kerkut, Shapira & Walker, 1965; Usherwood & Machili, 1966) and γ-aminobutyric acid (GABA) (Usherwood & Grundfest, 1964, 1965). Usherwood & Grundfest (1964, 1965) have demonstrated that GABA mimics the transmitter at the inhibitory neuromuscular synapses in the locust and the cockroach, while Usherwood & Grundfest (1965) and Usherwood & Machili (1966) have suggested a role for L-glutamate at the excitatory neuromuscular synapse in the locust. L-Glutamate is also thought to be involved in transmission at the excitatory neuromuscular synapse in the cockroach (Kerkut, Shapira & Walker, 1965). In view of the close phylogenetic relationships between crustaceans and insects it is perhaps not very surprising that the neuromuscular systems of these two animal groups have strikingly similar properties. Indeed many of the studies of the physiology and pharmacology of insect neuromuscular systems have been inspired by earlier investigations of crustacean nerve-muscle preparations (e.g. Robbins, 1958, 1959; van Harreveld, 1959; van Harreveld & Mendelson, 1959; Takeuchi & Takeuchi, 1963, 1964). The present study was undertaken to clarify the pharmacological properties of the excitatory neuromuscular synapse in the locust, the grasshopper and the cockroach. Many different amino acids and related compounds have been tested on a large number of nerve-muscle preparations from these insects and the results substantiate earlier claims for a role for L-glutamate as an excitatory transmitter in these insects. These results also provide a basis for speculation on the structural configuration of the chemical receptors on the excitatory postsynaptic membranes of insect muscle fibres.

METHODS

The metathoracic retractor unguis muscles of the locusts *Schistocerca gregaria* and *Locusta migratoria*, the grasshopper *Romalea microptera*, and the cockroach *Blaberus giganticus* were used. These small muscles can be isolated from the insect with their innervating axons and therefore have many advantages over the larger, complex *in situ* nerve-muscle preparations used in previous investigations of insect neuromuscular



Text-fig. 1. Diagrammatic representation of perfusion bath and isolated retractor unguis nerve-muscle preparation. Muscle not drawn to scale. The bath was constructed out of a block of clear Perspex. The over-all design of the perfusion system is illustrated in (*b*) while details of the arrangement of stimulating and recording electrodes and attachment of muscle to the perfusion chamber and transducer are illustrated in (*a*). (Drawings by the late S. L. Hill.)

physiology and pharmacology (e.g. Hill & Usherwood, 1961; Usherwood & Grundfest, 1964, 1965). The retractor unguis muscle is about 1 cm long and 300 μ in diameter in the locust and cockroach (slightly larger in the grasshopper) and rarely contains more than 18 fibres. The fibres are arranged in parallel and are intimately connected together by branches of the tracheolar respiratory system. The muscle is attached proximally to the lateral wall of the femoral segment of the hind leg and ends

in a long filamentous apodeme (tendon) which runs through the femoral and tibial segments to the tibial ('toe') joint.

Hind legs were removed from adult insects and the femoral segments were fixed horizontally in a perfusion bath by attaching the trochanteral segment to the wall of the bath with Tackiwax (Text-fig. 1). The flexor and extensor tibiae muscles were then removed from the femur, together with most of the exoskeleton, leaving the retractor unguis muscle and its innervating nerve. The muscle apodeme was then attached to a Grass FT 10 strain gauge with a short strand of Terylene, the muscle being stretched to maximal body length. The total volume of the bath, including inlet and outlet reservoirs, was about 2·2 ml. and the contents could be replaced within 1 sec. Mechanical recording artifacts, resulting from the flow of saline over the preparation and past the strain-gauge shaft, were reduced to a minimum by covering the part of the bath containing the muscle preparation with thin Polythene sheeting. This did not seriously affect the rate of replacement of saline around the nerve-muscle preparation. With practice, it was possible to dissect out the nerve-muscle preparation and arrange it in the perfusion bath in less than 10 min. The dissection and setting-up procedure were performed in continuously flowing saline. These isolated nerve-muscle preparations can obtain sufficient oxygen from the saline medium provided they are perfused slowly, but continuously, throughout the experimental period.

The muscles were stimulated indirectly through fine (40–80 μ) copper or platinum wire electrodes insulated to their tips and placed on the retractor unguis nerve. Intracellular recordings from the muscle fibres were made using 3 M-KCl glass microelectrodes. The microelectrodes were connected to a negative-capacitance d.c. preamplifier with an agar/AgCl bridge. A second agar/AgCl bridge was used to earth the perfusion bath. High-resistance ($>$ 20 MΩ) glass microelectrodes (tip diam. less than 1 μ) filled with molar sodium L-glutamate (pH 8·0) were used for iontophoretic application of glutamate.

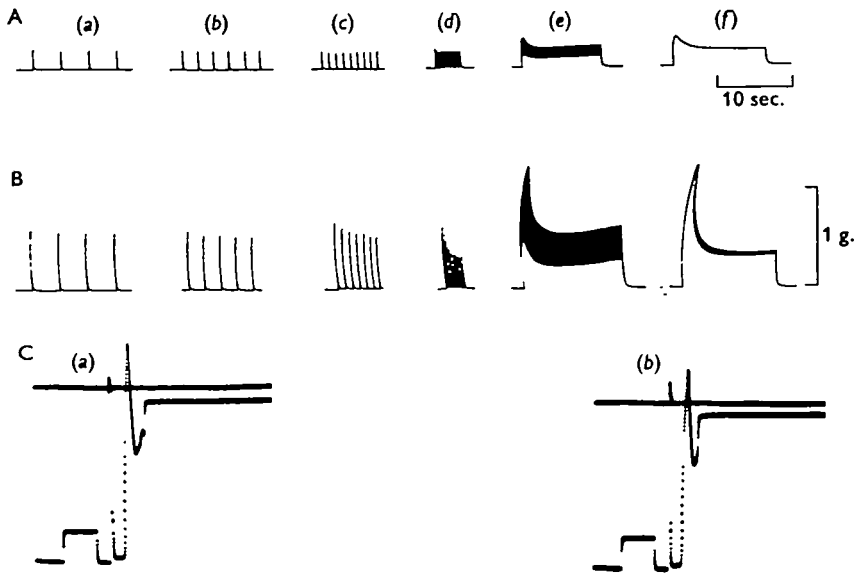
In most experiments drugs were applied to the nerve-muscle preparation by dissolving them in locust (10 K) saline of the following composition: NaCl, 140; KCl, 10; $CaCl_2$ 2; NaH_2PO_4, 4; Na_2HPO_4, 6 mM. All the salines were buffered at pH 6·8 and all experiments were carried out at 18–19° C.

RESULTS

Structure and physiology of the retractor unguis nerve-muscle preparation

The retractor unguis muscle is differentiated structurally and functionally into two units, although outwardly it has the appearance of a compact single bundle of fibres (Usherwood, 1967*b*). Part of the muscle is made up of a group of large white fibres (*c.* 70 μ diam.), with sarcomere lengths of about 4 μ when the muscle is at maximal body length. The rest of the muscle contains red fibres of about 40 μ diameter. These fibres are characterized by 7 μ sarcomeres at maximal body length and an abundance of mitochondria near the Z-lines. Significantly, the smaller fibres fatigue more slowly under sustained tetanic stimulation (Text-fig. 2A, B). The two bundles of fibres are innervated separately, each bundle receiving a single motor axon. In all fibres the electrical response to neural stimulation consists of a large excitatory postsynaptic potential (EPSP) plus graded electrically excited response (Text-fig. 2C). The mechani-

cal responses of both sets of fibres consist of twitch contractions at low frequencies and tetanic contractions at high frequencies of nerve stimulation, but the white fibres fatigue more quickly than the red fibres during sustained tetanic stimulation. The muscle undergoes a single brief (*c.* 4 sec.) phasic contraction during potassium depolarization. The retractor unguis muscle fibres are multiterminally innervated with synapses distributed along the surface of the fibres. The structure of the neuromuscular synapse is illustrated in Plate 1 and is characteristic of most insect neuromuscular synapses in the absence of any visible structural differentiation of the postsynaptic membrane. Other properties of the retractor unguis muscle have been described in three recent publications (Cochrane & Elder, 1967; Usherwood, 1967*a*, *b*).



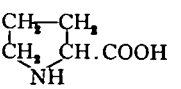
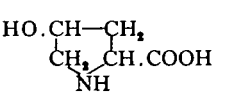
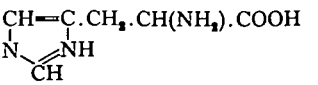
Text-fig. 2. (A–B) Mechanical responses of an isolated metathoracic retractor unguis nerve-muscle preparation from the locust. (A) Responses of pink fibres to neural stimulation at different frequencies. Note slow rate of fatigue of these fibres even during tetanic stimulation. (B) Combined responses of pink and white fibres to neural stimulation at different frequencies. The white fibres fatigued much more quickly than the pink fibres. Stimulation frequencies were: (*a*), 0·3/sec., (*b*) 0·5/sec., (*c*) 1/sec., (*d*) 3/sec., (*e*) 6/sec., (*f*) 15/sec. (C) Electrical responses from (*a*) a pink fibre and (*b*) a white fibre during neural stimulation. Each response consists of a large EPSP plus a graded electrically excited response. Calibration pulse at beginning of each trace was 10 mV. 20 msec.

Pharmacological properties

The presence of short-chained dicarboxylic amino acids, such as aspartate and glutamate, in insect tissues and blood in concentrations as high as 10^{-3} M (Frontalis, 1961) is suggestive of the important role of these amino acids in physiological and metabolic processes in insects.

Table 1 summarizes the effects of various amino acids and related compounds on the retractor unguis preparation. With few exceptions modifications of the structure or position of the distal carboxyl grouping of the α-amino-dicarboxylic acids appear to cause profound changes in the activities of the compounds. Of the dicarboxylic acids

Table 1. *Structure-activity relationships of the amino acids and related compounds tested on the insect nerve-muscle preparations*

Compound	Structure	Minimum concentration (w/v) required to evoke a contraction
L-Aspartic acid	$HOOC.CH_2.CH(NH_2).COOH$	10^{-3}
L-Glutamic acid	$HOOC.(CH_2)_2.CH(NH_2).COOH$	10^{-6}
D-Glutamic acid	$HOOC.(CH_2)_2.CH(NH_2).COOH$	—
DL-Aminoadipic acid	$HOOC.(CH_2)_3.CH(NH_2)\ COOH$	—
DL-aminopimelic acid	$HOOC.(CH_2)_4.CH(NH_2).COOH$	—
L-Asparagine	$NH_2CO.CH_2CH(NH_2).COOH$	10^{-3}
L-Glutamine	$NH_2CO.(CH_2)_2.CH(NH_2).COOH$	10^{-4}
Succinic acid	$HOOC.(CH_2)_2.COOH$	—
Glutaric acid	$HOOC.(CH_2)_3.COOH$	—
Glycine	$NH_2.CH_2.COOH$	—
β-Alanine	$NH_2.(CH_2)_2.COOH$	—
γ-Aminobutyric acid (GABA)	$NH_2.(CH_2)_3\ COOH$	—
Taurine	$NH_2.(CH_2)_2\ SO_3H$	—
DL-Ornithine	$NH_2\ (CH_2)_3.CH(NH_2).COOH$	—
L-β-Phenylalanine	 $.CH(NH_2)\ CH_2.COOH$	—
DL-Homocysteic acid	 $.(CH_2)_2.CH(NH_2).COOH$	10^{-3}
L-Arginine	 $.NH(CH_2)_3.CH(NH_2).COOH$	—
Lysine	$NH_2\ (CH_2)_4.CH(NH_2).COOH$	—
L-Cysteine	$CH_3S.CH(NH_2).COOH$	10^{-3}
DL-Cysteic acid	$HO_3S.CH_2.CH(NH_2).COOH$	10^{-3}
L-Cystine	$[CH_2S.CH(NH_2).COOH]_2$	—
DL-Methionine	$CH_3S.(CH_2)_2.CH(NH_2).COOH$	10^{-3}
L-Leucine	$(CH_3)_2CH.CH_2\ CH(NH_2).COOH$	—
L-Serine	$HO.CH_2.CH(NH_2)\ COOH$	10^{-3}
L-Threonine	$CH_3.CHOH.CH(NH_2).COOH$	—
L-Tyrosine	 $.CH_2.CH(NH_2)\ COOH$	
L-Valine	$(CH_3)_2CH.CH(NH_2).COOH$	—
α-Methyl-DL-aspartic acid	$HOOC.CH_2.C(CH_3NH_2).COOH$	10^{-3}
N-Methyl-DL-aspartic acid	$HOOC.CH_2.CH(NHCH_3).COOH$	—
L-Proline		—
Hydroxyproline		—
L-Histidine		—

tested in the present investigations, L-glutamic acid was the most active. When applied to the preparation in concentrations higher than 10^{-7} (w/v) this substance evoked a single phasic contraction followed by a decline in the neurally evoked contractions. Maximal contractions were obtained with concentrations of L-glutamic acid of about 10^{-6} (w/v). When preparations were perfused with L-glutamic acid in concentrations $> 5 \times 10^{-5}$ (w/v) the neurally evoked responses were completely abolished. The neurally evoked contractions were enhanced or potentiated during perfusion of the muscle with saline containing L-glutamic acid in concentrations between 10^{-7} (w/v) and 10^{-12} (w/v). The effects of L-glutamic acid were fully reversible and could be readily repeated whilst the preparation remained viable (about 30 hr. at 18° C.). The D-enantiomorph of glutamic acid was about 100 times less active than the naturally occurring L-form. L-Aspartic acid was even less active, having no effect at concentrations less than 10^{-4} (w/v). The amide derivatives of L-aspartic acid and L-glutamic acid were surprisingly active, although much less so than L-glutamic acid. Asparagine at a concentration of 10^{-5} (w/v) evoked a phasic contraction accompanied by abolition of the neurally evoked contractions, while the latter were enhanced by 10^{-8} (w/v) asparagine.

The α-decarboxylation products of the short-chained amino acids could be of special significance in insects in view of the possible implication of GABA at inhibitory neuromuscular synapses (Usherwood & Grundfest, 1964, 1965). However, since the muscles used in these studies do not receive an inhibitory innervation it is perhaps not very surprising that glycine, GABA, β-alanine and taurine have only minimal effects.

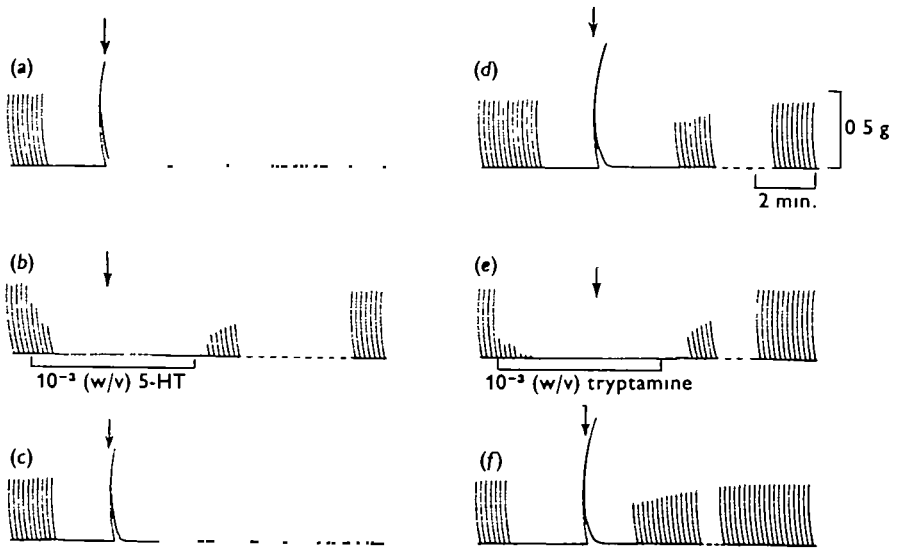
The effects of substitution in the carbon chain by methyl or ring groupings on the activity of amino acids on the retractor unguis preparation were examined by perfusing the preparation with *N*-methyl-DL-aspartate, L-proline, L-hydroxyproline and L-histidine. The effects of these substances were minimal, even at concentrations $> 10^{-3}$ (w/v). Some of the compounds, e.g. DL-cysteic acid, in which the distal carboxyl groupings of dicarboxylic amino acids are replaced by different configurations, at high concentrations were active, but most were inactive (i.e. $> 10^{-4}$ w/v). Succinic acid, which lacks the amino group characteristic of L-glutamic acid and other amino acids, was completely inactive.

Site of action of L-*glutamate*

There is little doubt that L-glutamic acid is by far the most active of all the amino acids and closely related compounds on insect (at least orthopteran) excitatory nerve-muscle preparations. In view of this the action of this substance on the retractor unguis preparation has been studied in some detail in an attempt to locate the specific site or sites of action of this substance.

In locust saline containing 10 mM potassium the normal resting membrane potential of the retractor unguis muscle fibre is about -60 mV. L-Glutamic acid in concentrations $> 10^{-7}$ (w/v) depolarizes these muscle fibres, the magnitude of the depolarization being directly proportional to the concentration of amino acid. Graded electrically excited responses accompany the larger glutamate depolarizations. The effective membrane resistance of the retractor unguis muscle fibre in 10 K saline is usually between 300–500 KΩ, but declines to about 30% of this value during treatment with 10^{-6} (w/v) L-glutamic acid. However, changes in membrane properties during prolonged expo-

sure to high concentrations of L-glutamic acid were only transitory since the membrane potential and effective resistance slowly returned to normal. Concomitant recovery of the neurally evoked contractions did not occur, however, yet following recovery of the membrane potential and effective resistance the muscle fibres contracted in response to direct electrical stimulation. Apparently L-glutamate has no effect on the electrically excitable membrane and contractile properties of the muscle fibres. It also has no effect on axonal conduction. Therefore the decline in magnitude of the neurally evoked contractions is indicative of failure of synaptic transmission.

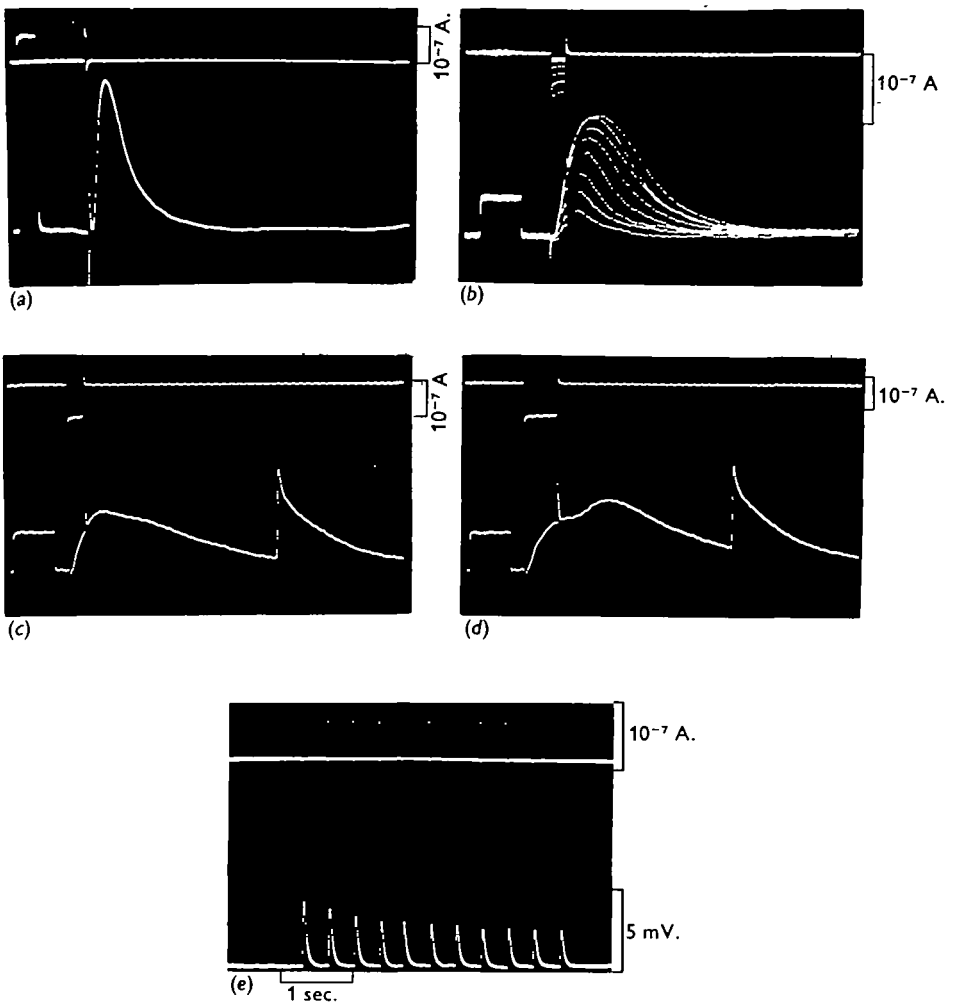


Text-fig. 3. Abolition of glutamate contractions of a locust retractor unguis muscle by tryptamine and 5-hydroxytryptamine. (*a*, *d*) Phasic contractions evoked by 10^{-6} (w/v) L-glutamic acid. Neurally evoked twitch contractions are shown at the beginning and end of each record; neural stimulation was discontinued for a short period before, during and after treatment with L-glutamic acid. (*b*, *e*) Absence of phasic contractions in response to L-glutamic acid (10^{-6} w/v) when preparation was perfused with (*b*) 10^{-3} (w/v) 5-HT and (*e*) 10^{-3} (w/v) tryptamine. These indolalkylamines are thought to block excitation of the excitatory postsynaptic membrane by competing with the excitatory transmitter for the postsynaptic receptor sites (Hill & Usherwood, 1961). After removal of the indolalkylamines phasic contractions could once again be evoked from the preparations by application of L-glutamic acid (10^{-6}, w/v) (*c.f.*). Brief applications of L-glutamic acid indicated by arrows above each trace. Time and tension calibrations same for (*a–f*).

High concentrations of 5-hydroxytryptamine (5-HT), tryptamine and a number of tryptamine analogues block neuromuscular transmission in locusts possibly by competing with the transmitter at the excitatory synapses for receptor sites on the excitatory postsynaptic membrane (Hill & Usherwood, 1961). These substances have no effect on either the axonal membrane or the electrically excitable membrane of the muscle fibre. Therefore if glutamate mimics the excitatory transmitter it should have no effect on preparations treated with 5-HT or tryptamine. To test this, preparations were perfused with either 10^{-3} (w/v) 5-HT or 10^{-3} (w/v) tryptamine. The neurally evoked contractions were abolished in the presence of these indolalkylamines, and application of L-glutamic acid in concentrations as high as 10^{-3} (w/v) now had no effect on these preparations (Text-fig. 3). After removing the 5-HT or tryptamine the

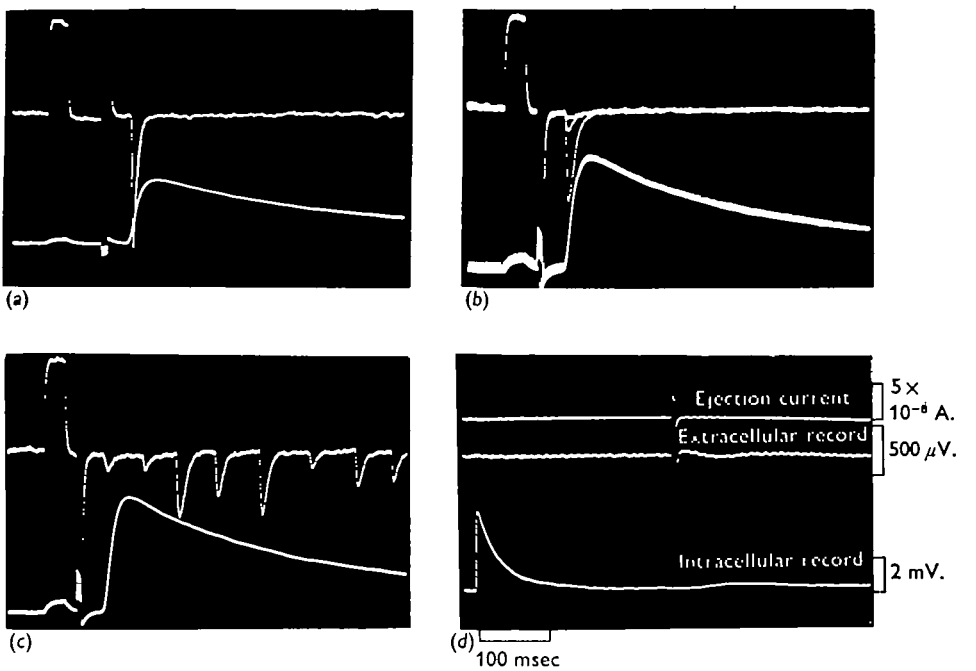
neurally evoked contractions reappeared and the muscle now responded normally to glutamate. It would appear therefore that the action of glutamate on the retractor unguis nerve-muscle preparation is limited mainly to the excitatory postsynaptic membrane of the muscle fibre, although the possibility of an additional presynaptic action cannot be ruled out.

Possibly the best way to pinpoint the site of action of glutamate is to apply it locally to the surface of the muscle fibre. Preliminary experiments using the ionto-



Text-fig. 4. Intracellular recordings (lower traces) of membrane depolarizations in response to iontophoretic application of L-glutamate. Upper traces, monitored ejection currents. (*a*) Large depolarization with the glutamate electrode close by a synaptic site. (*b*) Graded responses to increasing ejection currents. (*c–d*) Glutamate potentials followed by EPSPs. Muscle treated with saline containing 40 mM magnesium to reduce height of EPSPs. In (*d*) the duration of the ejection current was increased and a second glutamate potential was observed. Presumably the drug was reaching two synaptic sites. (*e*) De-sensitization of glutamate receptors by repetitive ejection of glutamate from a focally sited glutamate electrode. The intracellular recordings in (*a–d*) are preceded by a calibration pulse: (*a*) 10 mV., 10 msec., (*b*) 2 mV., 200 msec 2 mV., 100 msec.

phoretic technique for application of L-glutamate to retractor unguis muscle fibres were somewhat disappointing. Glutamate-sensitive sites were found on the fibres but usually only in the regions where two fibres made contact. In fact electron-microscope studies of the retractor unguis muscle have shown that most of the synapses are located on the inner faces of the fibres, with the result that they are more or less unavailable for quantitative iontophoretic studies. The glutamate-sensitive areas were very circumscribed, the 'glutamate-evoked' depolarizations disappearing when the tip of the drug-filled electrode was moved to a distance of a few microns. When glutamate



Text-fig. 5. Intracellular (lower traces) and extracellular (upper traces) EPSPs recorded from a locust retractor unguis muscle fibre (resting potential, 60 mV.). The extracellular EPSPs were recorded from glutamate-sensitive sites on the muscle fibre. When the extracellular recording electrode was moved a few microns away from the synaptic areas the extracellular EPSPs disappeared although the response to glutamate was less circumscribed. Preparation perfused with saline containing 40 mM magnesium (substituted for sodium) to reduce height of EPSPs and thereby abolish contractions of muscle. Retractor unguis nerve stimulated at a frequency of 1/sec. In (*b*) three traces are superimposed to illustrate the variable height of the extracellular EPSP. Note the response height varies in a stepwise fashion, suggesting quantal release of transmitter. This synapse failed to respond about 20% of the time during neural stimuli. In (*c*) the small neurally evoked extracellular EPSP is accompanied by a burst of spontaneous potentials. These are miniature potentials resulting from the spontaneous release of transmitter at the synapse below the extracellular recording electrode. The extracellular and intracellular responses to a small quantity of iontophoretically applied glutamate recorded from the same syapse as (*c*) are illustrated in (*d*). No synaptic current (extracellular EPSP) was recorded, indicating that transmission at this synapse had failed on this occasion. Calibration pulse at the begining of each record (*a–c*) was 1 mV., 5 msec.

was applied repeatedly to a sensitive site the response height was quickly diminished (Text-fig. 4), presumably through de-sensitization of the glutamate receptors. The glutamate sensitivity of these sites quickly returned following a short rest period. Glutamate-sensitive sites are also found on denervated muscle fibres after the endings

of the innervating excitatory axons have degenerated (Usherwood, Rees & Cochrane, 1968), neuromuscular transmission has failed and the miniature EPSPs have disappeared (Usherwood, 1963*b*, *c*), which confirms the suggestion that glutamate acts at least on the postsynaptic membrane of the excitatory synapse.

During a recent re-examination of the effects of iontophoretic application of L-glutamate on the retractor unguis preparation a few sensitive spots have been found on the 'free' outer surfaces of a few fibres and, significantly, it is only at these sites that synaptic currents can be recorded during neural stimulation and iontophoretic application of glutamate (Text-fig. 5). It seems reasonable to conclude therefore that the glutamate-sensitive sites and the excitatory synaptic sites are identical. Beránek, R. & Miller, P. L. (personal communication) have found glutamate-sensitive spots on the metathoracic coxal adductor muscle fibres of the locust, and although these glutamate spots have not yet been demonstrated as synaptic sites* they have found that the reversal potentials of the glutamate depolarizations and miniature EPSPs are identical. Their results are in line with those obtained by Takeuchi & Takeuchi (1964) from crayfish muscle fibres.

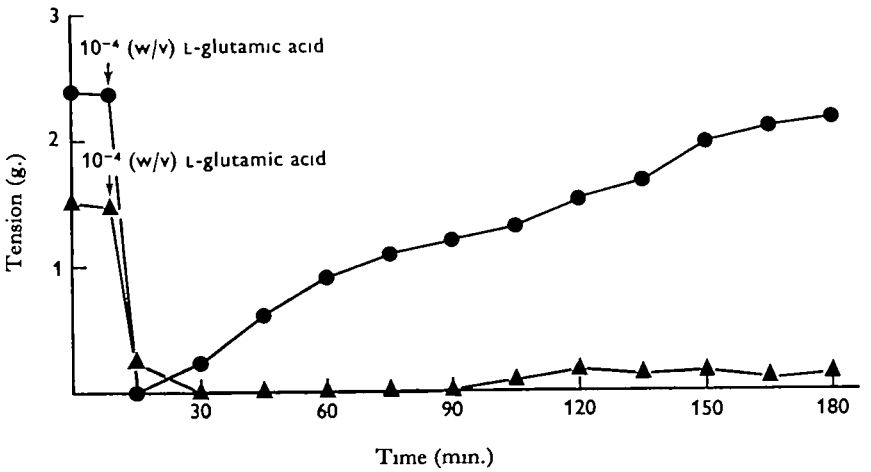
There is little doubt that retractor unguis nerve-muscle preparations become insensitive to glutamate and to the excitatory transmitter during topical application of high concentrations of L-glutamic acid. Similar effects of acetylcholine on vertebrate striated muscle have been observed and it has been suggested that these are due to desensitization of the receptors on the post-synaptic membrane of the muscle fibre by acetylcholine (Thesleff, 1955; Katz & Thesleff, 1957). It has been further suggested that this de-sensitization arises from a gradual transformation of the drug-receptor compound into a neutral form (Thesleff, 1955).

Further evidence for de-sensitization of the excitatory postsynaptic receptors on the insect retractor unguis muscle fibre was obtained in the following way. Nerve-muscle preparations were perfused with saline containing 5×10^{-5} (w/v) L-glutamic acid. After the neurally evoked contractions had disappeared the preparations were perfused briefly at 30 min. intervals with saline containing 5×10^{-5} (w/v) L-glutamic acid plus a high concentration of potassium (100 mM) (Text-fig. 6). The high-K saline was isotonic with the normal 10 mM-K-saline, potassium being substituted for sodium. The chloride concentration of the high-K saline was reduced, by substituting propionate for chloride, so as to maintain the product of the potassium and chloride concentrations the same as in the 10 mM K-saline. This was necessary to ensure fast reversibility of the potassium effects, since the muscle fibres are readily permeable to potassium chloride and show anomalous rectification (Usherwood, 1967*a*, *b*). The normal response of the retractor unguis nerve-muscle preparation to 100 mM K-saline consists of a large phasic contraction. In the presence of 5×10^{-5} (w/v) L-glutamic acid there was initially no response to the high-K saline, presumably because the muscle fibres were already depolarized and the membrane conductance was high as a result of glutamate activation of the postsynaptic membrane component. This is not an unreasonable assumption if it is remembered that insect muscle fibres are multiterminally innervated. When the membrane conductance slowly returns to its initial low resting value during prolonged treatment with a high concentration of

* Beránek & Miller have now demonstrated that 'the peaks of L-glutamate-sensitivity are very close to or identical with neuromuscular junctions.' (*J. Physiol* **196**, 71–2*P*, 1968).

glutamic acid, the potassium response would be expected to reappear. The fact that the potassium response returned whilst the neurally evoked contractions remained depressed suggests that the main site of glutamate action is the postsynaptic membrane and that the loss of the neurally evoked contractions is due to de-sensitization of the receptors on this membrane. Also, it is significant that, even after 3–4 hr. in saline containing 5×10^{-5} (w/v) glutamic acid, the neurally evoked contractions quickly reappeared when the muscle was perfused with drug-free saline. This underlines the very rapid reversibility of the glutamate response.

When the retractor unguis preparation was perfused with concentrations of glutamic acid $< 10^{-7}$ (w/v) but $> 10^{-12}$ (w/v) the neurally evoked contractions were potentiated. In the absence of nerve stimulation miniature EPSPs can be recorded from insect muscle fibres (Usherwood, 1961, 1963*a*). Low concentrations of L-glutamic



Text-fig. 6. Effect of L-glutamic acid (10^{-4}, w/v) on the mechanical responses of the locust retractor unguis muscle to neural stimulation (▲) and to potassium (100 mM) (●). The twitch contractions and potassium contractures were initially reduced during glutamate treatment. The potassium response then slowly recovered until eventually the phasic contracture was as large as the response recorded in the glutamate-free saline. The neurally evoked twitch contraction was completely abolished throughout almost the entire period of glutamate treatment. The slight recovery of the twitch response seen after about 2 h. perfusion with glutamate-saline is not considered significant.

acid also increase the frequency but not the amplitude of these miniature EPSPs (Usherwood & Machili, 1966). Presumably concentrations of L-glutamic acid greater than 10^{-7} (w/v) also increase the miniature EPSP frequency although this change would be masked to some extent by the increased conductance of the muscle fibre, which occurs during treatment with high concentrations of L-glutamic acid. Of course considerable care must be exercised in interpreting measurements of miniature frequency and amplitude from multiterminally innervated insect muscle fibres, since the amplitude distribution in fibres with small length constant/length ratios is markedly skewed and many potentials are lost in the amplifier noise (Usherwood, 1963*a*). Changes in membrane conductance could therefore lead to errors in interpretation of mean amplitudes and frequencies of the miniature EPSPs. For this reason we have re-examined the effect of low concentrations of L-glutamic acid on fibres of the locust extensor tibiae muscle which have large length constant/length ratios (Usherwood,

1963*a*). The results from these studies confirmed those obtained from our previous experiments of the retractor unguis muscle, i.e. low concentrations of L-glutamic acid increase miniature frequency but not miniature amplitude. It has been proposed (e.g. del Castillo & Katz, 1954*a*, *b*) that changes in miniature EPSP frequency reflected some alteration in presynaptic rather than postsynaptic properties. Possibly glutamate facilitates the release of transmitter from the excitatory nerve terminals. This could account for potentiation of the neurally evoked contractions in low concentrations of L-glutamate (Usherwood & Machili, 1966).

Enzymes and enzyme inhibitors

At vertebrate neuromuscular junctions there is clear evidence for the presence of high concentrations of a powerful enzyme, cholinesterase, which is thought to hydrolyse the transmitter released from the motor nerve terminals and acetylcholine applied either topically or iontophoretically. Administration of potent cholinesterase inhibitors like prostigmine, eserine and edrophonium leads to increases in amplitude of the miniature EPSPs, EPSPs and neurally evoked twitch contractions of the skeletal muscle fibres (e.g. del Castillo & Katz, 1957; Axelsson & Thesleff, 1958). In view of

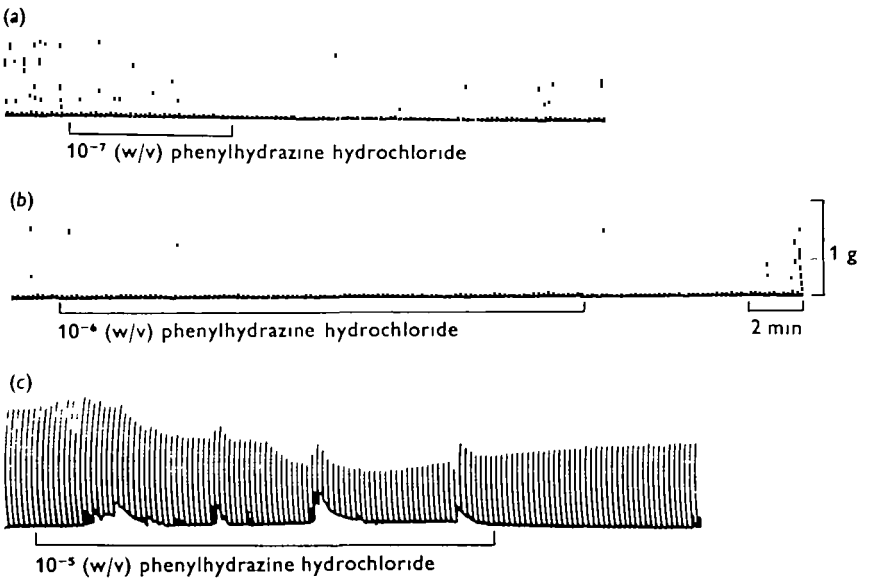
Table 2. *Effect of enzyme inhibitors**

Compound	Enzyme	Minimum concentration (w/v) required to affect indirect mechanical responses
Semicarbazide HCl	Glutamic-pyruvic transaminase Glutamic decarboxylase Diamine oxidase	10^{-3}
Hydroxylamine HCl	Glutamic-pyruvic transaminase Glutamic decarboxylase Diamine oxidase	10^{-3}
Thiosemicarbazide HCl	Glutamic-pyruvic transaminase Glutamic decarboxylase Diamine oxidase	10^{-3}
Phenylhydrazine HCl	Glutamic-pyruvic transaminase Glutamic decarboxylase Diamine oxidase	10^{-3}
p-Phenylenediamine HCl	Glutamic-oxaloacetic transaminase Glutamic pyruvic transaminase	10^{-3}

* Adapted from Curtis, Phillips & Watkins, 1960.

the undoubted enzyme-transmitter relationships in vertebrate neuromuscular systems it seemed worth while to search for parallel systems in the insect, working on the assumption that the chemical mediator at the excitatory nerve-muscle synapse in insects is L-glutamate. The enzyme glutamic decarboxylase occurs in relatively high concentrations in some insect tissues (Frontalis, 1961). This enzyme mediates the

α-decarboxylation of L-glutamic acid to GABA. In view of the very narrow limits required for optimal activity of this and other enzymes which use glutamate as a substrate, it is perhaps not very surprising that the effects of perfusion of the retractor unguis nerve-muscle preparation with this enzyme were minimal. Nevertheless glutamic decarboxylase depresses the neurally evoked contractions of the locust retractor unguis muscles (Usherwood & Machili, 1966). Glutamic transaminases also slightly depress the mechanical responses, apparently more effectively than glutamic decarboxylase. Glutamic dehydrogenase has very little effect on the contraction height. As an alternative approach a number of inhibitors of glutamic decarboxylase were



Text-fig. 7. Effect of phenylhydrazine hydrochloride, a carbonyl reagent, on the neurally evoked twitch contractions of a locust retractor unguis muscle. With low concentrations of enzyme inhibitor the twitches were potentiated (*a*) but with higher concentrations (*b*, *c*) the twitches were initially potentiated and then depressed, the depression of the mechanical responses being accompanied in (*c*) by some repetitive activity. Time and tension calibrations same for (*a*–*c*).

tested on our preparation and the results are summarized in Table 2. Phenylhydrazine HCl was by far the most potent inhibitor. It greatly increased the amplitude of the neurally evoked mechanical responses and evoked repetitive contractions in response to a single nerve impulse (Text-fig. 7) and was effective at concentrations as low as 10^{-9} (w/v). If phenylhydrazine inhibits a naturally occurring decarboxylase or transaminase and glutamate is the excitatory transmitter, then this inbibitor would be expected to prolong and enhance the EPSP, which in turn would cause repetitive firing of the electrically excitable membrane of the muscle fibre. These changes would be expressed as a potentiation of the neurally evoked contractions with intermittent repetitive contractions, i.e. exactly the type of response recorded in the present experiments. Unfortunately inhibitors like phenylhydrazine HCl are not very specific and could be acting on enzymes other than glutamic decarboxylase or a glutamic transaminase.

Analysis of amino acids in locust haemolymph (blood)

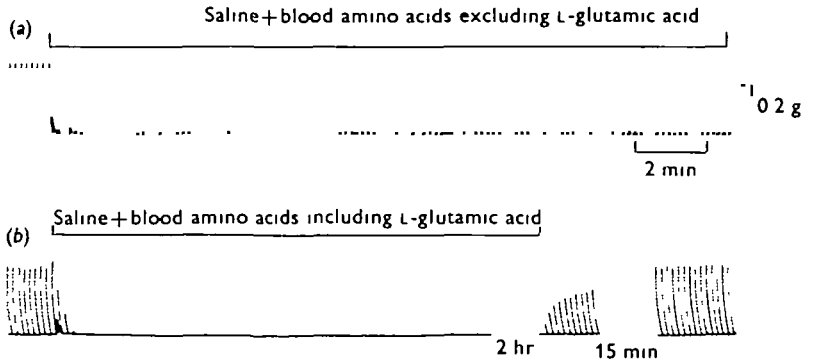
The fact that insect nerves and muscles are bathed in haemolymph poses problems for the advocates of amino acid involvement in insect neuromuscular synaptic transmission. For example, it has been reported on a number of occasions (e.g. Florkin, 1959; Sutcliffe, 1963) that the blood of many insects contains high concentrations of short-chained amino acids including glutamate and glutamine, the two substances known to be most active at the excitatory nerve-muscle synapse in the locust, grasshopper and cockroach. Using an amino acid analyser we have recently re-investigated the amino acids of locust and grasshopper haemolymph and two results from the locust are illustrated in Table 3. If glutamate is the excitatory transmitter then it is

Table 3. *Molar concentrations of free amino acids in the haemolymph (blood) of the locust*, Locusta migratoria

Amino acid	Sample 1	Sample 2	Sample 3
β-Alanine	$9{\cdot}8 \times 10^{-4}$	$1{\cdot}7 \times 10^{-4}$	$1{\cdot}7 \times 10^{-3}$
Arginine	$5{\cdot}7 \times 10^{-4}$	$7{\cdot}2 \times 10^{-5}$	$1{\cdot}2 \times 10^{-3}$
Asparagine	$7{\cdot}0 \times 10^{-4}$	$3{\cdot}1 \times 10^{-4}$	—
Aspartic acid	$8{\cdot}4 \times 10^{-5}$	$1{\cdot}8 \times 10^{-5}$	—
Cystine	$6{\cdot}6 \times 10^{-5}$	$6\,4 \times 10^{-5}$	$3\,8 \times 10^{-5}$
Glutamic acid glutamine	$3{\cdot}7 \times 10^{-4}$	$5{\cdot}4 \times 10^{-4}$	$5{\cdot}1 \times 10^{-4}$
Glycine	$5{\cdot}1 \times 10^{-3}$	$5\,1 \times 10^{-3}$	$2{\cdot}4 \times 10^{-3}$
Histidine	$5\,4 \times 10^{-4}$	$1{\cdot}3 \times 10^{-5}$	$1{\cdot}7 \times 10^{-3}$
Isoleucine	$2{\cdot}8 \times 10^{-4}$	$6{\cdot}4 \times 10^{-5}$	$4{\cdot}2 \times 10^{-4}$
Lysine	$1{\cdot}0 \times 10^{-3}$	$3{\cdot}3 \times 10^{-4}$	$1{\cdot}4 \times 10^{-3}$
Methionine	$1{\cdot}7 \times 10^{-4}$	$5\,6 \times 10^{-5}$	$2{\cdot}6 \times 10^{-4}$
Phenylalanine	$1{\cdot}2 \times 10^{-4}$	$4{\cdot}6 \times 10^{-5}$	$1{\cdot}5 \times 10^{-4}$
Proline	$2{\cdot}9 \times 10^{-3}$	$9\,8 \times 10^{-4}$	$1{\cdot}2 \times 10^{-3}$
Serine	$6{\cdot}4 \times 10^{-4}$	$6{\cdot}0 \times 10^{-5}$	$5{\cdot}9 \times 01^{-4}$
Threonine	$7{\cdot}0 \times 10^{-4}$	$3{\cdot}1 \times 10^{-4}$	$5{\cdot}7 \times 10^{-4}$
Tyrosine	$2{\cdot}8 \times 10^{-4}$	$2{\cdot}5 \times 10^{-4}$	$2{\cdot}2 \times 10^{-4}$
Valine	$7{\cdot}0 \times 10^{-4}$	$1{\cdot}5 \times 10^{-4}$	$1{\cdot}5 \times 10^{-3}$

difficult to explain why the high concentration of glutamate in the haemolymph of the locust does not de-sensitize the postsynaptic receptors on the muscle fibres. In other words, transmission at the excitatory neuromuscular synapses of the locust does not seem possible *in vivo* if glutamate is the chemical mediator. It is not possible to invoke either structural or enzymic barriers around the neuromuscular junctions to protect the synapses from the glutamate in the blood, since the postsynaptic receptors are excited by very low concentrations of topically and iontophoretically applied glutamate. Since it was not possible to separate the glutamine and glutamate fractions in the blood, it is possible that locust blood contains little 'free' glutamate. This could of course mean that there is a lot of glutamine present, but since this substance is also relatively active at the excitatory neuromuscular synapses, this would not solve the problem. In view of this it was decided to examine the effects of glutamate on the perfused retractor unguis preparation using saline containing all of the amino acids shown in Table 3 in concentrations found in the blood. When L-glutamic acid was omitted from this saline, the neurally evoked contractions were initially depressed but then recovered (Text-fig. 8*a*) and were often slightly larger than in normal 10 K

saline without the amino acids, even though the 'amino acid' saline contained 10^{-4} (w/v) glutamine. In the absence of the other amino acids, glutamine at this concentration markedly de-sensitizes the excitatory receptors. When 10^{-4} (w/v) L-glutamic acid was added to this 'amino acid' saline a typical large glutamate phasic contraction was recorded followed by immediate disappearance of the neurally evoked contractions, presumably as a result of receptor de-sensitization (Text-fig. 8*b*). When the glutamic acid was removed the neurally evoked contractions reappeared. In another series of experiments isolated retractor unguis nerve-muscle preparations were perfused with normal 10 K saline. This saline was then replaced with locust haemolymph extracted from about 100 adult locusts. Significantly there was no lasting change in the charac-



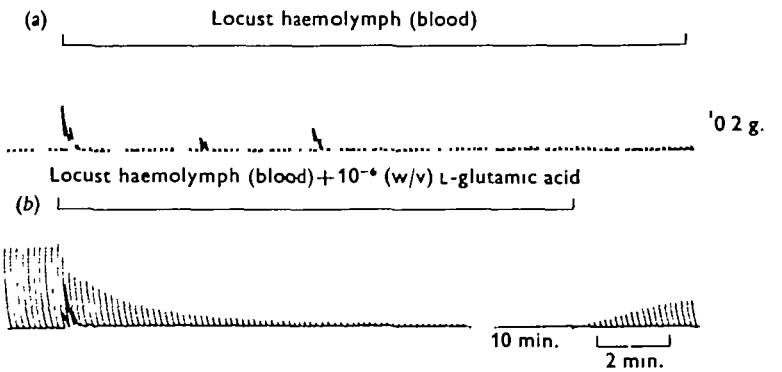
Text-fig. 8. Changes in the neurally evoked contractions of a locust retractor unguis muscle during perfusion with saline containing all the amino acids found in locust haemolymph (Table 3): (*a*) with the exception of L-glutamic acid; (*b*) including L-glutamic acid. The concentrations of the amino acids were approximately the same as in locust haemolymph. The preparation was perfused with normal (i.e. amino acid-free) locust saline for 3 hr. before treatment with the experimental saline. In the absence of L-glutamic acid the 'amino acid saline' caused only a small *transient* change in the response pattern, whereas when L-glutamic acid was included in the saline there was a sustained abolition of the mechanical responses. Time and tension calibrations same for (*a–b*).

teristics of the neurally evoked contractions during haemolymph perfusion (Text-fig. 9). However, when 10^{-6} (w/v) L-glutamic acid was added to the haemolymph the muscle gave a typical large phasic contraction, followed by a sustained decline in the height of the neurally evoked responses, presumably as a result of de-sensitization of the postsynaptic receptors (Text-fig. 9*b*). It seems probable therefore that the haemolymph of locust, grasshopper and cockroach contains very little 'free' glutamate. The lowered excitatory activity of topically applied glutamine in the presence of other amino acids remains to be explained.

Release of glutamate during nerve stimulation

The results of preliminary studies show that when retractor unguis nerve-muscle preparations from the locust are perfused with saline, four amino acids, aspartate, glutamate, glycine and alanine appear in the perfusate. During nerve stimulation only the glutamate concentration increases significantly. At low frequencies of stimulation the amount of glutamate recovered in the perfusate is roughly proportional to

the number of stimuli applied to the retractor unguis nerve. Kerkut, Leake, Shapira, Cowan & Walker (1965) obtained L-glutamate from cockroach leg preparations during nervous stimulation, the amount recovered being proportional to the number of stimuli applied to the leg nerves. It seems probable therefore that glutamate is released by motor nerve endings in the grasshopper, locust and cockroach and that this amino acid is the transmitter at the neuromuscular excitatory synapses in these insects. Significantly the amount of glutamate appearing in the perfusate from the locust retractor unguis nerve-muscle preparation during neural stimulation is increased by blocking the excitatory synapses with 5-hydroxytryptamine. This will be discussed more fully in a later publication.



Text-fig. 9. (*a*) Absence of any permanent change in the magnitude of the neurally evoked contractions of a locust retractor unguis muscle when perfused with locust haemolymph (blood). However, when 10^{-4} (w/v) L-glutamic acid was added to the haemolymph the contractions were abolished (*b*), presumably as a result of de-sensitization of the glutamate (excitatory transmitter?) receptors. Time and tension calibrations same for (*a–b*).

DISCUSSION

It is clear from these studies that glutamic acid is the most potent amino acid at the excitatory synapses in the locust, grasshopper and cockroach. It is perhaps significant in this respect that the α-decarboxylation product of glutamic acid, GABA, mimics the transmitter at inhibitory neuromuscular synapses in insects (Usherwood & Grundfest, 1964, 1965). There are striking similarities between the effects of amino acids on insect neuromuscular preparations, on the one hand, and crustacean neuromuscular preparations (Robbins, 1959, van Harreveld & Mendelson, 1959; Takeuchi & Takeuchi, 1964) on the other. There are also similarities between insect neuromuscular preparations and cat spinal neurones (Curtis & Watkins, 1960; Curtis, Phillis & Watkins, 1960) with respect to their responsiveness to amino acids. Curtis & Watkins (1960) concluded that for optimal excitation of cat spinal neurones the amino acids must have two acidic groups and one basic group, with two or three carbon atoms between the amino group and one of the acidic groups (distal). The other acidic group is optimally situated α with respect to the amino group. Activity is abolished if the acidic or basic groups are not free and is reduced or abolished by substitution within the intermediate carbon chain or within the amino group, depending on the size, number and position of the constituents. In insects the presence of the distal acid

group in the amino acid chain is absolutely essential for activity. This group, as well as the amino group, could of course be almost completely ionized in the physiological medium. There is a clear relationship between optimum activity and the position of the amino group with respect to the distal acid group. For example, the α-dicarboxylic amino acids aspartate, glutamate, α-aminoadipate and α-amino pimelate carry similar charges on their molecules (a positive charge on the amino group and a negative charge on each of the two carboxyl groups) and yet their activities are considerably different. The only structural variation among these amino acids is of course the length of the carbon chain and therefore the distance of the distal acid group from the positively charged amino group. Reduction of the negative charge on the distal acid group, for instance by substitution of OH^- by an amino group (e.g. glutamine), also reduces responsiveness, whereas complete removal of the acidic group (e.g. GABA, β-alanine) or its replacement with neutral groups (e.g. tyrosine, valine and leucine) abolishes activity altogether. Absence of the amino group, as in succinic acid, abolishes activity completely. So also does any substitution within the carbon chain. From these studies it seems possible that glutamate is the only compound with the necessary chain length and distribution of the ionized groups suitable for interaction with ionic charges on the receptor sites. Steric orientation of the transmitter molecule could also be decisive for optimal activity, which could account for the fact that D-glutamic acid is much less active than the naturally occurring L-isomer. L-Glutamate is also the most active amino acid at crustacean excitatory neuromuscular synapses and here, as in the insects, activity of the D-enantiomorph is considerably lower (Robbins, 1959; Takeuchi & Takeuchi, 1964).

The effects of L-glutamate on insect neuromuscular preparations raises the question of whether this substance is the transmitter at the excitatory synapses. There are several criteria for identification of a substance as a chemical synaptic transmitter (Paton, 1958; Florey, 1960; Werman, 1966) among which the most fundamental being that the substance must have the same action on the transmitter and that it should be recoverable in the perfusion fluid during neural stimulation. Additional support for the role of a substance as a chemical mediator at synapses would be the demonstration of enzyme systems capable of synthesis and destruction of the substance at the synaptic sites. There seems little doubt that L-glutamate mimics the transmitter at excitatory neuromuscular synapses in the locust, cockroach and grasshopper. By applying L-glutamate topically and iontophoretically to neuromuscular preparations from these insects it has been shown that this substance acts specifically at the excitatory synapses and that its mode of action is identical to that of the excitatory transmitter. For example, the identification of glutamate-sensitive sites on the muscle fibres with the excitatory synapses, the excitatory effects of potassium on glutamate-de-sensitized muscle fibres where neuromuscular transmission was completely blocked, and the absence of glutamate responses in preparations treated with the excitatory synaptic blocking agents 5-HT and tryptamine point conclusively to a specifically synaptic action for L-glutamate. Furthermore, the positive effects of L-glutamate on denervated muscle fibres in which the nerve endings have demonstrably degenerated and the positive effects of L-glutamic acid on muscle fibres treated with magnesium ions in concentrations which block synaptic transmission presynaptically (Usherwood, 1963*a*), confirm at least the postsynaptic action of this amino acid. In other words, it appears

from this evidence that the excitatory receptors on the postsynaptic membrane are in fact glutamate receptors. This does not necessarily imply that the excitatory transmitter is L-glutamate, although the recovery of L-glutamate in the perfusate from neuromuscular preparations of locust, grasshopper and cockroach following nerve stimulation makes this extremely likely. It will of course be necessary to demonstrate the synthesis and/or storage of glutamate in the terminals of these insect excitatory motoneurones before this can be conclusively established.

The relatively brief depolarizations recorded in response to low levels of iontophoretically applied L-glutamate could be due to enzymic removal of this substance although it is possible that it simply diffuses away from the synaptic region and enters the ordinary metabolic pool. Removal of GABA, a possible inhibitory transmitter in insects, is thought to occur by diffusion only, at least at inhibitory neuromuscular synapses of cockroach, grasshopper and locust (Usherwood & Grundfest, 1965). Enzymic destruction of glutamic acid could, however, facilitate the removal of glutamate *in vivo* from the excitatory neuromuscular synapses. The reduction of the neurally evoked contractions during treatment with glutamic decarboxylase and glutamic transaminases gives added weight to the argument that glutamate is the transmitter and the potentiation of these responses in the presence of decarboxylase and transaminase inhibitors like phenylhydrazine hydrochloride is suggestive of some enzymic role at insect excitatory neuromuscular synapses. In fact the responses of insect neuromuscular preparations to these inhibitors are strikingly reminiscent of the effects of anticholinesterases on nerve-striated muscle preparations in vertebrates. The effects of carbonyl reagents on insect neuromuscular preparations must, however, be interpreted with some caution. An inhibitor completely specific for either glutamic decarboxylase or transaminases has not yet been tested on our insect preparations. Furthermore, although insect nervous tissue is known to contain high concentrations of glutamic decarboxylase (Frontalis, 1961), neither this enzyme nor transaminases have not been shown to occur at the excitatory neuromuscular synapses.

There remains the problem of the potentiating effects of low concentrations of L-glutamic acid on the neurally evoked contractions of insect muscles. It has been suggested that this is due, at least in part, to facilitated release of the excitatory transmitter as a result of some action of glutamate on the presynaptic axonal membrane (Usherwood & Machili, 1966; Usherwood, 1967*b*). This suggestion was based on the observation that in locust muscle fibres the frequency but not the amplitudes of the miniature EPSPs was increased in the presence of low concentrations of L-glutamic acid. The neurally evoked contractions of crustacean muscle fibres are also potentiated by low concentrations of L-glutamic acid (Robbins, 1958). However, Takeuchi & Takeuchi (1964) have demonstrated that this could be, at least in part, postsynaptic in origin. They found that the depolarization of crutacean muscle fibres resulting from brief iontophoretic applications of glutamate to the excitatory synapses were transiently potentiated when a small conditioning dose of this amino acid was applied to the synapse. Possibly, both presynaptic and postsynaptic changes contribute to the potentiation of the neurally evoked contractions of these arthopodean muscle fibres.

Suggestions that the transmitter at insect excitatory neuromuscular synapses is L-glutamate have been viewed in the past with some degree of scepticism. This was

due to the apparent presence of very high (10^{-4}, w/v) concentrations of L-glutamate in the haemolymph of many insects, i.e. glutamate concentrations which should completely de-sensitize the postsynaptic receptors at the excitatory synapses. The results of the present investigations should remove some of this uncertainty, since it has been shown that isolated locust neuromuscular preparations function quite well when perfused with haemolymph and contract phasically when 10^{-6} (w/v) L-glutamic acid is added to the haemolymph. Apparently locust haemolymph contains very little 'free' glutamate. There seems to be no reason, therefore, why L-glutamate should not be the transmitter at excitatory neuromuscular synapses in the locust, grasshopper and cockroach. Indeed, glutamate-sensitive sites have recently been found on muscle fibres of mealworm (*Tenebrio molitor*) larvar (Usherwood, unpublished). The mealworm belongs to the Coleoptera, an insect group far removed from the orthopteroid groups to which the cockroach, locust and grasshopper belong. Perhaps, therefore, glutamate is the excitatory transmitter at all insect excitatory neuromuscular synapses.

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SUMMARY

1. The effects of a wide range of amino acids and related compounds on retractor unguis nerve-muscle preparations from the locust, grasshopper and cockroach have been investigated.

2. L-glutamate is the most active excitatory substance. The presence of two acidic groups and one amino group is essential for excitatory activity while the position of the amino group is of some importance in determining the level of activity.

3. When L-glutamate is applied iontophoretically to the muscle fibres, 'glutamate' depolarizations are recorded only at the synaptic sites. Other evidence that the action of glutamate is restricted to the synaptic sites is presented.

4. Perfusion of isolated locust retractor unguis nerve-muscle preparations with locust haemolymph does not markedly affect the neurally evoked mechanical responses. It appears that locust haemolymph contains little 'free' L-glutamate.

5. Four acidic amino aids have been identified in the perfusate from isolated retractor unguis preparations namely, glycine, alanine, aspartate and L-glutamate. However, only L-glutamate increases in concentration during stimulation of the retractor unguis nerve.

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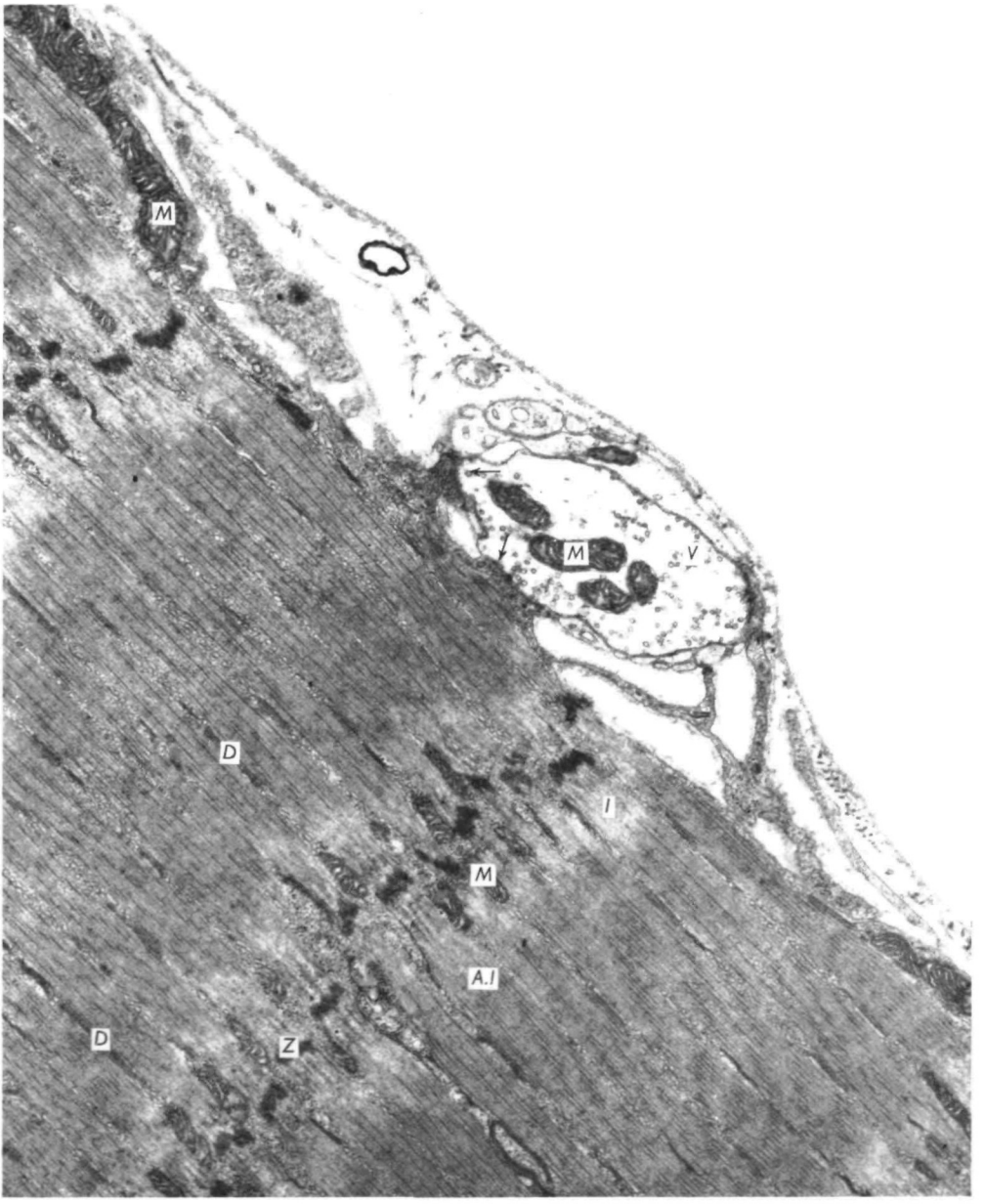

P. N. R. USHERWOOD AND P MACHILI

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EXPLANATION OF PLATE

Electron micrograph of a neuromuscular synapse on a metathoracic retractor unguis muscle fibre from the locust. A nerve ending containing mitochondria (*M*) and presynaptic vesicles (*V*) makes intimate contact with a muscle fibre. The axonal membrane and membrane of the muscle fibre are separated by a gap of less than 200 Å (arrows). This is presumably the synapse proper. The synaptic gap contains some dense material. Note absence of any visible structural differentiation of postsynaptic membrane. The nerve ending is situated in a depression on the surface of the muscle fibre and is partly surrounded by glial cells. Some details of the structure of the phasic striated muscle fibre of the locust are seen in longitudinal section. *M*, Mitochondria; *Z*, Z-line; *I*, actin filaments; *A.I*, actin and myosin filaments at junction of A band and I band; *D*, diad.