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THE ACTION OF IONTOPHORETICALLY APPLIED GLUTAMATE ON INSECT MUSCLE FIBRES

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INTRODUCTION

After it had been established that glutamate has excitatory transmitter-like action on crustacean neuromuscular junctions (Robbins, 1958, 1959; van Harreveld, 1959; van Harreveld & Mendelson, 1959; Takeuchi & Takeuchi, 1964) several authors considered this substance as a possible neuromuscular transmitter also in insects. Kerkut, Shapira & Walker (1965*a*) were able to show that low concentrations of glutamate caused a cockroach muscle to develop tension and Kerkut *et al.* (1965*b*) reported that after indirect stimulation of the perfused muscle, glutamate could be detected in the perfusate. In locust muscles kept *in vitro*, Usherwood & Machili (1966) produced a depolarization of the resting membrane by L-glutamate in concentrations greater than 10^{-8} (w/v) and at concentrations 10^{-7} (w/v) the muscles reacted by single contractions which reached a maximum at 10^{-5} (w/v). High concentration brought about de-sensitization. Low concentrations (10^{-9}—10^{-12}, w/v) of L-glutamate potentiated the indirect response and raised the frequency of miniature excitatory junction potentials (min. e.j.p.'s) and the authors suggested that glutamate exerts both a pre- and post-synaptic effect. Increased spontaneous electrical activity in cockroach muscles on local application of L-glutamate was reported by Kerkut & Walker (1966, 1967) and McCann & Reece (1967) observed depolarization of the flight muscle fibres of *Sarcophaga* on local or systemic application of glutamate.

In these studies glutamate and other drugs were applied diffusely over a number of muscle fibres, usually added to the perfusing fluid or the bath solution, or applied through a micro-pipette without precise localization. Continuous and diffuse application of the drug, however, besides complicating the experiments by de-sensitization, does not localize the depolarizing effect. These difficulties can be obviated by local transient iontophoretic applications of the drug on the fibre surface. Such applications closely imitate the release of transmitter from nerve endings (del Castillo & Katz, 1955) and this technique combined with microelectrode recording provides details of the synaptic action of a drug.

In the present experiments we have applied L-glutamate to the surface of muscle fibres from a locust metacoxal muscle using the iontophoretic technique. By various procedures, similar to those used by Takeuchi & Takeuchi (1964) in crustacea, we have established that the membrane is sensitive to L-glutamate only in a small number of highly discrete sites, that it affects these sites directly rather than via a pre-synaptic

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structure, that the depolarizations so produced share characteristics with those produced naturally and that the sensitive sites are very close to and probably identical with neuro-muscular junctions. We believe that these experiments support the hypothesis that glutamate is the natural excitatory transmitter at insect neuromuscular junctions. A brief account of the main results was given during a demonstration at the Symposium of the Society of Experimental Biology on 'Aspects of cell motility' in Oxford, September 1967, and before the Physiological Society (Beránek & Miller, 1968).

METHODS

The experimental animals and preparations

The experiments were performed on twenty-eight neuromuscular preparations of the metathoracic adductor coxal muscle (m. 130) from adult specimens of *Schistocerca gregaria* Forskål and *Locusta migratoria* (L.) (Hoyle, 1966). The muscles with or without their nerves were dissected out after brief CO_2 anaesthesia, tested for direct and indirect excitability and kept during the experiment in a Ringer solution (Hoyle, 1953) at room temperature (18–22° C.). In these conditions the muscles preserved their resting potentials and gave twitches to single-shock stimulation for several hours.

Procedure with micro-electrodes

For recording, the muscles were stretched slightly with their ventromedial face upwards on a plane-parallel glass plate which formed the bottom of a Perspex chamber containing the Ringer solution; they were kept in position by means of magnetic pinholders. Ag–AgCl reference electrodes were connected by agar bridges with the solution in which also, when necessary, a suction electrode was placed for nerve stimulation. The recording chamber was screwed to a firm horizontal Perspex stage which was in turn bolted to the base of a double Zeiss (Jena) micro-manipulator placed in a shielded box in a vibration-free suspension. The muscle was trans-illuminated through the glass bottom of the recording chamber from a source with heat and dispersion filters, and it was observed through a binocular dissecting microscope under high (× 100) magnification. Before recording, tracheae were removed to expose as much as possible of the surface of the muscle fibres. Microelectrodes of the standard glass capillary type filled with 3 M-KCl were used throughout the experiments. The micro-pipettes were prepared so as to have 30–50 MΩ resistance after filling, but their tips were broken before recording to obtain 5–20 MΩ, sharp, low-noise microelectrodes. For extracellular recording the electrodes were filled with 3 M-NaCl. The electrodes were mounted on micro-manipulator holders, and the electrolyte was connected by means of a thin AgCl-coated silver wire to the input of a high-impedance differential pre-amplifier (Ujec & Beránek, 1967) or to a source of polarizing current. The recorded potential changes were photographed on the screen of a 502A Tektronix oscilloscope.

Procedure with electrophoretic micro-pipettes

The technique used was essentially that described by other authors (Nastuk, 1953; del Castillo & Katz, 1955; Takeuchi & Takeuchi, 1964). Micro-pipettes of the same dimensions as the KCl-filled electrodes were filled with distilled water which was then

replaced by 1·5 M sodium salt of L-glutamic acid (British Drug Houses, Biochemical Reagent), the pH of which was adjusted to 8. For some experiments the pipettes were filled with D-glutamate. The electrodes were used fresh or stored in a refrigerator for up to 10 days. During experiments they were mounted on a micro-manipulator holder and the inside was connected by a silver wire with the source of electrophoretic current pulses. At pH 8 the glutamate is completely anionic so that to extrude the substance the tip of the electrophoretic pipette has to be made negative. A steady tip-positive 'braking' voltage (1–5 V.) was used to oppose the free diffusion of glutamate anions out of the pipette between the electrophoretic pulses. A 100 KΩ resistor was placed in the pipette circuit to monitor the electrophoretic current.

The electrodes were brought into position under continuous visual control. In order to secure a close apposition of the electrophoretic pipette to the fibre surface, the intracellular electrode was used to record, at high gain, the first detectable potential change appearing when the pipette touches the fibre. The recording of electrotonic potentials was used as an indication that both the electrophoretic and recording pipettes were placed on the same fibre. A wide range of electrophoretic pulses was used (1–1000 msec., 10^{-8}—10^{-7} A.) and the sensitivity to glutamate was expressed in mV./nC. units (Miledi, 1960), i.e. mV. of depolarization per nC. of charge used to displace the ionized substance from the pipette.

RESULTS

Resting potentials

The middle of a fibre was penetrated by an intracellular electrode. Preliminary estimation of the length constant of the fibre indicated values of approximately 2·5 mm. As the length of the average fibre is about 1·1 mm. little distortion would be expected for potential changes originating at any part of the membrane. The resting potentials measured immediately on piercing of the fibre membrane by a micro-electrode were between 50 and 60 mV. but built up to 70–80 mV. during the experiment due probably to spontaneous sealing of the membrane around the electrode. In some fibres slow (about 1–2/sec.) spontaneous oscillations of the membrane potential occurred which made the measurements difficult. The origin of these oscillations was not investigated and fibres were used in which they were absent or small.

Glutamate sensitivity

When the glutamate-filled micro-pipette was moved along the fibre surface and L-glutamate was applied iontophoretically, very circumscribed spots could be found where the applications of glutamate produced depolarizations with a fast-rising and a slow-falling phase (Fig. 1). Focal points could be found at these places where with close apposition of the micro-pipette the sensitivity reached tens of mV./nC. units. The sensitivity decreased sharply with distance from the focal point and within 10–35 μ it fell either to zero or to a low 'background' sensitivity which was detected all over the fibre. When the surface of a fibre was examined systematically in 10 μ steps in a longitudinal track several such spots were usually revealed (Fig. 2), though in a few fibres no sensitive spots could be found in spite of a thorough search. The sensitive spots were predominantly (but not exclusively) found near the line of

apposition of two neighbouring fibres and sometimes in order to reach the point of maximum sensitivity the electrode had to be inserted between the fibres. In order to produce a glutamate potential the iontophoretic pipette had to be in close contact with the fibre surface, practically prodding the membrane, so that simple shifts of the pipette from place to place by horizontal movements of the manipulator were seldom possible and 'jumps' had to be made to investigate the sensitivity along the fibre.

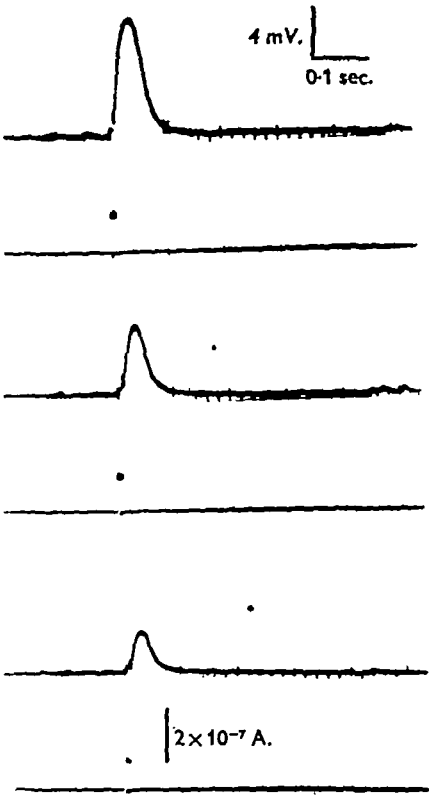

Fig. 1

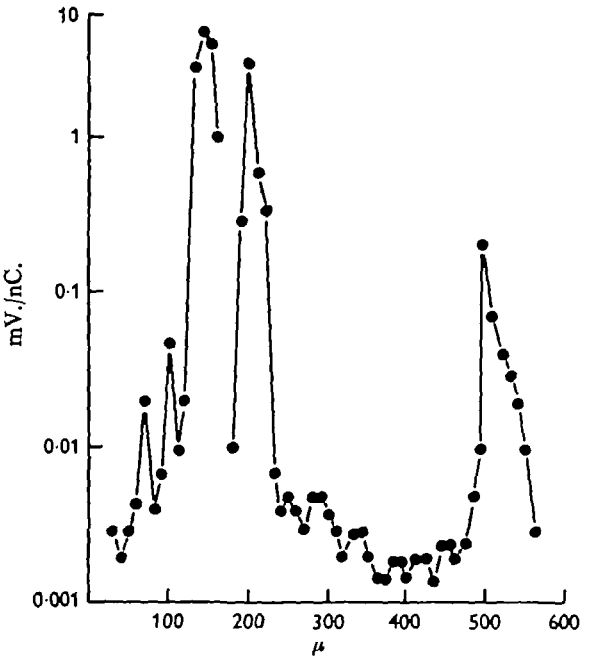

Fig. 2

Fig. 1. Depolarizations of the muscle-fibre membrane produced by brief electrophoretic applications of L-glutamate (glutamate potentials). The upper trace in each pair of records: membrane potential measured by an intracellular electrode. Lower trace: current flowing through electrophoretic pipette placed on the fibre surface. From top to bottom: 1, response from a spot of peak sensitivity; 2, the electrode moved 4 μ from position in 1; 3, electrode moved 4 μ farther away. Temperature 22° C.

Fig. 2. Distribution of sensitivity along a part of a muscle fibre. Sensitivity was measured in mV./nC. units as the microphoretic pipette was moved in 10 μ steps along the fibre surface with the exception of a small portion covered with a tracheole.

The relation of glutamate-sensitive spots to the excitatory junctional potentials (e.j.p.'s)

In order to record the e.j.p.'s undisturbed by twitches the Mg^{2+} concentration was raised to 5 mM and the Ca^{2+} concentration was halved; this reduced the e.j.p.'s to below the spike-triggering level. This effect, observed already at normal Ca^{2+}

and higher Mg^{2+} concentration by Usherwood (1963), appears to be due to the reduction of the amount of transmitter released from the nerve endings, since the reduced e.j.p.'s oscillate in a quantal manner as do the Mg-reduced e.p.p.'s of vertebrate skeletal muscles. When an extracellular electrode was positioned over a glutamate-sensitive spot and the nerve was stimulated, the electrode recorded a negative deflexion coinciding in time with the intracellularly recorded e.j.p. (Fig. 3 *a*, *b*). Elsewhere on the membrane the extracellular electrode recorded either zero or a positive-going response (Fig. 3 *c*, *d*). From this it can be concluded that glutamate-sensitive spots are closely related to and probably identical with neuromuscular junctions.

Fig. 3

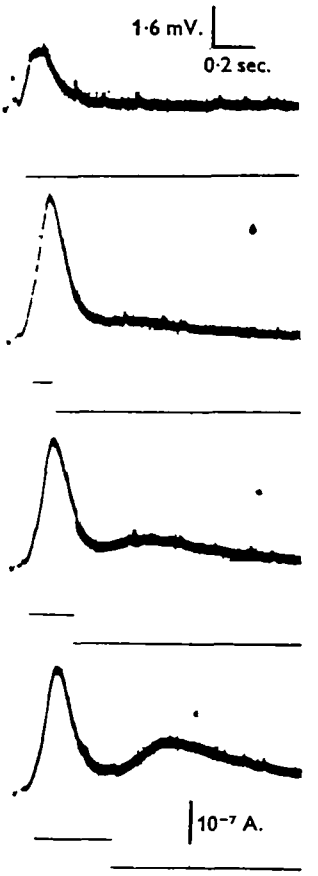

Fig. 4

Fig. 3. Extracellularly (upper traces) and intracellularly (lower traces) recorded e.j.p.'s from a glutamate-sensitive spot (*a*, *b*) on the fibre surface and outside this spot (*c*, *d*). Calibration: *a*, upper trace 0·3 mV., lower trace 7·5 mV.; *b*, upper trace 0·3 mV., lower trace 3 mV.; *c*, *d*, upper trace 0·9 mV., lower trace 7·5 mV. Time: 10 msec.

Fig. 4. Depolarizations recorded by an intracellular electrode from an insect muscle fibre on the surface of which L-glutamate was applied electrophoretically in increasing amounts (from top to bottom). Note the increase in amplitude and appearance of an additional wave of depolarization in the second trace and partial de-sensitization of the membrane in trace 3 and 4. The pulses were applied in 10 sec. intervals. Temperature 22° C.

The dose–response curve of glutamate potentials reaches a plateau of 15–25 mV. at the points of peak sensitivity (Fig. 4) and shows no discrete steps. The membrane is sensitive only to applications of glutamate from the outside and not from the inside of the fibres.

Large doses may produce additional peaks of depolarization at 500–1500 msec. intervals, probably due to the successive diffusion of glutamate to neighbouring foci (Fig. 4). Large doses readily de-sensitize the membrane reversibly to the action of glutamate and the de-sensitization may last several tens of seconds. A spot of low sensitivity is thus frequently lost after its first appearance when hit by a large dose of

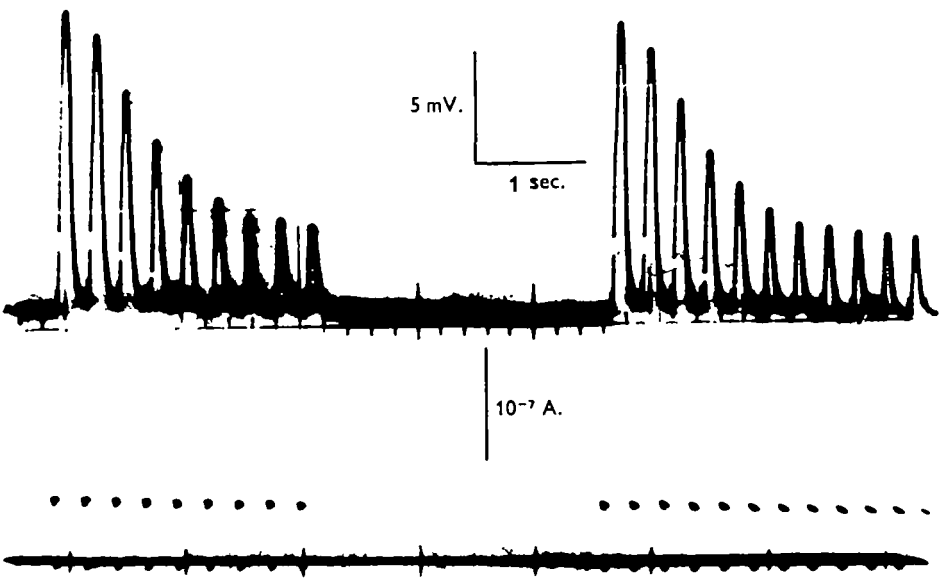

Fig. 5. De-sensitization of a highly sensitive spot on the muscle fibre surface by repetitive applications of L-glutamate from an electrophoretic pipette. Note the almost complete recovery after a brief pause. Temperature 21° C.

glutamate which has been used for exploring the fibre membrane. At focal points of sensitivity repetitive applications of brief glutamate pulses produce potentials of gradually decreasing amplitude, but the response recovers in a few seconds after the glutamate application is discontinued (Fig. 5). Large doses, however, produce a longer-lasting de-sensitization even in spots of high sensitivity (Fig. 6). Potentiation was not observed, but no systematic study of short-interval conditioning was made.

Denervation experiments

To eliminate the possible participation of nerve endings in the production of glutamate potentials, the nerve to the muscle was cut 3–10 days before the experiments. During the denervation period the animals were kept at *ca.* 35° C. The muscles did not respond to electrical stimulation of the nerve after 2–3 days and no miniature end-plate potentials were observed throughout the whole experiment. From denervated muscle fibres glutamate potentials could be elicited (Fig. 7) disclosing peaks of sensitivity against a low- or zero-sensitivity background. As with normal muscles,

some fibres showed no sensitivity. These denervation experiments were not designed to provide data on the quantitative differences in the sensitivity distribution of normal and denervated fibres, but they indicate that at least a considerable part of the glutamate effect revealed by local iontophoretic application is of post-synaptic origin.

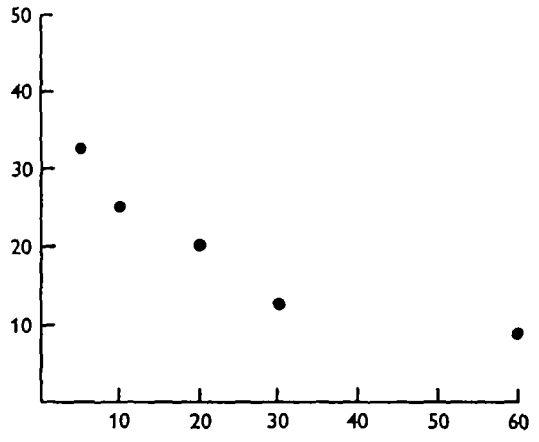

Fig. 6

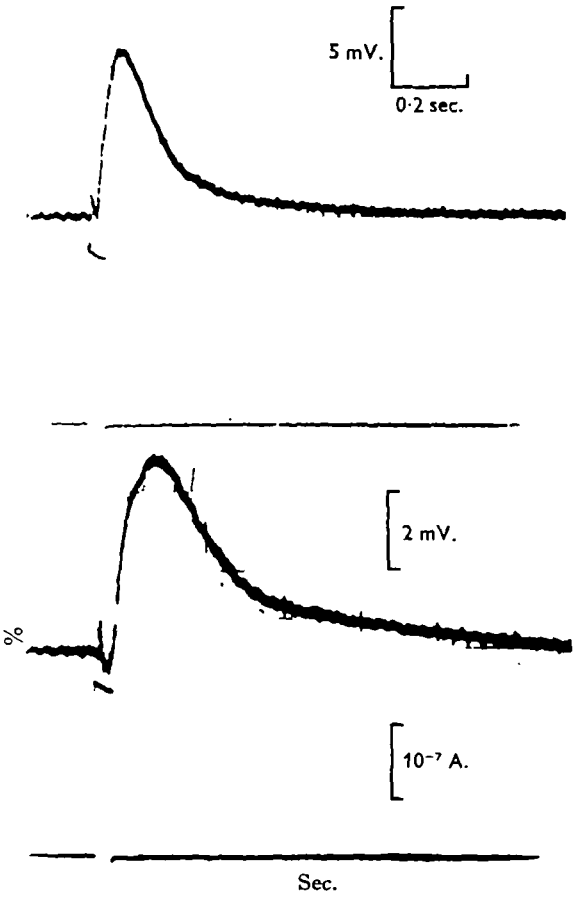

Fig. 7

Fig. 6. De-sensitization recovery curve measured on a spot of medium sensitivity. The ordinate: reduction of glutamate potential amplitude expressed in percentage of an 18 mV. conditioning control potential. Abscissa: time between conditioning and test pulses in seconds. Temperature 21° C.

Fig. 7. Potentials obtained by application of L-glutamate to denervated fibres. The two records are from two different fibres. Temperature 23° C.

Application of D-glutamate

A separate micro-pipette was used to apply D-glutamate to an L-glutamate-sensitive spot on the fibre surface. While it was usually possible to return to a point sensitive to L-glutamate after the micro-pipette had been displaced, attempts to depolarize these spots with D-glutamate failed, though up to 100 times larger doses were used. When an L-glutamate pipette and a D-glutamate pipette were both positioned on a point known to be sensitive to L-glutamate, no interaction could be detected either by mutual conditioning or overlapping of glutamate ejections from the two pipettes.

This indicates that D-glutamate has a much smaller, if any, depolarizing action on the L-glutamate-sensitive points of the membrane and that it does not compete with L-glutamate for the same sites.

Equilibrium point

To displace the resting membrane potential during the local application of L-glutamate, an additional 3 M-KCl-filled micro-electrode was introduced near the recording electrode; both were in the close vicinity (within 100 μ) of the iontophoretic pipette, which was on a glutamate-sensitive spot. Direct current of different

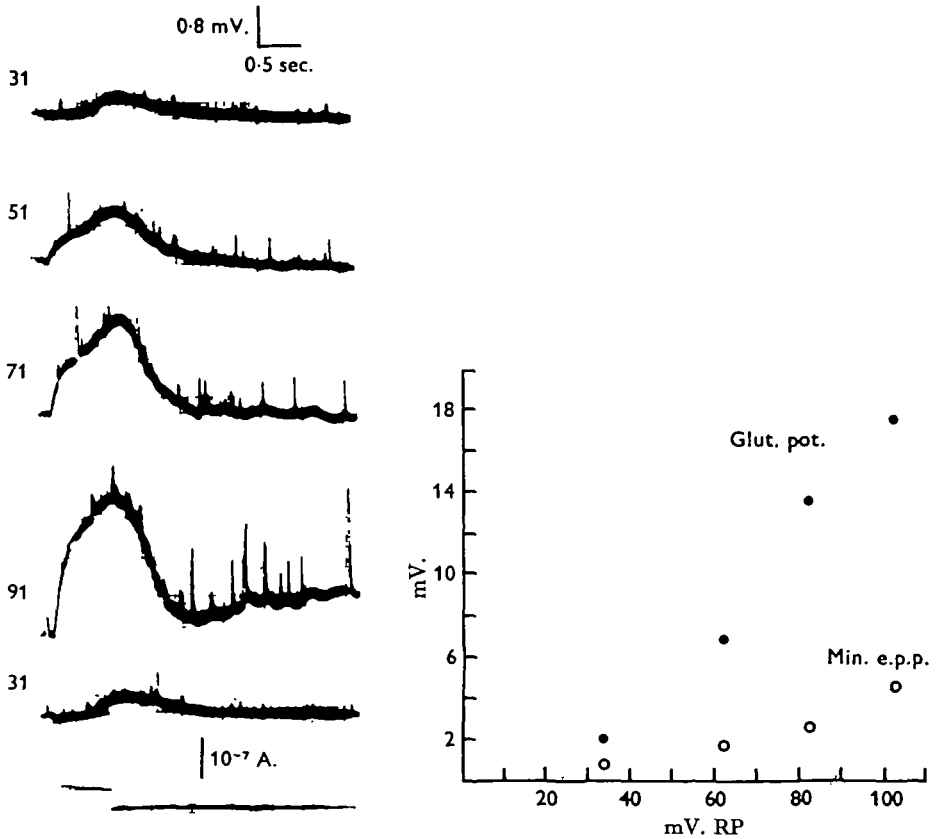

Fig. 8. Glutamate potentials and min. e.j.p.'s recorded simultaneously by an intracellular electrode, while the resting membrane potential is displaced to various values, which are indicated in millivolts over the corresponding recordings. Note that changes in amplitude for both glutamate and min. e.j.p.'s are proportional.

Fig. 9. Diagrammatic representation of the experiment from Fig. 9. Ordinate: amplitude of glutamate potentials and min. e.j.p.'s in mV. Abscissa: corresponding RPs.

intensities and directions was passed through the polarizing electrode to move the resting potential through a large range of levels. While it was easy to hyperpolarize the fibre, with increasing depolarization the current requirements set a limit to the membrane potential displacement. For this reason we were not able to see a true reversal point either for glutamate or for junction potentials and we had to reach

our conclusions from extrapolations. The amplitudes of 100–150 miniature junction potentials were averaged at each level and compared with a simultaneously recorded glutamate potential (Fig. 8). The extrapolation of the curves obtained from these measurements indicated that the equilibrium point of glutamate potentials coincides with the equilibrium point of miniature e.j.p.'s and lies between -10 and -25 mV. (Fig. 9).

DISCUSSION

Our experiments have shown that L-glutamate reproduces in detail a number of actions required to qualify a substance as a chemical synaptic transmitter. Its depolarizing action, described by previous authors in bath-application experiments, is confined to very circumscribed spots which, as indicated by extracellular recording of e.j.p.'s, coincide with neuromuscular junctions. As no discrete steps are observed in dose–response curves and as the sensitivity persists after denervation it can be concluded that the main site of action is the muscle fibre membrane itself. That the binding of L-glutamate to the receptor is similar to the action of other known transmitters is indicated by the course of desensitization and by the fact that only the outside of the membrane is sensitive. The coincidence of the equilibrium potential of glutamate and of miniature e.j.p.'s is further support for the hypothesis that glutamate is the transmitter substance. The behaviour and locations of L-glutamate-sensitive spots in the insect muscle studied bear a close resemblance to those of glutamate potentials in crustacea as described by Takeuchi & Takeuchi (1964). In the crayfish the analysis was extended to iontophoretic application of GABA and the substance was qualified as a likely inhibitory transmitter acting on different receptors from L-glutamate (Takeuchi & Takeuchi, 1965). The work on inhibitory systems in insects (Usherwood & Grundfest, 1965) makes a similar analysis desirable. Though it was shown in the present experiments that the degeneration of nerves does not deprive the muscle fibres of their sensitivity to L-glutamate, more exact studies are advisable to show whether any changes occur in the distribution of chemosensitivity of the fibre surface in conditions of chronic denervation. The insect muscles appear to be a suitable object for these studies as the peripheral stumps of the sectioned motor nerves undergo rapid Wallerian degeneration (Roeder & Weiant, 1950; Beránek & Novotný, 1959). This does not seem to be the case in crustacea. Eckert & Beránek (1957, unpublished observation) found that the peripheral stumps of severed crayfish nerves were capable of conducting impulses for as long as 5 months, after which the experiment was discontinued.

Another question, open to experimental study, concerns the specificity of glutamate receptors. Though experiments with drugs applied in a bath indicate that glutamate is the most potent of depolarizing substances, it would be desirable to check these results in local application experiments. A search for competitive inhibitors and possible enzymes which inactivate the transmitter may also be pursued with advantage by the iontophoretic technique. It should be noted, however, that there is no available evidence for the presence of an inactivating enzyme on insect neuromuscular junctions. On the contrary, if the glutamate obtained by Kerkut *et al.* (1965*b*) in the perfusate from stimulated muscles were derived from the junctions this result might suggest that little or no inactivating enzyme is present and that diffusion from the synaptic

cleft is sufficient to terminate the action of the transmitter. The ease with which the glutamate-sensitive spots on the fibre membrane are de-sensitized and frequently lost, a phenomenon described in prostigminized frog muscle fibres by del Castillo & Katz (1955), could also be explained by the lack, on the insect neuro-muscular junctions, of an inactivating enzyme.

An important problem is how to reconcile the idea of L-glutamate as a neuromuscular transmitter with the relatively high concentrations of this substance which are believed to occur in the haemolymph of many insect species (Florkin & Jeuniaux, 1964). Concentrations of L-glutamate of the order of 10^{-2} M in the haemolymph of locusts would seriously interfere with concentrations of glutamate reached at the receptor sites by iontophoretic applications in our experiments (approximately 10^{-8}–10^{-4} M as calculated from the diffusion equation after del Castillo & Katz, 1955). A natural transmitter would either have to reach a very high concentration in the synaptic cleft or the junction would require some protective mechanism for preventing the glutamate of the haemolymph from reaching the subsynaptic receptor sites. Analyses should, however, be carried out to discover how much of the haemolymph glutamate is bound to cells and how much of it is available in plasma.

SUMMARY

1. Electrophoretic application of L-glutamate from glass capillary micro-pipettes was used to investigate the 'spot sensitivity' of the membrane of coxal adductor muscle fibres from adult specimens of *Schistocerca gregaria* Forskål and *Locusta migratoria* (L.).

2. Circumscribed spots could be detected on the fibre surface where brief applications of L-glutamate produced transient depolarizations (glutamate potentials).

3. Extracellular recording of excitatory junction potentials revealed that focal points of glutamate sensitivity are closely related to, and probably identical with, neuromuscular junctions.

4. Large doses readily de-sensitized the membrane to L-glutamate for periods greatly exceeding the duration of the glutamate potentials.

5. In chronically denervated muscles peaks of sensitivity could still be detected.

6. Spots sensitive to L-glutamate were not depolarized by D-glutamate.

7. The equilibrium point for glutamate potentials coincides with the equilibrium point of miniature excitatory potentials and lies between -10 and -25 mV.

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