J. Physiol. (1964), **170**, *pp.* 296–317
With 15 *text-figures*
Printed in Great Britain

THE EFFECT ON CRAYFISH MUSCLE OF IONTOPHORETICALLY APPLIED GLUTAMATE

BY A. TAKEUCHI AND NORIKO TAKEUCHI

From the Department of Physiology, School of Medicine, Juntendo University, Hongo, Tokyo, Japan

(*Received* 30 *July* 1963)

It has been observed that glutamate and some related compounds produce contraction and depolarization of crustacean muscle in relatively low concentrations (Robbins, 1958, 1959; van Harreveld, 1959; van Harreveld & Mendelson, 1959). The pharmacological effects of glutamate have also been investigated on the gut of crayfish (Florey, 1961; Jones, 1962), on the mammalian spinal cord (Curtis, Phillis & Watkins, 1960; Curtis & Watkins, 1960), on the mammalian cerebral cortex (Hayashi, 1954; Purpura, Girado, Smith, Callan & Grundfest, 1958; van Harreveld, 1959; Krnjević & Phillis, 1963) and on the crayfish stretch receptor (Elliot & Florey, 1956).

In crustacean muscles glutamate is considered to have its action at the neuromuscular junction (van Harreveld & Mendelson, 1959). On the other hand, in mammalian spinal cord Curtis *et al.* (1960) concluded from the iontophoretic application of drugs that glutamate is a non-specific excitant. The action of drugs on the crustacean muscle has been investigated by perfusing the preparation with solutions containing drugs (Robbins, 1958, 1959; van Harreveld, 1959; van Harreveld & Mendelson, 1959). With such methods the sites of action of drugs cannot be decided precisely and any rapid time course of drug action is uncertain. For these reasons it seemed desirable to determine the site and duration of action of drugs by use of more refined experimental technique.

In the present experiments the neuromuscular junction was located by recording extracellularly the excitatory junctional potential produced by nerve stimulation (Dudel & Kuffler, 1961, *a*,*b*,*c*), and glutamate was applied electrophoretically from micropipettes (e.g. del Castillo & Katz, 1955; Curtis & Eccles, 1958).

In this way drugs could be applied locally to the neuromuscular junction. It will be shown that L-glutamate has a localized action on the junctional membrane of the crayfish muscle. A preliminary report on the location of L-glutamate sensitive sites of crayfish muscle has already appeared (Takeuchi & Takeuchi, 1963).

METHODS

The abductor muscle of the dactyl in the 1st or 2nd walking leg of the crayfish (*Cambarus clarkii*) was used. The adductor of the dactyl was removed and the leg was placed in a bath containing physiological saline solution, with the dactyl and the carpopodite fixed. Excitatory and inhibitory axons were exposed in the meropodite and stimulated separately with fluid electrodes, glass tubes being used. The nerve twigs running on the surface of the muscle were followed under the dissecting microscope. This procedure was helpful in locating glutamate-sensitive spots or single junctional areas.

The composition of van Harreveld's solution (1936) used was (mM): NaCl 207·5; KCl 5·4; $CaCl_2$ 13·5; $MgCl_2$ 5·3. Tris buffer was added to keep pH about 7·5. In some cases the concentration of $CaCl_2$ was lowered (7 mM) to reduce the movement artifact. In most cases experiments were performed on muscles in flowing fluid.

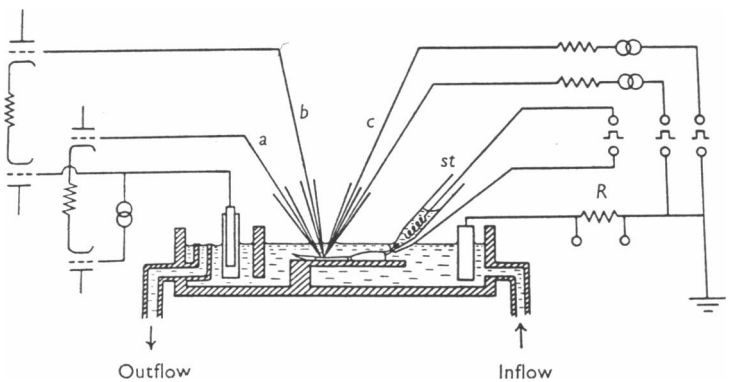

Fig. 1. Diagram showing method of injection of glutamate and potential recording. Abductor of the dactyl is exposed and is placed in flowing fluid. *a* and *b* are intra- and extracellular recording electrodes; *c*, double-barrelled micropipette for injection; *st*, fluid electrode for nerve stimulation; *R*, resistor for monitoring the injection current.

The experimental arrangement is shown in Fig. 1. For potential recording micropipettes were generally filled with 3 M-KCl and had resistance of about 10 MΩ. 3 M-NaCl filled micropipettes of small MΩ resistance were also used for extracellular recording. When the membrane potential was altered, 3 M-K citrate-filled micropipettes were sometimes used for current electrodes. Bath electrodes were composed of Ag-AgCl agar. Potential changes were recorded intracellularly as well as extracellularly through high input impedance amplifiers. The resting potential was measured from a backing potential which brought the beam of the oscilloscope back to the original level.

L- and D-glutamate solutions were prepared by adjusting the pH of L- and D-glutamic acids (Tokyo Kasei Kogyo, Co.) to pH 8 by adding NaOH, and single- or double-barrelled micropipettes were filled with a solution of about 1 M. The iso-electric point of glutamic acid is at pH 3·22. Thus glutamate ion is an anion at pH 8. The diffusional outflow of glutamate from the micropipette was stopped by making the inside of the pipette about 1 V positive relative to the bathing fluid. The glutamate was injected electrophoretically by applying a negative pulse to the pipette, the current passing through a 50 MΩ resistance. The current through the pipette was monitored by the potential drop through a resistor (30 kΩ, *R* in Fig. 1) inserted between the bathing fluid and earth, and was recorded on the third beam of the oscilloscope (Nihon Kohden Kogyo Co.).

Double-barrelled micropipettes (Coombs, Eccles & Fatt, 1955; del Castillo & Katz, 1957*a*) were used for comparing effects of drugs or for testing the sensitivity of the receptor. There are possible interactions between barrels and this was tested by filling one barrel with L-glutamate and the other with 3 M-NaCl. After locating the L-glutamate-sensitive spot, a strong inward or outward current was passed through the NaCl barrel, and no appreciable change was observed in the membrane potential. However, when a strong inward current was passed through the NaCl barrel while L-glutamate was injected through another barrel, the L-glutamate-induced depolarization had a tendency to increase (sometimes up to 10 %). When outward current was passed through the NaCl barrel, L-glutamate-induced depolarization tended to decrease. The interaction between barrels was small when inward current was passed through the NaCl barrel, but rather larger when outward current was passed. In order to avoid the interaction, in some cases two micropipettes were used to apply drugs.

Experiments were done at room temperature (21–23° C).

RESULTS

Glutamate-induced depolarization

The recording micropipette was usually inserted near the middle of the muscle fibre. As the fibre was only about 1·5 mm long any potential change along it could be recorded with little distortion (Dudel & Kuffler, 1961*a*).

The L-glutamate-filled micropipette was lowered to the surface of the muscle fibre and a strong inward current was passed through the pipette. Usually no potential change was observed. After a search along the surface of the muscle fibre, a place was found where the injection of L-glutamate produced a slow depolarization. By adjustment of the tip of the micropipette a site could be found which produced a faster and larger depolarization. Figure 2 shows an example in which the L-glutamate-filled micropipette was moved along muscle fibre in 8 μ steps. In Fig. 2*a* the response was large and its peak was not recorded. A record at a lower gain showed that it reached 16 mV. As the pipette was moved along the muscle length the depolarization became smaller and slower. In Fig. 2*e*, where the glutamate pipette was about 32 μ away from the focus, the potential change almost disappeared. After locating the approximate position of the glutamate-sensitive site with strong pulses, a finer search was undertaken with weak and short pulses. The glutamate-sensitive spot was very circumscribed and movement of less than 10 μ of the tip of the pipette abolished or very much distorted the response. Outward current through the glutamate pipette produced no potential change. The chances of finding these spots, when the glutamate pipette was moved along a muscle fibre, varied from fibre to fibre. Usually only a few such spots were detected along the whole length of the fibre.

L-glutamate-induced depolarization (glutamate potential) applied near a focus is shown in Fig. 3, where the upper trace shows the monitored

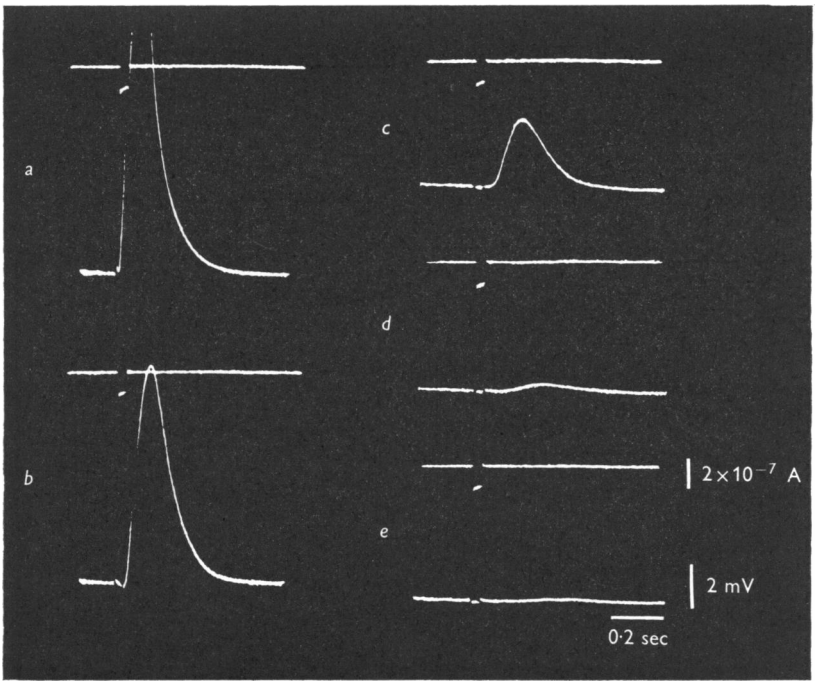

Fig. 2. Lower traces, L-glutamate induced depolarizations. Upper traces, monitored injection current. *a*, L-glutamate filled pipette was located near a focus. The amplitude of the depolarization was about 16 mV in a record at a lower gain. From *a* to *e* the L-glutamate pipette was moved along muscle fibre in 8 μ steps.

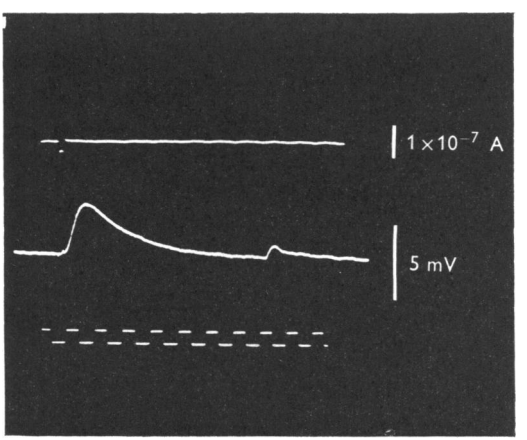

Fig. 3. An example of glutamate potential recorded when the injection pipette was adjusted near a focus. A spontaneous e.j.p. is seen on the falling phase. Upper trace, monitored injection current. Time scale, 50 c/s.

current pulse for the injection (downward deflexion indicates inward current through the pipette). After a current pulse was passed, the membrane potential slowly fell. Depolarization attained its peak in about 18 msec and then declined gradually, the total duration of depolarization being about 120 msec. On the falling phase of the glutamate potential a spontaneous miniature potential may be seen. Both time course and amplitude of glutamate potential were dependent on the position of the pipette. The time course may be mainly determined by the diffusion time of glutamate from the tip of the pipette to the sensitive spot on the muscle fibre. The rough approximation of the probable concentration of applied glutamate can be calculated from the diffusion equation (del Castillo & Katz, 1955; Krnjević & Phillis, 1963). When a substance is applied instantaneously at a point source in an infinite homogeneous medium, the peak maximum concentration at point of distance r from the point source is given by $C = (Q \exp(-1{\cdot}5))/8(\pi DT)^{1{\cdot}5}$, where Q is amount of substance applied instantaneously, D the diffusion constant, and T is the time to the peak. The peak time T is related to the distance r according to $6DT = r^2$ (del Castillo & Katz, 1955). If the diffusion constant of glutamate is taken as of the same order of that of glutamin ($7{\cdot}62 \times 10^{-6}$ cm^2/sec at 25° C; Longsworth, 1953) the diffusion distance r in the case of Fig. 3 is given as 9 μ. In this instance about 9×10^{-11} coulombs was passed through the pipette. If the transport number of glutamate is assumed as 0·5, $4{\cdot}5 \times 10^{-16}$ mole of L-glutamate was injected and produced the depolarization of about 3·5 mV. The peak maximum concentration is calculated from the above equation as 0·045 mM. This value is of the same order of the threshold concentration for the contraction (0·035 mM in *Cambarus clarkii*; van Harreveld, 1959).

When the amount of injection current was increased, the amplitude of the depolarization increased without appreciable change in its time course and attained a maximum of about 20 mV. In rare cases the depolarization produced a spike potential of the muscle fibre.

Site of action of L-*glutamate*

The amplitude and the time course of glutamate potential were measured along the axis of the muscle fibre, and an example is presented in Fig. 4. The pitch of the screw of the micromanipulator was calibrated and the distance along the muscle fibre was read from the scale on the screw. In Fig. 4 the abscissa represents the distance along the muscle fibre, filled circles represent the peak amplitude of the glutamate potential, and open circles its time to the peak. In this instance $1{\cdot}3 \times 10^{-7}$ A was passed for 40 msec. Thus, the dose injected was large and the responses were obtained from rather broad area. Two peaks are observed in the amplitude of the

response. At these points the rise time of the depolarization is shortest. The relation between the amplitude and the time to peak is in rough agreement with that obtained from the diffusion equation (amplitude $\propto$ (peak time)$^{-1 \cdot 5}$; del Castillo & Katz, 1955). This suggests that at these points the glutamate-sensitive sites are localized. Some irregular humps are observed on the curves. These irregularities may be due to diffusion of glutamate to the glutamate-sensitive spot some distance away from the track of the pipette.

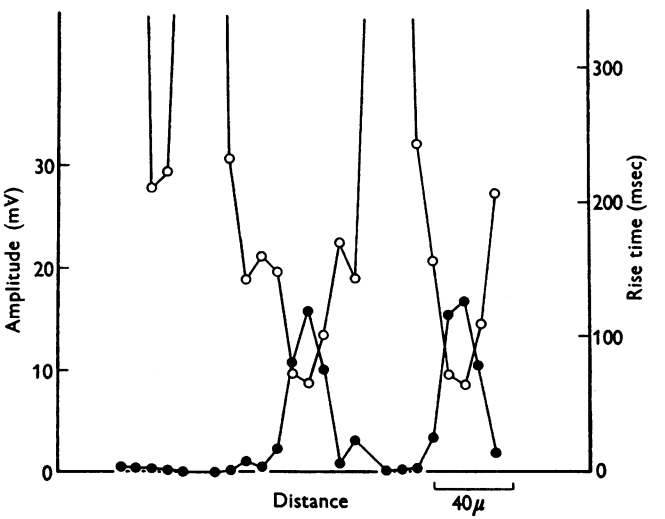

Fig. 4. Distribution of glutamate potential along a muscle fibre. ●, amplitudes of glutamate potentials obtained by injecting the same doses at spots on the surface; ○, time to the peak of glutamate potential. Abscissa, distance along the muscle surface.

This result indicates that the glutamate-sensitive site is a discrete spot on the muscle fibre. Whether or not this spot coincides with the junctional area is another problem.

The excitatory nerve was stimulated repetitively and the recording electrode was placed on the surface of the muscle. A spot was located where the negative-going potential change, recorded extracellularly, coincided with the rising phase of the intracellularly recorded excitatory junctional potentials (e.j.p.s.). The localization of the spot was critical and the potential disappeared on the movement of the tip of the electrode by about few microns (Dudel & Kuffler, 1961*a*). Two examples of simultaneously recorded extracellular and intracellular potential changes are presented in Fig. 5*a* and *b*. The upper traces of *a* show the extracellularly recorded e.j.p.s and the lower traces the simultaneous e.j.p.s. Downward

intermittent deflexions observed in the upper traces coincided with the rising phase of the e.j.p.s of the lower traces. In both traces downward deflexions represent negativity of the tip of the electrode. After a single junctional area was thus located, a L-glutamate-filled micropipette was brought near the tip of the extracellular electrode and a strong inward current pulse was passed through the pipette. Middle traces of Fig. 5*b* present the extracellularly recorded potential changes from the same junctional areas of those in *a*, produced by iontophoretically injecting

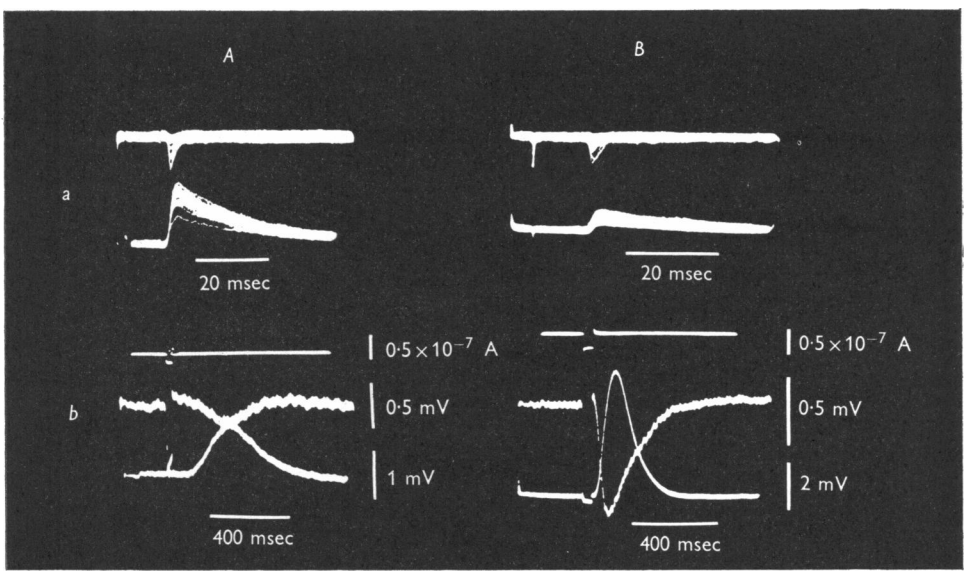

Fig. 5. Two examples (*A* and *B*) of potential changes recorded at single junctional areas. *a*: upper traces, extracellular e.j.p.s at stimulation rate of 15/sec; lower traces, simultaneous intracellular records; about 15 sweeps were superimposed. *b*: upper traces, monitored injection current; middle traces, extracellularly recorded glutamate potentials obtained at the same spots with those of *a*; lower traces, simultaneous intracellular records.

L-glutamate. The lower traces are the simultaneously recorded intracellular glutamate potentials. The amplitude calibrations are the same for *a* and *b*, but the time scales are slower in *b*. Upper traces of *b* represent monitored injection current. After the injection of L-glutamate downward deflexion was observed in the extracellular records, with somewhat faster time course than that of the intracellular glutamate potentials. Negativity of the extracellularly recorded potential change would indicate inwardly directed current through the membrane close to the tip of the recording electrode. Thus this result shows that the membrane of the junctional area is activated by the application of L-glutamate.

The glutamate-filled micropipette, through which inward current pulses were passed, was lowered perpendicularly to the muscle surface over a glutamate-sensitive spot. As the micropipette was brought nearer to the surface of the muscle, the amplitude of the glutamate potential became larger and its time course faster. When the micropipette was further advanced a rapid hyperpolarization appeared, in some cases with a small delayed depolarization which may arise from an incomplete insertion of the pipette allowing a fraction of the current to by-pass the fibre membrane. Further lowering of the pipette abolished the slow depolarization, leaving only the rapid hyperpolarization. This result is similar to that observed in the application of acetylcholine inside the muscle fibre at the vertebrate end-plate (del Castillo & Katz, 1955). It may be concluded, as in the case of the vertebrate end-plate, that the glutamate-sensitive receptor is located on the outer surface of the membrane and that glutamate injected inside the muscle fibre does not diffuse through the membrane.

There remains a possibility that glutamate activates the nerve terminal, causing the release of transmitter. The nerve trunk was cut at the joint between carpopodite and melopodite and 3 days after denervation the preparation was dissected. The injection of L-glutamate produced the usual glutamate potential. Furthermore, in normal preparations, the iontophoretic application of glutamate produced a continuously graded depolarization and no discrete unitary components. This was also true for the extracellularly recorded responses. In contrast, the release of the transmitter from the nerve terminal has been shown to be quantal (Dudel & Kuffler, 1961*a*).

'Desensitization' and 'potentiation'

It has been observed that L-glutamate-induced contraction and depolarization are transient and the response declines while the muscle is perfused with glutamate (van Harreveld & Mendelson, 1959; Robbins, 1959). Figure 6 shows the depolarization produced by the steady iontophoretic application of L-glutamate. The depolarization is not maintained, but declines in a few hundred milliseconds (Fig. 6*a*). A short pulse of L-glutamate was applied through another barrel to the same spot (*b*). When the short pulse was superimposed on the steady efflux, the response due to the short pulse almost disappeared (*c*). This suggests that the decline of glutamate potential, during the steady application of glutamate, is due to 'desensitization' of the receptor (Katz & Thesleff, 1957). The time course of the decline of the glutamate potential depended on the amount of steady efflux, being faster when larger doses were applied.

The change in sensitivity of the receptor was tested by applying brief pulses of glutamate while glutamate was applied by d.c. current through

another barrel of a double-barrelled micropipette or a separate micropipette. In Fig. 7 steady doses of varying amount were applied through one micropipette, while the sensitivity was tested by brief application from another pipette. After the start of steady efflux the membrane depolarized slowly and superimposed test responses showed at first an increase in amplitude, which then declined gradually. As the steady dose

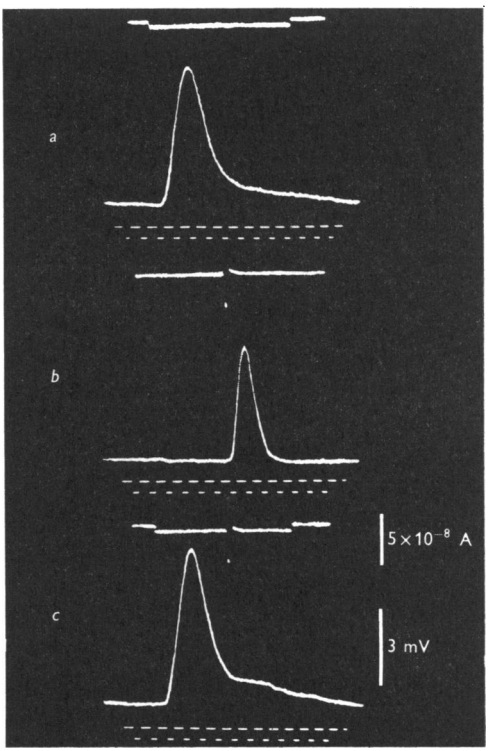

Fig. 6. Glutamate potentials produced by steady application (*a*) and brief application (*b*) through each barrel of a double-barrelled pipette. In *c* both steady and brief applications were superimposed. Time scale, 10 c/s.

of glutamate was increased, the amplitude of conditioning depolarization became larger and the densensitization was accelerated. After the cessation of steady glutamate application the amplitude of test responses recovered in few seconds.

In the case of Fig. 7 separate micropipettes were used, and the one for steady application of glutamate might have been further away from the receptor than the pipette applying glutamate by brief pulses. This could be the reason why the time courses of conditioning depolarization and desensitization were rather slow in this case. When a double-barrelled

pipette was critically adjusted to a receptor and the steady dose of L-glutamate was applied from one barrel while test pulses were applied from the other barrel, the onset and the decline of the conditioning depolarization were faster than those in Fig. 7. The time course of the

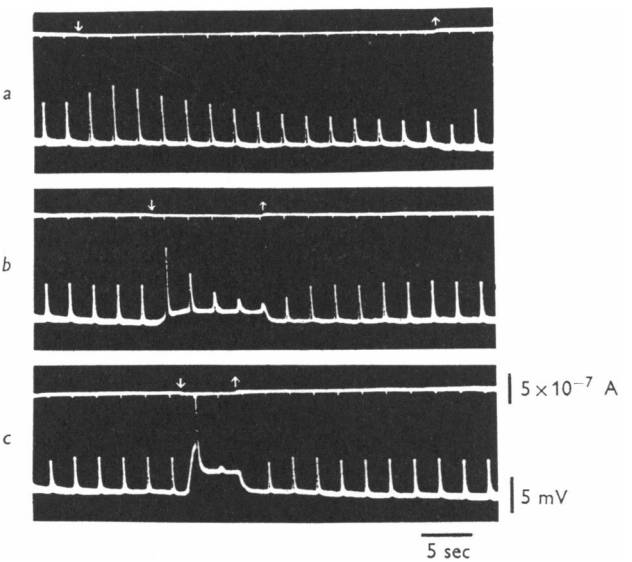

Fig. 7. Desensitization and potentiation of glutamate potential. Brief test responses were recorded and during the period indicated by arrows steady conditioning dose was injected through another pipette. From *a* to *c* the strength of conditioning dose was increased.

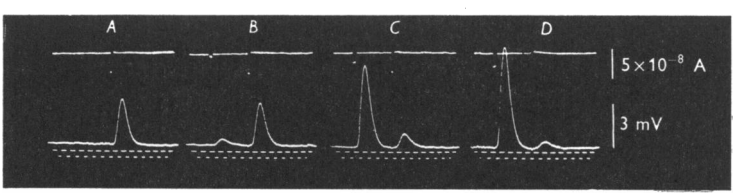

Fig. 8. Desensitization observed after brief conditioning application of L-glutamate at a constant pulse interval (440 msec). *A* is control. From *B* to *D* conditioning dose was increased. Time scale, 10 c/s.

desensitization was then also rather rapid and best measured by a double-pulse technique. Test and conditioning pulses of various strengths were applied at varying intervals to a junctional area from each barrel of a double-barrelled micropipette. An example is shown in Fig. 8, where the strength of conditioning pulses was changed at a constant pulse interval

(at 440 msec). As the amplitude of the conditioning response was increased, that of test response decreased.

The amplitude of test response was also dependent on the pulse interval. The relation between the amplitude of test response and the pulse interval is presented in Fig. 9. The abscissae represent the interval between the conditioning and test pulses and ordinates the relative amplitude of test

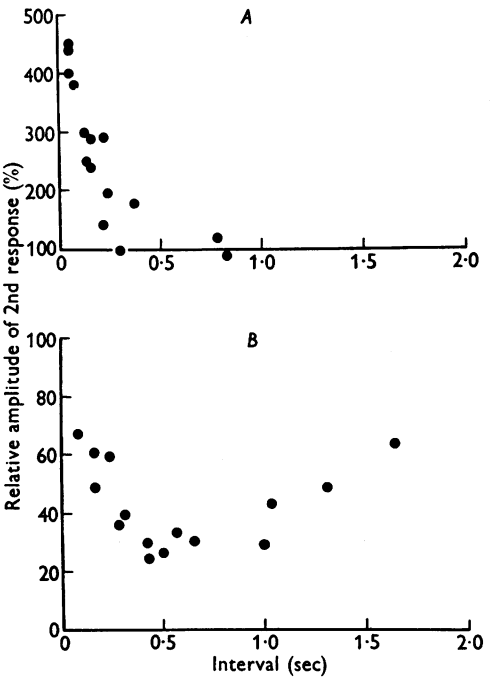

Fig. 9. Relations between the relative amplitude of test (second) response and the time after the conditioning (first) pulse. *A*, conditioning and test depolarization were both about 1 mV. *B*, conditioning response about 8 mV and test response 5 mV. Abscissae, intervals between the conditioning and test pulses.

responses to the control. *A* was obtained when the conditioning and the test responses were small (both 1 mV) and *B* was obtained when larger conditioning and test responses (conditioning 8 mV, test 5 mV) were used. In *B* the amplitude of test responses is smaller than that of the control and it has a minimum at about 0·5 sec after the conditioning pulse. Thereafter the amplitude gradually recovered. When the conditioning dose was small (Fig. 9*A*) no appreciable decrease in the amplitude of test response was observed, but rather a remarkable increase in its amplitude which returned to the original amplitude in approximately half a second.

The relation between amplitudes of conditioning and test responses at two different intervals between the conditioning and test pulses is shown in Fig. 10. Circles on the ordinate show amplitudes of unconditioned test responses. At an interval of 440 msec (*B*), the amplitude of test responses was decreased as the conditioning response increased, whereas at an interval of 170 msec (*A*), the amplitude of the test response increased and then fell to near control values as conditioning responses were increased.

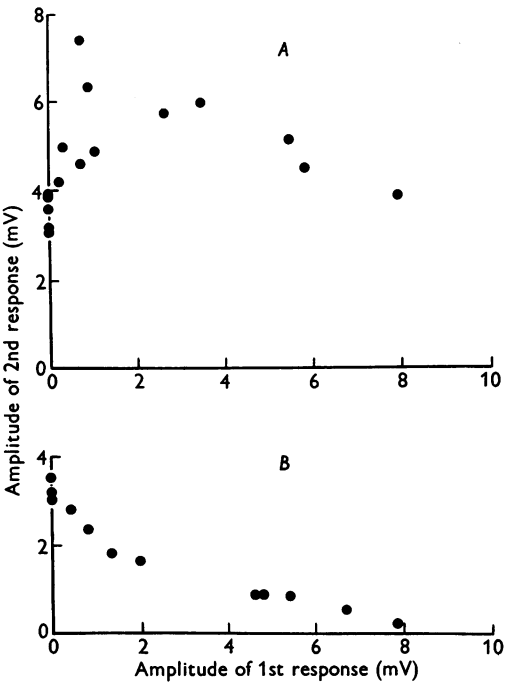

Fig. 10. Influences of the conditioning (first) dose on the amplitude of test (second) response at constant intervals. *A* was obtained at an interval of 170 msec, and *B* at 400 msec. Circles on the 0 mV axis show amplitudes of unconditioned test responses.

'Potentiation' of glutamate potential, when a small conditioning dose was applied, is of considerable interest. This result is not due to the movement artifact, because 'potentiation' was observed even when the conditioning dose was so small that little or no depolarization was observed. Nor is it the result of the electrical interactions between barrels, because even when two separate micropipettes were used for the injection, the effect could still be obtained (Fig. 7). Similar 'potentiation' has also been observed with acetylcholine receptor of the frog end-plate (Katz & Thesleff, 1957).

The relation between the strength of the injection pulse and the amplitude of the depolarization produced was also examined. Approximately equal doses of glutamate were applied from each barrel of a double-barrelled micropipette, separately and simultaneously. Starting with very small doses, the dose–response relation was traced in increasing steps. The sum of responses produced separately is plotted on the abscissa of Fig. 11. The ordinate is the amplitude produced by simultaneously applied glutamate from both barrels, the abscissa the sum of the amplitudes

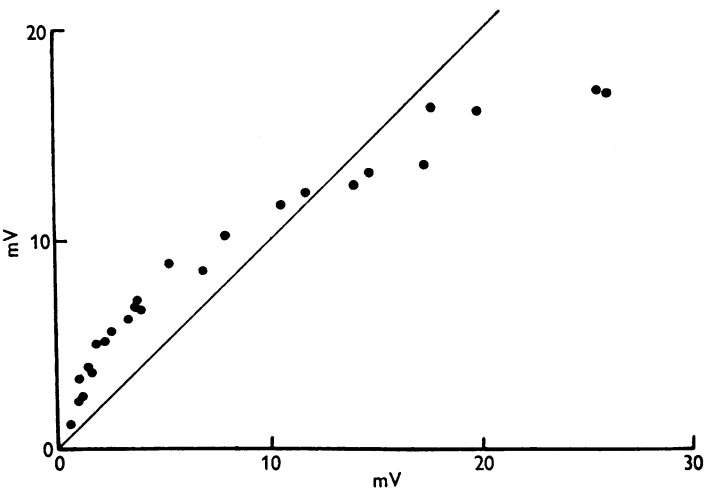

Fig. 11. Relation between the strength of injection pulse and the amplitude of depolarization produced. Abscissa, sum of the amplitudes obtained by separate injection of approximately equal doses from each of double barrels. Ordinate, the amplitude obtained by simultaneous application from both barrels. The straight line was drawn at 45°.

obtained by separate, approximately equal, injections. The line drawn at 45° represents linear summation. Combined response showed more than a simple additive effect for small doses, and less than linear summation for large doses. The response became almost saturated at about 18 mV. In the frog a non-linear dose–response relation has been observed at the acetylcholine receptor (Katz & Thesleff, 1957).

Interaction between L-*glutamate and e.j.p.s*

Results in preceding sections show that L-glutamate has its action on the neuromuscular junction. The question arises whether the receptors which respond to L-glutamate are identical with the normal neuro-receptors. It is conceivable that L-glutamate combines with receptors which are near the junctional areas, but that these are different from

normal neuroreceptors and produce a different mode of response. It has been observed that the glutamate potential shows a characteristic change in amplitude following a conditioning application of glutamate. If the conditioning injection of glutamate also produced a similar change in the

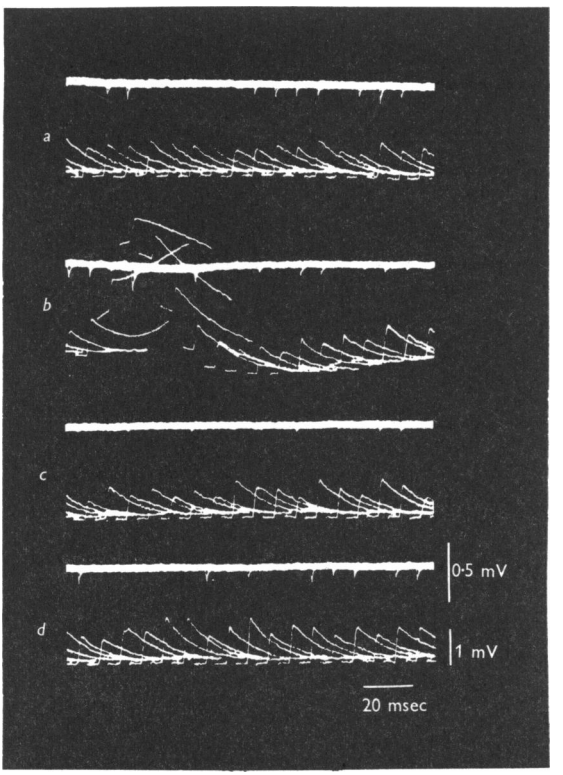

Fig. 12. Interaction between L-glutamate and e.j.p.s. Upper traces show extracellular potential changes at a junctional spot and lower traces simultaneously recorded intracellular e.j.p.s produced by nerve stimuli at 15/sec. Intra- and extracellular potentials were recorded both by a.c. amplifiers and traces were superimposed on the moving film. L-glutamate pipette was adjusted near the same junctional spot. *a*, before injection. At the onset of *b*, injection of L-glutamate is started; *c* is about 10 sec after start of the injection; *d*, about 10 sec after the cessation of the injection. Average amplitude of e.j.p.s is somewhat larger in *d* than in *a*. This is due to the facilitation of e.j.p.s by continued stimulation.

amplitude of e.j.p.s, one might consider the receptors which respond to L-glutamate to be identical with the normal neuroreceptor.

E.j.p.s were recorded both intra- and extracellularly and L-glutamate was applied iontophoretically at the same junctional area. The upper traces of Fig. 12 present extracellular potential changes and the lower traces simultaneously recorded intracellular responses. Stimuli were

applied at 15/sec and traces were superimposed on moving film (*a*). In Fig. 12*b* the injection of L-glutamate was started and this caused depolarization in the intracellular record and a slow downward deflexion in the extracellular record. Responses during the continued application of

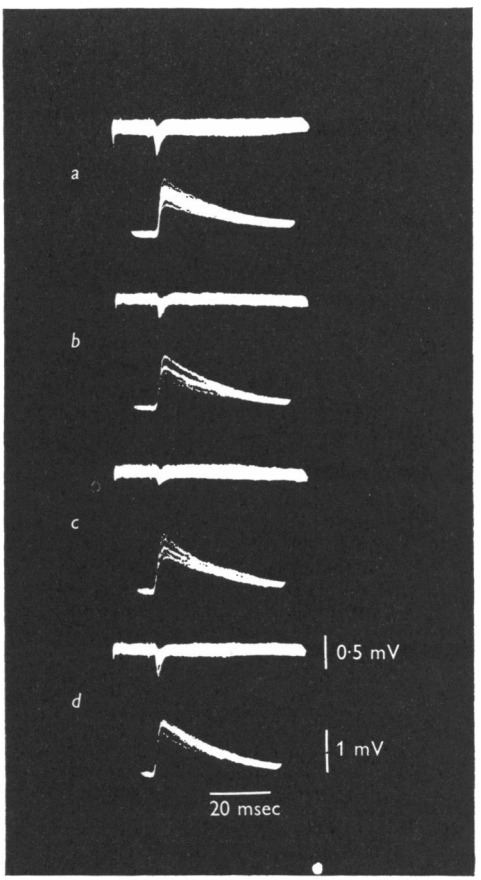

Fig. 13. Interaction between L-glutamate and e.j.p.s. Upper traces of each record, extracellularly recorded e.j.p.s; lower traces, intracellular e.j.p.s recorded simultaneously. Nerve stimuli at 15/sec, and about 30 sweeps were superimposed. *a*, before injection; *b*, immediately after start of the L-glutamate injection to the same junctional spot where the extracellular e.j.p.s were recording; *c*, about 10 sec after start of the glutamate injection and *d* after cessation of the injection.

glutamate and after cessation of the injection are shown in Fig. 12*c*, *d*. Both intra- and extracellular responses were recorded by a.c. amplifiers. Soon after the start of injection the amplitude of extracellular e.j.p.s (upper trace) tended to decrease and remained small during the application of L-glutamate. A few seconds after the cessation of the injection

the amplitude gradually regained its original value. Another example is shown in Fig. 13, where intra- and extracellular e.j.p.s were superimposed. Upper traces are extracellular recordings and lower traces intracellular records. The amplitude of the extracellular e.j.p.s decreased gradually during the L-glutamate injection, its time course being of the same order of that of desensitization of glutamate potential. This result suggests that the neuroreceptor is desensitized by prolonged application of L-glutamate. The decrease in the amplitude of extracellular e.j.p.s is not due to the decrease in the membrane conductance which is produced by the applied L-glutamate, because the change in the amplitude of e.j.p.s showed a different time course from that of glutamate potential which might be parallel with the change of the membrane conductance.

'Potentiation' in the amplitude of e.j.p.s would also be expected, if the above supposition was true. This test was rather difficult because 'potentiating effect' was transient and the amplitude of extracellular e.j.p.s obtained from a single junctional area was rather irregular even with high frequency of stimulation. It was observed that if a small dose of L-glutamate was applied from some distance away from the receptor, 'potentiation' obtained with L-glutamate injection lasted for some 5–10 sec.

A small dose of L-glutamate was applied to a junctional area, while e.j.p.s were recorded extracellularly (at a stimulation rate of 15/sec). Mean amplitude of responses to every ten stimuli was plotted in Fig. 14. During the hatched periods steady doses of L-glutamate were iontophoretically applied to the same junctional area. The numbers attached show the current strength used for the injection. Mean amplitude showed large variations but during the application of a small dose of L-glutamate the mean value showed a tendency to increase. When rather strong current was used, immediately after the start of the application the amplitude was augmented, but it soon declined. The decrease in the amplitude was larger when stronger current was used. After the cessation of drug application the amplitude gradually recovered. In Fig. 14 the recovery of the amplitude was rather slow when large doses were injected and it took some 10 sec. This is slower than the recovery observed with the glutamate potential. A simple explanation may be that in this case the glutamate pipette was some distance away from the receptor site; thus at the cessation of the injection the ejected L-glutamate had to diffuse some distance before reaching the receptor. This explanation is supported by the fact that 'potentiation' attained its peak 5–10 sec after the start of the injection when weak current was used, in contrast to the more rapid potentiation of the glutamate potential (Fig. 9).

There remains a possibility that L-glutamate acts on the presynaptic

terminal by changing the amount of the transmitter released from the nerve ending. If the action of L-glutamate was on the presynaptic terminal, it is conceivable that the quantum content was changed without change in the quantum size. However, the quantum size calculated from the extracellular e.j.p.s, recorded during steady application of

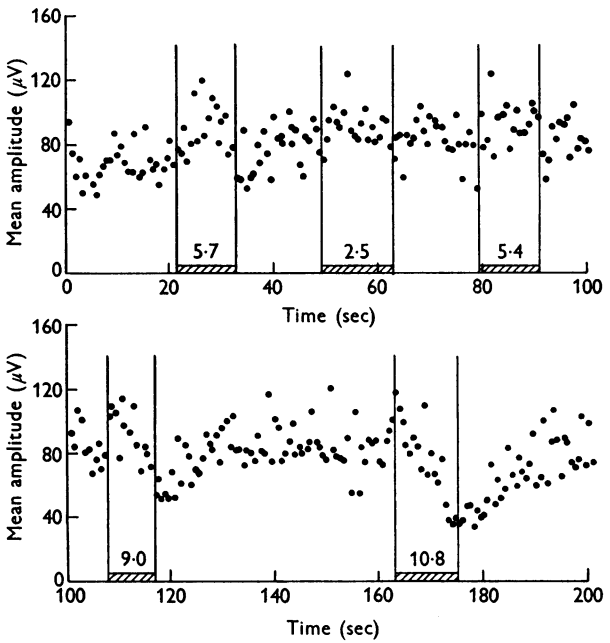

Fig. 14. Effect of application of the small dose of L-glutamate on mean amplitude of extracellularly recorded e.j.p.s. Ordinates, mean amplitude of extracellular e.j.p.s of every 10 stimuli at stimulation rate of 15/sec. Abscissae, time, continued from upper to lower. During hatched periods steady doses of L-glutamate were injected to the same junctional area. The numbers attached show the current strength for iontophoretic injection in 10^{-8} A.

L-glutamate, became small, while no appreciable change in the quantum content was observed (Takeuchi and Takeuchi, unpublished). This supports the view that the interaction between L-glutamate and e.j.p.s is of post-synaptic nature.

Effect of D-*glutamate*

D- and L-glutamate in a concentration about 0·7 M were loaded into separate barrels of a double-barrelled micropipette. After locating a spot sensitive to L-glutame, D-glutamate was injected to the same spot from the other barrel. In Fig. 15*A*, $4{\cdot}4 \times 10^{-10}$ coulombs through a L-glutamate-filled barrel produced a depolarization of 1·8 mV, while $1{\cdot}25 \times 10^{-8}$ coulombs through a D-glutamate-filled barrel produced no appreciable

potential change (Fig. 14 *B*). When D- and L-glutamate were injected simultaneously to the same spot, no appreciable change in the amplitude of L-glutamate-induced depolarization was observed (Fig. 14 *C*). In some cases a tendency to increase in amplitude of the L-glutamate potential was observed when D- and L-glutamate were injected simultaneously. This result was not obtained constantly, and may have been due to the electrical interaction between barrels (see Methods).

If the smallest potential change which is detectable in the present instance is assumed to be 200 μV, the present result shows that the injec-

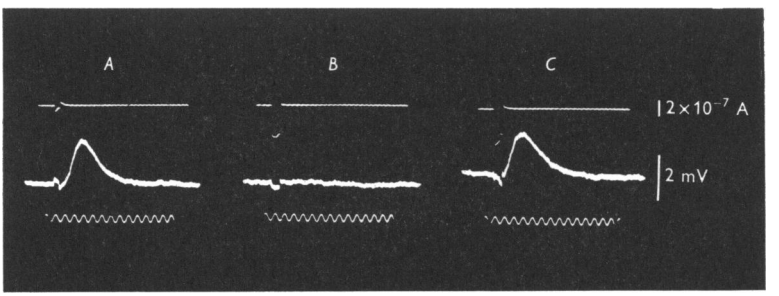

Fig. 15. Effects of L- and D-glutamate. *A*, depolarization produced by L-glutamate injection from one barrel of a double-barrelled pipette. *B*, D-glutamate was injected at the same spot from another barrel. *C*, L- and D-glutamate of the same doses with those in *A* and *B* were applied simultaneously to the same spot. Upper traces, monitored injection currents. Middle traces, intracellular potential changes. Time scale, 25 c/s.

tion of about 28 times more D-glutamate produced at least 9 times less potential change. This suggests that D-glutamate is at least 250 times less effective than L-glutamate. It also shows that D-glutamate does not become competitively attached to receptors.

DISCUSSION

The present experiments provide evidence that L-glutamate has its action on the neuromuscular junction of the crayfish. The action of L-glutamate is not on the presynaptic terminal, because the drug produced the smoothly graded depolarization without any unitary responses and had no influence on the frequency of the miniature discharge. Furthermore, after denervation the drug still produced its effect. On the other hand, in the mammalian spinal neurones several amino acids are considered as non-specific excitants: they act on other membrane than synaptic membrane (Curtis *et al.* 1960). A remarkable difference in glutamate action on spinal neurones and on crayfish muscle is that in the former case D-glutamate had a stimulating action (Curtis & Watkins, 1960) but had no appreciable effect in the latter. However, cortical

neurones discriminate between D- and L-glutamate more effectively than spinal neurones (Krnjević & Phillis, 1963).

The result that D-glutamate has no depolarizing action on the crustacean muscle nor any interaction with the glutamate potential suggests that D-glutamate has no appreciable affinity for the receptor. This also suggests that the steric configuration at α carbon is important for the binding. pK_2 and pK_3 of glutamic acid are 4·25 and 9·67 respectively. Therefore in the normal condition of pH 7·5 more than 99% of glutamate ion has negative charges on both carboxyl groups and positive charge on the amino group. It might be noted that iontophoretic application of gamma-aminobutyric acid (GABA), which is a decarboxylated form of glutamic acid, produces a different mode of response from glutamate potential, suggesting that different receptors are concerned (Takeuchi & Takeuchi, unpublished observation). Another difference between the actions of L-glutamate on neurones and on the crayfish muscle is that no appreciable desensitization was observed in the former (Curtis *et al.* 1960; Krnjević & Phillis, 1963) but in the crayfish muscle desensitization was striking.

In the motoneurone the equilibrium potential for the depolarizing action of the acidic amino acids was at a different, less depolarized, level than that of the synaptic potential (Curtis, see Eccles, 1962). Preliminary experiments of voltage clamping of crustacean muscle during glutamate action showed that the reversal potential in the normal van Harreveld's solution was near zero membrane potential. Further investigation will be needed, however, to decide the exact value of the reversal potential, because of the experimental difficulties, especially the movement artifact and the necessity of a rather large current for changing the membrane potential. Another difficulty in comparing the reversal potential for the glutamate potential and that for e.j.p.s arises from the fact that the crayfish muscle has a widely distributed innervation (Fatt & Katz, 1953). When the polarizing current is applied at a point on the muscle fibre the measured reversal potential of e.j.p.s may therefore show a more depolarized value than that of the equilibrium potential (cf. Burke & Ginsborg, 1956).

The interaction observed between applied L-glutamate and e.j.p.s strongly suggests that the receptor which responds to the transmitter also reacts to L-glutamate. Interaction between applied L-glutamate and contraction of the muscle produced by nerve stimulation has been also observed (van Harreveld & Mendelson, 1959; Robbins, 1959).

The present experiments showed that L-glutamate mimics the transmitter. However, there are several criteria for identification of a substance suspected as the transmitter (Paton, 1958; Florey, 1960). Most fundamental requirements of these are (1) the substance must have the same

action as the transmitter when artificially applied; (2) the substance should be recoverable in the perfusing fluid; (3) the substance should exert its action in doses comparable with the concentration found in the perfusion fluid; (4) the presence of an enzyme system capable of synthesis of the substance; and (5) the presence of a enzyme system necessary for the inactivation of the substance is also desirable. These requirements can be tested principally by biochemical or pharmacological experiments. The present results show only that L-glutamate fulfills the first criterion. However, the interaction of L-glutamate with e.j.p.s suggests that the receptors which respond to L-glutamate are identical with the normal neuroreceptors and that the steric configuration of L-glutamate has some similarities with that of the transmitter. The suggestion that some acidic amino acids are related to the transmitter in the central nervous system has been made by Eccles (1962).

The experiments described here show that small doses of glutamate have a 'potentiating effect' and large doses an 'inhibitory effect'. The 'inhibitory effect' may be reasonably considered as the desensitization of the receptor, which has been fully described for the acetylcholine receptor of the vertebrate motor end-plate (Thesleff, 1955; Katz & Thesleff, 1957). 'Potentiation' was observed even when the conditioning depolarization was hardly appreciable and it was also observed when the conditioning and test doses were applied with separate micropipettes. Thus, this effect was not due to movement artifact, nor to interaction between barrels.

There are some uncertainties in accurately estimating the time course of drug application, as discussed by del Castillo & Katz (1957*a*). But the time course of 'potentiation' was much longer than that of the glutamate potential, which was of the order of one-tenth of a second. Furthermore, after large pulses, when desensitization was dominant, the interval-amplitude relation of test responses showed a minimum (Fig. 9*B*). These results suggest that the conditioning dose leaves a facilitatory effect for a short period after its cessation.

There are several possible mechanisms of 'potentiation'. But they are quite speculative and until the contribution of enzyme for the removal of glutamate is determined, we have to wait to decide whether 'potentiation' is due to a specific interference with the enzymic destruction of L-glutamate (del Castillo & Katz, 1957*b*) or to other causes (Katz & Thesleff, 1957).

SUMMARY

1. The effect of glutamate on the abductor of the dactylopodite in the crayfish was investigated. Iontophoretic microapplication was used for the injection of the drug and the resulting potential changes were recorded extra- as well as intracellularly by capillary electrodes.

2. Momentary application of L-glutamate to a sensitive spot on the surface of the muscle produced transient depolarization.

3. L-glutamate-sensitive spots on the muscle were very circumscribed and these coincided with single junctional areas located by extracellularly recording excitatory junctional potentials (e.j.p.s). Injection of L-glutamate in the interior of the muscle fibres produced no appreciable depolarization.

4. Injection of D-glutamate to L-glutamate-sensitive spots produced no appreciable potential change. Simultaneous injection of L- and D-glutamate to the same spot showed no appreciable interaction.

5. The changes in the sensitivity of the receptor during or after the conditioning dose of L-glutamate were investigated. Conditioning and test doses of the drug were applied from the two barrels of a double-barrelled pipette or from separate pipettes. When a relatively small conditioning dose was applied, a transient increase of amplitude was observed in test responses. As the conditioning dose was increased, 'desensitization' of the receptor became dominant.

6. L-glutamate was iontophoretically applied to a single junctional area, while e.j.p.s were recorded extracellularly. A small steady dose produced a transient increase in the amplitude of e.j.p.s, followed by the decrease in its amplitude.

7. It was concluded that the receptors which respond to L-glutamate are identical with normal neuroreceptors.

The authors are indebted to Professors C. C. Hunt and B. Katz for their helpful criticisms of the manuscript. Thanks are also due to Misses Shigeko Ikeda and Keiko Kamimura for their help in preparing the manuscript. The work was supported in part by grants from the Ministry of Education and the Rockefeller Foundation.

REFERENCES

BURKE, W. & GINSBORG, B. L. (1956). The action of the neuromuscular transmitter on the slow fibre membrane. *J. Physiol.* **132**, 599–610.

COOMBS, J. S., ECCLES, J. C. & FATT, P. (1955). The electrical properties of the motoneurone membrane. *J. Physiol.* **130**, 291–325.

CURTIS, D. R. & ECCLES, R. M. (1958). The excitation of Renshaw cells by pharmacological agents applied electrophoretically. *J. Physiol.* **141**, 435–445.

CURTIS, D. R., PHILLIS, J. W. & WATKINS, J. C. (1960). The chemical excitation of spinal neurones by certain acidic amino acids. *J. Physiol.* **150**, 656–682.

CURTIS, D. R. & WATKINS, J. C. (1960). The excitation and depression of spinal neurones by structurally related amino acids. *J. Neurochem.* **6**, 117–141.

DEL CASTILLO, J. & KATZ, B. (1955). On the localization of acetylcholine receptors. *J. Physiol.* **128**, 157–181.

DEL CASTILLO, J. & KATZ, B. (1957*a*). A study of curare action with an electrical micromethod. *Proc. Roy. Soc.* B, **146**, 339–356.

DEL CASTILLO, J. & KATZ, B. (1957*b*). Interaction at end-plate receptors between different choline derivatives. *Proc. Roy. Soc.* B, **146**, 369–381.

DUDEL, J. & KUFFLER, S. W. (1961*a*). The quantal nature of transmission and spontaneous miniature potentials at the crayfish neuromuscular junction. *J. Physiol.* **155**, 514–529.

DUDEL, J. & KUFFLER, S. W. (1961*b*). Mechanism of facilitation at the crayfish neuromuscular junction. *J. Physiol.* **155**, 530–542.

DUDEL, J. & KUFFLER, S. W. (1961*c*). Presynaptic inhibition at the crayfish neuromuscular junction. *J. Physiol.* **155**, 543–562.

ECCLES, J. C. (1962). Spinal neurones: synaptic connexions in relation to chemical transmitters and pharmacological responses. *Proc. first int. pharmacological Meeting*, vol. 8, 157–182. Oxford: Pergamon Press.

ELLIOT, K. A. C. & FLOREY, E. (1956). Factor I. Inhibitory factor from brain. *J. Neurochem.* **1**, 181–191.

FATT, P. & KATZ, B. (1953). Distributed 'end-plate potentials' of crustacean muscle fibres. *J. exp. Biol.* **30**, 433–439.

FLOREY, E. (1960). Physiological evidence for naturally occurring inhibitory substances. *Inhibition in the Nervous System and γ-aminobutyric acid*, ed. ROBERTS, E. Oxford: Pergamon Press.

FLOREY, E. (1961). A new test preparation for bio-assay of Factor I and gamma-amino butyric acid. *J. Physiol.* **156**, 1–7.

HAYASHI, T. (1954). Effects of sodium glutamate on the nervous system. *Keio J. Med.* **3**, 183–192.

JONES, H. C. (1962). The action of L-glutamic acid and of structurally related compounds on the hind-gut of the crayfish. *J. Physiol.* **164**, 295–300.

KATZ, B. & THESLEFF, S. (1957). A study of the 'desensitization' produced by acetylcholine at the motor end-plate. *J. Physiol.* **138**, 63–80.

KRNJEVIĆ, K. & PHILLIS, J. W. (1963). Iontophoretic studies of neurones in the mammalian cerebral cortex. *J. Physiol.* **165**, 274–304.

LONGSWORTH, L. G. (1953). Diffusion measurements, at 25°, of aqueous solutions of amino acids, peptides and sugars. *J. Amer. chem. Soc.* **57**, 5705–5709.

PATON, W. D. M. (1958). Central and synaptic transmission in the nervous system (pharmacological aspects). *Ann. Rev. Physiol.* **20**, 431–470.

PURPURA, D. P., GIRADO, M., SMITH, T. G., CALLAN, D. A. & GRUNDFEST, H. (1958). Structure-activity determinants of pharmacological effects of amino acids and related compounds on central synapses. *J. Neurochem.* **3**, 238–268.

ROBBINS, J. (1958). The effects of amino acids on the crustacean neuro-muscular system. *Anat. Rec.* **132**, 492–493.

ROBBINS, J. (1959). The excitation and inhibition of crustacean muscle by amino acids. *J. Physiol.* **148**, 39–50.

TAKEUCHI, A. & TAKEUCHI, N. (1963). Glutamate-induced depolarization in crustacean muscle. *Nature, Lond.*, **198**, 490–491.

THESLEFF, S. (1955). The mode of neuromuscular block caused by acetylcholine, nicotine, decamethonium and succinylcholine. *Acta physiol. scand.* **34**, 218–231.

VAN HARREVELD, A. (1936). A physiological solution for freshwater crustaceans. *Proc. Soc. exp. Biol., N.Y.*, **34**, 428–432.

VAN HARREVELD, A. (1959). Compounds in brain extracts causing spreading depression of cerebral cortical activity and contraction of crustacean muscle. *J. Neurochem.* **3**, 300–315.

VAN HARREVELD, A. & MENDELSON, M. (1959). Glutamate-induced contractions in crustacean muscle. *J. cell. comp. Physiol.* **54**, 85–94.