J. Exp. Biol. (1974), **60**, 783–794
With 6 text-figures
Printed in Great Britain

L-GLUTAMATE IN ARTHROPOD BLOOD PLASMA: PHYSIOLOGICAL IMPLICATIONS

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(*Received 15 October 1973*)

INTRODUCTION

When a skeletal muscle from a crayfish or locust is perfused with saline containing L-glutamate its fibres become depolarized and go into contraction. After a while, in the continued presence of L-glutamate, the contraction declines, probably because the L-glutamate receptors in the muscle become desensitized. *In vivo* the muscles of arthropods are bathed in blood which may contain relatively high concentrations of L-glutamate. How the blood L-glutamate is prevented from interfering with neuromuscular transmission in these animals has remained something of a mystery. For the locust, *Schistocerca*, it has been suggested that a binding factor (Miller, Leaf & Usherwood, 1973) prevents the otherwise supra-threshold amounts of L-glutamate from being physiologically active. For the lobster, *Homarus*, it appears that the serum concentration is too low to cause interference with neuromuscular transmission (Kravitz *et al.* 1970).

Our goal was to learn more about the physiology of L-glutamate in the blood of arthropods, specifically, whether there is sufficient L-glutamate in blood plasma to present apparent difficulties for neuromuscular transmission. Accordingly, we have obtained data on the concentration of L-glutamate in the blood plasma from three types of arthropods – a crayfish, a locust, and a spider. We then determined whether neuromuscular transmission in a nerve–muscle preparation from each animal is affected by the concentration of L-glutamate that occurs in its blood plasma.

The results indicate that plasma L-glutamate poses no problems for neuromuscular transmission in the crayfish and spider. With the locust, however, the plasma level of L-glutamate would appear to be high enough to cause some depression of transmission; evidently, in this case, additional protective mechanisms must be at work *in vivo*.

MATERIALS AND METHODS

Experimental animals were the locust *Locusta migratoria* (L.), the tarantula *Dugesiella hentzii* (Chamb.), and the crayfishes *Astacus leptodactylus* (Eschsholz) and *Astacus astacus* (L.). The locusts were reared as described by Hunter-Jones (1966). Their diet consisted of sedge or fresh grasses, wheat germ and water *ad lib.* The spiders were a gift of Professor W. Rathmayer. They were fed on mealworms three times a week. The crayfish were purchased from commercial suppliers and held as described previously (Murdock, 1971); they were provided with carrot slices as food, *ad lib.*

Blood samples from the locust were taken by puncture of the dorsal cervical

membrane. Adult animals which had passed the imaginal ecdysis 3 weeks or more before the experiment were used. Fifty μl samples of blood could usually be obtained in 20–30 sec. Spider blood and crayfish blood were removed from the pericardial space with a hypodermic syringe.

Blood samples were transferred to siliconized micro centrifuge tubes (capacity *ca.* 200 μl) made from Pasteur pipettes. The blood was de-proteinized with an equal volume of 0·4 N-$HClO_4$ at 0 °C. After standing for 10 min in an ice-bath the precipitated proteins were centrifuged off (2700 *g*, 5–7 °C, 3 min). An aliquot of the supernatant was neutralized with a predetermined volume of 5 N-KOH and allowed to stand for 10 min at 0 °C. The precipitate was centrifuged off and the supernatant was analyzed for L-glutamate by the enzymic-fluorometric method of Graham & Aprison (1966). L-Glutamine was determined as L-glutamate after hydrolysis with glutaminase (Lund, 1970). All analyses were completed on the same day as that on which the blood was sampled.

Blood plasma was prepared by centrifugation of whole blood (600 *g*, 3 min, 5–7 °C). Precipitated haemocytes were taken up in a volume of H_2O equal to the original plasma volume and then extracted as for whole blood.

L-Glutamate recoveries were not significantly different from 100% when 2·5 n-moles of L-glutamate were added to 50 μl crayfish blood which was then carried through the extraction and analysis. Similar results were obtained with spider blood. With locust blood the recovery averaged 89%, and the experimental values were corrected using this factor. When nine individual samples of haemolymph from the same crayfish were separately extracted and analysed, the coefficient of variation was 0·12; for standard solutions containing 0·4 n-moles of L-glutamate the value was 0·10; for 2·0 n-moles it was 0·026.

The effect of L-glutamate on neuromuscular transmission was assessed by perfusing nerve–muscle preparations with physiological saline containing the amino acid while stimulating the motor axon serving the muscle and recording the excitatory junction potentials (e.j.p.s). With the crayfish the dactyl abductor preparation (Dudel & Kuffler, 1961) was used, with the spider the promotor tibiae (Rathmayer, 1966; Brenner, 1972), and with the locust the metathoracic extensor tibiae (Usherwood & Grundfest, 1965). The respective physiological salines have been described previously: crayfish (Murdock, 1971); spider (Brenner, 1972); locust (Usherwood & Machili, 1968). The e.j.p.s were recorded intracellularly using conventional intracellular electrode techniques. The e.j.p.s from the crayfish and spider muscles were averaged with a signal-averaging computer (Biomac 1000, Data Laboratories Ltd., London), and recorded with a Clevite Mark 220 Recorder (Clevite Corporation, Cleveland, Ohio). The e.j.p.s from the locust muscle were registered directly on the Clevite recorder.

The e.j.p. size was determined before, during and after perfusion with saline containing L-glutamate. When a preparation was perfused with a concentration of L-glutamate which had no observable effect on neuromuscular transmission, perfusion was continued for 15–30 min so as to ensure adequate time for the amino acid to reach the receptors. With concentrations of L-glutamate at which an effect was observed, it usually began within 30 sec of the beginning of the solution change. Responses with the spider muscle were often slower to appear, probably because a larger bath volume

Table 1. L-*Glutamate in whole blood, plasma, and blood cells from the crayfish* A. leptodactylus, *the tarantula* E. hentzii *and the locust* L. migratoria

(The concentration units for whole blood and plasma are μmoles/l, while the value given for cells is the number of micromoles of L-glutamate in the cells from 1 l of blood (μmoles in cells/l). Values given are means ± standard error of the means, with the number of observations in parentheses.)

	L-Glutamate		
Animal	Whole blood (μmole/l)	Plasma (μmole/l)	Cells (μmole in cells/l)
Crayfish	7·1 ± 1·6 (8)	1·4 ± 0·6 (8)	7·2 ± 1·5 (8)
Tarantula	52 ± 9 (6)	7 ± 2 (6)	—
Locust	104 ± 10 (9)	88 ± 9 (9)	28 ± 7 (8)

was used and the geometry of the muscle was less favourable. Even so, responses to higher concentrations of L-glutamate were often obtained in 2–3 min after beginning perfusion.

RESULTS

Crayfish blood. The concentration of L-glutamate in whole crayfish (*A. leptodactylus*) haemolymph is at least 5 times higher than in blood plasma (Table 1). This suggests that most of the amino acid is associated with an element of blood which is removed by centrifugation. When the residue after centrifugation was analysed, it was found to contain a high percentage of the total blood L-glutamate. Microscopic examination of the residue revealed that it consists mainly of packed blood cells, so it is probable that most of the total blood L-glutamate is contained within these cells.

Only a few successful analyses of *A. astacus* blood plasma could be obtained, because the blood had a far greater tendency to gel during sampling and centrifugation than did *A. leptodactylus* blood. Three values showed an average plasma concentration of *ca.* 2×10^{-6} M, i.e. not very different from *A. leptodactylus* blood.

The value given for the L-glutamate concentration in blood plasma in Table 1 is probably an overestimate. With several blood samples the plasma concentration was below the limits of detection. In those cases where measurable L-glutamate was retained in the plasma, it may have happened that the separation of cells from the plasma was incomplete. We often observed that if the blood had begun to gel before centrifugation it was not possible to detect differences in the concentration of L-glutamate between whole blood and plasma. When gelation was detected the samples were rejected. However, in some cases gelation may have occurred and remained undetected; this would have led to an overestimate of the plasma L-glutamate. Overestimation of the plasma level of the amino acid would also have occurred if cell lysis occurred, or if some of the L-glutamate leaked from the cells during blood removal and centrifugation.

In contrast to L-glutamate, a second amino acid, L-glutamine, was not found to be associated with crayfish haemocytes. The concentration of L-glutamine in blood varied greatly in different animals, even when these had the same apparent nutritional history. Values ranging from 0·05 to 0·5 mM have been observed in various batches of crayfish. Measurements made on ten animals in June gave an average L-glutamine concentration of 0·075 ± 0·015 (S.E.) mM in both plasma and whole blood, where paired values were

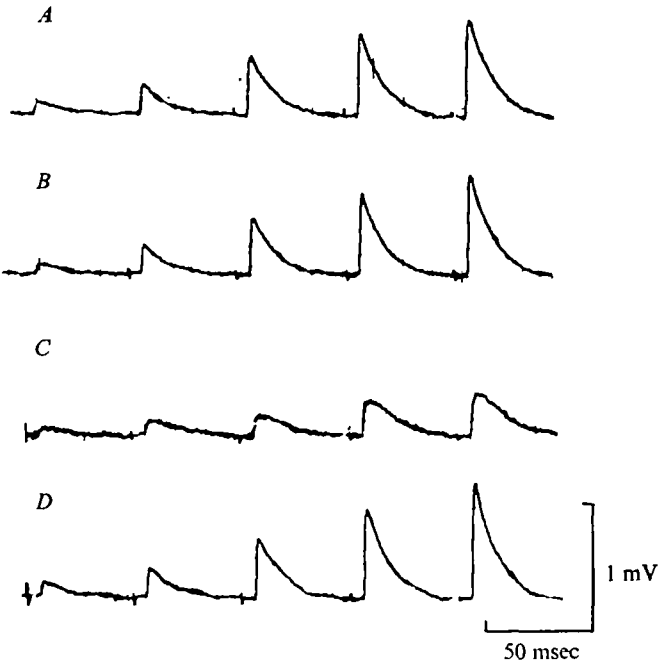

Fig. 1. Intracellularly recorded e.j.p.s from a crayfish (*A. astacus*) dactyl abductor muscle fibre during trains of five indirect stimuli. Each trace is the average of 32 traces taken during the preceding 1½ min. The initial e.j.p.s of each train are small, and subsequent e.j.p.s show considerable facilitation. Records *A–D* inclusive show the effects of perfusion with control or L-glutamate (L-Glu) salines. (*A*) Perfusion with control saline. (*B*) Perfusion with $1{\cdot}5 \times 10^{-5}$ M L-glutamate (*ca.* 10 times the mean plasma concentration) for *ca.* 9 min when the record was taken. There are no visible changes in the size of the e.j.p.s. (*C*) After an intermediate period of perfusion with control saline, the preparation was perfused with L-glutamate, $1{\cdot}5 \times 10^{-4}$ M for 5 min before the record was made. Depression of the e.j.p.s is marked. (*D*) Seven min after beginning of wash with control saline, the e.j.p.s had returned to the original size. Vertical lines in each trace are stimulus artifacts. Calibrations: 50 msec, 1 mV.

determined for each animal. Statistical analysis by the *t* test for paired data failed to reveal a significant difference.

Tarantula. An association of L-glutamate with blood cells was also found with the spider. The plasma concentration of L-glutamate averaged one-seventh of that of whole blood (Table 1). Direct assays of blood cells were not performed.

Locust. In contrast to the spider and crayfish, it was not possible to show a strong association of L-glutamate with locust haemocytes. Despite attempts to separate the haemocytes by centrifugation at different speeds (400–2700 *g*) and at two different temperatures (6 and 22 °C) for different lengths of time (0·5–5 min), a high percentage of the L-glutamate was always associated with the plasma fraction.

The values for the average amount of L-glutamate associated with the haemocytes (Table 1) is probably too high, because residual plasma (containing L-glutamate) would have remained with the haemocytes after the main bulk of the plasma was decanted off. This would have led to spuriously high estimates of the amount of L-glutamate associated with the haemocyte fraction. Assuming that one-half of the total L-glutamate in the haemocyte fraction is due to retained plasma, then about 8–12 % of the total L-glutamate in locust blood is associated with the blood cells.

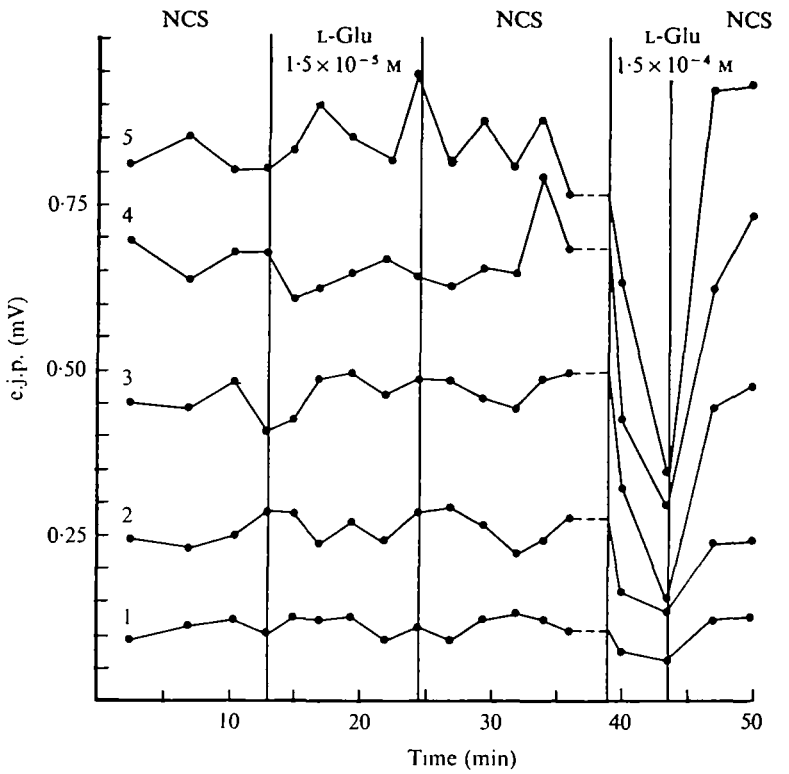

Fig. 2. Same experiment as Fig. 1. Traces 1–5 inclusive represent the 1st–5th inclusive e.j.p.s of the train. NCS, Control saline; L-Glu, L-glutamate containing saline, concentration as given. Vertical lines indicate a switch to a different saline which was perfused until the next vertical line. The resting potential of the muscle fibre was −80 to −81 at the beginning and −78 to −79 at the end of the experiment. Transient depolarization of a few mV occurred during exposure to the higher concentration of L-glutamate.

PHARMACOLOGICAL EXPERIMENTS

Crayfish. Neuromuscular transmission in the saline-perfused dactyl abductor preparation of the crayfish, as judged by the average e.j.p. size, was unaffected by L-glutamate at $1{\cdot}5 \times 10^{-6}$ M; that is, by the estimated average plasma concentration of the amino acid (Figs. 1, 2). The 10-fold higher concentration also failed to affect the e.j.p. size, even when the perfusion with the amino acid saline was continued as long as 25 min (Fig. 1). When the L-glutamate concentration in the saline was 100 times higher than the average plasma level there was a substantial diminution of the average e.j.p. size (Fig. 1). The depression, which presumably is due to post-synaptic desensitization, continued as long as perfusion with the L-glutamate saline continued. The control e.j.p. size was re-attained quickly, however, usually within 1–3 min, after the wash with control saline was begun.

Although the dactyl abductor muscle is innervated by a single motoneurone, this neurone forms synapses in different parts of the muscle which have different quantal contents and facilitation properties (Bittner, 1968). A spectrum of such synapse types was examined in this study (Figs. 1, 3) and no marked differences in sensitivity could be

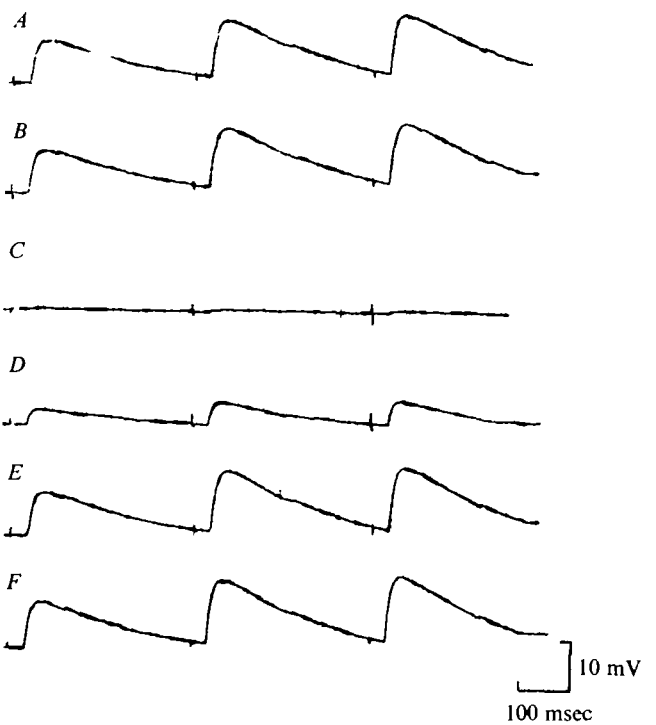

Fig. 3. Experiment as in Fig. 1, but using trains of three stimuli. Each trace is the average of 16 traces taken during the preceding minute. Initial e.j.p.s of each train were large, and showed little facilitation. (*A*) Perfusion with control saline. (*B*) Perfusion with L-glutamate, $1{\cdot}5 \times 10^{-5}$ M had continued for 4 min when the record was made. (*C*) After an intermediate control saline wash, the preparation was perfused with L-glutamate, $1{\cdot}5 \times 10^{-4}$ M for the 5 min prior to the record. The e.j.p.s are nearly abolished. (*D*) After a brief intermediate wash during which the e.j.p.s returned approximately to the control size, perfusion with L-aspartate, $1{\cdot}5 \times 10^{-3}$ M, was begun. The record was taken 4 min later. (*E*) After an intermediate wash with control saline, during which the e.j.p. returned to normal, the preparation was perfused with L-arginine, 10^{-3} M. The record was made 8 min later. There is no marked effect on the e.j.p.s. (*F*) e.j.p.s at the end of the experiment, 3 min after returning to control saline. Vertical lines in each trace are stimulus artifacts. Calibrations: 100 msec, 10 mV.

ascertained. All types appeared to be unaffected by $1{\cdot}5 \times 10^{-5}$ M L-glutamate (10 times the average plasma concentration) and to be substantially depressed by $1{\cdot}5 \times 10^{-4}$ M L-glutamate. In a single experiment (Figs. 3, 4), L-aspartate ($1{\cdot}5 \times 10^{-3}$ M) was observed to cause depression, but clearly less than that produced by one-tenth as much L-glutamate. L-Arginine ($1{\cdot}5 \times 10^{-3}$ M) had little or no effect on the preparation (Fig. 3).

Tarantula. Perfusion of the spider promotor tibiae preparation with 7×10^{-6} M L-glutamate (the average plasma concentration) had no measurable effect on the mean e.j.p. size (Fig. 5) during perfusions lasting as long as 20 min. Increasing the concentration to 7×10^{-5} M was also always without measurable effect. In one preparation, 1×10^{-4} M caused some depression (Fig. 5), in others it had no obvious effect. L-Glutamate at 10^{-3} M always caused severe depression of the e.j.p.s within 1–2 min of beginning perfusion (Fig. 5). The depression lasted as long as perfusion with the amino acid was continued, but returned to approximately its original (control) size after beginning the wash with control saline.

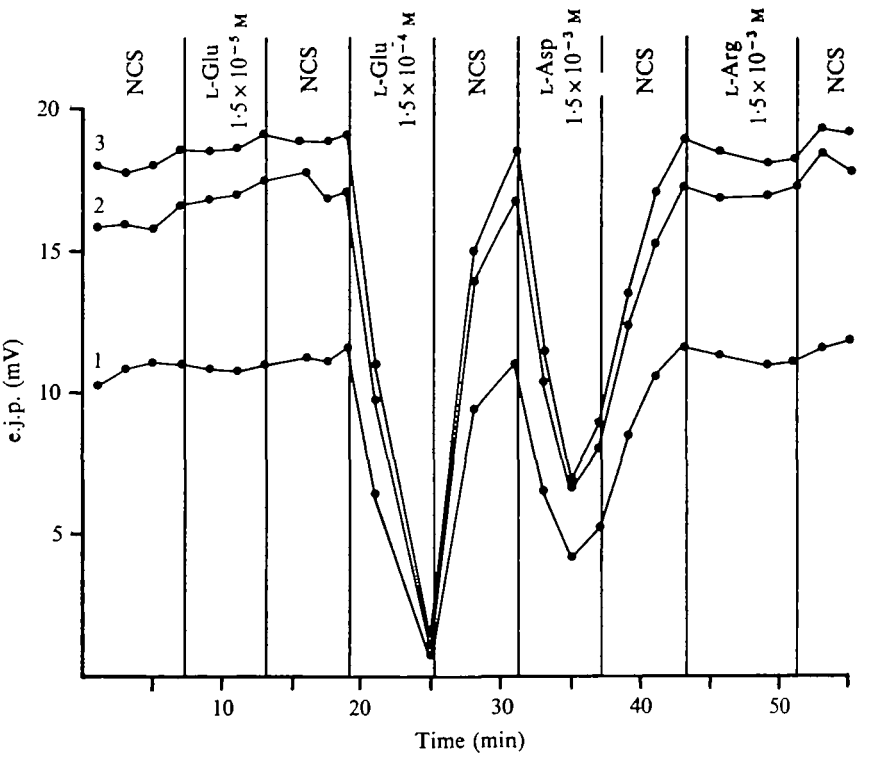

Fig. 4. Same experiment as Fig. 3. Traces 1–3 inclusive represent the 1st–3rd inclusive e.j.p.s of the train. NCS, Control saline; L-Glu, L-glutamate saline; L-Asp, L-aspartate saline; L-Arg, L-arginine saline. Vertical lines indicate switch to the next saline, which was perfused until the next vertical line.

Locust. When the extensor tibiae muscle of the locust was perfused with $1{\cdot}5 \times 10^{-4}$ M L-glutamate in saline, there was substantial depolarization and a marked decrease in the size of the e.j.p.s (Fig. 6). The magnitude of the effect seemed to vary considerably even in fibres of the same muscle. The e.j.p.s returned to nearly their control size within a minute or so of the beginning of the wash with control saline. It would appear that locust neuromuscular synapses are substantially depressed by a concentration of L-glutamate which can occur in locust blood plasma.

DISCUSSION

In the crayfish and the tarantula the blood L-glutamate appears to be concentrated in the haemocytes, as has previously been observed for *Carcinus maenas* (Evans, 1972; Miller *et al.* 1973). We have been unable to demonstrate an association of a second amino acid, L-glutamine, with haemocytes of the crayfish, whereas 40–50% of the L-glutamine in *Carcinus* blood is found in the haemocyte fraction.

Our finding that only a small fraction of L-glutamate in *Locusta* blood is associated with the haemocytes is in basic agreement with the results of Miller *et al.* (1973), who studied *Schistocerca* blood. They found that *ca.* 4% of the total L-glutamate of blood is present in the cells; we estimate the value for *Locusta* blood to be *ca.* 8–12%. Recently Holden (1973) has reported that only 12% of the total amino acids of *Periplaneta*

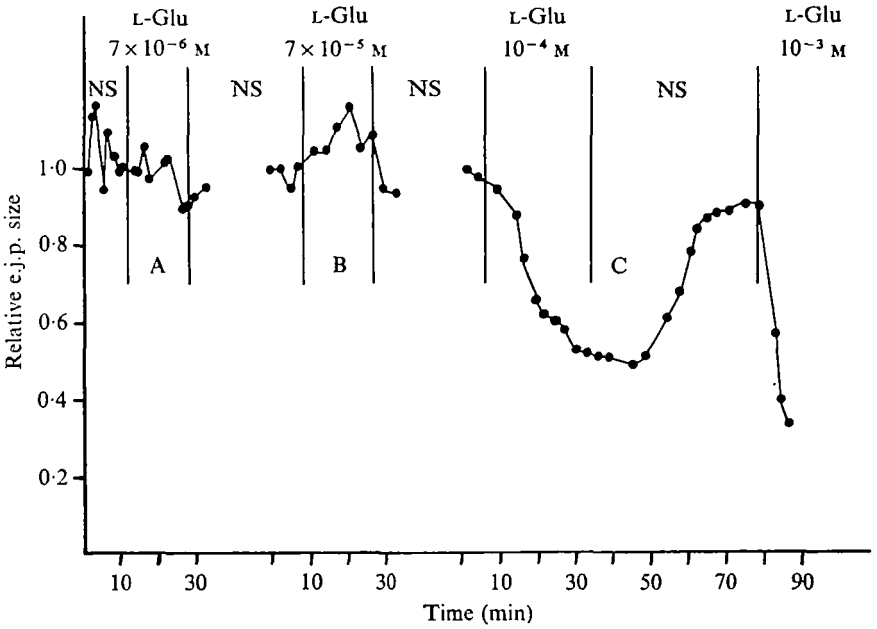

Fig. 5. The effect of L-glutamate on the relative e.j.p. size of the tarantula promotor tibiae muscle fibres during indirect stimulation. (A), (B) and (C) represent different preparations. NS refers to control saline perfusion, L-Glu to perfusion with L-glutamate.

(A) Stimuli were given every 2 sec. Each point is the average of 32 e.j.p.s taken during the preceding minute. 7×10^{-6} M L-glutamate has no obvious effect on neuromuscular transmission. Resting potential remained unchanged at 68–69 mV through the test.

(B) Stimuli were given every 2 sec. Each point is the average of 64 e.j.p.s taken during the preceding 2 min. 7×10^{-5} M L-glutamate had little, if any, effect on the average e.j.p. size. The resting potential of the fibre remained unchanged at 70–71 mV throughout the experiment.

(C) Stimuli were given every 2 sec. Each point is the average of 32 e.j.p.s taken during the preceding minute. Perfusion with L-glutamate (10^{-4} M) caused a marked depression of the e.j.p.s, but recovery occurred after washing with control saline. Perfusion with 10^{-3} M L-glutamate caused a more extensive depression of the e.j.p.s, with an apparent faster onset of action. The resting potential was 66–67 mV initially, dropped to 61–62 mV during maximal action of L-glutamate (10^{-4} M) and returned to 66–67 mV during the saline wash. Absolute e.j.p. sizes (equal to relative value of 1·0) were (A) 16·2 mV, (B) 10·8 mV, (C) 7·7 mV.

blood are bound to the haemocytes. These results suggest the conclusion that the haemocytes of orthopteroid insects contain only a small fraction of the total free amino acids in the blood and that, therefore, most of the amino acids are associated with plasma.

It would appear that with the crayfish and the tarantula blood plasma L-glutamate is present in too low a concentration to hinder synaptic transmission. Concentrations even 10-fold higher than the average plasma level are without suppressive effect, so there may be a considerable safety factor involved which ensures that small variations in plasma L-glutamate do not affect neuromuscular performance. Kravitz *et al.* (1970) have previously indicated that the concentration of L-glutamate in blood serum of the lobster, *Homarus americanus*, is too low to markedly reduce synaptic transmission.

In contrast to the crayfish and the tarantula, the quantity of L-glutamate in *Locusta* blood plasma would indeed appear to pose a problem for normal neuromuscular transmission, should the amino acid be truly free *in vivo*. Miller *et al.* (1973) have pre-

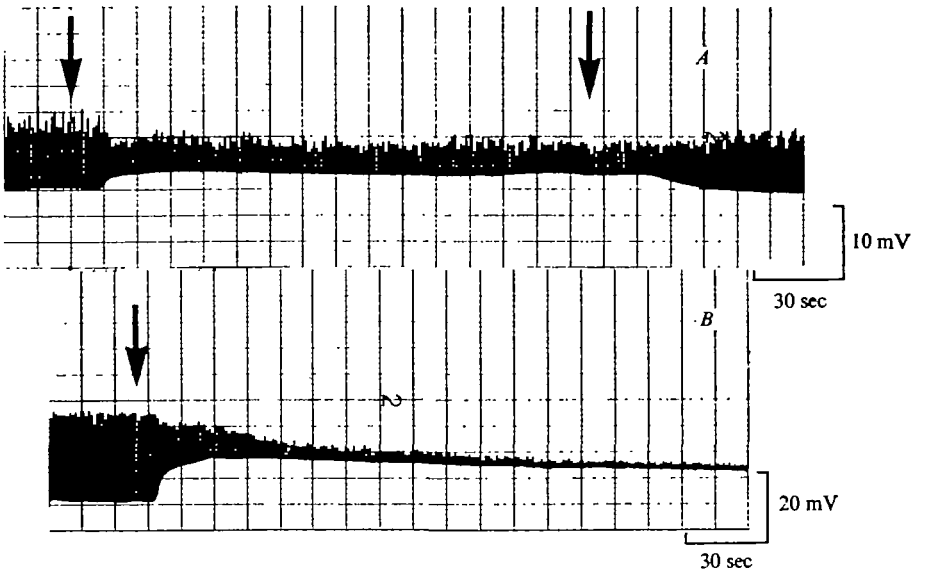

Fig. 6. Intracellularly recorded e.j.p.s from the extensor tibiae muscle fibres of *Locusta*. Records read from left to right. (*A*) and (*B*) represent two fibres from different preparations. At the first arrow L-glutamate, $1{\cdot}5 \times 10^{-4}$ M was perfused. Thereafter there was depolarization of several mV and decrease in the e.j.p. size. After washing with control saline e.j.p.s returned nearly to their original size (second arrow). Resting potentials: (*A*) 50 mV, (*B*) 38 mV. Calibrations: (*A*) 10 mV, 30 sec; (*B*) 20 mV, 30 sec.

sented evidence that fresh *Schistocerca* blood contains a mechanism which inactivates the otherwise supra-threshold levels of L-glutamate. The nature of the mechanism is unknown, although it seems to be labile *in vitro*. Presumably a similar mechanism is present in *Locusta*.

It appears that one tactic used by arthropods to protect L-glutamate-sensitive neuromuscular synapses from extra-synaptic L-glutamate is to maintain a subthreshold level of the amino acid in the blood plasma which bathes the muscles. Regulation of blood L-glutamate at a low level is indicated by experiments showing unchanged levels of the amino acid in blood of crayfish absorbing L-glutamate from the intestinal lumen (Murdock & Chapman, unpublished). Furthermore, preliminary experiments in which L-glutamate was injected into the haemocoel of crayfish indicate that the excess amino acid is rapidly removed from the blood.

Physiological inactivation of the plasma L-glutamate, perhaps via protein binding or binding to other blood components, represents a second tactic to maintain a subthreshold level of the free amino acid in plasma. This appears to be necessary in blood of *Locusta* or *Schistocerca*, for the average plasma concentration would otherwise be sufficient to cause substantial depolarization and desensitization of the muscle fibres. Usherwood & Machili (1968) observed that although locust blood contained considerable L-glutamate it did not cause a contraction when tested on a muscle preparation. When, however, L-glutamate (10^{-6} w/v) was added to the blood, a strong contraction occurred, followed by desensitization to indirect stimulation. This result suggests that the capacity of the blood to inactivate L-glutamate is limited, and indicates

that it would be advantageous to the locusts to prevent large rises in blood glutamate. That the L-glutamate concentration in locust blood does not rise during intestinal absorption of large quantities of the amino acid was indicated in previous experiments (Murdock & Koidl, 1972*a*, *b*). We have recently confirmed this result by direct assay, finding no significant change in blood L-glutamate even after 2 h of absorption of 100 mM L-glutamate. By contrast, when L-glutamine was being absorbed, the L-glutamine concentration in blood rose by 5–10 mM (Murdock & Chapman, in preparation).

An alternative to the maintenance of low levels of 'free' L-glutamate in blood plasma is the concept of a barrier system, either chemical or physical, between main-haemocoel plasma and the extracellular fluid surrounding the neuromuscular synapses. This implies that the extracellular fluid can differ substantially from that of main-haemocoel plasma. The basement membrane that surrounds muscle fibres and encloses nerve terminals (cf. Usherwood, 1969) might conceivably have a regulatory role as a physical barrier, but evidence is lacking. The close packing of muscle fibres, especially in thicker muscles, would presumably retard exchange of materials from the bathing fluid to the extracellular fluid in the centre of the muscle, and might thus act as a physical barrier.

Chemical barriers may take the form of uptake systems which remove L-glutamate which enters the extracellular fluid either from main-haemocoel blood or through leakage from cells lining the extracellular channels of the muscle. Uptake systems in arthropod nerves or nerve–muscle preparations have been observed (Faeder & Salpeter, 1970; Salpeter & Faeder, 1971; Iversen & Kravitz, 1968; Baker & Potashner, 1971; Evans, 1973) which might perform the role of maintaining a low concentration of L-glutamate in extracellular fluids.

Based on these considerations, we suggest that (1) maintenance of relatively low levels of L-glutamate in blood plasma, (2) physiological inactivation of some or all of the plasma L-glutamate, (3) physical barriers, and (4) chemical barriers all contribute to preventing body L-glutamate from interfering with synaptic transmission at L-glutamate-sensitive synapses. It appears that the relative importance of each of the mechanisms will vary in different species. Acting together, these mechanisms provide a large safety factor preventing extra-synaptic L-glutamate from interfering with normal synaptic transmission.

It is interesting to note that such defences are necessary even if L-glutamate is not the transmitter at many arthropod neuromuscular junctions, because desensitization caused by exogenous L-glutamate causes depression of synaptic transmission at these junctions.

SUMMARY

1. The L-glutamate concentration in blood plasma of the crayfish, *Astacus leptodactylus* and *A. astacus*, was found to be about $1{\cdot}5 \times 10^{-6}$ M, as measured by a specific enzymatic method. Even 10 times this concentration of L-glutamate in saline failed to significantly affect neuromuscular transmission in the dactyl abductor muscle, although higher concentrations caused depression.

2. L-Glutamate in the blood plasma of the spider, *Dugesiella hentzii*, amounted to 7×10^{-6} M. As in the crayfish, even 10 times this concentration of L-glutamate in saline

failed to affect neuromuscular transmission in the promotor tibiae muscle, while higher concentrations caused depression.

3. The concentration of L-glutamate in *Locusta migratoria* blood plasma, *ca.* 7×10^{-5} M, would appear to be sufficient to cause depression of neuromuscular transmission *in vivo*. Perfusion of the extensor tibiae preparation with 15×10^{-5} M L-glutamate caused marked depolarization and decreases in the size of e.j.p.s.

4. It appears that in the tarantula and the crayfish, blood plasma L-glutamate does not pose difficulties for neuromuscular transmission. In the locust, additional protective mechanisms which are not able to function in the saline-perfused *in vitro* preparation must be at work in the living animal.

The studies with the crayfish, tarantula and locust neuromuscular preparations could not have been made without the expert help of the Drs T. Linder, H. R. Brenner, and C. Walther. We thank Professor Ernst Florey for criticizing the manuscript. The work was supported by the Deutsche Forschungsgemeinschaft (Forschergruppe 138: Biologische Grenzflächen und Spezifität).

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