J. Physiol. (1982), **322**, *pp.* 529–539
With 6 text-figures
Printed in Great Britain

ON THE QUANTAL RELEASE OF ENDOGENOUS GLUTAMATE FROM THE CRAYFISH NEUROMUSCULAR JUNCTION

BY R. KAWAGOE, K. ONODERA AND A. TAKEUCHI

From the Department of Physiology, School of Medicine, Juntendo University, Hongo, Tokyo 113, Japan

(*Received 22 June 1981*)

SUMMARY

1. The abdominal slow flexor muscle was isolated from the crayfish (*Cambarus clarkii*) and placed in 150 μl. Harreveld solution. The concentrations of glutamate and aspartate in this solution were measured by mass fragmentography.

2. Application of black widow spider venom (BWSV) produced a marked increase in the frequency of miniature excitatory post-synaptic potentials (m.e.p.s.p.s). During the high frequency discharge of m.e.p.s.p.s, the glutamate content in the solution was significantly increased. There was an approximately linear relationship between the increase in the glutamate efflux produced by BWSV and the variance of the membrane potential fluctuation during high frequency discharge of m.e.p.s.p.s.

3. In most cases, the efflux of aspartate during control rest periods was smaller than that of glutamate. During the discharge of m.e.p.s.p.s produced by BWSV, the increase in the aspartate efflux was very small compared to glutamate.

4. Nerve stimulation caused a significant increase in the efflux of glutamate, but the change in the aspartate efflux was very small and not significant.

5. Application of methylene blue increased the frequency of m.e.p.s.p.s and glutamate efflux, but little, if any, increase was found in aspartate efflux.

6. It is concluded that glutamate is preferentially released from nerve terminals in a quantal fashion.

INTRODUCTION

In the crayfish neuromuscular junction, nerve stimulation caused a significant increase in the efflux of glutamate, which was dependent on the external concentration of Ca^{2+} (Takeuchi, Onodera & Kawagoe, 1980; Kawagoe, Onodera & Takeuchi, 1981). This result, together with previous observations that L-glutamate mimics the action of excitatory transmitter, strongly supports the idea that L-glutamate is the excitatory transmitter at the crayfish neuromuscular junction (Takeuchi & Takeuchi, 1964; Gerschenfeld, 1973; Takeuchi, 1976). Since the discharge of the excitatory post-synaptic potentials (e.p.s.p.s) in the crayfish neuromuscular junction is quantal (Dudel & Kuffler, 1961), one would expect that glutamate is released in a quantal fashion. In order to test this hypothesis the frequency of miniature e.p.s.p.s (m.e.p.s.p.s) was increased by applying black widow spider venom (BWSB) and the

efflux of glutamate was measured with mass fragmentography using a gas chromatograph-mass spectrometer (GC-MS). It has been shown that exposure to BWSV causes a very large increase in the frequency of m.e.p.s.p.s and finally depletes synaptic vesicles in vertebrate and invertebrate neuromuscular junctions (Longenecker, Hurlbut, Mauro & Clark, 1970; Kawai, Mauro & Grundfest, 1972; Cull-Candy, Neal & Usherwood, 1973; Griffiths & Smyth, 1973; Gorio, Hurlbut & Ceccarelli, 1978; Fritz, Atwood & Jahromi, 1980). It was found that the glutamate efflux was significantly increased by treatment with BWSV.

Another question regarding the transmitter release at the crayfish neuromuscular junction is whether or not other amino acids are released in addition to glutamate. Since the concentration of aspartate in crustacean motor axons is several times higher than that of glutamate (for review see Kehoe & Marder, 1976), it seems important to measure the release of aspartate under the conditions in which the glutamate release is increased. It was found that the release of aspartate was very small compared to that of glutamate. Some of these results have been presented in preliminary form (Takeuchi, Onodera & Kawagoe, 1981).

METHODS

The experimental procedures were essentially the same as those described in previous reports (Takeuchi, Onodera & Kawagoe, 1980; Kawagoe, Onodera & Takeuchi, 1981). The abdominal slow flexor muscle was isolated from the crayfish (*Cambarus clarkii*) and placed in a small bath. After washing for 30–60 min, the solution was removed with a suction pump and 150 μl. of a solution were put into the bath with a pipette. After 10 min, the solution was collected with a syringe and stored in a prechilled test tube. The bath solution was cooled to 9 °C, and nerve stimulation was applied with a pair of silver wires. The membrane potential of the muscle fibres was recorded intracellularly and stored on magnetic tape. The variance of membrane potentials was calculated with a computer (PDP 11/60, Digital Equipments Corporation).

The composition of the perfusion fluid was (mM): NaCl, 207·5; KCl, 5·4; $CaCl_2$, 18·8; *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES), 5·7 and pH was adjusted to 7·2. In preparing the crude BWSV extract, 1·5 mg of whole dessicated poison apparati (Sigma, grade II) were homogenized in 120 μl. Harreveld solution, and centrifuged at 3000 rev/min for 30 min at room temperature. The extract was diluted 10 times with Harreveld solution or it was applied directly to the preparation. In some cases the extract was dialysed with a membrane filter (Diafilter G-01T, Bio Engineering) to remove small molecules (molecular weight less than 1000). In the series of experiments in which the frequency of m.e.p.s.p. was increased by BWSV and methylene blue, tetrodotoxin (TTX, Sankyo) was added to the solution (2×10^{-7} g/ml.) to prevent the appearance of nerve spikes (Narahashi, Moore & Shapiro, 1969).

Measurement of glutamate and aspartate. The method of analysis used in previous reports (Kawagoe, Onodera & Takeuchi, 1981; Murayama, Shindo, Mineki & Ohta, 1981) was not sensitive enough for the measurement of aspartate in the sample, and a slightly modified method was employed. From each test tube 120 μl. of the solution were sampled. After adding 200 p-mole of deuterium-labelled L-glutamic acid ([D]L-glutamic acid, Aldrich Chemicals) and L-aspartic acid ([D_3]L-aspartic acid, Merck) as the internal standards, the solution was evaporated to dryness under a vacuum. The sample was dissolved in 250 μl. methanol and 100 μl. thionyl chloride was added at −80 °C. After incubation at 45 °C for 30 min, the solution was evaporated to dryness by blowing N_2 gas over the surface. The sample was then dissolved in 100 μl. heptafluorobutyric anhydride (HFBA) and allowed to react at 60 °C for 15 min. The reagent was evaporated to dryness with N_2 gas. The residue was dissolved in 100 μl. acetone and centrifuged at 3000 rev/min for 5 min. One μl. of supernatant was injected in the GC-MS (Finnigan Type 4000 with INCOS 2000 Data System). The gas chromatographic column used was the same as that described in the previous report and the column temperature was 125 °C.

Mass spectra of HFB glutamate methyl ester gave major peaks at *m/e* 312, 340, 372 and those

of HFB aspartate methyl ester at *m/e* 298, 326, 358. Among these peaks, *m/e* 312 and 298 were used. The concentration of amino acid in the sample was calculated from the ratio of the area of the mass fragmentogram for amino acid to that of deuterium-labelled amino acid (*m/e* 312/313 for glutamate and 298/301 for aspartate) using the calibration curve, which was obtained from standard solutions containing 0, 2, 5, 10, 20, 40 and 100 p-mole L-glutamate and L-aspartate plus

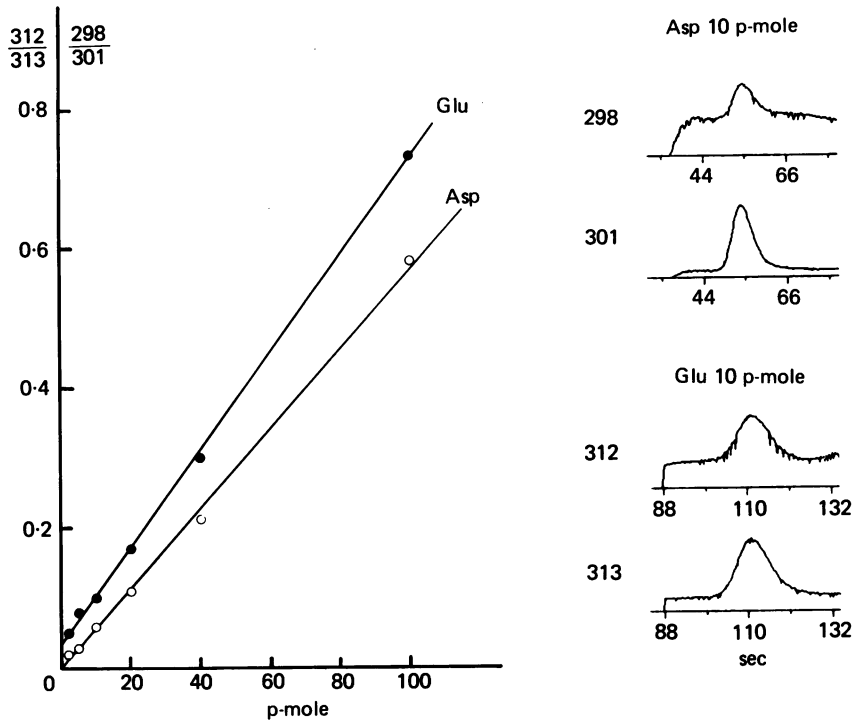

Fig. 1. Calibration curves for the measurement of glutamate and aspartate. Ordinate: the ratio of the area under mass fragmentograms for L-glutamate and deuterium-labelled L-glutamate (*m/e* 312/313, ●) and that for L-aspartate and deuterium-labelled L-aspartate (*m/e* 298/301, ○). Abscissa: the amount of L-glutamate and L-aspartate in 150 μl. solution. Mass fragmentograms for 10 p-mole L-aspartate (298) and L-glutamate (312) are shown on the right. Numbers on the abscissa of the mass fragmentograms represent the retention time (sec). Internal standards are 200 p-mole deuterium-labelled L-aspartate (301) and L-glutamate (313).

internal standards (Fig. 1). The efflux of amino acids was indicated as p-mole per sample. The sensitivity of this method was 1 p-mole/150 μl. for glutamate and aspartate.

Drugs used were HEPES (Wako Junyaku), thionyl chloride (Nakarai), HFBA (Pierce), L-glutamic acid (Ajinomoto) and L-aspartic acid (Ajinomoto).

RESULTS

Effects of BWSV on the spontaneous e.p.s.p.s

It has been well documented that BWSV increases the frequency of m.e.p.s.p.s in vertebrate and invertebrate neuromuscular junctions (Longenecker, Hurlbut, Mauro & Clark, 1970; Kawai, Mauro & Grundfest, 1972; Cull-Candy, Neal &

Usherwood, 1973; Griffiths & Smyth, 1973; Gorio, Hurlbut & Ceccarelli, 1978; Fritz, Atwood & Jahromi, 1980). When BWSV was applied to the abdominal slow flexor muscle, the frequency of m.e.p.s.p.s rose progressively and reached an extremely high level in about 10 min. Although the frequency of m.e.p.s.p. declined gradually, the high rate of discharge lasted for 20–30 min after washing away the venom (Fig. 2*Ab*). In most cases, the frequency of m.e.p.s.p.s returned to approximately the

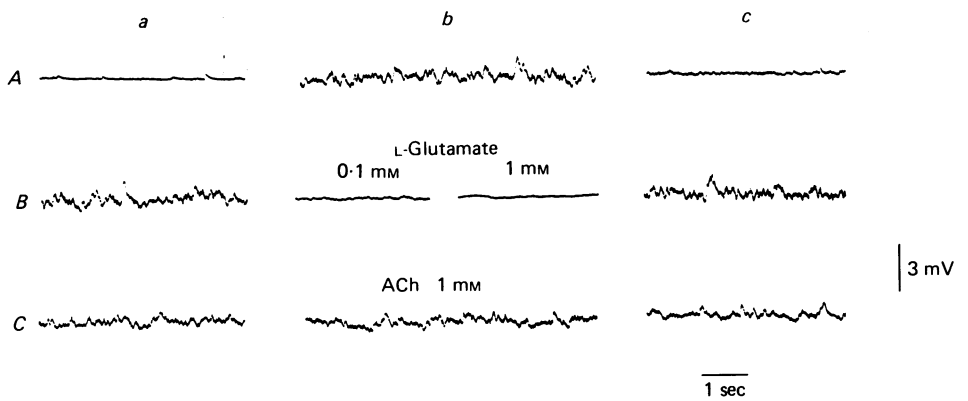

Fig. 2. Effects of black widow spider venom (BWSV) on the miniature e.p.s.p.s. *Aa*, before application of BWSV. *Ab*, after treatment with BWSV. *Ac*, after further washing with standard solution for 2 h. *B*: desensitization of miniature e.p.s.p.s by application of L-glutamate. *a*, before application of L-glutamate. *b*, during application of 0·1 and 1 mM-L-glutamate. *c*, after washing away L-glutamate. The preparation had been treated with BWSV. *C*: effect of ACh on miniature e.p.s.p.s induced by BWSV. *a*, before, *b*, during application of 1 mM-ACh and *c*, after washing away ACh. All records were obtained from the same preparation.

original level after washing for 2–3 hr (Fig. 2*Ac*). At later stages of the venom action, large potentials (giant miniature potentials) appeared sporadically superimposed on small m.e.p.s.p.s (e.g. see Kawai, Mauro & Grundfest, 1972). Application of 0·1 mM-L-glutamate solution gradually depressed the amplitude of m.e.p.s.p.s, and 1 mM-L-glutamate almost completely desensitized the m.e.p.s.p.s (Fig. 2*Bb*). However, the application of 1 mM-acetylcholine (ACh) to the preparation did not change the discharge of m.e.p.s.p.s (Fig. 2*Cb*). These observations indicate that potential changes produced by BWSV are due to the action of the transmitter on the glutamate receptors and that ACh is probably not contained in the transmitter (Kawagoe, Onodera & Takeuchi, 1981, see, however, Futamachi, 1972).

Effects of BWSV on the release of glutamate and aspartate

In Fig. 3 the glutamate contents (Glu) in 150 μl. solution are shown. Each column represents the amino acid content of a 10 min collection period, and the filled columns are those during the control rest period. In these experiments, standard Harreveld solution contained TTX 2×10^{-7} g/ml. to prevent the appearance of nerve spikes. After three collections from the resting preparation, the crude venom extract was applied for 20 min. Then, the venom was washed away with standard Harreveld

solution for 7 min at the rate of 0·75 ml./min and two collections were made in the standard solution (stippled columns). It was found that the glutamate efflux was increased above the background efflux. During this period, the frequency of m.e.p.s.p.s was extremely high, as shown at the top (Fig. 3*B*). When the preparation was washed with standard Harreveld solution for an additional 100 min, the frequency of m.e.p.s.p.s returned to approximately the original level (Fig. 3*C*) and

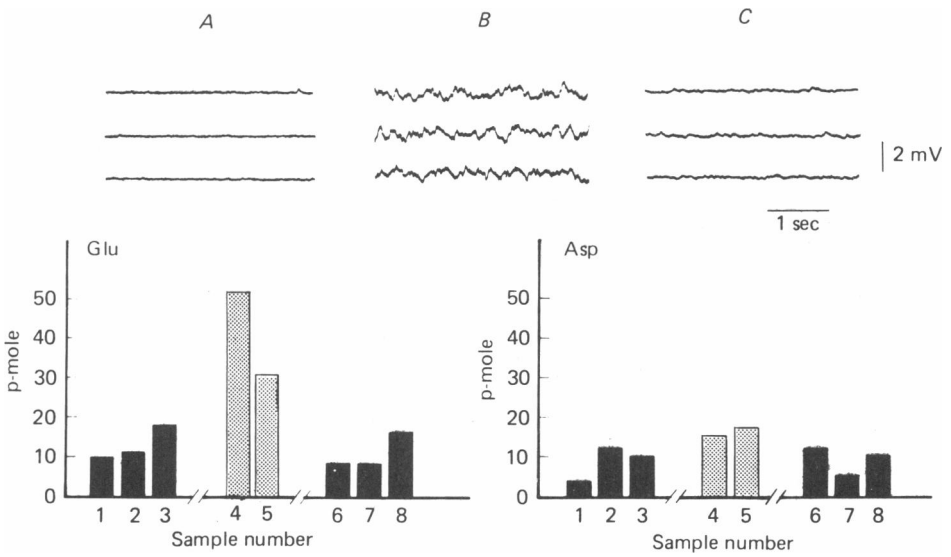

Fig. 3. Release of glutamate and aspartate induced by BWSV. Each column represents the content of glutamate (Glu) and aspartate (Asp) in 150 μl. solution for a collection period of 10 min. Filled columns are those during the control rest period. Between sample numbers 3 and 4, the preparation was treated with BWSV. Stippled columns show the glutamate release when the miniature e.p.s.p. frequency was increased by BWSV. Between sample numbers 5 and 6, the preparation was washed for 100 min with standard solution. Sample records of miniature e.p.s.p.s are shown at the top. *A*, before, *B*, after treatment with BWSV and *C*, after further washing with standard solution. All solutions contained TTX.

the glutamate release was also decreased (sample numbers 6–8). Average values of glutamate content in 150 μl. solution at control rest periods and after treatment with venom are shown in Table 1. The glutamate content was significantly increased by treatment with BWSV ($P < 0{\cdot}005$; Student's t test).

The above results suggest that glutamate is released in packets from the nerve terminals and produces a quantal response. However, it is also possible that BWSV induces the release of glutamate from other stores than nerve terminals, such as nerve branches and muscle fibres. In the latter case, the release sites are far from the glutamate receptors and the glutamate release may escape the detection by electrical recording. In order to test this possibility, the relationship between the frequency of m.e.p.s.p.s and the amount of glutamate released was studied.

Discharge of m.e.p.s.p.s after treatment with BWSV was extremely high and it was

TABLE 1. Release of glutamate and aspartate induced by treatment with BWSV (mean ± S.E., p-mole)

	Control before	After treatment	Control after washing
Glutamate	11·6 ± 1·0 (n = 14)	25·7 ± 5·4* (n = 10)	9·2 ± 0·9 (n = 15)
Aspartate	7·7 ± 1·4 (n = 14)	11·2 ± 2·2** (n = 10)	8·8 ± 2·4 (n = 15)

* $P < 0{\cdot}005$ compared with control before; ** $0{\cdot}10 < P < 0{\cdot}25$ compared with control before.

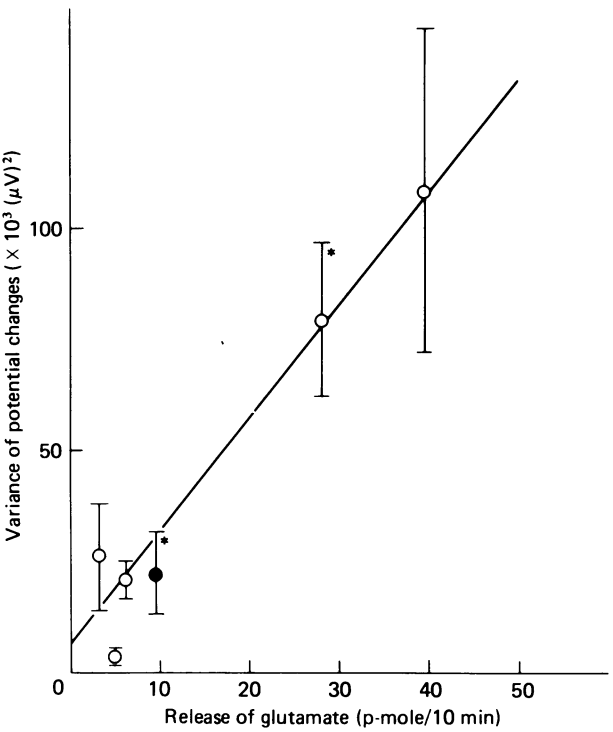

Fig. 4. The relationship between the glutamate release and the variance of membrane potential fluctuations after treatment with BWSV (○) and with methylene blue (●). Ordinate: mean value of the variance of membrane potentials recorded from two to four muscle fibres in the preparation. Bars indicate S.E. of the mean. Bars with asterisks show the range. Abscissa: release of glutamate.

almost impossible to measure its frequency. Since the variance of the membrane potential fluctuation during the high frequency discharge of m.e.p.s.p.s may be proportional to the mean frequency of quantal release (for theoretical analysis, see Katz & Miledi, 1972), the membrane potential was recorded from two to four muscle fibres in the preparation treated with BWSV and the variance was calculated with a computer. The mean value of the variance was plotted against the release of glutamate induced by BWSV above the resting release (Fig. 4). Although considerable

variations occurred, an approximately linear relation was found between the variance and the glutamate release. The line was drawn according to the method of least squares and the correlation coefficient was 0·66 ($n = 28$, $P < 0·005$).

The effect of BWSV on the release of aspartate is shown in Fig. 3 (Asp) and the average values are summarized in Table 1. The contents of glutamate and aspartate were measured from the same samples. After treatment with BWSV, the aspartate release was slightly increased (stippled columns), but the average increase was small and not very significant ($0·1 < P < 0·25$).

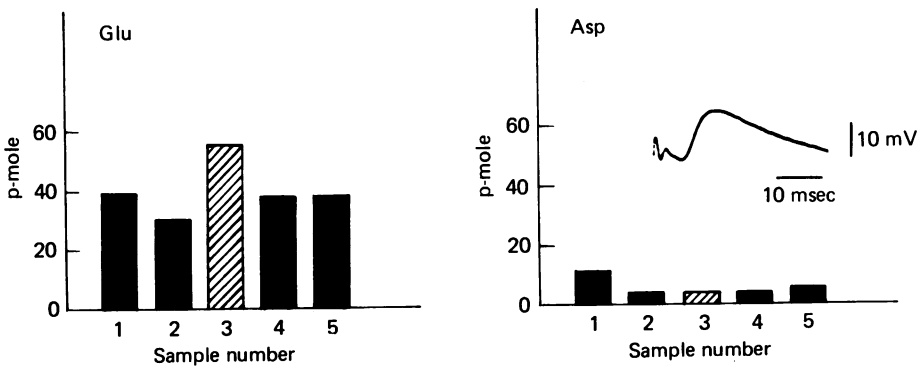

Fig. 5. Release of glutamate and aspartate in response to nerve stimulation. Nerve stimulation was applied at 20 Hz for 9 min and the contents of glutamate (Glu) and aspartate (Asp) in the sample are shown in the hatched columns. Sample record of e.p.s.p. is shown in the inset.

Effect of nerve stimulation on the release of glutamate and aspartate

In Fig. 5, the release of glutamate (Glu) and aspartate (Asp) is shown. The filled columns represent the content of amino acids during the control rest period. Application of nerve stimulation at 20 Hz for 9 min increased the glutamate release above the background release by about 20 p-mole, but no change was observed in the aspartate release (hatched column). A sample record of e.p.s.p. is shown in the inset.

The average values of the glutamate and aspartate release are shown in Table 2. The glutamate release was significantly increased by nerve stimulation at 20 Hz ($P < 0·05$), but the increase in the aspartate release was very small and not significant ($0·25 < P < 0·5$). Aspartate and glutamate were measured using the same samples.

Effect of methylene blue on the release of glutamate and aspartate

When a methylene blue solution (0·025 mg/ml.) was applied to the nerve-muscle preparation, the nerve terminals took up the dye and a marked discharge of min. e.p.s.p.s was produced in 5–10 min (Onodera & Takeuchi, 1980). The high frequency discharge of min. e.p.s.p. lasted for about 20 min after washing away the methylene blue. The frequency gradually decreased during further washing and after 2–3 hr, the discharge of min. e.p.s.p. returned to almost the control level. An example is shown

in Fig. 6. The glutamate efflux was increased after treatment with methylene blue, but the increase in the aspartate efflux was small (stippled columns). In three preparations, the resting release of glutamate was 4·4±0·9 p-mole/10 min (mean± S.E.) ($n = 8$) and, after treatment with methylene blue, the release was significantly increased to 14·6±2·0 p-mole/10 min ($n = 7$) ($P < 0·005$). However, the increase in the aspartate release was not significant: 4·0±1·2 p-mole/10 min ($n = 8$) at

TABLE 2. Release of glutamate and aspartate induced by nerve stimulation (mean±S.E., p-mole)

	Control before	20 Hz stimulation	Control after
Glutamate	20·3±2·5 ($n = 12$)	34·1±7·4* ($n = 6$)	19·3±3·0 ($n = 12$)
Aspartate	6·6±1·6 ($n = 12$)	9·8±3·5** ($n = 6$)	8·3±3·3 ($n = 12$)

* $P < 0·05$ compared with control before; ** $0·25 < P < 0·5$ compared with control before.

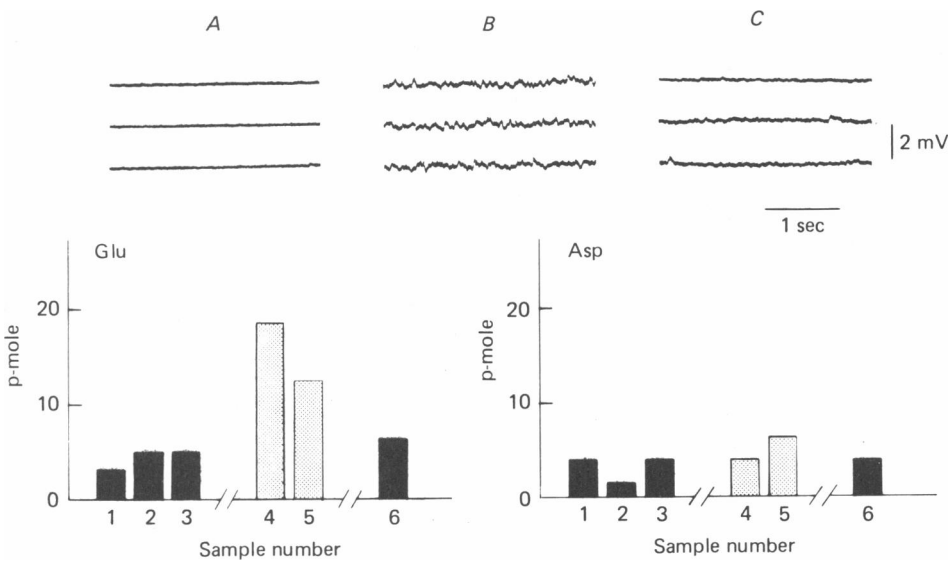

Fig. 6. Release of glutamate and aspartate induced by treatment with methylene blue. Between sample numbers 3 and 4, methylene blue was applied and the contents of glutamate (Glu) and aspartate (Asp) are shown as stippled columns. Between sample numbers 5 and 6, the preparation was washed for 2 hr. Sample records of membrane potentials are shown at the top. *A*, before, *B*, after treatment with methylene blue and *C*, after washing for 2 hr. All solutions contained TTX.

rest and 4·9±1·9 p-mole/10 min ($n = 7$) after treatment with methylene blue ($0·25 < P < 0·5$). In these experiments, all the solutions contained TTX (2×10^{-7} mg/l.). The variance of the membrane potential was measured in one preparation and it is shown in Fig. 4 (●).

Resting release of glutamate and aspartate

Resting release of glutamate was found in all the preparations tested and it was usually about 10–20 p-mole/10 min, whereas the resting release of aspartate was usually smaller than that of glutamate in the same preparation. In six out of eighteen preparations, the resting release of aspartate was not detected by the present method (less than 1 p-mole). In these preparations, the average value of the resting glutamate release was $10{\cdot}6 \pm 2{\cdot}3$ p-mole/10 min ($n = 12$).

When one compares the resting glutamate release in Table 1 and that in Table 2, it is noted that glutamate release in Table 1 is smaller than that in Table 2. The experiments in Table 1 were performed mainly in the winter season and those in Table 2 were done in the autumn. Therefore, the different values of the resting glutamate release may be partially attributed to seasonal differences. Another possibility may be that in the experiments in Table 1, the solutions contained TTX, whereas no TTX was used in those in Table 2, and TTX may have some depressing effects on the glutamate release. Although TTX had no apparent effect on the spontaneous e.p.s.p.s, more detailed experiments may be necessary to clarify the above possibility, e.g. comparison of its action, using the same preparations.

DISCUSSION

The present experiments showed that glutamate efflux from the crayfish nerve-muscle preparation was increased under the conditions in which the frequency of m.e.p.s.p.s was elevated. The amount of glutamate released was linearly related to the variance of the membrane potential during high frequency discharge of m.e.p.s.p.s. This result suggests that the glutamate release is proportional to the frequency of m.e.p.s.p.s. Thus, glutamate may be released as packets of molecules, as are ACh molecules in vertebrate neuromuscular junctions (Katz, 1969; Kuffler & Yoshikami, 1975).

The concentration of aspartate in the motor axon is three to five times higher than that of glutamate in the lobster and crab (McBride, Shank, Freeman & Aprison, 1974; Sorenson, 1973). A high concentration of aspartate in the motor axons was also found in the crayfish. In our preliminary experiments, single motor axons were isolated from nerve branches which innervate the opener muscle of the crayfish claw, and the concentrations of glutamate and aspartate in the axon were measured by mass fragmentography. The mean ratio of aspartate to glutamate concentrations was $3{\cdot}3 \pm 0{\cdot}6$ ($n = 8$).

It has been reported that aspartate enhances the conductance change produced by bath application or superfusion of glutamate at the crustacean neuromuscular junction (Kravitz, Slater, Takahashi, Bownds & Grossfeld, 1970; Kerkut & Wheal, 1974; Shank & Freeman, 1975; Shank, Freeman, McBride & Aprison, 1975; Crawford & McBurney, 1977; however, for ionophoretic application of glutamate, see Takeuchi, 1976 and Dudel, 1977). These observations, together with the high concentration of aspartate in motor axons, have prompted several authors to propose that glutamate and aspartate are synchronously released from the excitatory nerves of crustacea (for

review, see Freeman, 1976). Our experiments, however, showed that the amount of aspartate released was very small compared to that of glutamate. Since glutamate is released as quanta, glutamate may have been accumulated selectively in the vesicles. Thus, aspartate may be contained in compartments other than those in which the releasable glutamate is stored. Another possibility may be that both amino acids are contained in the axon terminals and that the terminal membrane has a permeability property that preferentially releases glutamate. Since amino acids are universal metabolic intermediates, only a small part of the amino acids in the axon may be used as transmitters. Thus, the mere presence of a putative transmitter in an axon, even in a considerable concentration, may not be considered as proof of its transmitter role.

It has been reported that nerve stimulation causes an increase in the release of glutamate and other amino acids as well in perfused legs of crab and locust (Daoud & Miller, 1976; also for discussion to earlier release experiments). Preferential release of glutamate observed in the present experiments may be attributed to the different experimental procedures and materials. In particular, the nerve-muscle preparation used was a much simpler system than perfused legs and, with this preparation, the background release of glutamate and aspartate could be kept low and stable (see Kawagoe *et al.* 1981).

The resting efflux of glutamate and aspartate may be partly attributed to non-specific leakage from neural and non-neural tissues. However, the resting release of glutamate was found in all the preparations tested, whereas in about 35% of the preparations, the resting release of aspartate was not detected. In these preparations, the resting release of glutamate averaged 10·6 p-mole/10 min. Therefore, at the resting state, glutamate is likely to be released preferentially among amino acids in the axons. By analogy with frog neuromuscular junctions (Katz & Miledi, 1977), a part of the resting release of glutamate might be a non-quantal leakage of the transmitter.

We thank Dr K. Murayama, Ms R. Mineki, N. Shindo and K. Ohta, Central Laboratory of Medical Sciences, Juntendo University, for their expert help in the amino acid analysis. Thanks are also due to Dr K. Yana, Department of Physiology, Juntendo University, for the computer analysis and to Dr N. Kawai, the Metropolitan Institute for Neurosciences, for the gift of BWSV. This work was supported in part by grants from the Ministry of Education, Sciences and Culture, Japan.

REFERENCES

CRAWFORD, A. C. & MCBURNEY, R. N. (1977). The synergistic action of L-glutamate and L-aspartate at crustacean excitatory neuromuscular junction. *J. Physiol.* **268**, 697–709.

CULL-CANDY, S. G., NEAL, H. & USHERWOOD, P. N. R. (1973). Action of black widow spider venom on aminergic synapse. *Nature, Lond.* **241**, 353–354.

DAOUD, A. & MILLER, R. (1976). Release of glutamate and other amino acids from arthropod nerve-muscle preparations. *J. Neurochem.* **26**, 119–123.

DUDEL, J. (1977). Aspartate and other inhibitors of excitatory synaptic transmission in crayfish muscle. *Pflügers Arch.* **369**, 7–16.

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

FREEMAN, A. R. (1976). Polyfunctional role of glutamic acid in excitatory synaptic transmission. *Prog. Neurobiol.* **6**, 137–153.

FRITZ, L. C., ATWOOD, H. L. & JAHROMI, S. S. (1980). Lobster neuromuscular junctions treated with

black widow spider venom: correlation between ultrastructure and physiology. *J. Neurocytol.* **9**, 699–721.

FUTAMACHI, K. J. (1972). Acetylcholine: possible neuromuscular transmitter in crustacea. *Science, N.Y.* **175**, 1373–1375.

GERSCHENFELD, H. M. (1973). Chemical transmission in invertebrate central nervous systems and neuromuscular junctions. *Physiol. Rev.* **53**, 1–119.

GORIO, A., HURLBUT, W. P. & CECCARELLI, B. (1978). Acetylcholine compartments in mouse diaphragm. Comparison of the effects of black widow spider venom, electrical stimulation and high concentrations of potassium. *J. Cell Biol.* **78**, 716–733.

GRIFFITHS, D. J. G. & SMYTH, T. Jr. (1973). Action of black widow spider venom at insect neuromuscular junctions. *Toxicon* **11**, 369–374.

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

KATZ, B. & MILEDI, R. (1972). The statistical nature of the acetylcholine potential and its molecular components. *J. Physiol.* **224**, 665–699.

KATZ, B. & MILEDI, R. (1977). Transmitter leakage from motor nerve endings. *Proc. R. Soc.* B **196**, 59–72.

KAWAGOE, R., ONODERA, K. & TAKEUCHI, A. (1981). Release of glutamate from the crayfish neuromuscular junction. *J. Physiol.* **312**, 225–236.

KAWAI, N., MAURO, A. & GRUNDFEST, H. (1972). Effect of black widow spider venom on the lobster neuromuscular junctions. *J. gen. Physiol.* **60**, 650–664.

KEHOE, J. S. & MARDER, E. (1976). Identification and effects of neural transmitters in invertebrates. *A. Rev. Pharmac.* **16**, 245–268.

KERKUT, G. A. & WHEAL, H. V. (1974). The excitatory effects of aspartate and glutamate on the crustacean neuromuscular junction. *Br. J. Pharmac.* **51**, 136–137*P*.

KRAVITZ, E. A., SLATER, C. R., TAKAHASHI, K., BOWNDS, M. D. & GROSSFELD, R. M. (1970). Excitatory transmission in invertebrates – Glutamate as a potential neuromuscular transmitter compound. In *Excitatory Synaptic Mechanisms*, ed. ANDERSEN, P. & JANSEN, J. K. S., pp. 85–93. Oslo: Universtetsforlaget.

KUFFLER, S. W. & YOSHIKAMI, D. (1975). The number of transmitter molecules in a quantum: an estimate from iontophoretic application of acetylcholine at the neuromuscular synapse. *J. Physiol.* **251**, 465–482.

LONGENECKER, H. E., HURLBUT, W. P., MAURO, A. & CLARK, A. W. (1970). Effects of black widow spider venom on the frog neuromuscular junction. *Nature, Lond.* **225**, 701–703.

MCBRIDE, W. J., SHANK, R. P., FREEMAN, A. R. & APRISON, M. H. (1974). Levels of free amino acids in excitatory, inhibitory and sensory axons of the walking limbs of the lobster. *Life Sci. Oxford*, **14**, 1109–1120.

MURAYAMA, K., SHINDO, N., MINEKI, R. & OHTA, K. (1981). Determination of glutamic acid and gamma amino butyric acid in Ringer's solution without desalination at femtomole level by gas chromatography chemical ionization mass spectrometry. *Biomed. Mass Spectrometry* **8**, 165–169.

NARAHASHI, T., MOORE, J. W. & SHAPIRO, B. I. (1969). Condylactis toxon: Interaction with nerve membrane ionic conductances. *Science, N.Y.* **163**, 680–681.

ONODERA, K. & TAKEUCHI, A. (1980). Distribution and pharmacological properties of synaptic and extrasynaptic glutamate receptors on crayfish muscle. *J. Physiol.* **306**, 233–250.

SHANK, R. P. & FREEMAN, A. R. (1975). Cooperative interaction of glutamate and aspartate with receptors in the neuromuscular excitatory membrane in walking limbs of the lobster. *J. Neurobiol.* **6**, 289–303.

SHANK, R. P., FREEMAN, A. R., MCBRIDE, W. J. & APRISON, M. H. (1975). Glutamate and aspartate as mediators of neuromuscular excitation in the lobster. *Comp. Biochem. Physiol.* **50***C*, 127–131.

SORENSON, M. M. (1973). The free amino acids in peripheral nerves and in isolated inhibitory and excitatory nerve fibres of *Cancer magister*. *J. Neurochem.* **20**, 1231–1245.

TAKEUCHI, A. (1976). Excitatory and inhibitory transmitter actions at the crayfish neuromuscular junction. In *Motor Innervation of Muscle*, ed. THESLEFF, S., pp. 231–261. London: Academic.

TAKEUCHI, A., ONODERA, K. & KAWAGOE, R. (1980). Release of endogenous glutamate from the neuromuscular junction of the crayfish. *Proc. Jap. Acad. B* **56**, 246–249.

TAKEUCHI, A., ONODERA, K. & KAWAGOE, R. (1981). The quantal nature of glutamate release at the crayfish neuromuscular junction. *Proc. Jap. Acad. B* **57**, 105–108.

TAKEUCHI, A. & TAKEUCHI, N. (1964). The effect on crayfish muscle of iontophoretically applied glutamate. *J. Physiol.* **170**, 296–317.