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THE OCCURRENCE IN AND ACTIONS OF AMINO ACIDS ON ISOLATED SUPRA ORAL SPHINCTER PREPARATIONS OF THE SEA ANEMONE *ACTINIA EQUINA*

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SUMMARY

1. L-glutamic acid reversibly inhibits the contractions of isolated circular muscle preparations from different body regions of the sea anemone *Actinia equina* to electrical stimulation.

2. L-glutamic acid does not cause inhibition of contraction by conversion to ammonia or γ-amino-butyric acid, but appears to act on a glutamate receptor.

3. It is postulated that in *Actinia equina* there is a receptor for glutamic acid whose structural requirements for activation are met by comparatively few analogues of glutamic acid. Of thirty-four analogues tested, only seven were active, most were less active, and only homocysteic acid had a greater activity than L-glutamic acid.

4. Glutamic acid does not depress conduction of contraction in circular muscle preparations.

5. The inhibition produced by L-glutamic acid does not appear to be conducted from one part of a preparation to another.

6. Glutamic acid occurs in high concentrations in supra oral sphincter preparations of *Actinia equina*.

7. Glutamic acid is released into sea water from sphincter preparations at rest and the amount released is increased by electrical stimulation of the preparation. However, many other amino acids are released at rest and following electrical stimulation, so that it is difficult to suggest that glutamic acid may be involved as a neurotransmitter in the sea anemone.

INTRODUCTION

The Coelenterates are the simplest multicellular animals that possess a recognizable and organized nervous system. The physiology of the nervous

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system and the problems of neuromuscular transmission in this phylum have been perhaps most intensively investigated in sea anemones (Pantin, 1952; Ross, 1960*a*, *b*; Josephson, 1966; Robson & Josephson, 1969; McFarlane, 1970). There have also been some investigations of the occurrence of pharmacologically active substances in the anemones and the possible nature of the neurotransmitter substances (Mathias, Ross & Schachter, 1960; Östland, 1954; Carlyle, 1969*a*, *b*). The nature of the transmission process in sea anemones is, however, still unknown. One of the greatest difficulties in analysis is that few classical autonomic drugs produce any effect on whole animals or on isolated preparations, even in high concentrations (Ross, 1960*a*, *b*; Carlyle, 1969*b*). It is therefore of interest that certain amino acids or derivatives produce complex physiological responses in some coelenterates.

Lenhoff (1961) showed that in hydra glutathione produced a complex feeding response involving movement of the tentacles, mouth and body. Steiner (1957) applied pieces of filter paper, soaked in various amino acids, to the mouth and tentacles of the sea anemone *Actinia equina*. Of the common amino acids tested only glutamic acid produced an opening of the mouth. Although the glutamic acid may in this case be acting only on a chemoreceptor cell to initiate these responses, it seemed possible that this action of glutamic acid might indicate a role of this, and possibly other amino acids, as neurotransmitters in the anemone.

There is evidence that amino acids may be transmitters in the nervous systems of mammals and other vertebrates and at the neuromuscular junctions of crustacea (Hebb, 1970; Phillis, 1970). It was therefore thought to be of interest to investigate the occurrence and actions of amino acids on isolated preparations of the sea anemone *Actinia equina*.

Some of the experiments in this paper have been communicated previously to the Physiological Society (Carlyle, 1970, 1971*a*, *b*, 1972).

METHODS

Animals. Anemones (*Actinia equina*) were obtained from Plymouth Marine Biological Association or collected from the sea shore adjacent to Hastings. The animals were kept at 8° C in 120 l. of aerated and filtered sea water. Animals required for experimental use were removed from the tank 7–10 days before required and stored at 8° C in 5 l. of filtered fresh sea water and were starved. Animals required for extraction and biochemical estimation of amino acids or other substances were further rinsed in filtered sea water immediately before dissection, and their body cavities washed out thoroughly with 30–50 ml. sea water, particular care being taken to remove any mucus adhering to the outside of the animal, and any fragments of food from the body cavity. In the experiments reported in this paper animals weighing between 2·8 and 5·4 g have been used.

Dissection. Before dissection animals were immobilized by the addition of an equal

volume of 0·4 M magnesium chloride solution to the sea water (Batham & Pantin, 1954). 8–12 hr later the animals were dissected. Following dissection preparations were allowed to recover in fresh sea water for 8–12 hr before use. Control experiments using preparations cut quickly from non-immobilized animals established that magnesium chloride treatment, followed by recovery in normal sea water did

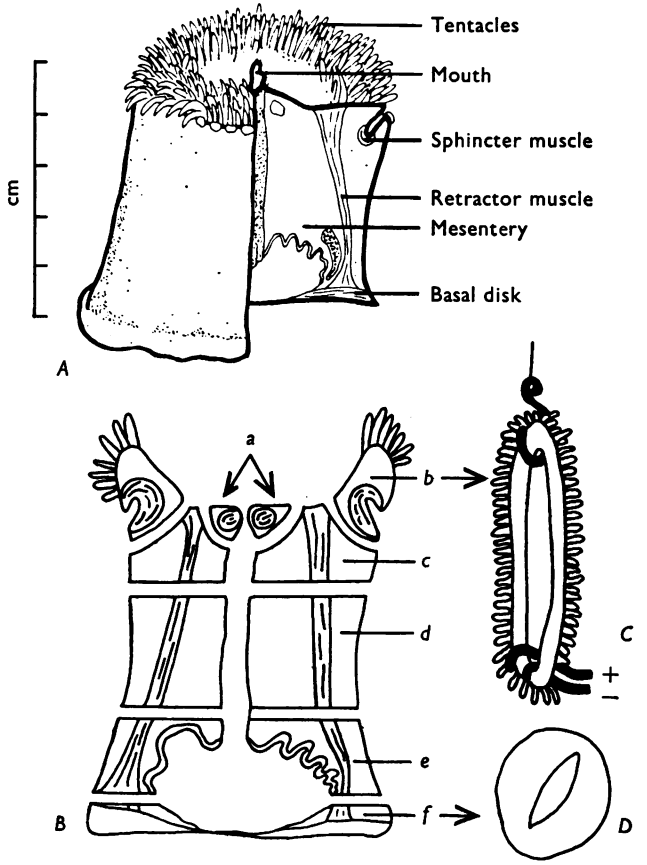

Fig. 1. General anatomy of the sea anemone (*Actinia equina*) and method of cutting the various circular body wall preparations.

Fig. 1*A* shows the main external anatomical features of *Actinia equina* and some of the internal structures, especially the sphincter muscles and basal disk.

Fig. 1*B* is a sagittal section through the body showing the cuts needed to yield the mouth sphincter preparations (cut *a*), the supra oral sphincter (cut *b*), the upper, middle and lower body ring preparations (cuts *c*, *d* and *e*), and the basal disk (cut *f*). The basal disk is converted to a ring by cutting a wide slit in its middle (Fig. 1*D*).

Fig. 1*C* shows the supra oral sphincter, in this case with intact tentacles, suspended from a pair of platinum electrodes (16 S.W.G. 3 mm apart). The upper platinum hook (16 S.W.G.) is attached to the recording lever or transducer. The scale given is approximate and does not apply to Fig. 1*D*.

not affect the responses to electrical stimulation or amino acids. Immobilization with magnesium chloride, however, had the advantage of preventing strong contraction of the animal and excessive production of mucus during dissection.

Isolated preparations. Most of the experiments reported in this paper have been made on rings of muscle produced by cutting in a circular fashion around the body wall above the level of the mouth. This ring of body wall which bears the bulk of the tentacles will be called the supra oral sphincter. If it is not otherwise stated, it may be assumed that an experiment is performed on a supra oral sphincter preparation. In a similar manner rings can be obtained from the upper, mid and lower body wall as well as from the mouth region. Also the basal disk of the animal can be detached, slit centrally and converted to a ring preparation. The cuts involved in making the various preparations are summarized in Fig. 1 *B* and *D*.

The rings so prepared were used in several ways. Most were placed on a pair of platinum hook electrodes (16 S.W.G., 3 mm spacing) in aerated sea water at 18–24° C. A platinum wire hook was passed through the top of the loop and connected to the recording apparatus (Fig. 1 *C*). Electrical stimulation was delivered through the platinum hook electrodes from an SRI stimulator (Model No. 6053).

Recording. Contraction or relaxation of the preparation was recorded isotonically using a frontal writing lever and a smoked drum, or a photo-electric transducer with its output fed to a Rikedenki 3-channel potentiometric pen recorder (model no. 2–341). In some experiments semi-isometric recordings were made by using a Grass FT 03 strain gauge fitted with a 120 mm long lever arm, and recording on a Grass model 7 polygraph. In most experiments the preparations were suspended in a bath containing 100 ml. sea water, but in a few instances, where amino acids were only obtainable in small amounts, a 3 ml. bath was used. Control experiments established that the responses of preparations to electrical stimulation or to amino acids in this small volume of sea water were not different from preparations suspended in 100 ml. sea water for a similar experimental period.

Three-chambered bath experiments. Experiments have also been made with ring preparations cut open to form a long strip which was then passed through a pair of rubber membranes spaced 10 mm apart, subdividing it into three sections. The arrangement is diagrammatically represented in Fig. 3.

The preparation was arranged so that a long length of the strip (C) protruded beyond one membrane. This long part was gently clamped at one end between Perspex jaws (C′) as it emerged from the membrane (A″) and the other end attached to the recording apparatus by the thread (D). The shorter length of preparation protruding from beyond the other membrane (A′) was laid over platinum hook electrodes, but no tension was applied to this section of the preparation. Platinum electrodes were also placed on the long strip (C) as it left the Perspex clamp. This arrangement allowed contractions of the long length of preparation (C) to be elicited by electrical stimulation of the strip (C) itself, or by stimulation of the short section (A), the contraction probably being transmitted to the longer section through the nerve net. As the shorter length of the preparation was under no tension contraction was not transmitted mechanically from the shorter to the longer section. Contractions have been recorded semi-isometrically or isotonically, the results being qualitatively and quantitatively the same in each case. During exposure of any one section of the strip to L-glutamic acid, the sea-water level was raised in each of the other chambers until it was at least 20 mm above that in the test chamber. This enabled any leakage between chambers to be detected during an experiment, and if present, the experiment was rejected. As a further precaution dye was added to each of the chambers in turn at the end of an experiment as a test for any leaks.

Loading and electrical stimulation. In all preparations the tension on the ring or strip of body wall was 300–500 mg. Ring preparations were usually set up in groups

of three with one preparation acting as an untreated control throughout the course of an experiment. The parameters of electrical stimulation were a square-wave pulse width of 1 ms with a voltage of 140 V (35 V p.d. measured across the preparation) at frequencies of between 0·5–20 Hz. Electrical stimulation was delivered automatically at intervals of 7·5–9·0 min for a period of 30 s. After setting up a preparation in the bath 2 hr was allowed for the contractile responses to electrical stimulation to become constant and the experimental procedures carried out in the next 6–8 h only. Preparations may respond to electrical stimulation for 3–4 days, but the nature and time course of the contractile response changes slowly over this time, particularly during days 2–3, so that experimental procedures were confined to the first 10 hr of electrical stimulation.

Effects of amino acids on isolated preparations. In preliminary experiments amino acids were placed in contact with preparations for periods up to 12 hr. It was found that active amino acids produced their effects in 0·5 hr or less, so that in all subsequent experiments a contact time of 0·5–1·5 hr was used. At the end of the contact period the preparation was washed twice with fresh sea water and then washed at 15 min intervals until recovery of the contractile responses; this normally becomes complete within 1 hr. Amino acids were added up to a bath concentration of 10^{-2} M. If an amino acid was ineffective in this concentration it was considered to be inactive.

In the absence of electrical stimulation preparations rarely show spontaneous contractions, and the rings or strips do not seem to be in a state of partial contraction. It is therefore difficult to demonstrate drug inhibition of muscle contractility in the resting preparation. However, if contractions are produced by regular electrical stimulation, these may be strongly inhibited by some amino acids.

Comparison of the potencies of amino acids. An approximate comparison of the potencies of amino acids in depressing the contractile response to electrical stimulation was made by taking a $1{\cdot}5 \times 10^{-3}$ M solution of L-glutamic acid as a standard. This concentration of glutamic acid produces a submaximal inhibition of the contractile responses to electrical stimulation, usually of the order of 60–70% inhibition. The concentration of the test amino acid was then varied until the magnitude of the depression produced was equivalent to that produced by the standard glutamic acid solution. Amino acids were applied at intervals of 1 hr, test solutions alternating with standard glutamic acid solution. No change in sensitivity to glutamic acid was noted on repeated applications, and previous exposure to glutamic acid did not affect the subsequent response to another amino acid. Each amino acid was tested on at least eight preparations. All amino acid solutions were made immediately before use. This was particularly important with α-amino pimelic acid where solutions that had been kept overnight were less potent in causing depression of the electrical response than when freshly made.

Washing. In all the *in vitro* experiments washing with fresh sea water, or replacement with sea water containing amino acids, was by displacement. Natural sea water and Instant Ocean (Aquarium Systems Inc. Ohio, USA) have both been used with success. No differences in the responses to electrical stimulation or amino acids have been noted in the artificial sea water (Instant Ocean). The pH of the aerated sea water was 7·8–8·0.

Biochemical methods; extraction and separation of amino acids

Preparations were dissected in the usual way, but the supra oral sphincters had their tentacles removed before being plunged into liquid air and homogenized in 0·4 N perchloric acid at 0° C using an Ultra Turrax homogenizer. The homogenate was then stored at 0° C for 0·5 hr during which time it was shaken twice for 1 min. The homogenate was then spun at 0° C for 1 hr at 38,000 *g* and the supernatent removed. The pellet was resuspended in 0·4 N perchloric acid (5 ml./g tissue) and

respun at 38·000 *g* at 0° C for a further 0·25 hr. The supernatent from the second spin was removed. The pooled supernatent was adjusted to pH 6·5 with a saturated solution of potassium hydroxide and the precipitate removed by centrifugation at 38·000 *g* at 0° C for 0·25 hr.

The amino acids were isolated from the supernatent by the method of Kurosky & Bars (1968), but using a column 120 mm × 7 mm of Zeo Carb 225 (8% DVB) and eluting the amino acids with 50 ml. N ammonium hydroxide. The final dried extract was used for preparation of derivatives suitable for gas-liquid chromatography (Islam & Darbre, 1969), Analytical Ion-exchange Chromatography on a Technicon Auto Analyser (Nunn & Vega, 1968) or thin layer chromatography (Jones & Heathcote, 1966).

Glutamine is converted to glutamic acid during the procedures necessary for gas–liquid chromatography so giving false high levels of glutamic acid. Glutamic acid was, therefore, separated from glutamine by a method based on that of Ersser, Gibbons & Seakins (1968), using Deacidite FF1P resin in the acetate form and eluting the glutamic acid with 50 ml. N hydrochloric acid. Thus glutamine and glutamic acid were separately estimated by gas–liquid chromatography.

Release of amino acids. In experiments on the release of amino acids into the sea water the supra oral sphincters had their tentacles removed before the ring was suspended in 100 ml. natural sea water, or Instant Ocean. The preparation was then stimulated at regular intervals for 2 hr as detailed above, then stimulation was stopped and the preparation washed twice with fresh sea water. The preparation was then left to rest for 30 min at the end of which time the bath fluid was removed for assay. Electrical stimulation, producing four contractions in 30 min, was then applied, and the bath fluid subsequently removed for assay.

On removal from the bath the sea water was made acid (pH 6·5) by the addition of a small quantity (about 50 µl.) of 0·1 N hydrochloric acid and the amino acids separated and estimated in the usual way.

Ammonia determinations. Preparations were frozen, homogenized in 0·4 N perchloric acid and centrifuged as described for estimation of amino acids. Ammonia was measured in the supernatant by the method of Kaplan (1969). Sea water blanks were treated in a similar way.

Amino acids. Most of the amino acids were obtained from commercial sources, and their homogeneity checked, where possible, by running samples in thin layer, ion exchange, or gas–liquid chromatographic systems. β-amino-glutaric acid was kindly supplied by Dr D. R. Curtis, Department of Physiology, Australian National University Canberra, Australia. Erythro-3-chloro-L-glutamic acid and threo-3-chloro-L-glutamic acid were kindly supplied by Dr J. Kollonitsch, Merck Sharp & Dohme Research Laboratories, New Jersey, U.S.A. If necessary, the pH of the sea water in these experiments was adjusted to pH 7·8–8·0 after addition of amino acid, unless otherwise stated.

RESULTS

Amino acids in the supra oral sphincter

Glutamic acid is found in the supra oral sphincter together with sixty-six to seventy other ninhydrin-positive materials of which thirty-six have been identified and estimated. It is readily apparent that the predominant amino acid is glutamic acid. Although insufficient experiments have been made, it seems likely that the highest concentration of glutamic acid in the animal may occur in the supra oral sphincter region. It seems that

γ-amino-butyric acid (GABA) is absent, or if it occurs it is in a concentration of less than 1 p-mol/g tissue. The data represented in Table 1 was taken from a group of animals collected in October and November 1970. As yet, no systematic study of any seasonal variations in the amino acid content has been made, although there is evidence that tissue levels of other substances in actinia, such as tetramethylammonium, may show seasonal variations (Carlyle, 1969*b*). The presence af 3·4 dihydroxyphenylalanine (DOPA) in *actinia* was first reported by Carlyle (1969*a*) using paper chromatography and fluorescence assay. The levels were only a two-hundredth of the level found in the present study. The reasons for this difference are not known.

TABLE 1. Concentrations of some free amino acids and related substances (in n-mole ± S.E. mean per g wet tissue) in the supra oral sphincter of *Actinia equina*. Values given are the mean of seven experiments

Cysteic acid	82 ± 16	Cystine	372 ± 54
Taurine	47 ± 9	Valine	87 ± 17
Aspartic acid	23 ± 7	Methionine	128 ± 24
Hydroxyproline	38 ± 11	Isoleucine	Trace
Threonine	470 ± 44	Leucine	100 ± 14
Serine	370 ± 62	Norleucine	347 ± 38
Asparagine	154 ± 28	Tyrosine	174 ± 32
Glutamic acid	1590 ± 370	Dopa	17 ± 4
Glutamine	14 ± 6	Phenylalanine	148 ± 16
Sarcosine	Trace	Ethanolamine	80 ± 7
Proline	40 ± 8	Tryptophan	872 ± 84
Glycine	380 ± 47	Ornithine	87 ± 23
Alanine	310 ± 38	Lysine	272 ± 103
Citrulline	16 ± 7	Histidine	107 ± 42
α-Amino-butyric acid	12 ± 4	3-Methyl histidine	Trace
α-Amino-iso-butyric acid	42 ± 8	Arginine	122 ± 34
α-Amino-iso-butyric acid	Trace	Homarginine	Trace
β-Alanine	727 ± 102	Cysteine	37 ± 9

The actions of amino acids other than glutamic acid on supra oral sphincter preparations. The amino acids detected in the sphincter together with γ-amino butyric acid were applied in concentrations of up to 10^{-2} M to electrically stimulated preparations. Most were inactive. DL-proline, DL-methionine and DL-arginine caused a slight increase in the response to electrical stimulation, but this has not been investigated further.

Effect of L-*glutamic acid.* L-glutamic acid (10^{-3}–5×10^{-3} M) reduced or abolished the response to electrical stimulation. The onset of the effect was rapid and was easily reversed on washing (Fig. 2*b*). In some preparations the response to electrical stimulation was reduced by L-glutamic acid, while the normally quiescent preparation became spontaneously active (Fig. 2*c* and *d*). This effect was more often seen if the sea water containing

glutamic acid was not adjusted to pH 7·8–8·0, so that the pH might be as low as 4·25 with 5×10^{-3} M-L-glutamic acid present. This low pH on its own had no effect on the ability of the preparation to respond to electrical stimulation and did not produce spontaneous contractions (Fig. 2*a*). The low pH merely seemed to enhance the ability of glutamic acid to produce spontaneous contractions. Fig. 2 demonstrates that this effect may, however, be marked in one preparation (2*d*) and absent in another (2*b*). Of eighteen preparations examined, seven showed a consistent increase in L-glutamic acid induced spontaneous activity by lowering the pH to 4·25. In eleven no difference was noted between the effect at pH 7·8–8·0 and pH 4·25. Switching off the electrical stimulation did not abolish the slow spontaneous contractions.

No experiments have been made on the effect of pH on the action of L-glutamic acid or active derivatives, and subsequent experiments reported in this paper were made at pH 7·8–8·0.

No systematic investigation has been made on the effect of changes in the ionic content of the sea water on the L-glutamic acid response.

Effect of L-glutamic acid on preparations from different parts of the anemone body. In eight experiments L-glutamic acid reversibly inhibited the response to electrical stimulation of the isolated oral sphincter, circular preparations of upper, mid and lower body wall, and of the basal disk. The action of L-glutamic acid on longitudinal preparations of body wall were not investigated.

The effect of removal of tentacles from the supra oral sphincter on the responses to electrical stimulation and glutamic acid. Experiments were made with supra oral sphincters before and after removal of the tentacles. No change in the response to electrical stimulation or the response to glutamic acid was noticed in any of the eight preparations tested.

Effects of glutamic acid applied to different regions of a supra oral sphincter strip preparation in a three-chambered bath. These experiments were made to test the hypothesis that L-glutamic acid applied to one part of a preparation might act on a chemoreceptor cell to initiate inhibiting processes which could spread through the nerve net to other parts of the preparation and so inhibit contraction. There was also the possibility that L-glutamic acid might interfere with conduction through the preparation, perhaps by a neurotransmitter blocking, or local anaesthetic action on the underlying nerve net.

The arrangement of the bath is diagrammatically represented in Fig. 3. Application of L-glutamic acid to the short section of the strip preparation *A* did not prevent initiation of a contraction in the distant section C (Fig. 3, trace *a*) so that L-glutamic acid presumably did not affect the electrical excitability of the conducting system. This observation also

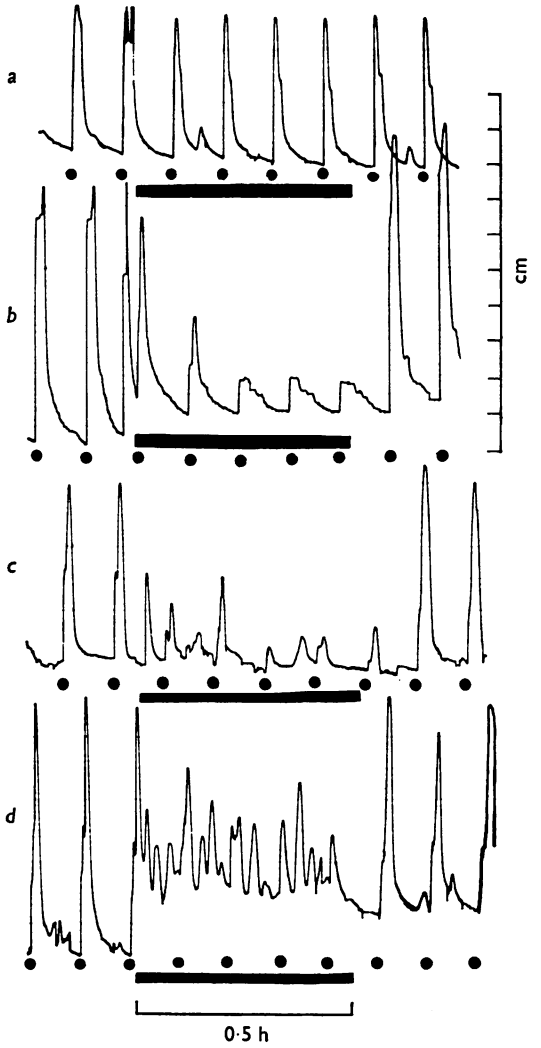

Fig. 2. Actions of L-glutamic acid on the contractile response of the supra oral sphincter to electrical stimulation.

Supra oral sphincter preparations suspended in 100 ml. of aerated sea water at 22° C, pH 7·8. Contractions were produced by electrical stimulation (140 V, 1 ms duration, at 2 Hz) for 30 s at intervals of 7·5 min (filled circles). Contractions recorded isotonically by means of a photo-electric transducer with a load of 500 mg applied to the tissue. Contraction is indicated by an upward movement of the trace.

Traces *a* and *b* are from two different preparations and traces *c* and *d* from the same preparation. In trace *a* the sea water was made acid (pH 4·25) by addition of 0·1 N-HCl. In trace *b* 5×10^{-3} M-L-glutamic acid was present, the pH being unadjusted at pH 4·25. In trace *c* 5×10^{-3} M-L-glutamic acid was present at pH 7·8 and in *d* 5×10^{-3} M-L-glutamic acid at pH 4·25. Experimental procedures were applied during the duration of the filled bars.

suggests that L-glutamic acid was unable to initiate any process in section A which could be transmitted to section C to cause inhibition, as might be expected if L-glutamic acid were exciting chemoreceptors which then passed inhibitory impulses through the nerve net to other sections of the preparation.

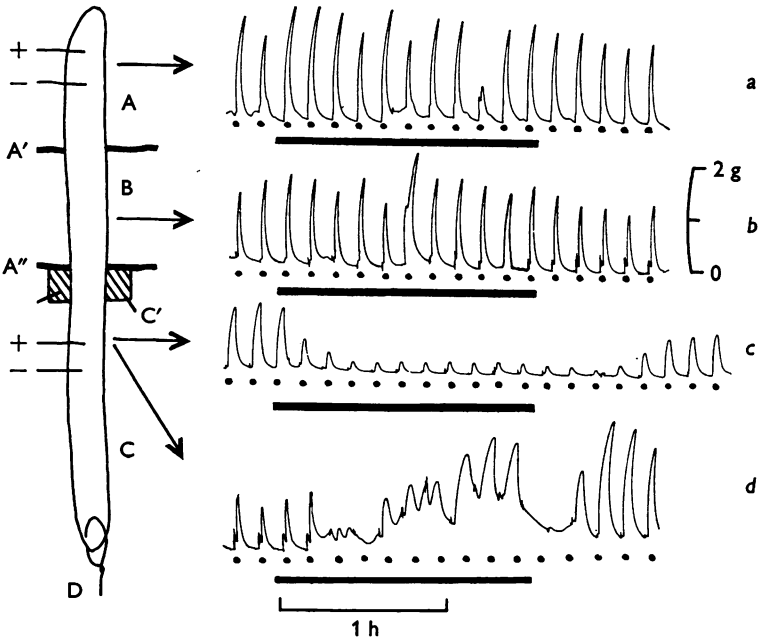

Fig. 3. The 3-chambered bath as used with a sphincter strip preparation and some effects of L-glutamic acid applied to different parts of the preparation.

On the left-hand side of the Figure is shown the arrangement of the membranes, clamps and electrodes used to produce the three-bath preparation. The preparation is passed through the membranes A′ and A″, sections A and B of the preparation being untensioned. The end of length C is sewn to a thread and connected to the recording apparatus under a load of 300 mg. Electrodes, 16 S.W.G., and 3 mm apart are gently placed on sections A and C of the strip and section C immobilized at the membrane end by the Perspex clamp C′. In the experiment above the tracings, *a*, *b*, *c* and *d* are records of the contractions of section C in response to stimulation of section A. Electrical stimulation, as detailed in Fig. 2, was applied at the filled circles. The preparation was suspended in aerated sea water (pH 8·0) at 18° C. Contractions were recorded semi-isometrically. Tracings *a*, *b* and *c* are from the same preparation and show the effect on the contractions of section C of applying 5×10^{-3} M-L-glutamic acid (filled bars) to section A (trace *a*) then section B (trace *b*) and lastly to section C (trace *c*) of the muscle strip.

Trace *d* shows the effect of L-glutamic acid applied to section C of a second preparation and shows the marked slow spontaneous contractions which may occur, the conditions of pH, load on the tissue and temperature being identical to those in traces *a*, *b* and *c*.

Placing L-glutamic acid in the middle section B of the strip did not prevent the impulse initiated in section A passing through B to cause contraction of section C (Fig. 3, trace *b*). Thus L-glutamic acid does not seem to possess a local anaesthetic action on the conducting system, nor does it seem capable of inhibiting neurotransmission in the underlying nerve net. Application of L-glutamic acid to section C promptly abolished the contractile response conducted from section A (Fig. 3, trace *c*.). Fig. 3 (trace *d*) shows that the inhibition of the contractile response to electrical stimulation may be accompanied by production of large slow spontaneous contractions. In other experiments stimulation of section C during the inhibitory effect of L-glutamic acid was, as might be expected, also unable to initiate contraction of section C.

In Fig. 3 it will also be noted that the contractions produced by electrical stimulation may show occasional irregularities; this is seen particularly in tracing *a* and *d* where several contractions fail to reach the normal height. This was attributed to the pressure of the membranes on the preparation, causing some impairment of conduction through the central part of the bath. Such behaviour was never seen in preparations freely suspended in sea water. It will also be noted that in trace *d* after washing out the glutamic acid the responses to electrical stimulation were markedly increased, and this increase was sustained for some hours. This effect was sometimes seen in ring preparations, but was much more common in strip preparations in the 3-chambered bath.

The effect of glutamic acid on ammonia levels in sphincter preparations. Ammonia (5×10^{-6}–10^{-5} M) caused a depression or abolition of the contractile response to electrical stimulation. In the higher concentrations it was difficult to reverse the effect on washing. This observation suggested that perhaps deamination of some of the glutamic acid applied to the tissue might yield sufficient ammonia to cause inhibition of the electrical response. The ammonia level in the supra oral sphincter was found to be high, $0{\cdot}53 \pm 0{\cdot}06$ (S.E. mean) μmole/g ($n = 12$). On exposing the preparation to 5×10^{-3} M-L-glutamic acid for 1 hr the ammonia level fell slightly to $0{\cdot}41 \pm 0{\cdot}12$ (S.E. mean) μmole/g ($n = 8$). It seemed unlikely, therefore, that glutamic acid was exerting its effect through production or release of ammonia.

The effect of L-*glutamic acid on γ-amino butyric acid (GABA) levels in the sphincter.* As mentioned earlier, GABA has not been detected in the sphincter. However, it was possible that the high levels of glutamic acid applied to the tissue might in some way yield or release GABA which might then cause inhibition. To test this hypothesis preparations were exposed to 5×10^{-3} M-glutamic acid for 1 hr then frozen and extracted in the usual way for amino acids. Using gas–liquid chromatography the extracts were carefully examined for the presence of GABA. In eight experiments no GABA was detected (limit of assay 0·1 p-mole wet wt. of tissue).

In these preparations there was also no apparent change in the tissue amino acid pattern on ion exchange or gas-liquid chromatographs. This suggests that the glutamic acid was not causing the synthesis, or release of an unidentified amino acid. It does, not of course, rule out the possibility that the glutamic acid was converted into, or caused the release of some compound not detectable in the chromatographic systems used in these experiments.

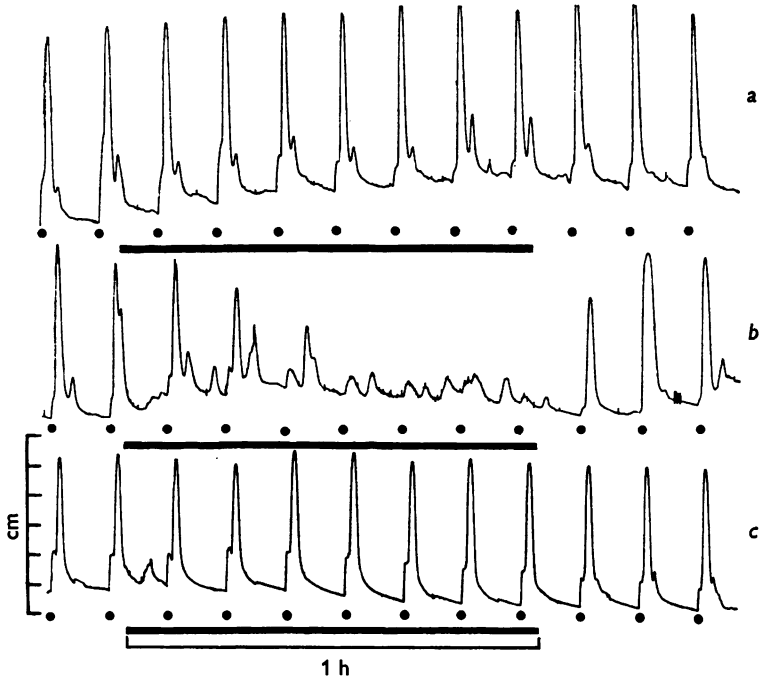

Fig. 4. Effects of glutaric acid, L-glutamic acid (α-amino glutaric acid) and β-amino glutaric acid on the contractile responses of sphincter preparations to electrical stimulation.

Supra oral sphincter preparation suspended in 3 ml. aerated sea water (pH 8·0) at 21° C. Contractions were produced by electrical stimulation (parameters as in Fig. 2) for 30 s every 9 min (filled circles). Contractions were recorded isotonically by a photo-electric transducer. Drugs were added during the period of the filled bars. Trace *a* shows the effect of glutaric acid (10^{-2} M), trace *b* L-glutamic acid (5×10^{-3} M) and trace *c* β-amino glutaric acid (10^{-2} M). The pH in each experiment was 8·0.

The effect of substances related to glutamic acid on the response to electrical stimulation. Thirty-four compounds with varying degrees of structural similarity to L-glutamic acid were tested on electrically stimulated preparations (see also Carlyle, 1971*a*). Apart from glutamic acid none of the compounds produced spontaneous contractions and no amino acid, in-

cluding glutamic acid, increased the contractile response. Those seven amino acids, including glutamic acid, which were active caused reversible depression of the contractile response to electrical stimulation.

As the distance between the amino and carboxyl group of glutamic acid is increased activity disappears with α-amino adipic acid, reappears with α-amino pimelic acid (activity equal to that of glutamic acid) and is absent in α-amino suberic and amino α-sebacic acid. Aspartic acid is also inactive.

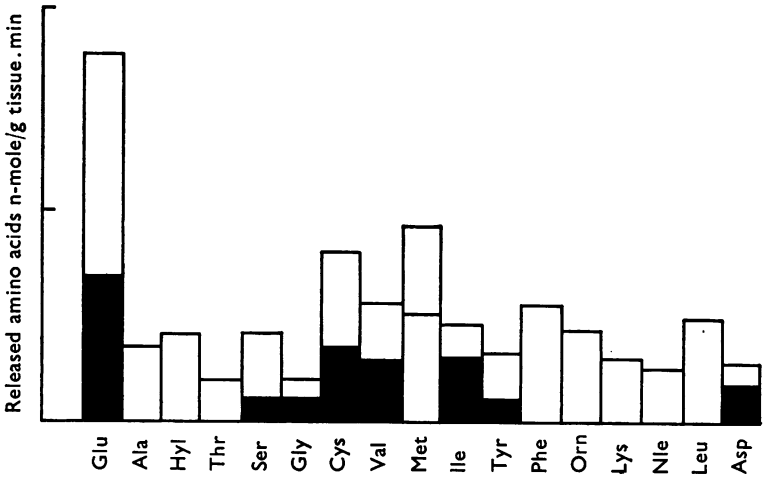

Fig. 5. Release of some amino acids from a supra oral sphincter into sea water at rest and after electrical stimulation.

Stimulation was at 7·5 min intervals for a period of 0·5 hr. Parameters of stimulation were as in Fig. 2. The resting release was collected after a preceding period of 0·5 hr in the absence of stimulation. The filled blocks represent release of amino acid at rest, and the open blocks the release after electrical stimulation. Released amino acid is expressed as n-mole/g wet wt. tissue-min.

The amino acids are: glutamic acid (Glu), alanine (Ala), hydroxyproline (Hyl), threonine (Thr), serine (ser), glycine (Gly), cysteine (Cys), valine (Val), methionine (Met), isoleucine (Ile), tyrosine (Tyr), phenylalanine (Phe), ornithine (Orn), lysine (Lys), norleucine (Nle), leucine (Leu) and aspartic acid (Asp).

Removal of the amino group to form glutaric acid or movement of the amino group to form β-amino glutaric acid completely abolished activity (Fig. 4). The γ-hydroxy derivative of glutamic acid, has only one tenth of the activity of glutamic acid, whereas the presence of a double band in the γ position does not reduce activity. The activity of glutamic acid is approximately halved by the substitution of a chlorine in the β position.

The formation of a ω-amide link in glutamine reduces the activity to one twentieth of glutamic acid.

Replacement of the ω-carboxyl group by a sulphonic group to form homocysteic acid results in an increase of fifteenfold in the depressant activity.

It is perhaps surprising that there is no difference in activity between the D or L forms of glutamic acid.

Release of amino acids from the supra oral sphincter. In the absence of electrical stimulation, the supra oral sphincter releases into the sea water glutamic acid and other substances amongst which are various amino acids. After stimulating the preparation electrically 4 times in 30 min, the amount of glutamic acid and many other amino acids released was inincreased. This pattern of release was seen in seventeen experiments, a representative one of which is seen in Fig. 5. Some amino acids only appeared in the bath fluid after electrical stimulation, though this may only mean that the initial level in the resting release was below the level of the assay used in that experiment. Apart from amino acids, other unidentified peaks were observed on gas liquid and ion exchange chromatography of samples obtained at rest and during electrical stimulation. Many of these unidentified substances also increased in amount on electrical stimulation, and their significance is not clear. This problem is currently under investigation.

DISCUSSION

Glutamic acid inhibits the contractile response of the supra oral sphincter to electrical stimulation. This response is not through deamination to GABA, ammonia, or conversion to some other active amino acid.

Circular preparations from *Actinia equina* can produce fast or slow contractile responses to different frequencies of electrical stimulation (Carlyle, 1969*b*). In the experiments reported here the effects of amino acids have only been tested on fast contractile responses to electrical stimulation. No systematic study of the effect of amino acids on slow responses has been made. It has been noted, though, that after inhibiting fast responses with L-glutamic acid slow responses may occur spontaneously.

Ross (1966) suggested two mechanisms for inhibition of coelenterate muscle by glutamic acid and related substances. First, an action on chemosensitive cells on the body surface mediating their effect through the nerve net, or secondly, a direct inhibitory action on the muscle cell.

A primary action on a chemosensitive cell is unlikely as removal of the tentacles, a rich source of such cells, does not affect the action of glutamic acid. Also glutamic acid applied to one section of the preparation cannot cause inhibition in a distant part. Glutamic acid does not appear to interfere with transmission in the nerve net or to have a local anaesthetic action. We must postulate, therefore, an action directly on the contractile

cell, an inhibitory effect on neuromuscular transmission, or an excitatory action on some inhibitory neurone in close proximity to the muscle cell.

As the site of the glutamate receptor is unknown we do not know whether comparison can be made with the glutamate receptors of the vertebrate brain or spinal cord, the crustacean neuromuscular junction, or of the molluscan neurone (Hebb, 1970; Curtis *et al.* 1961; Takeuchi & Takeuchi, 1964; and Gerschenfeld & Lasansky, 1964). Despite this limitation, it is interesting to compare the structure action relationships of glutamic acid analogues in this preparation with those described by Curtis, Phillis & Watkins (1961) for the excitatory action of glutamic acid analogues on toad spinal cord neurones. Of twenty-one amino acids tested in both the toad cord and the anemone, seventeen showed activity on the toad cord, whereas only six were active on the anemone preparation. This suggests that the anemone glutamate receptor may be more specific than the vertebrate receptor. Curtis *et al.* (1961) suggested that the requirements for activity in the glutamic acid molecule were an amino group and two carboxyl groups with an optimal separation of two or three carbon atoms. Within these restrictions they showed that a remarkable number of substitutions in the carbon chain, N-alkylation, changes in the position of the amino group and other changes could be tolerated with little loss in activity, or even with increased activity. This situation does not seem true of the anemone receptor. Movement of the amino group to the β position halves the activity on the toad spinal cord but abolishes activity in the anemone. Similarly N-methylation of glutamic acid enhances activity in the toad cord but abolishes it in the anemone. Even N, N-dimethyl-glutamic acid has considerable activity in the toad cord, but none in the anemone.

In the toad cord D-glutamic acid is more active than L-glutamic acid. No such difference is seen in the anemone. This could be used to argue against the specificity of the anemone glutamate receptor. However differences in enzymic destruction and uptake for D or L glutamic acid may mask the true potency ratio (Steiner & Ruff 1966; Tsukada, Nagata Hirano & Matsutani 1963).

There are, however, similarities between the actions of certain glutamic acid analogues on the toad spinal cord and the anemone preparation. Increasing the carbon chain length to yield α-aminoadipic acid reduces activity in the toad and abolishes it in the anemone. Activity reappears in the anemone with α-amino pimelic acid and also increases again in the toad before disappearing again in both species with α-amino sebacic acid. Conversion of the ω-carboxyl group to give homocysteic acid greatly increases activity, both in the case of the toad where activity is increased twenty-five fold and in the anemone where there is a fifteenfold increase.

Cysteic acid is almost as potent as glutamic acid on the toad, but inactive in the anemone, but this is not surprising as tartaric acid, the analogue of cysteic acid, is also inactive in the anemone but active on the toad cord.

I. K. M. Morton (personal communication) has made an interesting comment on the possibly higher structural specificity of the glutamate receptor in *Actinia equina* as compared to that in the toad spinal cord. He has suggested that in lower animals the nerve endings may release a large number of substances, including amino acids, as a result of metabolic activity. In order to make use of one of these substances, say an amino acid, as a transmitter, a highly specific receptor substance may have evolved. In higher animals neural evolution may have led to fewer molecules being released by a nerve ending, so that the structural specificity of the receptor could be lower without detriment to the transmission process. This idea could be tested by comparing the number of substances released from a nerve ending and receptor specificity at that synapse in different phyla.

Is glutamic acid a transmitter in *Actinia equina*? Glutamic acid occurs in high concentration in the supra oral sphincter, although possibly much of this may be contained in structures other than nerves (Lane & Dodge, 1958). Glutamic acid is released from preparations at rest, and the amount is increased on electrical stimulation. However, many amino acids and other substances also appear in the bath fluid, making interpretation difficult (Carlyle 1971*b*). Nematocysts and mucus secreting cells may be potential sources of release. Study of changes in tissue levels of amino acids during electrical stimulation might be more useful than measurement of bath amino acid content in analysing this problem. Some preliminary experiments in the supra oral sphincter have been reported by Carlyle (1972). These experiments suggest that rapid changes of glutamate concentration take place within the preparation following electrical stimulation, but as in the release experiments, changes also take place in other amino acids. It seems that it is too early to ascribe a definite physiological role to the glutamate receptor in *Actinia equina*.

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