J. Exp. Biol. (1974), 61, 421–442
With 2 figures
Printed in Great Britain

PHARMACOLOGICAL STUDIES ON A LOCUST NEUROMUSCULAR PREPARATION

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(*Received* 13 *March* 1974)

SUMMARY

1. The structure–activity relationships of agonists of the locust excitatory neuromuscular synapse have been reinvestigated, paying particular attention to the purity of compounds, and to the characteristics and repeatability of the muscle response. The concentrations of compounds required to stimulate contractions of the retractor unguis muscle equal in force to the neurally evoked contractions provided a measure of the relative potencies.

2. Seven amino acids were capable of stimulating twitch contractions, glutamic acid being the most active, the others being analogues or derivatives of glutamic or aspartic acid. Aspartic acid itself had no excitatory activity.

3. Excitatory activity requires possession of two acidic groups, separated by two or three carbon atoms, and an amino group α to a carboxyl. An L-configuration appears essential. The ω-acidic group may be a carboxyl, sulphinyl or sulphonyl group. Substitution of any of the functional groups generally causes total loss of excitatory activity, but an exception is found in kainic acid in which the nitrogen atom forms part of a ring.

4. The investigation of a wide variety of compounds revealed neuromuscular blocking activity among isoxazoles, hydroxylamines, indolealkylamines, β-carbolines, phenazines and phenothiazines. No specific antagonist of the locust glutamate receptor was found, but synaptic blocking agents of moderately high activity are reported.

INTRODUCTION

The study of arthropod neuromuscular physiology has been impeded by the lack of an antagonist which can be used to block excitatory synaptic transmission by a specific postsynaptic effect. This paper reports the results of a search for compounds that block the postsynaptic receptors of locust excitatory neuromuscular synapses, and for compounds that affect neuromuscular transmission in other ways. L-Glutamic acid is an effective agonist at locust excitatory neuromuscular synapses, and an attempt was made to define the chemical structure necessary for interaction with the glutamate receptors. Subsequently, the blocking activities of a wide range of compounds were measured, and in all more than 250 compounds were tested. In most cases the actions of the compounds on the mechanical responses of a locust nerve–muscle preparation provided the method of assay.

MATERIALS AND METHODS

Nerve–muscle preparation

All experiments and assays were carried out with males of the desert locust, *Schistocerca gregaria* Forskål, which had fledged 2–4 weeks earlier. A culture was maintained as described by Hunter-Jones (1966), and the locusts were given fresh green food daily, supplemented with stabilized wheat germ.

The retractor unguis muscle from the hind femur was used in all experiments, for this small muscle, when dissected out and irrigated with saline, is readily accessible to drugs. It consists of two bundles of muscle fibres which are innervated by two small branches of nerve $5b_2$, a division of the crural nerve. Each branch contains two excitatory axons, but no inhibitory axons (Hoyle, 1955; Rees & Usherwood, 1972). The preparation was essentially that described by Usherwood & Machili (1968), which was called an 'isolated preparation' by Clements & May (1974) to distinguish it from their alternative 'perfused-femur preparation'. A hind leg was removed, secured with wax in a narrow channel in a block of perspex, and dissected to leave the retractor unguis muscle, still attached at its origin to cuticle, and its nerve. The apodeme of the retractor unguis muscle was tied with a fine terylene fibre to the insulated arm of a force transducer (Grass Instrument Co., FT.03C) and the muscle adjusted to its natural rest length. Saline was pumped past the preparation by a peristaltic pump (Watson-Marlow Ltd., MHRE.72) at a rate which was varied between 0·4 and 1·4 ml per min. Preparations were stimulated electrically every 10 sec, either by a suction electrode connected to the crural nerve or by pin electrodes placed in the saline, and the deflexions of the force transducer caused by muscle contractions were recorded by pen recorder.

The 'standard saline' described by Clements & May (1974) was used in all experiments. It contained NaCl 140, KCl 10, NaH_2PO_4 4, Na_2HPO_4 6, $CaCl_2$ 2 and sucrose 90 mmol/l. This saline was approximately iso-osmotic with the haemolymph of adult male locusts. The pH of all test solutions was measured before use, and when necessary was adjusted to 6·8 with NaOH or HCl. The saline used for producing potassium contractures contained K_2SO_4 24, Na_2SO_4 55, NaCl 28, NaH_2PO_4 4, Na_2HPO_4 6, $CaCl_2$ 2 and sucrose 90 mmol/l. To obtain submaximal potassium contractures the high potassium saline was mixed with standard saline to give potassium concentrations of 25 m-equiv./l or less.

Pharmacological assays

The agonistic activity of compounds was assessed by measuring their ability to stimulate muscle contractions in the absence of neural stimulation. Potential agonists were applied in 2 ml solution through a small funnel held over an isolated preparation. The funnel was arranged so that the solution fell directly on to the muscle but without causing any mechanical disturbance visible on the force transducer record. The stimulator was stopped immediately before each test, the recorder chart being run at 5 mm/sec during the test so that both the form and the force of the muscle contraction were recorded. Dose/response curves were prepared for all effective agonists. It was found that the maximum force developed in response to stimulation with L-glutamic acid was approximately twice that developed in the neurally evoked contractions of the same preparation: it was thus convenient to record as the E_{50} the

molar concentration of an agonist that was required to produce a muscle twitch equal in amplitude to the neurally evoked contractions.

The neuromuscular blocking activity of compounds was assessed by measuring the depression of the force of the neurally evoked contractions after 20 min exposure at each of a range of concentrations. Dose/response curves were prepared for all compounds showing depressant activity. It was found convenient to record as the I_{50} the molar concentration of a compound causing 50% reduction in the force of neurally evoked contractions after 20 min exposure. Records were made of rates of recovery on washing. The prolonged exposure of nerve–muscle preparations to agonists such as the excitatory amino acids also caused depression of neurally evoked contractions, presumably through desensitization of the postsynaptic receptors, and both E_{50} and I_{50} values were obtained for such compounds.

Sources and purity of compounds

Most compounds were obtained from commercial sources. L-Glutamic acid was used as the sodium salt (B.D.H.). DL-2-Amino-4-sulphinobutyric acid was synthesized by the method of Watkins (1962). Indolealkylamines and alkaloids were normally used as the hydrochlorides. 5-Hydroxytryptamine was used as the creatinine sulphate complex. The fluorinated compounds were obtained from Koch-Light Laboratories Ltd. The isoxazoles were synthesized by Dr J. H. Davies, Dr P. Hackett and Mr R. H. Davies of this Laboratory. *N*-phenoxycarbonyltryptamine was synthesized by Dr P. Hackett, and *β*-*N*-oxalyl-DL-*α*,*β*-diaminopropionic acid by Mr M. Pearson of this Laboratory. 1-Hydroxy-3-amino-2-pyrrolidone was given by Dr G. A R. Johnston. Avenaciolide was given by the Pharmaceutical Division of ICI.

All amino acids that affected neuromuscular transmission were analysed for contamination with L-glutamic acid. In most cases the concentration of L-glutamic acid was measured enzymically with L-glutamic dehydrogenase, using the procedure described by Bergmeyer (1965), the formation of DPNH being measured with a Zeiss PMQ II spectrophotometer. These assays were supplemented by thin-layer chromatography or electrophoresis, and by automatic amino acid analysis using a Technicon NC-1 Analyser with Type A resin and gradient elution. Commercial samples of L-amino acids were often contaminated with glutamic acid, and the esters and amides of glutamic acid were also frequently found to be contaminated with the parent compound, sometimes heavily. Whenever possible, contaminated samples were discarded and pure samples from other sources were used, but when samples of sufficient purity could not be obtained allowance was made for the activity of the L-glutamic acid known to be present before assessing the pharmacological activity of the compound.

RESULTS

The actions of amino acids

L-Glutamic acid is a potent agonist which stimulates muscle contraction in insects, acting at the excitatory neuromuscular synapses (Usherwood & Machili, 1968; Beranek & Miller, 1968). The invariable response of an isolated retractor unguis preparation to a 2 ml pulse of L-glutamate of sufficient concentration was a muscle twitch, a linear relationship being found between twitch force and log molar con-

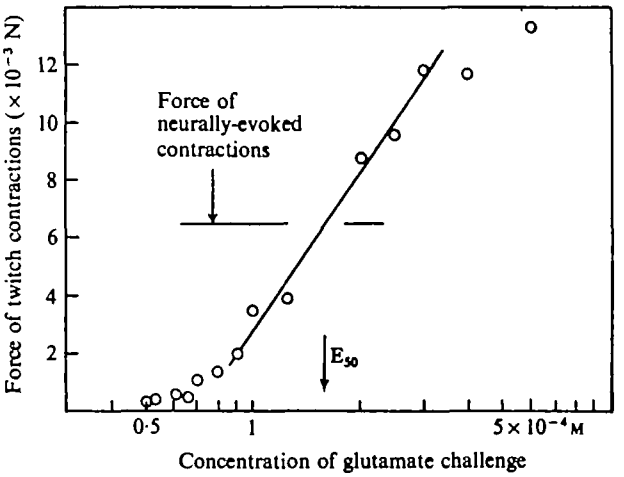


Fig. 1. The force of muscle contractions given by a retractor unguis preparation when challenged with 2 ml pulses of glutamate solution.

centration of glutamate (Fig. 1). Some isolated preparations also gave a brief, slightly delayed, contracture following the twitch when challenged with glutamate and other excitatory amino acids, but the mechanism of the contracture is uncertain (Clements & May, 1974). Another action of L-glutamate on isolated retractor unguis preparations was to depress the force of the neurally evoked contractions (Usherwood & Machili, 1968), a linear relationship again being found between the extent of the depression and the log molar concentration of glutamate (Fig. 2).

In an early study, Usherwood & Machili (1968) found that several naturally occurring amino acids would stimulate the locust retractor unguis muscle to contract. Later, McDonald & O'Brien (1972) reported that the 3-sulphino analogue of aspartic acid is an agonist for the retractor unguis muscle of the locust *Romalea microptera* and also assessed the pharmacological activities of a number of amino acids and peptides by irrigating retractor unguis preparations with 10 mM solutions and measuring the depression of the force of the neurally evoked contractions. They considered that the percentage depression provided a satisfactory measure of the relative potencies of these compounds at the excitatory neuromuscular synapse because McDonald (1971) had found that the threshold concentrations for excitation and depression by L-glutamic acid were the same. However, it was found in the present study of a number of amino acids that there is not always a constant ratio between the concentrations that excite and depress. It was also found that commercial samples of certain L-amino acids and of derivatives of glutamic acid are often contaminated with glutamic acid. It was considered, therefore, that if the structure–activity relationships of the excitatory amino acids were to be established with accuracy the following refinements of technique were necessary.

(1) The force of the twitch contraction should be the parameter measured whenever possible, and depression of the force of neurally evoked contractions should be used only to provide supporting data.

(2) The form of the muscle contraction should be recorded in such a way that the twitch component could be distinguished from the contracture.

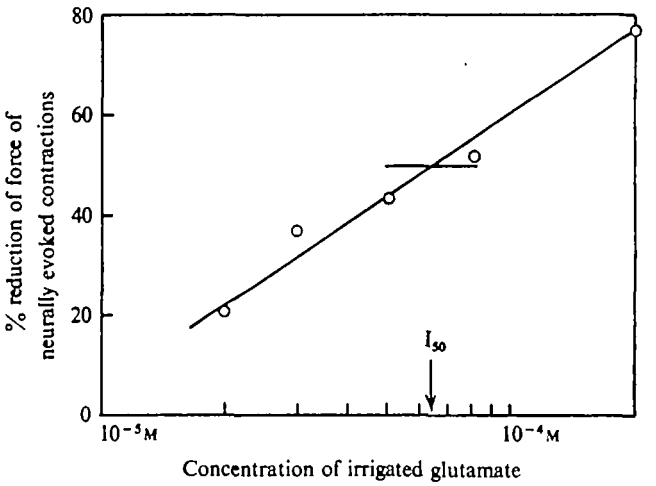


Fig. 2. Depression in force of the neurally evoked contractions of a retractor unguis preparation caused by L-glutamic acid, and measured after 20 min exposure at each concentration.

(3) Active compounds should be assayed on a number of preparations and at sufficient concentrations to provide dose/response curves.

(4) Active compounds should be analysed for contamination with L-glutamic acid.

About 40 analogues or derivatives of glutamic acid or aspartic acid were tested for pharmacological activity on the retractor unguis preparation. All amino acids that showed some activity are listed in Table 1, where the mean concentrations required to cause a muscle contraction equal in force to the neurally evoked contractions (E_{50}), or to depress the force of the neurally evoked contractions by 50% (I_{50}), are also given. For L-glutamic acid (I) the E_{50} was $2{\cdot}4 \times 10^{-4}$ M (S.D. = $1{\cdot}7 \times 10^{-4}$ M; $n = 36$) and the I_{50} $6{\cdot}4 \times 10^{-5}$ M (S.D. = $1{\cdot}8 \times 10^{-5}$ M; $n = 12$). The E_{50} and I_{50} values recorded for the other amino acids listed in Table 1 were each derived from the dose/response curves of two to four nerve–muscle preparations, a high consistency being found for the response to each compound. The relative activities of the amino acids are expressed as the 'equipotent molar ratios' (Barlow, 1964); that is, as the number of molecules of the amino acid producing the same effect as one molecule of L-glutamic acid (I). Those amino acids and closely related compounds that caused less than 25% depression in the force of the neurally evoked contractions after 20 min exposure at 10 mmol/l were classed as inactive, representative examples of such inactive compounds being listed in Table 2. From Tables 1 and 2 it will be seen that a number of amino acids which were previously considered to be agonists at the locust excitatory neuromuscular junction proved inactive or, at most, to show blocking activity. An interesting example is L-aspartic acid (VI) which, when free of contamination with L-glutamic acid, possessed weak blocking activity but showed no agonistic activity, even at very high concentrations. However, a number of analogues or derivatives of aspartic acid possessed excitatory activity, in some cases to a greater extent than the equivalent glutamate analogues. All compounds that were excitatory also depressed the force of neurally evoked contractions on prolonged exposure.

Curtis *et al.* (1972) found that L-glutamic acid diethyl ester antagonized the

Table 1. *The excitatory and blocking activities, on locust nerve–muscle preparations, of certain amino acids structurally related to glutamic acid and aspartic acid*

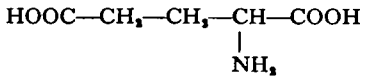
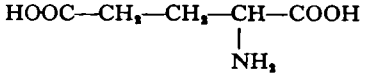
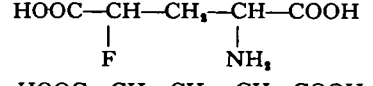
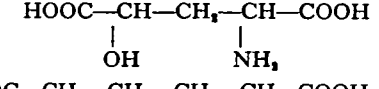
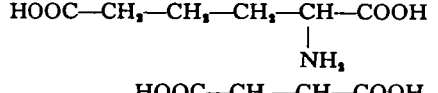
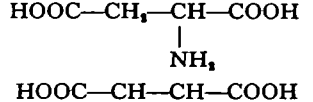
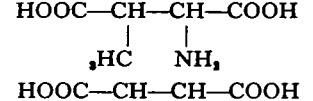
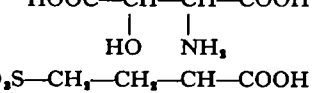
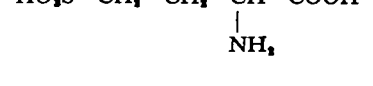
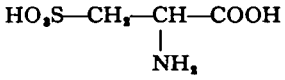
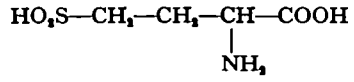
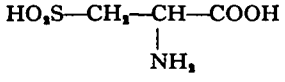
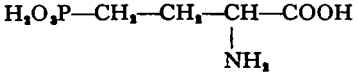
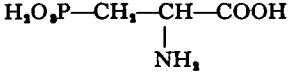
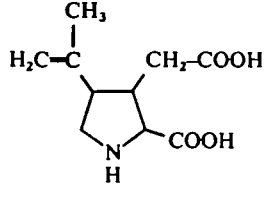
		Excitation		Blockade		$\frac{\text{E.M.R.}_{E_{50}}}{\text{E.M.R.}_{I_{50}}}$
		E_{50} (M)	E.M.R.†	I_{50} (M)	E.M.R.†	
I. L-Glutamic acid		$2{\cdot}4\times10^{-4}$	1	$6{\cdot}4\times10^{-5}$	1	1
II. D-Glutamic acid		***	>2080	$5{\cdot}5\times10^{-3}$	86	—
III. DL-4-Fluoroglutamic acid		$8{\cdot}9\times10^{-4}$	3·7	$2{\cdot}3\times10^{-4}$	3·6	1
IV. L-*allo*-4-Hydroxyglutamic acid		$3{\cdot}8\times10^{-3}$	16	$2{\cdot}6\times10^{-4}$	4·1	4
V. L-2-Aminoadipic acid		‡	—	$7{\cdot}7\times10^{-3}$	120	—
VI. L-Aspartic acid		****	>4160	$1{\cdot}5\times10^{-3}$	23	—
VII. DL-3-Methylaspartic acid		***	>2080	$3{\cdot}4\times10^{-3}$	53	—
VIII. DL-*threo*-3-Hydroxyaspartic acid		$3{\cdot}7\times10^{-3}$	154	$1{\cdot}7\times10^{-3}$	26	6
IX. DL-Homocysteic acid		***	>2080	$4{\cdot}9\times10^{-3}$	76	—

Table 1 (*cont.*)

		Excitation		Blockade		$\frac{\text{E.M.R.}_{B_{50}}}{\text{E.M.R.}_{I_{50}}}$
		E_{50} (M)	E.M.R.†	I_{50} (M)	E.M.R.†	
X. L-Cysteic acid		$2{\cdot}8 \times 10^{-3}$	12	$3{\cdot}5 \times 10^{-4}$	5·5	2
XI. DL-2-Amino-4-sulphinobutyric acid		**	>830	$1{\cdot}7 \times 10^{-3}$	26	—
XII. L-2-Amino-3-sulphinopropionic acid		$8{\cdot}2 \times 10^{-4}$	3·4	$2{\cdot}3 \times 10^{-4}$	3·6	1
XIII. DL-2-Amino-4-phosphonobutyric acid		***	>2080	$3{\cdot}0 \times 10^{-3}$	47	—
XIV. DL-2-Amino-3-phosphonopropionic acid		**	>830	$1{\cdot}2 \times 10^{-2}$	187	—
XV. L-*xylo*-Kainic acid		$3{\cdot}1 \times 10^{-1}$	1290	$1{\cdot}3 \times 10^{-3}$	20	65

Asterisks. No muscle contraction was obtained at the highest concentration tested. **** = M; *** = 5×10^{-1} M; ** = 2×10^{-1} M.

† E.M.R. = Equipotent molar ratio relative to L-glutamic acid.

‡ Activity could be ascribed to contamination with L-glutamic acid.

Table 2. *Compounds related to glutamic acid and aspartic acid which had a negligible effect on the locust nerve-muscle preparation, i.e. which caused less than 25% reduction in the force of the neurally evoked contractions after 20 min exposure at 10 mmol/l*

XVI	$CH_3—OOC—CH_2—CH_2—CH(NH_2)—COOH$	L
XVII	$C_2H_5—OOC—CH_2—CH_2—CH(NH_2)—COOH$	L
XVIII	$CH_3—SO_2—CH_2—CH_2—CH(NH_2)—COOH$	L
XIX	$NH_2—OC—CH_2—CH_2—CH(NH_2)—COOH$	L
XX	$HO—CH_2—CH_2—CH(NH_2)—COOH$	L
XXI	$NH_2—CH_2—CH_2—CH(NH_2)—COOH$	L
XXII	$CH_2{=}CH—CH_2—CH(NH_2)—COOH$	DL
XXIII	$CH_3—CH_2—CH_2—CH(NH_2)—COOH$	L
XXIV	$CH_3—CH_2—CH(NH_2)—COOH$	L
XXV	$HOOC—CH_2—CH_2—C(CH_3)(NH_2)—COOH$	DL
XXVI	$HOOC—CH_2—C(CH_3)(NH_2)—COOH$	DL
XXVII	$HOOC—CH_2—CH_2—CH(NH_2)—CONH_2$	L
XXVIII	$HOOC—CH(F)—CH_2—CH(NH_2)—CONH_2$	DL
XXIX	$C_2H_5—OOC—CH_2—CH_2—CH(NH_2)—COO—C_2H_5$	L
XXX	$HOOC—CH_2—CH_2—CH(NH—CH_3)—COOH$	DL
XXXI	$HOOC—CH_2—CH_2—CH(NH—CO—NH_2)—COOH$	L
XXXII	$HOOC—CH_2—CH_2—CH(NH—Ph)—COOH$	L
XXXIII	$HOOC—CH_2—CH(HO)—CH_2(NH_2)$	
XXXIV	$HOOC—CH_2—CH_2—CH_2(NH_2)$	
XXXV	$HOOC—CH_2—CH_2—CH_2(NH—CH_3)$	
XXXVI	$HO_3S—CH_2—CH_2—CH_2(NH_2)$	
XXXVII	$HOOC—CH_2—CH_2—CH_2—COOH$	

excitation of various spinal neurones by L-glutamic acid and DL-homocysteic acid. Irrigation of the locust nerve–muscle preparation with $2{\cdot}5 \times 10^{-3}$ M L-glutamic acid diethyl ester (XXIX) had no effect on the force of muscle contractions stimulated by 2 ml pulses of 2×10^{-4} M L-glutamic acid.

The following conclusions can be drawn about the structure–activity relationships of the open-chain analogues and derivatives of glutamic acid and aspartic acid (see Tables 1 and 2).

(1) Excitatory activity is found in the sulphonic (X) and sulphinic (XII) analogues of aspartic acid, although it is not found in L-aspartic acid itself (VI) nor in the sulphonic (IX) and sulphinic (XI) analogues of glutamic acid. The phosphonic analogues (XIII, XIV) of glutamic and aspartic acids do not show excitatory activity, but at high concentrations show blocking activity.

(2) The steric configuration at carbon-2 is very important, as D-glutamic acid (II) has no excitatory activity and only weak blocking activity.

(3) Shortening the carbon chain of L-glutamic acid by one atom, as in L-aspartic acid (VI), or lengthening it by one atom, as in L-2-aminoadipic acid (V), causes loss of excitatory activity and greatly reduces blocking activity.

(4) All activity is lost when the 5-carboxyl group of L-glutamic acid is converted to a methyl ester (XVI), ethyl ester (XVII) or amide (XIX). Replacement of the 5-carboxyl group with a methyl group, as in L-norvaline (XXIII), or loss of this group, as in 2-amino-*n*-butyric acid (XXIV), causes loss of all activity.

(5) Conversion of the 1-carboxyl group of L-glutamic acid to an amide, as in L-isoglutamine (XXVII), or its loss, as in 4-amino-*n*-butyric acid (XXXIV), causes loss of all activity.

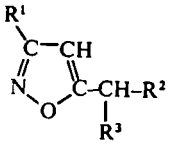
(6) Replacement of one hydrogen atom of the amino group in glutamic acid by methyl (XXX), carbamoyl (XXXI), or phenyl (XXXII) substituents causes loss of all activity. Removal of the amino group, as in glutaric acid (XXXVII), causes loss of all activity.

(7) All activity is lost on introducing a methyl group into the 2 position, as in DL-2-methylglutamic acid (XXV) and DL-2-methylaspartic acid (XXVI).

(8) Excitatory activity is retained when a fluoro or hydroxyl substituent is introduced into the 4 position in glutamic acid. Relative to L-glutamic acid (I), the activity is slightly lower in DL-4-fluoroglutamic acid (III) and appreciably lower in L-*allo*-4-hydroxyglutamic acid (IV). In the case of aspartic acid derivatives, the introduction of a hydroxyl group on the carbon atom next to the ω-carboxyl group, as in DL-*threo*-3-hydroxyaspartic acid (VIII), leads to weak excitatory activity which is absent in the parent compound. DL-3-Methylaspartic acid (VII) shows weak blocking activity.

Ls-*xylo*-Kainic acid (XV) is an anthelmintic present in seaweed, and its structure was determined by Nitta, Watase & Tomhe (1958). It contains the three functional groups of glutamic acid (I). The carboxyl groups are unsubstituted, and the nitrogen atom, which is slightly more basic than that of glutamate, is contained within a ring. A dreiding model of L-glutamic acid can be superimposed precisely over the relevant part of the Ls-*xylo*-kainic acid model. Some configurations of the glutamic acid molecule, but not all, are available to the kainic acid molecule. Ls-*xylo*-Kainic acid was a weak depressant and a very weak excitant of the locust nerve–muscle preparation (Table 1). Dr A. Daoud, using microelectrode techniques, has recently demonstrated

Table 3. *The neuromuscular blocking activity of certain isoxazoles*



No.	Compound			Blockade	
	R^1	R^2	R^3	I_{50}	Reduction of force at 10^{-2} M
XXXVIII	OH	NH_2	COOH DL	1×10^{-2} M	50%
XXXIX	OH	NH_2	H	—	5%
XL	OH	$N(CH_3)_2$	H	—	14%
XLI	OH	$NH.COCH_3$	H	—	11%
XLII	OH	—	=NOH	—	43%
XLIII	CH_3O	NH_2	COOH DL	—	20%
XLIV	CH_3O	NH_2	H	4×10^{-3} M	81%
XLV	CH_3O	OH	H	—	44%
XLVI	Br	NH_2	H	5×10^{-3} M	70%
XLVII	C_6H_5	NH_2	H	8×10^{-4} M	100%

that kainic acid acts postsynaptically at the locust excitatory neuromuscular synapse, and that it may have an additional non-synaptic action (personal communication).

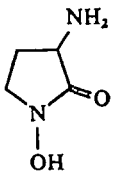
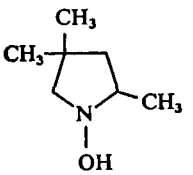
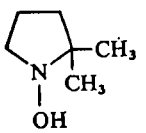
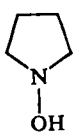
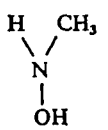
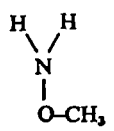
The activity of certain isoxazoles

During the past decade three groups of workers have investigated the insecticidal constituents of the fungi *Amanita muscaria* Fr. and *Tricholoma muscarium* Kawamura, and have shown that the isoxazoles ibotenic acid, muscimol, muscazone and tricholomic acid are present and are toxic to flies (Takemoto, Nakajima & Yokobe, 1964; Eugster, Müller & Good, 1965; Bowden, Drysdale & Mogey, 1965). Ibotenic acid (XXXVIII, Table 3) can be considered an analogue of glutamic acid (I), and muscimol (XXXIX) an analogue of 4-aminobutyric acid (XXXIV), and it appeared possible that the insecticidal actions of ibotenic acid and muscimol were due to their interaction with the excitatory and inhibitory neuromuscular synapses. This hypothesis later received support from the report that ibotenic acid is a powerful excitant of cat spinal interneurones and that muscimol is a depressant of the same cells (Johnston *et al.* 1968). A number of isoxazoles, including DL-ibotenic acid and muscimol, were synthesized in this Laboratory and tested on the retractor unguis preparation.

The results of the assays are summarized in Table 3. At high concentrations DL-ibotenic acid (XXXVIII) depressed the force of the neurally evoked contractions ($I_{50} = 10^{-2}$ M). As expected (since the retractor unguis muscle receives no inhibitory innervation) muscimol (XXXIX) was inactive. Most of the other compounds had negligible effects, but antagonistic activity was found in three muscimol derivatives in which the hydroxyl group was replaced by a methoxy (XLIV), bromo (XLVI), or phenyl (XLVII) substituent. Muscazone and tricholomic acid were not available for testing.

Samples of DL-ibotenic acid and muscimol were sent to Dr P. N. R. Usherwood and Dr T. J. Lea of Glasgow University for more detailed studies with the locust coxal

Table 4. *The neuromuscular blocking activity of certain hydroxylamines*

No.	Compound	Blockade	
		I_{50}	Reduction of force at 10^{-3} M
XLVIII		—	10%
XLIX		—	26%
L		$2{\cdot}2 \times 10^{-4}$ M	—
LI		$4{\cdot}5 \times 10^{-3}$ M	—
LII		$2{\cdot}3 \times 10^{-4}$ M	—
LIII		$2{\cdot}8 \times 10^{-4}$ M	—
LIV		—	0

adductor muscle, which has both excitatory and inhibitory innervation. From measurements of the effects of ibotenic acid on the EPSP, and on the responses to iontophoretically-applied and to bath-applied L-glutamate, it was concluded that ibotenic acid has no effect on locust excitatory neuromuscular synapses but reacts with receptors on the non-synaptic muscle membrane to increase chloride permeability, leading to an increase in membrane conductance (Lea & Usherwood, 1970, 1973*a*, *b*). Cull-Candy & Usherwood (1973) later showed that DL-ibotenic acid and L-glutamic acid react with the same extra-junctional receptor on locust muscle, to affect chloride permeability. Lea & Usherwood (1973*a*) also found that muscimol is an agonist at inhibitory neuromuscular synapses, acting as a mimic of 4-aminobutyric acid.

The activity of certain hydroxylamines

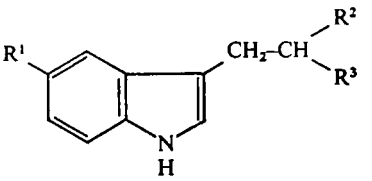
1-Hydroxy-3-amino-2-pyrrolidone has been shown to reduce the excitatory effects of L-glutamate and L-aspartate on neurones in the cerebral cortex of the cat, but to have no effect or only a weak depressant effect on cortical cells excited by acetylcholine. In the cuneate nucleus of the cat brain, this compound reduced the responses of synaptically excited neurones and reduced the chemical excitation of the neurones by L-glutamate and L-aspartate (Davies & Watkins, 1973). Working with Renshaw cells in the cat spinal cord, Curtis *et al.* (1973) found that at lower concentrations 1-hydroxy-3-amino-2-pyrrolidone reduced the excitatory effects of L-glutamate and L-aspartate without affecting sensitivity to acetylcholine, but that at higher concentrations it also depressed the action of acetylcholine. To explain the partial selectivity shown by this compound, Davies & Watkins (1973) suggested that it may block the access of excitatory amino acids to the membrane receptor sites.

A small sample of 1-hydroxy-3-amino-2-pyrrolidone (XLVIII, Table 4) was available for testing on the locust nerve–muscle preparation. Applied to a single preparation, a 10^{-3} M solution caused 10% depression of the force of the neurally evoked contractions after 20 min exposure. In contrast to this essentially negative result, a number of other 1-hydroxypyrrolidines (XLIX, L, LI) were found to possess pharmacological activity (Table 4). Since activity was also present in methylhydroxylamine (LII) and hydroxylamine (LIII) but not in methoxylamine (LIV), there is reason to suspect that, in the case of the locust nerve–muscle preparation, the activity present in certain 1-hydroxypyrrolidines is due to the $\rangle$N—OH moiety.

The activity of certain indolealkylamines, β-carbolines and phenothiazines

From detailed studies of the effects of tryptamine and 5-hydroxytryptamine on locust nerve–muscle preparations, Hill & Usherwood (1961) concluded that these compounds block neuromuscular transmission, acting either presynaptically to prevent transmitter release or postsynaptically by blocking receptor sites. Subsequently, Usherwood & Machili (1968) found that tryptamine and 5-hydroxytryptamine blocked the action of glutamate on the postsynaptic membrane. Table 5 summarizes the blocking actions of tryptamine and certain tryptamine derivatives on the mechanical response of the locust retractor unguis preparation in the present study. Tryptamine (LV) was among the most active of this group of compounds with an I_{50} of 8×10^{-4} M. The introduction of fluoro- (LVI) or methyl (LVII) substituents

Table 5. *The neuromuscular blocking activity of certain indolealkylamines*

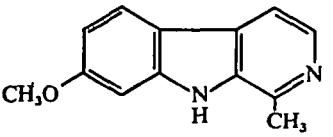
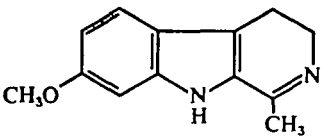
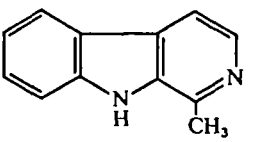
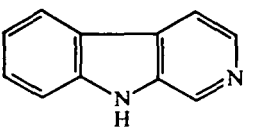
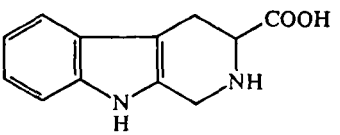
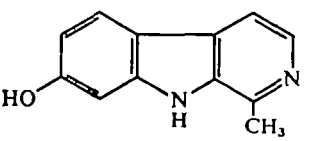
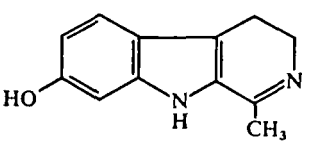
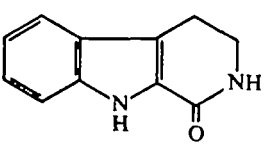
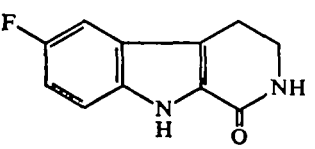


No.	Compound	R^1	R^2	R^3	Blockade I_{50}
LV	Tryptamine	H	H	NH_2	8×10^{-4} M
LVI	5-Fluorotryptamine	F	H	NH_2	8×10^{-4} M
LVII	5-Methyltryptamine	CH_3	H	NH_2	8×10^{-4} M
LVIII	5-Hydroxytryptamine	OH	H	NH_2	8×10^{-3} M
LIX	5-Methoxytryptamine	OCH_3	H	NH_2	2×10^{-3} M
LX	L-Tryptophan	H	COOH	NH_2	Inactive at 10^{-2} M
LXI	L-Tryptophanamide	H	$CONH_2$	NH_2	4×10^{-3} M
LXII	L-Tryptophanbenzylester	H	$COO.C_6H_5$	NH_2	4×10^{-4} M
LXIII	DL-5-Hydroxytryptophan-ethylester	OH	$COO.C_2H_5$	NH_2	1×10^{-3} M
LXIV	*N,N*-Dimethyltryptamine	H	H	$N(CH_3)_2$	2×10^{-3} M
LXV	Tryptophol	H	H	OH	1×10^{-3} M
LXVI	*N*-Phenoxycarbonyltryptamine	H	H	$NH.COO.C_6H_5$	1×10^{-4} M

into the 5-position did not affect activity, but activity was diminished by the introduction of hydroxy (LVIII) or methoxy (LIX) substituents. L-Tryptophan (LX) was inactive, but activity was restored by formation of the amide (LXI) and of esters (LXII, LXIII). *N,N*-Dimethyltryptamine (LXIV) and tryptophol (LXV) had moderate activity. The most active tryptamine derivative was *N*-phenoxycarbonyltryptamine (LXVI), which can be considered an acylating agent. This compound rapidly blocked muscle contraction at concentrations above 10^{-4} M but most preparations recovered well, if slowly, on washing.

A number of compounds with a β-carboline skeleton, collectively known as the harmala alkaloids, have been extracted from plants of several genera and are known both as amine oxidase inhibitors and as hallucinogens (Naranjo, 1967). These compounds affected the isolated retractor unguis preparation at rather lower concentrations than did the indolealkylamines. Irrigation with harmine (LXVII), harmaline (LXVIII), harman (LXIX) or norharman (LXX) caused a progressive reduction in force of neurally evoked contractions which did not level off with time, and the I_{50} values after 20 min irrigation ranged from 6×10^{-5} M to 2×10^{-4} M (Table 6). Preparations generally recovered well from these compounds on washing. Irrigation with harmol (LXXII) and harmalol (LXXIII), compounds containing a 7-hydroxyl group, caused first a very strong enhancement of the force of neurally evoked contractions (up to 260% of the normal force) and later depression followed by blockade. Washing off the harmol and harmalol generally led to a temporary recovery, sometimes accompanied by a very powerful enhancement of the neurally evoked contractions (up to 340%), but this was often followed by a subsequent decline. The complex alkaloid ibogaine caused a temporary slight potentiation of the mechanical response when irrigated at 10^{-3} M but depressed it at lower concentrations. Irrigation with the alkaloid yohimbine caused a maintained and very strong enhancement of the

Table 6. *The neuromuscular blocking activity of certain β-carbolines*

No.	Compound	Blockade I_{50}
LXVII	Harmine 	6×10^{-5} M
LXVIII	Harmaline 	2×10^{-4} M
LXIX	Harman 	9×10^{-5} M
LXX	Norharman 	$1{\cdot}5 \times 10^{-4}$ M
LXXI	Tetrahydronorharman-3-carboxylic acid 	4×10^{-3} M
LXXII	Harmol 	($1{\cdot}5 \times 10^{-4}$ M causes strong enhancement of contractions followed by decline)
LXXIII	Harmalol 	(5×10^{-4} M causes strong enhancement of contractions followed by decline)
LXXIV	Tetrahydronorharman-1-one 	$2{\cdot}5 \times 10^{-4}$ M
LXXV	6-Fluorotetrahydronorharman-1-one 	$1{\cdot}5 \times 10^{-4}$ M

mechanical response. For example, 3×10^{-4} M yohimbine caused a 290% increase in force after 3 min. Yohimbic acid was inactive at 5×10^{-4} M.

The potencies of these compounds are on the whole rather similar but a few comments can be made on their structure–activity relationships. Hydrogenation in the 3 and 4 positions slightly reduces biological activity. The presence of a 1-methyl or 1-keto substituent causes little change. The presence of a 7-methoxy substituent increases activity slightly, and a 7-hydroxy substituent changes the nature of the effect by inducing strong enhancement of contractions followed by a decline which is not reversed on washing. The presence of a 3-carboxyl substituent depresses biological activity. From their structure it appeared possible that the harmala alkaloids might be electron donors capable of forming charge transfer complexes, and so the interaction of harmine (LXVII) with a number of electron acceptors was examined spectrophotometrically. With such acceptors as trinitroanisole, chloranil and tetracyanoethylene charge transfer bands were seen, and with tetracyanoethylene a reaction ensued. It is clear that harmine can form charge transfer complexes, but it is not possible to say whether complex formation is involved in its action on the locust neuromuscular system.

When nerve–muscle preparations were irrigated with 10^{-4} M harmine or harman the neurally evoked contractions quickly declined and became blocked. Similarly, both glutamate contractions and potassium contractures also declined and became blocked, showing no recovery during the continued irrigation of the drug for periods up to 135 min. Irrigation with $2{\cdot}5 \times 10^{-3}$ M tryptamine had similar, if slightly less marked, effects. This blockade of potassium contractures might suggest that the drugs were affecting the non-synaptic muscle membrane. However, it is possible that the drugs were acting as partial agonists, for glutamate also would block potassium contractures. In one experiment, for example, irrigation with 7×10^{-5} M glutamate depressed and later blocked both neurally evoked contractions and potassium contractures, no recovery occurring during the 135 min exposure. On one occasion challenge with a 2 ml pulse of 3×10^{-4} M harmine caused a slightly delayed and rather feeble contraction, and this also implies a weak agonistic action. In experiments using microelectrodes it was found that irrigation with 5×10^{-4} M solutions of harmine or harman caused a rapid and strong reduction of the response to electrophoretically applied glutamate and an even more marked reduction of EPSPs. These changes were accompanied by a slight increase in the conductance of the muscle membrane. Irrigation with 5×10^{-4} M harman caused irregular fluctuations in both amplitude and frequency of miniature EPSPs which were impossible to interpret. These results indicate that the compounds had acted postsynaptically at the excitatory synapses, but the marked effect on EPSPs and the effect on frequency of miniature EPSPs suggest a possible presynaptic action also (Dowson & Clements, 1974).

A number of tricyclic compounds with a phenazine or phenothiazine nucleus were found to affect the mechanical response of the retractor unguis preparation. These included *N*-methylphenazonium methosulphate ($I_{50} = 5 \times 10^{-5}$ M), phenosafranine ($I_{50} = 2 \times 10^{-4}$ M), chlorpromazine ($I_{50} = 1 \times 10^{-4}$ M) and trifluoperazine ($I_{50} = 6 \times 10^{-5}$ M). Isolated preparations irrigated with 2×10^{-4} M chlorpromazine failed to respond to challenges with L-glutamate and potassium chloride, indicating some form of postsynaptic block.

The activity of inhibitors of glutamate metabolism and glutamate transport

Dowson & Usherwood (1973) gave reasons (see Discussion, below) for thinking that at the insect excitatory neuromuscular synapse the natural transmitter is removed from the synaptic cleft by enzymatic degradation or active transport. There is evidence which suggests that L-glutamic acid may be the natural transmitter at these synapses (Pitman, 1971), and so it was considered important to investigate the effect, on the neurally evoked contractions of the nerve–muscle preparation, of fairly prolonged exposure to known inhibitors of glutamate-metabolizing enzymes and of glutamate transport systems.

Two types of glutamic decarboxylase are known from mammalian tissues: GAD I from synaptosomes, and GAD II from mitochondria. GAD I is inhibited by 10^{-2} M solutions of the carbonyl-trapping agent aminooxyacetic acid (NH_2—O—CH_2COOH), whereas GAD II is stimulated by such solutions (Haber, Kuriyama & Roberts, 1970). The GAD which is present in *Sarcophaga* flight-muscle mitochondria is stimulated by aminooxyacetic acid (Langcake & Clements, 1974). Irrigation of two retractor unguis preparations with 10^{-2} M aminooxyacetic acid for 20 min caused 33 % and 12 % reduction in the force of the neurally evoked contractions. Both preparations failed to recover on washing and slowly declined. In contrast, two reversible inhibitors of glutamic decarboxylase with K_i's of about 10^{-2} M, DL-*C*-allylglycine (XXII) (Alberici, Arnaiz & De Robertis, 1969) and 2-methyl-DL-glutamic acid (XXV) (Fonda, 1972), had negligible effects on the nerve–muscle preparation after irrigation for 20 min at 10^{-2} M.

4-Iodoacetamidosalicylic acid is an alkylating agent which is an active-site-directed and irreversible inhibitor of glutamate dehydrogenase (Baker, Lee & Tong, 1962*b*). Irrigation of retractor unguis preparations with a $2{\cdot}5 \times 10^{-3}$ M solution of this compound for 20 min caused a variable reduction in the force of the muscle contractions with poor recovery on washing. When washed preparations were treated a second time with the compound they fairly rapidly entered on a marked and irreversible decline. 1-Hydroxy-2-naphthoic acid is a reversible inhibitor of glutamate dehydrogenase, with a K_i of 10^{-4} M in the presence of 10^{-3} M substrate (Baker *et al.* 1962*a*). It showed moderately high activity on the retractor unguis preparation, with an I_{50} of 3×10^{-5} M. Irrigation of the preparation with this compound sometimes caused a sudden fall in the force of the neurally evoked contractions which could be reversed by raising the stimulating voltage. This action of the compound was tentatively interpreted as a presynaptic effect, and it was later found that 2×10^{-4} M 1-hydroxy-2-naphthoic acid would block conduction of action potentials in the locust crural nerve after 25 min exposure. 1-Hydroxy-2-naphthoic acid was also found to cause complete uncoupling of oxidative phosphorylation in *Sarcophaga* flight muscle mitochondria at 10^{-4} M. L-Methionine-DL-sulphoximine, which is an irreversible inhibitor of glutamine synthetase (Ronzio, Rowe & Meister, 1969) and which antagonizes the excitation of cat spinal neurones by L-glutamate (Curtis *et al.* 1972), had no effect on the neurally evoked contractions at 10^{-2} M.

Treatment of a nerve–muscle preparation with compounds which inhibited active transport of the natural transmitter away from the synaptic region could cause accumulation of the transmitter, and this might lead to enhancement or depression o

neurally evoked contractions. However, if these compounds were not specific inhibitors of the uptake system, but had other actions also, it would require detailed studies to interpret their actions. Mammalian brain and spinal cord contain a high affinity uptake system for L-glutamate which is possibly associated with synaptosomes (Balcar & Johnston, 1972, 1973; McLennan & Haldeman, 1973). A number of amino acids are powerful inhibitors of this high affinity uptake system, and the most active of these are DL-*threo*-3-hydroxyaspartic acid (VIII), L-2-amino-3-sulphinopropionic acid (XII), L-cysteic acid (X), and D-aspartic acid, with IC_{50} values between 4 and 9 μM. These compounds also excite cat spinal neurones when administered iontophoretically (Balcar & Johnston, 1972, 1973). Table 1 shows that all of these amino acids, except D-aspartate which was not tested, depressed the neurally evoked contractions of the locust nerve–muscle preparation, and were also excitants of that preparation. McLennan & Haldeman (1973) reported that 10^{-3} M glutamic acid diethylester (XXIX) inhibited the high affinity uptake of glutamate by rat brain synaptosomes. In the present work this compound had negligible effects on the nerve–muscle preparation at 10^{-2} M. Balcar & Johnston (1972) found that juglone, chlorpromazine and *p*-chloromercuriphenylsulphonate inhibited high affinity uptake of glutamate by rat brain slices, with IC_{50} values of 50–100 μM. Faeder & Salpeter (1972) reported that chlorpromazine inhibits glutamate uptake by glial cells in the cockroach. In the present work, juglone irreversibly depressed the neurally evoked contractions, having an I_{50} of 2×10^{-4} M, and chlorpromazine reversibly depressed the neurally evoked contractions, with an I_{50} of 10^{-4} M. *p*-Chloromercuriphenylsulphonate was not tested.

Avenaciolide is an effective inhibitor of glutamate transport by rat liver mitochondria (Meyer & Vignais, 1973). It has a low water solubility, but a saturated solution of avenaciolide had no effect on locust nerve–muscle preparations during 20 min exposure. Balcar & Johnston (1973) found that avenaciolide did not influence glutamate uptake by rat brain slices. β-*N*-Oxalyl-L-α,β-diaminopropionic acid, a neurotoxin from *Lathyrus* seeds, at concentrations between 0·5 and 5×10^{-3} M, competitively inhibits glutamate uptake by bovine mitochondria (Duque-Magalhaes & Packer, 1972). It is also a powerful excitant of spinal interneurones in the cat (Watkins, Curtis & Biscoe, 1966). Irrigation of retractor unguis preparations for 20 min with a 10^{-3} M solution of the mixed DL isomers of this compound caused 6% enhancement of the force of the neurally evoked contractions.

DISCUSSION

The actions of amino acids and other compounds

L-Glutamic acid interacts with locust neuromuscular systems at a number of sites and in different ways. At excitatory synapses it acts both presynaptically, affecting transmitter release (Dowson & Usherwood, 1972), and postsynaptically, mimicking the natural transmitter (Usherwood & Machili, 1968). It also acts on the non-synaptic muscle membrane to affect chloride permeability (Cull-Candy & Usherwood, 1973). Special methods are needed to distinguish at which of these sites any compound is acting. In the present work the actions of amino acids and drugs on a locust nerve–muscle preparation were studied by measuring their ability to stimulate muscle

contraction or to depress the force of neurally evoked contractions. The location the receptors mediating the responses could not be identified with certainty, but it may be assumed that when amino acids stimulated the muscle to twitch their action was predominantly on the excitatory postsynaptic membrane. The concentration of L-glutamic acid required to stimulate contraction of the retractor unguis muscle was close to the concentration of acetylcholine reported to stimulate contraction of the frog rectus abdominis muscle by bath application (Su & Lee, 1960). The sensitivities of locust neuromuscular synapses to electrophoretically applied glutamate (Beranek & Miller, 1968) and of frog neuromuscular synapses to electrophoretically applied acetylcholine (Feltz & Mallart, 1971) are also very similar.

As far as definition of the molecular structure needed to stimulate the locust neuromuscular synapse is concerned, this work confirmed what was already largely known: that maximum potency is found in glutamic acid, that only the L-enantiomer is active, and that, in general, the three functional groups must carry a charge and be unsubstituted. Some excitatory activity is retained if the ω-carboxyl group is replaced by a sulphinyl or sulphonyl group, but in such cases excitatory activity is present only in aspartic acid analogues, possibly reflecting the larger size of the sulphur atom. Excitatory activity is lost if the carbon chain of the dicarboxylic amino acids is lengthened or shortened. Kainic acid (XV) has weak excitatory activity although its nitrogen atom is substituted. However, in this compound the nitrogen atom forms part of a ring, so there may be less risk of steric hindrance than in a compound such as *N*-methyl glutamic acid (XXX). It was found that for some amino acids there is a similar, and small, ratio between the concentrations that stimulate contraction and depress neurally evoked contractions, but that for some other amino acids the concentrations that stimulate and depress are very different. In general, the results emphasized the high specificity of the glutamate receptor. The steric requirements of this receptor make the design of antagonists very difficult.

The structure–activity relationships indicate that at the locust excitatory neuromuscular synapse, L-glutamic acid interacts electrostatically with a receptor containing three charged centres, much as was first proposed for mammalian glutamate receptors by Curtis & Watkins (1960). The locust receptor appears to differ to some degree from the various types of glutamate receptor known to be present on mammalian central neurones. For example: mammalian spinal neurones are strongly excited, and mammalian cortical neurones are weakly excited, by D-glutamic acid (Curtis & Watkins, 1960; Krnjević & Phillis, 1961); L-4-fluoroglutamate can be a more potent excitant of spinal neurones than L-glutamate (Curtis *et al.* 1972); and L-glutamic acid diethylester antagonizes the excitation by L-glutamate of spinal neurones (Curtis *et al.* 1972). The locust synaptic glutamate receptor is very much more specific than some other types of receptor, e.g. the various mammalian acetylcholine receptors (Barlow, 1964). Such high specificity may be essential where an α-amino acid functions as a transmitter, and so may reflect the force of natural selection, or it may be due simply to the trifunctional structure of the transmitter molecule.

It has long been known that, in addition to their effects on the central nervous system, phenothiazines and β-carbolines can block neuromuscular transmission reversibly. Such a blockade has been demonstrated in the rat with tranylcypramine, harmaline and other compounds (Anderson & Ammann, 1963); in the frog and cock,

ach with chlorpromazine (Muchnik & Yaryura, 1969; Faeder & O'Brien, 1970); and in the crab with phenothiazine (Collier, 1940). These compounds have a wide range of pharmacological actions, including inhibition of monoamine oxidase and of Na/K-ATPase, inhibition of the release and resorption of transmitter substances, etc. (Glick, 1972), and without a very detailed investigation it is impossible to assign any particular mechanism to their actions on neuromuscular transmission. The present study of β-carbolines and phenothiazines has revealed a number of neuromuscular blocking agents with moderate potency, and the preliminary results suggest that the actions of harmine and harman may be predominantly on the postsynaptic membrane.

Inactivation of the natural transmitter

The natural transmitter could be lost from the synaptic cleft by enzymic degradation, by active transport, or by diffusion, and it is interesting to consider whether there is any evidence, direct or indirect, which indicates how transmitter molecules are lost from the insect excitatory neuromuscular synapse. At such synapses in locusts the synaptic current flows only very briefly, in marked contrast to the prolonged duration of the conductance increase at inhibitory synapses during the IPSP (Usherwood & Machili, 1968; Usherwood & Grundfest, 1965). This indicates that the duration of transmitter action at the excitatory synapses is brief, and has been taken to suggest that the transmitter is rapidly removed from the synaptic cleft by enzymic degradation or active transport, or that the receptors are rapidly desensitized (Dowson & Usherwood, 1973).

An enzyme which was located on the postsynaptic membrane and which had the function of degrading the excitatory transmitter might be expected to have similar properties to acetylcholinesterase, i.e. to be membrane bound, to have a high specific activity, to have a moderately low Michaelis constant, and to require no other substrate for the reaction than the transmitter molecule itself. Detailed studies of the glutamate-metabolizing enzymes present in the indirect flight muscles of *Sarcophaga* revealed none which had all these properties (Donnellan, Jenner & Ramsey, 1974; Langcake & Clements, 1974). Usherwood & Machili (1968) suggested that the pharmacological actions of enzyme inhibitors might help to identify the enzyme postulated to degrade the excitatory transmitter. In the present study the carbonyl-trapping agent amino-oxyacetic acid, which is an inhibitor of one form of glutamate decarboxylase, affected the nerve–muscle preparation at high concentration, but two reversible inhibitors of glutamate decarboxylase had no effect. 4-Iodoacetamidosalicylic acid and 1-hydroxy-2-naphthoic acid, which are inhibitors of glutamate dehydrogenase, both affected the nerve–muscle preparation. However, the former is a chemically reactive compound, and the latter was found to produce a number of effects, including uncoupling oxidative phosphorylation, so there is no reason to ascribe their pharmacological actions to inhibition of a particular enzyme. L-Methionine-DL-sulphoximine, an irreversible inhibitor of glutamine synthetase, had no effect at high concentrations. The absence from the insect muscle of a glutamate-metabolizing enzyme with suitable properties suggests that, if glutamate is the excitatory transmitter, it may well be removed from the synaptic cleft by a process other than enzymic degradation. The variable actions of the inhibitors of glutamate-metabolizing enzymes provide only very uncertain evidence.

High-affinity binding systems for L-glutamate are present in rat and cat central nervous tissue, where L-glutamate is believed to be a transmitter. Balcar & Johnston (1972, 1973) have given reasons which suggest that this binding is not to postsynaptic receptors but to another system, possibly one which removes glutamate from the synaptic environment. Lunt (1973) extracted two proteins from *Schistocerca* flight muscle which showed high affinity binding of L-glutamate, but gave no evidence to indicate whether these were derived from glutamate receptors on the postsynaptic membrane or from another source. In the present study, a number of compounds which had been reported to inhibit glutamate transport systems were tested on the nerve–muscle preparation, and some affected neuromuscular transmission. However, since these compounds were not specific inhibitors but had other pharmacological actions also, the results cannot be taken to prove that active transport has a role in the removal of glutamate from the synaptic environment.

Using autoradiography, Faeder & Salpeter (1970) and Salpeter & Faeder (1971) demonstrated a stimulation-enhanced, high-affinity uptake system for L-glutamate in cockroach skeletal muscle. It was absent from most regions of the muscle and its associated axons, but was found at the neuromuscular junctions and in the glial cells and tracheole cells which surrounded the axon terminals. If the excitatory transmitter is L-glutamate, this system may be sufficient to inactivate transmitter molecules which are released into the synaptic cleft.

I should like to express my thanks to Dr P. Hackett, Dr J. H. Davies, Mr R. H. Davies and Mr M. Pearson for the synthesis of compounds, and also to Dr G. A. R. Johnston and to ICI for the gift of compounds. This work has benefited greatly from the interest and advice of Dr P. N. R. Usherwood and Dr R. J. Dowson.

REFERENCES

ALBERICI, M., ARNAIZ, G. R. L. & DE ROBERTIS, E. (1969). Glutamic acid decarboxylase inhibition and ultrastructural changes by the convulsant drug allylglycine. *Biochem. Pharmac.* **18**, 137–43.

ANDERSON, E. G. & AMMANN, A. (1963). The effects of monoamine oxidase inhibitors on neuromyal transmission. *J. Pharmac. exp. Ther.* **140**, 179–82.

BAKER, B. R., LEE, W. W., TONG, E., ROSS, L. O. & MARTINEZ, A. P. (1962*a*). Non-classical antimetabolites. V. Further factors in the design of exo-alkylating enzyme inhibitors, particularly of lactic dehydrogenase. *J. theor. Biol.* **3**, 446–58.

BAKER, B. R., LEE, W. W. & TONG, E. (1962*b*). Non-classical antimetabolites. VI. 4-(Iodoacetamido)-salicylic acid, an alkylating irreversible inhibitor. *J. theor. Biol.* **3**, 459–83.

BALCAR, V. J. & JOHNSTON, G. A. R. (1972). The structural specificity of the high affinity uptake of L-glutamate and L-aspartate by rat brain slices. *J. Neurochem.* **19**, 2657–66.

BALCAR, V. J. & JOHNSTON, G. A. R. (1973). High affinity uptake of transmitters: studies on the uptake of L-aspartate, gaba, L-glutamate and glycine in cat spinal cord. *J. Neurochem.* **20**, 529–39.

BARLOW, R. B. (1964). *Introduction to Chemical Pharmacology*, 2nd ed. London: Methuen.

BERANEK, R. & MILLER, P. L. (1968). The action of iontophoretically applied glutamate on insect muscle fibres. *J. exp. Biol.* **49**, 83–93.

BERGMEYER, H. U. (1965). *Methods of Enzymatic Analysis*. New York, London: Academic Press.

BOWDEN, K., DRYSDALE, A. C. & MOGEY, A. C. (1965). Constituents of *Amanita muscaria*. *Nature, Lond.* **206**, 1359–60.

CLEMENTS, A. N. & MAY, T. E. (1974). Studies on locust neuromuscular physiology in relation to glutamic acid. *J. exp. Biol.* **60**, 673–705.

COLLIER, H. O. J. (1940). Depression of neuro-muscular transmission in a crab by phenothiazine. *Nature, Lond.* **146**, 232–3.

CULL-CANDY, S. G. & USHERWOOD, P. N. R. (1973). Two populations of L-glutamate receptors on locust muscle fibres. *Nature New Biol.* **246**, 62–4.

RTIS, D. R., DUGGAN, A. W., FELIX, D., JOHNSTON, G. A. R., TEBECIS, A. K. & WATKINS, J. C. (1972). Excitation of mammalian central neurones by acidic amino acids. *Brain Res.* **41**, 283–301.

CURTIS, D. R., JOHNSTON, G. A. R., GAME, C. J. A. & MCCULLOCH, R. M. (1973). Antagonism of neuronal excitation by 1-hydroxy-3-aminopyrrolidone-2. *Brain Res.* **49**, 467–70.

CURTIS, D. R. & WATKINS, J. C. (1960). The excitation and depression of spinal neurones by structurally related amino acids. *J. Neurochem.* **6**, 117–41.

DAVIES, J. & WATKINS, J. C. (1973). Micro-electrophoretic studies on the depressant action of HA-966 on chemically and synaptically excited neurones in the cat cerebral cortex and cuneate nucleus. *Brain Res.* **59**, 311–22.

DONNELLAN, J. F., JENNER, D. W. & RAMSEY, A. (1974). Subcellular fractionation of fleshfly flight muscle in attempts to isolate synaptosomes and to establish the location of a number of glutamate enzymes. *Insect Biochem.* **4** (in the Press).

DOWSON, R. J. & CLEMENTS, A. N. (1975). The actions of some harmala alkaloids on transmission at a glutamate-mediated synapse. *J. Neurochem.* (in the Press).

DOWSON, R. J. & USHERWOOD, P. N. R. (1972). The effect of low concentrations of L-glutamate and L-aspartate on transmitter release at the locust excitatory nerve–muscle synapse. *J. Physiol., Lond.* **299**, 13–14*P*.

DOWSON, R. J. & USHERWOOD, P. N. R. (1973). The mode of action of phenylhydrazine hydrochloride on the locust neuromuscular system. *J. Insect Physiol.* **19**, 355–68.

DUQUE-MAGALHAES, M. C. & PACKER, L. (1972). Action of the neurotoxin *β*-*N*-oxalyl-L-*α*,*β*-diaminopropionic acid on glutamate metabolism of brain mitochondria. *FEBS Lett.* **23**, 188–90.

EUGSTER, C. H., MÜLLER, G. F. R. & GOOD, R. (1965). Wirkstoffe aus *Amanita muscaria*: Ibotensäure und Muscazon. *Tetrahedron Lett.* **23**, 1813–15.

FAEDER, I. R. & O'BRIEN, R. D. (1970). Responses of perfused isolated leg preparations of the cockroach, *Gromphadorhina portentosa*, to L-glutamate, gaba, picrotoxin, strychnine and chlorpromazine. *J. exp. Zool.* **173**, 203–14.

FAEDER, I. R. & SALPETER, M. M. (1970). Glutamate uptake by a stimulated insect nerve muscle preparation. *J. Cell Biol.* **46**, 300–7.

FAEDER, I. R. & SALPETER, M. M. (1972). Chlorpromazine induced inhibition of glutamate uptake by insect nerve. *A. Mtg. Soc. Neurosci.* **2** (Abstract).

FELTZ, A. & MALLART, A. (1971). An analysis of acetylcholine responses of junctional and extrajunctional receptors of frog muscle fibres. *J. Physiol., Lond.* **218**, 85–100.

FONDA, M. L. (1972). Glutamate decarboxylase. Substrate specificity and inhibition by carboxylic acids. *Biochemistry, Easton* **11**, 1304–9.

GLICK, N. B. (1972). Inhibition of transport reactions. In *Metabolic Inhibitors* (ed. R. M. Hochster, M. Kates and J. H. Quastel), vol. 3, pp. 1–45. London: Academic Press.

HABER, B., KURIYAMA, K. & ROBERTS, E. (1970). Mitochondrial localization of a new L-glutamic acid decarboxylase in mouse and human brain. *Brain Res.* **22**, 105–12.

HILL, R. B. & USHERWOOD, P. N. R. (1961). The action of 5-hydroxytryptamine and related compounds on neuromuscular transmission in the locust *Schistocerca gregaria*. *J. Physiol., Lond.* **157**, 393–401.

HOYLE, G. (1955). The anatomy and innervation of locust skeletal muscle. *Proc. R. Soc. Lond.* B **143**, 281–92.

HUNTER-JONES, P. (1966). *Rearing and Breeding Locusts in the Laboratory*. London: Anti-Locust Research Centre.

JOHNSTON, G. A. R., CURTIS, D. R., DE GROAT, W. C. & DUGGAN, A. W. (1968). Central actions of ibotenic acid and muscimol. *Biochem. Pharmac.* **17**, 2488–9.

KRNJEVIĆ, K. & PHILLIS, J. W. (1961). The actions of certain amino acids on cortical neurones. *J. Physiol., Lond.* **159**, 62–63*P*.

LANGCAKE, P. & CLEMENTS, A. N. (1974). L-Glutamic acid decarboxylase of fleshfly flight muscle, its properties and subcellular location. *Insect Biochem.* **4**, 225–41.

LEA, T. J. & USHERWOOD, P. N. R. (1970). Increased chloride permeability of insect muscle fibres on exposure to ibotenic acid. *J. Physiol., Lond.* **211**, 32–33*P*.

LEA, T. J. & USHERWOOD, P. N. R. (1973*a*). The site of action of ibotenic acid and the identification of two populations of glutamate receptors on insect muscle fibres. *Comp. gen. Pharmac.* **4**, 333–50.

LEA, T. J. & USHERWOOD, P. N. R. (1973*b*). Effect of ibotenic acid on chloride permeability of insect muscle fibres. *Comp. gen. Pharmac.* **4**, 351–63.

LUNT, G. G. (1973). Hydrophobic proteins from locust (*Schistocerca gregaria*) muscle with glutamate receptor properties. *Comp. gen. Pharmac.* **4**, 75–9.

MCDONALD, T. J. (1971). Studies of the physiology and pharmacology of insect neuromuscular transmission. Ph.D. Thesis, University of California, Riverside. University Microfilms, 71–27,078.

MCDONALD, T. J. & O'BRIEN, R. D. (1972). Relative potencies of L-glutamate analogs on excitatory neuromuscular synapses of the grasshopper, *Romalea microptera*. *J. Neurobiol.* **3**, 277–90.

MCLENNAN, H. & HALDEMAN, S. (1973). The actions of the dimethyl and diethyl esters of glutamic acid on glutamate uptake by brain tissue. *J. Neurochem.* **20**, 629–31.

MEYER, J. & VIGNAIS, P. M. (1973). Kinetic study of glutamate transport in rat liver mitochondria. *Biochim. Biophys. Acta* **325**, 375–84.

MUCHNIK, S. & YARYURA, A. (1969). The action of chlorpromazine on the neuromuscular junction. *Acta physiol. latinoam.* **19**, 94–100.

NARANJO, C. (1967). Psychotropic properties of the harmala alkaloids. In *Ethnopharmacologic Search for Psychoactive Drugs* (ed. D. H. Efron, B. Homstedt and N. S. Kline). U.S. Public Health Serv. Publ. no. 1645, pp. 385–91.

NITTA, I., WATASE, H. & TOMHE, Y. (1958). Structure of kainic acid and its isomer, allokainic acid. *Nature, Lond.* **181**, 761–2.

PITMAN, R. M. (1971). Transmitter substances in insects: a review. *Comp. gen. Pharmac.* **2**, 347–71.

REES, D. & USHERWOOD, P. N. R. (1972). Fine structure of normal and degenerating motor axons of nerve–muscle synapses in the locust, *Schistocerca gregaria*. *Comp. Biochem. Physiol.* **43***A*, 83–101.

RONZIO, R. A., ROWE, W. B. & MEISTER, A. (1969). Studies on the mechanism of inhibition of glutamine synthetase by methionine sulfoximine. *Biochemistry, Easton* **8**, 1066–75.

SALPETER, M. M. & FAEDER, I. R. (1971). The role of sheath cells in glutamate uptake by insect nerve muscle preparations. *Progr. Brain Res.* **34**, 103–14.

SU, C. & LEE, C. Y. (1960). The mode of neuromuscular blocking action of chlorpromazine. *Br. J. Pharmac. Chemother.* **15**, 88–94.

TAKEMOTO, T., NAKAJIMA, T. & YOKOBE, T. (1964). Structure of ibotenic acid. *Yakugaku Zasshi* **84**, 1232–3.

USHERWOOD, P. N. R. & GRUNDFEST, H. (1965). Peripheral inhibition in skeletal muscle of insects. *J. Neurophysiol.* **28**, 497–518.

USHERWOOD, P. N. R. & MACHILI, P. (1968). Pharmacological properties of excitatory neuromuscular synapses in the locust. *J. exp. Biol.* **49**, 341–61.

WATKINS, J. C. (1962). The synthesis of some acidic amino acids possessing neuropharmacological activity. *J. med. pharmac. Chem.* **5**, 1187–99.

WATKINS, J. C., CURTIS, D. R. & BISCOE, T. J. (1966). Central effects of β-*N*-oxalyl-α,β-diaminopropionic acid and other *Lathyrus* factors. *Nature, Lond.* **211**, 637.