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ACIDIC AMINO ACIDS WITH STRONG EXCITATORY ACTIONS ON MAMMALIAN NEURONES

BY D. R. CURTIS AND J. C. WATKINS
From the Department of Physiology, Australian National University, Canberra, Australia

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It has been demonstrated recently that, when applied extracellularly, glutamic acid and certain structurally similar compounds depolarize mammalian and amphibian neurones and crustacean muscle fibres (Robbins, 1959; Van Harreveld, 1959; Curtis, Phillis & Watkins, 1960, 1961; Curtis & Watkins, 1960). In the case of mammalian neurones this effect superficially resembles that produced by excitatory transmitter substances, and the excitant amino acids are effective in concentrations which are similar to those of acetylcholine required to excite central cholinoceptive neurones (Curtis & Eccles, 1958). Unlike choline esters, however, members of the glutamic-acid group of excitants have actions which are non-specific with regard to neuronal type; they excite many functionally different types of neurone, including both cholinoceptive and non-cholinoceptive cells in the spinal cord (Curtis, Phillis & Watkins, 1960), brain stem (Curtis & Koizumi, 1961), and cerebral cortex (Curtis & Watkins, 1961; Phillis & Krnjević, 1961), as well as cells of the lateral geniculate nucleus which have been shown to be a site of action of drugs possessing an indole nucleus (Curtis & Davis, 1962). Furthermore, there is evidence (Curtis, 1962) that the equilibrium potential for the conductance change, induced in the membrane of spinal motoneurones by L-glutamic acid, differs from that associated with excitatory transmitter action.

An extensive investigation of compounds related to glutamic acid was previously undertaken in order to define the structural requirements for this type of excitatory action and also to determine whether greater specificity could be achieved by structural change (Curtis & Watkins, 1960). The present paper is an extension of the previous studies, and, in particular, is concerned with the effects of N-alkylation and of replacing the ω-carboxylic acid group by sulphonic or sulphinic acid groups. Differences in the actions of D and L forms of compounds were also investigated. The substances were administered by the electrophoretic technique, and the responses of neurones were monitored by an improved method which facilitated comparison of relative potencies. A preliminary report has been published (Curtis & Watkins, 1961).

METHODS

The experiments were performed upon neurones located in various regions of the central nervous systems of cats lightly anaesthetized with pentobarbital sodium. The methods whereby amino acids were applied electrophoretically to single neurones have been detailed previously (Curtis & Watkins, 1960), the use of five-barrel electrodes permitting a comparison of the actions of four different agents upon one cell. The central recording barrel contained 5 M-NaCl and the over-all tip diameter of electrodes varied between 4 and 10 μ. The outer barrels of the electrodes contained solutions of the amino acids which were adjusted to pH 8–8·5 (glass electrode) with NaOH, and from which the acids were passed electrophoretically as anions. Molar solutions were most commonly used in order to ensure that the electrical resistance of the filled barrels would be low (Curtis & Watkins, 1960). In order to control leakage caused by diffusion from the electrode tips, a 'backing' or retaining voltage of 0·5 V was usually applied to each amino-acid barrel. This was reduced or turned off, in a particular case, if the diffusional efflux of the compound was insufficient to modify the behaviour of the cell under observation. That such voltages did not entirely eliminate diffusional escape of the active anions from the electrodes was apparent when the electrodes were filled with molar solutions of the very strong excitants. In these cases a suitable retaining voltage often could not be found to prevent a nearby cell from being excited, and frequently an increase in the voltage produced an increased leakage, presumably due to electro-osmosis (Curtis & Watkins, 1960; Curtis, Perrin & Watkins, 1960). This difficulty was overcome by reducing the concentration of the compound to 0·1–0·5 M, and in such cases all the other test solutions in the electrode were similarly diluted. The electrodes were filled either by the conventional diffusional method (Curtis & Eccles, 1958) or by centrifugation. In the latter method, dry electrodes were filled from the top by means of fine polyethylene tubing and then centrifuged for 5 min with the tips outermost (approximately 1600 *g*).

The frequency of firing of neurones was monitored continuously by electronically counting the number of spikes over period of 1 sec, the interval between the counts being 0·2 sec. The counts were displayed on a paper recorder and the neuronal spikes were simultaneously observed upon an oscilloscope, from which photographic records could be made as required.

The relative potencies of four substances in an electrode were assessed by the ratios of the respective electrophoretic currents which were necessary to evoke the same frequency of spike discharge of the particular neurone being investigated. It was then assumed that equi-effective concentrations of the different agents had been attained in the extracellular environment of the cell. The currents required varied from 2 to 200 nA. The onset of excitatory action was sometimes slow, owing in some cases to the prolonged use of a retaining potential, which diminished the concentration of the test substance within the electrode tip, and in others to the structure of the particular compound which was ejected. When comparing potencies it was extremely important to ensure that the response of a neurone to a certain electrophoretic dose of a substance had reached a maximum before terminating the application. The continuous monitoring of spike frequency allowed direct observation of the attainment of maximal response, after which the electrophoretic ejecting current was maintained for 10–20 sec. The most satisfactory frequencies of firing for the comparisons ranged between 20 and 60 spikes/sec.

Most of the experiments were performed upon spinal interneurones and each of the four amino acids in a particular electrode was tested upon at least three different cells in the same animal, several different overlapping combinations of acids being used, both in the same and in different animals. In this way each amino acid was tested on 10–50 different interneurones in 3–10 animals, and the potency of each substance relative to between 5 and 15 others was eventually estimated. In making comparisons of potencies by the electrophoretic method some allowance should be made for the different distances which may

separate the orifices of the individual micropipettes from the particular neurone under investigation. These distances cannot be measured, and in previous studies such parameters were neglected, particularly as the maximal distance between the centres of two diametrically opposite barrels was only of the order of 5–6 μ, and thus probably relatively small when compared with the distance of the cell from each of them (Curtis, Perrin & Watkins, 1960). Greater experience with the electrophoretic technique has revealed that repeated comparisons upon different neurones of two or more substances in a multibarrelled electrode leads to some variation in the relative potencies. This is due largely, no doubt, to variations in the relative spatial disposition of the cells and the electrode orifices. Thus, although results on any one cell gave a reproducible order of relative potencies of the four substances in the electrode, a different order of potencies sometimes emerged when the same substances were tested on another cell. When all four barrels of a composite electrode contained the same substance, the measured potencies varied by as much as 30 %. There appears to be no way of overcoming such difficulties and of thus rendering the technique more quantitatively reliable. By testing all substances repeatedly and in such a way that each was the object of many different four-way comparisons, the importance of the variations in cell-tip distances was minimized, but it must be emphasized that the observed relative potencies can only be approximate. Other factors which probably influence the potencies include the possibility of inactivation of the amino acids, during and after the period of ejection from the micropipette, by enzymic destruction or by interaction with tissue components.

Relative latencies of onset and durations of action of the substances following the commencement and termination respectively of their equi-effective electrophoretic currents were also determined in certain cases. Several factors are involved in the time of onset of excitant action and when four compounds were being compared, it was assumed that the time for diffusion from the electrode orifice to the neuronal membrane was similar for them all. However, when comparing the times necessary for the excitant molecules to diffuse to the receptor sites and to interact with them, it was necessary to be certain that there was minimal delay between the onset of the applying current and the ejection of the ions from the electrode tip. Since the application of a 'backing' voltage would reduce the amino-acid ion concentration within the orifice, comparisons of the onset of excitant action were made in the absence of such voltages. Several prolonged applications (20–30 sec) of each compound were initially made, following which the minimal times were determined between the onset of current flow and the attainment of the maximal rate of firing. For a comparison of the duration of action of compounds once the flow of current ceased, the appropriate backing voltage was immediately reapplied in order to reduce to a minimum the 'after diffusion' of the compound (del Castillo & Katz, 1957).

Experiments were also carried out upon Renshaw cells of lumbar segments of the spinal cord of the cat and upon neurones of the lateral geniculate nucleus (Curtis & Davis, 1962). In addition, some of the amino acids were applied to spinal motoneurones and the effect observed either by the recording barrel of the multibarrelled electrode (Curtis, Phillis & Watkins, 1960), or by recording the motoneuronal discharge in the appropriate ventral root.

Of the amino acids used in these investigations, DL-homocysteic acid, D- and L-glutamic acid, D- and L-aspartic acid, L-cysteic acid and L-2-amino-3-sulphinopropionic acid (L-cysteine sulphinic acid) were obtained from commercial sources and the rest were synthesized (Watkins, 1962). One sample of DL-α-amino-adipic acid was a gift from Dr J. G. Brockman of the American Cyanamid Co.

RESULTS

Table 1 lists 31 amino acids in groups of descending order of potency as excitants of spinal interneurones. An approximate group rating is given in each case, these having been obtained from the observed ratios of

equi-effective electrophoretic currents, with N-methyl-D-aspartic acid, the most potent excitant, given an arbitrary rating of 100. For example, in ten separate comparisons it was found that a current within the range 70–120 nA had to be passed through a barrel containing N-methyl-L-aspartic acid to cause the test interneurones to fire with the same frequency as when a current of 10 nA applied N-methyl-D-aspartic acid from another barrel of the same electrode. The L-isomer was thus estimated to be between one-seventh and one twelfth as strong as the D-isomer, and hence of potency 8–14. Substances which were not directly compared with N-methyl-D-aspartic acid were compared with other compounds of known potency relative to the standard. Within each group the amino acids are arranged in descending order of average potency, but, owing to the scatter of the results, little significance can be attached to this order other than that substances situated at the bottom of a group proved more frequently to be less potent than those at the top. The demarcation between groups from *F* to *I* is less well defined than that between the other groups.

TABLE 1. Order of potency of excitatory amino acids

General potency	Group	Group Rating	No.	Compound	Structure
Very strong to strong	*A*	100	1	N-Methyl-D-aspartic acid	$HO_2C.CH_2.CH(NHCH_3).CO_2H$
	B	50–80	2	D-Homocysteic acid	$HO_3S.(CH_2)_2.CH(NH_2).CO_2H$
	C	45–65	3	N-Methyl-DL-aspartic acid	See 1
	D	30–55	4	DL-Homocysteic acid	See 2
	E	15–50	5	N-Iminomethyl-D-aspartic acid	$HO_2C.CH_2.CH(NH.CH{=}NH).CO_2H$
			6	N-Ethyl-D-aspartic acid	$HO_2C.CH_2.CH(NH.CH_2CH_3).CO_2H$
	F	10–30	7	N-Ethyl-DL-aspartic acid	See 6
			8	DL-2-Amino-4-sulphino-*n*-butyric acid	$HO_2S.(CH_2)_2.CH(NH_2).CO_2H$
Strong to medium	*G*	8–20	9	L-Cysteic acid	$HO_3S.CH_2.CH(NH_2).CO_2H$
			10	L-2-Amino-3-sulphinopropionic acid	$HO_2S.CH_2.CH(NH_2).CO_2H$
			11	N-*n*-Propyl-D-aspartic acid	$HO_2C.CH_2.CH[NH(CH_2)_2CH_3].CO_2H$
			12	L-Homocysteic acid	See 2
			13	L-Glutamic acid	$HO_2C.(CH_2)_2.CH(NH_2).CO_2H$
			14	L-Aspartic acid	$HO_2C.CH_2.CH(NH_2).CO_2H$
			15	N-Methyl-DL-glutamic acid	$HO_2C.(CH_2)_2.CH(NHCH_3).CO_2H$
			16	N-Methyl-L-glutamic acid	See 15
			17	N-Methyl-D-glutamic acid	See 15
			18	N-Methyl-L-aspartic acid	See 1
Medium to weak	*H*	4–10	19	DL-2-Amino-5-sulpho-*n*-valeric acid	$HO_3S.(CH_2)_3.CH(NH_2).CO_2H$
			20	N,N-Dimethyl-DL-aspartic acid	$HO_2C.CH_2.CH[N(CH_3)_2].CO_2H$
			21	D-Glutamic acid	See 13
			22	D-Aspartic acid	See 14
			23	D-Cysteic acid	See 9
			24	N-Methyl-DL-cysteic acid	$HO_3S.CH_2.CH(NHCH_3).CO_2H$
			25	N-Methyl-DL-homocysteic acid	$HO_3S.(CH_2)_2.CH(NHCH_3).CO_2H$
Weak to very weak	*I*	0–6	26	N,N-Dimethyl-D-aspartic acid	See 20
			27	N-Iminomethyl-L-aspartic acid	See 5
			28	N-Ethyl-L-aspartic acid	See 6
			29	N-Methyl-L-cysteic acid	See 24
			30	DL-α-Aminoadipic acid	$HO_2C.(CH_2)_3.CH(NH_2).CO_2H$
Inactive	*J*	0	31	N-*n*-Propyl-L-aspartic acid	See 11

Structure-activity relationships are more apparent from Table 2, in which the compounds are arranged horizontally according to the extent of N-alkylation, and vertically, in three main sections, according to the type of ω-acidic group. The observations may be briefly summarized as follows:

Variation of ω-acidic groups

Replacement of the ω-carboxylic group of amino-alkane-α,ω-dicarboxylic acids by the sulphonic acid group had a very pronounced effect in some cases but not in others (Table 2, col. 1). Thus, the potencies of L-cysteic and L-homocysteic acids were not greatly dissimilar from those of L-aspartic and L-glutamic acids. Similarly D-aspartic and D-cysteic acids were similar in potency to each other, and both a little less effective than their L-enantiomers. On the other hand, a very marked difference was seen between D-glutamic acid and D-homocysteic acid, the latter being approximately ten times more potent than the former, and the second most powerful amino acid excitant yet encountered. The separate optical isomers of α-aminoadipic and of 2-amino-5-sulpho-*n*-valeric acids were not available for comparison, but the DL forms of these compounds were both only weak excitants, the dicarboxylic acid being practically inactive. These two compounds are both very much weaker than their respective lower homologues, DL-glutamic acid and DL-homocysteic acid, showing that, in both series, potency falls off sharply when the acidic groups are separated by more than three carbon atoms. The L-series of aminoalkane-α-carboxylic-ω-sulphonic acids parallels both the D and L series of amino alkane-α, ω-dicarboxylic acids, in that there appears to be relatively little difference in the potencies of the compounds having a 2-carbon atom connecting chain from those having a 3-carbon atom connecting chain, that is, between aminoethane and aminopropane derivatives in these three series. This is in contrast to the high and very sharp peak shown by D-homocysteic acid, the aminopropane derivative, in the D-series of aminoalkane-α-carboxylic-ω-sulphonic acids. Insufficient compounds were available in the aminoalkane-α-carboxylic-ω-sulphinic acid series for complete comparison with substances within the other two major groups, but it was evident that L-2-amino-3-sulphinopropionic acid was about equal in potency to both L-aspartic and L-cysteic acids, whilst DL-2-amino-4-sulphino-*n*-butyric acid was somewhat stronger than DL-glutamic acid but definitely weaker than DL-homocysteic acid.

N-Alkylation

Mono-N-methyl derivatives. In a previous study (Curtis & Watkins, 1960) it was reported that N-methyl-DL-aspartic acid, whilst having an action of longer duration than DL-aspartic acid, was nevertheless the weaker of the two excitants on mammalian neurones because it seemed to

TABLE 2. Variation of excitatory potency of acidic amino acids with chain length, optical form, type of ω-acidic group and N-alkylation

General type	Specific type	Row	Optical form	Specific compounds* and potency rating† from Table 1: Col. 1 $R_1{=}R_2{=}H$	Col. 2 $R_1{=}H$; $R_2{=}CH_3$	Col. 3 $R_1{=}H$; $R_2{=}CH{:}NH$	Col. 4 $R_1{=}H$; $R_2{=}C_2H_5$	Col. 5 $R_1{=}H$; $R_2{=}n\text{-}C_3H_7$	Col. 6 $R_1{=}R_2{=}CH_3$
Aminoalkane-α,ω-dicarboxylic acids	$n = 1$	1	D	**7** (22)	**100** (1)	**33** (5)	**33** (6)	**14** (11)	**3** (26)
$HO_2C.(CH_2)_n.CH.CO_2H$	Aspartic acid	2	L	**14** (14)	**14** (18)	**3** (27)	**3** (28)	**0** (31)	[**11**]
$\lvert$	group	3	DL	[**11**]	**55** (3)	[**18**]	**20** (7)	[**7**]	**7** (20)
NR_1R_2	$n = 2$	4	D	**7** (21)	**14** (17)				
	Glutamic acid	5	L	**14** (13)	**14** (16)				
	group	6	DL	[**11**]	**14** (15)				
	$n = 3$ α-Aminoadipic acid	7	DL	**3** (30)					
Aminoalkane-α-carboxylic-ω-sulphonic acids	$n = 1$	8	D	**7** (23)	[**11**]				
	Cysteic acid	9	L	**14** (9)	**3** (29)				
	group	10	DL	[**11**]	**7** (24)				
$HO_3S.(CH_2)_n.CH.CO_2H$	$n = 2$	11	D	**65** (2)					
$\lvert$	Homocysteic	12	L	**14** (12)					
NR_1R_2	acid group	13	DL	**43** (4)	**7** (25)				
	$n = 3$ 2-Amino-5-sulpho-*n*-valeric acid	14	DL	**7** (19)					
Aminoalkane-α-carboxylic-ω-sulphinic acids	$n = 1$ 2-Amino-3-sulphino-propionic acid	15	L	**14** (10)					
$HO_2S.(CH_2)_n.CH.CO_2H$									
$\lvert$									
NR_1R_2	$n = 2$ 2-Amino-4-sulphino-*n*-butyric acid	16	DL	**20** (8)					

* Numbers in parentheses relate to position of compound in Table 1. † The potencies given are the means of the values indicated in Table 1. Insertion of a potency between square brackets indicates that it is inferred from the potencies of the other modifications.

require the larger electrophoretic current to evoke the same frequency of interneuronal spike response. In the same communication it was reported that N-methyl-DL-glutamic acid was completely inactive. Following the observation (Curtis *et al.* 1961) that both these N-methyl derivatives, but more particularly the former, had a stronger action on neurones of the isolated toad spinal cord than had aspartic or glutamic acid, the earlier findings with respect to mammalian cells were re-investigated (Table 2, col. 2). In the case of N-methyl-DL-glutamic acid it was found that the gift sample previously used was one which had substantially cyclized into N-methyl-DL-pyroglutamic acid during removal of solvent of crystallization at elevated temperatures (Watkins, 1962). When new samples of N-methyl-DL-glutamic acid of analytical purity were used, it was established that the previous report of complete inactivity was incorrect and that in fact the methyl derivative was approximately equi-potent with DL-glutamic acid when applied to spinal interneurones. The reported weaker action of N-methyl-DL-aspartic acid, compared with aspartic acid, likewise proved erroneous. It is now apparent that in the more qualitative earlier experiments the durations of application were too brief, and insufficient time had been allowed for the maximal effect of N-methyl-DL-aspartic acid to become manifest. In the present investigation this substance was found to be about five times as potent as DL-aspartic acid.

This pronounced potency-increasing effect of N-methylation in the case of DL-aspartic acid was unique, in that it was not seen with other racemic excitants of potency equal to or greater than aspartic acid. Thus, N-methyl-DL-glutamic acid was found to be of similar potency to DL-glutamic acid, whilst N-methyl-DL-cysteic and N-methyl-DL-homocysteic acids were considerably weaker than the unmethylated parent compounds. It was necessary to establish whether there was any difference in the potencies of the two enantiomers of N-methylaspartic acid, and, following the synthesis of these compounds (Watkins, 1962), it was shown that the high potency of the racemic modification was due mostly to the D-isomer, which was about 10 times as potent as the L isomer. N-methyl-D-aspartic acid, being about 1·5 times as strong as D-homocysteic acid, was thus the most potent of the excitant amino acids on cat spinal interneurones, this result parallelling those upon neurones within the toad spinal cord (Curtis *et al.* 1961) and cat cerebral cortex (Curtis & Watkins, 1961). No such pronounced differences were observed in the potencies of the enantiomers of N-methylglutamic acid, nor in the comparative actions of N-methyl-L- and N-methyl-DL-cysteic acids. The separate enantiomers of N-methyl-homocysteic acid were not synthesized for comparison in view of the low potency of the racemate.

Other N-alkyl derivatives of aspartic acid. In view of the high potency of N-methyl-D-aspartic acid, it was of interest to determine the effects of other N-alkyl substituents, and, in particular, whether a marked enantiomeric difference was a characteristic feature of such compounds (Table 2, rows 1–3, cols. 3–6). N-Iminomethyl-D-aspartic acid proved to be only about one third as strong as the methyl analogue, but still considerably stronger than aspartic acid. In this case the higher potency of the D- over the L-form was even more marked than with the N-methyl derivatives. The two enantiomers of N-ethyl aspartic acid were of similar potency to the corresponding iminomethyl analogues, and thus also displayed a very large enantiomeric difference. N-*n*-Propyl-D-aspartic acid was considerably weaker than N-ethyl-D-aspartic acid, but had a very prolonged action following termination of its application, as is discussed in greater detail below. Again a marked enantiomeric difference was observed, the L-form of the propyl derivative being inactive. It was not possible to assess with any certainty the relative potencies of the D- and DL-forms of N,N-dimethylaspartic acid, both of which were only weak excitants.

Time factors in the onset and termination of amino-acid excitation

Previous investigations indicated that N-methyl-DL-aspartic acid continued to excite cells for several seconds following the termination of its electrophoretic application. This was in contrast to the very short duration of action of most of the other excitatory amino acids (Curtis, Phillis & Watkins, 1960; Curtis & Watkins, 1960). The present experiments revealed that the prolonged action was characteristic of mono-N-alkyl derivatives of aspartic acid, and that the effect increased with increase in the length of the N-alkyl chain.

Figure 1 illustrates the excitation of an interneurone by D-aspartic acid (*A*), N-methyl-D-aspartic acid (*B*), N-ethyl-D-aspartic acid (*C*) and N-*n*-propyl-D-aspartic acid (*D*). By repeated trials electrophoretic currents were found for each substance such that the neurone responded with the same maximal frequency of spike discharge. In the experiment illustrated these currents were continued until the maximal frequency had been maintained for a fixed period, after which the currents were terminated and replaced by currents in the reverse direction which, in themselves, were adequate to control diffusional escape from the individual barrels of the electrode. In this way the 'after-diffusion' of the compounds from the electrode was reduced to a minimum and differences in the durations of the excitant action of the compounds could be ascribed to differences in the time course of their interaction with membrane receptors. From Fig. 1 it is apparent that the descending order of potency,

based on the magnitudes of the equi-effective currents, is N-methyl-D-aspartic acid (rating 100), N-ethyl-D-aspartic acid (rating 42), N-*n*-propyl-D-aspartic acid (rating 13) and D-aspartic acid (rating 7), whereas the post-application durations of action are in the order N-propyl- > N-ethyl- > N-methyl- > unsubstituted D-aspartic acid. The actual durations varied considerably from cell to cell, but the order was always consistent. N-Alkyl derivatives of L-aspartic acid likewise had longer actions than the parent amino acid (although in these cases the derivatives were weaker than the unsubstituted substance), whilst other compounds with somewhat prolonged actions included D-homocysteic acid and DL-2-amino-4-sulphino-*n*-butyric acid. N-*n*-Propyl-D-aspartic acid had the longest duration of action of all the amino acids in the present series.

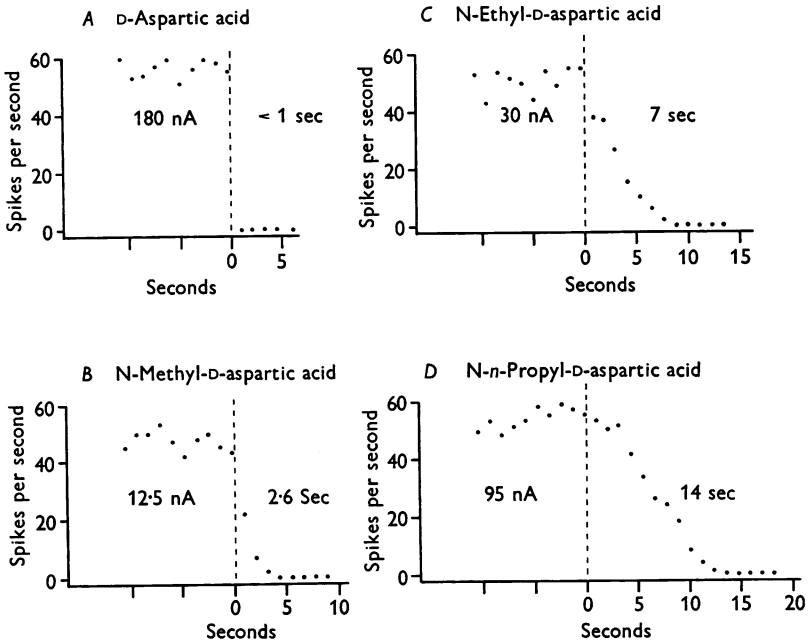

Fig. 1. Plottings of the firing frequency of an interneurone produced by the electrophoretic application of D-aspartic acid (*A*), N-methyl-D-aspartic acid (*B*), N-ethyl-D-aspartic acid (*C*) and N-*n*-propyl-D-aspartic acid (*D*). The spikes, counted for periods of 1 sec, were recorded by means of the central barrel of a 5-barrel electrode. The 'backing' voltage of 0·5 V which was applied to each amino-acid barrel (molar solutions) was, in this instance, adequate to prevent the neurone from being fired by the diffusional escape of any of the compounds. Each substance was applied, by means of the currents indicated, until a maximum frequency had been attained. The current was then terminated (vertical broken line and zero time) and the backing voltage immediately re-established. The time of cessation of firing is indicated to the right of the vertical line. *Abscissae*—time in seconds.

It was of interest to determine whether there was a relationship between the durations of onset and offset of amino acid action upon inter-neurones. For this comparison, substances of nearly equal potency were selected, so that similar electrophoretic currents could be used to obtain a particular frequency of spike response. The onset of the excitant action was measured from the beginning of the electrophoretic current until the final maximal frequency was attained. Figure 2 shows the time course of the development and cessation of firing of an interneurone in the presence of D-homocysteic acid (*A*) and N-iminomethyl-D-aspartic acid (*B*). D-Homocysteic

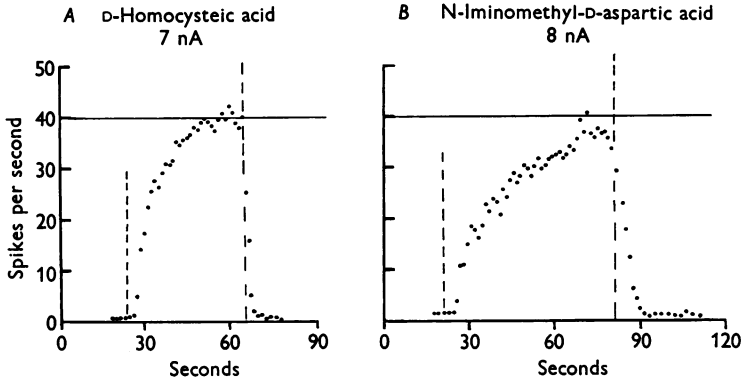

Fig. 2. The frequency of firing of an interneurone evoked by D-homocysteic acid (*A*) and N-iminomethyl-D-aspartic acid (*B*). The respective electrophoretic currents (7 and 8 nA) began and terminated as indicated by the vertical broken lines. In each case several prior applications had been made in the absence of retaining voltages but these voltages (0·5 V) were immediately applied on the termination of the electrophoretic currents in the test applications illustrated. *Ordinates*—the number of spikes counted over periods of 1 sec, there being 0·2 sec between counts. *Abscissae*—time in seconds.

acid, applied with a current of 7 nA, fired the cell at a final frequency of 39 spikes/sec and was therefore slightly more potent than the D-aspartic acid derivative (current, 8 nA; spike frequency, 36/sec). No backing potential had been applied to either barrel for about 5 min before these applications, and in each case several initial applications, each of 30 sec, were made, the last ceasing 15 sec before the test. At the cessation of each of these illustrated applications a backing potential of 0·5 V was immediately applied to the appropriate barrel. In the case of D-homocysteic acid, the maximal effect occurred approximately 28 sec after the onset of current flow, and firing had virtually ceased within 2 sec of its termination. On the other hand, the onset and offset times of N-iminomethyl-D-aspartic acid were 48 and 9 sec respectively. The number of compounds with which such a test could be performed was rather limited, but the

comparison of two such substances, several different interneurones being used, invariably showed that the one which required the longer time to evoke a certain spike frequency also had the longer post-application duration of action.

Non-specificity of amino-acid excitatory action

In order to investigate whether the actions of the more potent excitants of the present series were as non-specific as, for example, those of glutamic, aspartic and cysteic acids (Curtis, Phillis & Watkins, 1960) many of the substances were ejected not only near spinal interneurones but also near spinal motoneurones, Renshaw cells and cells of the lateral geniculate nucleus. Each of the substances excited all the cells on which it was tested: the particular tests which were tried are shown in Table 3. Although precise estimations of potency were not attempted for motoneurones, Renshaw cells or lateral geniculate neurones, the relative potencies of the active excitants appeared to be similar to those found when they

TABLE 3. Types of neurones tested

	Inter-neurones	Moto-neurones	Renshaw cells	Geniculate cells
N-Methyl-D-aspartic acid	+	+	+	+
N-Methyl-L-aspartic acid	+		+	+
N-Ethyl-D-aspartic acid	+		+	+
N-Iminomethyl-D-aspartic acid	+		+	+
N-*n*-Propyl-D-aspartic acid	+		+	+
D-Homocysteic acid	+			+
DL-Homocysteic acid	+	+	+	+
DL-2-Amino-4-sulphino-*n*-butyric acid	+		+	+
L-2-Amino-3-sulphinopropionic acid	+		+	

were tested upon spinal interneurones. One finding of interest was that both N-methyl-D-aspartic acid and DL-homocysteic acid were sufficiently potent to evoke the firing of motoneurones. The firing was detected both by the extracellular recording electrode in the same electrode assembly as the barrels containing amino acid, and by recording electrodes upon the segmental ventral root. Since for this effect it was necessary to apply N-methyl-D-aspartic acid with currents as large as 40–100 nA, it was not surprising that neither L-glutamic nor D-aspartic acid produced firing of these cells, even though currents in the region of 500 nA were used. However, it has been shown previously that both glutamic and aspartic acids do depolarize spinal motoneurones (Curtis, Phillis & Watkins, 1960) and their failure to fire these large cells can be ascribed to the limited area of membrane affected by the electrophoretic method of application.

DISCUSSION

The earlier survey of structure-activity relationships concerning the actions on cat spinal neurones of substances within the excitatory amino acid series (Curtis & Watkins, 1960) led to several conclusions, some of which may be revised and developed in the light of the present results. It was evident from the previous investigations that the main requirement for high excitatory activity lay in the possession by a compound of one amino and two acidic groups, the latter being separated from each other by a distance equal to that occupied by a chain of two to three carbon atoms, whilst the amino group should optimally be situated in the *alpha* position with respect to one of the acidic groups. It was also inferred, from observations on a limited number of compounds, that substituents attached to carbon atoms in the chain, or to the nitrogen atom of the amino group, lowered excitatory potency, and there was some evidence that sulphonic- and sulphinic-acid groups were interchangeable with carboxyl groups, at least at one end of the chain. Two pairs of epimeric excitants tested at that time showed little difference in the potencies of D and L forms. The present study has extended the range, in both the D and L series, of compounds containing sulphonic- and sulphinic-acid groups, and of those containing secondary and tertiary amino groups. Several newly synthesized compounds have been found to be of exceptionally high potency. These include D-homocysteic acid and the N-methyl, N-imino-methyl and N-ethyl derivatives of D-aspartic acid. The presence on this list of the latter three substances makes necessary a revision of the earlier conclusion that N-alkylation invariably reduces excitatory potency. Moreover, the marked difference in potency observed in enantiomeric pairs of the new substances indicates that the lack of similar enantiomeric difference observed earlier is not general.

The only previously examined excitant containing a sulphonic acid group was L-cysteic acid, which was found to be of potency similar to that of L-glutamic and L-aspartic acids (Curtis, Phillis & Watkins, 1960; Curtis & Watkins, 1960). The synthesis of D-cysteic acid, D-and L-homocysteic acid and DL-2-amino-5-sulpho-*n*-valeric acid (Watkins, 1962) permitted the completion of a series analogous to the aminoalkane-α, ω-dicarboxylic acids previously examined, and showed that peak activity was associated with a chain length of three carbon atoms between the two acidic groups, coupled with the D-configuration around the asymmetric carbon atom. The large difference in the excitatory potencies of D- and L-homocysteic acids contrasts with the small difference between D- and L-glutamic acids. In the two cases where comparisons could be made, the ω-sulphinic acid group proved to be less effective than the ω-sulphonic, but more effective

than the ω-carboxylic acid group. It is not known whether there are enantiomeric differences in potency in the ω-sulphinic acid series.

The N-alkyl-D-aspartic acids comprise the other group of compounds amongst which exceptionally high excitatory activity was found. The introduced alkyl group was optimally methyl, activity falling off as the methyl group was replaced by iminomethyl, ethyl and *n*-propyl groups. The latter compound, N-*n*-propyl-D-aspartic acid, was of potency only slightly higher than that of L- and D-aspartic acid, but, in contrast to the unsubstituted compounds, had a very long duration of action. In the L series, potency declined somewhat with the introduction of the methyl group and fell off sharply as the introduced alkyl group became larger, the *n*-propyl compound being inactive. N-Methylation of D- and L-glutamic acids, of DL- and L-cysteic acids, and of DL-homocysteic acid produced compounds of potency approximately equal to or lower than the parent compounds. The striking increase in potency following N-methylation of D-aspartic acid was thus unparalleled in any other case.

When tested upon spinal single interneurones the most potent substances, N-methyl-D-aspartic acid and D-homocysteic acid, were only 7–10 times stronger than aspartic, glutamic and cysteic acids. This contrasts with the very much larger difference in potency observed when these compounds were applied to the cerebral cortex to produce spreading depression (Curtis & Watkins, 1961). The explanation of this discrepancy probably lies in the non-specificity of amino-acid depolarization, each of the individual cells in an affected population being rendered more excitable and thus more likely to fire when excited by impulses from chemically or synaptically activated neurones in interconnected chains. Consequently, differences in the abilities of substances to depolarize individual neurones may be magnified to an extent which depends upon the size of the affected population and the complexity of the synaptic interconnexions. This could account for the approximately seven-hundredfold difference between the minimum concentrations of N-methyl-D-aspartic acid and of D- or L-aspartic acid which are required to produce spreading depression in the cerebral cortex of the cat (Curtis & Watkins, 1961).

It has been proposed that the amino-acid action on central neurones involves an initial interaction between the amino acids and specific regions of the external surface of the neuronal membrane which are important in permeability control (Curtis & Watkins, 1960). The present results support the conclusion that an amino-acid–surface interaction is involved and further elucidate the three-dimensional geometry of the site of action. The non-specificity of the excitatory action is in accord with earlier results and suggests that the site of action is a fundamental structural component of the membranes of all central neurones.

SUMMARY

1. A series of compounds structurally related to aspartic and glutamic acids has been applied electrophoretically into the extracellular environments of single neurones within the central nervous system of cats lightly anaesthetized with pentobarbital sodium.

2. The relative potencies of the substances as excitants of spinal interneurones were obtained by comparison of the electrophoretic currents of application which produced a standard frequency of spike response.

3. N-Methyl-D-aspartic acid and D-homocysteic acid were very much stronger excitants than all others previously tested under these conditions.

4. Other mono-N-alkyl derivatives of D-aspartic acid also had strong actions, these being in some cases quite prolonged. In particular N-*n*-propyl-D-aspartic acid continued to excite cells for many seconds following termination of its application.

5. The actions of the amino acids were non-specific with regard to the functional type of the neurones upon which they were tested.

6. The results are discussed in terms of the structure–activity relationships previously elucidated.

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