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The Specificity of Strychnine as a Glycine Antagonist in the Mammalian Spinal Cord

D.R. Curtis, A.W. Duggan and G.A.R. Johnston

Department of Physiology, Australian National University, Canberra (Australia)

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Summary. An investigation was made of the influence of strychnine on the depression of the firing of spinal interneurones and Renshaw cells by glycine, GABA, *nor*-adrenaline and 3-hydroxytyramine. Administered electrophoretically or intravenously, strychnine blocks the effect of glycine more readily than that of the other depressants. Such specific antagonism of glycine action by relatively low concentrations of strychnine may be competitive in nature, but technical difficulties precluded a full assessment of the type of antagonism. The effects of relatively high concentrations of strychnine on the action of the other depressants probably result from interference with membrane permeability changes. The findings are considered to support previous proposals that glycine is the transmitter at spinal strychnine-sensitive inhibitory synapses.

Key Words: Spinal neurones — Glycine — GABA — Strychnine

Strychnine, administered electrophoretically, suppresses the inhibitory action of glycine upon neurones of the spinal cord (Curtis, Hösli, Johnston, Johnston, 1968b; Curtis, Hösli and Johnston, 1968a; Davidoff, Aprison and Werman, 1969; Larson, 1969; de Groat, 1970), cerebral cortex (Curtis *et al.*, 1968a; Johnson, Roberts and Straughan, 1970), brain stem, including Deiters nucleus (Bruggencate and Engberg, 1969a; Hösli and Tebēcis, 1970; Curtis, Duggan and Felix, 1970), the oculomotor nucleus (Obata and Highstein, 1970) and the cuneate nucleus (Galindo, 1969).

The activity of all of these neurones is also depressed by electrophoretically administered gamma-aminobutyric acid (GABA), but it has been a common finding that strychnine more readily suppresses the effects of glycine than those of GABA. Indeed, the degree of selectivity of strychnine as a glycine antagonist has been considered to be reasonably high (Curtis *et al.*, 1968a), since concentrations of the alkaloid exceeding those which were adequate to abolish the just-maximal inhibitory action of glycine did not diminish the action of equally effective amounts of GABA upon spinal neurones. Similarly, in the brain stem and cerebral cortex, it has proved possible to block with strychnine near-maximal inhibition of neurones by glycine without reducing the effects of GABA (Bruggencate and Engberg, 1969a; Hösli and Tebēcis, 1970; Johnson *et al.*, 1970)

These findings have suggested that strychnine interacts in a relatively specific fashion with membrane sites at or near glycine receptors (Curtis *et al.*, 1968a;

Curtis, 1969; see also Larson, 1969). In contrast, an investigation of the influence of strychnine on the dose response curves relating the inhibition of firing of spinal neurones to the electrophoretic currents ejecting glycine and GABA has resulted in the proposal that strychnine is a non-competitive antagonist of both glycine and GABA, and that the degree of selectivity of strychnine as an antagonist of the effects of these amino acids is relatively low (Davidoff *et al.*, 1969). Such a proposal raises doubts regarding the relevance of amino acid antagonism by strychnine to the function of glycine as a transmitter at strychnine-sensitive inhibitory synapses (Curtis *et al.*, 1968a, b). Consequently we have re-investigated the influence of strychnine on the depression of spinal neurones by glycine, GABA, *nor*-adrenaline (NA) and 3-hydroxytyramine (dopamine, DA), all of which have been considered as possible spinal inhibitory transmitters. In view of the difficulties inherent in the microelectrophoretic method of drug administration, strychnine has been administered intravenously in some experiments.

A preliminary report of these findings has been published (Curtis, Duggan and Johnston, 1969).

Methods

The experiments were performed on spinal interneurones and Renshaw cells of lumbar segments of cats (see Curtis and Watkins, 1960). The majority of animals were anaesthetised with pentobarbitone sodium (35 mg/kg intraperitoneally, initially); some animals were decerebrated by coagulation of the brain stem during halothane anaesthesia (Crawford and Curtis, 1966), and were unanaesthetised during the experiment. All spinal cords were transected at the lower thoracic level, and the temperature of the animals was maintained between 37—38°C. In experiments in which strychnine was administered intravenously the animals were paralysed with gallamine triethiode and artificially respired, the end-tidal CO_2 levels being maintained between 4.0—4.5%.

Extracellular action potentials (positive-negative, 100—500 μV in amplitude) were recorded by means of the centre barrel (4 M NaCl) of seven barrel micropipettes of tip diameter 4—6 μm. The potentials were monitored on an oscilloscope, and the rate of firing of neurones was displayed continuously on a rectilinear ink-writing paper recorder. The effects of depressant substances were assessed on the spontaneous firing of neurones, or, when necessary, on firing resulting from continuous chemical stimulation by DL-homocysteate (interneurones and Renshaw cells) or acetylcholine (Renshaw cells).

The outer barrels of the micropipettes contained aqueous solutions of the following molarity, adjusted to the stated pH with the listed alkali or acid: acetylcholine (ACh, M, chloride); glycine (0.5 M, pH 3, HCl), gamma-aminobutyrate (GABA, 0.5 M, pH 3, HCl); *nor*-adrenaline (NA, 0.2 M, bitartrate); 3-hydroxytyramine (dopamine, DA, 0.2 M, HCl); DL-homocysteate (DLH, 0.2 M, pH 8, NaOH); strychnine (hydrochloride, 2 mM or 10 mM in 165 mM NaCl).

The drugs were administered electrophoretically by appropriately directed electrical currents, cationic (+) or outwardly directed currents ejecting cations, anionic (—), anions. The magnitudes of currents are expressed in nano-amps (nA, 10^{-9} A), zero (0) current indicating removal of the retaining current which was usually chosen to produce a retaining voltage of 0.5 V for each drug-containing barrel (see Curtis, 1964). Strychnine and each depressant were administered for sufficient periods of time to attain maximal effects for the particular ejecting currents. Thus drug effects were studied under equilibrium conditions. Furthermore, care was taken to assess the action of depressants before and during the action of strychnine upon a constant firing rate: a constant "dose" of depressant invariably produced *less* percentage depression as the basal firing rate of a neurone increased, contrary to the observation of Johnson *et al.* (1970).

Difficulties associated with the study of dose-response relationships, and the effects of drug antagonists upon them, are discussed in the Appendix of this paper.

Results

The Effect of Strychnine on the Glycine Dose-Response Curve. Electrophoretic Administration

Although dose-response relationships were studied for both glycine and GABA primarily to determine the specificity of strychnine as an amino acid antagonist over a range of amino acid concentrations, it was hoped that the effect of strychnine on such curves would provide evidence for the nature of the antagonism between the alkaloid and glycine. The difficulties outlined in the Appendix limited the number of observations which could be made for each curve, and detailed results were obtained with only 5 interneurones and 2 Renshaw cells. These indicated that relatively low concentrations of electrophoretically administered strychnine displaced the glycine dose-response curve to the right, in a roughly parallel fashion,

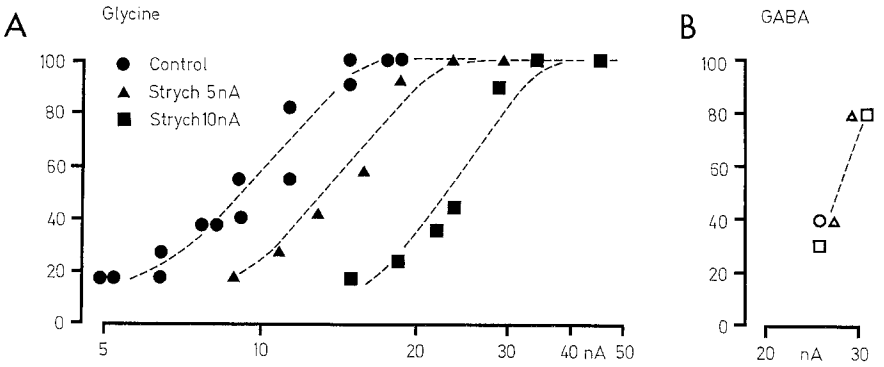

Fig. 1. *Effect of strychnine on the sensitivity of a spinal interneurone to glycine and GABA.* The firing rate of the neurone was maintained at a frequency of approximately 60 spikes per second by continuously administered DLH, and the dose-response curves relate percentage inhibition of firing (*ordinate*) to the electrophoretic current (logarithmically plotted as *abscissae*) used to eject the depressant amino acids — see text. Filled symbols (A), glycine; corresponding hollow symbols (B), GABA. Circles, control observations; triangles, during the electrophoretic ejection of strychnine (+5 nA, 2 mM in 165 mM NaCl); squares, during the administration of strychnine with a current of +10 nA

without reducing the plateau level, and with little or no change in the slope of the straight line portion of the curve. Contrary to the observations of Davidoff *et al.* (1969), increased amounts of glycine could "overcome" the decrease in maximal depression produced by strychnine.

Results from one interneurone are plotted in Fig. 1A. Firing was maintained at approximately 60 spikes per second by continuously administered DLH (—15 nA), and the response (ordinate) is the percentage decrease of this rate produced by the administration of glycine for a period of 10—30 sec, until maximum inhibition was achieved for that particular ejecting current. Glycine was retained within the micropipette by a current of —9 nA (anionic). As the rate of firing was not enhanced by increasing this current, and reduction to —4 nA resulted in approximately 20% inhibition prior to the administration of strychnine, zero "dose" was taken as —9 nA (anionic) and ejecting currents (dose, abscissa) were measured

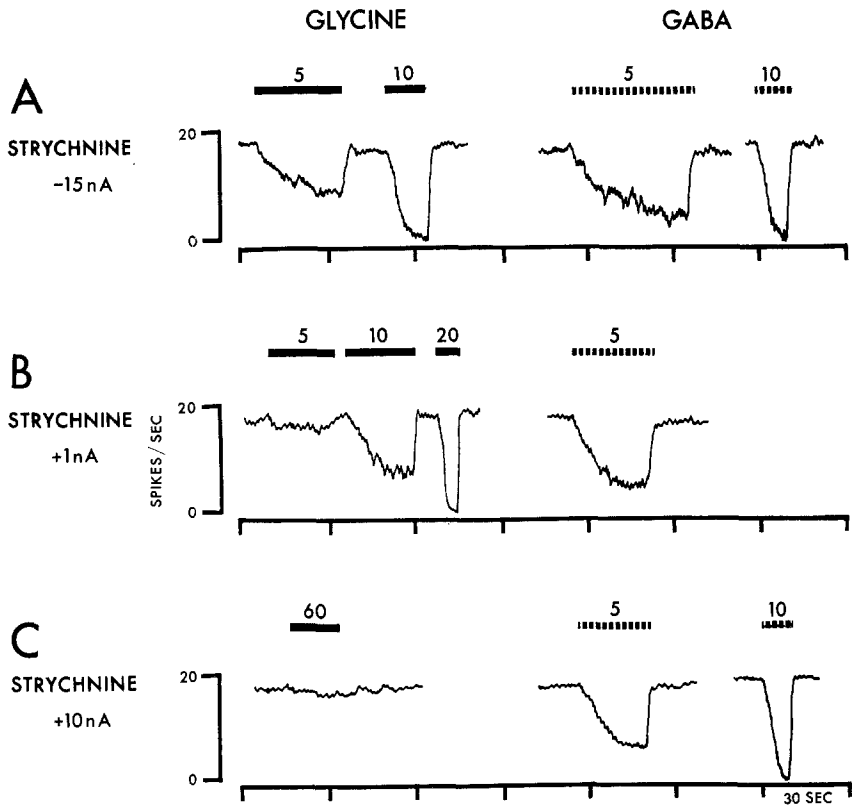

Fig. 2. *The effect of electrophoretically administered strychnine on the inhibition of a spinal interneurone by glycine and GABA.* The cell was firing spontaneously at a rate of 18—20/sec. *Ordinates:* spikes/sec. *Abscissae:* time, 30 sec. Electrophoretic ejections of amino acids are indicated by horizontal markers: solid line, glycine; broken line, GABA; the figures indicate cationic electrophoretic currents (nA). A. Before the administration of strychnine (10 mM in 165 mM NaCl, retaining current —15 nA). B, C — during the administration of strychnine with currents of +1 and +10 nA respectively

relative to this value. Thus an actual cationic ejecting current of +10 nA is indicated as a "dose" of 19 nA. Three curves are plotted in Fig. 1A, from observations made before (filled circles), and during the electrophoretic administration of strychnine (2 mM in 165 mM NaCl, retaining current —12 nA) using currents of +5 nA (filled triangles) and +10 nA (filled squares). In each case strychnine was ejected for at least 6 min in order to reach equilibrium conditions, and the overall effect was to increase the "dose" of glycine required to halve the firing rate of this neurone by a factor of 2.7. If extracellular concentrations of glycine and strychnine attained during electrophoretic ejection can be compared on the basis of current ejecting these cations, and if the amount of current administering strychnine is approximately 2/165 of the current passed through the strychnine-NaCl mixture, then the concentration of strychnine (approx. 1/8 nA) required for this almost threefold reduction in glycine sensitivity was less than 1% of the initial glycine concentration which produced complete suppression of firing (approx. 15 nA).

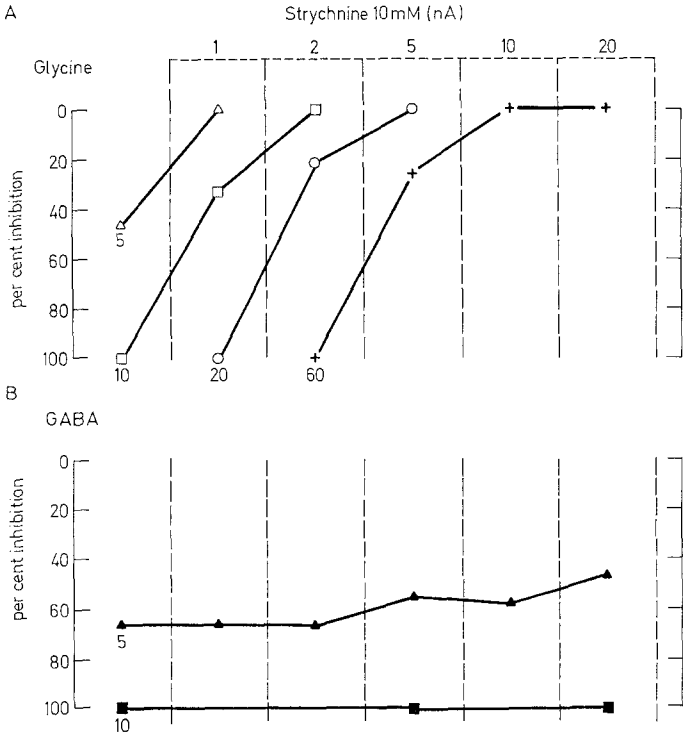

Fig. 3. *Graphical presentation of results obtained from the neurone of Fig. 2. Ordinates:* percentage inhibition of firing. *Abscissae:* cationic current ejecting strychnine. A. Glycine ejected with different currents, +5 nA (open triangles), +10 nA (open squares), +20 nA (open circles), +60 nA (crosses). B. GABA ejected with currents of +5 nA (closed triangles) and +10 nA (closed squares)

The interaction between strychnine and glycine upon another interneurone is illustrated in Figs. 2 and 3, Fig. 2 being a tracing of records from which the points of Fig. 3 were derived. The cell was firing spontaneously at a rate of 18—20 spikes per sec (Fig. 2), and prior to the administration of strychnine (Fig. 2A), glycine ejected with a current of +10 nA (retaining current —10 nA) was just adequate to suppress completely this firing. The response to this amount of glycine (and to GABA) was slow because of the relatively large retaining current. Approximately 48% inhibition followed the ejection of glycine with a current of +5 nA. Strychnine, having been retained with an anionic current of 15 nA, was then administered with cationic currents which were increased in a step-wise manner (Fig. 3A) from a micropipette containing 10 mM strychnine hydrochloride and 165 mM NaCl. Each "dose" level of strychnine was maintained for 5—6 min, and the amount of glycine required for just-maximal inhibition was then determined, together with an amount producing submaximal inhibition. Tracings of the effects of strychnine, +1 nA and +10 nA, are reproduced in Fig. 2B and C respectively, and the results of the full series are plotted in Fig. 3A. The maximum current which could be passed through the glycine micropipette was +60 nA, and although the number of observations made was limited, the findings are consistent with those of Fig. 1,

namely a displacement of the dose-response curve to the right by low concentrations of strychnine, rather than a diminution of the maximum effect of glycine. On a comparison of electrophoretic currents, the concentration of strychnine abolishing the depressant effect of glycine (60 nA) was approximately 1% of that of glycine.

Similar results were obtained with all neurones so studied; in some, recovery with restoration of the dose-response curve was also observed. In each case the reduction by strychnine of inhibition of firing by a particular "dose" of glycine could be restored by increasing the current used to eject the amino acid. However the maximum current which could be passed through glycine-containing barrels of 60—100 MΩ resistance was often limited to 60—80 nA, and thus reliable information could not be obtained regarding the influence of relatively high concentrations of strychnine.

Intravenous Administration

Several experiments were carried out to determine the effect of intravenously administered strychnine on the glycine dose-response curve. Since strychnine administered in this fashion generally increased the spontaneous firing rate of spinal neurones, and the sensitivity to excitants, control observations were made on the firing of neurones by DLH at rates slightly exceeding the spontaneous rate. Subsequent to the injection of strychnine, the amount of DLH ejected was then reduced to maintain a rate of firing similar to that of the control period. Results from one experiment are illustrated in Fig. 4A and B. The firing rate of a spinal interneurone was maintained at a frequency of 25 spikes per sec, and two doses of strychnine hydrochloride (each of 0.1 mg/kg) were administered intravenously. Such doses of strychnine, which would be expected to reduce but not abolish the synaptic inhibition of spinal neurones (Bradley, Easton and Eccles, 1953), clearly displaced the glycine dose response curve to the right. Throughout the period of observation the amplitude of the extracellularly recorded action potentials was not altered. These doses of strychnine (Fig. 4B) and even an additional 0.2 mg/kg (not illustrated) did not modify the sensitivity of the neurone to GABA.

In another experiment, glycine (0 nA) halved the spontaneous rate of firing of an interneurone (30—40 spikes/sec). This effect was abolished after the administration of 0.2 mg/kg of strychnine hydrochloride. The reduction of the frequency of firing of a Renshaw cell from 80 spikes/sec to zero by glycine (+4 nA) was also effectively diminished in another animal by the same dose of strychnine, the rate being reduced from 80—90 spikes/sec to only 60—70 spikes/sec. Similar results were obtained in two other experiments.

The Effect of Strychnine on the Depression of Spinal Neurones by GABA, NA and DA

Since strychnine reduces the magnitude of inhibitory postsynaptic potentials recorded from spinal motoneurones (see Curtis, 1963), and must thus be considered as a possible antagonist of an inhibitory transmitter, the effects of strychnine should be determined on the postsynaptic action of substances known to hyperpolarize spinal neurones. These are glycine and GABA (Curtis *et al.*, 1968b; Bruggencate and Engberg, 1968; Larson, 1969), and *nor*-adrenaline (Engberg and Ryall, 1966; Biscoe and Curtis, 1966; Biscoe, Curtis and Ryall, 1966; Weight and

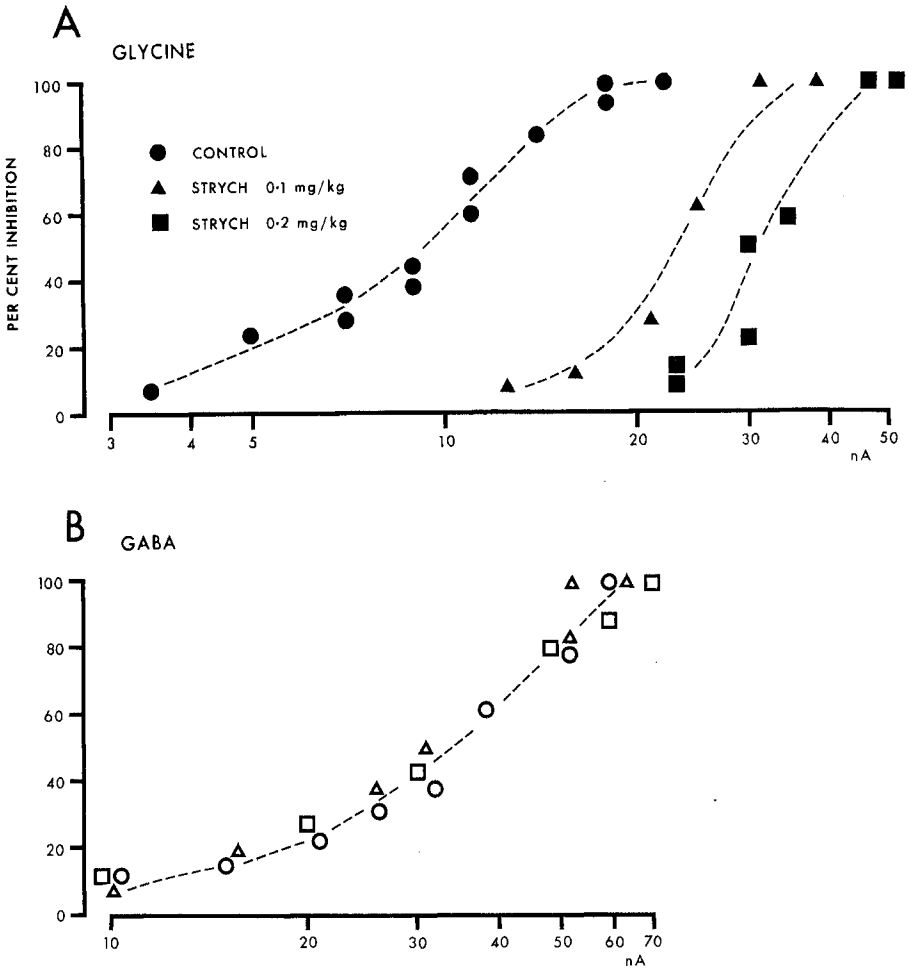

Fig. 4. *Dose-response curves for the depression of firing of an interneurone by glycine and GABA.* A. Glycine (filled symbols); B. GABA (open symbols): before (circles), 20 min after the intravenous injection of strychnine hydrochloride, 0.1 mg/kg (triangles) and 20 min after a second dose of strychnine, 0.1 mg/kg (squares) administered 25 min after the first. The rate of firing of this neurone was maintained at approximately 25 spikes per second by adjusting the current used to eject DLH. Currents of —7 nA and —12 nA retained glycine and GABA respectively within the micropipettes, and the cationic ejecting currents (*abscissae*) are measured from these values

Salmoiraghi, 1966, 1967; Curtis, 1968; Phillis, Tebēcis and York, 1968; Bruggencate and Engberg, 1969b). In addition, dopamine (DA) has also been considered as a possible spinal inhibitory transmitter (McLennan, 1961; Biscoe *et al.*, 1966).

Detailed observations of the effect of strychnine on glycine and GABA dose-response curves were possible with only a few neurones. The most usual experiment in both anaesthetised (27) and decerebrate non-anaesthetised (5) cats concerned the effects of strychnine on "doses" of glycine and GABA which were just adequate to suppress spontaneous or chemically evoked firing, and "doses" which had submaximal effects (30—60% inhibition).

One comparison of dose-response curves is illustrated in Fig. 1A and B. The electrophoretic ejection of strychnine (2 mM) with a current of +10 nA displaced the glycine curve to the right (Fig. 1A). In contrast, the "doses" of GABA required for approximately 45% and 85% inhibition were not altered (Fig. 1B). In the case of the neurone illustrated in Figs. 2 and 3, administration of strychnine (10 mM, +20 nA) abolished the depressant effect of a "dose" of glycine six times that initially required for just-total inhibition, whilst apparently reducing the effect of +5 nA of GABA from approximately 66% inhibition to 47% inhibition: there was no appreciable reduction in the rate of onset of depression by GABA. Throughout the period of strychnine administration +10 nA of GABA was sufficient to suppress completely the firing of this particular interneurone (Fig. 3B).

Results are illustrated in Fig. 5 from another neurone upon which the effects of glycine, GABA, DA and NA were tested. The firing of this interneurone was maintained in the range of 30—40 spikes per second by the continuous electrophoretic ejection of DLH, initially —10 nA. During the control period (Fig. 5A), this firing was just abolished by GABA +5 nA, and was reduced to 50% by +2 nA. Glycine, ejected with a current of +3 nA reduced the rate of firing by approximately 70%.

Strychnine was then administered for 27 min with step-wise increasing currents from a solution of 2 mM strychnine hydrochloride in 165 mM NaCl, and the figure includes tracings of the effects of the depressants 2—6 min after these currents were changed. The effect of glycine +3 nA was abolished during the ejection of strychnine by a current of +10 nA (Fig. 5B), yet depression by GABA was unaltered. However, further increases in the strychnine current resulted in a small diminution of the effectiveness of GABA such that a current of +5 nA was required to achieve 50% inhibition when strychnine was being ejected with +80 nA (Fig. 5E), at which time less than 20% inhibition followed the administration of glycine with a current of +80 nA. At this stage, and even earlier (Fig. 5B, C and D), the onset of GABA depression was slowed, but care was taken in experiments such as this to administer GABA for several successive periods in order to exclude infrequent ejection from a pipette having a slightly excessive retaining current as a factor in producing slow responses.

Following the termination of the strychnine administration, the depressant action of GABA recovered almost completely within 2—3 min (Fig. 5F), whereas recovery of that of glycine was still incomplete after 17 min. When strychnine was subsequently administered with a current of +160 nA, 22 min after the previous test (not illustrated), the firing rate of the neurone was increased and the amplitude of the action potential reduced. Glycine administered with a current of +40 nA

Fig. 5. *Effect of strychnine administered electrophoretically on the depression of the firing of a spinal interneurone by glycine, GABA, dopamine and nor-adrenaline.* Firing was maintained throughout by the continuous ejection of DLH from one barrel of a seven barrel micropipette. Electrophoretic ejections (cationic currents) are signalled by horizontal lines, currents being expressed in nA: GL, glycine; GA, GABA; DA, dopamine; NA, *nor*-adrenaline. A. Control observations. B. During the administration of strychnine with a current of 10 nA for $4^1/_2$ min. (2 mM solution in 165 mM NaCl). C. Strychnine 20 nA (total time 7 min). D. Strychnine 40 nA (8 min). E. Strychnine 80 nA ($7^1/_2$ min). F. Two minutes after the current ejecting strychnine was terminated. *Ordinates:* spikes/sec. *Abscissae:* time, minutes

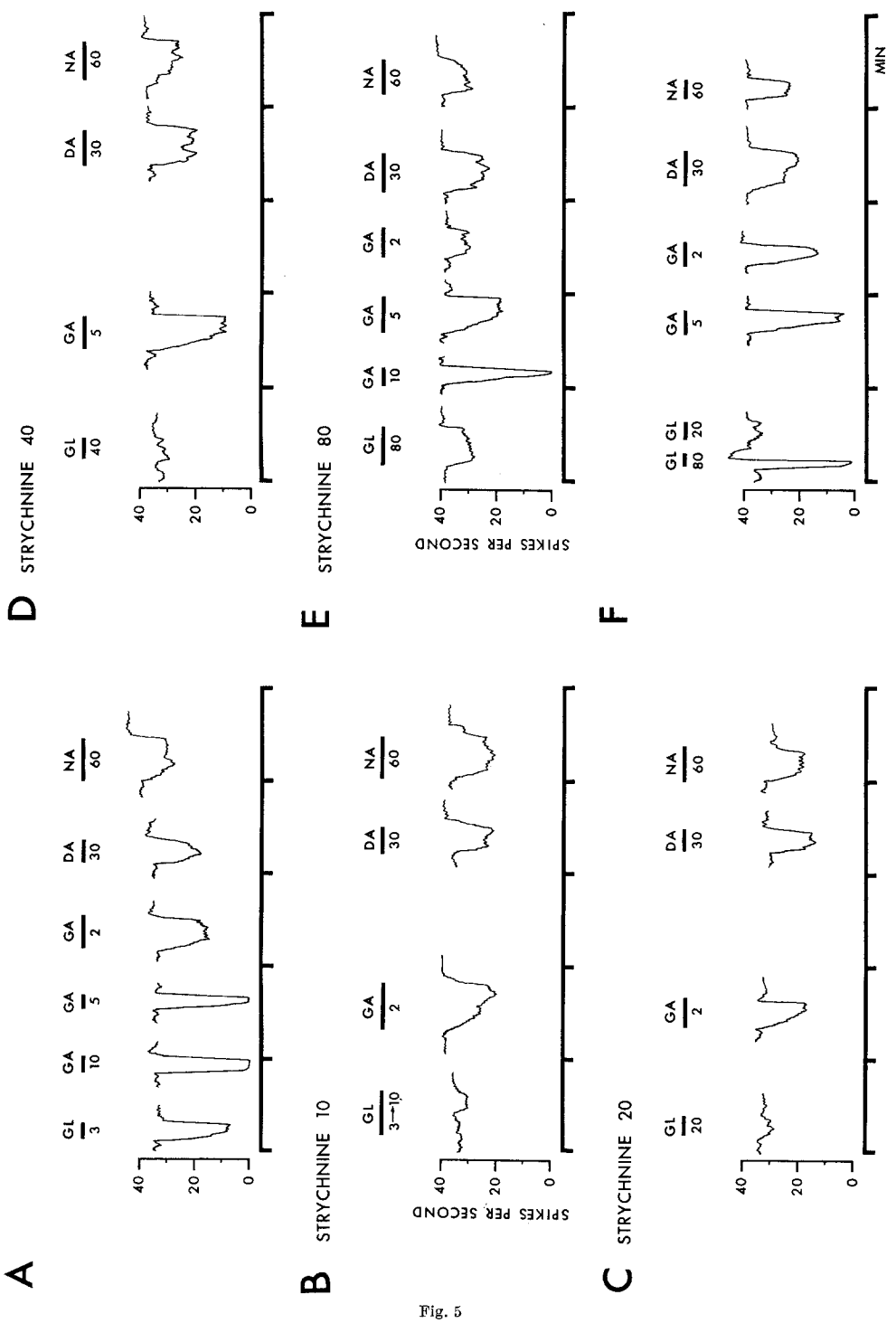
A
GL 3
GA 10
GA 5
GA 2
DA 30
NA 60
B
STRYCHNINE 10
GL 3→10
GA 2
DA 30
NA 60
C
STRYCHNINE 20
GL 20
GA 2
DA 30
NA 60
D
STRYCHNINE 40
GL 40
GA 5
DA 30
NA 60
E
STRYCHNINE 80
GL 80
GA 10
GA 5
GA 2
DA 30
NA 60
F
GL GL 80 20
GA 5
GA 2
DA 30
NA 60
SPIKES PER SECOND
40
20
0
MIN

Fig. 5

did not alter the rate of firing; the depressant effect of GABA +5 nA was reduced from 100% to approximately 10%, but complete inhibition of firing followed ejection of this amino acid with a current of +20 nA. Furthermore this "dose" of strychnine also reduced the effects of DA and NA, although the earlier lower concentrations of the alkaloid had little or no influence on the effects of these amines (Fig. 5B—E).

Of the total number of spinal neurones tested (27 interneurones, 26 Renshaw cells), strychnine administered in amounts adequate to suppress completely the depressant action of glycine (100% or 30—60% inhibition), reduced the action of initially equally effective amounts of GABA only on 3 neurones, and by less than 20%. When the concentrations of strychnine were increased, the action of GABA on 2 additional cells was reduced and slowed in onset, but was not abolished.

Several factors interfered with a complete assessment of the degree of selectivity of strychnine as an antagonist of glycine and GABA. Currents exceeding +80 nA could not be readily passed through glycine-containing micropipettes, and hence an estimate could not be made of how many times the "dose" of glycine needed to be increased in the presence of strychnine. Secondly, the direct excitation of neurones by relatively high concentrations of strychnine, and the depression of firing with alterations of the amplitude and shape of the action potentials by even higher concentrations, frequently limited the current used to eject this alkaloid, and hence presumably reduced the possibility of influencing the action of GABA. Thus, whilst it was clear in all experiments that strychnine was a highly specific glycine antagonist, the specificity could not be defined numerically with any great degree of confidence. Many cells were found in which a concentration of strychnine 15—20 times that abolishing the action of glycine was without effect on the action of GABA. For example, the firing of one interneurone was depressed to approximately 20% of the initial rate by glycine +5 nA and GABA +30 nA. The effect of glycine was abolished when strychnine was ejected with +5 nA from a 2 mM solution. However, the depressant action of GABA remained unaltered when strychnine was administered from a 10 mM solution in another barrel of the micropipette with a current of +80 nA. If it be assumed that a given electrophoretic current ejects approximately five times more strychnine from a 10 mM solution than from a 2 mM solution (both in 165 mM NaCl), in this particular experiment strychnine would be more than 80 times less effective as a GABA antagonist than as a glycine antagonist.

Intravenously administered strychnine was also tested as a possible GABA antagonist. In the case of the cell illustrated in Fig. 4A and B, 0.2 mg/kg of strychnine hydrochloride displaced the glycine dose-response curve to the right, reducing the effect of glycine by a factor of approximately 3 (Fig. 4A) without influencing the depressant action of GABA (Fig. 4B). In another experiment 0.8 mg/kg of strychnine hydrochloride, which reduced the effect of glycine upon an interneurone by a factor of 5, assessed on the "dose" of glycine for 50% inhibition, did not modify the effects of GABA. Larger doses of strychnine could not be administered because of changes in the excitability and firing pattern of the neurone being studied. Hence experiments of this type, while clearly establishing a difference between the susceptibility to strychnine of the effects of glycine and GABA, were not suitable for estimating the degree of specificity.

Difficulties were also experienced in determining the relative activity of strychnine as an antagonist of the depressant effects of the amines. In some instances, as illustrated in Fig. 5, no clear antagonism was demonstrated with concentrations of strychnine which did not alter cell excitability and which exceeded those adequate to suppress the effects of glycine. Observations of this type were made with both NA (3 interneurones, 5 Renshaw cells) and DA (3 interneurones). On the other hand the depressant action of NA on 7 Renshaw cells (4 decerebrate cats) could be reduced by concentrations of strychnine which, whilst not affecting the action of GABA, abolished that of initially equally effective "doses" of glycine. After the termination of the strychnine ejecting current the effect of NA usually recovered more rapidly than that of glycine.

Discussion

Although a variety of effects of strychnine on neuronal membranes have been described (see Ajmone-Marsan, 1969), the investigations have been performed on a number of different tissues and many may not be relevant to the hyperexcitability produced in mammals by doses of the alkaloid of the order of 0.01—0.2 mg/kg, doses which if uniformly distributed in body water, produce concentrations of the order of 2.5×10^{-8}—5×10^{-7} M.

Strychnine increases the excitability of nerve fibres, and in higher concentrations has a stabilizing effect similar to that of "local" anaesthetics (Peugnet and Coppée, 1936; Heinbecker and Bartley, 1939; Coppée and Coppée-Bolly, 1941; Erlanger, Blair and Schoepfle, 1941; Maruhashi, Otani, Takahashi and Yamada, 1956; Alving, 1961; Curtis and Ryall, 1966; Sabelli and Gorosito, 1969). Complex effects have been observed on the ionic permeability of neuronal membrane (Washizu, Bonewell and Terzuolo, 1961; Araki, 1965; Pollen and Ajmone-Marsan, 1965; Stefanis and Jasper, 1965; Pollen and Lux, 1966), and on the operation of cholinergic synapses (Fromherz, 1933; Bouman, 1937; Lanari and Luco, 1939; Alving, 1961; Takagi and Takayanagi, 1966; Neal, 1967; McKinstry and Koelle, 1967; Landau, 1967). Electrophoretically administered strychnine excites, and higher concentrations depress, many different types of central neurone including Purkinje cells (Crawford, Curtis, Voorhoeve and Wilson, 1966), cortical neurones (Krnjević, Randić and Straughan, 1966; Biscoe and Curtis, 1967; Phillis and York, 1967; Johnson *et al.*, 1970), thalamic neurones (Phillis and Tebēcis, 1967) and spinal neurones (Curtis, 1967; Curtis *et al.*, 1968a, b). Depression is usually accompanied by a reduction in the amplitude of extracellularly recorded action potentials. The direct excitation of neurones is presumably the basis of strychnine neuronography (see Chang, 1951; Frankenhaeuser, 1951; Wall and Horwitz, 1957).

In microelectrophoretic experiments both excitation and depression usually occur with strychnine concentrations exceeding those required to reduce the sensitivity of neurones to glycine and to reduce spinal inhibitory postsynaptic potentials (Curtis, 1962; Curtis *et al.*, 1968a, b). Moreover, subconvulsive doses of strychnine which diminish the amplitude of intracellularly recorded recurrent and "direct" inhibitory potentials of spinal motoneurones neither depolarize these cells, alter the membrane resistance nor influence excitatory synaptic transmission (Eccles, Fatt and Koketsu, 1954; Coombs, Eccles and Fatt, 1955; Curtis, 1962;

Fuortes and Nelson, 1963; Araki, 1965; Curtis *et al.*, 1968a, b; Larson, 1969). It thus seems reasonable to interpret the effect of relatively low concentrations of strychnine on inhibition in the spinal cord (Bradley *et al.*, 1953) and medulla (Morimoto, Takata and Kawamura, 1968; Kidokoro, Kubota, Shuto and Sumino, 1968) in terms of a relatively specific action at inhibitory synapses, an effect which satisfactorily accounts for the subsequent hyperexcitability and widespread disturbance of the nervous system.

A number of mechanisms have been proposed by which strychnine could interfere with the operation of certain spinal inhibitory synapses, and discussion of these is relevant to the present and past observations of antagonism between strychnine and glycine. When administered close to motoneurones, strychnine suppresses inhibitory potentials generated by impulses in a number of different spinal pathways (Curtis, 1962). Furthermore strychnine does not affect the firing of inhibitory interneurones of either the "direct" or recurrent pathways to motoneurones (Eccles *et al.*, 1954; Curtis, 1962, 1967; Larson, 1969), and thus rather than interfering with inhibitory processes by blocking the firing of inhibitory interneurones, strychnine acts in the vicinity of inhibitory synapses. Suppression of transmitter synthesis seems unlikely since the latency of strychnine action is extremely short, and although evidence has been presented that strychnine alters the excitability of primary afferent terminals within the spinal cord (Wall, McCulloch, Lettvin and Pitts, 1955; also Curtis and Ryall, 1966), a difference between the properties of preterminal excitatory and inhibitory fibres would need to be demonstrated in order to explain the selective effect of strychnine upon inhibitory transmission. No direct evidence has been obtained to support proposals that strychnine interferes with the cholinergic excitation of inhibitory nerve terminals, a process suggested as necessary for transmitter release (McKinstry and Koelle, 1967); in fact concentrations of strychnine adequate to suppress the inhibition of spinal Renshaw cells facilitate transmission at the cholinergic axon collateral terminals which excite these neurones (Curtis, 1967).

Strychnine may reduce the amount of transmitter released from spinal inhibitory nerve terminals, but such a proposition becomes less tenable when considered in relation to neurochemical and physiological evidence that glycine could be the transmitter at these inhibitory synapses (Werman, Davidoff and Aprison, 1968; Curtis *et al.*, 1968a, b; Aprison and Werman, 1968; Curtis and Johnston, 1970; Hebb, 1970). Since both glycine and the inhibitory transmitter at synapses affected by strychnine appear to produce an identical change in postsynaptic membrane permeability, it seems reasonable to conclude that glycine receptors on spinal neurones could be identical with those activated by the transmitter, and that strychnine interferes with the action of glycine and the transmitter by a common mechanism. Although strychnine could combine with glycine on a molecular basis, effectively reducing the subsynaptic concentration of the amino acid, the inhibitory action of glycine is blocked by much lower concentrations of the alkaloid, generally of the order of less than 1% if extracellular concentrations can be compared on the basis of measured electrophoretic currents. Furthermore, physicochemical studies *in vitro* have provided no evidence for the existence of stable glycine-strychnine complexes other than simple salts. Thus it seems likely that strychnine, rather than reducing the effective concentration of glycine in the

immediate vicinity of receptors, has an action confined to the postsynaptic inhibitory membrane.

Several observations suggest that concentrations of strychnine adequate to reduce spinal postsynaptic inhibition, and the effects of glycine, do not modify the *nature* of the ionic conductance change induced in the postsynaptic membrane. Thus both hyperpolarizing and depolarizing inhibitory potentials (depolarizing after manipulation of intracellular ion concentrations) are blocked by strychnine (Coombs *et al.*, 1955; Curtis *et al.*, 1968b; Larson, 1969); the "reversal" potential for the inhibitory potential is not altered by strychnine (Larson, 1969); and although both glycine and GABA apparently induce the same change in ionic permeability, the action of glycine is much more sensitive to strychnine than that of GABA (Curtis *et al.*, 1968a, b; Larson, 1969). Higher concentrations of strychnine may indeed interfere with or even alter the nature of the change in ionic permeability (Araki, 1965; Pollen and Lux, 1966), providing in part an explanation for the modification of the inhibitory effects of GABA, DA and NA on a variety of central neurones (Phillis and York, 1967; Phillis and Tebēcis, 1967; Tebēcis, 1967; Davidoff *et al.*, 1969; Johnson *et al.*, 1970), and even higher concentrations may involve changes in sodium permeability accounting for excitation and depression of nerve cell activity.

Changes in the properties of cortical neurones induced by topically administered strychnine, interpreted in terms of alterations in sodium ion permeability (Pollen and Ajmone-Marsan, 1965; Stefanis and Jasper, 1965), need not necessarily have arisen from a direct action of strychnine at inhibitory synapses on pyramidal tract neurones. The inhibitions studied were polysynaptic in nature, the postsynaptic potentials recorded probably consisted of a mixture of excitatory and inhibitory potentials, and strychnine may have modified the operation of inhibitory and excitatory interneurones synapsing with pyramidal cells. The conclusion of Araki (1965) concerning the abolition of a potassium conductance change by strychnine is incompatible with the observation that low concentrations do not affect the hyperpolarization of motoneurones by GABA.

Thus, strychnine most probably reversibly interacts with membrane structures at or near inhibitory receptors on spinal neurones so preventing effective interaction with glycine molecules. The examination of the structures of a number of strychnine-like compounds, all of which selectively reduce the sensitivity of spinal neurones to glycine and block spinal inhibition (Curtis *et al.*, 1968a, Curtis and Duggan, 1969; T.J. Batterham, D.R. Curtis, A.W. Duggan and G.A.R. Johnston, unpublished observations) indicates common molecular features which could interact with receptors having sites suitably spaced and charged for combination with glycine. Although a number of factors may be involved in the differential sensitivity of glycine and GABA receptors to electrophoretically administered strychnine, as for example a difference in the location on neurones of these receptors in relation to the juxta-somatic sites of strychnine administration, intravenously administered strychnine also blocks the inhibition of neurones by glycine more readily than that produced by GABA, suggesting that the antagonism is a receptor phenomenon. Differences between glycine and GABA receptors are also indicated by the remarkably clear cut distinction between glycine-like amino acids (α-alanine, β-alanine, cystathionine, serine, taurine) and GABA-like amino acids (GABA, γ-amino-β-hydroxybutyric acid, 3-amino-propane sulphonic acid, muscimol) on the basis of antagonism by strychnine (Curtis *et al.*, 1968a; John-

ston, Curtis, de Groat and Duggan, 1968), a classification supported recently by observations that bicuculline blocks the action of GABA (and GABA-like amino acids) but not that of glycine (and glycine-like amino acids) upon spinal neurones (Curtis, Duggan, Felix and Johnston, 1970).

A major aim of this study was to establish whether the specificity of strychnine as a glycine antagonist was sufficiently high to justify its use in determining the nature of the transmitter at inhibitory synapses. Because of the complexities involved in studying the amino acid sensitivity of single neurones, an accurate figure cannot be given for the concentration range over which strychnine discriminates between receptors for glycine (and glycine-like amino acids) and for the other depressants studied, but a rough estimate of 10—100 seems not unreasonable. Such a figure is probably quite adequate to enable strychnine to be used in determining whether glycine is involved in a particular inhibitory process. The results obtained with intravenous strychnine are consistent with, but by no means establish, strychnine as a competitive antagonist of glycine. However, full analysis of the type of antagonism would require the use of much higher doses of the alkaloid (Ariëns and Simonis, 1964). Experiments of this type were precluded by the increase in neuronal excitability which followed doses of strychnine exceeding even 0.1—0.2 mg/kg, and by limitations usually experienced in the amount of glycine which could be administered electrophoretically.

The reduction of the inhibition of spinal neurones by GABA, NA and DA by relatively high concentrations of strychnine may have resulted from an interaction between the alkaloid and the appropriate membrane receptors, but modification of membrane permeability changes as discussed above, a type of non-competitive antagonism, seems more likely. An explanation of the apparently non-competitive effects of strychnine on glycine and GABA receptors of spinal neurones (Davidoff *et al.*, 1969) has been provided in terms of excessive concentrations of strychnine such that there were probably no control observations made of glycine responses in the absence of strychnine (see Curtis *et al.*, 1969). The effect of strychnine on the sensitivity of cortical neurones to GABA, both the slower attainment of maximum depression which accompanied marked reduction of glycine inhibition by certain concentrations of strychnine, and the diminished equilibrium effectiveness of GABA with higher concentrations (Johnson *et al.*, 1970), may also reflect an interference with the action of GABA at or beyond the receptor level.

Appendix

Microelectrophoretic Dose-Response Relationships

The "classical" sigmoid dose-response curve, used as a basis for the analysis of drug-receptor interactions, for comparisons of the effectiveness of different agonists, and in studying the actions of antagonists, is derived under conditions in which a finite number of receptors are exposed to drug molecules uniformly distributed in the external medium, as for example in the fluid bathing an isolated piece of tissue or an organ. In contrast, when drugs are administered electrophoretically from the 1—2 μm orifice of a micropipette located near a neurone, and the response is recorded either as an alteration in firing frequency (extracellular recording), or as a change in membrane potential, conductance or excitability (intracellular recording), a number of factors need to be considered when analysing dose-response relationships (see also del Castillo and Katz, 1955; 1957; Curtis and Crawford, 1969).

1. All of the neuronal membrane is not exposed to a uniform concentration of a drug. In general, because of their attachment to extracellular or intracellular recording microelectrodes,

drug-administering micropipettes are most probably located closer to the soma of neurones than to dendrites. Drug concentrations will not be uniform around the soma, and dendritic receptors will be exposed to lower concentrations than are those on the soma.

Alterations in nerve cell activity may arise from the involvement of, and perhaps saturation of, receptors upon membrane lying closest to the drug source, an area which may be a small fraction of the total neuronal surface. The extent of the membrane influenced will depend on the diffusion of the drug through the extraneuronal space, and on enzymic and other processes of drug inactivation or removal from this space. More distant receptors will be exposed to lower concentrations which attain peak levels more slowly (see Curtis, Perrin and Watkins, 1960; Jaeger, 1965). Receptors far out on dendrites may even be unaffected by drugs administered electrophoretically. Cell size and geometry may thus be an important factor in any assessment of drug effects on neurones.

2. An increase in the rate of drug ejection raises the concentration in the vicinity of the proximal receptors, and, in addition, involves even more distant receptors which were previously unaffected by the drug. The response to increasing "doses" (rates of drug ejection) thus encompasses an increasing number of receptors exposed to the drug as well as an increasing concentration in the vicinity of some of the receptors.

A comparison of the sensitivity of different agonists to a given antagonist depends on the assumption that the appropriate receptors are similarly distributed in relation to the orifice of the micropipette from which the antagonist is ejected. The effect of a particular agent may not be influenced by an antagonist merely because the antagonist fails to gain access to the appropriate receptor sites. Furthermore, an agonist may appear to surmount the influence of an antagonist because with increasing rates of ejection it gains access to more remote receptors which are unaffected by the antagonist, rather than by displacing antagonist molecules from receptors. Thus dose-response curves may be displaced in a parallel fashion even in the presence of a non-competitive antagonist until all receptors on a neurone are affected by the agonist.

3. Alterations in firing rate of a neurone are largely determined by the membrane potential of the initial segment. Since receptors upon a neurone are not uniformly and simultaneously exposed to a drug, a given response may be achieved by drug effects having magnitudes inversely related to the proximity of the affected membrane to this segment. Thus, the effect on the firing frequency of an administered excitant or depressant will usually be a combination of a relatively short latency action at membrane sites close to the pipette, together with effects of smaller magnitude, which are less rapid in onset because of the time taken for the drug to diffuse to its site of action, but perhaps of equal effectiveness in altering the firing rate. Accordingly, antagonism of receptors near the pipette orifice may merely slow the onset of the action of an agonist without influencing its effect at equilibrium. Such antagonism would not be apparent if the dose-response relationship was evaluated only on the eventual equilibrium effect of a certain dose of agonist.

4. The difficulties outlined above would be least likely when agonist and antagonist were administered from neighbouring micropipettes at similar rates. However, a complete analysis of the agonist-antagonist interaction requires systemic administration of the latter, in order to achieve a more uniform pericellular concentration, provided that the substance passes through the "blood brain barrier", that it does not indirectly modify the activity of the neurone under observation by influencing cells with which it is synaptically connected, and that the amount administered is not limited by effects elsewhere in the experimental animal, as for example on the cardiovascular or pulmonary system. In addition, the possibility exists that a systemically administered antagonist of an electrophoretically administered agonist will be relatively ineffective in comparison with its action when ejected near neurones. In general, tissue concentrations attained after systemic injection, although uniform, will be lower than those after local administration, particularly in the vicinity of the micropipette orifice. Hence difficulty may be experienced in demonstrating antagonism between two compounds, one administered microelectrophoretically, the other systemically, even though antagonism can be demonstrated readily when both are ejected from different barrels of a multibarrel micropipette.

5. Antagonists may have complex concentration-dependent effects on membranes and receptors, and relatively highly specific actions at receptors located at a distance from the micropipette orifice may be obscured by less specific effects at more proximal receptors subject

to higher concentrations of the antagonist. Again, systemic administration may be of advantage to achieve uniform and relatively low pericellular concentrations.

6. In the absence of a direct measure of concentration, the "dose" is expressed as the rate of drug ejection. Under equilibrium conditions the concentration at a given distance from the micropipette orifice is directly related to the ejecting current, and inversely related to the distance. The sensitivities of different neurones to a particular substance are not directly comparable since the orifice-cell distance can neither be measured nor expressed in a simple manner. Thus neurones differ in shapes and sizes, and the relationship between current and rate of ejection of any one substance may vary from one micropipette with changing conditions, and between different micropipettes.

7. Under some conditions (drugs ejected as identical ions from solutions of the same ionic concentration, from micropipettes of relatively low and stable electrical resistance) more or less meaningful comparisons are possible of the relative effectiveness of a number of depressants (or excitants) ejected from different barrels of a multibarrel micropipette assembly upon the one neurone, based on the currents required to achieve similar submaximal responses by interaction with the same receptors. These findings can be related to experimentally determined values of transport numbers for the individual ions, and when carried out on a number of neurones of a particular type provide a basis for a comparison with the action of the same substances on neurones of other types.

8. A number of technical difficulties also need to be considered, including the maintenance of stable recording conditions and a constant micropipette-neurone distance for a time adequate to complete a study of dose-response curves; difficulties inherent in measuring small effects, excitant or depressant, on a fluctuating "base-line", be it firing frequency or membrane parameters; limitations placed on the magnitude of electrophoretic currents, and hence on attainable drug concentrations, by the generation of electrical noise and fluctuation of micropipette resistance values; difficulty in determining the current corresponding to zero concentration since the diffusional efflux of drugs is controlled by a retaining current which is usually excessive in order to reduce "after-diffusion" (del Castillo and Katz, 1957a); and problems arising from the uncontrolled diffusional efflux of antagonists, associated generally with the use of too highly concentrated solutions.

In general, therefore, dose-response relationships obtained microelectrophoretically probably reflect a balance between over-saturation effects at receptors close to the pipette orifice, near-saturation effects at receptors slightly further away, and minimal involvement of receptors at the periphery of the volume of tissue influenced by the ejected agent. The shape of the dose-response curve, and the effects of antagonists upon it, will be the result not only of the kinetics of drug receptor interactions at individual receptors, but also a complex function of the number of receptors involved and their spatial arrangement relative to the soma of the cell. In addition consideration should also be given to effects on cell function of alterations of extra- and intracellular ion concentration which may follow relatively prolonged activation of transmitter receptors.

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Prof. D.R. Curtis
Dept. of Physiology
John Curtin School of Med. Reseach
P.O. Box 334
Canberra City, A.C.T. 2601 (Australia)