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THE INHIBITORY ACTION OF NORADRENALINE AND OTHER MONOAMINES ON SPINAL NEURONES

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SUMMARY

1. L-Noradrenaline (NA), 5-hydroxytryptamine (5-HT) and acetylcholine (ACh) were administered micro-electrophoretically to feline lumbar neurones while recording their spike potentials extracellularly.

2. There was no evidence to suggest that NA acts as an excitatory transmitter in the spinal cord.

3. NA had potent inhibitory effects on some interneurones as revealed by a depression of spontaneous and synaptic firing and on the firing to a local application of an excitant amino acid. The effects on Renshaw cells and motoneurones were less marked.

4. The depressant actions of 5-HT were less marked than those of NA. ACh and carbamylcholine had depressant effects on some NA-sensitive interneurones but were invariably far less potent and on other NA-sensitive cells were completely inactive.

5. NA had no detectable effect on the normal spike amplitude but when the action potentials were reduced by excessive depolarization then both NA and synaptic inhibition increased the spike amplitude; this effect could be due to a hyperpolarization of the cell membrane.

6. There was a correlation between the distribution of NA-sensitive cells and the relative densities of NA-containing terminals in various layers of the grey matter.

7. It was postulated that NA acts as an inhibitory transmitter released from the terminals of descending pathways in the spinal cord. Other possible mechanisms were discussed but lacked experimental support.

INTRODUCTION

Recent experiments (Andén, Lundberg, Rosengren & Vyklický, 1963; Andén, Jukes & Lundberg, 1964; Andén, Jukes, Lundberg & Vyklický, 1964, 1966) have clearly shown that the intravenous injection of the amine

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precursors dihydroxyphenylalanine (DOPA) and 5-hydroxytryptophane have marked and specific effects on flexor reflexes in unanaesthetized spinal cats: the actions of the precursors seem to be due to a conversion to the corresponding amines noradrenaline and 5-HT within and their subsequent release from the terminals of descending fibres in the spinal cord. When considered in conjunction with the facts that these amines are only located within the terminals of descending fibres in the spinal cord (Carlsson, Falck, Fuxe & Hillarp, 1964; Dahlström & Fuxe, 1965), that reserpine produces an effect similar to that of the precursors in the acute but not in the chronically spinalized cat (I. Engberg, A. Lundberg & R. W. Ryall, unpublished work), and that the decerebrate control of inhibition from the flexor reflex afferents (FRA) is partially released by agents which block the peripheral actions of 5-HT and is increased by the amine oxidase inhibitor, nialamide (I. Engberg, A. Lundberg & R. W. Ryall, unpublished work), the evidence suggests that noradrenaline and 5-HT are normally liberated from the terminals of the descending fibres and serve as transmitter substances at spinal levels. Nothing was known about the actions of these substances at the cellular level in the spinal cord. Therefore the following experiments were carried out in an attempt to find out whether their actions are likely to be those of excitatory or inhibitory transmitter substances. The results are overwhelmingly in favour of the latter suggestion. Preliminary reports have been published (Ryall, 1965; Engberg & Ryall, 1965).

METHODS

The experiments were carried out upon twenty-nine unanaesthetized cats with the spinal cord transected in the lower thoracic region. The cats were prepared under ether anaesthesia. At the end of the dissection anaemic decortication was performed according to the procedure described by Andén, Jukes, Lundberg & Vyklický (1966). This method includes ligation of the basilar artery in some cats and procures a functional decortication in every preparation. Ether anaesthesia was then discontinued and at least 2 hr elapsed before the recordings were commenced. Neuromuscular blockage was maintained throughout the experiments by the intravenous injection of gallamine triethiodide, and the animals were artificially respired on air or a mixture of air and oxygen. Extracellular recordings of action potentials were obtained from 294 neurones, consisting of 247 interneurones, nineteen motoneurones and twenty-eight Renshaw cells in the lower lumbar segments of the spinal cord. Motoneurones and Renshaw cells were identified by stimulation of the transected ventral roots. The interneurones, including cells giving rise to ascending tracts, were located by their response to stimulation of afferents in the hind-limb nerves (posterior biceps-semitendinosus, anterior biceps-semimembranosus, sural, gastrocnemius, knee joint, flexor digitorum longus, tibial, superficial peroneal and deep peroneal), by their spontaneous firing or by their firing in response to a micro-electrophoretic application of an excitant amino acid, DL-homocysteic acid (Curtis & Watkins, 1963). These various procedures were routinely used in combination in order to sample as many different types of neurones as was possible.

The spike potentials were recorded through one barrel containing NaCl of a multi-barrelled micro-electrode and were amplified through a very high impedance transistorized

input stage and displayed on a Tektronix oscilloscope. The output from the Y-amplifier of the oscilloscope was fed through an amplitude discriminator into a frequency meter. The reciprocal of each consecutive spike interval was displayed on an ink-writing recorder (Mingograph) together with the average frequency of firing (time constant, 750 msec). Spikes larger than 75–100 μV were adequately discriminated from the background noise.

The method of administering substances electrophoretically to single neurones from multi-barrelled micropipettes has been adequately described in recent publications (Andersen & Curtis, 1964; Curtis, 1964; Curtis & Ryall, 1966). In the present investigation the substances were dissolved in aqueous solutions of the following concentrations: L- or D-noradrenaline, 0·5M, pH 3 or 5; 5-hydroxytryptamine creatinine sulphate, saturated solution, less than 0·12M, pH 3; acetylcholine chloride, 1 or 2M, pH 4; carbamylcholine chloride, 1M, pH 4; DL-homocysteic acid, 0·1M, pH 8 with NaOH; phentolamine hydrochloride, saturated solution, about 0·12M, pH 3; propranolol hydrochloride, saturated solution, less than 0·12M, pH 3; NaCl, 3M; sodium bitartrate, 0·5M, pH 3; procaine hydrochloride, 1M, pH 3.

RESULTS

The excitability of about half of the neurones tested was reduced by L-noradrenaline (NA). The depression was observed on either spontaneous firing, synaptic responses, or on the firing induced by the electrophoretic application of DL-homocysteic acid (DLH), often on more than one of these responses. An attempt to classify those neurones on which the effect of stimulation of afferent nerves was studied is presented in Table 1. The classification of interneurones on the basis of extracellular records was particularly difficult and is therefore rather arbitrary and serves only to give some indication of the types of neurones which were studied. Although the method of locating neurones was designed to avoid selection as far as possible, some selection was unavoidable, as in all studies with micro-electrode recording (see Discussion), and it is probable that some types of cells, particularly small ones, have not been investigated. For this reason, little significance can be attached to the exact proportion of neurones affected by NA. Therefore the results will mostly be given in a qualitative fashion.

Noradrenaline

Effect of noradrenaline on the synaptic activation of interneurones. An impressive effect of NA was the reduction of the synaptically evoked responses of some interneurones. One example of such an action was illustrated in a preliminary report (Engberg & Ryall, 1965).

The graph in Fig. 1 and the filmed records in Fig. 11 show the effects obtained upon another interneurone when the common peroneal nerve was stimulated repeatedly at a frequency of 1/sec; this neurone also fired in response to stimulation of the tibial nerve, but it was not activated from any of the other hind-limb nerves or from the contralateral L7 dorsal root. The diffusion of NA from the micropipette was reduced by the application of a small retaining current (5×10^{-9} A, 5 nA) and, under these conditions

TABLE 1. Classification and sensitivity of spinal neurones to noradrenaline

(*a*) Interneurones

	Synaptically activated									
	1	2	3	4	5	6	7	8	9	
	High spont.	FRA	Late disch.	Contra	Cutan.	Gp I muscle	Others	Not activated (inhib.)	Not identified	Total
Depressed	(7)	19	6	1	7	3	33	21 (9)	6	96
Not depressed	(25)	38	5	0	5	3	30	13 (2)	14	108

(*b*) Motoneurones and Renshaw cells

	Moto-neurones	Renshaw cells	Total neurones
Depressed	7	8	111
Not depressed	2	5	115

1. High spont. Number of neurones with spontaneous rate of firing in excess of 10/sec.
2. FRA. Cells which fired to stimulation of cutaneous, joint and high threshold muscle afferents.
3. Late disch. (greater than 30 msec latency), usually irregular, after stimulation of afferent nerves.
4. Contra. Only fired from contralateral nerves or dorsal root.
5. Cutan. Only fired to stimulation of cutaneous afferents.
6. Gp I muscle. Fired by volleys in Gp I muscle afferents.
7. Other interneurones which were fired by stimulation of afferent nerves but not easily fitted into other categories.
8. Not activated from peripheral nerves. The number in brackets shows how many of these were inhibited.
9. Not identified. The effects of afferent nerve stimulation were not tested on these cells.

Figures in brackets (columns 1 and 8) are not included in the totals since these cells are also represented in other places.

the firing remained fairly constant for about 3 hr. The passage of sodium ions with a current of 40 nA from a solution containing sodium bitartrate had no effect on the synaptic responses (Fig. 11*D*). In contrast, there was a marked reduction when NA was passed with the same current and a slightly smaller effect when only 10 nA were employed. The effect of NA commenced within a few seconds and increased progressively for about 2 min. Recovery was slow, particularly after the ejection of relatively large amounts of NA. The gradual increase in the effect and the prolonged

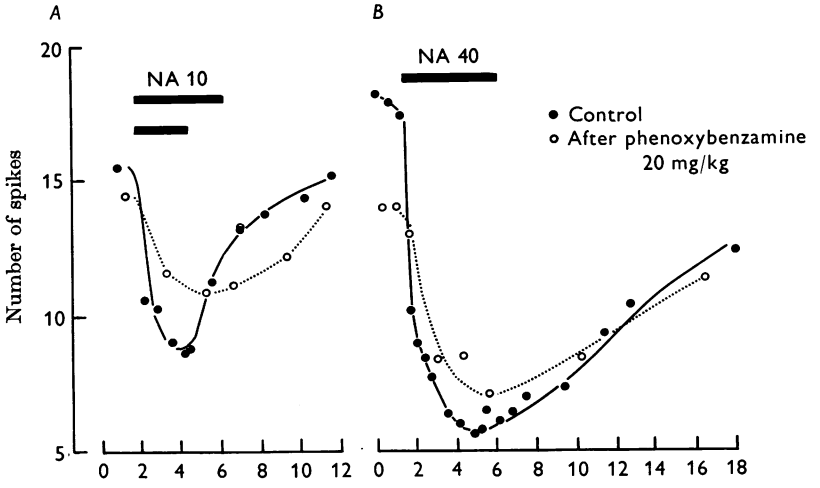

Fig. 1. Graph showing effect of NA ejected by an electrophoretic current of 10 nA (*A*) and 40 nA (*B*) on the synaptic discharge of an interneurone located 1·45 mm from the cord dorsum (NA retaining current was 5 nA). The duration of the NA application is indicated by the horizontal bars; the shorter bar in *A* refers to the continuous curve. Curves obtained before (●-continuous lines) and after (○-interrupted lines) the intravenous injection of 20 mg/kg of phenoxybenzamine. Ordinates: number of spikes following a single volley (10 times threshold) to the peroneal nerve; each point is the average for four consecutive volleys. Abscissae: time in minutes. Filmed records of the synaptic responses of this neurone are shown in Fig. 11.

action may have been due to the slow attainment of an equilibrium concentration around the neurone and to a slow rate of removal respectively. These rates may be determined by factors limiting the rate of diffusion to or from the cell membrane.

In addition to interneurones which responded with a long train of impulses to synaptic activation, as in the examples cited, interneurones were found with other types of synaptic responses which were reduced by NA. Among these were cells located 1·5–1·75 mm from the cord dorsum (see below and Fig. 10), which fired only a few spikes with short central

latencies (1·5–5 msec). Other cells responded with a late (greater than 30 msec latency) and usually irregular discharge to stimulation of high threshold afferents and the spontaneous firing was sometimes elevated after nerve stimulation. This discharge was suppressed by NA but on some occasions the effects were not clear-cut, possibly due to the irregular nature of the discharge.

In contrast with these types of discharge, which were reduced by NA, other neurones, most frequently located at a depth of 2·0–2·5 mm from the cord dorsum, responded to afferent nerve stimulation with a brief train of impulses at 10–15 msec latency and this type of response was never reduced by NA. There was also little effect on the short latency (less than 1·5 msec) discharge of interneurones to stimulation of the group I muscle afferents (the DLH-induced firing of some of these neurones was reduced by NA, see Table 1).

Thus, there appeared to be a selective action of NA on certain types of neuronal discharge.

Effects of noradrenaline on the spontaneous activity of interneurones. When interneurones were firing spontaneously or when the spontaneous firing was increased for prolonged periods by the occasional stimulation of afferent nerves, NA sometimes reduced the rate of firing. However, the spontaneous firing was often resistant to the depressant action of NA, even though the higher firing rate caused by the electrophoretic application of DLH on the same cell was reduced (see below).

The spontaneous activity of the neurone from which the records in Fig. 2 were obtained was particularly sensitive to NA. A retaining current of 30 nA was necessary to prevent the small amounts of NA which diffused from the pipette from reducing the rate of firing. Reducing this retaining current to 20 nA (Fig. 2*D*) produced a slight diminution in the frequency of firing, whereas reducing it to 10 nA (Fig. 2*C*) caused a marked diminution; the current changes were compensated by passing equal and opposite currents through a barrel containing NaCl, but this cell was relatively insensitive to current changes of the magnitude employed. In contrast, ACh had no effect, even when ejected with a current of 20 nA (Fig. 2*A*). The time course of the depressant action of NA is shown at a faster recording speed in Fig. 2*E*. The effect commenced in less than 1 sec, but a maximum effect was only obtained after about 5 sec. Recovery, after the re-application of the retaining current, followed a similar time course. When larger currents were used to eject the NA (Fig. 2*B*) the recovery was slower than with the ejection of small amounts. This cell was fired at 0·9 msec latency by a stimulus to the hamstring nerve, which was just threshold for the nerve. This synaptic firing was only slightly reduced by NA (firing index decreased from 0·86 to 0·71).

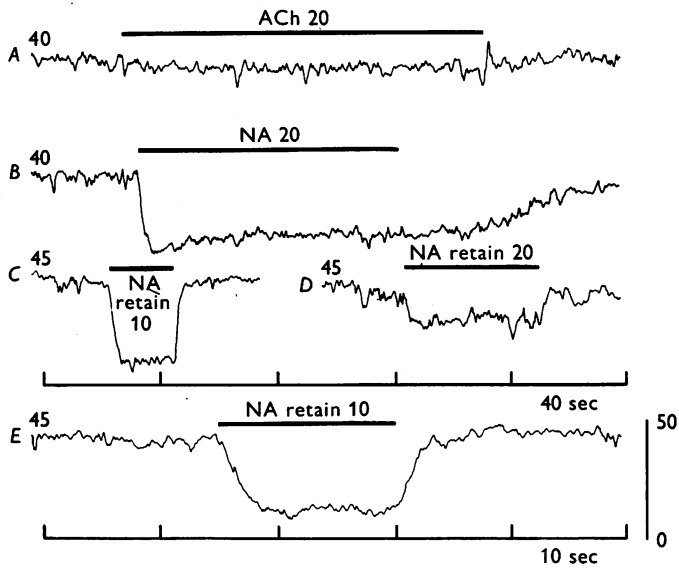

Fig. 2. Effects of NA and ACh on the spontaneous firing of an interneurone located 2·55 mm from the cord dorsum. *A*, ACh ejected with a current of 20 nA. *B*, NA, 20 nA (retaining current for ACh and NA was 30 nA). In *C* the NA retaining current was reduced from 30 to 10 nA and in *D* it was reduced from 30 to 20 nA. *E* shows the same effect of NA as in *C* but at a faster recording speed. Ordinates: frequency of firing. In this and subsequent illustrations, the initial firing frequency is indicated to the left of every record and the scale is shown to the right of the lowest record. Abscissae: intervals of 40 sec in *A–D* and of 10 sec in *E*. This interneurone was activated at 0·9 msec central latency by stimulation of group I muscle afferents but this synaptic activation was only slightly reduced by NA.

Effect of noradrenaline on amino acid induced excitation of interneurones. Many cells were located by their firing in response to the continuous or intermittent ejection of DLH while tracking through the spinal cord. Often they lacked or had a low spontaneous activity and could not be fired by any other means. A few of these neurones could be activated from peripheral nerves when the excitability was raised by DLH. Others were inhibited by stimulation of peripheral nerves. The DLH-induced firing of many of these cells was diminished by NA, which also reduced the frequency at which other neurones, activated from peripheral nerves, fired in response to DLH, even though no effect was seen on the synaptic activation. Thus, the DLH-induced excitation of these cells was a more sensitive test for depression than were the synaptic responses or even spontaneous activity. Retaining currents of about 20 nA were frequently required to prevent the diffusion of NA from the micropipette from reducing

the rate of firing. Marked depressant effects were obtained by a reduction in this retaining current of only 5–10 nA, but more usually removing the retaining current entirely or even ejection with small currents was necessary (Figs. 3*C*, *D* and 4*E*).

By applying suitable current controls it was established that these effects were not due to changes in potential imposed across the cell membrane. When tartrate from a barrel of the pipette containing sodium bitartrate had an action on neurones, the effect was negated by applying compensating currents through another barrel containing NaCl. Thus

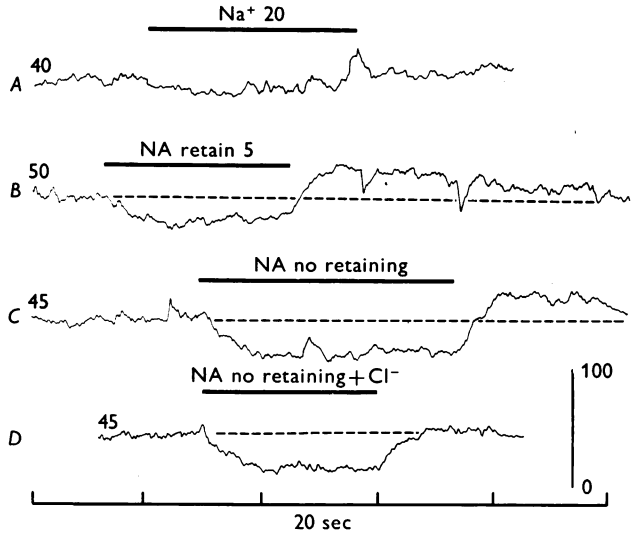

Fig. 3. Effect of NA on the DLH-induced firing of an interneurone located 1·8 mm from the cord dorsum. The firing was produced by the continuous ejection of DLH from one barrel of the micropipette. *A*, control effect of sodium ions ejected with a current of 20 nA. The effect of reducing the NA retaining current from 19 to 5 nA, and to zero is shown in *B* and *C* respectively. In *D* the current change in the barrel containing NA was compensated by an equivalent ejection of chloride ions from another barrel of the micropipette. Ordinates: frequency of firing as in Fig. 2. Abscissae: intervals of 20 sec. The DLH-induced firing of this neurone was inhibited by stimulation of cutaneous, joint and high threshold muscle afferents from both the ipsilateral and contralateral sides.

the effects were not caused by the removal of an excitatory action of tartrate anions with which the NA was associated. Furthermore, NA was still effective when compensation was applied through a barrel which contained sodium bitartrate, i.e. with no change in the rate of ejection of tartrate anions during the ejection of NA. Hydrogen ions in the solution did not modify the action of NA since it was equally effective when passed from solutions of either pH 3 or pH 5.

Electrophoretic currents up to 20 nA (Fig. 4*E*) were sometimes required to demonstrate marked depressant actions on the amino acid-induced firing. When larger currents were required, the reduction in firing rate was usually small and the effects could often be attributed to potential changes across the cell membrane. Effects due to current were more troublesome in the presence of excitation produced by DLH than they were on either spontaneous or synaptically induced excitation. The effects of current were similar to those observed in other investigations (see Krnjević & Phillis, 1963; Curtis, Ryall & Watkins, 1966) and could usually be distinguished from those of NA by a rapid onset and offset. The usual effect was a depression of firing when cations were ejected and excitation with anion ejection. Rarely, the time course of the depression by the ejection of sodium ions was slow. These unusually slow effects are of unknown origin, but they could be due to a modification of the extraneuronal milieu or to changes in the flow or distribution of the DLH anions as a result of a change in the local electric field. They are not likely to have been of great importance in the assessment of the results since they were adequately controlled by the use of a compensating current through an adjacent barrel of the electrode. On a few 'current-sensitive' cells, some action of NA persisted in the presence of an exactly equal and opposite compensating current, but the effect was completely abolished by a small increase in the compensating current. Such results were impossible to assess, but were in any case of little interest in view of the very marked and unequivocal results obtained on many other neurones. There were many cells on which NA was completely without action on the DLH-induced firing. This result is of the utmost importance in interpreting these data (see Discussion).

Occasionally excitatory effects were produced by the ejection of NA and other cations. This may have been due to the close proximity of the pipette to the cell membrane and the consequent depolarization (Krnjević, 1964).

More pertinent to the question as to whether NA has excitatory actions on neurones in the spinal cord is the observation that, following the depression of amino acid firing during the application of NA, the rate of firing rose above the control level when the ejection was terminated (see Figs. 3*B*, *C* and 4*E*). This 'facilitation' was extremely variable and was seen after the administration of 5-HT or ACh (Fig. 4*C*, *D*), of other cholinomimetics or even of simple organic cations (Curtis *et al.* 1966). It is not interpreted as a specific excitatory action of NA. A 'facilitation' also followed current depression (Fig. 4*A*) or inhibitory synaptic bombardment (Fig. 4*F*) and was seen following the lower firing frequency produced by a temporary reduction in the amount of DLH ejected from the micropipette (Fig. 4*B*).

There were probably two factors mainly responsible for this 'facilitation' of DLH-induced firing. The first was the excitation due to the depolarizing action of the cation retaining current (or of a cessation of the ejecting current) on current sensitive cells. This effect could be counteracted by the application of a compensating current through a barrel containing NaCl (compare Fig. 3*C* with 3*D* and 4*C* with 4*D*). The explanation of the 'facilitation' persisting after compensation for current effects is more complicated. When excitant amino acids are applied to some neurones the frequency of firing gradually decreases from its initially high value to a lower one and this effect has been attributed to desensitization (Curtis & Ryall, 1966). This desensitization is more marked at high frequencies of firing than at low frequencies. Thus, when the frequency of firing to DLH was reduced by the application of substances such as NA, ACh, or 5-HT, it could be expected that there would be a concomitant reduction in the desensitization. When the ejection of the depressant ceased,

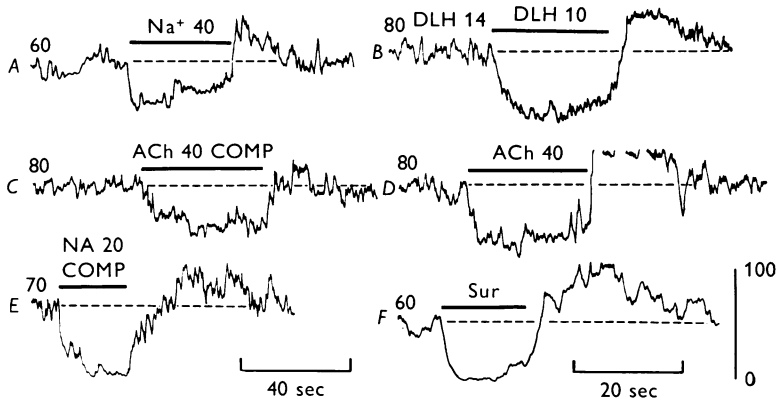

Fig. 4. Records to show the 'facilitation' which follows a reduction in DLH-induced firing of interneurones. *A*, ejection of sodium ions with a current of 40 nA. In *B* the current ejecting DLH was reduced by a very small amount (from 14 to 10 nA). In *C*, ACh was ejected with a current of 40 nA and the current was compensated (COMP) by a current change in the barrel containing NaCl but in *D* the compensation was omitted. Record *E* was obtained from another interneurone and shows the effect of an ejection of NA (20 nA) with current compensation. Record *F*, from a third neurone, shows the 'facilitation' which followed the inhibitory effect of a train of stimuli (5/sec) to the sural nerve (Sur). Ordinates: frequency of firing as in Fig. 2. Abscissae: interval of 40 sec for *A*–*E* and 20 sec for *F*. Retaining current for NaCl, NA and ACh was 10 nA in all records.

the firing rate could rise above the control level and could then again decline as desensitization was re-established. This explanation is supported by the observation that a temporary reduction in the amount of DLH ejected from the micropipette, and the consequent decrease in firing frequency, could be followed by 'facilitation' which had a time course similar to that seen after the application of depressant substances. This mechanism may also explain the 'facilitation' seen after inhibitory synaptic bombardment. An alternative hypothesis concerning the production of a 'facilitation' by cholinomimetic substances has been given (Curtis *et al.* 1966).

To determine whether the receptors involved in the inhibitory effects of NA on spinal neurones differed from peripheral NA receptors, the potency

of L- and D-noradrenaline was compared upon nine interneurones, but no difference between these two isomers was noted.

Effect of noradrenaline upon motoneurones and Renshaw cells. A limited number of observations were made upon motoneurones and Renshaw cells since these neurones are easily identified and experiments on them could give more information on the mechanism of action of NA. On five of nine motoneurones there was a definite depression of the DLH-induced firing. On another two cells there was possibly a slight action. No effects on orthodromic activation were noted.

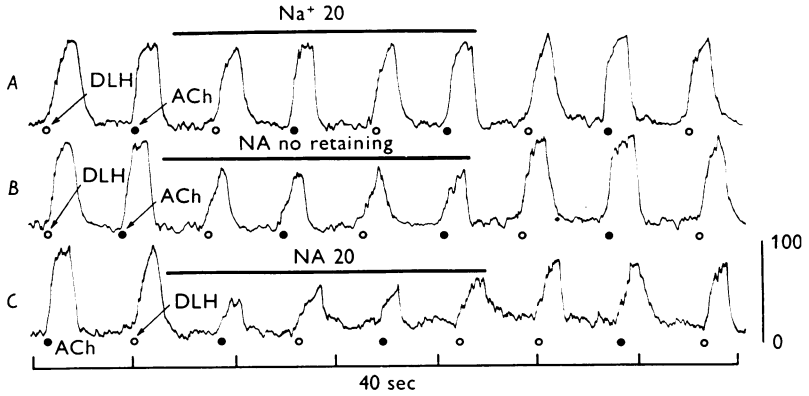

Fig. 5. Effect of NA on the firing of a Renshaw cell to alternate ejections of DLH (○) or ACh (●) from the micropipette. *A*, Ejection of sodium ions with a current of 20 nA (retaining current 20 nA). *B*, Diffusion of NA by removing the retaining current of 20 nA, and *C*, ejection of NA with a current of 20 nA. Ordinates: frequency of firing as in Fig. 2. Abscissae: intervals of 40 sec.

In tests upon thirteen Renshaw cells, the effects produced by NA were usually not very marked. No reduction in the firing to ACh or to DLH was observed on five neurones. On the remaining eight cells, electrophoretic currents of 20–80 nA were usually required to reduce the responses to ACh or to DLH. The records in Fig. 5 were obtained from one of two cells which were particularly sensitive to small amounts of NA and show that the responses to both ACh and DLH were similarly depressed. This observation is of interest since it shows that the effects on interneurones was unlikely to be due to a specific interaction of NA with the amino acid receptors on the cell membrane. However, on one cell there was apparently a specific reduction of the response to ACh, leaving that to DLH unaffected. This was also observed with 5-HT on two occasions.

NA did not affect the high frequency discharge of Renshaw cells to stimulation of the ventral root, but spontaneous firing was depressed on one occasion. An increase of the spontaneous firing was noted on two cells.

As on other interneurones, the significance of the occasional increase in firing rate is not clear.

Effect of noradrenaline on spike amplitude. NA did not modify the size or shape of synaptically evoked or spontaneous action potentials (extracellular recording) or those evoked by the micro-electrophoretic ejection of DLH in concentrations which themselves had no effect on the spike. When interneurones were depolarized by the application of excessive amounts of

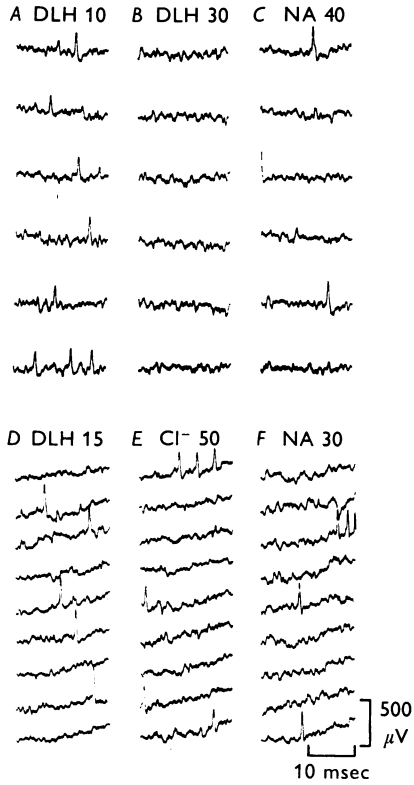

Fig. 6. The action of NA on the size of the action potentials produced by electrophoretic applications of DLH to two interneurones located 2·3 mm (records *A–C*) and 2·4 mm (records *D–F*) from the cord dorsum. *A* and *D* are control records obtained during the ejection of DLH with currents of 10 and 15 nA respectively. In *B*, the DLH current was increased to 30 nA and in *E* the current ejecting DLH was maintained at 15 nA but chloride ions were ejected with a current of 50 nA from an adjacent barrel. Both procedures caused a reduction in spike amplitude and in *B* this resulted in a cessation of spike discharge. Records *C* and *F* were obtained as in *B* and *E* but, in addition, NA was ejected with currents of 40 nA and 30 nA respectively (these current changes were compensated by passing opposite currents through an adjacent barrel containing NaCl). Calibrations: 500 μV and 10 msec. The records in *A–C* were taken with single sweeps at a frequency 1/sec, whereas those in *D–F* were taken on moving film with a sweep frequency of 25/sec.

DLH, the spikes became progressively smaller, sometimes reversed in polarity and then disappeared (see also Curtis, Phillis & Watkins, 1960). The micro-electrophoretic application of NA at this time caused an increase in the amplitude of negative spikes or even caused a re-appearance of negative spikes when these had been completely suppressed. The sequence of events is depicted in Fig. 6*A–C*. With the reduction in spike amplitude, the recording system failed to detect spikes and the recorded frequency of firing fell. NA was applied when no spikes were visible (Fig. 6*C*) and there was a gradual re-appearance of negative spikes and a progressive increase in the frequency indicated on the recorder. With the cessation of NA ejection, the spike amplitude again decreased and the recorded frequency fell. These effects also were not attributable to the flow of current from the micropipette since they were not negated by the application of compensating currents through an adjacent barrel. Such effects have not so far been observed with ACh and were clearly in contrast to those of the local anaesthetic, procaine, which decreased the normal spike amplitude, as found in previous investigations (Curtis & Phillis, 1960; Krnjević & Phillis, 1963). These effects with NA were not seen on every occasion on which they were looked for and conditions seemed to be critical for demonstration. One incidental observation which may be of relevance to the mechanism of action is that on a few 'current-sensitive' neurones the ejection of chloride ion with 'anionic' currents from a neighbouring barrel of the electrode at first increased the frequency of firing to DLH and then produced a reduction in spike amplitude which was also counteracted by NA (Fig. 6*D–F*). Now, DLH was also ejected as an anion and it is possible that on some cells the changes in the spike size when the ejection was increased was due not only to the DLH *per se* but also to the 'anionic' current with which it was ejected. Since it is impossible to analyse and interpret these data with any certainty, more direct investigations of the action of NA on the membrane potential are required. However, it was noted that when the spike size was decreased by DLH an increase in the amplitude of negative spikes could be obtained following inhibitory synaptic bombardment of interneurones (Fig. 7) and, in one instance, following recurrent inhibition of a motoneurone by stimulation of the ventral root. These observations suggest that the action of NA could be similar to that of inhibitory synaptic transmitters.

Effect of blocking agents on the depressant action of noradrenaline. It has not been possible to produce any convincing evidence of a blockade of the depressant action of NA on interneurones, either by intravenously injected phenoxybenzamine or by locally applied phentolamine (an α-adrenergic blocking agent) or by propranolol (a β-blocking agent). The lack of effect with phenoxybenzamine on the depression of synaptic activation by NA

is seen in Fig. 1. Small effects were seen but these could easily have been due to small movements of the micropipette away from the cell. The doses of phenoxybenzamine were similar to those effective in antagonizing the actions of DOPA in the spinal cat (Andén, Jukes & Lundberg, 1964).

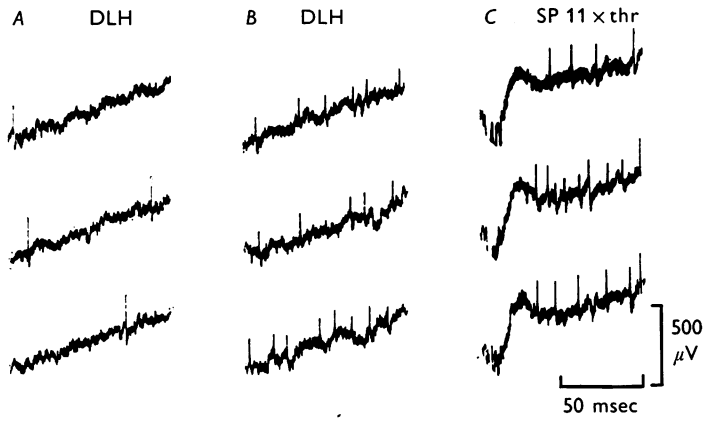

Fig. 7. The effect of inhibitory synaptic volleys on the size of the action potentials of an interneurone located 2·55 mm from the cord dorsum. Record *A* shows the initial size of the action potentials produced by the ejection of DLH. In *B* there was some decrease in amplitude with continued ejection of DLH. In *C* the spike amplitude increased following stimulation of the superficial peroneal nerve (11 times threshold) once per sweep. Calibrations: 50 msec, 500 μV. Sweep frequency: 2·5/sec.

When phentolamine or propranolol were ejected with relatively large electrophoretic currents (up to 80 nA), the frequency of firing to DLH gradually declined and the spikes became smaller. Recovery of spike size and frequency after the termination of the ejection took up to 20 min. The mechanism by which the blocking agents decreased the frequency of firing and the spike amplitude could be similar to the action of some substances such as ephedrine and amphetamine on the cerebral cortex (Krnjević, 1964). It was observed that the rate of recovery from the blocking agents was increased by an interruption of the ejection of the amino acid. Although the mechanism of action of the blocking agents need not concern us here, these effects indicate that it will be extremely difficult to test these substances effectively by local electrophoretic application. It may be impossible by this technique to obtain an adequate concentration to block the receptors without producing these undesirable actions. In concentrations which had no effect on the spike amplitude, the blocking agents failed to modify the depressant actions of NA on the DLH firing. Locally applied phentolamine also failed to block the depression by NA of orthodromic activation of interneurones.

The classification and anatomical location of noradrenaline sensitive neurones. It was impossible to include all neurones depressed by NA in a single functional category (Table 1). About three times as many insensitive as sensitive interneurones fired spontaneously at a rate in excess of 10/sec. There were no other obvious differences, except that about two thirds of the neurones which were not activated by the stimulation of peripheral nerves (column 8, Table 1) were depressed by NA; on these cells the depression of the DLH induced firing was the only criterion available.

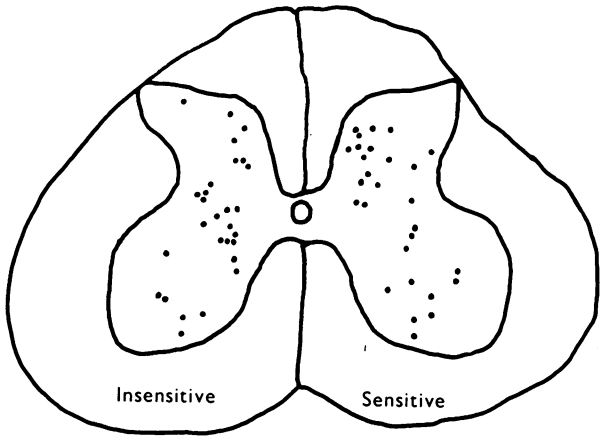

Fig. 8. Outline diagram of spinal cord section in the lumbar region (L7) showing the approximate positions of fifty-seven interneurones located in five experiments. On the left hand side are shown those neurones which were insensitive to NA. The positions of neurones on which NA depressed firing to DLH, spontaneous activity or synaptic activation are shown on the right side of the figure.

A map of the approximate distribution of NA-sensitive and insensitive neurones in five experiments is shown in Fig. 8. The points on the diagram were obtained from angle and depth records by reference to an electrode left in place at the end of each experiment. This map gives an approximate indication of the area of spinal cord which was sampled. Sensitive neurones were located over quite a large area of the grey matter.

Some indication of a more specific localization was obtained when the results of 175 neurones in twenty-nine experiments were analysed in terms of depth from the cord dorsum. This analysis is presented in Fig. 9. The depths were divided into ranges of 0·25 mm and are those actually recorded in the experiments; no allowances for variations in surface contours have been made. Alongside the analysis is a diagram of a cord section on approximately the same scale. Interneurones were located at depths from 0·5 to 4·25 mm, with a highest frequency of occurrence at 2·0–2·5 mm. No NA-sensitive cells were found above 1·25 mm. Below this depth the

frequency distribution of sensitive cells showed three peaks. Of these three peaks, that at 1·5–1·75 mm was the most prominent and nearly all, eighteen of nineteen cells, were depressed by NA. χ^2 tests showed that the distribution of NA sensitive cells was not significantly different from the total cell distribution except at the depth of 1·5–1·75 mm, where there were significantly more sensitive cells than could be expected from the distribution of all of the cells located ($\chi^2 = 16{\cdot}7$, $P < 0{\cdot}001$).

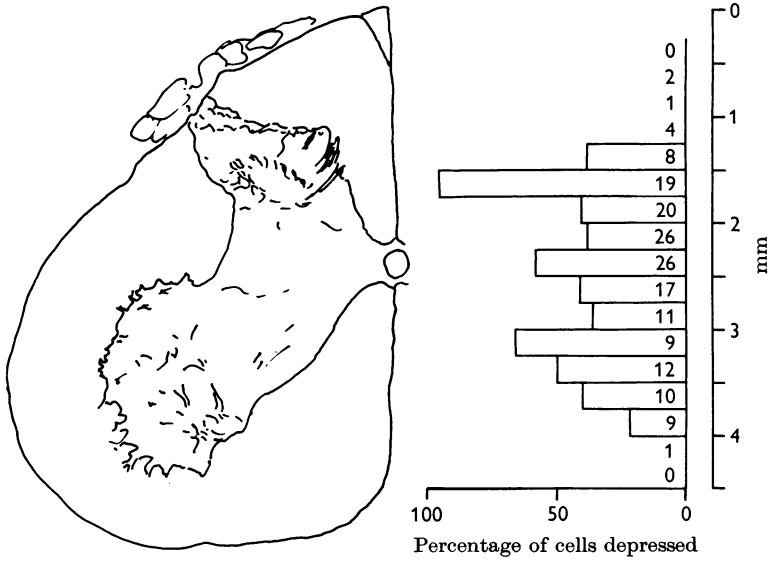

Fig. 9. Histogram showing the depth distribution of 175 interneurones located in twenty-nine experiments together with a schematic diagram of the cord in the lumbar region (L 7). The depths were divided into 0·25 mm ranges and the number of neurones located at each depth is shown. The histogram shows the percentage of cells on which depressant effects of NA were observed at each depth.

The synaptic activation of neurones in the 1·5–1·75 mm region was also reduced by NA. Figure 10 shows two neurones where the administration of NA inhibited the spike discharge to afferent nerve stimulation. In *B* the effect was produced by mere diffusion of NA from the micropipette, whereas in *E* ejection with a current of 30 nA was required. These effects were rapid in onset but took about 30 sec to become fully developed. Recovery took 30–120 sec depending upon the duration of the application of NA. Ach was also tried upon one of these cells, but was ineffective.

The action of pentobarbital sodium

Since Curtis, Phillis & Watkins (1961) were unable to demonstrate any effect of NA on the synaptic activation of spinal interneurones in cats

anaesthetized with pentobarbital, the action of this anaesthetic was briefly investigated.

An intravenous injection of 5–30 mg/kg of pentobarbital caused a marked decrease in the excitability of the two interneurones upon which it was tested. This decrease in excitability was revealed in two ways. First, there was a reduction in the number of impulses in response to stimulation of afferent nerves. The neurone depicted in Fig. 11 fired a long train of impulses following a single shock to the common peroneal nerve (Fig. 11*A*).

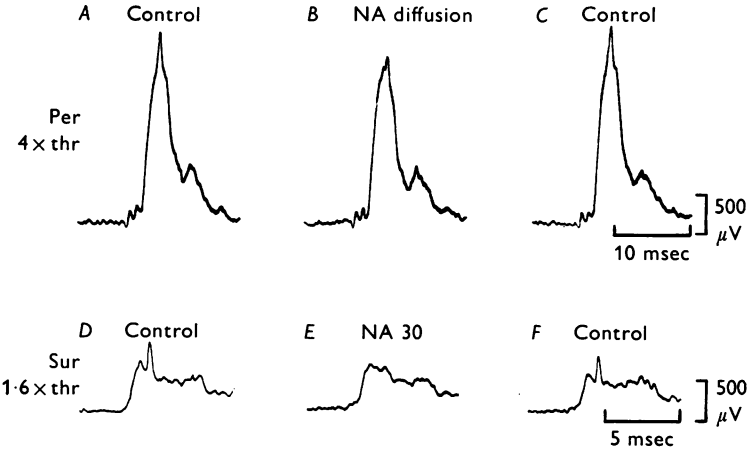

Fig. 10. Effect of NA on the short latency (4 and 1·7 msec central latencies) synaptic discharges of two interneurones located 1·5 mm (records *A–C*) and 1·65 mm (records *D–F*) from the cord dorsum. Records *A–C* show the action potentials superimposed on the focal synaptic potentials generated by stimulation of the peroneal nerve (Per, 4 times threshold for the nerve). Records *D–F* show similar potentials when the sural nerve (Sur) was stimulated at 1·6 times threshold. *A* and *D* are control responses. Record *B* was obtained when NA diffused from the micropipette by removing the retaining current. Record *E* was obtained during ejection of NA with a current of 30 nA. Records *C* and *F* are control records obtained when the application of NA had ceased. All current changes were compensated by currents passed through a barrel of the micropipette which contained NaCl. Calibrations: 500 μV, 10 msec for records *A–C* and 5 msec for *D–F*.

After pentobarbital (10 mg/kg), the number of spikes gradually decreased (Fig. 11*E*, *F*) and the few spikes remaining were unaffected by the administration of NA (Fig. 11*G*, *H*), which previously had a marked depressant effect on the discharge (Fig. 1 and 11*B*). This decrease in synaptic activation was not entirely due to a reduction in transmission through the polysynaptic pathways (see also Brooks & Eccles, 1947; Løyning, Oshima & Yokota, 1964; Shapovalov, 1964) since there was also a decrease in sensitivity to locally ejected DLH. In one experiment, four neurones were tested before the slow injection of pentobarbital (30 mg/kg) and all were

depressed by NA. During the injection of pentobarbital, the DLH-induced firing of the neurone under observation gradually decreased and finally the neurone could not be fired at all by DLH. Subsequently, four other neurones were located in the same region but the amino acid sensitivity of these neurones was unaffected by NA. However, it is not certain that these neurones would have been sensitive to NA in the absence of pentobarbital. In a third experiment, 7 mg/kg of pentobarbital intravenously caused an eight-fold decrease in the sensitivity of a Renshaw cell to ACh. NA still depressed the ACh induced firing after pentobarbitone, but the effect was less marked than before the injection.

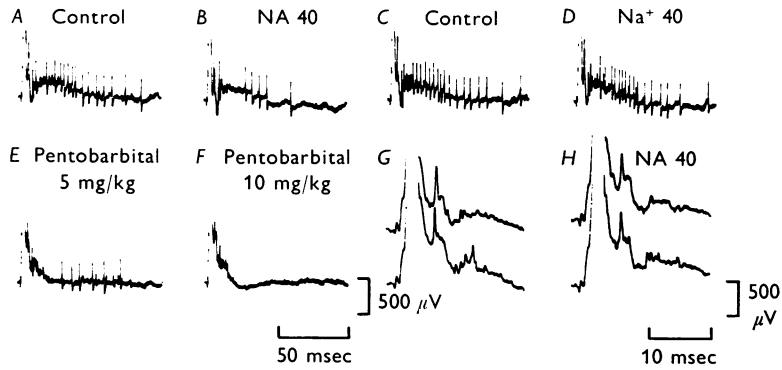

Fig. 11. Same interneurone as illustrated in Fig. 1. Action potentials generated by single shock stimulation of the peroneal nerve (10 times threshold). *A* and *C* are control records. NA was ejected with a current of 40 nA in *B*. Sodium ions (40 nA) were ejected from a solution of sodium bitartrate in *D*. Between *D* and *E*, 5 mg/kg of pentobarbital were injected. Record *F* was obtained after the injection of another 5 mg/kg of pentobarbital. Calibrations for records *A–F*: 50 msec and 500 μV. *G*: Moving film records at a faster sweep speed after 10 mg/kg of pentobarbital. *H*: as in *G* but during ejection of NA (40 nA). Calibrations for records *G* and *H*: 10 msec and 500 μV.

Thus the previous failure to demonstrate any action of NA on spinal neurones (Curtis *et al.* 1961) may have been due to the use of anaesthetics which may have modified the synaptic discharges recorded and thus rendered them insensitive to NA. Alternatively, they may not have located any neurones with a synaptic discharge which was sensitive to NA.

5-*Hydroxytryptamine*

5-HT was tested upon forty-eight interneurones, ten motoneurones and fifteen Renshaw cells. A reduction in the rate of firing to DLH was produced on thirteen interneurones but there was no effect on the other interneurones or motoneurones. On five interneurones, the effectiveness of NA and 5-HT was compared. Three of these neurones were depressed by

NA and to a lesser extent by 5-HT, but a more detailed study is required to show whether all neurones depressed by NA are also depressed by 5-HT. However, these investigations were hampered by the relative insolubility of 5-HT. The concentration of the saturated solution of 5-HT was less than 0·12M in comparison with NA which was dissolved at a concentration of 0·5M. Thus the diffusion of NA from the micropipette would have been more than 4 times as large for NA as for 5-HT and quantitative comparisons are clearly impossible. Furthermore, 5-HT was associated with creatinine and the latter would have carried a considerable proportion of the electrophoretic current. This again would have reduced the apparent effectiveness of 5-HT. Electrodes containing 5-HT tended to have high resistances and often would not pass current well without generating noise.

In preliminary experiments on Renshaw cells 5-HT was without any effect. In subsequent experiments the 5-HT was dissolved in 0·06M-NaCl in order to increase the conductance of the solutions. Depressant effects on the firing to ACh or DLH were observed on six cells, but on two of these the response to ACh was reduced whereas the effect of DLH was unchanged. The spontaneous firing of two cells was increased during prolonged application of 5-HT.

Acetylcholine and carbamylcholine

Cholinomimetic substances depress the excitability of some interneurones in some cats (see Fig. 4*C*, *D* and Curtis *et al.* 1966). In the present experiments NA-sensitive cells were found in all cats and the cholinomimetics ACh and carbamylcholine were either ineffective or much less effective than NA in reducing the firing to DLH. This observation is based upon results on twenty-four interneurones on which the effects of ACh (from a 2 or 1M solution) were compared with the effect of NA (from a 0·5M solution) and on a further six interneurones upon which NA and carbamylcholine (from a 1M solution) were compared. The excitability of twenty-one of these cells was depressed by NA but only eight of them were depressed by ACh and two were depressed by carbamylcholine. Depressant effects of NA and ACh are shown in Fig. 4. It is of interest that, on these cells, a 'facilitation' followed the depression by ACh of DLH-induced firing (Fig. 4*C*, *D*). This 'facilitation' was partly due to 'current effects' since it was reduced by the application of simultaneous compensating currents through a NaCl-containing barrel. However, the 'facilitation' was not entirely due to an action of the electrophoretic current *per se*, but also to the preceding period during which the frequency of firing was low, and it probably represents a decrease in the desensitization to DLH which occurs when DLH is administered to neurones electrophoretically (see p. 307).

DISCUSSION

The excitability of many neurones in the spinal cord was depressed by NA. On interneurones, NA produced a marked depression of the firing induced by the electrophoretic application of an excitant amino acid, DLH, of some types of synaptic activation and less commonly of spontaneous firing. The action of NA was characterized by a particularly high potency. Mere diffusion from a 0·5M solution, or ejection with small electrophoretic currents, was often sufficient to markedly reduce the firing.

Clear demonstrations of the inhibitory effect of NA on synaptic activation were seen on neurones which fired a long train of impulses in response to a single afferent nerve volley and on the brief discharge of neurones located 1·5–1·75 mm from the cord dorsum. Inhibitory effects, although less clear-cut, were also obtained upon the late and irregular synaptic discharges seen with other neurones. On many cells, particularly those which fired a brief burst of impulses at about 10–15 msec delay in response to afferent nerve stimulation, NA did not affect the synaptic excitation, even though in some instances it reduced the amino acid sensitivity. Such a differential effect could be due either to the greater intensity of synaptic action or to the fact that DLH was applied to the same part of the cell membrane as the NA. The failure to reduce the synaptic discharge, spontaneous firing or DLH-induced firing of many cells shows that there were no NA receptors on these neurones.

Although NA occasionally increased slightly the spontaneous firing of spinal neurones, the electrophoretic currents were high and excitation by the ejection of sodium ions was also observed in a few instances. The rebound facilitation observed with NA was unlikely to have been due to a specific excitatory action (see Results). Thus there was no evidence for an excitatory action of NA on spinal neurones. Since a large number of cells, of presumably many different types, were sampled it seems unlikely that NA functions as an excitatory transmitter in the spinal cord, and the excitant effect of intravenously or intra-arterially injected NA (Skoglund, 1961) may be due to disinhibition or may be secondary to circulatory changes.

The most interesting effect of noradrenaline was therefore the depression of neuronal excitability and the mode of action and possible significance deserve more detailed discussion.

Since NA inhibited the synaptic activation of interneurones the inhibitory effect on the amino acid induced firing was unlikely to have been due to an interaction of NA with the amino acid receptor. (Although it is conceivable that an amino acid could be an excitatory transmitter acting on interneurones, this has been considered to be unlikely, Curtis &

Watkins, 1960, 1963; Curtis *et al.* 1960; Curtis, 1965.) This conclusion is also supported by the observation that the excitatory effect of both DLH and ACh on Renshaw cells was reduced by NA.

The effects of NA were not counteracted by intravenous injections of phenoxybenzamine, or by the local electrophoretic administration of phentolamine or propranolol (α- and β-andrenergic-blocking-agents respectively). Phenoxybenzamine blocks the action of dihydroxyphenylalanine (DOPA) on reflexes in the spinal cat (Andén *et al.* 1964; Andén *et al.* 1964). Since DOPA is thought to act by increasing the release of noradrenaline from the terminals of descending fibres (see Introduction), the lack of effect with phenoxybenzamine in the present study may indicate that the receptors involved are different. An alternative explanation is that phenoxybenzamine may interfere with the mechanism by which DOPA is taken up, converted to noradrenaline and subsequently released from the terminals. Blakeley, Brown & Geffen (1964) have shown that phenoxybenzamine reduces noradrenaline output from nerve terminals by blocking the uptake and re-use of the transmitter. Therefore phenoxybenzamine may reduce the action of DOPA by this mechanism and the receptors upon which the noradrenaline acts when released from descending tracts may, like those for noradrenaline in the present study, be unaffected by phenoxybenzamine or β-blocking agents. This and the fact that there was no difference in potency between the D and L stereoisomers of noradrenaline indicates that the noradrenaline receptors in the spinal cord differ from the peripheral receptors (see also Marley, 1964). However, the effect of the local application of α- and β-blocking agents was complicated by changes in the size of the recorded action potentials and a decline in firing frequency, which may be of similar origin to the effects produced by local anaesthetics (Curtis & Phillis, 1960; Krnjević & Phillis, 1963; Krnjević, 1964) or by substances such as ephedrine and amphetamine on cortical neurones (Krnjević & Phillis, 1963; Krnjević, 1964). Thus, it may not be possible to achieve, by the micro-electrophoretic technique, a sufficiently high concentration of the blocking agent at the receptors without causing these undesirable actions on the cell membrane at the same time.

Some interneurones were also depressed by 5-HT or ACh. Although this may indicate that more than one inhibitory transmitter substance can act on the same neurone, it is more probable that the actions of these two substances and of ACh in particular, which had a very low potency compared with NA, are due to different mechanisms. It has been suggested (Curtis *et al.* 1966) that the depressant action of ACh may be due to interaction with a membrane receptor which is not acted upon by transmitter substances.

Little importance can be attached to the actual number of neurones

depressed by NA since there was unavoidable selection of the cells; the extracellularly recorded action potentials had to be of sufficient amplitude (at least 75–100 μV) to permit good recording and had to be discriminated in size from others in the same electrode position. Nevertheless, about half of the neurones examined were completely insensitive to NA indicating some specificity of action. The effect was unlike that of γ-amino-*n*-butyric acid (see Curtis, 1965; Krnjević, 1964) and local anaesthetics (Curtis & Phillis, 1960; Krnjević & Phillis, 1963; Krnjević, 1964) which depress the excitability of all types of neurones which have so far been examined. Since there was no decrease in spike amplitude with NA there was clearly no similarity to the action of local anaesthetics. On some cells only a slight depression of DLH-firing was produced by NA and usually large electrophoretic currents were necessary to produce this effect. It is possible that these relatively weak actions were produced by a mechanism which differed from that of NA on the more sensitive cells. These small effects on some spinal neurones could be analogous to those seen with relatively large amounts of NA elsewhere in the central nervous system (see Krnjević & Phillis, 1963; Bloom, Oliver & Salmoiraghi, 1963; Krnjević, 1964; Bradley & Wolstencroft, 1965).

There are three possible explanations of the potent inhibitory effects of NA on some spinal interneurones. The most likely is that NA is an inhibitory transmitter released from the terminals of descending pathways in the spinal cord. Alternatively, NA may cause a release of inhibitory transmitter from presynaptic terminals. Such a mechanism could even be responsible for the effects of NA released from the terminals of descending fibres and would be difficult to differentiate from an inhibitory effect *per se*. A third explanation is that the receptors with which the NA combines are not concerned with synaptic transmission. The evidence regarding the localization of the NA sensitive cells (see below) does not support this last suggestion.

The effectiveness of NA in causing a reduction in excitability of sensitive neurones is at least equivalent to that of ACh in exciting Renshaw cells when the substances are applied micro-electrophoretically. Thus the high potency of NA is compatible with the hypothesis that these depressant effects may be related to a physiological function of NA in the spinal cord. The onset of action was fast (less than 1 sec) and the relatively slow attainment of a maximum effect may be attributed to the time taken to achieve equilibrium concentrations of ejected NA around the neurone. This time may be slow for NA compared with ACh on Renshaw cells if there is no enzymic destruction which proceeds at a rate comparable to the hydrolysis of ACh by cholinesterase. When NA is liberated subsynaptically from nerve terminals, then the rate of diffusion away from the synaptic cleft could be

sufficiently rapid to limit the duration of the effect (Eccles & Jaeger, 1958). Unfortunately, there is no direct evidence to prove that NA acts as an inhibitory transmitter in the spinal cord. Such evidence may be extremely difficult to obtain on interneurones where the depressant actions of NA were most marked. Although the depressant effects observed on different types of neurones need not be caused by the same mechanism, the high potency of NA on some neurones, the apparent specificity of action and some degree of localization of sensitive cells (see below) are certainly suggestive of an inhibitory transmitter function.

Another observation may indicate that NA, like inhibitory transmitters, may cause a hyperpolarization, or repolarization, of the cell membrane. On some cells NA increased the spike amplitude when the spike size was decreased by an excessive application of DLH or by applying large anionic currents, and an increase was also observed following inhibitory synaptic bombardment. The analysis of this phenomenon is complex. The cause of the spike change by DLH itself may well be due to a combination of factors such as the depolarization caused by the current used to eject the DLH anions and the depolarization due to an action of DLH on the membrane permeability. If NA produces a hyperpolarization of the cell membrane, this would tend to increase the spike size (Eccles, 1964) but the conductance change involved should tend to decrease it. However, the relative importance of these two factors in determining the size of the extracellularly recorded action potential of depolarized cells is unknown and it is possible that the hyperpolarization could be the more important. Further investigation with intracellular recording techniques is required to provide more direct proof of a membrane hyperpolarization.

Noradrenaline-sensitive interneurones were located throughout the dorsal horn and some were found in the medial parts of the ventral horn. Analysis of the recordings of the depth of the cells from the cord dorsum showed that, in contrast with neurones elsewhere, those at 1·5–1·75 mm from the surface were all, with one exception, inhibited by NA. A reduction in the frequency of firing to orthodromic activation was also noted at this depth. This suggests that most of the cells in this region were especially sensitive to NA. Alternatively, it is possible that the NA receptors on these cells are ideally situated for demonstrating an action upon them by the micro-electrophoretic technique; if the receptors were located on or near the soma then they would be closer to the micropipette, which was positioned to record the maximum size of the action potentials, than if they were located far out on the dendrites. The neurones at this depth did not have a great deal of excitatory convergence upon them but more detailed studies are required before it will be possible to correlate functional organization with susceptibility to NA. However, this region corresponds

to layer IV (Rexed, 1954) and has recently been shown to contain a particularly large number of catecholamine-containing terminals (K. Fuxe, personal communication).

Although it has been considered that there may be no monosynaptic inhibitory transmission from descending pathways in the cord (Eccles 1964), the terminals containing NA and 5-HT are probably derived from small, unmyelinated nerve fibres (Dahlström & Fuxe, 1965) and nothing is known about transmission from these terminals. Thus a descending system operating by the release of NA with post-synaptic inhibitory effects upon spinal neurones is suggested by the present evidence, which shows for the first time that NA is extremely potent in reducing the excitability of some spinal neurones. Such an effect could explain the effects of DOPA on spinal reflex pathways (see Introduction). However, to establish this hypothesis on a firmer basis, it is necessary to show that the release of noradrenaline from the terminals of the descending fibres produces effects identical to those of electrophoretically administered NA on the same cells.

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