AN ANALYSIS OF THE PHYSIOLOGICAL AND BIOCHEMICAL PARAMETERS OF RELEASE, STORAGE AND SYNTHESIS OF TRANSMITTERS FROM SYNAPSES ONTO SINGLE IDENTIFIABLE NEURONES IN THE BRAIN OF HELIX ASPERSA

by

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ABSTRACT

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AN ANALYSIS OF THE PHYSIOLOGICAL AND BIOCHEMICAL PARAMETERS OF RELEASE, STORAGE AND SYNTHESIS OF TRANSMITTERS FROM SYNAPSES ONTO SINGLE IDENTIFIABLE NEURONES IN THE BRAIN OF HELIX ASPERSA

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Intracellular recordings were made from identifiable neurones in the suboesophageal ganglionic mass of Helix aspersa. A neurone was identified which gave an inhibiting post synaptic potential (ipsp) on stimulation of the anal nerve. Pharmacological evidence suggested that the synaptic transmitter was acetylcholine. The amplitude of the response was between 14-20 mV. The results from repetitive stimulation experiments at different frequencies suggest that acetylcholine is released from a single presynaptic store and the amount released is a constant fraction of the store by each stimulus. Repetitive stimulation either in the presence of hemicholinium or in snails pretreated with hemicholinium resulted in the almost complete disappearance of the ipsp. It is suggested that the release of acetylcholine is exponential and that hemicholinium blocks acetylcholine resynthesis.

Two other neurones were identified and one was found to have an inhibition of long duration (IID) and the other a driven ipsp via the anal nerve. From pharmacological analysis it can be suggested that dopamine is the transmitter in both cases. The IID neurone responded

to octopamine and noradrenaline at sufficiently low concentrations to suggest they might have physiological actions on this cell. On repetitive stimulation the events did not decline. Further in the presence of cocaine the synaptic event was potentiated and this also occurred in the presence of amphetamine. This suggests that the transmitter in these synapses has a high rate of turnover which could be due to the reuptake of the transmitter, dopamine.

The pathway of catecholamine synthesis was followed by incubating the snail brain with various radioactive precursors. It was found that the brain was unable to metabolise tyrosine to Dopa but the kidney was capable of this transformation. Tyrosine hydroxylase could only be assayed in the kidney and not in the brain. Using a radioactive assay noradrenaline and octopamine were found to be present in the brain and heart of Helix aspersa. Therefore it was suggested that the kidney converts tyrosine to Dopa and this is released into the blood where it is taken up by the brain and converted to dopamine and to noradrenaline. The breakdown of catecholamines in the brain is carried out by monoamine oxidase and catechol-o-methyl transferase.

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Abbreviations

ipsp	Inhibitory post synaptic potential
ILD	Inhibition of long duration
HC3	Hemicholinium
Ach	Acetylcholine
Dopa	Dihydroxyl phenyl acetic acid
Mao	Monoamine oxidase
Comt	Catechol-o-methyl transferase
Pnmt	Phenyl-N-methyl transferase
Atp	Adenosine triphosphate
Adp	Adenosine diphosphate
Mepps	Miniature end-plate potentials

C O N T E N T S

GENERAL INTRODUCTION

Acetylcholine

Distribution of Acetyltransferase

Analysis of nervous tissue by differential centrifugation enabled Gray and Whittaker (1962) to identify the tissue particles containing acetylcholine and choline acetyltransferase, as broken off nerve endings or synaptosomes. Further Whittaker (1964) suggested that acetylcholine was stored separately from other constituents such as the synthesising enzymes. De Robertis et al (1963) using another method suggested that acetylcholine was associated with choline acetyltransferase and with the vesicles. They explained the difference as being due to species variation. Tubbeck (1966) analysed six species for distribution of choline acetyltransferase and found some unbound in the brain of guinea pig and pigeon and bound enzyme in the brain of sheep, rabbit, cat and dog. Choline acetyltransferase was found bound mainly to the membrane fractions other than synaptic vesicles. Fonnum (1967, 1968) showed that binding of the enzyme was dependent on the pH; at low pH the enzyme binds to membranes while at high pH it is more soluble. Therefore Fonnum concluded that in intact nerve endings 'much if not all of the choline acetyltransferase must be in a free form'. The one problem with this idea is that it is difficult to account for accumulation into vesicles.

Acetylcholine Pools

The product of synthesis, acetylcholine, is found in both free and bound forms. Whittaker (1969) in a review suggested three pools. One is the extracellular pool or free acetylcholine, which is about 25 per

cent of the total. The other two fractions form the total bound acetylcholine and are recovered from the nerve ending fraction. One is thought to be cytoplasmic and released by hyposmotic shock or by mechanical pressure and is called the labile-bound fraction by Whittaker. The other is the stable-bound fraction, which is largely contained in the synaptic vesicles. From further studies of release of acetylcholine from preganglionic terminals into the venous circulation of perfused sympathetic ganglia (Perry, 1953; Birks and MacIntosh, 1961) another model of acetylcholine was suggested, in which acetylcholine was compartmentalised into two stores in the nervous system. The smaller store is readily depleted by nerve impulses, and is replenished from transmitter located in a larger store. Recently Bennett and Maclachan (1972a and b) using an electrophysiological method for measuring acetylcholine released on the stimulation of the cervical ganglia of guinea pigs, i.e. using the size of the post-synaptic event as a measure of the amount released, demonstrated the presence of one releasable store. Further Bourdis and Szerb (1972) using the prism preparation of the rat cerebral cortex suggested that the increase in acetylcholine incorporation into labile-bound fraction is due to reuptake of acetylcholine. This will also apply to the 'surplus acetylcholine' which accumulates in the presence of anticholinesterase (Birks and MacIntosh, 1961) which disappears from the ganglion during stimulation in the absence of synthesis. It is possible to explain the appearance of two releasable stores due to the presence of this surplus (Perry, 1953, and Birks and MacIntosh, 1961) since these experiments are carried out in the presence of anticholinesterases. It would appear that there is one releasible store although on analysis acetylcholine is found in two main regions, that associated with cytoplasm and that with the vesicles.

Substrates for Acetylcholine Synthesis

1. Acetate

Sollenberg and Sorbo (1970) found that a very small amount of acetylcholine can be formed from acetate by brain slices, but the rate of synthesis in this case is less than one per cent of the rate obtained when glucose is used. Further, Quastel (1962), using glucose, lactate pyruvate, a-glycerophosphate and glutamate found that all support some acetylcholine synthesis in slices of cerebral cortex but glucose is the most effective. Glucose was used to support acetylcholine synthesis in the perfused superior cervical ganglion by MacIntosh (1959).

2. Choline

Brain tissue is unable to synthesise choline, nevertheless the brain is estimated to contain 20 μmoles/gm/tissue choline. Therefore maintenance is dependent on the blood to supply the choline. Ansell and Spanner (1968 and 1971) showed that choline was carried around in the blood as phosphatidylcholine. There is also free choline present in the blood in a concentration of 10^{-5} M. Birks and MacIntosh (1961) calculated that during repetitive stimulation of the cervical ganglia the acetylcholine released required the uptake of 20 per cent of all free choline. Therefore choline must be added in order to maintain the output of acetylcholine at a reasonable value during prolonged stimulation.

Normal requirement of the nervous system is met by the free choline in the circulation. The chief reason for believing that this is so is the demonstration of a specific carrier mediated system that is operated at a low choline concentration and found to be concerned

in its entry into such tissues as the squid giant axon, (Hodgkin and Martin, 1965) slices of mammalian brain (Balfour and Hebb, 1952), nerve ending fraction of brain homogenates (Marchbanks, 1968) and some non-nervous tissue (Martin, 1969). Further this process could be inhibited competitively by hemicholinium (Butterbaugh and Spratt, 1960; Collier and MacIntosh, 1969 and Potter, 1970). This is in agreement with Birks and MacIntosh (1961) who found that hemicholinium inhibited synthesis. Therefore its effect would seem to act by preventing uptake of choline. Further evidence points to the fact that this effect is small compared with that on acetylcholine synthesis. Collier and Lang (1969) and recently Hemsworth (1972) suggest its effect is prejunctional due to its being acetylated instead of choline.

Therefore there is some doubt about the form in which choline is taken up into the cell, but little doubt that free choline can be recaptured from the synaptic cleft after release and hydrolysis of acetylcholine. This idea was first suggested by Ferry (1953) and has since been confirmed by Collier and MacIntosh (1969) and by Potter (1970).

Site of Synthesis of acetylcholine

Collier and Katz (1971) showed that transmitter released from the vesicular store must be replaced by acetylcholine synthesised at a site close to the vesicle. This would represent the extravesicular pool, although no pool has been identified. Evidence for the heterogeneity of cytoplasmic acetylcholine has been inferred from the experiments of Richter and Marchbanks (1971a and b). They showed that acetylcholine isolated from vesicles when incubated with labelled choline has a lower specific activity than acetylcholine from the cytoplasmic pool. This would suggest that there is some transport mechanism but only one

experiment has demonstrated this (Guth, 1969). Since it is difficult to show acetylcholine being transferred to vesicles there is at present considerable conjecture as to exactly where acetylcholine is synthesised, though evidence points to the cytoplasm.

Release of acetylcholine

Katz was the first to suggest that the vesicles in somatic motor nerve endings might constitute the storage system for acetylcholine released following nerve stimulation. This is reviewed by Katz (1969). To begin with they established that the miniature end-plate potentials (mepps) observed to occur spontaneously at the neuromuscular junction were due to the release of multimolecular amount, or quanta, of acetylcholine. Birks et al (1960) showed that they originated from the presynaptic nerve endings and further analysis showed that they are equivalent in size to the basic unit of transmitter release. Katz (1969) suggested the following hypothesis of release known as the 'vesicular hypothesis'. He suggested that the vesicles were normally in motion and frequently collided with the axon membrane but will not successfully release acetylcholine unless collision occurs in such a fashion that an active site on the vesicle collides with the appropriate receptor site on the membrane; fusion then occurs in such a way that the vesicle opens directly onto the synaptic cleft and releases the transmitter into the synaptic cleft. If the hypothesis holds the release of transmitter should follow a Poisson distribution, since a large number of collisions will occur with low individual expectation of success. This has been demonstrated under varying experimental conditions (Chiou and Sastry, 1968; Katz, 1969; and Katz and Miledi, 1965).

This theory does not seem to be able to account for the existence

of more than one pool of acetylcholine or the results of Collier (1969) and Collier and MacIntosh (1969) working on the perfused cervical ganglion who have evidence to show the preferential release of newly synthesised acetylcholine. Further studies on the phrenic-diaphragm preparation by Potter (1970) and on the cerebral cortex in vivo (Barker et al, 1970) have also indicated that this is happening. The existence of two populations of vesicles requires careful consideration.

Del Castillo and Katz (1956) suggested that some vesicles had a larger area or number of reactive sites, so that collision with the terminal membrane would be more likely to occur. Whittaker (1970) discussed this and suggested that it may explain the loss of a small 'hot pool' of acetylcholine that occurred in the preparation of synaptic vesicles, that is, the high turnover vesicles are more fragile than others. Such a difference in the vesicle structure might arise with age or usage, according to the number of cycles of filling and emptying it undergoes. Rodriguez (1970) has suggested that the mean life of a vesicle is twenty-one days. If this is so it may be expected that they will be used and reused many times.

Another argument by Hebb (1972) which can explain the discrepancies is that if all impulse-releasible or depot acetylcholine is contained in the synaptic vesicles, then that population of vesicles nearer the sympathetic membrane at the time of arrival of a nerve impulse will be more likely to collide with the membrane than the vesicles further away and hence have more opportunities to discharge their contents. This interpretation fits into the accepted division of depot acetylcholine into readily and less readily releasible pools; and further, that the release of transmitter from a vesicle is immediately followed by its filling up again with newly synthesised transmitter. With a slowly shifting population of vesicles that discharge their contents

more than once before more outlying vesicles would enter the active area.

Further evidence to support the vesicle hypothesis that exocytosis does occur has been carried out on cholinergic nerve endings by freeze-etching technique used by Nickel and Potter (1970). They revealed a picture that convincingly demonstrates the different stages of exocytosis and the involvement of synaptic vesicle-like structures in this process.

Catecholamines

Pathway for synthesis

The correct pathway was first proposed for catecholamine metabolism in mammals by Blascko (1939) after the demonstration of the decarboxylation of 3,4 dihydroxyphenylalanine (DOPA) by Holtz (1939) in guinea pig kidney. Blascko proposed that in the formation of adrenaline from tyrosine four steps are necessary, three of which must occur in the following order:

(i) introduction of a phenolic hydroxyl group
(ii) decarboxylation of the amino acid group
(iii) N-methylation;

the fourth step was the introduction of an hydroxyl group into the side chain, but the stage at which this happened could not be predicted. Gurin and Delluva (1947) showed that phenylalanine was a precursor of adrenaline using C14 and H3 phenylalanine. They administered the radioactive phenylalanine to rats and isolated radioactive adrenaline from their adrenal glands. Finally using isotopic dilution technique Goodall and Kirshner (1957) confirmed the proposed pathway. All that

remained was to isolate the various enzymes involved. In fact it was still difficult to prove dopamine was converted to noradrenaline until Levin et al (1960) succeeded in isolating and characterising the enzyme dopamine-B-hydroxylase. Further there was controversy on the nature of the phenolic hydroxylation, some groups suggested that a tyrosinase was responsible. But Nagastu and Udenfriend (1964) managed to isolate and characterise the enzyme tyrosine hydroxylase from beef adrenal medulla.

Inactivation of Catecholamines.

(a) Enzymic.

The major enzymes involved are termed 'monoamine oxidases' (MAO). This term is used to designate a group of enzymes concerned in catalysing the oxidative deamination of tyramine, tryptamine, serotonin, noradrenaline, dopamine and other amines. However MAO could not account for all the inactivation. Evidence for another enzyme being involved was demonstrated when Armstrong et al (1957) identified 3-methoxy-4-hydroxymandelic acid in the urine of subjects with adrenaline forming tumours. This observation immediately suggested that catecholamines might undergo an O-methylation reaction. Cantoni (1951) had shown that S-adenosylmethionine formed enzymatically from ATP and methionine can donate its methyl group to the nitrogen of a nicotinamide. Therefore Axelrod (1957) added ATP and methionine to rat liver fraction plus Mg and adrenaline and showed that this catecholamine was converted to a methylated derivative 3-methoxy-4-hydroxyphenylglycol. This was followed by the isolation of the enzyme catecholamine-O-methyl transferase (COMT) from rat liver by Axelrod and Tomchick (1958). This enzyme is present in all mammalian

species examined and exist in some plants. The liver and kidney exhibit highest activity and in the brain it is unequally distributed being highest in the area postrema and lowest in the cerebral cortex (Axelrod et al, 1959). COMT occurs mainly in the soluble fraction of the cell, but small amounts are present in the fat cell membranes (Traiger and Calvert, 1969) and in the microsomes (Inscoe et al, 1965). The function of the enzyme was suggested by Kopin (1964). Since this enzyme acts mainly outside the neurone, Kopin (1964) suggested that its function was to metabolise catecholamines released into the circulation and also to inactivate catecholamines in tissue with sparse adrenergic innervation (Levin and Furchgott, 1970). It also appears to be associated with an extraneuronal uptake mechanism (uptake 2) according to Eisenfield et al (1967). Uptake 2 is a term designated for the uptake of endogenous amines into certain non-neuronal tissue (Iverson, 1967), such as the transport of noradrenaline and other catecholamines into cardiac muscle cells (Farnebo and Malmfors, 1969; Clark et al, 1969). Recently COMT has also been shown to be present in mammalian erythrocytes by Axelrod and Cohn (1971). Whereas MAO is localised mainly within the neurone, the kidney and liver, (Axelrod, 1966). Investigation of the intracellular content of MAO by Braudhuin et al (1964) showed that mitochondria contain about 70 per cent of the total MAO content.

The pysiological significance of MAO is not clear, although it is certain that it plays a part in detoxification and degradation of monoamines formed by various organs. In a review Kopin (1964) it has been pointed out that there is experimental evidence that indicated that the noradrenaline more firmly bound to the storage granules in the nerve, and which is released either by physiological turnover or by reserpine treatment undergoes oxidative deamination.

The less firmly bound and exogenous noradrenaline, and that released by nerve stimulation or tyramine is acted on preferentially by COMT. Under physiological conditions a small portion of intracellular amines is present in an unbound form in the cell plasma and is in equilibrium with the firmly bound portion. The unbound catecholamine is being continually metabolised by MAO. Therefore it is conceivable that an important function of this enzyme is the maintenance of a diffusion gradient in the direction of the unbound amine. This intracellular regulatory mechanism is necessary to maintain a constant amine concentration in the cell in spite of continuous synthesis and storage of amines (Brodie and Beaven, 1963).

(b) Uptake.

Inactivation of neurotransmitter can be carried out by an uptake mechanism. Basically the transmitter following release from the synaptic cleft is removed by means of a membrane transport system located adjacent to that region. The ability of postganglionic sympathetic neurones to take up exogenous catecholamines is now well known and has been extensively reviewed by Iverson (1970a and b, 1967).

The work on this process was made possible by the synthesis of tritated noradrenaline and adrenaline of high specific activity at the 7 position. This made it possible for the administration of physiological amounts of the neurotransmitter and the study of the localisation and metabolism of circulating catecholamines. Axelrod et al (1959) found after intravenous injection of 3(H)adrenaline into cats the catecholamine was rapidly and unequally distributed in tissues. Injections of 3(H)noradrenaline by Whitby et al (1961) gave similar results. Hertting et al (1961) found that when sympathetic nerves to an organ were allowed to degenerate there was a sharp

reduction in the uptake of 3(H) noradrenaline by the organ. Iversen (1963) demonstrated uptake of 3(H) noradrenaline in the isolated perfused rabbit heart. Further evidence for uptake being restricted to sympathetic nerves was demonstrated by the observation that the uptake of 3(H) noradrenaline was reduced by more than 95 per cent in rat hearts after sympathetic innervation was removed through immunosympathectomy at birth (Iversen et al, 1966). The uptake process is inhibited by a wide range of sympathomimetic amines structurally related to noradrenaline, and the results of such experimental information has been obtained on the structural activity relationships required for the optimal binding of B-phenylethylamines to the noradrenaline uptake sites on the sympathetic nerve terminal (Burgen and Iversen, 1965). Such binding occurs with very high affinity for certain amines such as (-)-metaraminol, which binds with an affinity about four times higher than that of the natural substrate (-)-noradrenaline.

Uptake in the CNS

Neurones in the mammalian CNS which contain noradrenaline or dopamine are also capable of an uptake of exogenous catecholamines. The kinetics of these uptake processes have recently been studied in adrenergic nerve terminals (synaptosomes) derived by homogenisation of brain areas rich in either noradrenaline, e.g. hypothalamus, or dopamine, e.g. neostriatium (Colburn et al, 1968; Coyle and Synder, 1969 a and b; White and Keen, 1970). These results suggest that the catecholamine uptake process for noradrenaline containing nerves is similar to the peripheral sympathetic neurones. Dopamine containing nerve terminals accumulate external catecholamines by a different mechanism. The uptake process will accumulate both noradrenaline and

dopamine, but has a higher affinity for dopamine, and shows no preference for optimal isomers. This uptake process is only slightly inhibited by imipramine and its analogues, but is very greatly inhibited by (-)-amphetamine and by certain anti-Parkinsonian drugs such as benzatropine, an atropine-like drug, which are not so potent in inhibiting uptake by noradrenaline neurones (Hamberger, 1967; Coyle and Synder, 1969a and b).

Release of Catecholamines

The evidence already discussed for quantal release of acetylcholine has naturally led to the suggestion that the same system might hold true in the case of adrenergic neurones. The possibility that the entire amine content of an amine granule might be released following nerve stimulation is supported by the observation that on stimulation of the adrenal medulla there is release of catecholamine and AMP in the same ratio as catecholamines to ATP in the gland. The distinctive soluble proteins of the adrenal medullary amine granules, chromgranins and dopamine-B-hydroxylase are similarly released (Kirshner and Viveros, 1970; De Potter et al, 1969). Amine granules are re-utilised since the turnover of noradrenaline in the adrenergic neurones is far more rapid than the life span of the granules. Evidence by Glowinski (1970) that the vesicle is not lost in its entirety may be found in experiments in vivo in which persistently bound reserpine, presumably bound to the granular membrane, is not affected by treatment such as insulin hypoglycaemia which causes intense neurogenic stimulation.

Compartmentalisation of noradrenaline into pools within granules is in line with the concept of a functional or 'available' noradrenaline pool, that is more readily released by nerve impulse or by indirect

agents such as amphetamine (Kopin et al, 1968; Schildkraut et al, 1971 and Stjärne and Wennmalm, 1970). Kopin et al (1968) showed that rapid stimulation of the spleen perfused with labelled tyrosine with a high specific activity resulted in the appearance in the perfusate of labelled noradrenaline of higher specific activity than that retained by the organ. Schilkraut et al (1971) showed recently that either spontaneously or after electroshock a high percentage of intracellular noradrenaline is converted to normetanephrine shortly after injection of noradrenaline. Further the ability of a-methyl-tyrosine, a tyrosine hydroxylase inhibitor, to inhibit amphetamine induced locomotive activity and stereotype in the rat, even without complete depletion of brain catecholamine stores, has been shown by Weissman et al (1966). This supports the hypothesis that newly synthesised noradrenaline is more readily released by nerve stimulation and is uniquely important in neurotransmission. Hedqvist and Stjärne (1969) suggested that reuptake of released noradrenaline rather than newly synthesised noradrenaline is the major mechanism for the maintenance of neuronal noradrenaline concentration rather than newly synthesised noradrenaline. Therefore the existence of two pools, particularly in the adrenal medulla, has led to the suggestion that there exist two populations of vesicles which represent the two pools by Slotkin et al (1971).

In addition all the enzyme seems to come from the soluble dopamine-B-hydroxylase of the storage granules and none is particular bound (Kirshner and Viveros, 1970). Therefore the results suggest that release at least in the adrenal medulla occurs by exocytosis. Further it has been demonstrated that the entire contents have been released from the adrenal gland (De Potter et al, 1969; Serck-Hanssen, 1971 and Viveros et al, 1969). In the mammalian neurone Folkow and Haggendal (1970) have calculated that only a small percentage of noradrenaline

contents of one granule correspond to a single quantal packet. This indicates that the quantum released is equivalent to a small portion of the granule or 'functional' compartment.

S E C T I O N I

METHODS

(a) The Preparation

The suboesophageal ganglia of Helix aspersa contains mainly large neurones reaching a size of 200 μ in diameter, for example the Big D cell. The size of the neurones makes it relatively easy to insert glass electrodes into them, to study the properties of this particular nervous system. Snails used for experimental purposes had shells which were approximately 3 cm. in diameter.

Dissection of the snail to remove the brain was carried out by first removing the shell and then extending the foot out and pinning it ventrally to a wax block. A slit was made along the central line of the dorsal side. The viscera was pinned back to expose the ganglia which were then removed and placed on a glass slide and held in place by placing the cerebro-visceral commissures under a rubber band and the nerves not required for stimulation under a second band, see figure 1. They were first stained in 1 per cent methylene blue solution and placed in the bath. Using a low power on the binocular microscope the thick connective tissue over the suboesophageal ganglia was removed with fine forceps. The nerves to be stimulated were also freed from the connective tissue. A little methylene blue was added to the experimental bath to stain up the cells slightly so they could more easily be seen.

(b) The Experimental Bath

The experimental bath was constructed from perspex with a glass front as shown in figure 1. It was filled with wax and a platform at 45° to the horizontal was hollowed out to firmly support the glass slide with the brain attached to it. When changing solutions of

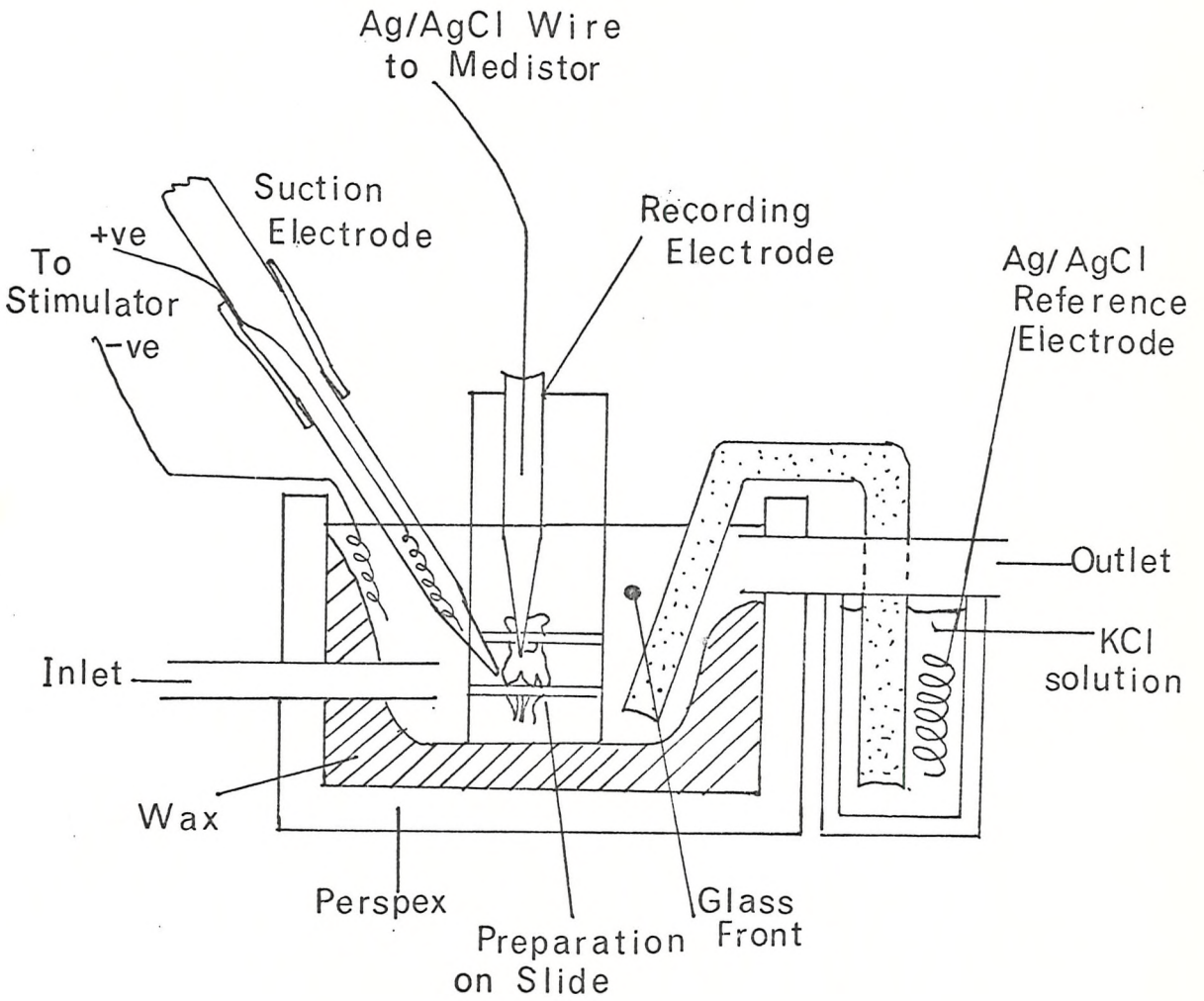

Fig. 1. Diagram of Experimental Bath.

Ringer about 300 ml. were run through; this was sufficient to remove a strong solution of methylene blue.

The reference electrode was separated from the bath by placing it in a separate container which was in electrical contact with the bath through an agar bridge, a tube containing agar gel saturated with KCl. The reference electrode consisted of a coil of silver wire coated with silver chloride, which was deposited by electrolysis from dilute HCl. The amount of wax was adjusted to give a bath volume of 20 ml.

(c) Observation and Illumination

The preparation was illuminated from above and slightly behind by a narrow beam of light focussed on the superficial cells by a moveable lens. In this way the lighting could be critically adjusted to show the cells against a dark background. A 'Prior' binocular microscope 10.20 was positioned to observe the preparation horizontally through the glass front of the bath.

(d) Ringer's Solution

The standard Ringer used throughout this investigation was that described by Kerkut and Meech (1966). The solution was prepared by mixing varying volumes of molar stock solutions and adding distilled water to give the final concentration. It was made up as follows:

1M NaCl	80 ml.
1M KCl	4 ml.
1M $CaCl_2$	7 ml.
1M $MgCl_2$	5 ml.
1M Tris/HCl	5 ml.

(pH 7.8)

Distilled water was then added to give a final volume of 1 litre. Chloride-free Ringer used in Section 1 was obtained by replacing chloride salts with their sulphate analogues. For potassium-free Ringer, sodium chloride was used to replace the potassium chloride.

(d) Microelectrodes

Microelectrodes were made from 'pyrex' glass tubing having an external diameter of 4 mm. The glass was cut into 7.5 cm. lengths, washed in hot distilled water, rinsed in acetone and finally dried in an oven. Single lengths of glass were pulled on a 'Narishige' vertical puller. Basically this consisted of a heating coil in which the glass was placed and held in position by two chucks. The bottom chuck was connected to a magnetic coil so that when the heating coil had heated the glass to the correct temperature the coil supplied a downward force which separated the glass in the centre leaving two very sharp tips on either end of the glass. The resistance of these tips was around 15 Meg ohms. The electrodes were normally filled with 1M potassium acetate. This was done by first boiling under pressure in methanol until the electrodes had filled. They were then placed in distilled water and boiled under pressure to remove the methanol. This was repeated twice to ensure that all the methanol had been removed. The electrodes were then left in molar potassium acetate overnight. Another method used was to introduce acetate into the shanks of the electrode by means of a syringe and allowing it to fill the tip of the electrode by capillary action. Electrodes having a resistance of 5-30 Meg. ohms were selected for use. They were held in a crocodile clip supported in a 'Prior' micromanipulator and could be accurately positioned over the preparation.

(f) Recording and Display of Potentials

The arrangement of apparatus used to display and record intracellular potentials is shown in figure 2. Potential changes in the neurone were recorded relative to the bath potential. From the recording electrode, the signal was led, via a chlorided silver wire to a Medistor A-35 Electrometer Amplifier. The reference electrode was also connected to the medistor. This electrode was a chlorided silver wire which was placed in a reference bath containing potassium chloride which was in contact with the Ringer in the experimental bath via an agar jelly/potassium chloride bridge. The separate reference bath was used to prevent the effect of changing Ringer in the bath from causing a shift in the resting potential and spurious potential changes. The amplified signal from the medistor was displayed on a Tektronix 502A oscilloscope. The oscilloscope was led to a 'Watanabe' WTR 211 pen recorder so that a permanent record could be obtained.

(g) Altering Membrane Potential

To alter the membrane potential required use of a bridge circuit, see figure 3. The bridge could be switched off or made to give either hyperpolarising or depolarising current to the impaled neurone by the three way switch S. The magnitude of the current to the impaled neurone could be determined by the position of the ten-turn potentiometer, Po.1.

If an accurate measurement of changes in the membrane potential was required, the bridge had to be balanced. This was carried out by placing the tip of the micro-electrode in contact with the bath solution. The bridge was switched off, switch S was placed in its central position and the oscilloscope beam adjusted to its reference

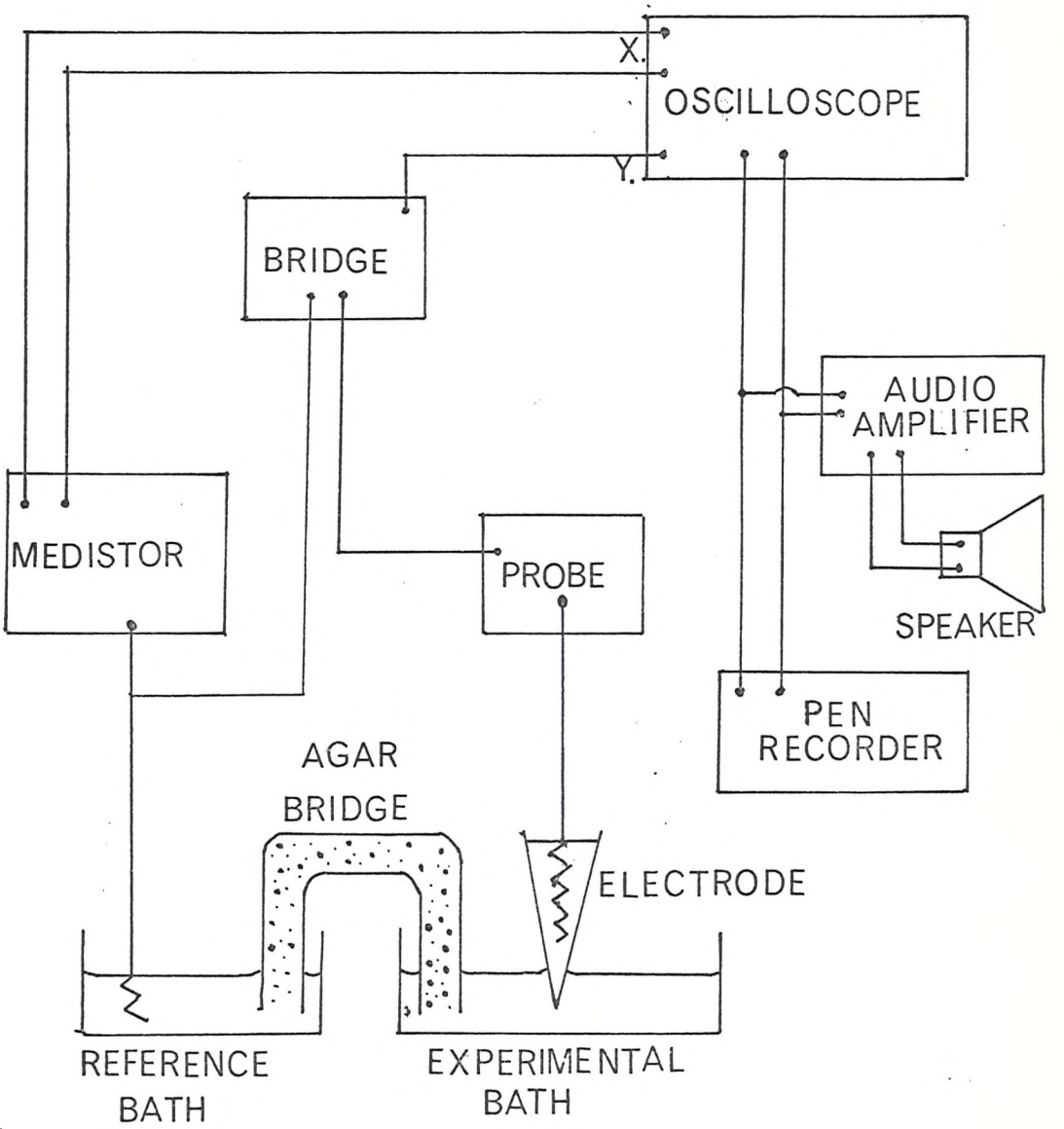

Fig. 2. Diagram showing Circuit used for Intracellular Recording from the Brain of Helix aspersa.

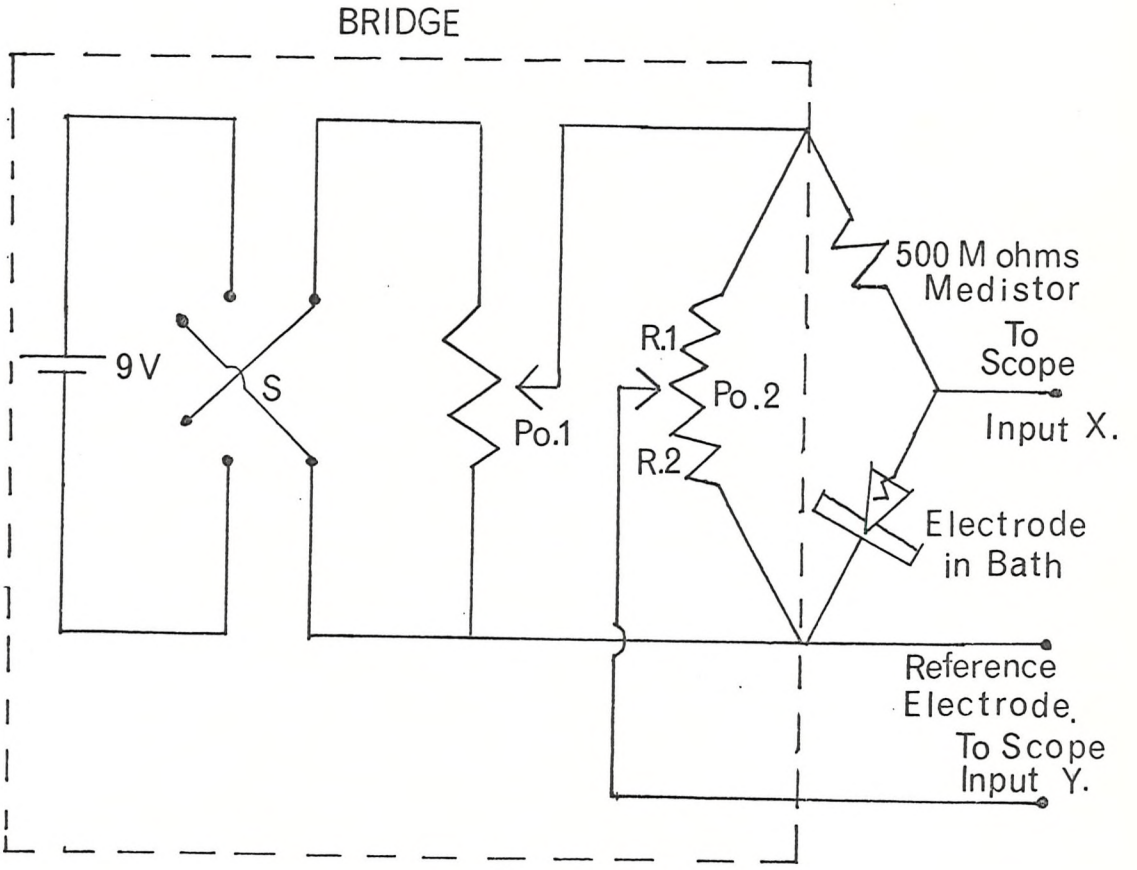

Fig. 3. Circuit Diagram of Bridge Circuit.
(Po-potentiometer, R-resistance and S
-switch selector,-ve or +ve.)

position. A hyperpolarising or depolarising current was applied from the bridge by switching S up or down with the current potentiometer adjusted to give maximum current. This would cause a deflection of the beam because of the different potential inputs being applied to the oscilloscope amplifier (inputs X and Y). The beam was returned to its original position on the screen by adjusting a second potentiometer Po.2. This brought the bridge into balance so that the ratio of the 500 Meg. ohm. resistance of the medistor probe and the electrode resistance was the same as the ratio of the resistance R1 and R2. When the cell was impaled any deflection of the oscilloscope beam caused by operation of the bridge circuit was a true measure of the membrane potential of the neurone, provided that the resistance of the recording electrode had not altered during impalement.

(h) Stimulation of Nerves

A 'Grass' S8 stimulator was used to provide single repetitive stimuli to the nerves. The stimulator was always used in conjunction with a 'Grass' Stimulus Isolation Unit. Suction electrodes were placed in the bath as in figure 1. The electrode consisted of a glass microelectrode with the tip broken down and flame polished, to avoid damage to the nerves on insertion into the electrode. The electrodes were connected to portex tubing. The chlorided stimulus wire inside the electrode was connected to a wire from the stimulus isolation unit at the connection between the glass and the tubing, see figure 4. This connection was cemented to ensure that it was leakproof. Suction was provided by a 20 ml. syringe connected to the tubing. The electrode was connected to a micromanipulator so that it could be easily transferred from one nerve to another. Before the nerves were sucked up the connective tissue around them was removed to

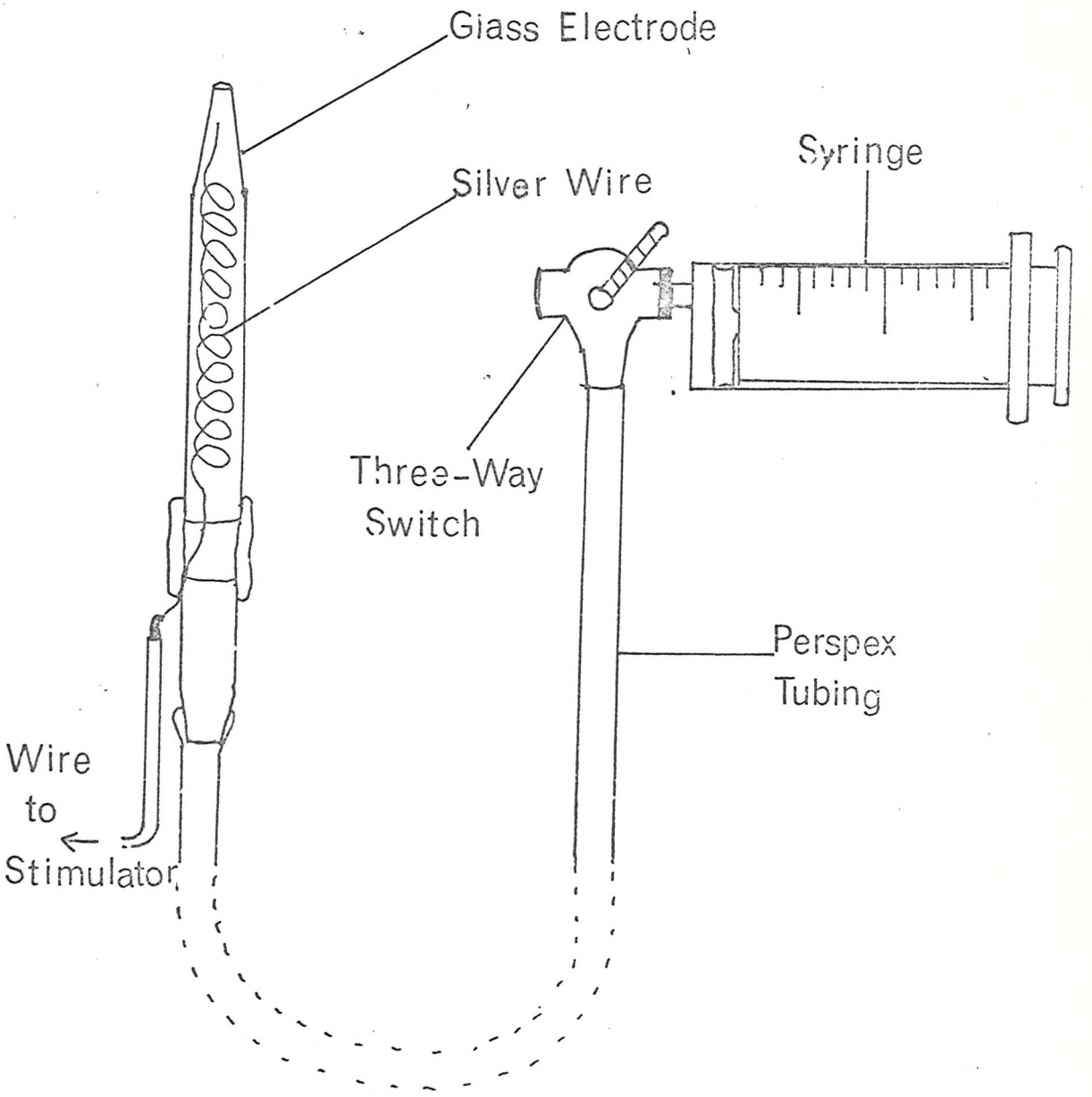

Fig.4. Diagram of suction electrode.

ensure a better contact. The voltage of the stimulation was between 0.5 - 5V and adjusted to give maximal amplitude of the response. The duration of the pulse was 2 msec.

(i) Identification of cells

The cells used in the study are situated in the visceral ganglion, see figure 5. The major difficulty in all experiments was the identification of the cell as it was found that the size of the cell varied considerably from preparation to preparation. However the approximate location of any one cell was constant. Cell identification was made on its spontaneous activity, pharmacological responses and synaptic inputs.

(j) Addition of Drugs

Putative transmitters were made up in Ringer at a known concentration and added directly over the preparation. The volume used was kept constant for each normally being 0.1 ml. Antagonists were added in a similar way but their concentration was expressed as a final concentration in the bath after equilibration with the volume of Ringer in the bath, which was 20 ml.

Iontophoresis was used to apply compounds to a specific neurone. Glass electrodes of low resistance (1 - 2 Meg. ohms) were employed and the pulse length controlled automatically. The electrodes were filled with molar concentration of the drug adjusted to a suitable pH to give good ionisation. The drug electrodes were filled as follows. The water in the shank of the electrode was removed with a syringe and replaced with the drug. The drug then diffused to the tip of the electrode.

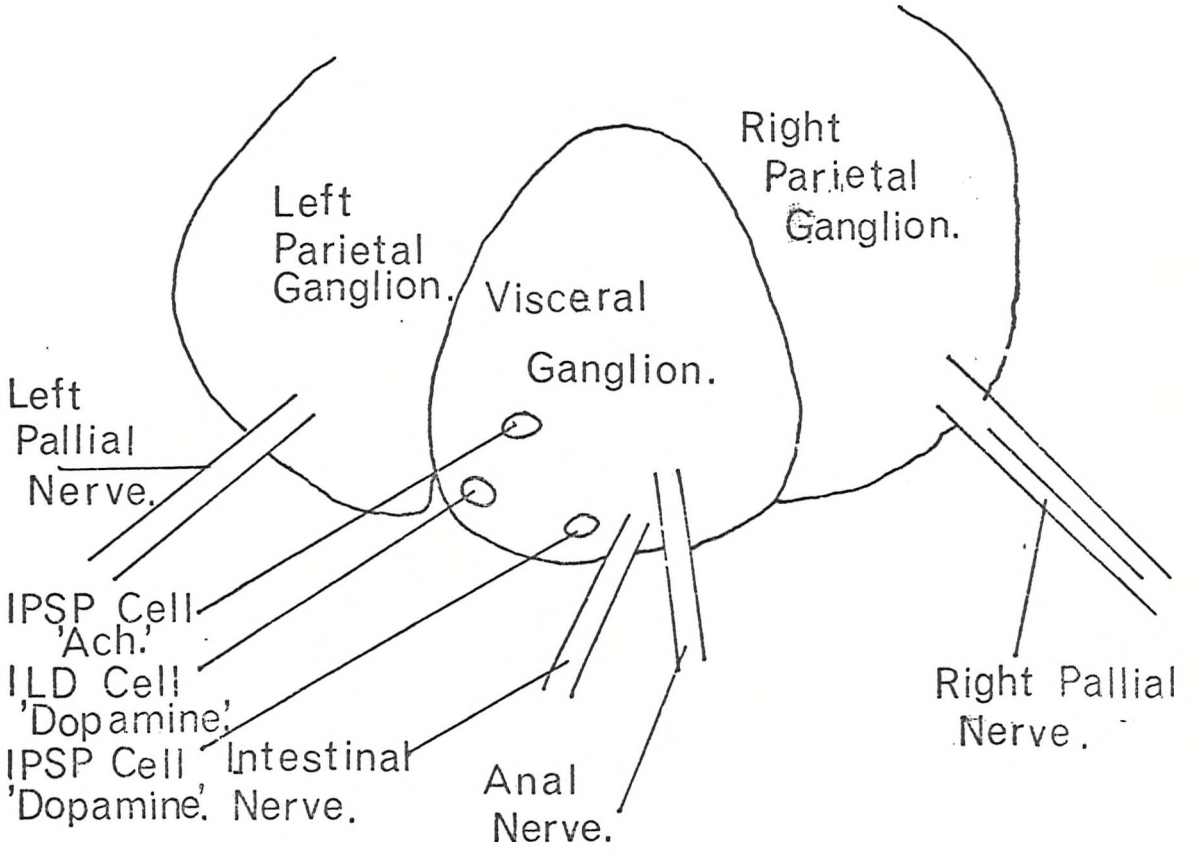

Fig. 5. Diagram of sub-oesophageal ganglia of Helix aspersa.

(k) Compounds used and their Sources.

The following compounds were used in the present study:

Acetylcholine chloride	BDH
Choline chloride	BDH
Hemicholinium 3	Aldrich Chemical Co. Inc.
Morphine chloride	BDH
Tubocurarine	Borroughs-Wellcome
Dopamine chloride	Koch-Light
Tyramine chloride	Koch-Light
Noradrenaline sulphate	Koch-Light
Adrenaline HCl	Koch-Light
Synephrine HCl	Lewis Laboratories, Leeds
Cocaine	BDH
Amphetamine sulphate	BDH
Ergometrine bisulphate	Halewood
NSD. 1024 HCl	Smith, Nephew Research
NSD. 1055 HCl	Smith, Nephew Research
3-methoxy-4-OH	Smith, Nephew Research
Phenylethylamine HCl	Calbiochem
4-OH, $3CH_3O$, Mandelic acid	Sigha
3,4 DiOH Phenylacetic acid	Koch-Light
Homovanillic Acid	Calbiochem
3,4 D_3iOH Mandelic Acid	Sigma

SECTION 1a

AN ELECTROPHYSIOLOGICAL ANALYSIS OF RELEASE, STORAGE AND SYNTHESIS OF TRANSMITTERS IN SINGLE IDENTIFIABLE NEURONES IN THE BRAIN OF THE SNAIL, HELIX ASPERSA

Introduction

In an attempt to study storage and release of acetylcholine, Perry (1953) and Birks and MacIntosh (1961) stimulated the preganglionic terminals and collected the acetylcholine released into the venous circulation of the perfused cervical ganglia of the cat. From these results the hypothesis has been built up that acetylcholine is comparmentalised into two stores in the nerve terminal: a smaller store which is readily depleted by stimulation and is refilled by the larger store. Similar results have been obtained for adrenergic nerves by von Euler (1965) and Kopin (1968) who stimulated the splenic nerve and measured the catecholamines released in the perfusate from the spleen, However, for the acetylcholine system an electrophysiological analysis has been carried out recently by Bennett and McLachlan (1972a) on the cervical ganglion of the guinea pig, and they have demonstrated that there is a single releasable store present in the nerve terminals. Release of acetylcholine from this store is expotential.

Electrophysiological techniques were used by Elmqvist and Quastel (1965a and b), to measure the size of the store of transmitter in the motor nerve terminal at the mammalian end plate. To measure the amount released by the nerve terminal they used the size of the postsynaptic response. Ralph (1970) used the time taken to reduce the postsynaptic response to a constant value when in the presence of different agents which block transmitter synthesis as a method of identifying the transmitter released at the synapse in the brain of

Helix aspersa. The technique used by Bennett and Maclachlan (1972a and b), was of intracellular recording from cells in the cervical ganglion of the guinea pig. However they were unable to identify the cells used. The synaptic event used is difficult to equate with the release of transmitter since the epsp's (excitatory post-synaptic potentials) tend to summate and a correction has to be made for this.

Using mammalian preparations presents certain problems, e.g. identification of cells. These can in part be overcome by using invertebrate preparations.

A considerable amount of our knowledge of cellular neurophysiology has been gained using simple, invertebrate preparations. The advantage of a molluscan preparation such as Helix aspersa is that it is relatively easy to insert intracellular electrodes into the larger cells and it is often possible to recognise the same cell from preparation to preparation. These properties facilitate the study of excitable membranes, with respect to ions and pharmacologically active substances.

In the present study an electrophysiological analysis was made by stimulating a single nerve and recording intracellularly the inhibitory post-synaptic potential in an identifiable neurone. The effect of frequency was studied on the time taken for the ipsp to be reduced to a constant height. For the acetylcholine system this was carried out in the presence of hemicholinium and morphine. It would appear from the results that each stimulus releases a constant fraction of the store. The amplitude of the ipsp is dependent on the amount of acetylcholine present in the presynaptic terminals. Release is also blocked by morphine. Resynthesis of acetylcholine depends on the amount released and could be blocked in the presence of hemicholinium. It is also suggested that acetylcholine is stored in a single

releasable store in Helix aspersa. The dopamine inhibitory events could not be reduced to a constant size by repetitive stimulation, but were potentiated by cocaine and amphetamine. These results suggested the presence of an amine pump in the brain of Helix aspersa.

Results of Ach. Neurone.

Pharmacology of identifiable Neurone

An addition of acetylcholine either directly to the bath or by iontophoresis causes the membrane potential to be hyperpolarised by up to 20 mV. The action of acetylcholine is reversibly blocked by $5 \cdot 10^{-5}$ gm. of tubocurarine. The activity of the cell is excited by the addition of GABA and glutamic acid, but fails to respond to dopamine or 5HT.

The membrane potential of the cell was found to be around 45 mV. When the membrane potential is hyperpolarised to -80 mV, the acetylcholine response disappears. The effect of chloride free Ringer on the acetylcholine response causes little or no change in the nature of the response. The addition of potassium free Ringer causes potentiation of the ipsp. The acetylcholine effect is therefore associated with an increase in potassium permeability.

Pharmacology of the Stimulated Event on the Identifiable Neurone

The neurone exhibits spontaneous inhibitory post-synaptic potentials (ipsp's). These are driven via the anal nerve, see figure 6, and range in size from 14 mV to 20 mV. Desensitisation of the membrane through addition of large doses of acetylcholine greatly reduces the amplitude of the ipsp. This ipsp is reversibly blocked by tubocurarine. As the membrane potential was gradually hyperpolarised,

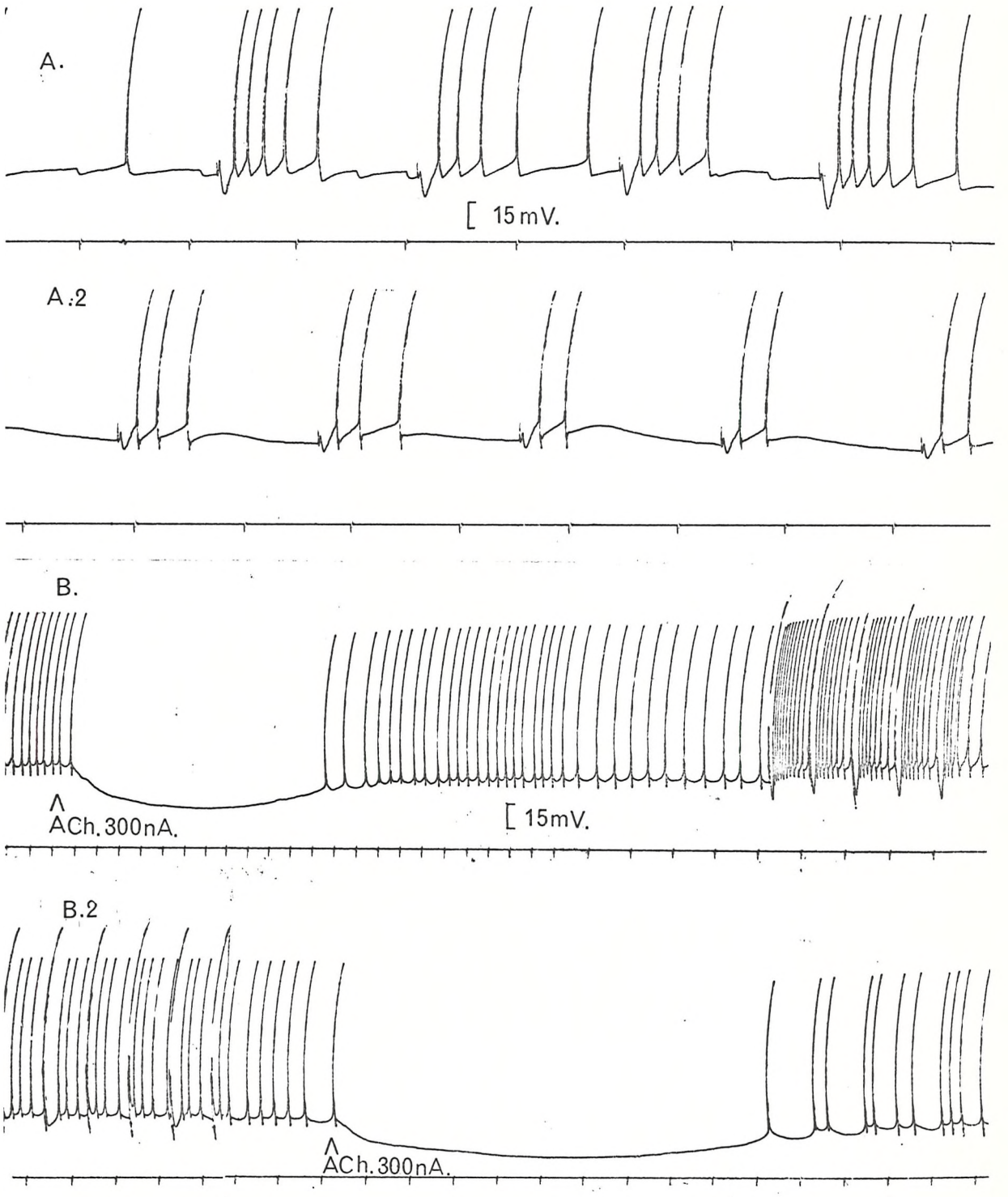

Fig. 6. Trace A : Initial effect of Repetitve Stimulation at one every 1.75sec.
Trace A2: Effect of 150 Stimuli.

Trace B : Effect of Iontophorectic Application of Ach before Stim.
Trace B2: After Stimulation.

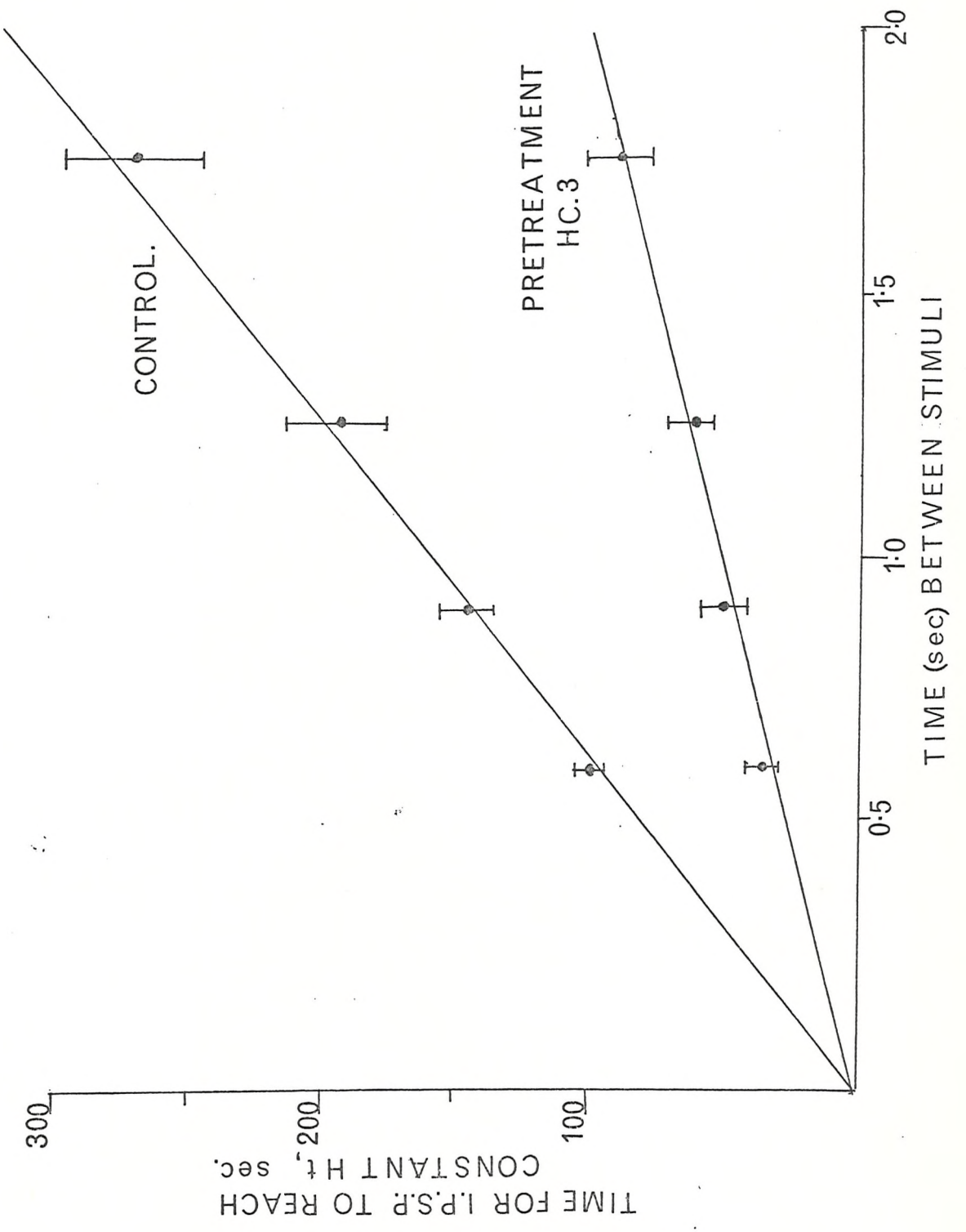

Fig. 7. Graph of Time of ipsp height to reach a Constant Level against time between stimuli. Also the effect of HC.3 pretreatment.

the ipsp was gradually reduced in size until between 80 mV and 90 mV it had been completely abolished, i.e. reached its equilibrium potential. Further, the ipsp is associated with an increase in permeability to K ions.

Effect of Stimulation on the Size of the IPSP

When the anal nerve is stimulated at low frequencies, e.g. 0.1 Hz, the height of the ipsp remained constant over a period of 20 minutes. This height varied slightly from preparation to preparation, but was in the range of 14 mV - 20 mV. This value was taken as the initial height. When the nerve was stimulated at a frequency of 1 Hz, the amplitude of the ipsp gradually fell until it reached a constant height. This final height was in the range of 4 mV - 6 mV, figure 6 A2. About 150 stimuli are required to reduce the ipsp to this final value.

Effect of Iontophoretically Applied Acetylcholine before and after Stimulation

It is possible that acetylcholine released during repetitive stimulation causes desensitisation of the receptor and so accounts for part of the decrease in amplitude of the ipsp. To check this, a standard dose of acetylcholine was applied iontophorectically before and after the period of repetitive stimulation. The amplitude of the response remained constant after a period of repetitive stimulation, though during this period the amplitude of the ipsp declined from 15 mV to 5 mV, see figure 6 B and B2.

TABLE I: RESULTS OF CONTINUOUS STIMULATION OF THE ANAL NERVE OF HELIX ON THE CHOLINERGIC IPSP OF A SINGLE CELL IN THE VIS. GANGLION

Time (in seconds) between Stimuli	No. Stimulation Reduce to Con. Ht.	Time (in seconds) to reduce to Constant Ht.	Height IPSP (mvs) Initial	Final
0.6	178	107	15	5
0.6	167	100	14	5
0.6	160	96	15	5
0.6	150	94	16	5
		99. sd ±5.7		
0.9	150	138	15	5
0.9	156	142	17	5
0.9	176	160	20	5
0.9	168	150	14	4
0.9	155	140	12	5
		146. sd ±9		
1.25	180	225	15	5
1.25	140	175	16	5
1.25	150	187	15	5
1.25	152	190	14	5
1.25	150	197	15	4
		194. sd ±19		
1.75	135	240	12	4
1.75	145	254	14	3
1.75	159	278	15	4
1.75	168	294	14	4
1.75	167	292	15	5
	158 sd ±12	271 sd ±24	14.9 sd ±2	4.7 sd ±.6

Effect of Variation of Stimulating Frequency

Repetitive stimulation was carried out at four different frequencies, i.e. one every 1.75 secs, 1.25 secs, 0.9 secs and 0.6 secs. Five values were obtained from the different preparations for each frequency. The results are expressed in a graph, see figure 7, or as a table, Table I. The number of stimuli required to reduce the ipsp to a constant final amplitude was found to be independent of frequency in the range used. The amplitude of the final height did not vary with frequency, and therefore it is also independent of the frequency range used. It can be seen from the figure7, that when the time taken to reduce the initial ipsp to a constant amplitude is plotted against frequency of stimulation, a linear relationship is obtained. This indicates that a constant number of stimuli are required to reduce the ipsp to a constant height. This value from the pooled data (see Table I) was 158, sd ±12. Therefore the rate of reduction in amplitude is directly dependent on the frequency of stimulation. This would indicate that each stimulus releases a constant fraction of the store.

Recovery of IPSP after Stimulation

The cell was left for a minute following stimulation and then restimulated. This was repeated several times at different intervals until the ipsp returned to near its normal size. The ipsp was found to recover to about 12 mV, but the results tended to vary considerably from preparation to preparation, probably due to the system deteriorating since the Ringer is just a basic physiological ion solution. Therefore no valid conclusions could be drawn from these results.

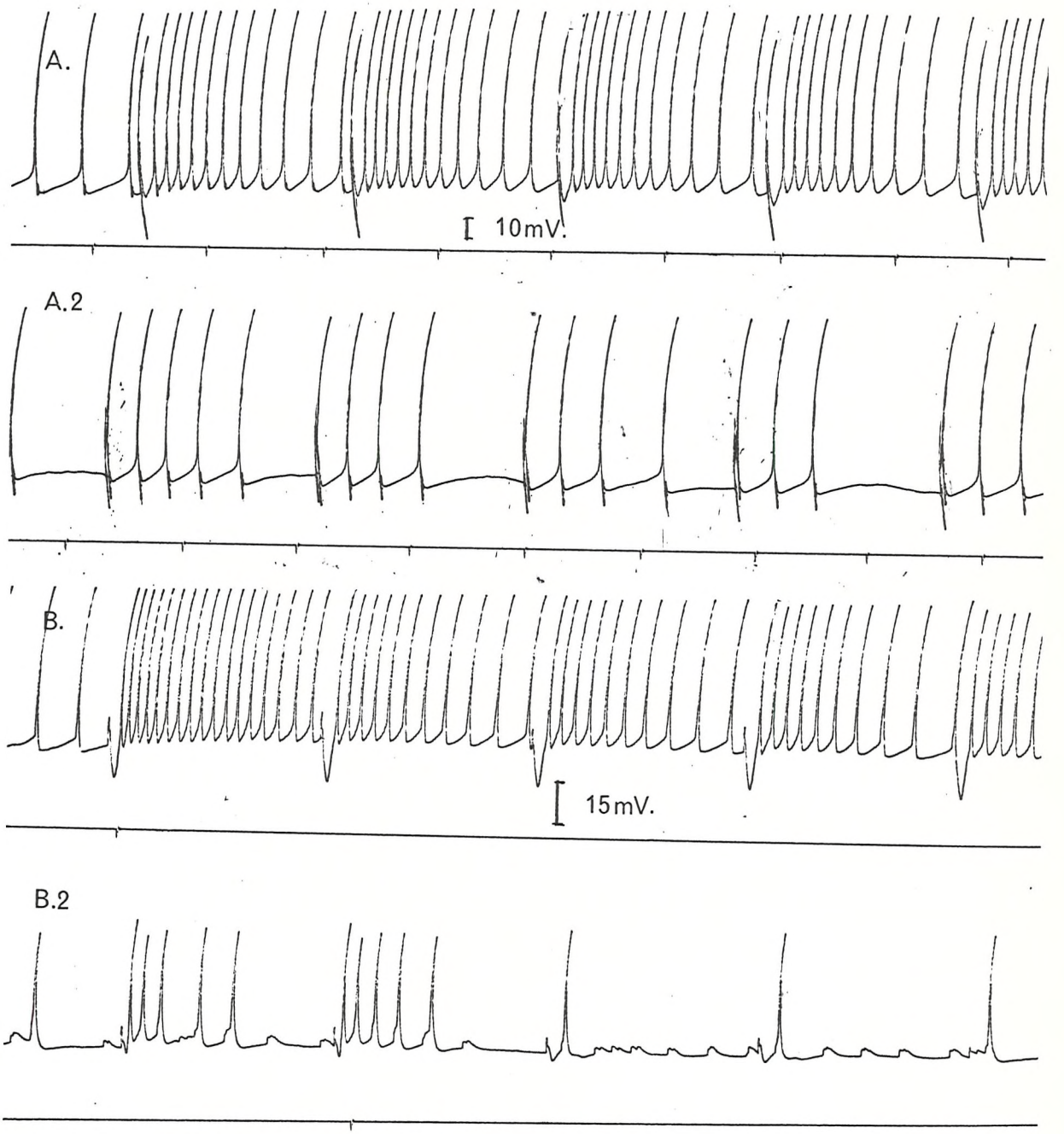

Fig. 8. Trace A : Initial effect of repetitive stimulation at one every 1.75sec after HC.3 pretreatment.
Trace A.2: Effect of 50 stimuli after HC.3 pretreatment.
Trace B. : Initial effect of repetitive stimulation at one every 1.75sec after addition of HC.3.
Trace B.2: Effect of 130 stimuli after addition of HC.3.

Effect of Hemicholinium Pretreatment

Snails were injected with 50 µg hemicholinium in 0.05 ml Ringer three hours prior to dissection. In these pretreated animals the initial height of the ipsp was between 4 mV - 6 mV and after repetitive stimulation the ipsp height fell to less than 1 mV, see figure 8 A and A2, indicating that in the presence of hemicholinium, nearly all resynthesis had been blocked. In pretreated animals repetitive stimulation was carried out at the same four frequencies as in the control untreated animals. The results are shown in figure 7. The slope of the graph was reduced, indicating that the number of stimuli required to reduce the ipsp to a constant height was smaller, the number being 53 ± 10, see Table II. The effect of hemicholinium pretreatment on the initial and final height and on the number of stimuli required to reach this constant height compared with the control values, is shown as a histogram, figure 9.

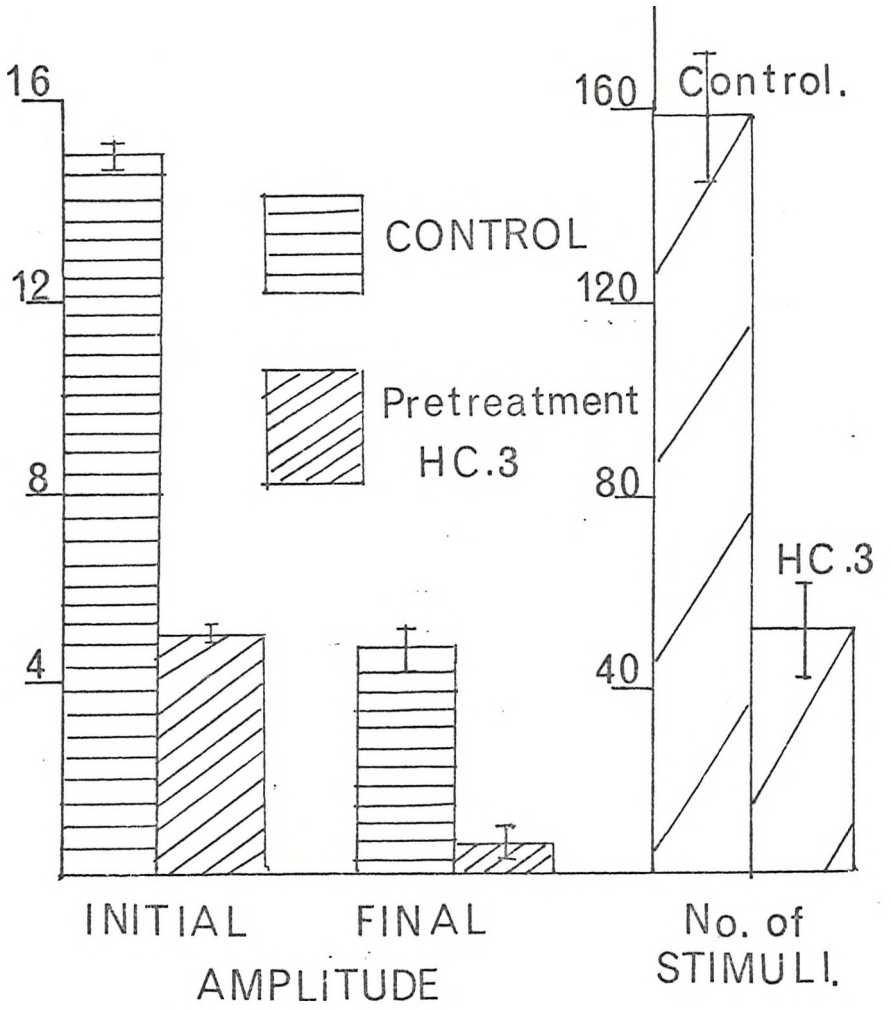

Fig. 9. Histogram showing effect of Hemicholinium pretreatment on amplitude of ipsp and number of stimuli required to reduce the height of the ipsp to a constant level.

TABLE II: EFFECT OF HEMICHOLINIUM ON THE DEPLETION RATE BY CONTINUOUS STIMULATION

Time (in seconds) between Stimuli	No. Stimulations reduce to Con. Ht.	Time (in seconds) to reduce to Constant Ht.	Height IPSP (mVs) Initial	Final
0.6	61	36.6	5	1.0
0.6	65	39.0	6	0.5
0.6	40	24.0	5	1.0
0.6	55	33.0	5	1.0
		33.2 sd ±6.6		
0.9	62	55.8	5	1.0
0.9	68	61.2	5	0.0
0.9	50	45.0	5	0.0
0.9	40	36.0	4	0.0
0.9	54	48.6	6	1.0
		49.2 sd ±9.8		
1.25	50	62.5	5	1.0
1.25	48	60.0	5	1.0
1.25	60	75.0	5	0.5
1.25	55	69.0	5	0.0
1.25	35	44.0	5	1.0
1.25	59	66.0	6	0.5
		62.1 sd ±11		
1.75	40	70.0	5	1.0
1.75	50	87.5	4	0.5
1.75	53	93.3	5	1.0
1.75	69	112.0	4	0.5
1.75	44	78.0	6	1.0
	53 sd ±10	88.0 sd ±16	5 sd ±0.6	0.7 sd ±0.4

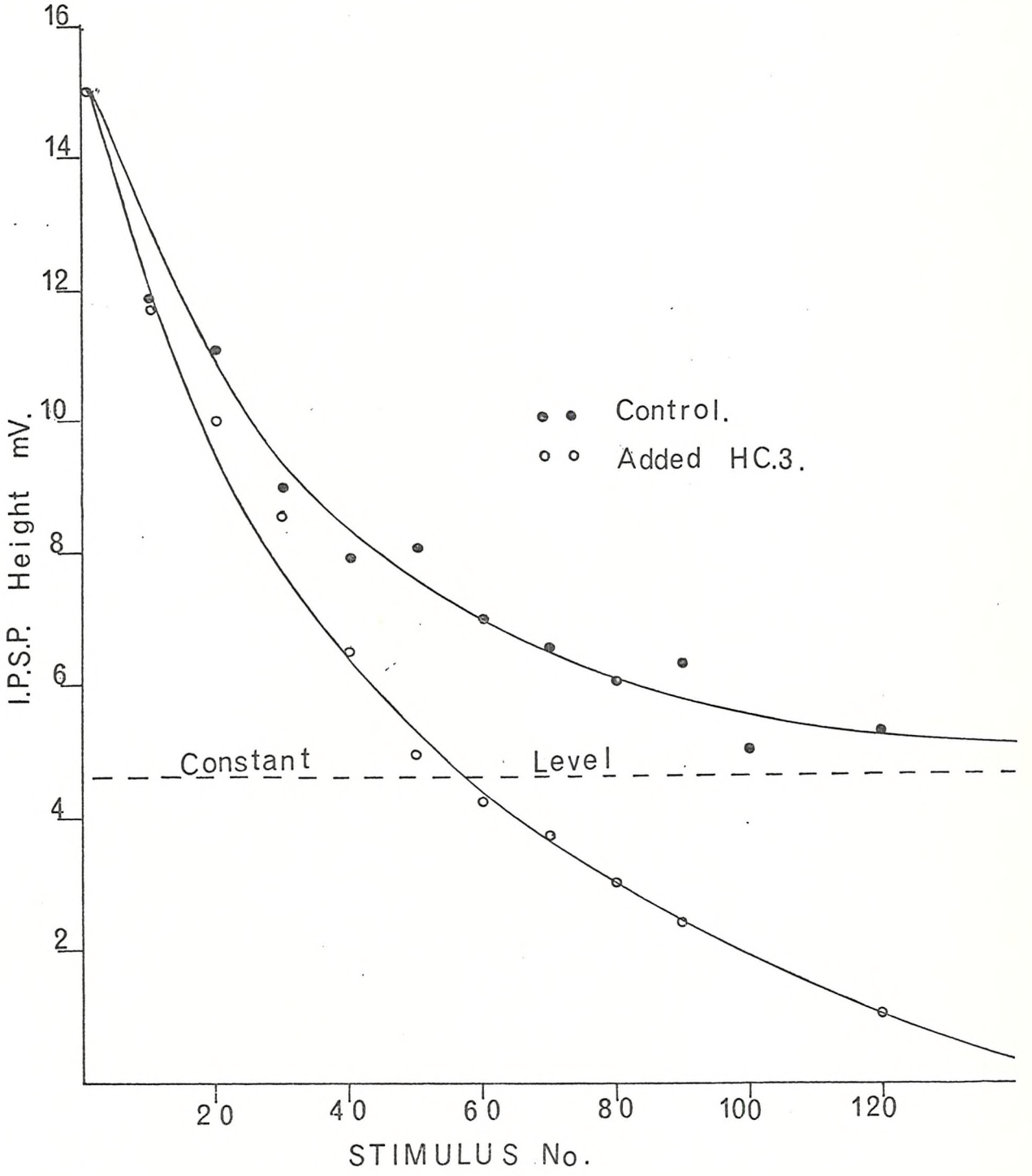

Fig.10. Graph of ipsp height against number of stimuli in the presence of Hemicholinium and in normal ringer.

Effect of Hemicholinium Incubation on IPSP

The cell was repetitively stimulated at one every 1.75 seconds in the presence of 2.5 µg/ml hemicholinium (50 µg in 0.05 ml was added over the preparation), and the decline in height of the ipsp noted. This dose of hemicholinium had previously been shown to have no effect on iontophoretic application of acetylcholine, figure 8 B1 and B2. Figure 10 shows the decline of ipsp amplitude in the presence of hemicholinium with repetitive stimulation and decline in the control. In the former case the ipsp amplitude declined more rapidly and was finally abolished. In the absence of hemicholinium the ipsp amplitude declined to a constant level between 4 mV - 6 mV. Therefore the difference between the two lines must represent the rate of resynthesis. If the values for the ipsp amplitude in the presence of hemicholinium are normalised and plotted on a log plot against stimulus number, a straight line is obtained, see figure 11. This indicates that the release of acetylcholine is exponential and therefore that each stimulus releases a constant fraction of the store.

Effect of Hemicholinium on the Cholinergic Receptor

Hemicholinium was added directly to the bath to study any possible direct effect between it and the cholinergic post-synaptic receptor. The addition of 1000 µg to the bath to give a final concentration of 50 µg/ml HC3, hyperpolarised the membrane potential by up to 8 mV and completely blocked iontophoretically applied acetylcholine, see figure 12. The amplitude of the ipsp was also reduced. When lower doses of hemicholinium, e.g. 50 µg, to give a final concentration of 2.5 µg/ml, were added to the preparation, they had no direct effect on iontophoretically applied acetylcholine, figure 12B. In animals pretreated with hemicholinium, the effective concentration at the receptor would

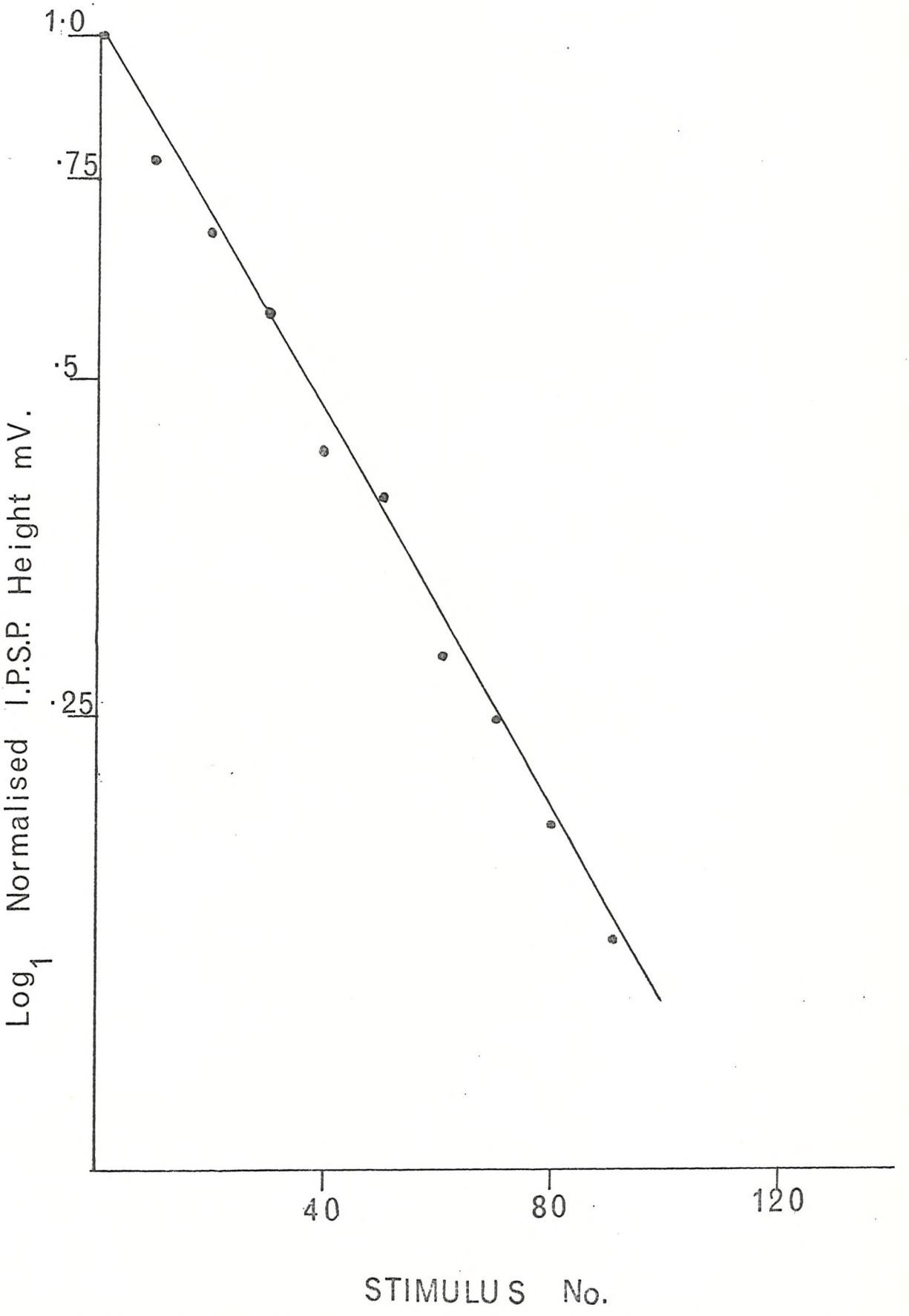

Fig.11. Graph of Log normalised height against number of stimuli in the presence of Hemicholinium

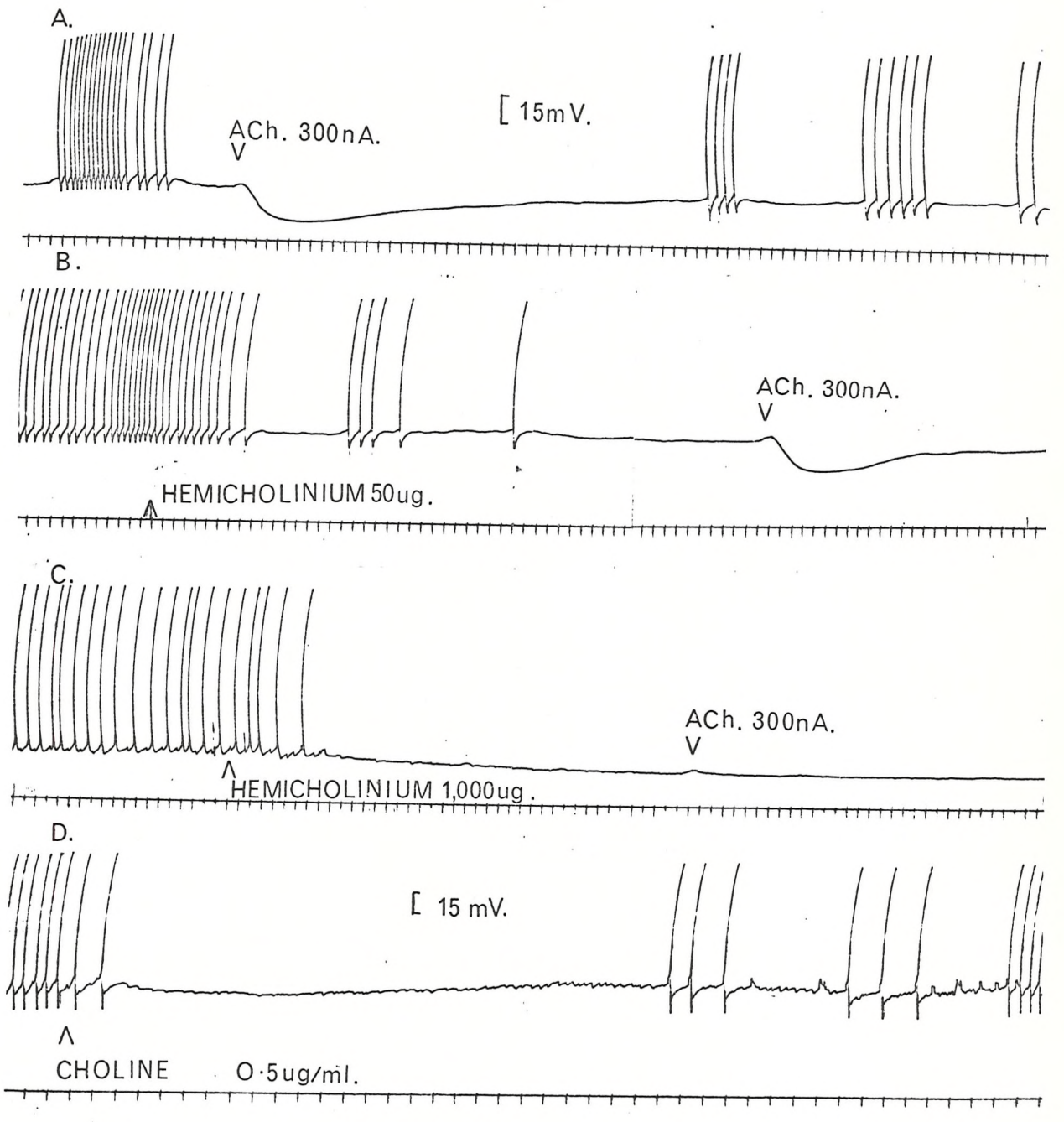

Fig.12. Trace A : Effect of iontophorectic application of Ach.
Trace B : Effect of iontophorectic application of Ach after addition of Hemicholinium.
Trace C : Effect of iontophorectic application of Ach after addition of a large dose of Hemicholinium.
Trace D : Effect of addition of choline to the bath.

be in the range where there would be little or no interaction between hemicholinium and the cholinergic receptor. Snails pretreated with hemicholinium respond to the iontophorectic application of acetylcholine quantitatively, as in the control untreated animals. Hemicholinium was also tested on cells which were excited by acetylcholine and was found to excite and depolarise the membrane potential when applied at high doses.

Effect of Choline on the Cholinergic Receptor

Attempts were made to incubate the brain with choline following repetitive stimulation and try to reverse the action of hemicholinium. However choline was found to interact with the cholinergic receptor at these doses, 0.5 μg/ml, and to mimic the action of acetylcholine, see figure 12D. This resulted in a partial desensitisation of the post-synaptic receptor and masked any possible enhancement of recovery following repetitive stimulation due to an increase in the supply of available choline.

Action of Morphine on the IPSP

The cell was repetitively stimulated one every 1.75 seconds in the presence of 100 μg of morphine. Initially the height of the ipsp was the same as that found in the controls, see figure 13B, but after forty or so stimuli, the height of the ipsp was virtually abolished, see figure 13C. Morphine added to the bath did not cause any hyperpolarisation of the membrane potential. The final level of the ipsp was also abolished but since it occurred in such a short time the action of morphine could not be explained in terms of preventing synthesis, but only by having a pre-synaptic action in blocking release of acetylcholine. Morphine had no effect on the amplitude of the

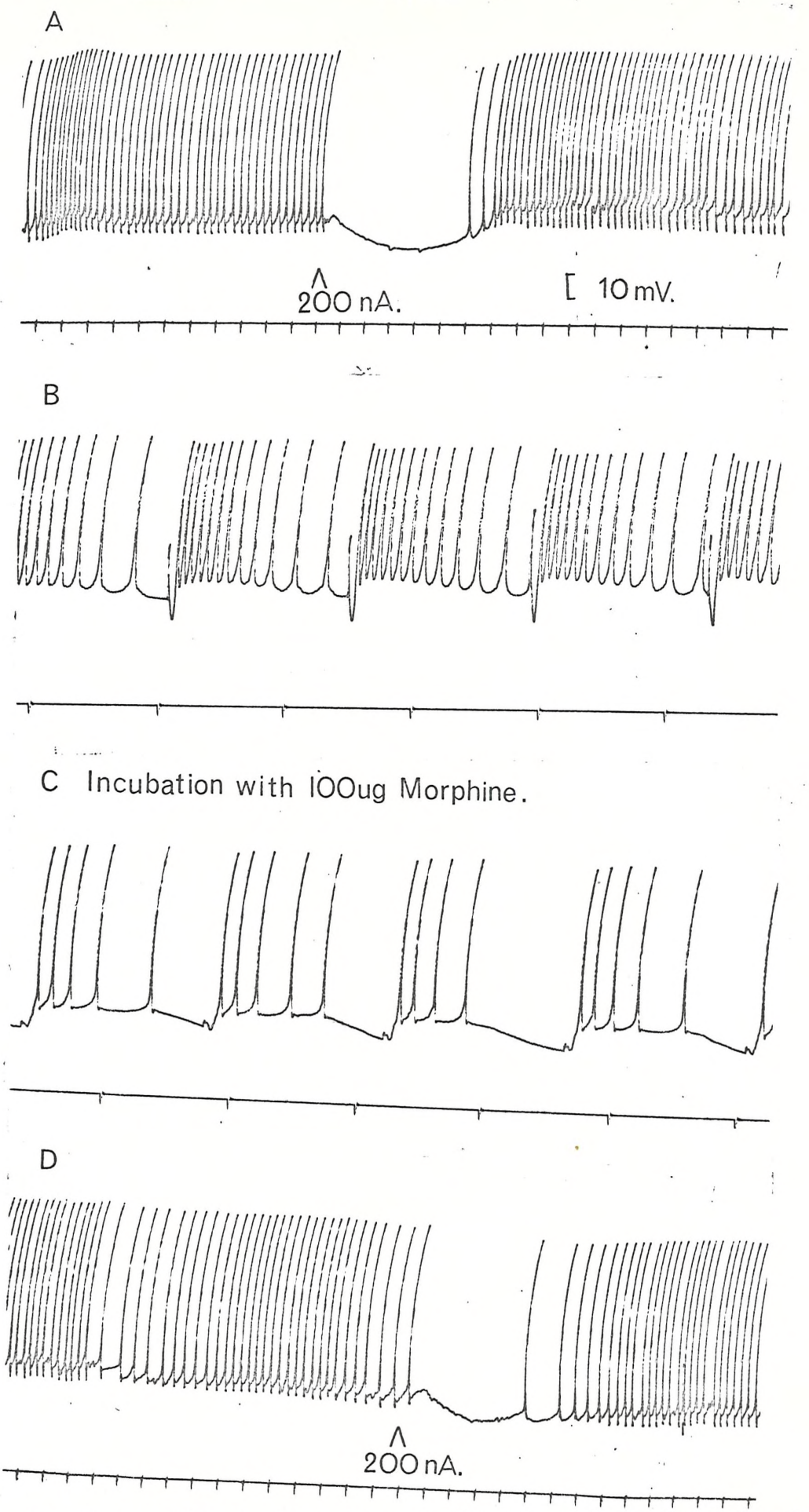

Fig.13. Effect of morphine on the driven ipsp, trace B and C. Trace A and D effect of iontophorectically applied Ach before and after stimulation.

response to iontophorectically applied acetylcholine, see figure 13A and D. Thus a direct post-synaptic action of morphine could be ruled out.

DISCUSSION

The response of the post-synaptic membrane can be taken as an indication of the amount of transmitter released, assuming that the sensitivity of the post-synaptic membrane remains constant. This has been shown for the neuromuscular junction by Otsuka, Endo, Nonomura (1962) and Elmqvist and Quastel (1965a). However, it is possible that in Helix the ipsp amplitude drops during repeated stimulation due to desensitisation of the post-synaptic membrane. The experiments in the present study in which acetylcholine was iontophoresced before and after stimulation, indicate that following stimulation there is no change in the membrane sensitivity to acetylcholine since the amplitude of the acetylcholine response remained constant. The size of the post-synaptic response is dependent on the amount of acetylcholine in the presynaptic store and on the rate of resynthesis of acetylcholine. Thus with stimulation the acetylcholine store will gradually be depleted, and so the height of the ipsp will decline to a constant value. This constant level will represent the rate of resynthesis. This pattern of release of transmitter during stimulation has been observed by several workers using different preparation: Brown and Feldberg, 1936; Perry, 1953; Straughan, 1960; Birks and MacIntosh, 1961; Paton, 1963; Kopin, Breese, Krauss and Weise, 1968; Bennett and McLachlan, 1972a and b.

Hemicholinium was found to reduce the final height of the Helix ipsp, both in pretreated animals and when it was added to the bath.

Hemicholinium has been shown to reduce the amount of acetylcholine stored by inhibiting the synthesis of acetylcholine (MacIntosh, Birks and Sastry, 1956). Hemicholinium acts by competing with choline for the presynaptic uptake mechanism for choline (Marchbanks, 1968). Therefore it will deplete the acetylcholine store in the nerve terminal and in turn the size of the post-synaptic event which is a function of the amount in the store. The finding that the decline in the ipsp is exponential supports the assumption that the amount of acetylcholine released by each stimulus would be a constant fraction of the releasable store. This was also observed by Bennett and McLachlan (1972a) in their experiments with the guinea pig cervical ganglion. Therefore the rate of release is dependent on the frequency of stimulation. The evidence both from the present study and that of Bennett and McLachlan (1972a) suggest a single store in the post-synaptic terminals rather than a two-component model developed from the overflow experiments on perfused cat ganglia (Perry, 1953; Birks and MacIntosh, 1961).

The constant height of the ipsp which is reached during repetitive stimulation, appears to be due to transmitter resynthesis (Birks and MacIntosh, 1961; Bennett and McLachlan, 1972a and b). Bennett and McLachlan suggest that synthesis is constant during stimulation since if they subtracted their maintained output value from their results, then these results were identical with those obtained when synthesis is blocked. When synthesis is blocked following addition of hemicholinium to the bath, the initial height of the ipsp is the same as the control untreated value, but the amplitude drops toward zero rather than reaching a constant value. In the present study it would appear that the rate of resynthesis is dependent on the rate of release since the final height does not vary with frequency. This is in agreement with the observations of Bennett and McLachlan and

suggests a simple homeostatic mechanism. The rates of acetylcholine synthesis in the cat sympathetic ganglion (Bannister and Scrase, 1950) and in the rat diaphragm (Hebb et al, 1964) are considerably below the rates suggested by the activity of choline acetylase or substrate concentration (Potter et al, 1968).

If the rate of acetylcholine synthesis is dependent on the law of mass action, then Bennett and McLachlan (1972b) suggest that the release of acetylcholine from the nerve terminals would decrease the acetylcholine concentration and so accelerate synthesis. This would also seem to occur in the Helix nerve terminal.

The present study confirms that hemicholinium has both pre- and post-synaptic actions on the system. At low doses there would appear to be no effect on the cholinergic receptors since the iontophoretic application of acetylcholine in the presence of hemicholinium was the same as in the control. However, large doses of hemicholinium, 1000 μg, added to the bath, clearly have a direct effect on the post-synaptic membrane of Helix neurones. This has been shown for mammalian tissue by Takagi et al, 1970, who demonstrated a curare-like effect at a high concentration. In Helix, hemicholinium at these doses acts as an acetylcholine agonist and also blocks the response to acetylcholine.

It is possible that hemicholinium could be acting by blocking the release of acetylcholine rather than its synthesis. However, this suggestion would not be compatible with the observation that following the addition of hemicholinium to the bath, release of acetylcholine declined in an exponential manner. It would have been useful in this context if the action of hemicholinium could have at least in part been reversed following application of choline to the bath, but the cell in the present study proved to be particularly sensitive to

choline. This direct agonist effect of choline masked any changes in amplitude of the ipsp following addition of choline to preparations treated with hemicholinium.

The presynaptic effect of morphine on this system is in agreement with the results, using guinea pig gut, obtained by Paton (1956), which suggested that its action was to inhibit the releasing mechanism. Further experiments are required to determine its precise action on the Helix system.

In conclusion it is suggested that the release of acetylcholine from presynaptic stores in Helix is exponential, and that this store can be depleted following treatment with hemicholinium. The release of acetylcholine can also be blocked presynaptically by morphine. These observations are in good agreement with the recent findings of Bennett and McLachlan (1972a and b) using guinea pig superior cervical ganglion, and others working on mammalian tissue.

S E C T I O N Ib

RESULTS: Dopamine Neurones.

Pharmacology of ILD Cell

The cell is positioned in the bottom left-hand corner of the visceral ganglion, see figure 5 and was found to give a driven inhibitory post-synaptic response called an ILD (inhibition of long duration) via the anal nerve, see figure 17 . The size of the response was found to vary between 10 and 15 mV. The cell responded to dopamine, noradrenaline and octopamine, see figure 14, at an equal concentration. All three compounds caused the membrane potential to hyperpolarise. The octopamine response was found to desensitise, but the cell still responded to a standard dose of dopamine. The cell responded to tyramine but octopamine was about 1000 times more potent than tyramine. Low doses of ergometrine blocked noradrenaline and dopamine, but larger doses were required to block octopamine. The synaptic event was also blocked by larger doses of ergometrine. Further on addition of dibenyline the response to all three compounds was blocked, but propanolol failed to block any of the three compounds. Cocaine, 10 μg/ml, blocked the effect of octopmaine at low doses of octopamine but not at high doses. The cell was also hyperpolarised by glutamate and depolarised by acetylcholine.

Pharmacology of IPSP Cell

The cell is situated in the visceral ganglion, see figure 5 . On stimulation of the anal nerve an inhibitory postsynaptic event known as an ipsp is obtained, see figure 16. The size of the response was found to vary between 10 mV - 14 mV in amplitude. The addition of dopamine to the bath caused a hyperpolarisation of the membrane whilst the addition of acetylcholine caused a depolarisation of the membrane.

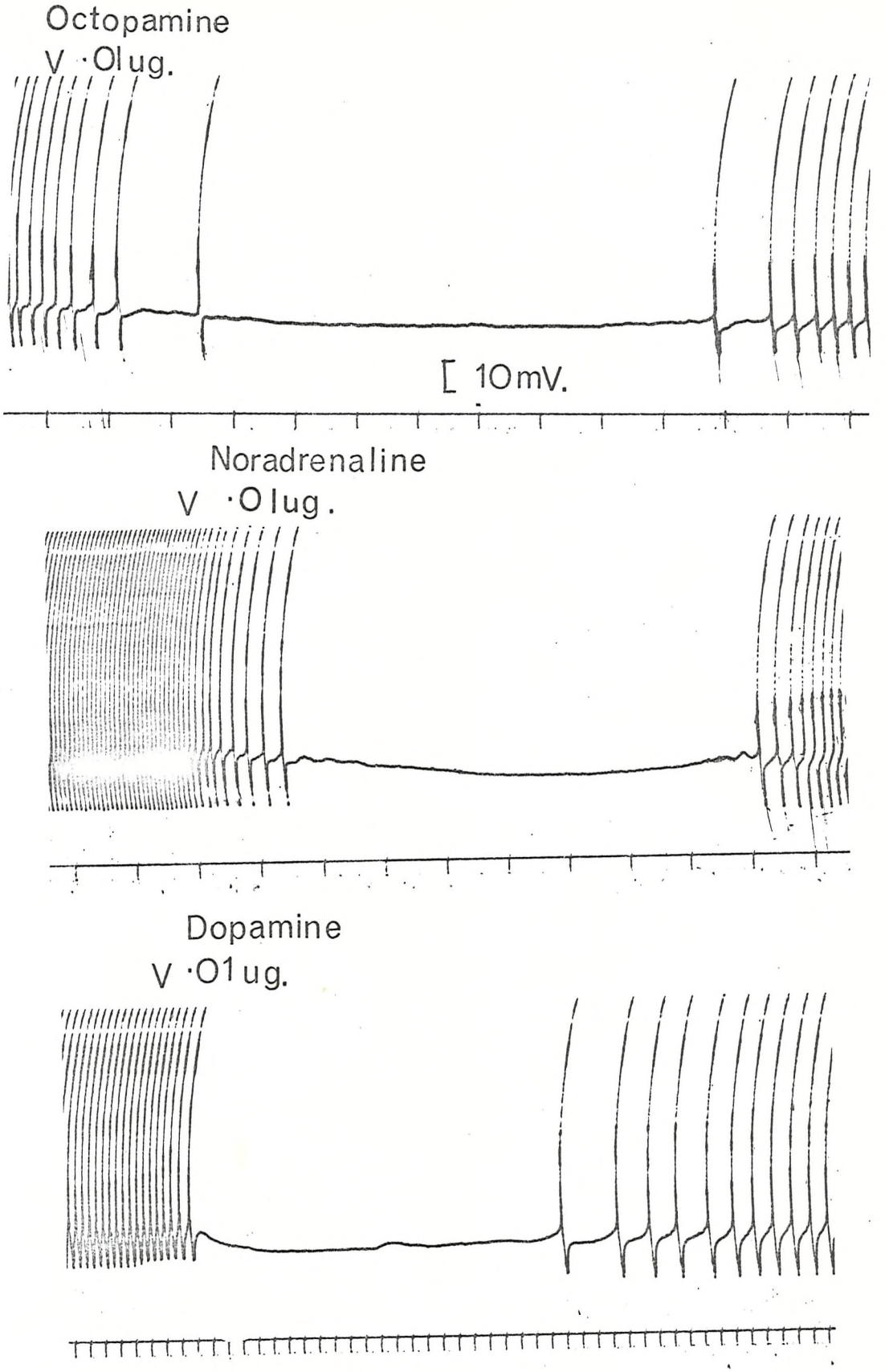

Fig.14. Effect of addition of Octopamine,Noradrenaline and Dopamine on ILD cell. (Concentrations are for one ml. of ringer.)

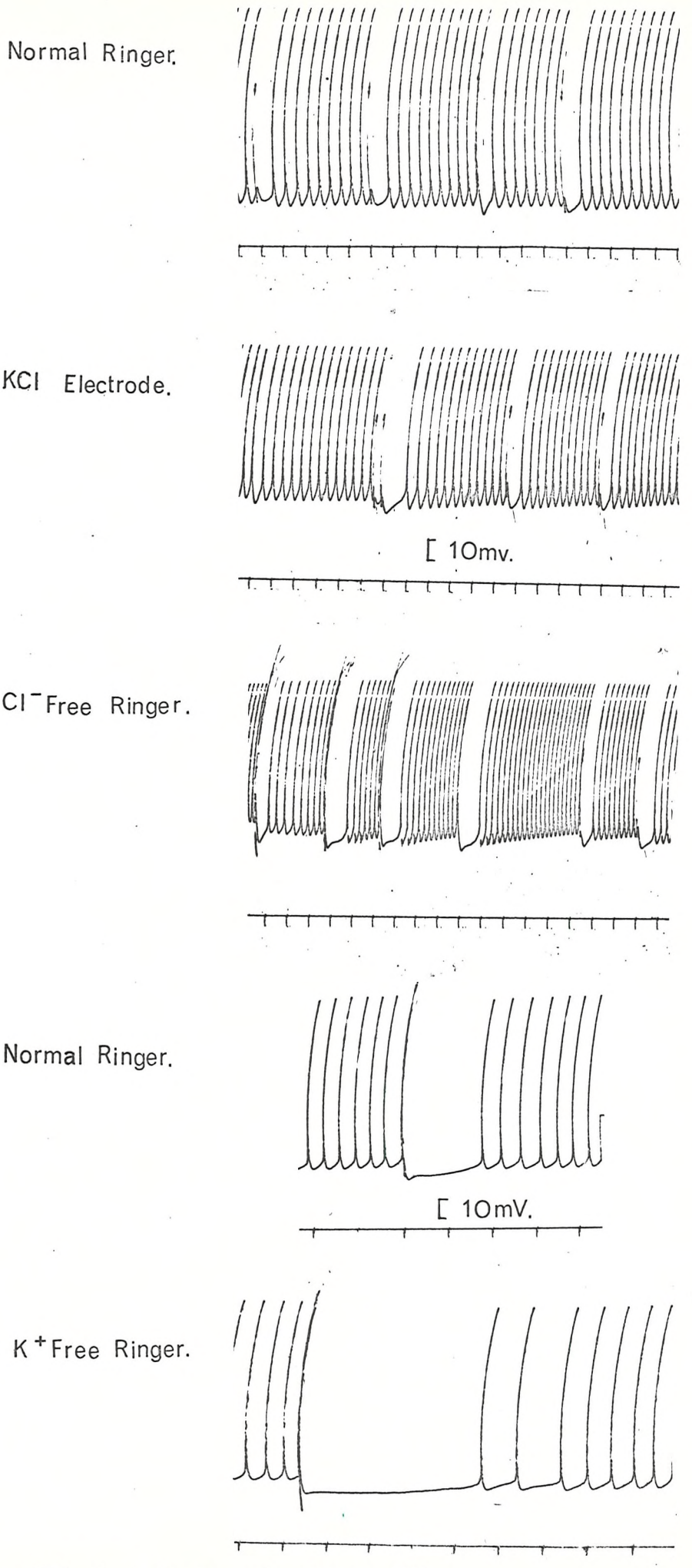

Fig.15. Effect of ions on the driven ILD.

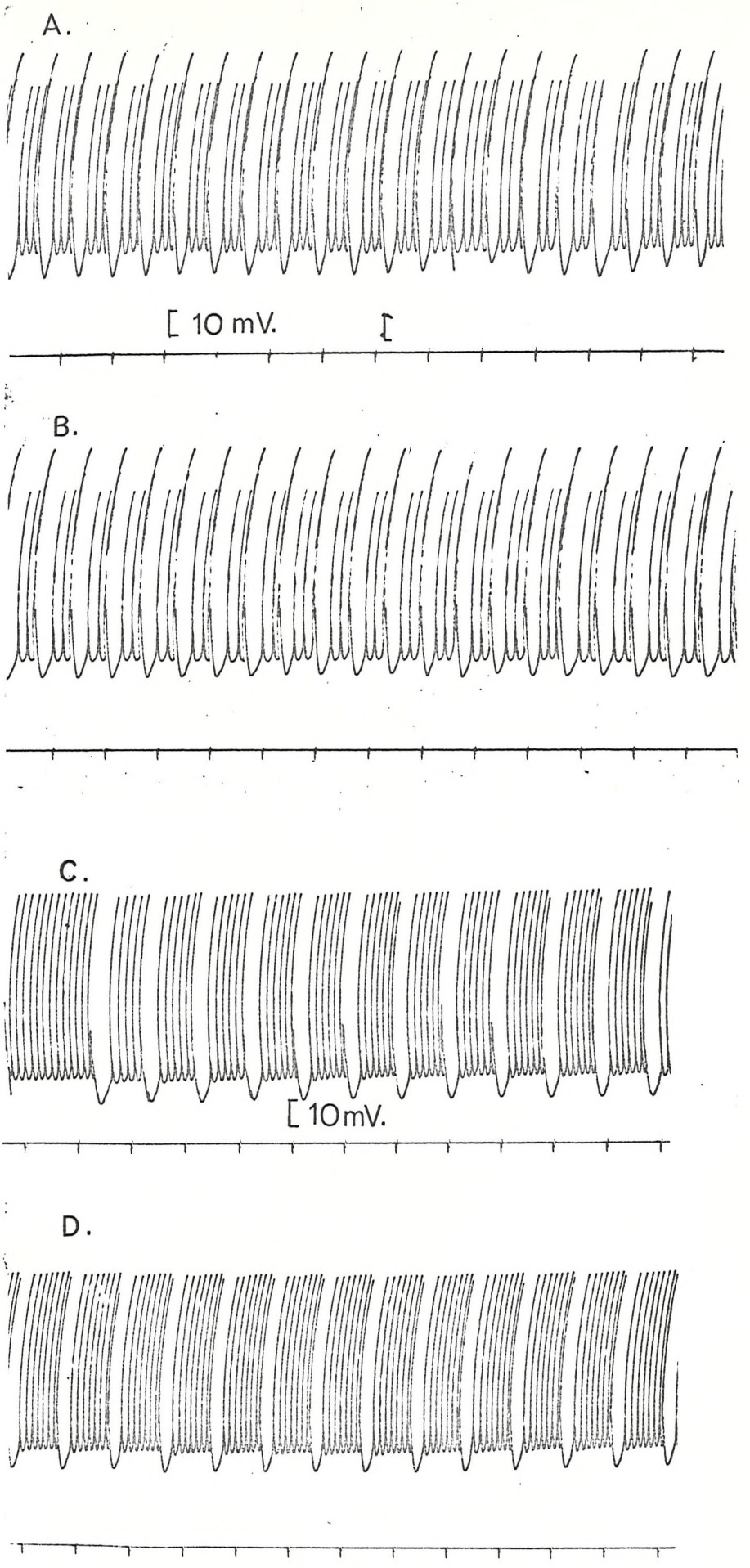

Fig.16. Effect of stimulation on 'Dopamine'ipsp.
Trace A and B stimulation at two a second.
Trace C and D stimulation at one a second.

Both the response to dopamine and the driven ipsp were reversibly inhibited by the addition of 5.10^{-5} gm/ml of ergometrine.

Effect of Different Ions on the Stimulated Synaptic Event

Stimulation in the presence of chloride free ringer or using a chloride electrode has no effect on the ILD, see figure 15. While in the presence of potassium-free ringer the ILD was potentiated. A similar effect was obtained for the ipsp cell.

As the membrane potential was hyperpolarised the ipsp and ILD were gradually reduced in amplitude until the membrane potential reached 80 mV - 90 mV, where the events reached zero and then gradually began to reverse as the membrane potential was further hyperpolarised. Therefore potassium ions are clearly involved in both events.

Effect of Repetitive Stimulation on the IPSP and ILD cell

Figure 16 shows the effect of repetitive stimulation at two different frequencies, one every 0.67 seconds and one every 0.96 seconds on the ipsp cell. The initial effect compared with the effect after 300 stimuli at a rate of one every 0.67 seconds can be seen in figure 16 A and B. After 300 stimuli at this frequency the amplitude had only slightly fallen from 10 mV to 8 mV. Further at the slower frequency, see figure 16 C and D, after the same number of stimuli there is no appreciable change in the size of the ipsp.

The ILD cell, due to the length of the ILD, could only be stimulated at slow rates since the membrane potnetial must return to normal after each event to give constant comparable results. Thus at the frequencies chosen the height of the ILD did not decline to a constant value.

Effect of Cocaine

Figure 17 A and B show the effect of repetitive stimulation on the ILD. Cocaine was added to the bath after 200 stimuli to give a final concentration of 50μg/ml. Twenty seconds after addition the stimulated ILD became potentiated in size, see figure 17 C. The amplitude of the ILD increased from 14 mV to 20 mV and the duration from 0.75 sec to 1.5 sec. In figure 17 D the amplitude declined after 50 stimuli to 8 mV. Therefore cocaine causes a potentiation and then a decline in amplitude. Similar results were obtained with the ipsp cell.

Effect of Amphetamine on the IPSP and ILD Cells

The ipsp cell was stimulated at a non-fatiguing frequency of one every 2.75 sec. The cell was found to give an ipsp of 8 mV in amplitude. Forty seconds following the addition of 100μg amphetamine, giving a final bath concentration of 5μg/ml, the amplitude had increased to 12 mV and the duration from 0.3 sec. to 0.4 sec. After 90 sec. the amplitude reached a maximum of 16 mV, duration 0.5 sec, see figure 18. Therefore amphetamine potentiation of the driven ipsp.

The effect of amphetamine on the ILD is to immediately cause a potentiation in the duration of the ILD. 30 sec. after addition of amphetamine the ILD duration has increased from 1 sec. to 4 sec. but these results show no change in the height of ILD, see figure 19.

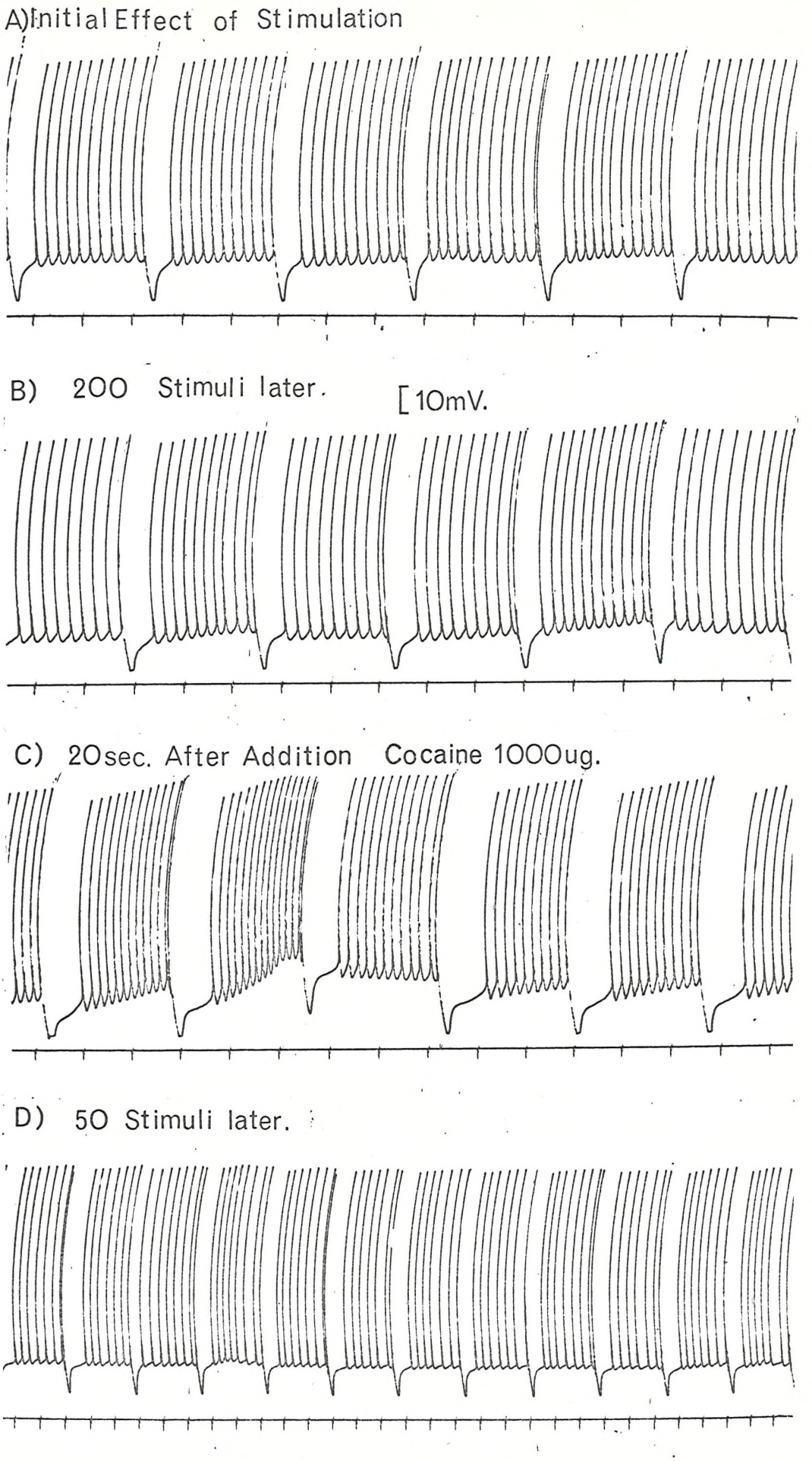

Fig.17. Effect of cocaine on the driven ILD.

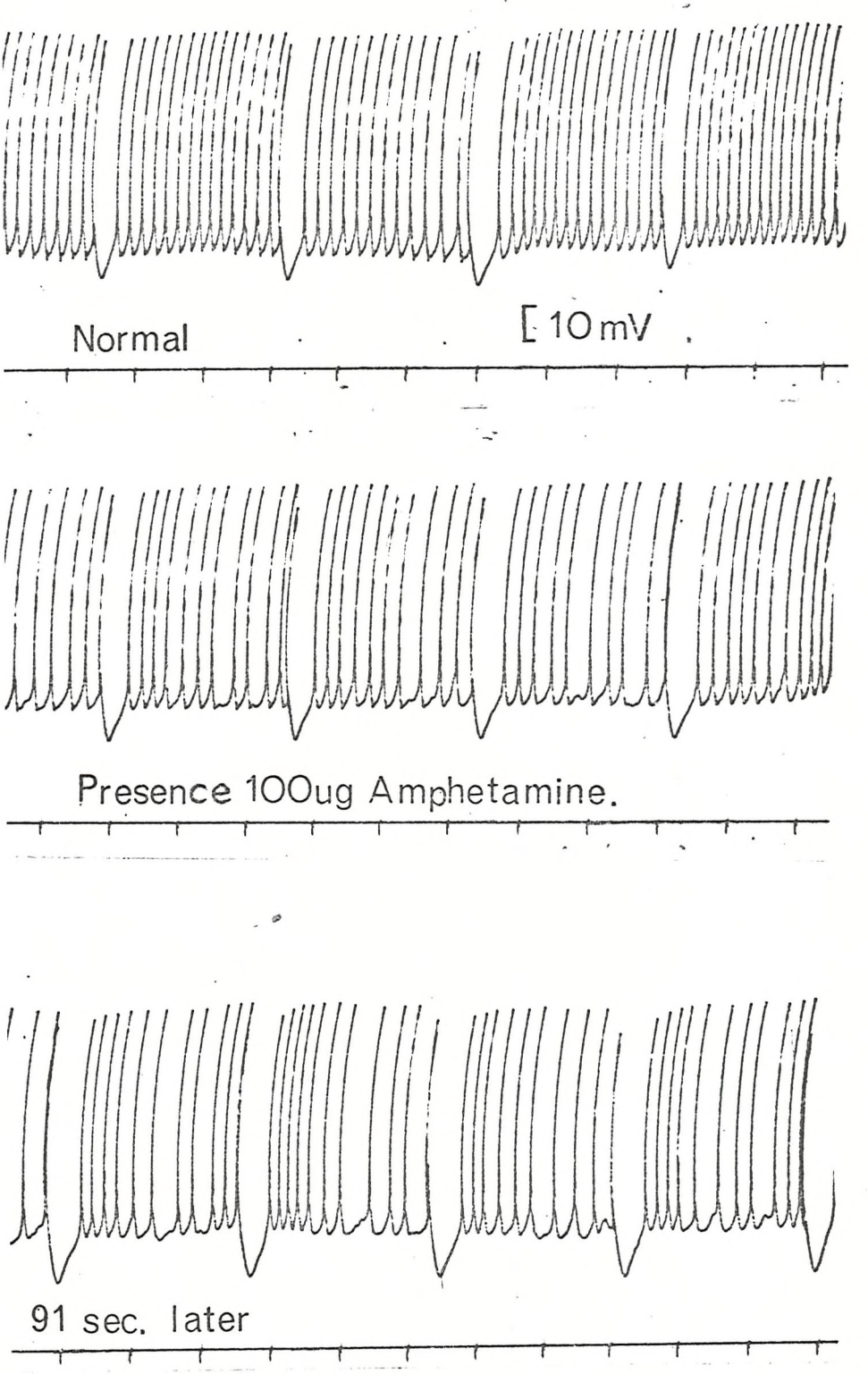

Fig.18. Effect of amphetamine on the 'Dopamine' ipsp.

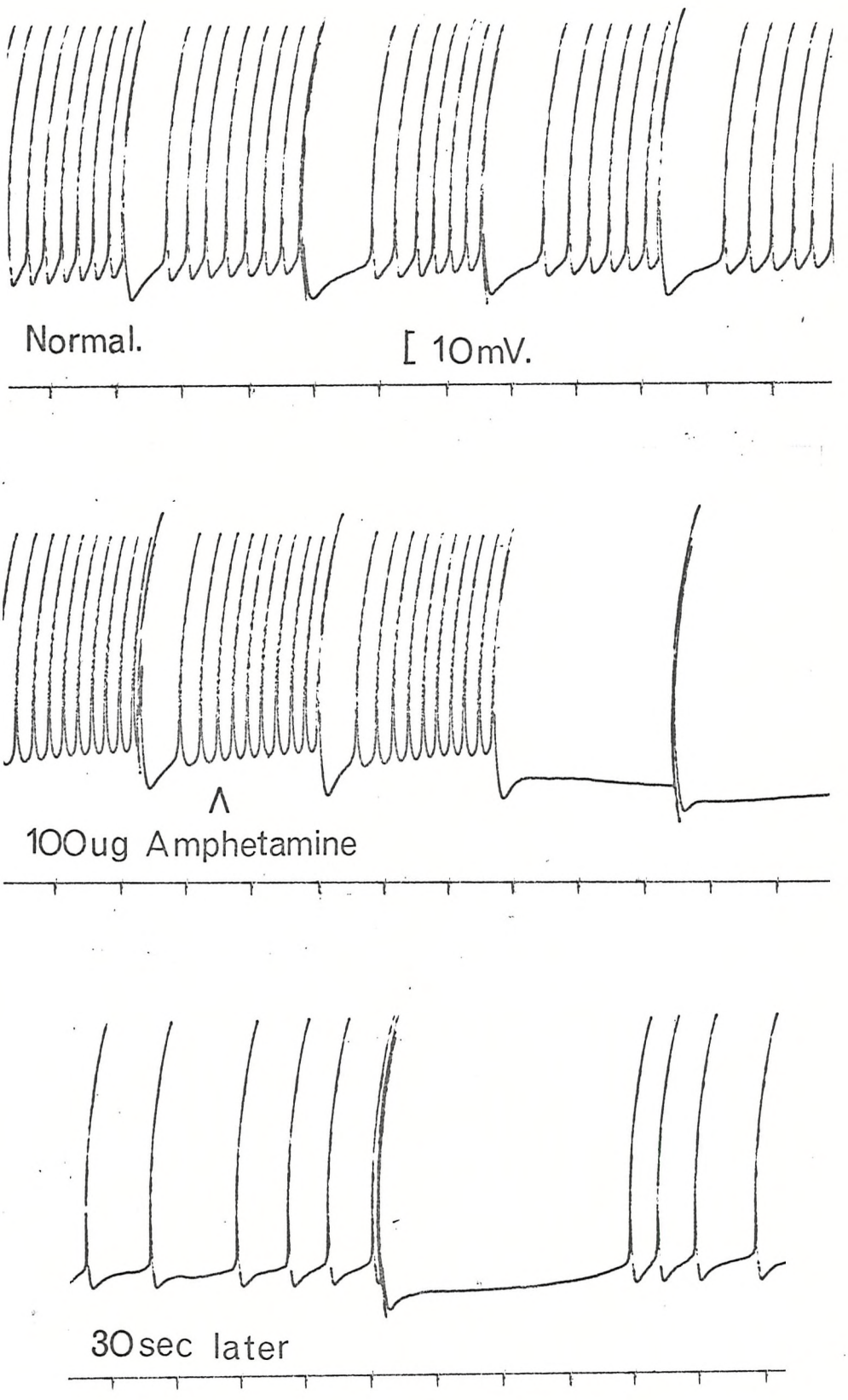

Fig.19. Effect of amphetamine on the driven ILD.

Page 59 was not present in the thesis at the time of scanning.

Discussion

The action of octopamine on identifiable neurones of Helix aspersa has not been reported by Walker et al (1968), Woodruff and Walker (1969) or Woodruff (1971).. They demonstrated that the dopamine receptor was capable of being blocked by alpha blockers but not by beta blockers. The dopamine receptor was reversibly blocked by ergometrine and lysergic acid; for further details see the review by Woodruff (1971). Further dopamine causes an increase in conductance probably due to an increase in potassium permeability (Kerkut et al, 1969). Both cells in the present study have dopamine receptors and dopamine would appear to be the transmitter released by the stimulation of the anal nerve onto both cells. The effect of octopamine has not been tested on the ipsp cell. There could be several explanations for the octopamine effect on the IID cell in contrast to its lack of effect on the cells studied by Walker and Woodruff. The cells used in their study of the snail dopamine receptor were not known to have any dopamine synaptic inputs, thus a true physiological receptor might not be present. Secondly there could be two distinct dopamine receptors in the snail brain. Alternatively octopamine could be acting presynaptically to cause the release of dopamine suggesting the presence of an amine pump at the synapses in these cells.

The later suggestion is of considerable interest since cocaine, shown by Iversen (1967) to inhibit catecholamine uptake in the mammalian heart, blocked the action of octopamine at a low concentration. This would suggest that octopmaine could be acting indirectly as already suggested by being taken up by the amine pump, since phenylethylamines have been shown to be readily taken up in the mammalian peripheral sympathetic nervous system (Iversen, 1967) and to

cause displacement of the transmitter. Therefore octopamine would be taken up and cause the release of dopamine in the snail thus explaining the action of dibenyline. The action of ergometrine also fits in with this hypothesis since it has been shown that higher doses of this antagonist are required to block synaptic events compared with the dose required to block addition of dopamine directly onto the cell. This could explain why higher doses of ergometrine are required to block octopamine.

Further the effect of repetitive stimulation of the ipsp and ILD cells caused only a slight decrease in the size of the postsynaptic event although fast repetitive stimulation could only be carried out on the ipsp cell. This observation is similar to that of von Euler (1955) who found that vigorous stimulation of the adrenergic nerves to the spleen did not appreciably lower the noradrenaline content of the organ in spite of considerable release. They attributed the effect to rapid resynthesis but further work by Hedqvist and Stjarne (1969) suggests that reuptake plays an important role in the maintenance of transmitter content in the nerve terminals. The presence of a rapid turnover mechanism for dopamine is also indicated in the snail brain, especially if these results are compared with the results of repetitive stimulation on the acetylcholine system.

The effect of high doses of cocaine on repetitive stimulation was initially to potentiate the amplitude of the event and subsequently to reduce it by half. The high dose used could cause an effect on conduction along the nerve, since cocaine is an anaesthetic. Further in the mammalian system the dopamine uptake mechanism does differ from the noradrenaline mechanism since imipramine has only a slight inhibiting effect while amphetamine inhibits quite considerably dopamine uptake (Coyle and Synder, 1969 a and b). The effect of

amphetamine on the driven ipsp and ILD is to potentiate the size very considerably of the post-synaptic event. Therefore evidence indicates the presence of an amine pump in the snail brain.

Cottrell (1971) has also demonstrated the existence of an uptake mechanism for 5HT. Although Gerschenfield and Stefani (1968) suggest that diffusion of 5HT is sufficiently rapid to account for the termination of the activity of 5HT in molluscan ganglia. This also plays an important role in the inactivation of transmitters in molluscan ganglia, but the presence of an amine pump could explain the high rate of turnover of dopamine compared with acetylcholine.

The effect of tyramine and noradrenaline could suggest that octopamine is acting directly on the receptor. This observation would be in agreement with the results obtained on mammalian tissue by Kopin (1968) although Lands and Grant (1952) showed it had only 1/50 of the pressor activity of noradrenaline in the mammal. Noradrenaline is more potent relative to dopamine on this cell than on the cells studied by Walker and Woodruff. Further studies are required concerning the effect of octopamine on this cell and to explain the differences obtained, but the results so far obtained indicate the presence of an amine pump.

S E C T I O N I I

SECTION II: Elucidation of the catecholamine pathway in Helix aspersa

Introduction

The occurence of catecholamines in molluscs was investigated by Sweeny (1963). He assayed for adrenaline, noradrenaline and dopamine by spectrofluorometry. The only catecholamine he detected was dopamine. Cardot (1963) also demonstrated the presence of dopamine in the brain of Helix pomatia. Further studies by Kerkut et al (1966) on Helix aspersa, Osbourne and Cottrell (1970) in Helix pomatia and recently by Juorio and Killich (1972) in both species have confirmed the presence of dopamine. The highest monoamine concentration was found ir the pedal ganglia by Juorio and Killich (1972). This is in agreement with Sedden et al (1968) who showed that the most intense fluorescence developed in the pedal ganglia. This is also true for Aplysia californica (Carpenter et al, 1971) and in the lamellibranchs (Dahl et al, 1966).

Noradrenaline has been demonstrated in Helix pomatia by Osbourne and Cottrell (1970) and Juorio and Killich (1972) who also demonstrated its presence in the octopus. The concentration of this substance in Helix is about one hundredfold less than dopamine. Further octopamine was first isolated by Erspamer and Boretti (1952) in the salivary glands of the common octopus, and its distribution in the brain of the octopus was determined by Juorio and Molinoff (1971). It has also been demonstrated in the mammalian nervous system by Molinoff and Axelrod (1969 and 1972) who demonstrated its presence in the rat sympathetic nervous system and brain. Also they have shown it to be present in the thoracic and abdominal nerve cord in the lobster. Further they demonstrated that it had a high rate of turnover and this is consistent with the high rate of excretion of its deaminated

metabolite, p-hydroxymandelic acid (Armstrong et al, 1956). The function of octopamine in the mammalian system could be as a co-transmitter and even as a primary one in invertebrates (Molinoff and Axelrod, 1972).

The distribution of catecholamine metabolites in the snail was also carried out by Osbourne and Cottrell (1970). They demonstrated the presence of acid metabolites of noradrenaline and dopamine and also the methoxylated metabolites. These results therefore suggest the presence of MAO and COMT in the nervous tissue of Helix pomatia. Kerkut and Cottrell (1963) were only able to show the presence of MAO in the kidney of Helix aspersa, and Cardot (1966) was also unable to demonstrate the existence of MAO in the nervous tissue of Helix pomatia. The enzyme has been found in the nervous tissue of Octopus vulgaris by Blascko and Hawkins (1952). Recently Juorioand Killich (1972) demonstrated the presence of acid metabolites in Helix but at very low concentrations, and have found that these very low concentrations are unaffected by MAO inhibitors. Therefore evidence for inactivation of catecholamine metabolites in Helix is not clear and the acid metabolites formed might be rapidly eliminated or suffer from metabolic transformations.

In the present study incubation with radioactive precursors have been used to elucidate the pathway. Also assays for noradrenaline and octopamine have been carried out and further tyrosine hydroxylase activity has been measured in the nervous tissue of the snail Helix aspersa. The results suggest that there are two distinct pathways, one in the kidney producing dihydroxyphenylalalanine (DOPA) and one in the brain converting DOPA to dopamine.

Methods

Incubation of Radioactive Catecholamine Metabolites and the Identification by Two-dimensional Autoradiography

The snails Helix aspersa prior to dissection were activated by placing in a beaker containing 15-20 ml. warm water. This activated the animals whose shells were then rapidly removed and the circumoesophageal ganglia, kidney and heart were dissected out.

The tissue was placed separately in 0.1 ml. Ringer solution at 25°C. Half a micro curie of L-3 (3,4-Dihydroxyphenyl) alanine-3-C14 (C14-DOPA) in the presence of ascorbic acid (50 μmoles per ml.) to prevent auto-oxidation of the L-DOPA in 0.1 ml. of Ringer was added to the incubation medium. The method differs from that of Huggins and Woodruff (1968) in that only half the amount of radioactivity is used since severe streaking occurred with the larger amounts.

Incubation was carried out in a shaking incubator for intervals of one and three hours. Each incubation was terminated by the addition of an equal volume of ethanol and boiled in a water bath for two to three minutes. The contents were left overnight at 5°C to extract. They were then centrifuged and the supernatant chromatographed using two-dimensional paper chromatography.

The radioactive intermediates were then visualised by autoradiography and the radioactivity in each compound was separately determined. The compounds were required for identification of the spots which were counted on a paper with a Geiger-Muller tube and not extracted for counting on a scintillation counter.

The solvents used were Butanol:Acetic acid:Water (4:1:1) for the long axis followed by Phenol:KCN:HCl (20 ml. of 0.02N HCl:80 gm. Phenol: solution saturated in sulphur dioxide plus a few mgm. KCN)

COMPOUNDS.	Rf's (x100) SOLVENTS			
	But/Ac/ H_2O	Isoprop /NH_4Ac	Phenol/ KCN/HCl	But/HCl
TYROSINE.	40	80	50	40
PHENYLALANINE.	49	70	83	-
TYRAMINE.	56	90	37	37
DOPAMINE.	43	84	50	20
OCTOPAMINE.	42	73	58	24
EPININE.	58	87	72	21
NORADRENALINE.	34	84	39	10
ADRENALINE.	45	86	58	14
DOPA.	20	-	22	-
VANILLIC ACID.	87	85	91	-
4OH, $3CH_3O$ MANDELIC ACID.	69	72	82	-
4OH, $3CH_3O$ PHENYLACETIC ACID.	55	73	80	-
3,4 OH MANDELIC ACID.	49	75	38	68
3,4 OH PHENYL-ACETIC ACID.	78	80	66	73
TYROSINE.	46	81	52	-

Fig.20. Rf's of various catecholamines metabolites.

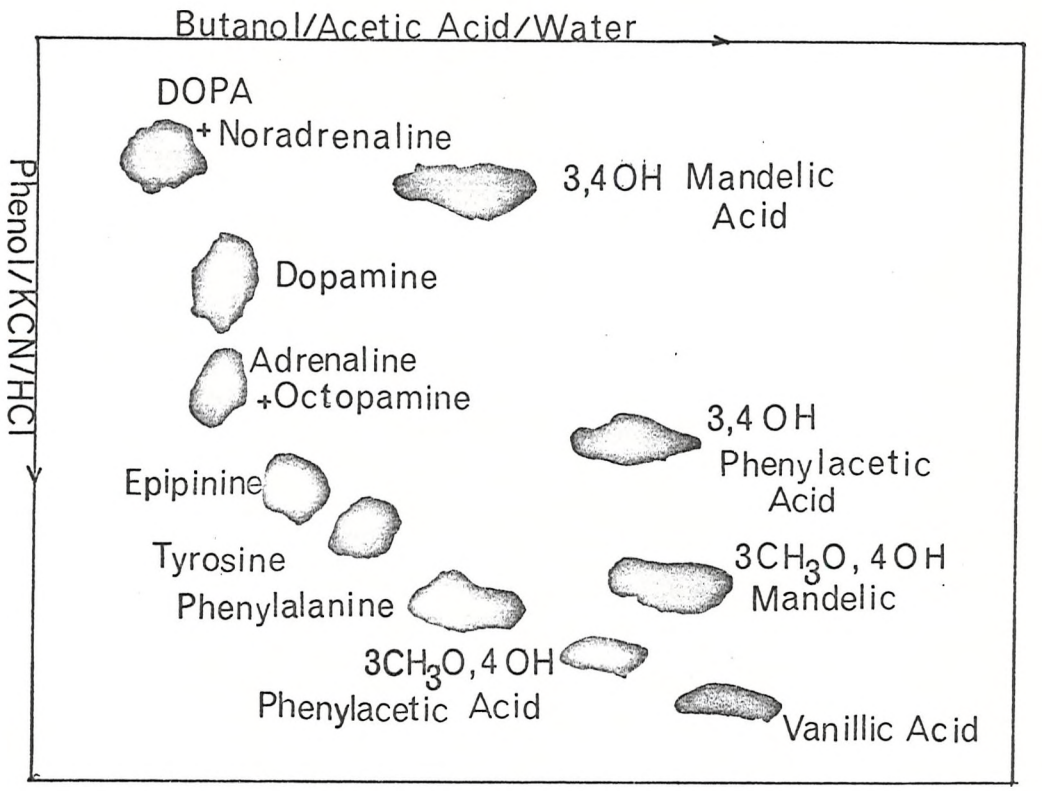

Fig. 21. Theoretical two dimensional chromatography map of catecholamine metabolites.

along the short axis. The Rf. values of various catecholamine metabolites are given in figure 20 and a two-dimensional map of cold metabolites used for identification is shown in figure 21 .

In vivo Studies

As described above the snails were first activated and 5μCi of C-4-DOPA were then injected in 0.1 ml. of Ringer into the haemocoel. The snails were left for three hours and the following organs dissected out: brain, heart, crop, salivary glands, kidney and hepto-pancreas. The metabolites were extracted as previously described.

In vitro Incubation with Inhibitors

The inhibitors were added to the incubation medium before the addition of the radioactive precursor at a concentration of 1 mg. The high amount selected was because of the failure of several workers to obtain any significant results with MAO inhibitors and DOPA decarboxylase inhibitors. The MAO inhibitor used was Nialamide and DOPA decarboxylase, N.S.D. 1024.

Identification

The metabolites were first tentatively identified by their position on the chromatogram and where possible by co-chromatography (Smith, 1960) with the authentic material being visualised by Nitroaniline Reagent II (Smith, 1960). The metabolites were extracted from the paper by one of the solvents systems used. The volume was reduced by evaporation under vacuum and the residue was then taken up in a small volume of the solvent. The solution was then

spotted on a chromatogram together with the possible cold metabolites and run in one of the solvents which gave the best separation. The radioactive intermediate was visualised by auto-radiography. If the cold spot and the radioactive spot coincide this indicates the possible identity of the material.

Incubation with C14 Phenylalanine and Tyrosine

The same procedure was used for these compounds as for C14 DOPA except for phenylalanine where incubations were carried out for three and six hours.

Method to Measure Tyrosine Hydroxylase Activity

The method used for assaying tyrosine hydroxylase activity was developed by Nagatsu et al (1964). The principle on which the assay is based is due to the availability of L-tyrosine-3,5-T, since the displacement of tritium from the 3 position will occur when tyrosine is converted to L-DOPA, as the reaction below:

$$\text{L-Tyrosine-3,5-T} + \tfrac{1}{2}O \xrightarrow[\text{Hydroxylase}]{\text{Tyrosine}} \text{L-DOPA-5-T} + T^{+}$$

$$T^{+} + H_2O \longrightarrow H^{+} + THO$$

TheTHO can be easily separated and the product very simply subjected to a radioassay.

The assay was first carried out on rat brain using a whole homogenate. The rat brain was homogenised in 0.32 M, sucrose and weighed. The volume used was 0.6 ml. As crude extracts were being used, an inhibitor of aromatic decarboxylase must be added. The compound used in this study was m-Hydroxy-p-bromobenzyloxyamine, NSD 1055.

Helix brain and kidney were also homogenised, but in Ringer containing 0.32 M sucrose. It was found that homogenisation of snail brain was difficult but if the amount used was reduced it became easier. The homogenate is spun at 15,000 rpm for fifteen minutes and the particular fraction is resuspended in the same volume.

For the assay tetrahydrofolic acid was prepared by catalytic reduction of folic acid and dissolved (0.05 M) in 1 M mercaptoethanol containing 0.1 M phosphate buffer, pH 7.4. The solution was prepared freshly once a week. A stock solution was stored at -20°C.

Procedure

The assay was carried out as follows: to a small test tube was added 100 μ L-tyrosine containing 1-2.10^{-5} cpm. tyrosine-3,5-T, 5 μmoles tetrahydrofolate, 0.5 μmoles Ferrous ammonium sulphate, 100 μmoles mercaptoethanol, 0.1 μmoles NSD 1055, 200 μmoles acetate, pH 6.0. The reaction is started by the addition of the crude homogenate and the final volume is made up to 1 ml. by the addition of distilled water. The test tube is then placed in a shaking water bath at 37°C for fifteen minutes. Following incubation 0.05 ml. glacial acetic acid is added to stop the reaction and the proteins are separated by centrifugation. The supernatant solution is transferred quantitively with a Pasteur pipette to a small Dowex 50 H^+ (0.5 cm x 3 cm) column. The test tube containing the protein precipitate is washed with 1 ml water and after the initial effluent has passed through, this is also added to the column. The combined effluents (2 ml) are collected in a counting vial to which are then added 10 ml of a flurophor solution for scintillation counting. A dioxane based scintillator was used since a polar liquid is being counted. Determination was carried out in triplicate.

A reagent blank in which all additions are made except the enzyme is used to correct for the small amount of tritium released non-enzymatically.

Enzyme Assay for Noradrenaline and Octopamine

This assay is based on the observation of Axelrod (1962) where the methyl-C14 is transferred to the primary nitrogen in the presence of the enzyme phenylethanolamine-N-methyl transferase (PNMT). The assay was developed by Saelens et al (1967) and further modified by Iversen and Jarrot (1970). The assay can also be adapted to measure octopamine since the Km is only slightly greater and the two products can be separated chromatographically. This method was described by Axelrod and Molinoff (1969).

Reagents

Homogenising solution

Solutions were made up in Ringer containing:

1 mM Pargyline (N-methyl-N-2-propynylbenzylamine 0.198 mgm/ml)
1 mM Pyrogallol (0.126 mgm/ml)
1 mM EDTA disodium salt (0.372 mgm/ml)
0.05% 2-mercaptoethanol.

Buffer

0.1 M potassium phosphate, pH 7.4
1 mM EDTA disodium salt.

S-adenosylmethionine-methyl-C14

Final concentration of this substance was made up to
0.3 - 0.4 μCi/ml.

The brains and hearts from a hundred snails were removed and placed in a ml. of homogenising solution; small batches of tissue were

homogenised separately and the final homogenised solution pooled. This only applied to the brain since the heart is very soft and can be easily homogenised. The weight of the tissue was recorded. Once the tissue was homogenised it was left in ice for thirty minutes. During this period the enzyme phenylethanolamine-N-methyl-transferase (PNMT) was made up in buffer at a concentration of 20 mgm/ml.

After thirty minutes an aliquote of each suspension was taken to determine the protein content using the method of Lowry (1951). Both suspensions are then centrifuged at 3,000 rpm for ten minutes.

The microtubes were prepared first by addition of 15 μl. of the extract. Further tubes were prepared with the addition of a known amount of octopamine and noradrenaline to prepare a standard curve. The concentration of standards used for the curve was as follows: 1.5 ng, 5.0 ng, 10 ng and 1.5 ng in 15 μl of distilled water. The microtubes containing extract and standards had added to them 10 μl of enzyme PNMT followed by 10 μl of S-adenosyl-L-methionine (methyl-C14) to each tube.

The caps were placed on each tube and the contents thoroughly shaken before being placed in a water bath for sixty minutes at 37°C. The reaction was stopped by being cooled rapidly to 0°C. The products synephrine (methyl-C14 and adrenaline methyl-C14) are isolated by ascending chromatography on Whatmann 3 MM paper. The tanks are all glass and sealed at the top by silicone and contain 500 ml of the solvent. The chromatogram paper is first spotted with 10 μl of carrier adrenaline (1 mgm/ml) and the same amount of synephrine. The sheet is then placed in the tank in Butanol/HCl 1N and left overnight to run.

The chromatogram is then removed and dried and the spots visualised with diaxo-nitroaniline as already described. The spots

are then dried, cut out, shredded and extracted in 4 ml of 95% ethanol. After at least four hours, 10 ml of toluene phosphor is added and the radioactivity counted by scintillation.

Results

Incubation of L-DOPA.C14 with the Brain of Helix aspersa

The radioactive intermediates are separated by two-dimensional chromatography and visualised by autoradiography. The incubation demonstrated that dopamine was formed and also an unknown intermediate spot 5, see figure 22 . Another compound was also identified as an inactivation product of dopamine, 3-methoxy,4-hydroxy phenylacetic acid. The presence of this compound was not always as clear as in figure 22 , but sometimes could not be shown. It suggests the presence of MAO and COMT in the snail brain. Incubation with L-DOPA.C14 in the brain and other tissues formed two spots. This occurred even in the control incubation where no tissue was present.

After incubation for an hour the Dopamine spot represented on average nine per cent of the total radioactivity present on the chromatogram while spot 5 represented about two per cent. After three hours the Dopamine spot declined to six per cent, while spot 5 increased to four per cent, suggesting that spot 5 was a metabolic product of Dopamine.

Incubation of L-DOPA.C14 with the heart of Helix aspersa

The heart gave a similar pattern to the brain but breakdown products of dopamine did not show up on the chromatogram. This suggested that the pathway did not have such a high turnover, but it

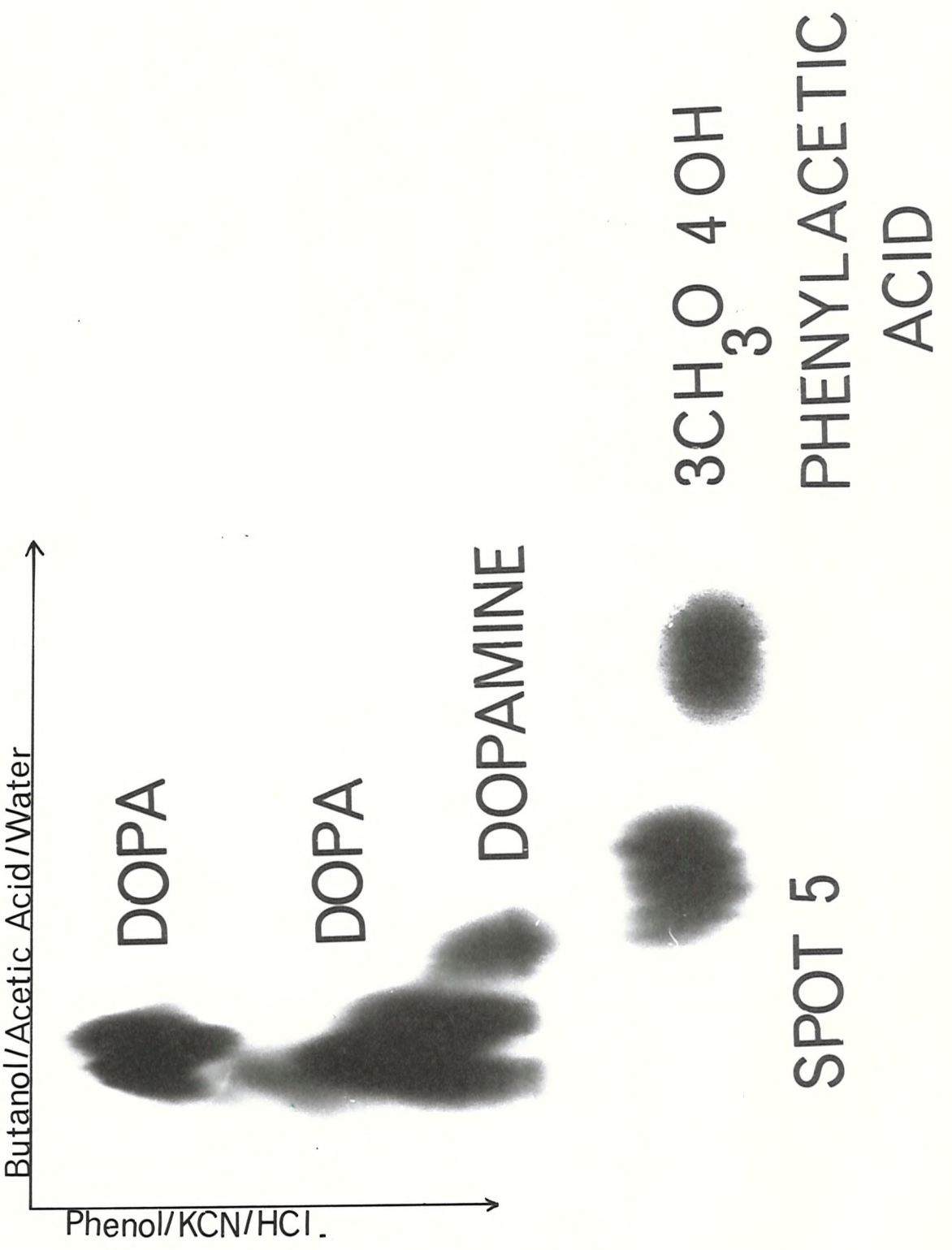

Fig.22. Two dimensional autoradiography of metabolites after L-DOPA.C14 incubation with the brain in vitro.

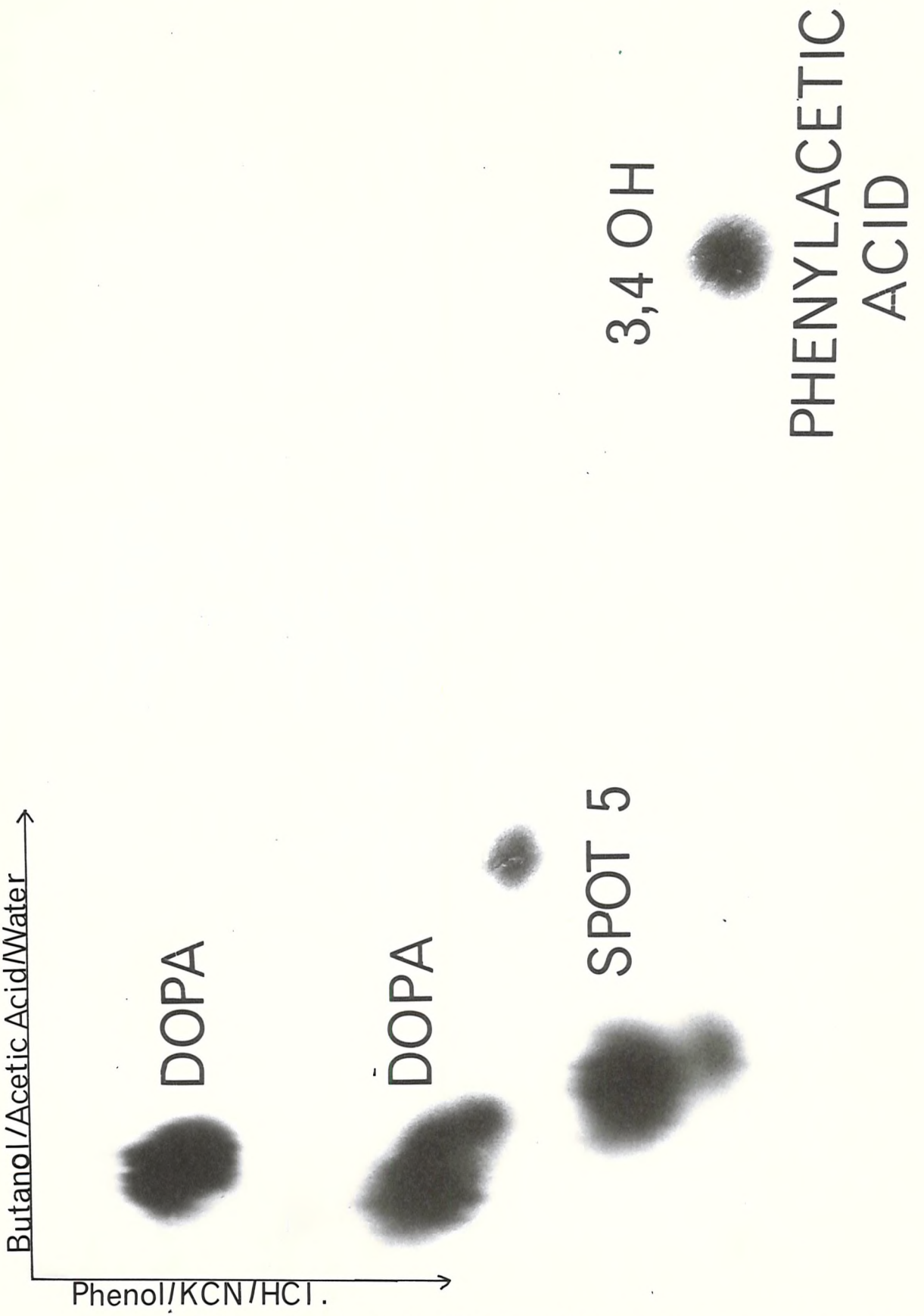

Fig.23. Two dimensional autoradiography of metabolites after L-DOPA.C14 incubation with the kidney in vitro.

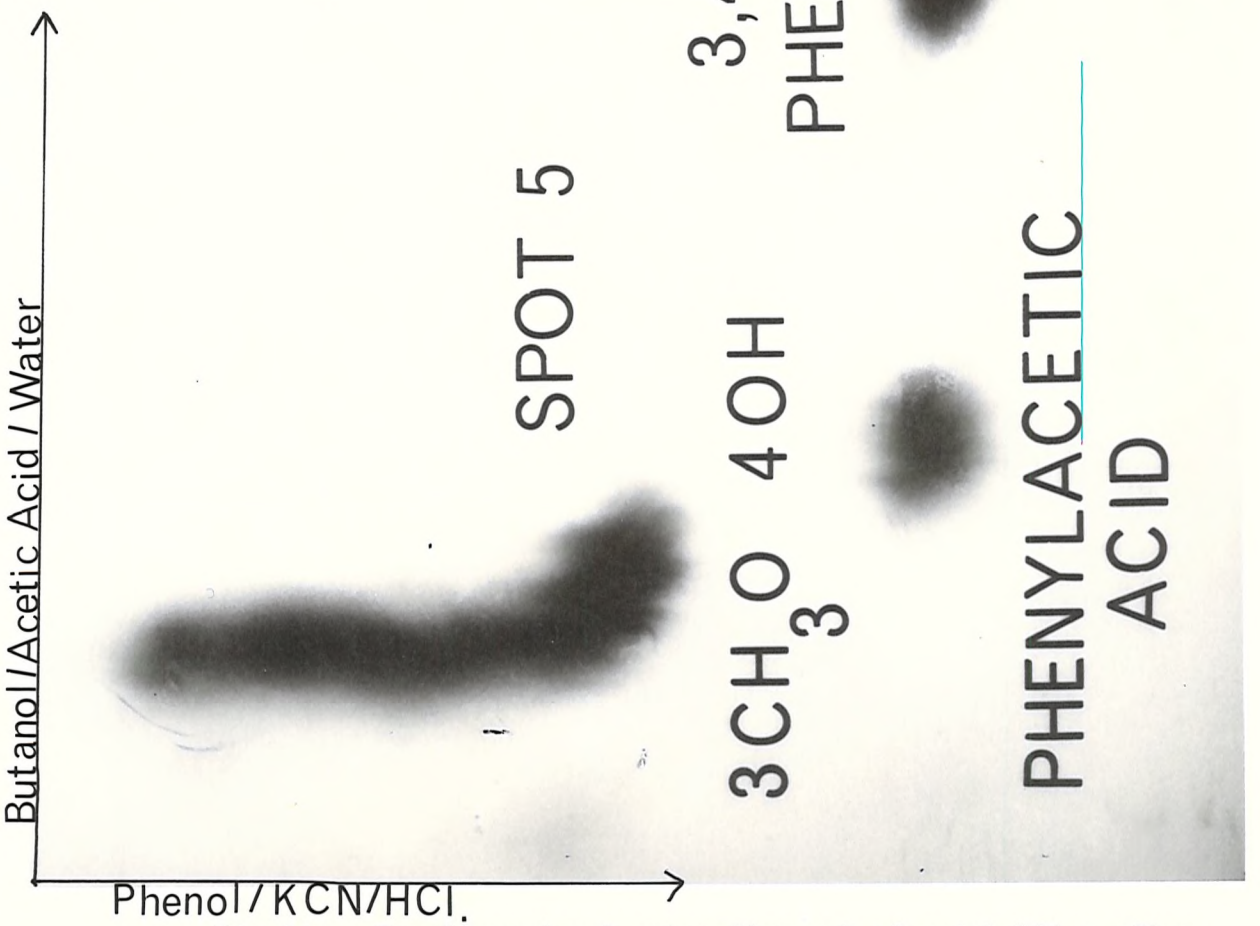

Fig.24. Two dimensional autoradiograph of metabolites after L-DOPA.C14 incubation with the kidney in vivo.

should be pointed out that the size of the heart is also about ten times less than the brain.

Incubation of L-DOPA.C14 with the kidney of Helix aspersa

Incubation of L-DOPA.C14 gave also similar distribution of intermediates as the brain, see figure 23 . The kidney differed in that it was easier to identify acid metabolites such as dihydroxyphenolacetic acid as a major metabolite from its position on the chromatogram. This tissue gave more consistent results for appearance of acid metabolites, compared with the other two tissues.

In vivo Incubation of L-DOPA.C14

The organs and tissues were dissected out after the animal had been injected with C-14 DOPA and had been left for three hours. The tissues removed were as follows: brain, heart, crop, salivary glands, crop and hepto-pancreas. The only significant results were those obtained from the kidney, see figure 24 . The metabolites could be tentatively identified from their position on the chromatogram as 3-methoxy,4-hydroxyphenylacetic acid, 3,4 dihydroxy phenylacetic acid and vanillic acid. Therefore indicating that the kidney is the main centre for inactivation of catecholamines.

Incubation of L-DOPA.C14 in the Presence of a DOPA Decarboxylase Inhibitor, MSD 1024

Only two spots were obtained on the chromatograms for brain, heart and kidney. These spots were the same as obtained in the control. Both were identified as DOPA, therefore indicating that spot 5 is formed from Dopamine.

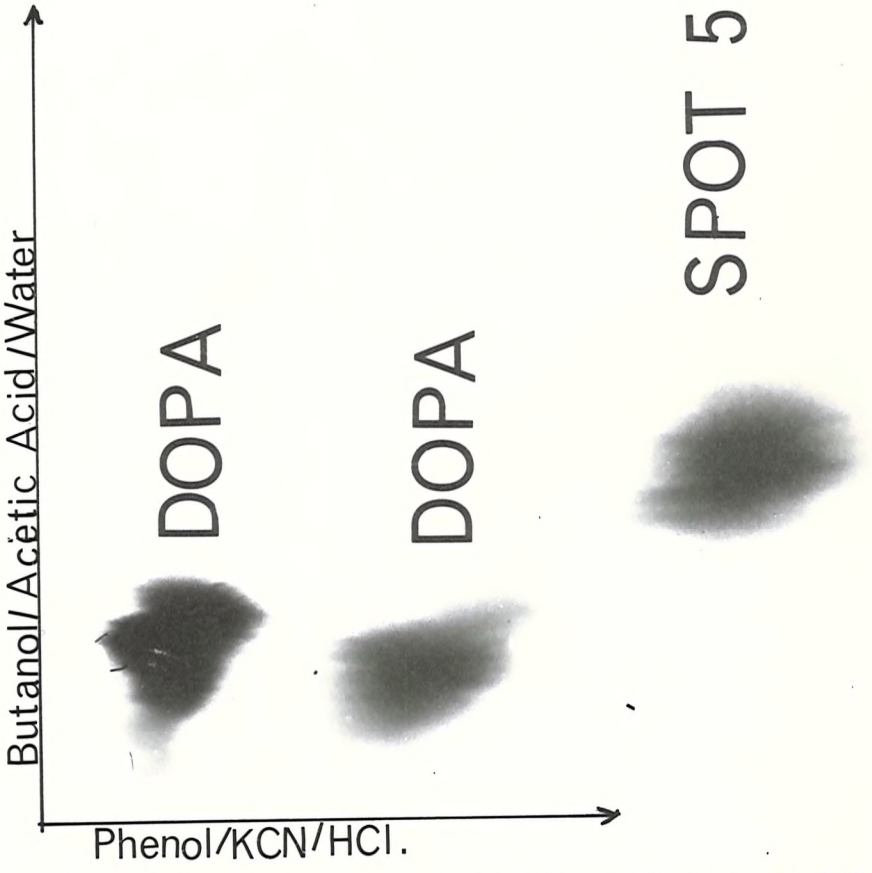

Fig.25. Two dimensional autoradiograph of metabolites after L-DOPA.^{14}C incubation in the presence of Nialamide in the kidney *in vitro*.

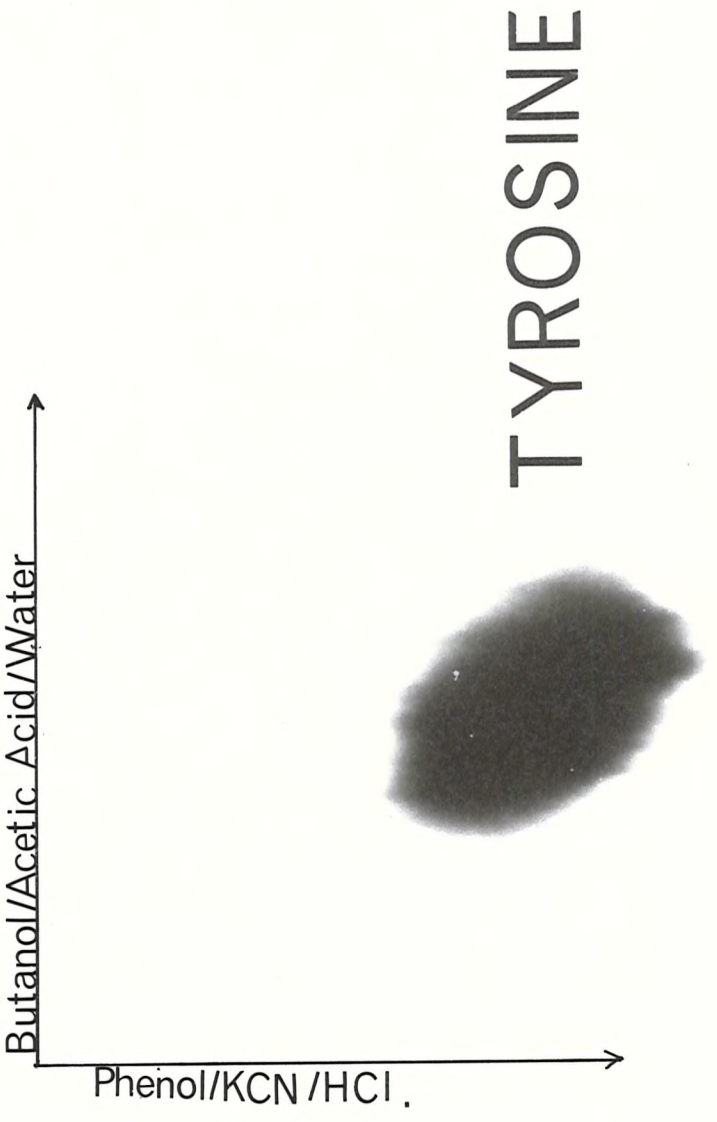

Fig.26. Two dimensional autoradiograph of metabolites after L-tyrosine.C14 incubation with the heart in vitro.

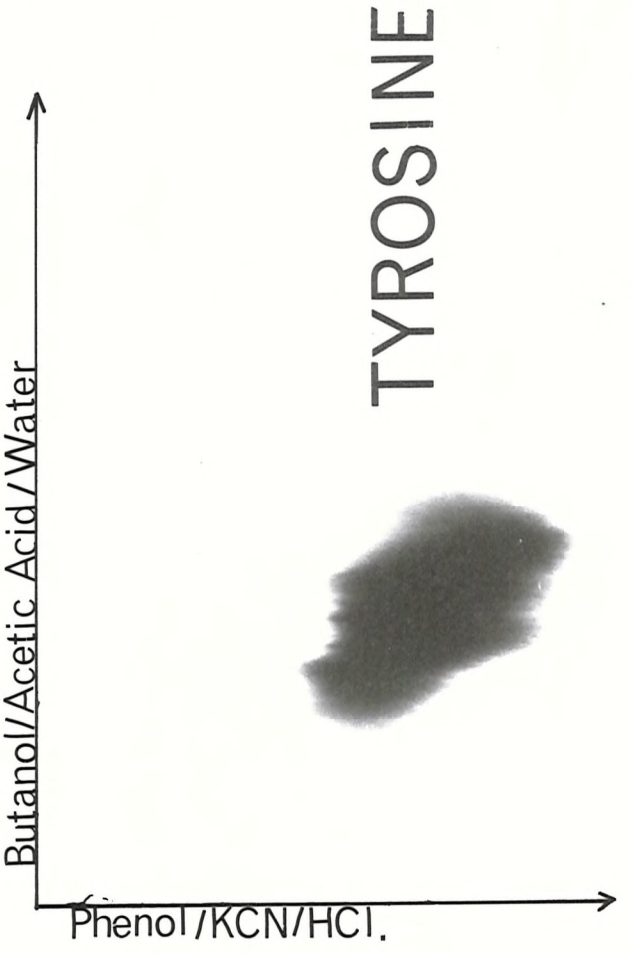

Fig.27. Two dimensional autoradiograph of metabolites after L-tyrosine.C14 incubation with the brain in vitro.

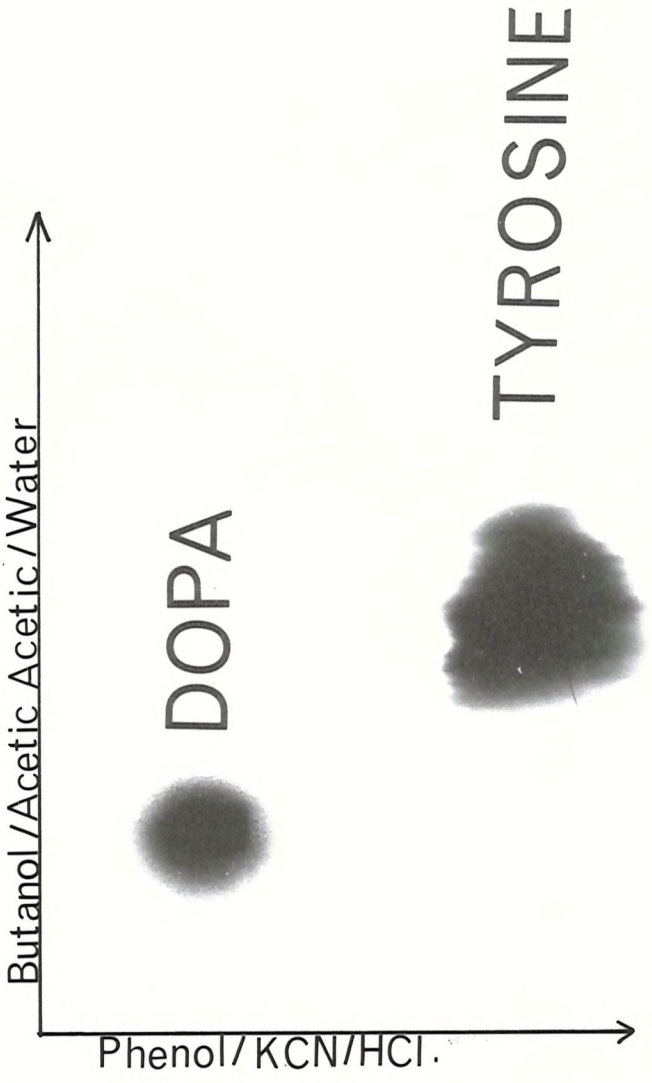

Fig.28. Two dimensional autoradiograph of metabolites after L-tyrosine.C14 incubation with the kidney in vitro.

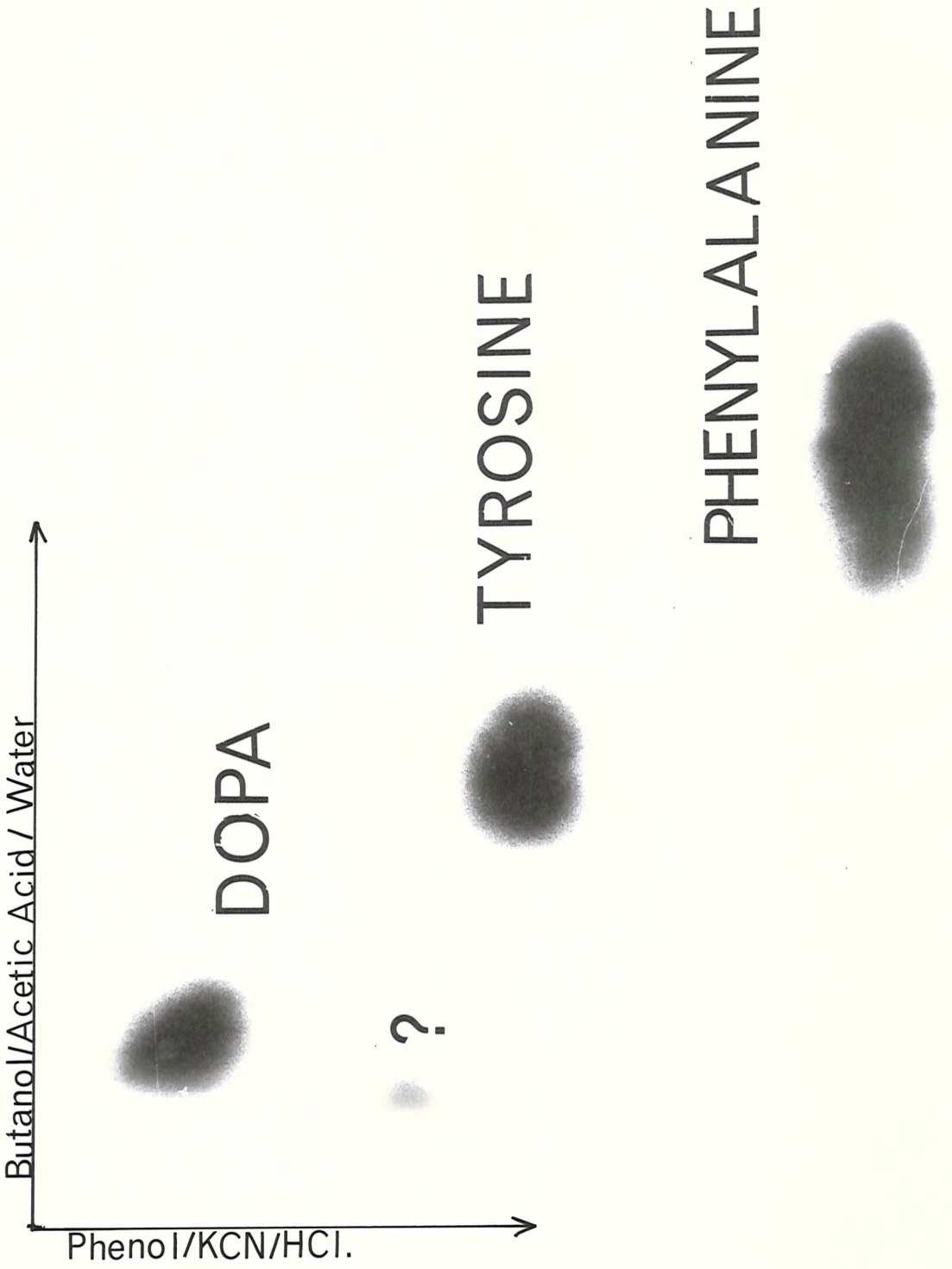

Fig. 29. Two dimensional autoradiograph of metabolites after L-phenylalanine.C14 incubation with the kidney *in vitro*.

Incubation of L-DOPA.C14 in the Presence of an MAO Inhibitor, Nialamide

The inhibitor had no effect on increasing the radioactivity in any of the spots from the brain. Although in the kidney spot 5, see figure 25, had a definite increase in the total percentage of radioactivity in it compared with the controls. This suggests that spot 5 is not a metabolite but probably a stored form of Dopamine, i.e. Dopamine in conjunction with some substance.

L-Tyrosine.C14 incubation with the Brain, Heart and Kidney of Helix aspersa

Incubation with tyrosine in the heart and brain - see figures 26 and 27 - revealed that these tissues do not metabolise tyrosine. The kidney was capable of metabolising tyrosine to DOPA, see figure 28.

L-Phenylalanine.C14 Incubation the Brain, Heart and Kidney of Helix aspersa

These incubations took place over a period of six hours. The brain metabolised phenylalanine to tyrosine. No other metabolites were present. While the kidney converted phenylalanine to tyrosine and then DOPA, see figure 29.

Results for Measurement of Tyrosine Hydroxylase Activity

Measurement was first carried out on whole rat brain. Activity of tyrosine hydroxylase (average of three results) is as follows:

Whole rat brain: 254μmoles/mg/15 mins tyrosine oxidase.

This result is four times greater than the results obtained by Cicero et al (1972) for the whole of the rat brain.

Measurement was then carried out on the brain and kidney of Helix

aspersa. No activity could be measured in the snail brain, but activity was found in the snail kidney:

Snail kidney: 162 μmoles/gm/15 mins tyrosine oxidase

This result is an average of three results obtained for the kidney. Therefore activity can only be measured in the snail kidney; no activity can be demonstrated to be present in the snail brain.

Noradrenaline and Octopamine Levels in Brain and Heart of Helix aspersa

The level of noradrenaline in the heart and brain of Helix aspersa are as follows:

Heart: $2.04 \pm 0.18 \mu$gm/gm protein

Brain: $0.82 \pm 0.35 \mu$gm/gm protein

(The results are the mean of twelve determinations)

Octopamine levels in the heart and brain were as follows:

Heart: $0.75 \pm 0.15 \mu$gm/gm protein

Brain: $1.32 \pm 0.54 \mu$gm/gm protein

(The results are a mean of six determinations)

The standard deviation is indicated in each case.

The greater levels of noradrenaline in the heart compared with the brain are similar to the results obtained by Osbourne and Cottrell (1970) who also found larger quantities in the heart compared with the brain.

Discussion

These results give an indication of the nature of the catecholamine pathway in Helix aspersa.

From the incubation studies it can be concluded that dopamine is probably converted to an unknown product, spot 5, which cannot be identified although it has been run against all possible metabolites. The main two inactivated products identified were dihydroxy phenylacetic acid and 3-methoxy,4 hydroxy phenylacetic acid. These are the normal metabolites expected for a dopamine pathway. This also agrees with the results of Osbourne and Cottrell (1970) for the dopamine pathway in Helix pomatia, and indicates the presence of MAO and COMT. Although evidence for the presence of MAO in the brain is fairly inconclusive, compared with the evidence for its existence in the kidney were Nialamide (MAO inhibitor) was effective in increasing the amount of radioactivity in spot 5. This further indicates that spot 5 is a metabolite of dopamine and this is also in agreement with the results obtained with the DOPA decarboxylase inhibitor (NSD 1024) which blocks the formation of dopamine and spot 5. Therefore the dopamine inactivation pathway is similar to that in mammals except for the existence of a possible dopamine conjugation product, spot 5. Also the kidney is the main site of inactivation of catecholamines.

Tyrosine is unmetabolised by the brain and heart, and only by the kidney where there is conversion to DOPA. Phenylalanine is converted to DOPA only by the kidney. Therefore with the results from the assay for tyrosine hydroxylase activity, where the only activity could be demonstrated in the kidney, it is likely that hydroxylation occurs outside the nervous system. This is in agreement with Edmonson (1965) who suggested that hydroxylation of tryptophan occurs outside the brain, and with the finding of Sedden (1967) that DOPA

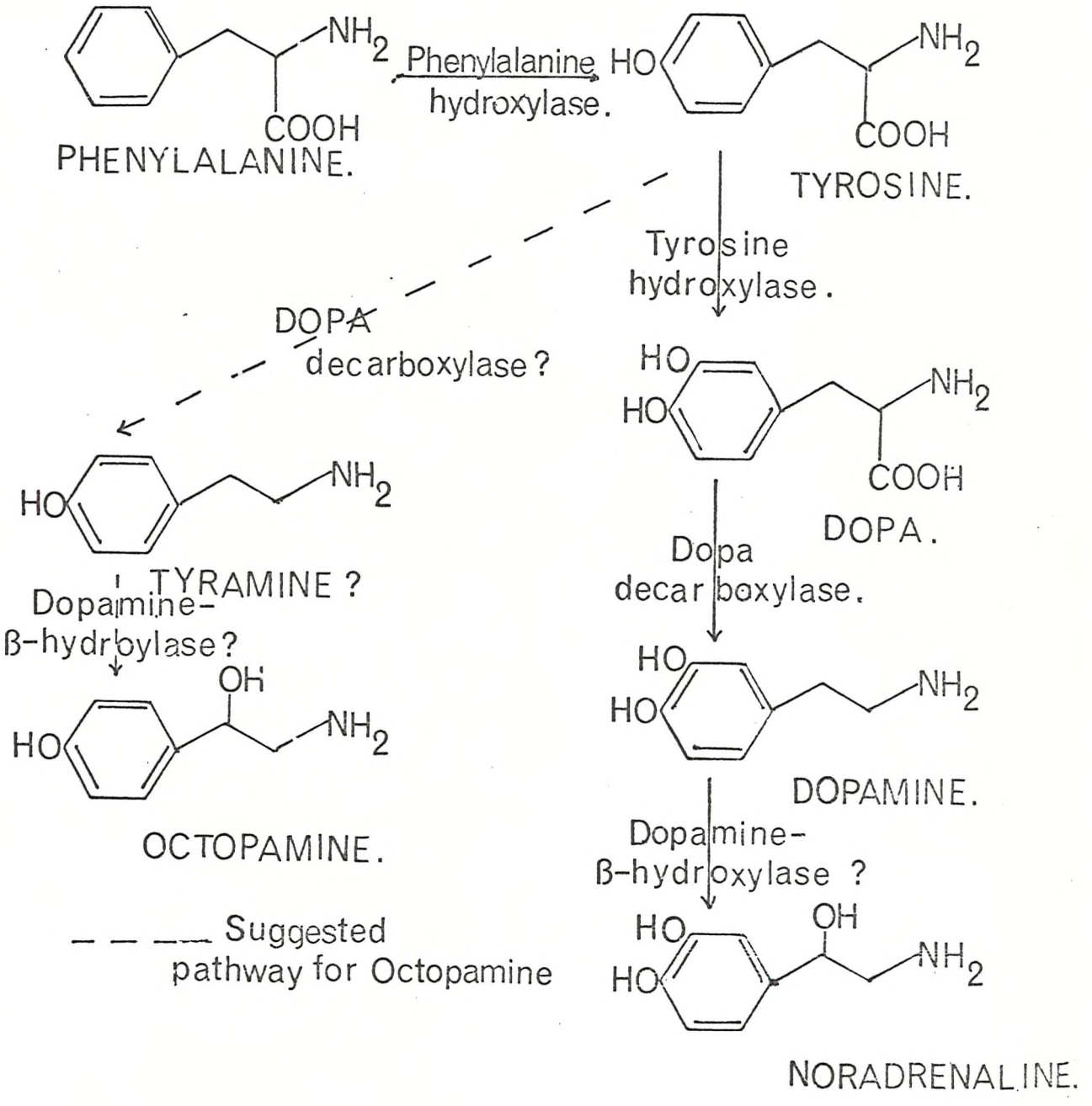

Fig.30. Proposed pathway for catecholamine synthesis in the snail, Helix aspersa.

(0.18 µg/ml) is present in the blood. Thus the site of hydroxylation would be the kidney in Helix aspersa.

The presence of noradrenaline has already been reported in other molluscan tissues (see Introduction). Its function is uncertain; Glaçnier (1967) has reported a cell that is affected by noradrenaline and not by dopamine and the cell used in section Ib is also hyperpolarised by noradrenaline, but is also affected by octopamine and dopamine. It could possibly be a transmitter in the snail nervous system, but only a minor one since its concentration in the nervous system is low. The action of octopamine is probably indirect but the physiological action of these compounds in the nervous system of molluscs is far from clear and further research is required into their physiological action. This applies especially to octopamine whose action even in the mammalian system is still unknown.

Therefore the results obtained indicate that the catecholamine pathway, see figure 30, can be divided into two main stages:

1. Conversion of phenylalanine to DOPA by the kidney which is either stored (spot 5) or passed into the bloodstream;
2. Uptake of DOPA by the brain and heart where it is converted to dopamine and stored as spot 5 and also possibly converted to noradrenaline. Dopamine is inactivated by MAO and COMT.

The pathway for octopamine could come direct from tyrosine which has been shown to occur in the blood of Helix by Cottrell (1963) but the presence of tyramine was not indicated in the incubations with tyrosine, but the level of octopamine is similar to that of noradrenaline and this compound could not be picked up by the method used. Its physiological action is uncertain although results from section Ib suggest it is involved in the uptake mechanism for dopamine in the brain.

Conclusion

The observations recorded in this thesis on the parameters of release, storage and synthesis of acetylcholine and dopamine show that both systems are similar in that they involve a single step in metabolism in the brain:

Acetylcholine: synthesised from choline and acetate

Dopamine: synthesised from decarboxylation of DOPA.

The release parameters of acetylcholine show the presence of a single store which fits in with the theory of vesicular release outlined in the general introduction. Other parameters for this nervous system are the same as those obtained by Bennett and Mclachlan (1972a and b) from the guinea pig cervical ganglion; that release is exponential, i.e. each stimulus releases a constant fraction of the store, also the amplitude and duration of the postsynaptic response is dependent on the amount of transmitter stored in the presynaptic terminals. These parameters could not be determined for Dopamine by the method used for acetylcholine, since the store could not be depleted by stimulation. This was prevented by a very quick turnover mechanism for the replacement of transmitter, probably due to the presence of an amine pump. Therefore the Dopamine system is more efficient in maintaining the store at a constant concentration compared with acetylcholine. This could be considered as major physiological difference between the two systems.

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