GABA IN THE GUINEA-PIG ENTERIC NERVOUS SYSTEM

A thesis submitted for the degree of

Doctor of Philosophy

by

ANTHONY KRANTIS, B.Sc.(Hons)

Department of Physiology,

University of Adelaide,

Adelaide, South Australia, 5000.

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To my wife Sandra

without whose love, support,

encouragement, understanding

and patience, this was not possible.

DECLARATION

I declare that this thesis is a record of original work and contains no material which has been accepted for the award of any other degree or diploma in any University.

To the best of my knowledge and belief, the thesis contains no material previously published or written by another person, except when due reference is made in the text of the thesis.

Anthony Krantis.
November, 1981.

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I am deeply indebted to my supervisor Dr D. I. B. Kerr without whose guidance, knowledge, critical discussion, keen eye and friendship this would not have been possible.

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CORRIGENDA

P8, line 10: delete 'actions'.

P10, para 2, last line should read: 'but this idea has never been subjected to further study'.

P19, line 17 should read: 'half of the myenteric neurones show'.

P27, para 2, last line: Insert reference (Olsen *et al.*, 1978).

P30, para 2, line 1: delete 'binding'.
para 2, last sentence: Insert reference (Breuker & Johnston, 1975).

Chapter II. Figs. 3, 5, 9, 11, 12, abscissa labels should read: '-log dose g/ml'.
Fig. 7, legend, add: 'stimulus period indicated by bar'.

Chapter III. Figs. 12, 13, legends, add new second sentence: 'Broken lines delineate ganglia'.

P70, para 1, last sentence should read: 'since β-alanine, which was present throughout, is a more effective inhibitor of glial than of neuronal GABA-uptake (Browery *et al.*, ...), yet only .....'.

P71, para 3, line 7, should read: 'the more usual criterion of Calcium dependence fails when veratridine is used as a stimulus'.

P80, para 2, last sentence, delete: 'coupling'.
para 3, first sentence should read: 'Following addition of bicuculline....'

P84, para 3, last sentence, should read: '... $GABA_A$ receptors or desensitization of $GABA_B$ responses ....'.

P95, para 2: 'Fig. 6a' should read 'Fig. 6b' and, last line 'Fig. 6b' should read 'Fig. 6a'.

P105, para 2, should read: ... 'antagonists are difficult to measure in central nervous preparations ....'.

SUMMARY

In this thesis a number of criteria for a neurotransmitter have been investigated for γ-amino butyric acid (GABA) in the guinea-pig intestine.

Chapter I is an introductory review of the biochemistry, physiology and pharmacology of GABA nervous transmission in vertebrates and invertebrates. This is followed by a detailed description of the nervous innervation of the mammalian gastrointestinal tract, in particular the small and large intestine, together with structural characteristics of the intestine wall. An overview of the pharmacology of the gastrointestinal tract is also given.

Chapter II is a pharmacological study of GABA actions in the guinea-pig ileum including determination of the chemical specificity of the receptors mediating GABA actions and their associated ionophore. The results confirm and extend the previously published pharmacological studies. GABA stimulation of intrinsic cholinergic motor nerves is via bicuculline sensitive receptors coupled with a Cl^- ionophore. The implications of the mode of GABA action is discussed.

Chapter III reports the autoradiographic investigation of a GABA high affinity uptake "inactivation mechanism" in the guinea-pig intestine. Evidence is presented to show the efficacy of [^{3}H] as an autoradiographic marker in laminar preparations of the myenteric plexus. The disposition of [^{3}H]-GABA and [^{3}H] β-alanine accumulating elements were determined by light microscopic autoradiography using specific neuronal and glial high affinity uptake inhibitors in both laminar and paraffin section preparations. [^{3}H]-GABA uptake sites are shown located to neurones of the myenteric plexus and associated nerve fibres ramifying in the circular muscle layer.

In Chapter IV the development of a relatively simple and rapid staining procedure is described, which affords good morphology of neurones in laminar preparations. This method allows deliniation of nerve types accumulating [^{3}H]-GABA, as shown by autoradiography. [^{3}H]-GABA labelled only a restricted population of myenteric neurones.

Chapter V describes the investigation of the evoked release criterion, using chromatographic and radiochemical analysis. Electrically evoked [^{3}H]-GABA release from laminar preparations of the guinea-pig ileum under various treatments, including neuronal and glial high affinity uptake blockers, tetrodotoxin, and calcium free medium, show [^{3}H]-GABA is released by myenteric neurones in a transmitter-like manner.

In Chapter VI current concepts of the mechanisms of peristalsis are discussed, together with results of experiments in which GABA-antagonism interfered with peristalsis, as measured in isolated preparations of the guinea-pig distal colon. The results suggest important implications for studies of intestinal motility.

Acetylcholine is the transmitter of the excitatory nerves stimulated by GABA but the transmitter released by the inhibitory nerves is not known, although it has been proposed to be adenosine 5'-triphosphate (ATP). In Chapter VII pharmacological investigation of this hypothesis using a new class of ATP antagonist is described. Furthermore, the involvement of ATP in transmission from non-cholinergic excitatory nerves was investigated. ATP is not the transmitter of these nerves.

Chapter VIII is an overview of the whole thesis and discussion of the results in regard to the various criteria for identification of a neurotransmitter substance. Conclusions and suggestions for future

research are also presented. It is concluded that GABA is a neurotransmitter in the guinea-pig myenteric plexus.

CHAPTER I

GENERAL INTRODUCTION

GENERAL INTRODUCTION

History

Since its synthesis in 1883 (Schotten, 1883) gamma-aminobutyric acid (GABA) has been confirmed to be an inhibitory neurotransmitter in vertebrates (Obata *et al.*, 1967; Obata and Takedo, 1969; Curtis and Johnston, 1974) and invertebrates (Kuffler and Edwards, 1958; Triggle and Triggle, 1976). The role of GABA as a major neurotransmitter in the peripheral and central nervous systems of these two 'phyla' is now generally accepted (for reviews see: Curtis and Watkins, 1965; Curtis and Johnston, 1974; Inversen *et al.*, 1975; Roberts *et al.*, 1976). In addition to its documented inhibitory role there is also evidence that GABA may function as an excitatory transmitter in at least some invertebrate and vertebrate preparations (Kerkut and Walker, 1962; Gerschonfeld and Lasansky, 1964; Walker *et al.*, 1971; Obata, 1976). Until recently there have been, however, surprisingly few investigations of any GABA functions in peripheral tissue although GABA has been observed to have pharmacological actions in mammalian intestine (Hobbiger, 1958a, b; Florey and McLennan, 1959; Inouye *et al.*, 1961; Takahashi *et al.*, 1961a, b; Bianchi *et al.*, 1968; Lewis *et al.*, 1972). Evidence has accumulated suggesting a neurotransmitter role for GABA in the mammalian enteric nervous system, where applied GABA stimulates myenteric neurones (Grafe *et al.*, 1979; Krantis *et al.*, 1980). GABA (10 nmol/g) and its synthezing enzyme glutamic acid decarboxylase (GAD) are reported to be localized to myenteric neurones of the guinea-pig taenia coli. Furthermore a small proportion of neurones in the myenteric plexus were shown to possess a high affinity uptake mechanism for GABA (Jessen *et al.*, 1979a). In light of the ubiquitos nature of GABA within central and peripheral neural tissue, together with the recent finings as to its pharmacological effects and apparent neuronal localization within the guinea-pig intestine, the work detailed in this

thesis sought to extend our knowledge about GABA and its function in the guinea-pig enteric nervous system.

Biochemistry

GABA is synthesised to a large extent from glutamic acid by the action of the enzyme glutamic-acid decarboxylase (GAD). Other synthetic pathways for GABA utilising different precursors and enzymes have recently been shown to exist (Roberts, 1981), however their contribution to the GABA neurotransmitter pool remains to be determined. GAD is present in non-neuronal tissue but is of low activity compared to GAD present in central nervous tissue (Baxter, 1970) where it is mainly concentrated in synaptosomal fractions (nerve terminals) (Wu, 1976; Barber and Saito, 1976; Wood *et al.*, 1976).

GABA is degraded by GABA-transaminase (GABA-T) which in the central nervous system is found chiefly in the mitochondria (Neal and Iversen, 1969; van den Berg *et al.*, 1975). The transamination of GABA forms succinic semi-aldehyde which is then oxidized to succinic acid which in turn can be oxidized by the reactions of the tricarboxylic acid cycle (Chase and Walters, 1976; van den Berg *et al.*, 1975). Glutamic acid formed by these reactions may then be converted to glutamine by the action of glutamine synthetase (GS) and shunted to the presynaptic nerve terminal where it is reconverted by phosphate-activated glutaminase back to glutamate for subsequent conversion to GABA (neurotransmitter pool) or enter the tricarboxylic acid cylce (Kvamme, 1979), Fig. 1.

Following its release at the synapse, the major mechanism of removal of GABA from the synaptic region is by transport or 'uptake' which occurs

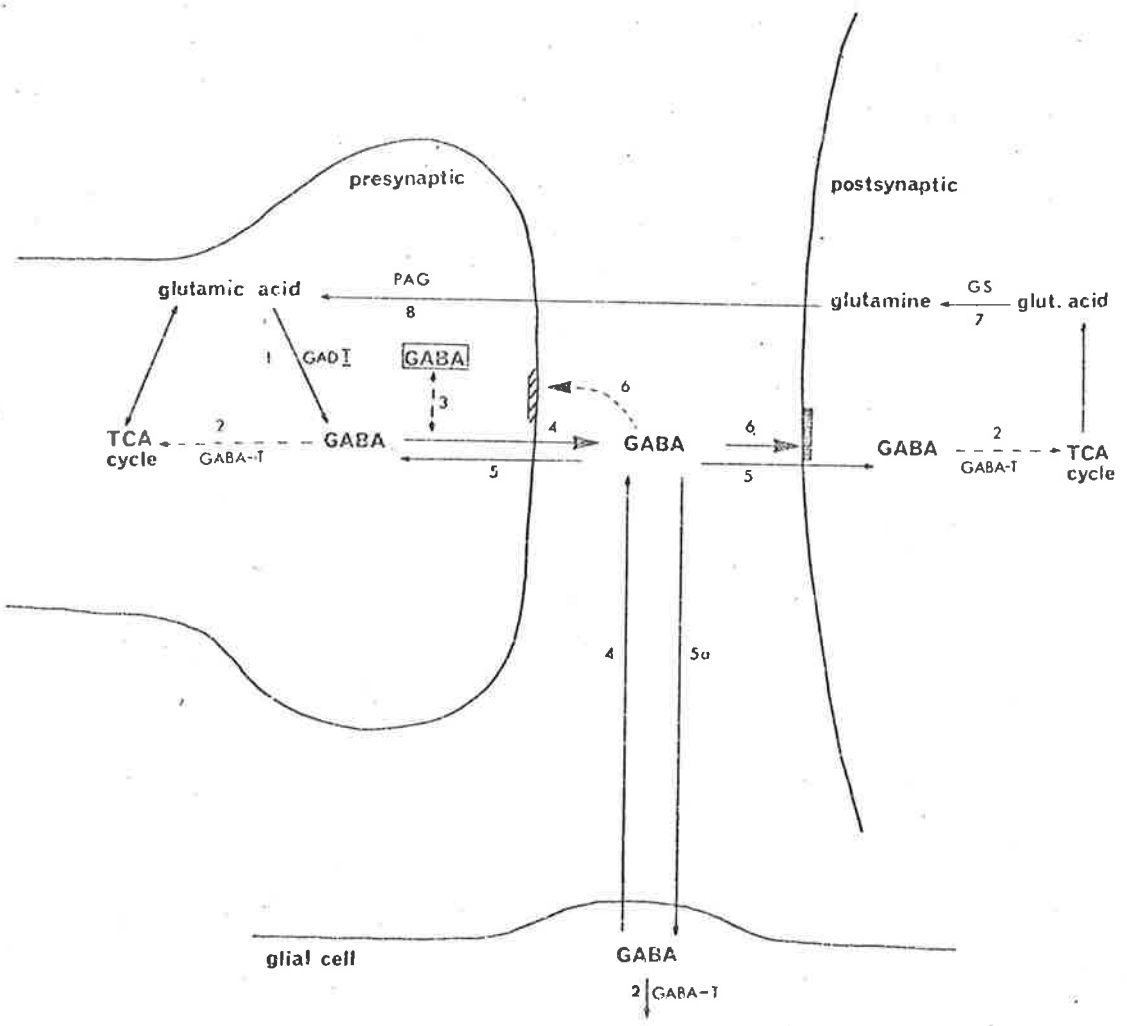


FIGURE 1. GABA-mediated synaptic function. Modified figure from Chase and Walters (1976) and van der Berg *et al.* (1975). 1: biosynthesis, 2: catabolism, 3: intraneuronal storage, 4: release, 5: uptake into presynaptic and postsynaptic neurones, 5a: uptake into perisynaptic glia, 6: receptor interactions, 7: glutamine synthesis, 8: glutamic acid synthesis.

in either neurones or the perisynaptic glia (Iversen and Kelly, 1975; Iversen, 1971). High and low affinity uptake systems have been described for GABA (see Fagg and Lane, 1979) and it has been suggested that both transport systems play a role in GABA uptake with the low affinity system thought to be associated with general metabolite supply to compartments within both glia and neurones (Bond, 1973). This low affinity uptake mechanism (Km 300-1600 M) (Martin, 1976) is evenly distributed in the brain. By contrast, the high affinity uptake mechanism (Km 0.3-40 M) (Elliot and van Gelder, 1958; Iversen and Neal, 1968) has a differential distribution within brain tissue (Hökfelt *et al.*, 1970) and in neurones is related to storage in synaptic vesicles within nerve terminals.

The function of glial cell high affinity uptake of GABA is not clear, however it may be related to the proposed 'cycle' of metabolites between neurones and glia (van den Berg *et al.*, 1975). Another possibility is that in addition to the release triggered by the nerve impusle, there could be a 'tonic' release of GABA from glia whereby GABA could function as a modulator (Tapia, 1974). This concept is supported by the observation that cells in peripheral ganglia, where synaptic release of GABA is virtually ruled out, possess receptors for GABA (De Groat *et al.*, 1972) and that on depolarization glia adjacent to these cells release previously accumulated [^{3}H] GABA (Bowery and Brown, 1972; Minchin and Iversen, 1974).

Although both nerve terminals and glial cells are capable of high affinity uptake of GABA, the substrate specificities of the respective uptake sites differ (Schon and Kelly, 1974; Iversen and Kelly, 1975; Martin, 1976). The GABA analogues 2,4 diaminobutyric acid (DABA), cis-3-aminocyclohexanecarboxylic acid (cis-ACHC) and nipecotic acid appear to be more selective for neuronal, compared to glial, uptake (Iversen and Kelly, 1975; Bowery *et al.*, 1976), where as β-alanine is substrate specific for

the glial GABA transport system (Schon and Kelly, 1974; Schousbe, 1979).

Physiology and Pharmacology of GABA

There is evidence for the release of endogenous and exogenously pre-loaded GABA from both vertebrate and invertebrate presynaptic terminals following appropriate stimulation (Jasper and Koyama, 1969; Bradford, 1970; De Feudis *et al.*, 1970; Iversen *et al.*, 1971; Curtis *et al.*, 1973; Levy *et al.*, 1973; Roberts, 1974). Furthermore, this release displays all the classic characteristics of that for a neurotransmitter. Following its release, GABA actions on the target membrane result in either hyper-polariazation or depolarization, both of which are due to an increased chloride ion (Cl^-) conductance.

The action of GABA in nervous tissue has been shown to result in the hyperpolarization of postsynaptic membranes with a reversal potential that is practically identical to that of the inhibitory postsynaptic potential, which led to its acceptance as a major inhibitory neurotransmitter. It is interesting to note however that the hyperpolarization of the spinal motor neurones is typically shorter than that for central neurones. Of the compounds known to antagonise the postsynaptic effect of GABA, picrotoxin and related compounds and bicuculline and related compounds (Curtis and Johnston, 1974; De Feudis, 1977) are the most effective and widely used. Membrane binding studies suggest that bicuculline (a convulsant phthaleido isoguinoline alkaloid) (De Feudis, 1977; Zukin *et al.*, 1974) competes for the postsynaptic receptor while picrotoxin (an equimolar mixture of picrotoxinin and picrotin) (Curtis and Johnston, 1974; Zukin *et al.*, 1974) acts not entirely at the GABA-receptor, but at the associated Cl^- ionophore, although there is some evidence for a direct receptor action as well.

In contrast to its observed effect upon spinal motor neurones, application of GABA onto primary afferent terminals in the spinal cord incuded depolarization, similar to the effect of presynaptic inhibition on dorsal root potential (Levy, 1977). In this type of inhibition GABA released at presynaptic axo-axonic synapses brings about depolarization of presynaptic terminals causing a reduction in the amplitude of the action potential in afferent terminals ant thus reduces transmitter release. Both presynaptic inhibition and GABA actions at the primary afferents are also blocked by the specific GABA antagonist bicuculline and by picrotoxin, or by desensitization to GABA actions. Taken together with the properties of GABA and the high concentration of GABA and its synthesizing enzyme (GAD) found in the dorsal horn of the spinal cord (Fahn, 1976; Miyata and Otsuka, 1975) this supports the hypothesis that GABA is a neurotransmitter mediating presynatpic inhibition (Levy, 1977; Obata, 1976).

The inhibitory action of GABA on vertebrate and invertebrate neurones, appears to be due to the same ionic mechanism (a pronounced increase in the permeability of the postsynaptic membrane to Cl^-) which clamps the membrane potential at a negative level, preventing depolarization of the membrane by any subsequent depolarizing influence. GABA-induced depolarizations are also mediated by an increased membrane permeability to Cl^-, however the direction of the Cl^- current is reversed.

GABA in Peripheral Nervous System

While effects of GABA on mammalian central neurones are in general inhibitory and hyperpolarizing (Roberts, 1976; Curtis and Johnston, 1974; Krnjevic, 1974), it has recently been found that GABA has some depolarizing actions on peripheral ganglion cells and their axons (De Groat, 1970;

De Groat *et al.*, 1971). Although the GABA induced depolarization of peripheral nerves results from an increased Cl^- conductance, the chloride equilibrium potential (E_{Cl}) is less than for the resting membrane potential (Em), suggesting that the electrochemical Cl^- diffusion gradient across the neuronal membrane is directed outward which is opposite to the inwardly directed Cl^- gradient mediating its central hyperpolarizing actions. This difference in Cl^- gradient is due to an inwardly directed Cl^- pump which maintains a high [Cl]i in afferent and peripheral neurones. Changes in external sodium or potassium ion concentrations do not significantly alter the chloride equilibrium potential (-33.7 or -20 mV) of afferent neurones (Gallagher *et al.*, 1975; Nishi *et al.*, 1974; Deschenes *et al.*, 1976). Studies involving intracellular and extracellular recording from the superior cervical ganglia, and dorsal root ganglia of the rabbit, cat, rat, bullfrog and chick embryo showed GABA in every instance to induce depolarization (see Obata, 1976). GABA actions in these cells showed analogous pharmacologic properties to those at central neurones in that picrotoxin and bicuculline reversibly antagonized GABA actions at low concentrations. Desensitization to GABA was marked and the depolarization declined during GABA application.

Depolarizing effects of GABA also occur at molluscan and echinoderm neurones (Florey *et al.*, 1975; Yarowsky and Carpenter, 1977) where GABA may have an excitatory function. Furthermore, it has been suggested that GABA may be an excitatory neurotransmitter in the inner ear of the bullfrog and skate (Flock and Lam, 1974) and olfactory nerve of garfish and pike (Roskoski *et al.*, 1974). GABA also appears to have a non-specific depolarizing action on peripheral C-fibres (Brown and Marsh, 1978).

Recently, 'GABA' receptors that are unaffected by the classical

'GABA' receptor antagonists and insensitive to many of the GABA-mimetics such as 3-aminopropanesulphonic acid (3-APS), isoguavicine and muscimol, have been described on peripheral autonomic nerve terminals and in mammalian brain (Bowery *et al.*, 1980, 1981; Bowery and Hudson, 1978). These receptors show high affinity for baclofen the β (-p-chlorophenyl) derivative of GABA (Fox *et al.*, 1978; Curtis *et al.*, 1974; Bowery *et al.*, 1979; Johnston *et al.*, 1980) and have been termed $GABA_B$ receptors by Hill and Bowery (1981). While not producing axonal depolarization as in classical presynaptic inhibition, the function of these baclofen ($GABA_B$) receptors is nevertheless similar to other axonal GABA receptors in that their activation causes a decrease in neurotransmitter release (Bowery *et al.*, 1980).

GABA in Enteric Nervous System

In previous studies (Hobbiger, 1958a, b; Florey and McLennon, 1959; Inouye *et al.*, 1960; Takahashi *et al.*, 1961a, b; Bianchi *et al.*, 1968; Lewis *et al.*, 1972) that investigated the effect of GABA on the isolated mammalian intestine; GABA was ineffective when administered alone, but in the guinea-pig ileum GABA (10^{-6} to 10^{-3} M) reduced the submaximal stimulatory effects of acetylcholine (ACh), nicotine, histamine (HA) and 5-hydroxy-tryptamine and depressed the peristaltic reflex (Hobbiger, 1958a, b). These effects were blocked by picrotoxin. According to Inouye *et al.*, (1961), in producing these effects GABA acted upon sites closely correlated with tryptamine receptors, but this work has never been subjected to a serious study.

In a recent and extensive pharmacological study of the actions of GABA in the guinea-pig small and large intestine by Krantis *et al.* (1980)

showed that GABA causes concentration-dependent transient relaxations of the distal colon, or contractions, or biphasic responses (contractions followed by relaxations) of the iluem. By using tetrodotoxin (TTX) which blocks nerve conduction (Kao, 1966), it was determined that GABA has little or no effect direct upon the muscle of the ileum or distal colon, rather GABA stimulates bicuculline sensitive receptors mediating excitation of cholinergic excitatory and enteric inhibitory neurones (the transmitter for which is presently unknown). Whereas Hobbiger (1958a, b) and Inouye *et al.* (1961) observed a depression of peristalsis by GABA itself, neither the ascending excitatory nor the descending inhibitory reflex (the primary reflex arcs underlying peristalsis, Costa and Furness, 1976) when elicited by localised stretch of the colon wall were affected by bicuculline. This apparent discrepancy in the effect of applied GABA upon motility and the induced intrinsic reflexes, is discussed elsewhere, along with a more detailed investigation of the pharmacological and ionic basis for GABA actions in the guinea-pig intestine.

Sites similar to the baclofen ($GABA_B$) receptors which are present in mammalian central and peripheral nervous tissue (see preceding section) have also been shown to occur in the guinea-pig ileum and vas deferens of mouse and guinea-pig (Bowery *et al.*, 1981). The action of GABA and baclofen at these receptors, depressed the twitch responses to electrical stimulation of these preparations, an action similar to that of both adenosine and the opiates (morphine, enkephalin) (Kosterlitz and Waterfield, 1975, North *et al.*, 1979) which inhibit the output of acetylcholine from cholinergic neruones in the guinea-pig.

The Enteric Nervous System

The mammalian enteric nervous system forms an extensive network of nervous elements totalling some 10^7 to 10^8 neurones comprising up to ten different neurone types (see Gabella, 1976) which, together with the different smooth muscle and non-neuronal layers with which these enteric nerve networks are interposed within the digestive tract wall, presents a rather complex arrangement. Therefore before any further discussion of GABA actions in the guinea-pig intestine, an explanation of the general structure of the intestine wall and in particular, the enteric nervous system, is necessary.

Functional Organization

The intestinal wall comprises a number of layers: muscle, nerve plexuses and mucosa in the general order described in Fig. 2. This organization is generally the same throughout the length of the gastro-intestinal tract, and although there are great changes in the size and shape of the various layers, there is considerable consistency of a given part of the tract in a given species (Schabadasch, 1930; Schofield, 1968; Stach, 1971; Gabella, 1976). In the small and large intestines, fibres of the outer longitudinal muscle coat run parallel to the axis of the intestine, and in the small intestine form a continous layer, whilst in the large intestine longitudinal muscle fibres are gathered into distinctive bands termed 'taenia coli'. The inner circular muscle coat consists of muscle fibres which are oriented perpendicular to the axis of the intestine. Intra-muscular blood capillaries (84 per 10,000 cross-sectioned smooth muscle cells, Gabella, 1976) are contained

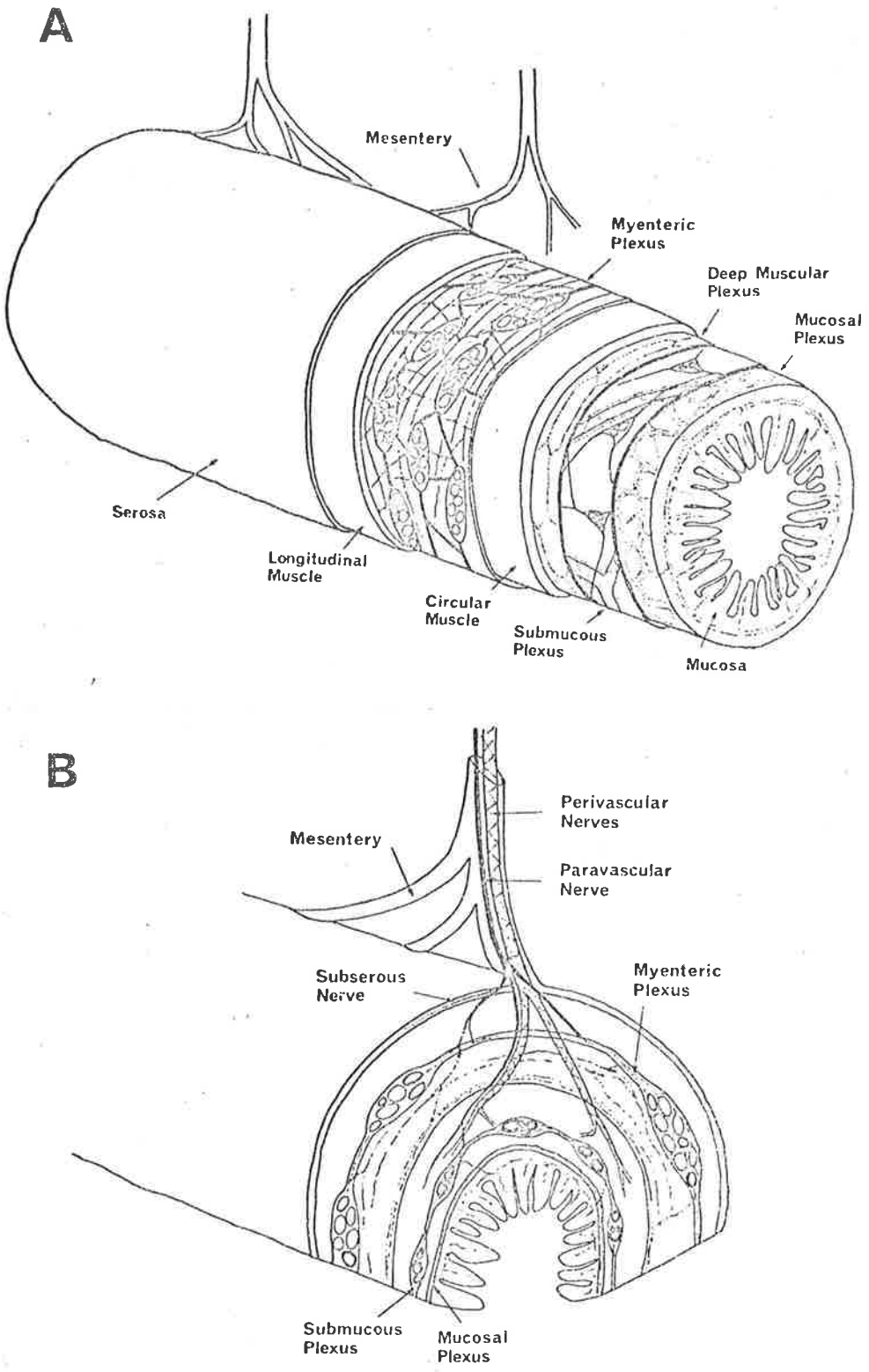


FIGURE 2. Diagrammatic representation showing the structure of the wall of a segment of intestine. In (A), the wall of the intestine is cut away to show the arrangement of the various layers. (B) The arrangement of extrinsic and intrinsic nerves in a section through the intestinal wall.

within the circular muscle layer, running approximately parallel to the muscle cells. Between the longitudinal and circular muscle layers are aggregates of ganglion cell bodies with interconnecting bundles (fasciculi) of axons. These aggregates and their associated connecting fibres form the myenteric plexus (Auerbach's plexus), which makes connections with an extrinsic innervation through the mesenteric nerve and a transitional subserous plexus also containing neurones into distinct ganglia.

The nervous innervation of the circular muscle coat consists of nerve bundles from the myenteric plexus which penetrate this muscle layer, some of which then run parallel to the smooth muscle cells, or pass through and connect with the submucous plexus (Cajal, 1911; Gabella, 1972). Nerve bundles from the myenteric plexus also connect with a nerve plexus 'muscularis profundus' (Cajal, 1911) which lies adjacent to the inner (luminal) aspect of the circular muscle. The nerve fibres of this 'deep muscular plexus' run parallel to the muscle cells. Distinct glanglia are absent.

Located between the circular muscle coat and mucosa, the submucous plexus consists of two nerve layers comprised of interconnected ganglia. These ganlia are generally smaller than the myenteric ganglia and generally contain fewer neurones. Extensions of the submucous plexus pass through the 'muscularis mucosae' to connect with the mucous plexus. Comprised of a variety of plexuses, variously associated with glands in the mucosa, these plexuses are continuous with and represent an extension of the submucous plexus. The plexus contains no neurone soma.

Nerve bundles are practically absent from the longitudinal muscle (Thaemert, 1963; Taxi, 1965; Paton and Vizi, 1969), however there are vesiculated nerve processes of the myenteric plexus which lie only a few tens of nanometres away, separated from the muscle by only a thin basal lamina (Gabella, 1972). This close apposition of nerve and muscle layers presumably allows rapid (milliseconds) diffusion of transmitter from the nerve plexus to muscle cells, and no doubt accounts for the variety of pharmacological responses observed in isolated longitudinal muscle myenteric-plexus preparations.

Myenteric Plexus

The neurones comprising this plexus are distributed into 3 plexiform meshworks (see Schofield, 1968). The large elongated ganglia with interconnecting bundles of unmyelinated fibres constitute the primary meshwork. Within, and continuous with this meshwork, is a secondary meshwork which, although lacking any discernable aggregates of cell bodies comparable to the ganglia of the primary meshwork, contains cell bodies along the course of the fasciculi. Branching from, and localised within, the secondary meshwork is the tertiary meshwork. There is some morphological evidence for the presence of cell bodies within this meshwork (Cajal, 1911; Gabella, 1979). Reciprocal innervation occurs between the myenteric plexus and large paravascular nerve bundles which ramify within the submucous plexus, and additional direct connections occur between the submucous plexus and elements of the myenteric tertiary meshwork. Innervation of the circular muscle coats by myenteric neurones arises from this tertiary meshwork. In contrast to the submucous plexus the myenteric plexus contains no blood vessels.

Myenteric Neurones

Individual neurones can be readily seen within the ganglion of the primary meshwork. These neurones range in size depending on the region of gastrointestinal tract and the species in question. In the rat caecum, the myenteric neurones range between 100 μm^2 to over 2000 μm^2 in cell surface area. Myenteric neurones in the guinea-pig measure between 10-100 μm in length and can number more than 100 per ganglion. Morphological characterization of myenteric neurones shows a broad spectrum of cell types conforming to Dogiel's (1899) classification: Type I, II and III cells, and to more recent classifications based on cell size (Gunn, 1959, 1968) and on the number of processes (Schofield, 1968).

The three types of ganglion cells described by Dogiel include: Type I cells, characterised by numerous short stout dendrites of irregular calibre (cogwheel in appearance) and a single slender axon which passes into the interconnecting fibre bundles (fasciculi). Type II cells are multipolar, however their dendrites are typically longer and usually extend beyond the ganglion. These dendrites are fewer in number than for the Type I neurones. Type III cells have dendrites of intermediate length which appear to terminate in the same or adjacent ganglion.

In addition to these neurones, which according to Gunn's classification are referred to collectively as large neurones, there are also smaller neurones present in relatively large numbers; smaller than any of Dogiel's cells.

Submucous Plexus

As with the myenteric plexus, the submucous plexus consists of several fine meshworks. However unlike the myenteric plexus, it is divided into two layers. The inner plexus (nearest the mucosa) termed Meissner's plexus, is characterised by a small, regular closed pattern meshwork, containing small ganglion cells. The outer layer, lying against the inner side of the circular muscle, is termed the Shabadasch plexus or Henle's plexus. In most part, this plexus is open meshed and very irregular with the ganglia sparsely distributed. The ganglion cells are of the same type as those of the myenteric plexus, however the ganglia of the submucous plexus contain fewer cells (approximately 8), and indeed there are fewer cells in total within this nerve plexus.

Extrinsic Innervation of the Enteric Plexuses

Although the gastrointestinal tract displays independence of action from central control there are nonetheless nervous connections with the central nervous system via three major efferent pathways: the vagus nerve; the splanchnic nerves, and the pelvic nerves (Fig. 2), through which the central nervous system modulates gastrointestinal activity.

On the motor side, the abdominal vagus accompanying blood vessels (paravascular), contains mostly parasympathetic cholinergic (ACh) preganglionic nerve fibres of which the majority innervate the stomach and distal portion of the large intestine. These cholinergic nerve fibres form excitatory connections with both intrinsic cholinergic and enteric inhibitory neurones located in the myenteric plexus (Daniel, 1968). There are also non-cholinergic inhibitory fibres (Langley, 1898) and a small

number of postganglionic adrenergic fibres (Nielson *et al.*, 1969; Lundberg *et al.*, 1976). In addition, the vagus also contains a variety of afferent fibres (Daniel, 1968).

Additional excitatory input to the intestine is evident on stimulation of the mesenteric nerves (Bartho and Szolcsanyi, 1979; Szolcsanyi and Bartho, 1979). According to these authors, the excitatory responses elicited in this way are the result of antidromic stimulation of sensory nerve processes causing the excitation of the intrinsic cholinergic nerves possibly by way of true synapses. The identity of the sensory neurotransmitter mediating these effects is unknown although there is evidence (Hökfelt *et al.*, 1976; Franco *et al.*, 1979a, b; Guillemein, 1976; Furness and Costa, 1979), precluding the involvement of either substance P (SP) or somatostatin.

Sympathetic innervation (adrenergic) of the intestine is largely postganglionic, eminating from three main groups of sympathetic ganglia: vertebral, pre-vertebral abdominal ganglia, terminal ganglia. Sympathetic input occurs via the mesenteric nerves which emerge from the abdominal plexus of the splanchnic nerves, the vagus, some of which may be preganglionic, and the pelvic nerve. These adrenergic (noradrenaline) nerve fibres have a prejunctional inhibitory action upon the release of acetylcholine from intrinsic cholinergic neurones (Furness and Costa, 1974; Hirst and McKirdy, 1974). This effect is mediated through alpha-adrenergic receptors. However there are also beta-adrenergic receptors present, located on the muscle which on stimulation cause relaxation of the intestine.

These paravascular nerves, can readily be distinguished from the fine anastomosing nerve plexus (perivascular) which supplies the arterial innervation. The latter include vasodilator nerves involved in the

intrinsic vasodilator reflexes, which release neither noradrenaline (NA) nor acetylcholine (ACh) (Biber, 1973; Fasth *et al.*, 1977).

Intrinsic Innervation of the Enteric Plexuses

The enteric nervous system of the mammalian intestine is complex, containing an intrinsic nervous network within which are sensory neurones, interneurones and motor excitatory and inhibitory neurones that provide the considerable integrative capacity necessary for the control of gastro-intestinal functions including the reflex movements of gut musculature (peristaltic reflex), where distension of the gut wall initiates enteric reflexes causing contractions which push the contents analward into an area of relaxation. As the contents move, the reflexes continue to be elicited, contraction behind and relaxation in front which maintain the propulsion of the contents. Reflexes also include secretion of gastro-intestinal hormones, and the regulation of blood flow. The gut also exhibits pendular non-propulsive movements which facilitate the mixing of gut contents for digestion.

A number of substances are proposed to be neurotransmitters in the mammalian enteric nervous system, including dopamine (DA), vasoactive intestinal peptide (VIP), somatostatin, substance P, enkephalin, cholecystokinin, bombesin, neurotensin, adenosine-5'-triphosphate (ATP), acetylcholine (ACh), noradrenaline (NA), and 5-hydroxytryptamine (5HT) (Burnstock, 1972; Gabella, 1976; Dreyfuss *et al.*, 1977; Costa *et al.*, 1980). Only ACh and NA have satisfied all of the criteria for a neuro-transmitter (Gershon, 1981) although there is considerable evidence for 5HT having such a function (Costa and Furness, 1979a; Gershon, 1981).

Low frequency transmural electrical stimulation of the intestine elicits a release of ACh and a concommitant contraction of both the circular and longitudinal smooth muscle coats (Bulbring *et al.*, 1958; Kosterlitz and Lees, 1964), which is antagonised by muscarinic receptor blockade. Furthermore, muscarinic antagonists inhibit peristalsis (Kosterlitz, 1968) suggesting that cholinergic motor neurones are important for the propulsive activity of the intestine. It has since been shown that the final neurones in the excitatory reflex component of peristalsis (Kosterlitz and Robinson, 1959; Furness, 1969) release ACh.

Ganglionic nicotinic blocking agents such as hexamethonium prevent both the excitatory and inhibitory components of the peristaltic reflex (Bulbring *et al.*, 1958; Kosterlitz, 1968). There are thus cholinoreceptive sites in the ganglia subserving the stimulation of both cholinergic and the enteric inhibitory motor neurones. In support of this Nishi and North (1973) and North and Henderson (1975) report that hexamethonium blocks fast EPSP's in guinea-pig ileal myenteric neurones. Approximately one half of myenteric neurones recorded from show fast EPSP's and are termed Type I cells (Nishi and North, 1973) or S cells (Hirst *et al.*, 1974), displaying a brief after-hyperpolarization.

The actions of enteric inhibitory neurones which supply the muscle are not prevented by adrenergic blockade, and hyperpolarization of the intestinal muscle still persists following degeneration of the noradrenergic innervation (Campbell, 1970; Burnstock, 1972). The identity of the inhibitory transmitter released by these neurones has proven elusive, although a number of substances have been proposed as candidates. According to Burnstock (1972, 1975) adenosine triphosphate (ATP) is the non-adrenergic non-cholinergic inhibitory transmitter, and the work by Burnstock and his colleagues has been the basis of the hypothesis where

these nerves have come to be termed 'purinergic'. There is however, considerable evidence available disputing the purinergic hypothesis, the details of which will be discussed in Chapter VII.

Recent immunohistochemical studies have demonstrated immunoreactivity toward the histologically localised polypeptides substance P, enkephalins, neurotensin, somatostatin, bombesin, vasoactive intestinal peptide (VIP) and gastrin/cholecystokinin tetrapeptide, within enteric neurones (Hökfelt *et al.*, 1975, 1976, 1977; Fuxe *et al.*, 1977; Schultzberg *et al.*, 1980).

In the mammalian gut, the enkephalins and morphine cause inhibition of neuronal acetylcholine release (Paton, 1957; de la Lande and Porter, 1967; Waterfield *et al.*, 1977) and inhibition of firing of myenteric neurones (North and Williams, 1976). These depressant effects of the opiates result from the depolarization of the nerve processes (North *et al.*, 1979), via naloxone sensitive opiate receptors which, in the guinea-pig inferior mesenteric ganglion, are proposed to be located on the cholinergic nerve terminals (Bornstein and Fields, 1979). This would explain the extensive distribution of enkephalin-like immunoreactive nerve fibres in the circular muscle and taenia coli (Jessen *et al.*, 1980) where a direct effect of the enkephalins on cholinergic nerve terminals would be possible.

Substance P (SP) neuronal processes densely innervate the myenteric ganglia projecting both in the oral and anal directions (Furness *et al.*, 1980). SP has been shown to depolarize enteric neurones (Katayama and North, 1978) and to cause contraction of the intestine by a direct action on the smooth muscle (Bury and Mashford, 1977; Szeli *et al.*, 1977; Yau, 1978; Franco *et al.*, 1979a, b). There is now evidence that substance P containing nerve fibres are also distributed throughout the circular muscle

layer of the small and large intestine, as well as in the longitudinal layer of the taenia coli and ascending colon where it is proposed to be the transmitter mediating non-cholinergic contractions of these preparations (Franco *et al.*, 1979a, b; Jessen *et al.*, 1980). Substance P is able to initiate and increase peristalsis in the guinea-pig ileum (Holzer and Lembeck, 1978) and although there are cholinergic inputs to substance P neurones (Franco *et al.*, 1979a, b) it is unclear whether substance P nerves are in anyway important for intestinal motility. Indeed most recently Costa *et al.* (1981) have shown that most of substance P released by electrical stimulation in fact originates from afferent fibres, not intrinsic neurones, whilst Chahl (1981) suggests that these afferent fibres make synaptic contact with the cholinergic motor neurones and that the substance P responses as a result of electrical stimulation are an artefact due to its overflow from these afferent synaptic regions.

Neurones displaying somatostatin immunoreactivity occur in both the submucosal and myenteric plexus (Furness *et al.*, 1980). Somatostatin has little direct effect on the smooth muscle. Rather somatostatin has been found to depolarize some myenteric neurones whilst hyperpolarizing others (Katayama and North, 1980). The types of myenteric neurones affected by somatostatin have not been fully investigated, however neurones hyperpolarized by somatostatin may be cholinergic-excitatory ones since somatostatin inhibits transmission from cholinergic neurones (Cohen *et al.*, 1978; Grafe *et al.*, 1978). There is pharmacological evidence (Furness and Costa, 1979) for intrinsic enteric inhibitory neurones being stimulated by somatostatin, which, taken together with the analward polarity of somatostatin immunoreactive neurones, suggests that this peptide may have a role in the descending inhibitory reflex (Furness and Costa, 1980).

VIP immunoreactive neurones have been identified within the guinea-pig

ileal myenteric and submucous plexuses (Schultzberg *et al.*, 1978). Within the myenteric plexus these neurones run in an oral to anal direction, ramifying to other myenteric neurones and to the circular muscle (Furness *et al.*, 1980). Furthermore, VIP relaxes smooth muscle (Furness and Costa, 1980) and is released from the gut following vagal stimulation (Fahrenkrug *et al.*, 1978). On this basis the non-adrenergic, non-cholinergic inhibitory neurones are proposed to be peptidergic rather than purinergic, releasing VIP (Costa *et al.*, 1980).

At present, however, there is a paucity of data to suggest that VIP is released by neuronal activity, or that it has pharmacological actions comparable to the non-adrenergic inhibitory transmitter. Indeed Cocks and Burnstock (1979) and Mackenzie and Burnstock (1980) have shown pharmacologically that exogenously administered VIP does not mimick the inhibitory response to non-adrenergic non-cholinergic nerve stimulation in the intestine. In addition, Williams and North (1979) report that VIP is ineffective upon ileal longitudinal muscle but rather depolarizes myenteric neurones. Although the neurotransmitter for non-adrenergic, non-cholinergic inhibition remains to be elucidated, the roles of these neurones are well defined (Furness and Costa, 1973; Hirst and McKirdy, 1974; Costa and Furness, 1976). They are involved in the descending inhibition, first described by Bayliss and Starling (1899) which produces the reflex relaxation ofthe gastrointestinal tract immediately in front of substances travelling through the digestive tube.

In addition to the intrinsic, cholinergic excitatory innervation of the smooth muscle coats of the intestine, nerve mediated contractions resistant to muscarinic blockade have been reported. These include a 'rebound' contraction of the muscle following an inhibitory influence (Bennett, 1966; Campbell and Burnstock, 1968; Furness, 1971) which may

appear as a primary response to stimulation, if the period of nerve stimulation is brief and the inherent muscle tone low. However, we cannot preclude the possibility that the depolarization leading to this type of contraction is in fact induced by an excitatory substance being released onto the muscle. For post-inhibitory 'rebound' this is almost certainly due to prostaglandin release (Burnstock *et al.*, 1975).

Studies by Ambache and Freeman (1968), Ambache *et al.* (1970) and Furness and Costa (1973) have shown slow contractions of the longitudinal muscle response to transmural stimulation (5-50 Hz) which have a different time course from the so called 'rebound' contraction described above, that could be blocked by tetrodotoxin (TTX). It has been suggested that a 5-hydroxytryptamine (5HT)-like substance may be mediating this response (Costa and Furness, 1973; Costa and Furness, 1979a). However, much more evidence is needed to substantiate this suggestion since in the distal colon, circular muscle is quite insensitive to 5HT (Costa and Furness, 1979b). Moreover, whilst cholinergic excitatory input to enteric neurones via nicotinic receptors is well established (Kosterlitz and Lees, 1964; Bulbring and Gershon, 1967; Crema *et al.*, 1970), there are synaptic actions where excitation is not blocked by nicotinic ganglionic blockers (Dingledine and Goldstein, 1976), but is antagonised by the 5HT antagonist methysergide or by desensitization to 5HT (Katayama and North, 1978; Wood and Mayer, 1979).

Sites of action for 5HT on myenteric neurones, distinct from nicotinic receptors have been identified (Browlee and Johnson, 1963; Gershon, 1967; North *et al.*, 1980) which together with evidence for its synthesis (Gershon *et al.*, 1965), storage (Gershon and Ross, 1966a, b) and release (Gershon, 1965; Bulbring and Gershon, 1967) has prompted speculation on the possibility that 5HT is a neurotransmitter in the enteric nervous

system (Costa and Furness, 1979a). However 5HT has widespread multiple effects in the mammalian intestine and the precise nature of 5HT actions in the mammalian gastrointestinal tract remains unclear.

Contractions of the ileal longitudinal muscle, distinct from post-inhibitory 'rebound' and the above slow excitation, are also elicited by transmural stimulation (5-50 Hz). According to Franco *et al.* (1979a, b) these are related to the action of substance P (SP). SP released by intestinal nerves, or applied exogenously, acts directly upon the muscle to induce a contraction. Whether any of these substances are released during the peristaltic reflex, remains to be determined.

According to Nishi and North (1973) and Hirst (1979) the slow depolarization potentials recorded from a small proportion of both Type 1 and Type 2 myenteric neurones are infact not mimicked by 5HT but have the same ionic mechanism as the potential change caused by SP. IPSP's have been recorded in myenteric neurones (Wood and Mayer, 1978) and approximately 45% of the submucous plexus (Hirst *et al.*, 1974; Hirst and McKirdy, 1975). The transmitter mediating IPSP's of enteric neurones is at present unknown. The ionic mechanism appears to be similar to the effects of somatostatin and enkephalin (Williams and North, 1979; Nishi and North, 1973; North *et al.*, 1979) although the hyperpolarizing mechanism has not been completely elucidated.

Although adrenergic (alpha and beta) receptors occur throughout the gastrointestinal tract, noradrenaline acting at these sites is in most part released from extrinsic axonal processes except for the proximal colon of the guinea-pig where intrinsic non-adrenergic cell bodies have been found, located the myenteric and submucous plexuses (Gabella, 1976). The peristence of IPSP's recorded from enteric neurones following

extrinsic denervation and subsequent loss of catecholamines (Johnson *et al.*, 1980), precludes the possibility of the intrinsic inhibitory motor neurones being noradrenergic (Furness and Costa, 1978). However, there is considerable evidence for noradrenaline to be released from extrinsic nerve terminals which impinge on cholinergic axons and exert a presynaptic inhibition of ACh release (Kosterlitz *et al.*, 1970; Holman *et al.*, 1972; Nishi and North, 1973; North and Nishi, 1974).

In addition to those compounds already described, a number of chemical substances have pronounced pharmacological actions on the intestine, including bradykinin, histamine and gastrins. Bradykinin (Day and Vane, 1963; Fishlock, 1966; Downing and Morris, 1981) elicit both contraction and relaxation, and histamine via direct and indirect actions upon the smooth muscle (Daniel, 1968; Downing and Morris, 1981).

The gastrins contract a number of mammalian gastrointestinal preparations and stimulate gastric and pancreatic secretions (see Daniel, 1968). At present the evidence for either neurotensin or bombesin to have major pharmacological or physiological actions in the mammalian alimentary canal is limited (Costa and Furness, 1980; Schultzenberg *et al.*, 1979), however, neurotensin contracts the guinea-pig ileum through a nerve mediated partly cholinergic process (Kitabgi and Freychet, 1978, 1979) and possibly a substance P-ergic process (Monier and Kitabgi, 1980).

C H A P T E R I I

THE PHARMACOLOGY OF GABA ACTIONS

Introduction

Recently Krantis *et al.* (1980) have shown that GABA has stimulating actions on enteric neurones, as seen in the isolated guinea-pig small and large intestine, in contrast to its recognised inhibitory actions in central and peripheral nervous tissue (Krnjevic, 1974). These excitatory actions were antagonised by the GABA antagonist bicuculline or by making the tissue tachyphylactic to GABA. Excitatory actions of GABA have also been observed when recording from neurones of the guinea-pig myenteric plexus (Grafe *et al.*, 1979), but apart from their demonstrated sensitivity to picrotoxin, which may suggest a hitherto unrecognised function of GABA in the intestine (e.g., enteric neurotransmitter), little is known of the pharmacology of these responses. However, for the study of a neurotransmitter some information about the site and mechanism of drug action is necessary (Orrego, 1979).

In the mammalian central nervous system, GABA actions at specific receptors result in an increased chloride conductance that is responsible for both pre and postsynaptic inhibition (Curtis and Johnston, 1974; De Feudis, 1977). When released on to postsynaptic receptors, GABA causes an increased chloride permeability of the target membrane resulting in a net influx of chloride (Cl^-) ions into the cells so that the membrane becomes hyperpolarized. Although presynaptic GABA actions also result in an increased Cl^- permeability, the Cl^- ions leave the cells instead of entering them, so that the cell membrane is depolarized. GABA depolarizing actions have also been observed in mammalian peripheral nervous tissue, including sympathetic ganglia (Adams and Brown, 1975), C-fibres (Brown and Marsh, 1978) and sensory neurones (Gallagher *et al.*, 1978). All of these GABA effects show analogous pharmacologic and ionic properties. In contrast, there is a class of receptors mediating GABA

effects selectively activated by baclofen (which is the β-p-chlorophenyl derivate of GABA). This effect, no coupled with Cl^- ionophores, is known to reduce transmitter release in a number of preparations (Bowery *et al.*, 1981).

Most experimental approaches to identifying the nature of GABA actions in various nervous tissue have involved isolated tissue preparations, since they permit the use of known concentrations of drugs and measured manipulation of the extracellular ionic environment. Picrotoxin and bicuculline reversibly antagonise the neuronal depressant actions of GABA in various vertebrate and invertebrate preparations (Galindo, 1969; Curtis *et al.*, 1970) and hence have been used extensively to examine GABA-synaptic transmission (De Feudis, 1977). Although there is some controversy as to the specificity of these antagonists (Dudel, 1965; Bowery and Brown, 1974; Barker *et al.*, 1975), it is generally agreed that bicuculline exerts its action at the GABA-receptor whilst picrotoxin acts at the associated ionophore.

A number of problems are associated with investigation of the ionic basis of GABA effects. For example, removal of Cl^- may result in an altered osmolarity, also the commonly used substituent anions may have the additional problem of binding calcium, usually to an undefined extent. Furthermore, if other than mono-valent substitutent anions are used (e.g., sulphate) the ionic strength of the solution is difficult to determine (A. H. Bretag, personal communication). The permeability of the membrane to the substituent anion may also result in equilibration of this anion across the membrane, so that it can substitute for Cl^- in the GABA-mediated conductance change. All in all this experimental approach would thus appear unsatisfactory. An alternative method involves the use of specific ion transport blockers (e.g., ammonium salts and furosemide, Nicoll, 1975, 1978), which in addition to avoiding many of the problems associated with ionic substitution, affords less interference with cationic permeability.

Furosemide selectively blocks hyperpolarizing and depolarizing responses to GABA in frog spinal cord, presumably by blocking the ionic channels operated by GABA (Nicoll, 1978).

A more detailed investigation of GABA-induced responses in the guinea-pig ileum using furosemide and a new and potent inhibitor of chloride transport, piretanide (Zeuthen *et al.*, 1978), together with the established GABA-antagonists bicuculline and picrotoxin is reported here.

Materials and Methods

Guinea-pigs of either sex weighing between 200-400 g were stunned by a blow to the head and bled. Segments of proximal ileum were quickly removed and placed in a modified Krebs solution of the following composition m mol/l: Na^+ 151.0; K^+ 4.6; Mg^{++} 0.6; Ca^{2+} 2.8; Cl^- 134.9; HCO_3^- 24.9; $H_2PO_4^-$ 1.3; SO_4^- 0.6; glucose 7.7. The solution was maintained at 36°C and gassed with a mixture of 95% O_2 and 5% CO_2, to maintain the pH at 7.3. Segments of ileum 4 cm in length were then attached at one end to the bottom of a 10 ml water-jacketed bath containing warmed Krebs solution, with the other end attached by a cotton thread to a Harvard rotary transducer and allowed to equilibrate in the organ bath for 60 min before commencing electrical stimulation or drug treatments. Mechanical reactivity of the ileal segments to the various treatments were displayed on a Grass Polygraph (Model 5D).

For experiments in which intramural nerves were stimulated and tissue responses recorded, two platinum wire ring electrodes 4 mm apart, were placed around the segments of intestine. Pulses of 0.2 msec duration were

delivered at a frequency of 0.2 Hz for a 10 sec stimulation period, or of 0.1 msec duration at 0.1 Hz for varying train lengths. The strength of stimulation was adjusted to give responses of near maximum amplitude.

Drug volumes administered were never more than 1% of the bath volume. Agonists were left in contact with the tissue for periods of 20 to 60 sec at intervals of 4 or more minutes. Antagonists were added at least 10 min before the agonist was tested. Drugs used were: acetylcholine chloride (ACh), atropine sulphate, bicuculline, gamma-aminobutyric acid (GABA), picrotoxinin, furosemide, histamine, bradykinin, piretanide, tetrodotoxin (TTX). Furosemide was a gift from Hoechst Australia and piretanide a gift from Dr Alan Bretag.

Statistical Analysis: Results are given in the figures as means ± s.e. means. The Student's t-test for paired and unpaired samples was used to assess the significance ($p<0.05$) of difference between mean values of the dose response effects. All regression lines were calculated by the least squares method. Regression line slopes of the Clark plot (Clark, 1928) were compared using the t value derived from $t = \frac{(b-b_o)\sqrt{N-2}}{\sqrt{1-r^2}}$ where b_o = slope of the control; N = sample size; r^2 = coefficient of determination. The regression line of the Schild plot was constrained to a slope of -1, and the 95% confidence limits of the pA_2 value, calculated according to Tallarida *et al.* (1979).

Results

When GABA was applied to the ileum in the organ bath it produced either contractions or else biphasic responses (transient contractions

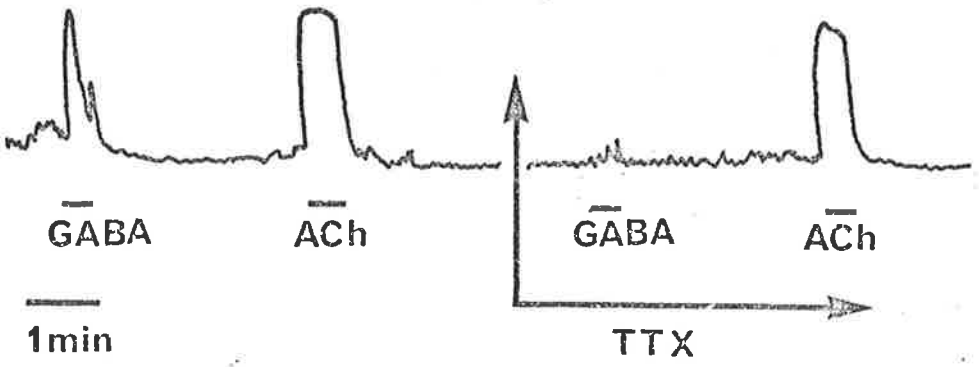


FIG. 1. The effect of tetrodotoxin (TTX) on contractions of the guinea-pig ileum. TTX 10^{-7} g/ml was added 15 min before the responses to GABA 3 x 10^{-6} g/ml and acetylcholine (ACh) 3 x 10^{-9} g/ml were retested.

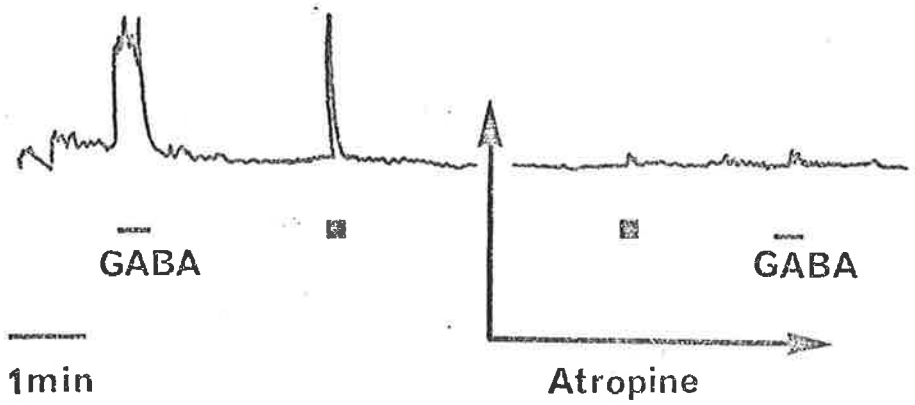


FIG. 2. The effect of atropine 2 x 10^{-7} g/ml on contractions of the guinea-pig ileum to GABA and transmural stimulation. Atropine was applied 15 min before the responses to GABA 3 x 10^{-6} g/ml and electrical stimulation ▪ (0.2 Hz, 0.2 ms, 60 V) were retested.

followed immediately by relaxation), as described by Krantis *et al.* (1980). Only those tissues showing purely contractile responses were subsequently used. The response of the ileum to applied GABA was immediate, with the contractions reaching maximum within 15-20 sec. The threshold concentration was about 10^{-7} g/ml and the maximum contraction was observed to occur between 6×10^{-6} - 10^{-5} g/ml. These concentration-dependent contractions were the result of GABA stimulation of neurones concerned with cholinergic innervation of the smooth muscle of the ileum, since GABA actions were abolished by tetrodotoxin 10^{-7} g/ml (a compound which interferes with nerve action potentials by blocking sodium channels, Kao, 1966) (Fig. 1), and the muscarinic antagonist atropine 2×10^{-7} g/ml (Fig. 2). GABA applied in the presence of prolonged application of the same concentration of GABA did not evoke any detectable response of the ileum. This effect is characteristic of the responses to GABA being desensitized. The contractions were antagonized in a concentration dependent manner by both picrotoxinin and bicuculline, which usually had no effect upon tissue activity when added to the bath although on occasion a transient contraction of the ileum was observed.

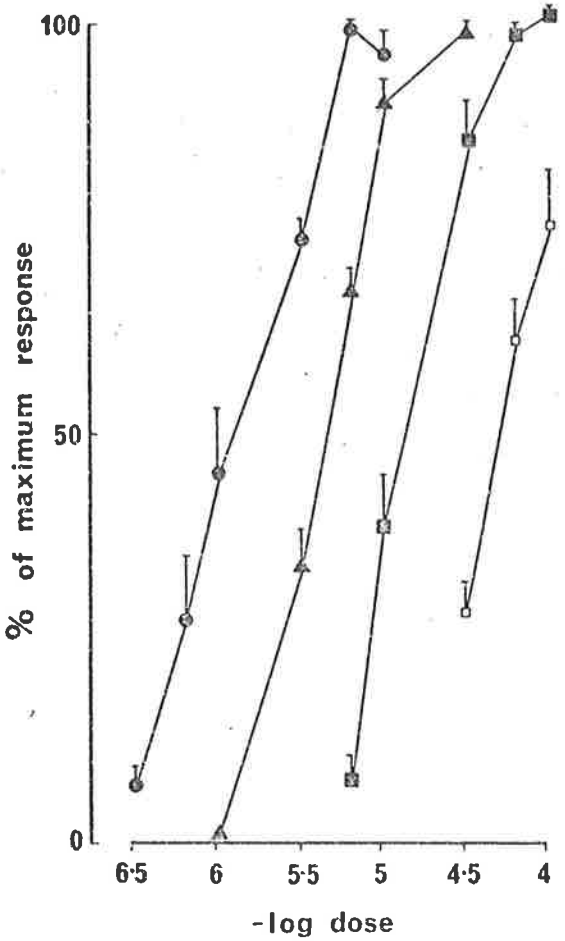
Bicuculline (a specific antagonist of GABA binding; Johnston, 1978), 10^{-6}, 2×10^{-6}, 5×10^{-6} g/ml elicited a parallel shift to the right (significant, d.f. = 6, $p<0.05$) in the dose response curve for GABA-induced contractions (Fig. 3a). In the Clark plot (Fig. 3b) bicuculline did not alter the slope (not significant d.f. = 6, $p>0.05$), but caused a parallel displacement, suggesting a competitive action of bicuculline at GABA receptors on the myenteric neurones involved in the response. From the Schild plot (constrained to a slope of -1), the pA_2 for bicuculline was 6.12 ± 1.39 (7.5×10^{-7} mol/l) (2.8×10^{-7} g/ml) (Fig. 4). Bicuculline itself has some cholinergic character which conceivably could be responsible for the observed actions on GABA-induced responses. To exclude this

FIG. 3.

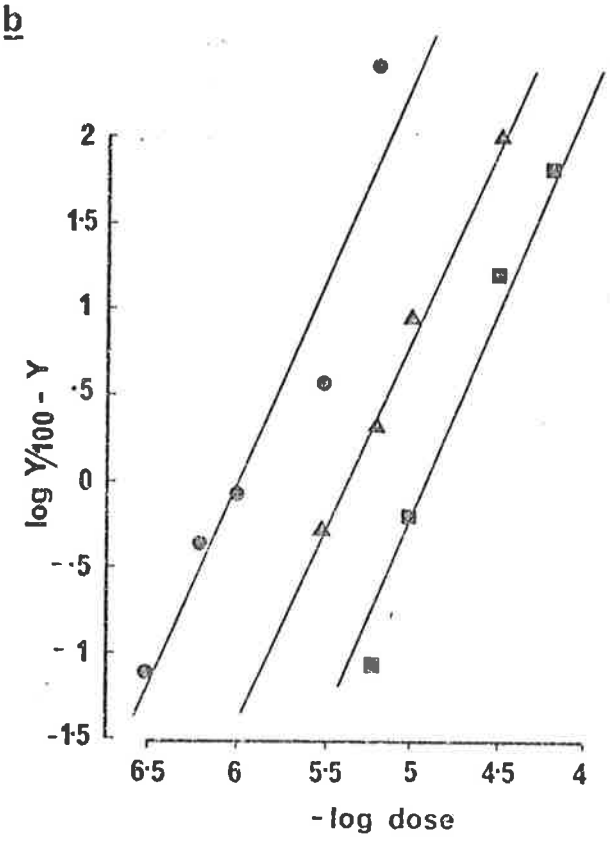
(a) The effect of bicuculline on the concentration response curves for the contraction elicited by GABA in the guinea-pig ileum. (●) Responses in normal solution; (▲) responses to GABA 20 min after the addition of bicuculline, 10^{-6} g/ml to the bathing solution; (■) in the presence of bicuculline, 2×10^{-6} g/ml; (□) in the presence of bicuculline, 5×10^{-6} g/ml. Each point is the mean and standard error of responses obrained in 5-8 preparations.

(b) Clark plot of the bicuculline induced antagonism of the GABA concentration response curve. (●) Control responses; (▲) responses in the presence of bicuculline, 10^{-6} g/ml; (■) 2×10^{-6} g/ml.

a


100
50
0
% of maximum response
6·5
6
5·5
5
4·5
4
-log dose

b


2
1·5
1
·5
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log Y/100 - Y
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4·5
4
-log dose

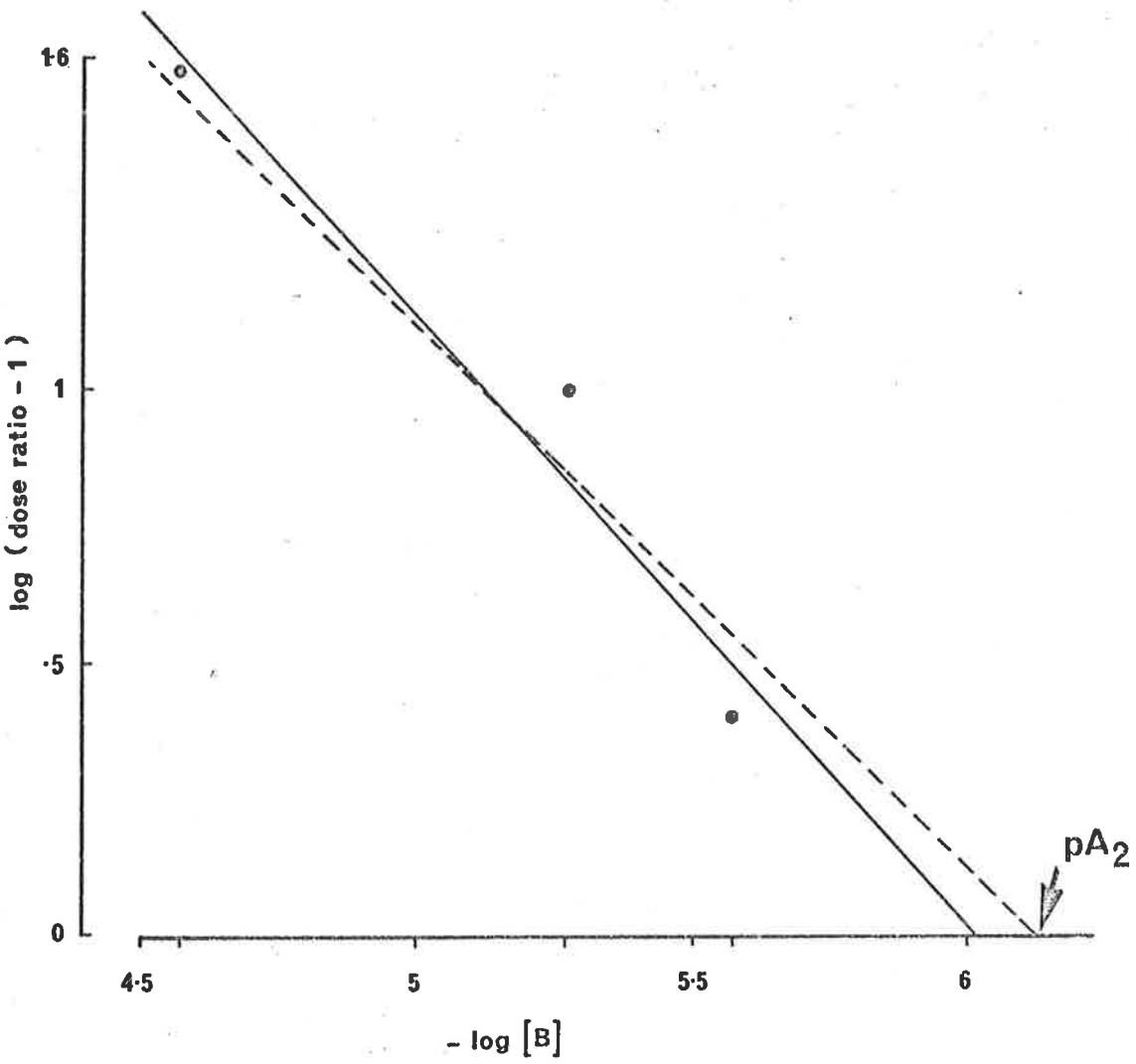


FIG. 4. Schild plot of the GABA/bicuculline dose response data. The plot is constrained to a slope of -1 (hatched line) in accordance with competitive theory. Ordinate: log of the dose ratio A'/A corresponding to each molar concentration of the antagonist [B]. Abscissa: log of the bicuculline [B] concentration. The 'best' straight line (solid line, slope = -1.12) was calculated by regression analysis. The pA_2 is determined from the intercept on the abscissa.

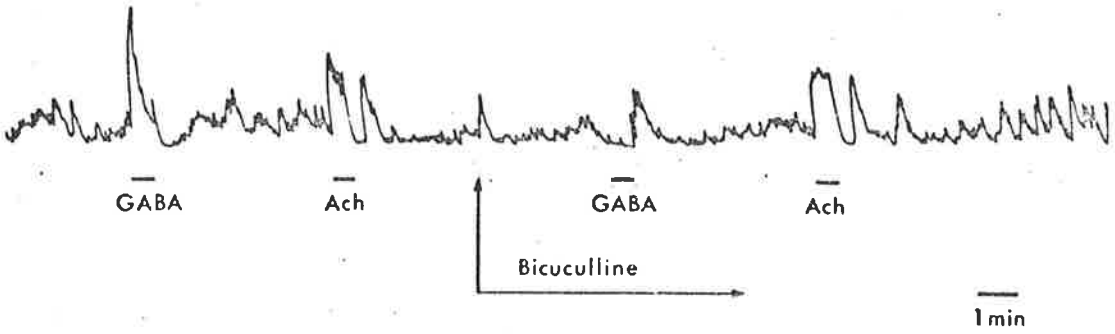


FIG. 5. The effect of bicuculline 10^{-5} g/ml on GABA 3 x 10^{-6} g/ml and acetylcholine (ACh) 3 x 10^{-9} g/ml induced contractions of the guinea-pig ileal longitudinal muscle. The response to GABA was abolished whereas the ACh induced contraction was not significantly affected.

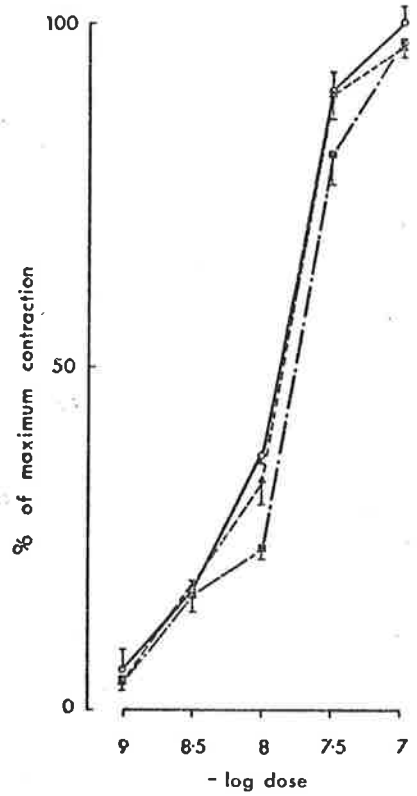


FIG. 6. The effect of bicuculline on guinea-pig ileal responses to acetylcholine (ACh). ● responses to ACh in normal solution; ▲ responses to ACh 20 min after the addition of bicuculline (10^{-6} g/ml); ■ responses to ACh 20 min after the addition of bicuculline (5 x 10^{-6} g/ml). Each point is the mean and standard error of responses obtained in 6-12 preparations.

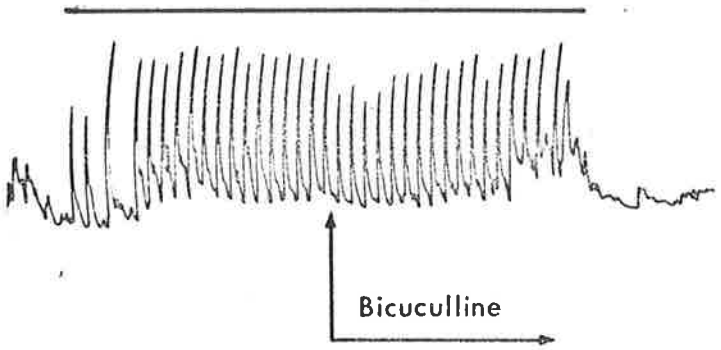


FIG. 7. The action of bicuculline 5 x 10^{-6} g/ml on the responses of longitudinal strip of guinea-pig ileum to field stimulation. Field stimulation 0.1 ms, 30 V, 0.1 Hz. Bicuculline had no significant effect upon the twitch response.

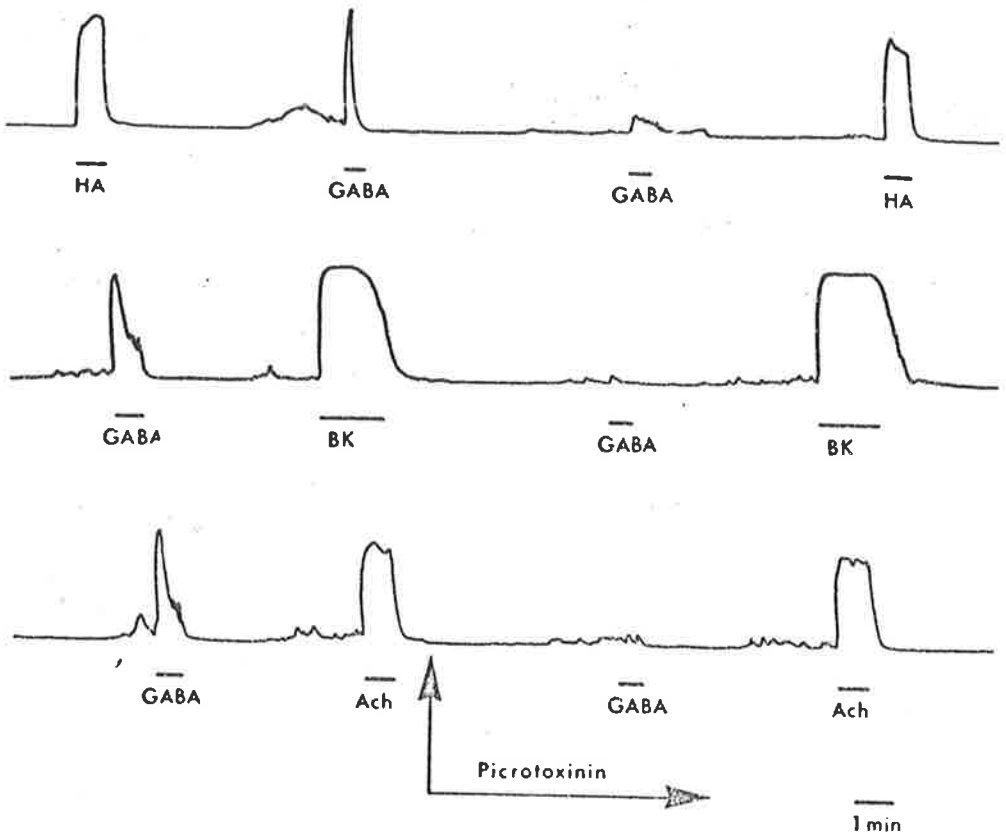


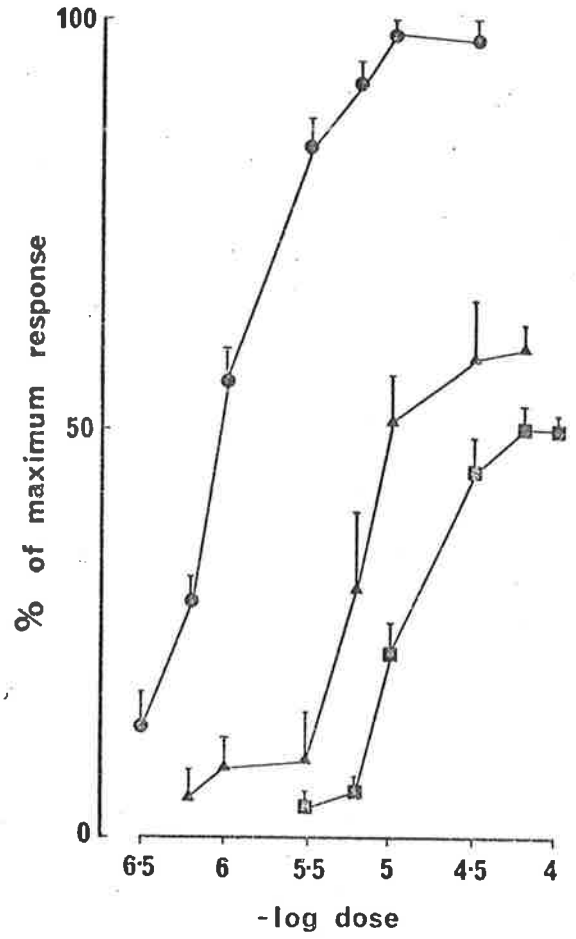
FIG. 8. The effect of picrotoxinin 10^{-5} g/ml on responses of the guinea-pig ileal longitudinal muscle to various substances. Histamine (HA) 10^{-8} g/ml, bradykinin (BK) 5×10^{-8} g/ml and acetylcholine (ACh) 3×10^{-9} g/ml induced contractions which were relatively unaltered by the GABA antagonist picrotoxinin. Contractions induced by equipotent concentrations of GABA, 3×10^{-6} g/ml, were abolished.

FIG. 9.

(a) The effect of picrotoxinin on the concentration dependent responses of the guinea-pig ileum to GABA. (●) Responses in normal solution; (▲) responses 20 min after the addition of picrotoxinin, 2×10^{-6} g/ml to the bathing solution; (■) responses in the presence of picrotoxinin, 4×10^{-6} g/ml. Each point is the mean and standard error of responses obtained in 5-8 preparations.

(b) Clark plot of the picrotoxinin induced antagonism of GABA concentration response curve (●) control responses; (▲) responses in the presence of picrotoxinin, 2×10^{-6} g/ml; (■) 4×10^{-6} g/ml.

a


100
50
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% of maximum response
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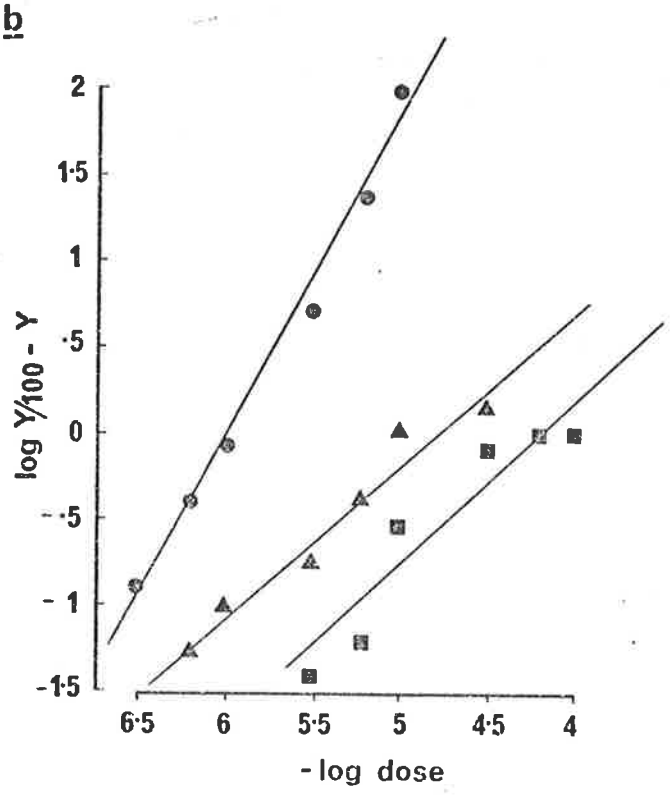
-log dose

b


2
1·5
1
·5
0
-·5
-1
-1·5
log Y/100 - Y
6·5
6
5·5
5
4·5
4
-log dose

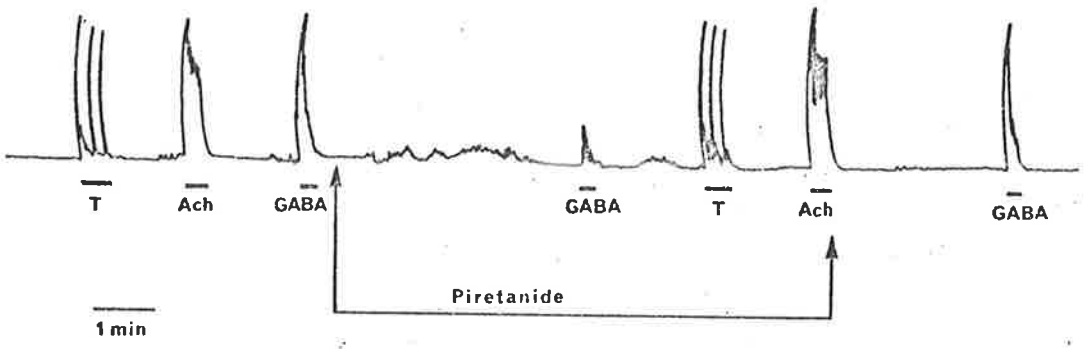


FIG. 10 (a) The effect of piretanide 2 x 10^{-5} g/ml on contractions of the guinea-pig ileal longitudinal muscle to various stimuli. Transmural stimulation (T), 0.1 Hz, 0.1 ms, 30 V and acetylcholine (ACh) 10^{-9} g/ml induced contractions were relatively unaltered. The GABA (3 x 10^{-6} g/ml) elicited contractions were substantially reduced.

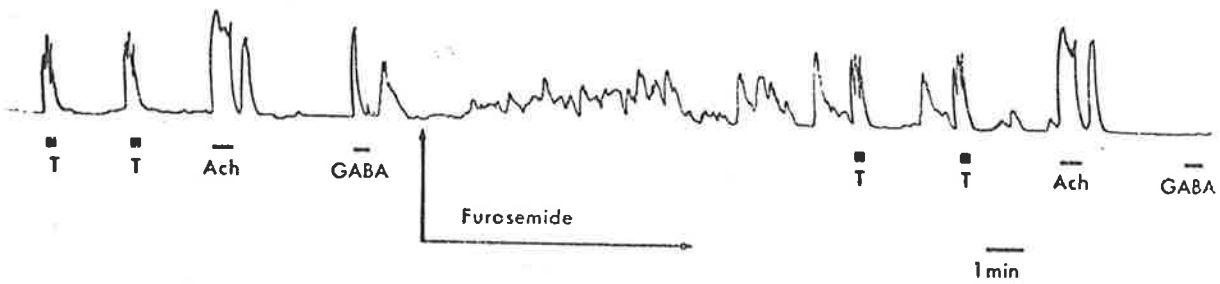


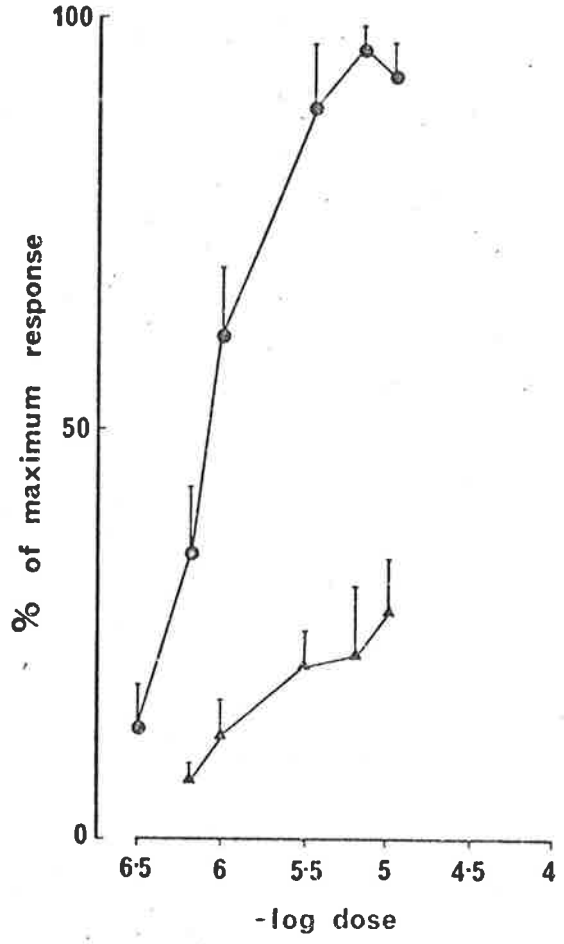
FIG. 10 (b) The effect of furosemide 7 x 10^{-5} g/ml on contractions of the guinea-pig ileal longitudinal muscle to various stimuli. Transmural stimulation (T) 0.2 Hz, 0.2 ms, 60 V and acetylcholine (ACh) 2 x 10^{-9} g/ml induced contractions were unaltered whereas responses to GABA 3 x 10^{-6} g/ml were substantially reduced.

FIG. 11.

(a) The effect of piretanide on the concentration response curve for GABA in the guinea-pig ileum. ● responses of the longitudinal muscle in normal solution; ▲ responses in the presence of piretanide 5×10^{-6} g/ml. Piretanide was present in the bath 20 min before adding GABA. Each point is the mean and standard error of responses obtained from 4 preparations.

(b) Clark plot of the piretanide induced antagonism of the GABA concentration response curve, ● control responses, ▲ responses in the presence of piretanide.

a


100
50
0
% of maximum response
6·5
6
5·5
5
4·5
4
-log dose

b

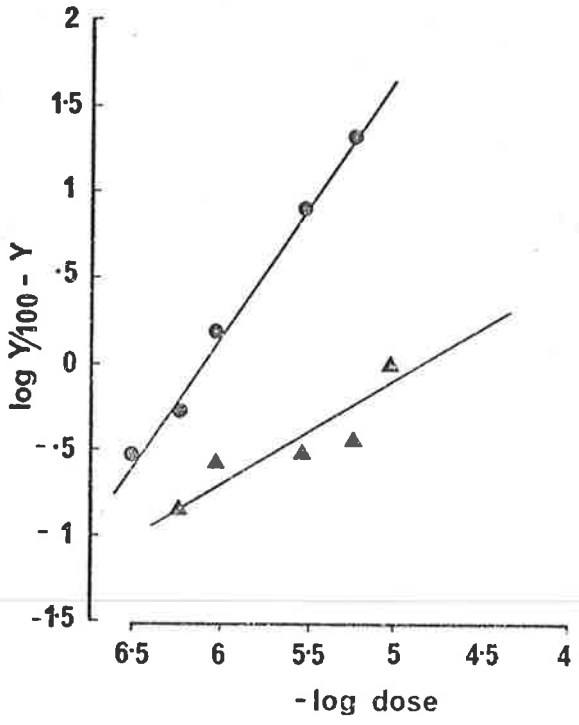
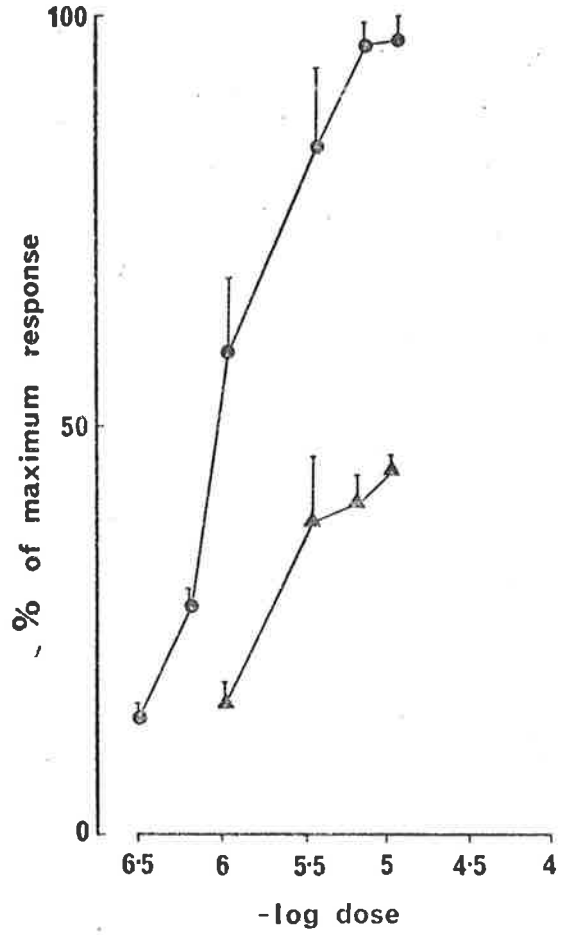

2
1·5
1
·5
0
-·5
-1
-1·5
log Y/100 - Y
6·5
6
5·5
5
4·5
4
-log dose

FIG. 12.

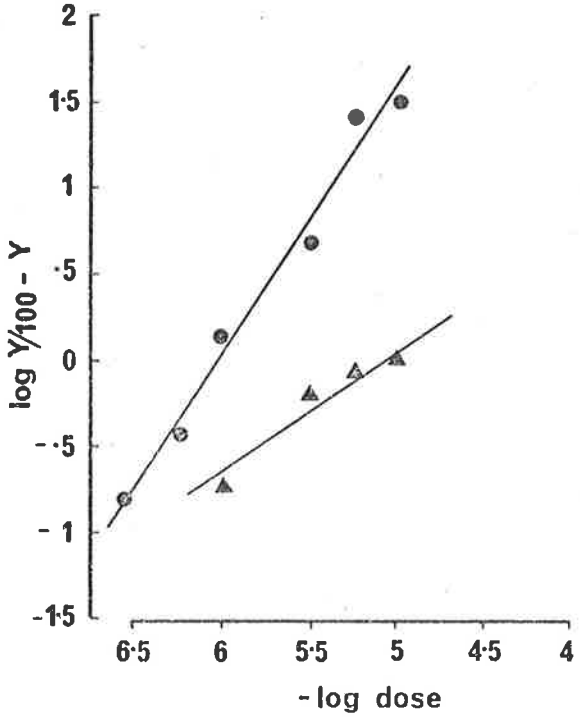
(a) The effect of furosemide on the concentration response curve for GABA in the guinea-pig ileum. ● responses of the longitudinal muscle in normal solution; ▲ responses in the presence of furosemide 2×10^{-5} g/ml. Furosemide was present in the bath 20 min before adding GABA. Each point is the mean and standard error of responses obtained from 4 preparations.

(b) Clark plot of the furosemide induced antagonism of the GABA concentration response curve, ● control responses, ▲ responses in the presence of furosemide.

a


100
50
0
% of maximum response
6·5
6
5·5
5
4·5
4
-log dose

b


2
1·5
1
·5
0
-·5
-1
-1·5
log Y/100 - Y
6·5
6
5·5
5
4·5
4
-log dose

possibility the effects of bicuculline on contractions elicited by acetylcholine (ACh) or by stimulation of intrinsic cholinergic neurones (Fig. 5) were tested, but no significant (d.f. = 6, $p>0.05$) alteration of the dose response curve for ACh in the presence of bicuculline, 10^{-6} or 5×10^{-6} g/ml (Fig. 6) nor alteration of the cholinergic twitch response elicited by transmural stimulation (Fig. 7) was observed.

The specificity of picrotoxinin for the GABA-induced contractile responses of the ileum is shown in Fig. 8. Whereas GABA responses were abolished, contractions elicited by acetylcholine, histamine or bradykinin remained relatively unaffected. Picrotoxinin, 2×10^{-6} and 4×10^{-6} g/ml produced a shift to the right (significant, d.f. = 6, $p<0.05$) of the dose response curve for GABA-induced contractions in the ileum and also had the effect of reducing the maximum response (Fig. 9a). The slope of the Clark plot (Fig. 9b) was altered (significant, d.f. = 6, $p<0.05$), thus the action of picrotoxinin appeared non-competitive, in keeping with the proposal that it acts to block GABA-induced chloride conductance at the ionophore (Johnston, 1978).

An interesting question raised by this study concerns the ionic basis of these excitatory actions of GABA in the guinea-pig ileum that are antagonised by bicuculline and picrotoxinin, since the latter antagonist would suggest that a chloride ionophore might be involved (Curtis and Johnston, 1974). Although excitatory, the responses to GABA were nevertheless chloride-dependent as shown by the use of diuretic chloride-ion channel blockers (Bretag *et al.*, 1980). Furosemide, 7×10^{-5} g/ml (Nicoll, 1978) or piretanide, 2×10^{-5} g/ml (Zeuthen *et al.*, 1978) substantially reduced GABA-induced responses but were without effect on those elicited by ACh or transmural stimulation of the ileum (Figs. 10a, b). The effectiveness of the channel blockers in antagonizing GABA actions can be seen in the dose response curves (Figs. 11 and 12), where,

in both instances, there was a shift to the right (significant, d.f. = 6, $p<0.05$) and a reduction in the maximum response, indicating a non-competitive nature for the antagonism by these Cl^- channel blockers.

Discussion

The present results confirm and extend the findings of Krantis *et al.* (1980) that GABA-induced contractions of the guinea-pig ileum are not the result of direct action in the smooth muscle, rather they result from stimulatory actions on cholinergic motor neurones since they are blocked by muscarinic antagonism and tetrodotoxin. These actions do not involve pre-ganglionic cholinergic nervous transmission, being unopposed by nicotinic antagonism (Krantis *et al.*, 1980) which further suggests that GABA directly stimulates the intrinsic cholinergic motor neurones.

Excitatory actions of GABA in peripheral nervous tissue are well documented. Intracellular and extracellular recordings from the superior cervical ganglion and dorsal root ganglion of the rabbit, cat, rat, bullfrog, and duck embryo show in every instance a GABA-induced depolarization through an increase in membrane permeability to Cl^- (De Groat, 1970, 1972; De Feudis, 1977). This effect is due to activation of 'true' GABA-receptors in that it is imitated by GABA analogues and blocked by picrotoxin and bicuculline (De Groat, 1970; Bowery and Brown, 1974; Obata, 1976). However, this depolarization does not cause sympathetic neurones to discharge. Rather, it is characteristic of presynaptic inhibition where the GABA-induced depolarization of nerve terminals results in a reduced transmitter output. Transmitter release by this GABA-induced terminal depolarization has never been described and is unlikely to be the cause of the

presently described persistent stimulation of ileal cholinergic neurones.

Grafe *et al.* (1979) showed the GABA induced depolarization of myenteric neurones to be through a picrotoxin sensitive mechanism which leads to myenteric excitation with firing of the intrinsic cholinergic motor neurones. Since the action of picrotoxinin seen here was reversed by washing the ileal segment with normal Krebs solution, it seems unlikely that the non-competitive nature of picrotoxinin is caused by its irreversible binding to the GABA-receptor. Alternatively, picrotoxinin could be interfering with the ionic mechanisms underlying the GABA induced depolarization. Indeed there is considerable evidence to suggest that picrotoxin (an equimolar mixture of picrotoxinin and picrotin) (Zukin *et al.*, 1974; Curtis and Johnston, 1974; Snyder and Goodman, 1980), blocks GABA inhibitory synapses in vertebrates and invertebrates non-competitively by actions at the Cl^- ionophore (Krnjevic, 1974; Shank *et al.*, 1974) and not at the GABA receptor although there is some evidence for a direct receptor action (De Feudis, 1977). In the present study furosemide and piretanide selectively antagonised GABA stimulatory actions in the guinea-pig ileum. Since furosemide (Montoreano *et al.*, 1975; Nicoll, 1975, 1978; Brazy and Gunn, 1976) and Piretanide (Zeuthen *et al.*, 1978) block chloride transport, and the Cl^- ionophore (Nicoll, 1978), it is very likely that picrotoxin actions in the intestine are at the Cl^- ionophore that is regulated by the GABA-receptor. Whether both furosemide and piretanide exert their effects by either blocking the ionic channels, or the chloride pump remains to be resolved. It would be interesting to look for a chloride pump in neurones of the guinea-pig ileal myenteric plexus, since excitation of cells by GABA through a chloride mechanism necessitates a high intracellular Cl^- to produce depolarization via an increased outward Cl^- conductance (Obata, 1976; Nicoll, 1978).

Where GABA has been proposed to be a true excitatory transmitter, in the sea urchin tube foot (Florey *et al.*, 1975) and aplysia (Yarowsky and Carpenter, 1977) its actions did not involve any alteration in Cl^- permeability, rather they appeared to be Na^+ or K^+ dependent. Therefore, the finding in this study that stimulation of myenteric neurones by GABA occurred through a Cl^- dependent mechanism is unexpected. Although the ionic mechanism mediating GABA effects in the sea urchin tube foot and guinea-pig ileum is different, GABA actions in these preparations are similar; the results of excitatory actions at cholinergic motor neurones. In addition to this action of GABA on ileal cholinergic excitatory neurones, the GABA-induced excitation of the non-cholinergic, non-adrenergic inhibitory neurones (Krantis *et al.*, 1980) persumably also acts through a Cl^- dependent mechanism, not examined for its details in this study.

Bicuculline has been reported to have a variety of effects other than blocking GABA actions, such as inhibition of acetylcholinesterase (AChE) (Svenneby and Roberts, 1973; Breuker and Johnston, 1975) and potentiation of acetylcholine action (Miller and McLennan, 1974), no doubt by this AChE inhibition, and a direct modification of nerve axon or muscle membrane conductance (Freeman, 1973; Shank *et al.*, 1974). Both picrotoxinin and bicuculline specifically antagonised GABA-induced responses in the ileum, although, in contrast to picrotoxinin, antagonism by bicuculline was of a competitive nature. The potency of this antagonist has been characterised by the derived pA_2. The similarity of action for bicuculline in central nervous tissue (Curtis and Johnston, 1974), guinea-pig large intestine (Krantis *et al.*, 1980) and the guinea-pig small intestine in this study, indicates the usefulness of the guinea-pig *in vitro* intestinal preparation as a model for investigation of GABA-receptors.

In contrast to the present results Bowery *et al.* (1981) and Ong (1981)

describe inhibitory actions of GABA in the guinea-pig intestine, a bicuculline insensitive depression of electrically evoked contractions of the ileum and distal colon. These actions mediated by baclofen-sensitive ($GABA_B$) receptors, are not influenced by furosemide and therefore considered by these authors to be not Cl^- dependent. Recently Kerr and Ong (1981) have shown stimulated release of endogenous GABA from intrinsic ileal myenteric neurones resulting in both $GABA_A$ and $GABA_B$ receptor mediated events. The presence of both excitatory and inhibitory responses to GABA in the intestine is similar to the variety of responses to other putative transmitter substances such as dopamine, noradrenaline and 5-hydroxytryptamine (Snyder and Goodman, 1980). The actions of GABA in the guinea-pig intestine could be an example of a transmitter from a single neurone being excitatory on one target cell and inhibitory on another (Kerkut and Meech, 1966) or an example of inhibitory and excitatory effects of one transmitter on a single cell (Kehoe, 1972).

The simplest model for the role of 'excitatory' GABA in the guinea-pig intestine is for GABA to be a transmitter of afferents, directly relaying on the effector neurones concerned with the peristaltic reflexes, without intervention of interneurones. Also, GABA could be a transmitter of myenteric interneurones which in turn impinge directly upon the respective motor neurones. Alternatively, GABA could be acting through 5HT, or a related amine, as 5HT receptors are also found on these same effector neurones (Costa and Furness, 1979). However, since there is at present controversy as to the role, if any, of 5HT or a related amine in the intrinsic excitation pathways, the final model for GABA actions cannot be decided here. Furthermore, the polysynaptic nature of various reflex pathways within the myenteric plexus is well known and it is quite feasible for two different interneurones, employing different transmitters, to intervene between the sensory and motor elements of the myenteric reflexes.

The findings of this study reinforce the notion that GABA may well have a transmitter function in the mammalian enteric nervous system, where it evidently acts through two specific GABA receptors, the one coupled to a Cl^- dependent excitatory mechanism, the other inhibiting transmitter output.

C H A P T E R I I I

LOCALIZATION OF [^{3}H] GABA HIGH AFFINITY UPTAKE SITES BY AUTORADIOGRAPHY

Introduction

Tracing suspected neurotransmitter substances e.g. monoamines, glycine glutamate and gamma-aminobutyric acid (GABA) has been carried out using various methods, including biochemical (Kuriyama *et al.*, 1966; Obata *et al.*, 1970) and neurophysiological (Obata *et al.*, 1967; Iversen *et al.*, 1971). Autoradiography is now recognized as a powerful tool for mapping GABA-ergic neurones (see Snyder *et al.*, 1970; Neal, 1971; Johnston and Iversen, 1971; Hökfelt and Ljundahl, 1972). This technique is based on the accumulation of radioactively labelled, exogenously applied substances to the nerve cells by specific sodium dependent high affinity uptake mechanisms localized to nerve cell membranes. Tissue relatively rich in synapses for a suspected transmitter, have a highly active high affinity membrane transport system which in contrast to low affinity processes, are of considerable physiological importance as an inactivation mechanism for the transmitter after its action at the receptor (Iversen, 1967; Orrego, 1979; Fagg and Lane, 1979). High affinity uptake mechanisms for GABA have been identified in the mammalian central (De Feudis, 1975; Johnston, 1978; Fagg and Lane, 1979) and peripheral nervous systems (Martin, 1976), where GABA is localized to nervous tissue. The [^{3}H]-GABA transported by this mechanism is accumulated in the same pool as the endogenous GABA, mainly in the synaptosome fraction although uptake of [^{3}H]-GABA also occurs into cell bodies of a number of brain neurones (Schon and Iversen, 1972). This uptake can be distinguished from binding of GABA identifying receptors on many neurones, since binding unlike GABA uptake occurs at 0°C and does not require the presence of sodium ions.

The application of autoradiography for the investigation of GABA in the central nervous system, has involved several methodologies of isotope administration. Homogenates incubated in [^{3}H]-GABA solutions can be used

to obtain information about the localization of [^{3}H]-GABA to nerve terminals (Iversen and Bloom, 1972). This allows the determination of the percentage of GABA accumulating terminals in different areas, however, there is a distinct lack of histological information. Thin slices of brain tissue incubated *in vitro* with [^{3}H]-GABA, have been used to give anatomical information about the localization of uptake sites (Iversen and Bloom, 1972; Hökfelt and Ljungdahl, 1970). However, this *in vitro* technique generally results in poor morphology, due to a partial destruction of normal cell bodies and swelling of perineuronal elements (e.g., glia) (Hökfelt and Ljungdahl, 1972). The specific uptake mechanism of GABA containing neurones may also be altered by damaging neuronal membrane, or both perikarya and/or its processes during the dissection and slicing procedure. Furthermore, penetration of the isotope from the surface is limited and could lead to erronous negative results (Hosli and Hosli, 1972). A third method of [^{3}H]-GABA administration is by *in vivo* injections (Hökfelt and Ljungdahl, 1972). [^{3}H]-GABA does not readily cross the blood brain barrier (see Rapoport, 1976), therefore the isotope must be administered by intraventricular or intracerebral injection. Intraventricular administration must be coupled with pretreatment with aminooxyacetic acid (AOAA), to prevent metabolism of the isotope (Wallach, 1961). Under these conditions GABA containing neurones are labelled (Ljungdahl and Hökfelt, 1973), however, there is limited diffusion of [^{3}H]-GABA from the ventricular system to the nervous perychyme and only the most superficial cells are labelled.

Intracerebral injection results in high isotope concentration in the tissue immediately surrounding the cannula, together with a swelling of the extracellular space. Under these circumstances significant radioactivity, together with good morphology is confined to a narrow zone

peripheral to the site of injection.

Another approach in the identification of GABA-containing neurones is using tissue culture, where the labelled amino acid can be directly added to the culture (Hosli and Hosli, 1979). Although diffusion of the transmitter in culture occurs more rapidly than in slices, thus allowing shorter incubation times, such preparations are not representative of the brain region under investigation since normal cellular investment is not maintained (e.g., transplants are devoid of there glial envelope) (Hosli and Hosli, 1978, 1979). This is particularly relevant when tracing GABA since this amino acid is also accumulated by a high affinity mechanism to perisynaptic glial cells (Hosli and Hosli, 1972), which may represent an important inactivation pathway for GABA (Henn and Hamburger, 1971). Furthermore, these preparations do not allow delineation of the anatomical arrangement of GABA neurones within the CNS.

Autoradiographic evidence, Schon and Kelly (1975) show the [^{3}H]-GABA high affinity transport systems in neurones and glial cells to differ in their substrate specificity, allowing differentiation of the uptake sites. Uptake into neurones is blocked by L-2,4-diaminobutyric acid (L-DABA), and nipecotic acid whilst β-alanine is a potent inhibitor of glial uptake (Iversen and Johnston, 1971; Iversen and Kelly, 1975).

Recently, Jessen *et al.* (1979) have shown high affinity uptake sites for [^{3}H]-GABA in cultured myenteric neurones of the guinea-pig intestine. In addition, glutamic acid decarboxylase (GAD) and endogenous GABA were also demonstrated to be present in the myenteric plexus.

Laminar preparations of this nerve network consisting of the myenteric plexus attached to the longitudinal muscle layer allow a more detailed

examination of nerve fibre ramification within this layer (Gabella and Costa, 1967; Furness, 1970). Such preparations of the intestine have been used for the immunohistochemical (Costa *et al.*, 1980; Schultzberg *et al.*, 1980), fluorescence histochemical (Furness, 1969; Costa and Furness, 1972; Olsen *et al.*, 1976), and autoradiographic (Diab *et al.*, 1976), investigations of the distribution of various substances within the mammalian enteric nervous system. The general organization of the mammalian myenteric plexus is reminiscent of the CNS rather than other autonomic ganglia. However, the wall of the gastrointestinal tract prevents no barrier to the diffusion of substances from the bathing solution to the various intrinsic nerve networks, lending itself to *in vitro* labelling for autoradiography.

Evidence is presented here to show the efficacy of [^{3}H] as an autoradiographic marker in laminar preparations of myenteric plexus using [^{3}H] proline and [^{3}H] leucine, two amino acids known to be actively taken up and transported by neurones. In addition, the disposition of [^{3}H]-GABA accumulating neurones within the guinea-pig enteric nervous system was determined by light microscopic autoradiography using specific inhibitors of [^{3}H]-GABA high affinity uptake mechanisms. The uptake of [^{3}H]-GABA was compared with that for [^{3}H] β-alanine which is substrate specific for glial cell high affinity uptake sites.

Methods

Preparations of myenteric plexus were obtained from freshly killed guinea-pigs of either sex, weighing 250-400 g. Segments of small and large intestine 8-10 cm long, were quickly removed and placed into Krebs solution

(the composition as described in Chapter II) maintained at (37°C) and gassed with a mixture of 95% O_2 and 5% CO_2. Two tissue preparations were used.

1. Longitudinal muscle with attached myenteric plexus dissected free from the segments of intestine and cut into short lengths (laminar preparations) (Fig. 1). Individual tissues were pre-incubated for 10 min in 350 µl Krebs at 37°C.

To block non-neuronal and neuronal uptake of ^{3}H GABA (Schon and Kelly, 1975), β-alanine, L-2,4-diaminobutyric acid or nipectoic acid were added to give a final concentration of 10^{-3} M, and the tissues were incubated for a further 5 min with shaking. Labelling of high affinity uptake sites was carried out using either tritiated GABA (66 Ci/mmole; 2,3-^{3}H GABA) or tritiated β-alanine (32 Ci/mmole; β-[3-^{3}H] alanine) at a final concentration of 5 x 10^{-9} M in the presence of uptake inhibitors where appropriate. In a further series of experiments, tritiated L-proline (40 Ci/mmole; 5-^{3}H proline) or tritiated L-leucine (52 Ci/mmole; 4,5-^{3}H leucine) were added to give a final concentration of 10^{-8} M. Tissues were incubated with shaking for 20 min, then washed in fresh Krebs solution, placed on subbed slides and fixed with 4% glutaraldehyde-phosphate buffer. GABA is a small easily diffusible substance and has to be immobilized during the preparation steps of autoradiography. To avoid dislocation of [^{3}H]-GABA together with good tissue preservation, glutaraldehyde is routinely used as the fixative (Ljungdahl *et al.*, 1975). Glutaraldehyde has the added advantage of binding GABA to the tissue affording high retention of [^{3}H]-GABA during fixation and dehydration steps.

2. Short segments of whole intestine 2-3 cm long were treated for [^{3}H]-GABA autoradiography as described for the laminar preparations,

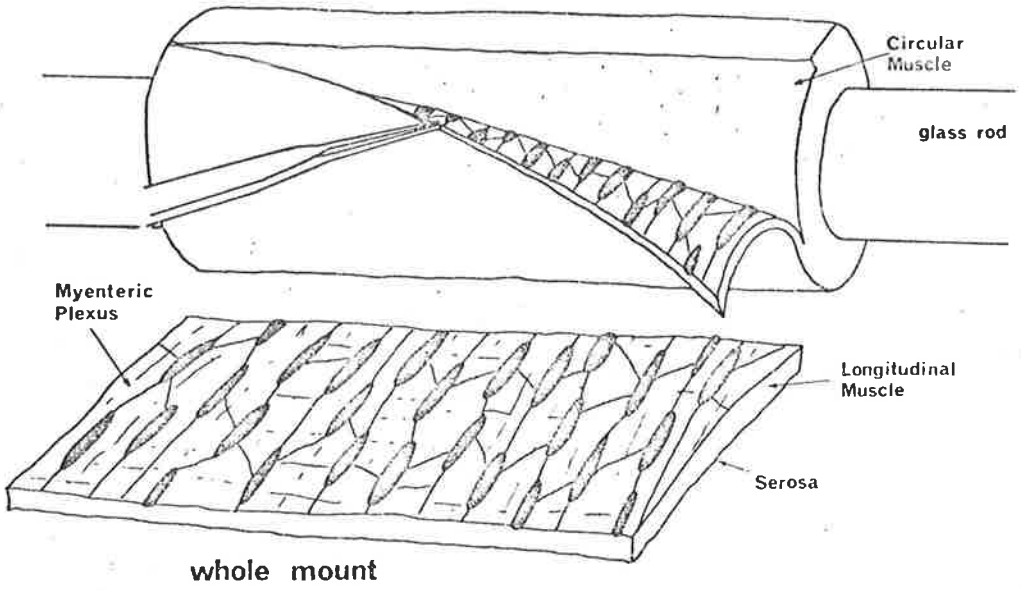


FIG. 1. Dissection of laminar preparations. A glass rod is positioned through the lumen of a segment of intestine and together secured by pins to a board to prevent movement of the tissue. Using a fine scalpel, a superficial cut is made along the length of the tissue segment close to the mesentery attachment. With fine forceps the serosa, longitudinal muscle and myenteric plexus as a whole are then carefully peeled off. Any adhering circular muscle fibres are removed with forceps.

however, before fixation a glass rod was positioned through the lumen in order to maintain the integrity of the 'tubular' nature of the intestinal segment. To determine whether enzymic degradation of GABA could alter the uptake and distribution of ^{3}H GABA, selected preparations were incubated in the presence of amino-oxyacetic acid (AOAA, 10^{-5} M), a specific inhibitor of the GABA metabolizing enzyme GABA-transaminase (GABA-T) (Da Vanzo *et al.*, 1964). Following fixation the 'tube' preparations were removed from the glass rod, washed, dehydrated and embedded in paraffin. Longitudinal sections (20 μm) cut perpendicular to the circular muscle cells, were placed on subbed slides for autoradiographic treatment.

Autoradiography

For light microscopic autoradiography Ilford K2 emulsion was used because of its adequate sensitivity for use with tritium. For coating, the emulsion was melted in a water bath at 41°C and diluted in the ratio of one part emulsion to one part distilled H_2O. The slides from both tissue preparations were dipped individually for 10 sec then placed on a level cold plate to set for 15-30 min. This procedure ensured an emulsion coat of uniform thickness. The slides were then removed to a bench and allowed to set for a further 3 hours whereupon they were packed in light tight boxes containing dessicant, sealed, encased in a light proof plastic bag and stored in a refrigerator at 4°C, for 14 days.

Slides were developed for 4 min in fresh undiluted Kodak D19 developed at 20°C. After rinsing in distilled H_2O the slides were fixed for 8 min in sodium thiosulphate, rinsed in distilled H_2O, then washed in gently running tap water for at least 15 min. Silver grains were almost totally absent from segments of nerve tissue treated for autoradiography without

tritium [^{3}H] present in the incubating medium, suggesting chemographic or physical artefact to be minimal.

Staining

In order to relate the deposition of silver grains associated with [^{3}H] uptake to the underlying cytoarchitecture tissue, preparations were stained with either cresyl violet or neutral red. This was carried out either directly after washing or the tissues were dried and stained at a later time. Further morphological characterization of the nerve cell types accumulating [^{3}H] amino acids, was carried out by comparing autoradiographs with laminar preparations of the myenteric plexus not treated for autoradiography, but stained by the Nitro BT method (see Chapter IV).

Compounds used were: β-alanine, amino-oxyacetic acid (AOAA), cis-3-aminocyclohexanecarboxylic acid (ACHC), L,2-4-diaminobutyric acid (L-DABA) (all Sigma). [^{3}H]-GABA, [^{3}H] β-alanine, [^{3}H] leucine, [^{3}H] proline (all Radiochemical Centre, Amersham).

Results

Uptake of ^{3}H-proline and ^{3}H-leucine into myenteric laminar preparations

Light microscopic autoradiographs showed an extensive uptake of both ^{3}H-proline and ^{3}H-leucine into laminar preparations of the myenteric (Auerbach's) plexus taken from all levels of the small intestine. No significant labelling was seen outside the plexus, on smooth muscle or

blood vessels. The most characteristic feature of the autoradiographs was the presence of numerous clusterings of silver grains, found more particularly over the ganglia and primary plexuses. Many of these were very dense and recognisably over neuronal cell bodies including emergent processes, most of which were dendrites, although some emergent axons were labelled for a short distance (Figs. 2, 3, 4). The extent of the cell labelling became more obvious when these autoradiographs were composed with similar laminar preparations stained by the Nitro-BT method (Fig. 5). In both, a great variety of neurones could be recognised, varying from unipolar to multipolar with distinctive dendritic ramifications, but a classification of these cell types was not attempted.

Less dense clusterings of silver grains were also to be found scattered over the ganglia and the primary and secondary plexuses. Little detail of the underlying labelled structures could be made out, but there was sufficient variation in form to suggest that these, too, were labelled cell bodies. In the tertiary plexus, at the intersections of the plexus, similar accumulations of silver grains were commonly found, some of which were rather intensely labelled such that processes could be recognised and their form was most suggestive of labelled cell bodies (Fig. 6).

In addition to the accumulations of silver grains obviously related to cellular uptake of these tritiated amino acids, there was also a faint, diffuse labelling found over the ganglia and plexuses. This was particularly obvious over the ganglia and primary plexuses, but could also be seen over the secondary and tertiary plexuses. In some areas the silver grains were sufficiently numerous that Auerbech's plexus was clearly recognisable, but the more dense accumulations due to cell labelling were nevertheless always recognisable against the relatively faint background labelling the nature of which is unknown.

Autoradiographs showing uptake of [^{3}H] into guinea-pig ileal myenteric plexus.

FIG. 2. [^{3}H] leucine (10^{-8} M). Labelled cell bodies are located throughout the plexus. Scale bar represents 50 μm.

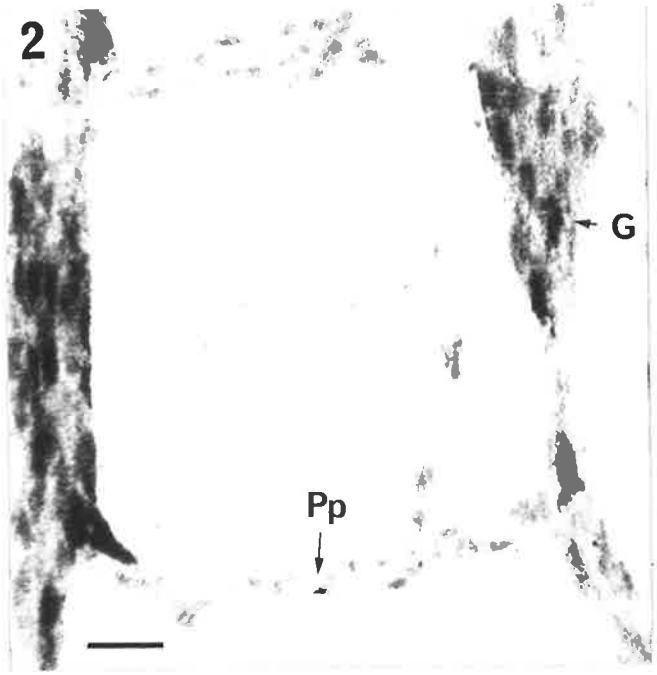
FIGS. 3 and 4. [^{3}H] proline (10^{-8} M). Labelled cells are located throghout the course of the primary (Pp) and secondary (Ps) meshworks. Labelled structures (B) can also be identified at intersections of the tertiary plexus (Pt). Scale bar represents 50 μm.

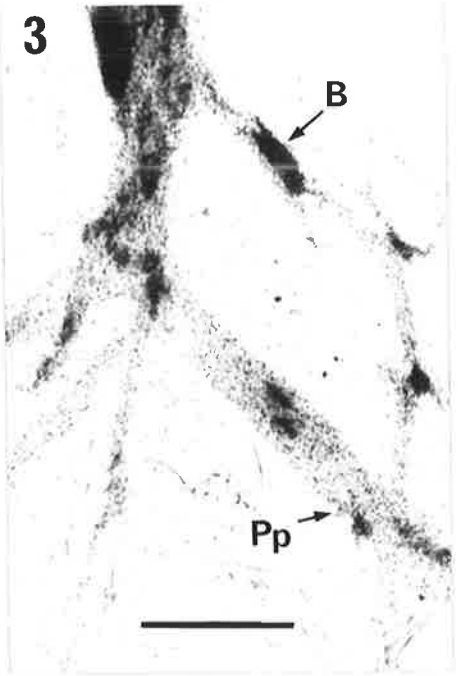
FIG. 5 (a) [^{3}H] leucine (10^{-8} M). Dense accumulations of silver grains can be seen over both the soma and emergent axons of extraganglionic neurones (N) located within fasciculi of the primary plexus (Pp).

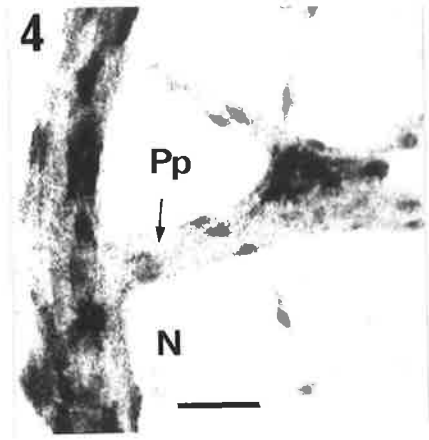
(b) Light micrograph of guinea-pig ileal myenteric plexus stained by the modified NADPH-dehydroganase method. A neurone (N) with long dendrites and single axon characteristic of a Dogiel Type II neurones can be seen located to a ganglion (G).

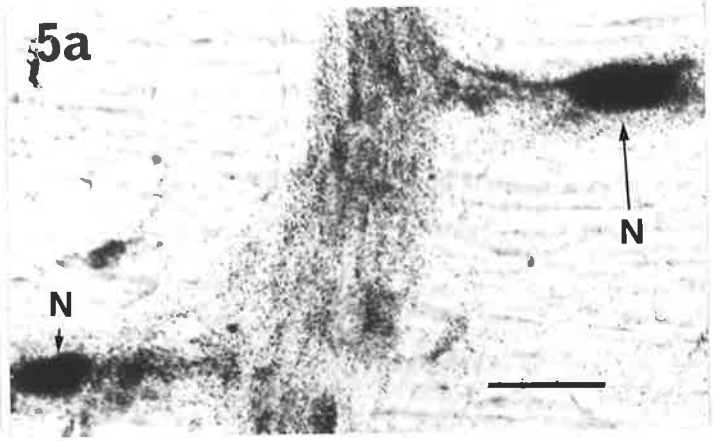
Scale bar represents 50 μm.

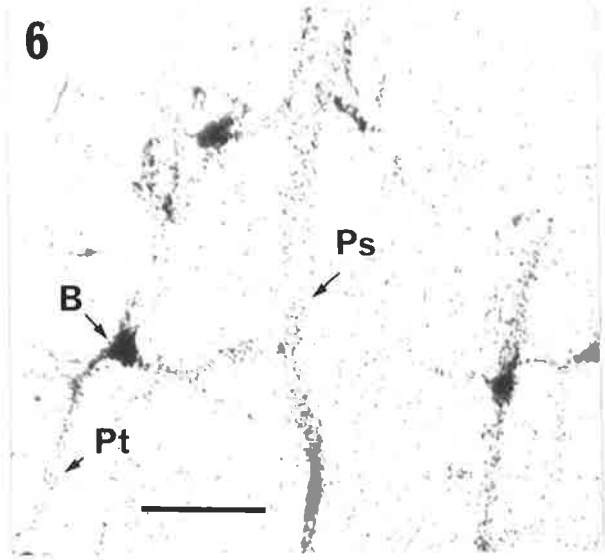
FIG. 6. [^{3}H] leucine (10^{-8} M). The secondary (Ps) and tertiary (Pt) meshworks can be seen with a diffuse cover of silver grains. Dense accumulations of silver grains (B) are present over intersections of the tertiary plexus (Pt). Scale bar represents 50 μm.


2
G
Pp


3
B
Pp


4
Pp
N


5a
N
N


6
Ps
B
Pt

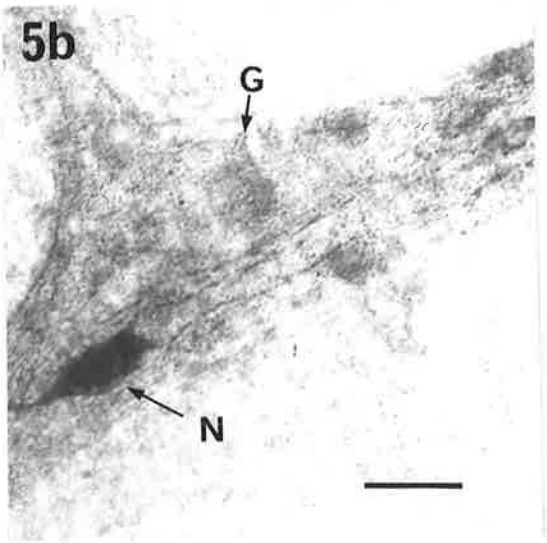

5b
G
N

FIG. 7. Light microscopic autoradiograph of guinea-pig ileal myenteric plexus treated with 5 x 10^{-9} M [^{3}H]-GABA in the presence of 10^{-3} M β-alanine. The three meshworks, primary plexus (Pp), secondary plexus (Ps) and tertiary plexus (Pt) are readily identified. Dense accumulations of silver grains (B) are clearly visible within one ganglia (G) and along the course of the secondary and tertiary meshworks. Scale bar represents 50 μm. Counter stained with neutral red.

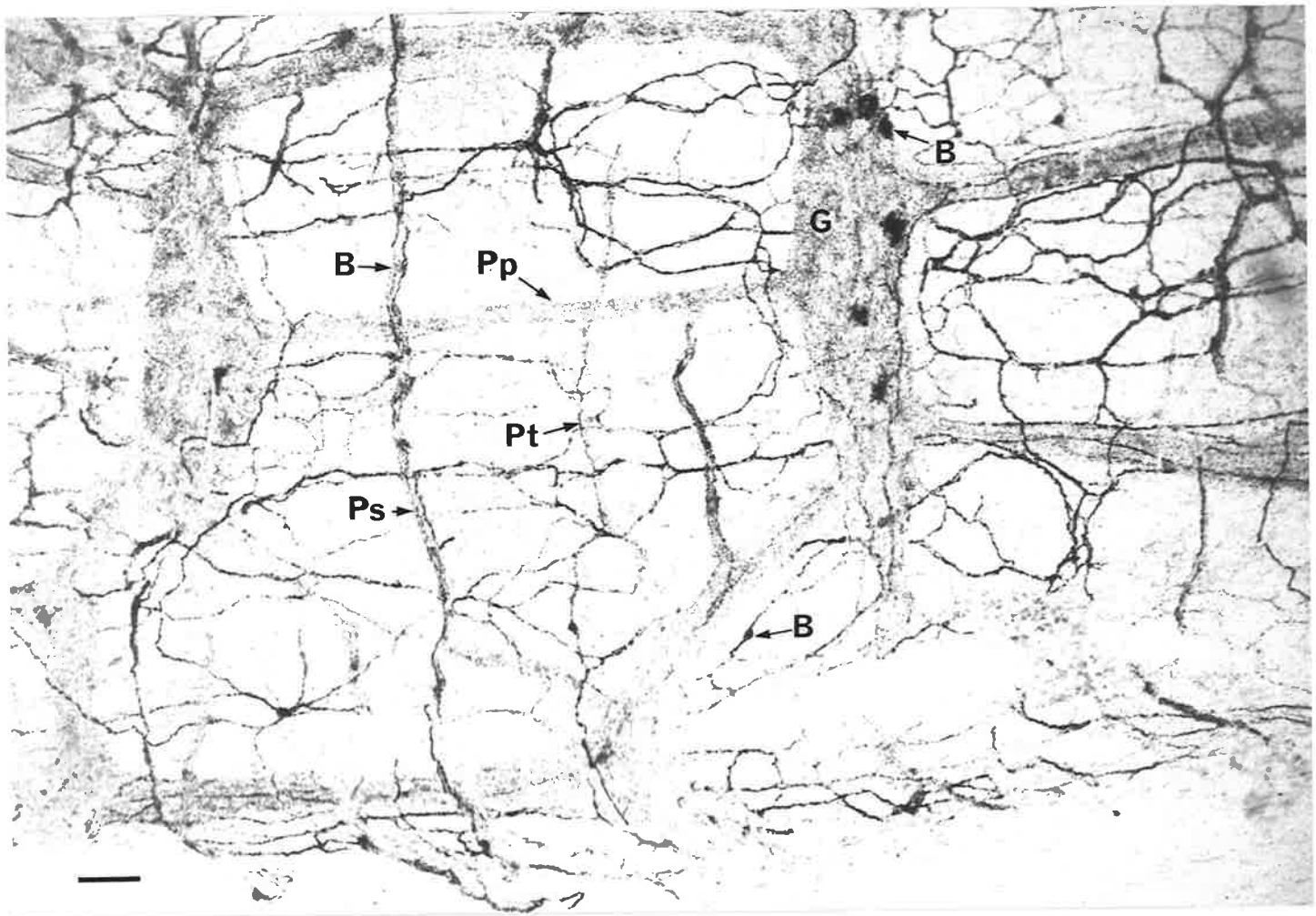
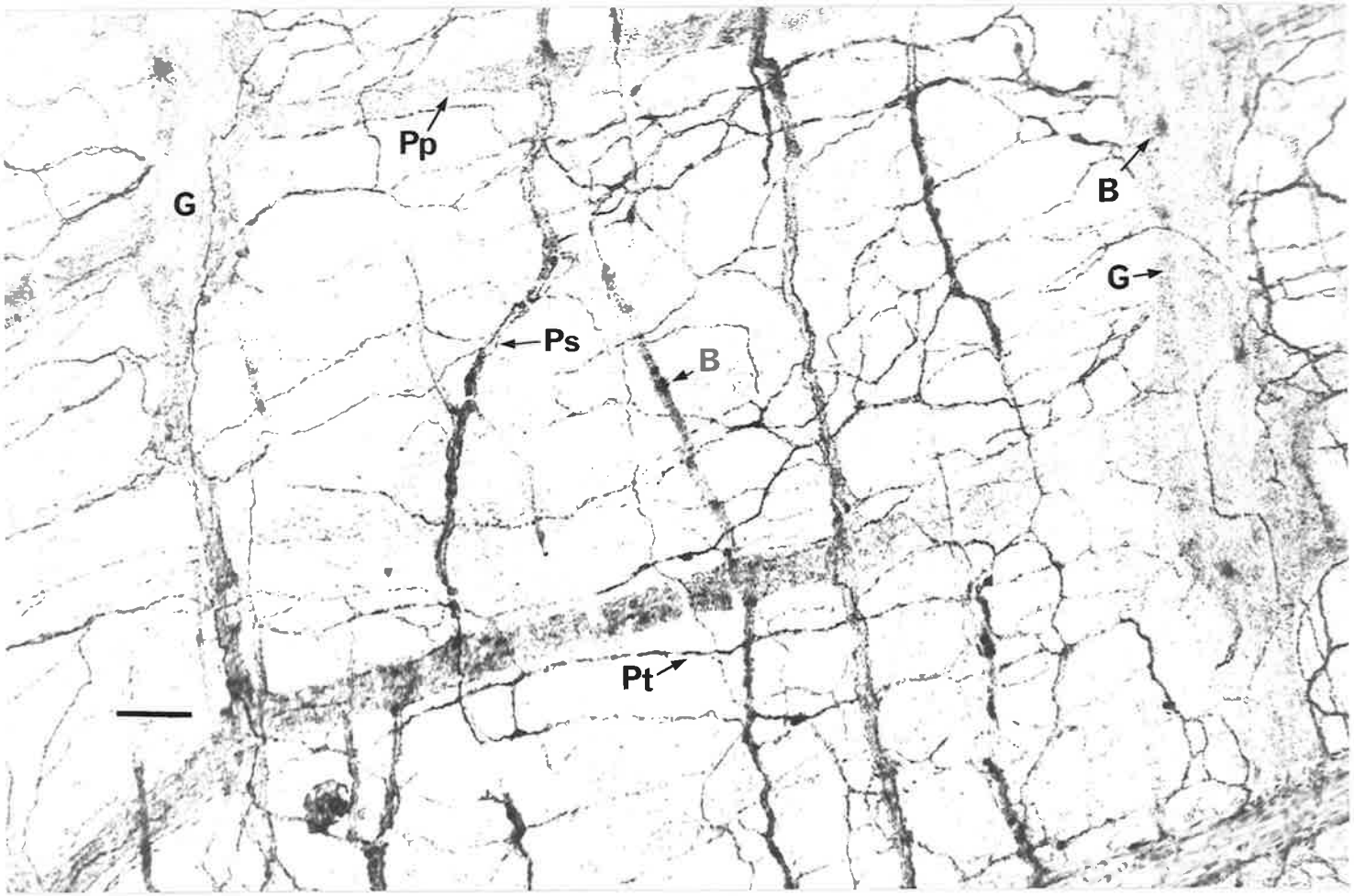

B
G
B
Pp
Pt
Ps
B

FIG. 8. Light microscopic autoradiograph of a myenteric plexus from the guinea-pig jejunum treated with 5×10^{-9} M [^{3}H]-GABA in the presence of 10^{-3} M β-alanine. The distribution of [^{3}H]-GABA uptake sites within the ganglia (G), and meshworks (primary Pp, secondary Ps, tertiary Pt) are readily identified. Counter stained with neutral red. Scale bar represents 50 μm.


Pp
G
B
G
Ps
B
Pt

Myenteric localization of [^{3}H]-GABA and ^{3}H-β-alanine in laminar preparations

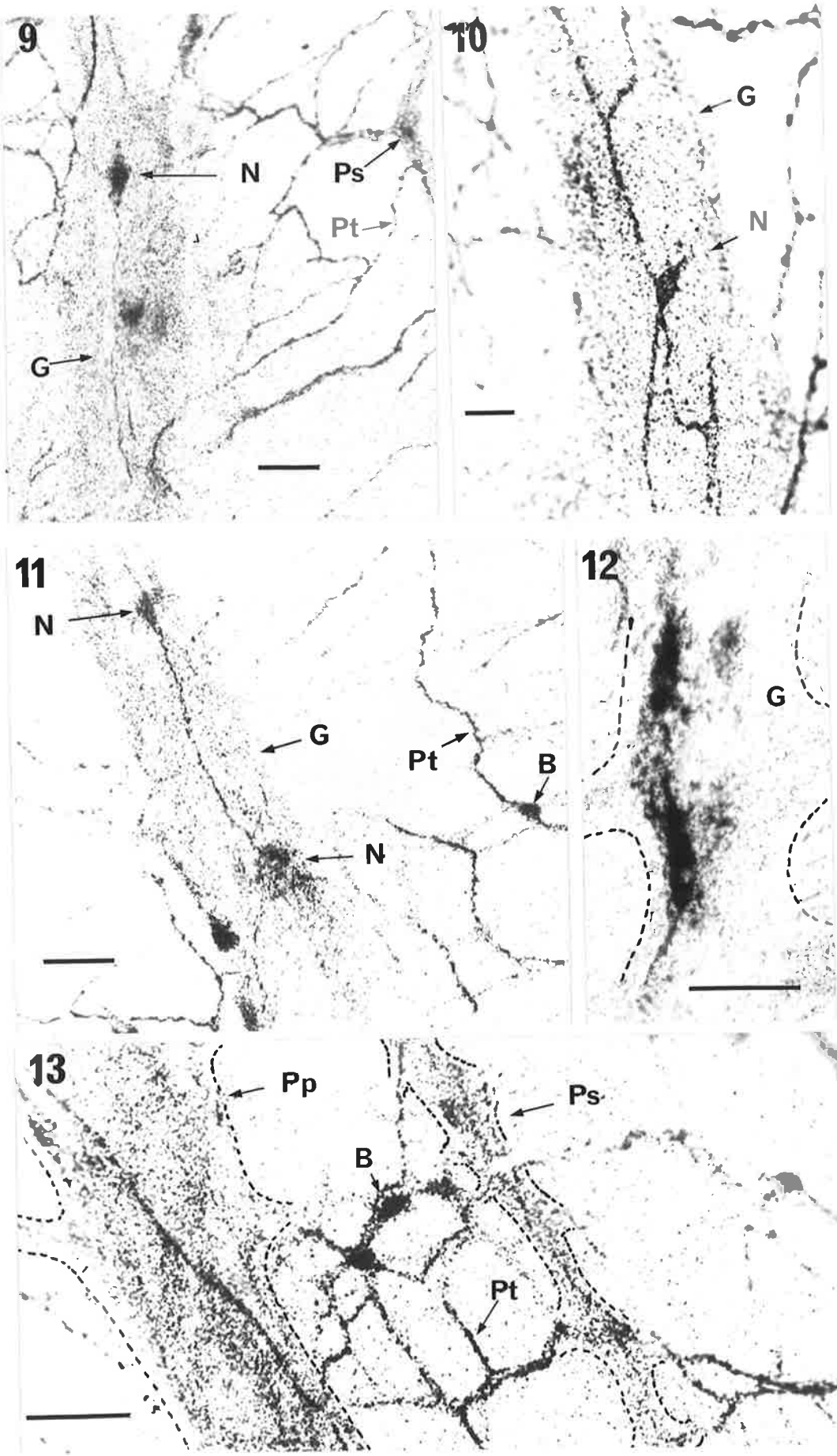
Autogradiographs of laminar preparation of longitudinal muscle with attached myenteric plexus from ileum or jejunum, were similar whether the tissues were incubated with [^{3}H]-GABA 5 x 10^{-9} M alone or in the presence of β-alanine 10^{-3} M (an inhibitor of glial GABA uptake). The characteristic plexiform meshworks of the myenteric plexus were revealed by the silver grains due to the ^{3}H-GABA labelling. In most part, there was a diffuse cover of silver grains over the ganglia and fasciculi of the primary plexus (Pp), giving a lightly shaded appearance, whilst the smaller fibre bundles of the secondary (Ps) and tertiary (Pt) meshworks were, in places, outlined by more dense accumulations of silver grains resembling distinct "tracts" (Figs. 7, 8). Some trails of silver grains were seen over the ganglia and primary meshwork, often as single fibre or fibre bundle. No dense accumulations of silver grains were observed in regions where the longitudinal muscle was devoid of myenteric plexus, neither was there labelling over adherent circular muscle fibres or of blood vessels.

In many preparations very dense accumulations of silver grains were found aggregated in patches over the ganglia, but were localized to only a few cells in each. In addition, similar accumulations were found scattered along the course of the primary, secondary and tertiary meshworks, particularly at junctions of the network. In some instances within the ganglia, these very dense accumulations were recogniseably localised over not only the soma but also dendrites and the emergent axon of individual neurones (Figs. 9, 10, 11). The types of ganglionic neurones labelled with ^{3}H-GABA included unipolar cells with an oval or 'tear-drop' shaped soma, or were multipolar, particularly those with a basal dendritic cluster and offset nucleus (Fig. 12).

FIGS. 9, 10, 11 and 12.

Dense accumulations of silver grains can be seen over both the soma and emergent processes of neurones (N) located within four different ganglia of the guinea-pig ileal myenteric plexus treated for [^{3}H]-GABA (5 x 10^{-9} M) autoradiography. β-alanine 10^{-3} M was present in the incubation medium. Individual preparations with the exception of Figure 12, were counter stained with neutral red. Scale bar represents 50 μm.

FIG. 13. Accumulations of silver grains associated with [^{3}H]-GABA uptake in the guinea-pig ileal myenteric plexus. Heavily labelled tracts and bodies (B) are present in the secondary (Ps) and tertiary (Pt) meshworks. [^{3}H]-GABA final concentration 5 x 10^{-9} M was used. β-alanine 10^{-3} M was present in the incubation medium. Tissue counter stained with neutral red. Scale bar represents 50 μm.


9
N
Ps
Pt
G
10
G
N
11
N
G
Pt
B
N
12
G
13
Pp
Ps
B
Pt

No specific cell types labelled by ^{3}H-GABA could be distinguished within the secondary and tertiary meshworks, although intense labelling of structures (mean length 25 μm, width 15 μm) occurred over intersections of the tertiary meshwork (Fig. 13) these were evidently part of the similar, larger population described above as being labelled with ^{3}H-leucine or ^{3}H-proline (Fig. 6). In autoradiographs of ileal laminar preparations, the distribution of the labelling with [^{3}H] β-alanine lacked any of the features of [^{3}H]-GABA uptake (Fig. 14). Very few silver grains were present, the cresyl violet counter stain predominating. The silver grains were not obviously accumulated over any cell bodies, glial or neural, nor was there any labelling over tracts. Where silver grains were present, they formed a light, diffuse cover over the ganglia and the primary and secondary meshworks but not the tertiary meshworks. This was in sharp contrast to the accumulations of silver grains seen over neurones of the primary meshwork and distinct tracts within all three meshworks in [^{3}H]-GABA treated tissue, indicating that [^{3}H] β-alanine was not taken-up by neuronal elements of the plexus.

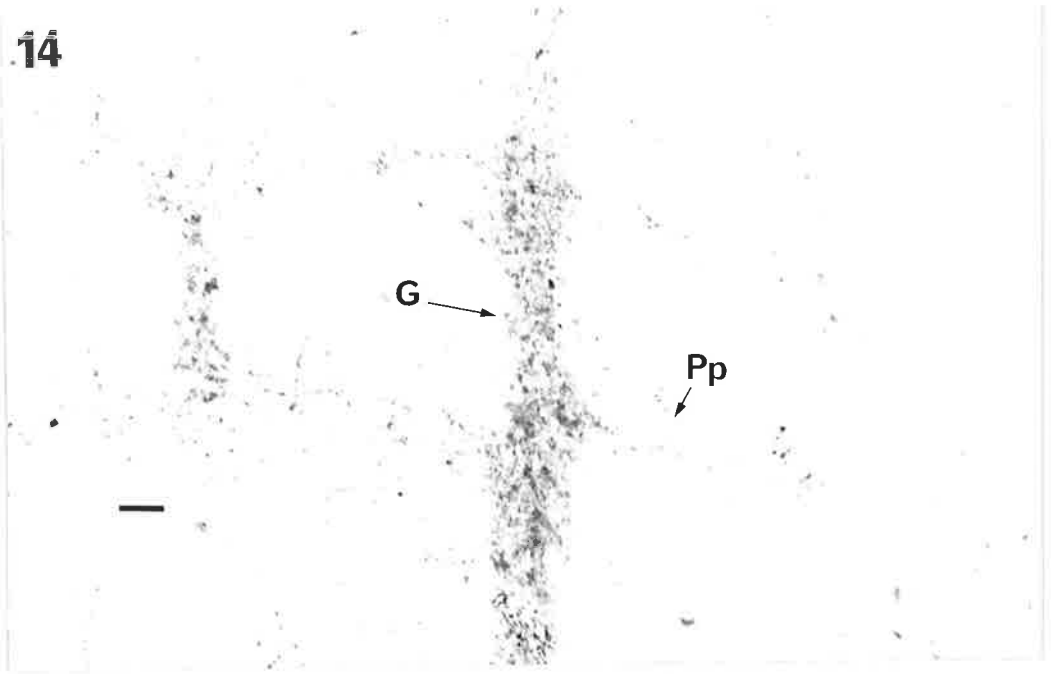
Effects of L-DABA, nipecotic acid and ACHC on [^{3}H]-GABA uptake

In tissues treated with [^{3}H]-GABA in the presence of L-DABA 10^{-3} M, nipecotic acid 10^{-3} M, or ACHC 10^{-3} M, there was almost a complete lack of dense accumulations of silver grains over the plexus, either in glia or the meshwork, indicating that neurones were no longer labelled (Fig. 15), due to inhibition of ^{3}H-GABA uptake. At the concentrations used, nipecotic acid appeared to be more effective than L-DABA or ACHC as an inhibitor of GABA uptake into neurones of the myenteric plexus, but quantitative analysis of the effects of uptake inhibition was not attempted. Such silver grains as were present in preparations treated with uptake inhibitor, formed a

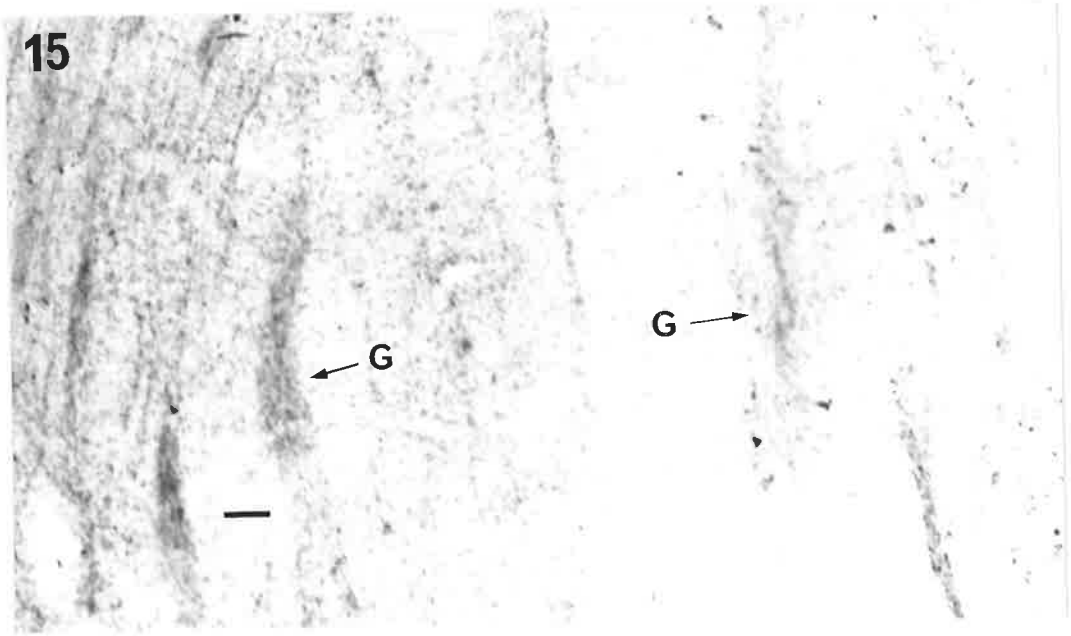
FIG. 14. Light microscopic autoradiograph of guinea-pig ileal myenteric plexus after incubation in [^{3}H] β-alanine (5×10^{-9} M). Silver grains are almost totally absent. Only the ganglia (G) are readily delineated, due in most part to counter staining the tissue with cresyl violet. Scale bar represents 50 μm.

FIG. 15. Autoradiograph of a guinea-pig ileal myenteric plexus treated with [^{3}H]-GABA 5×10^{-9} in the presence of L-DABA 10^{-3} M. The ganglia (G) of the primary meshwork (Pp) are readily identifiable, counter stained with cresyl violet. Only a very diffuse cover of silver grains was evident over these structures. Scale bar represents 50 μm.

14



15

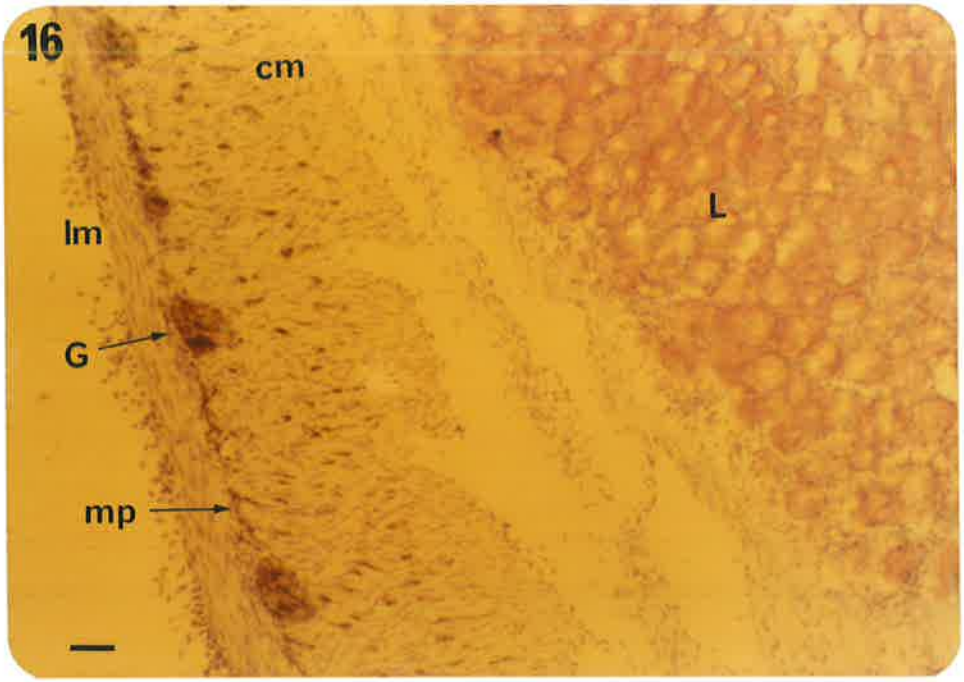


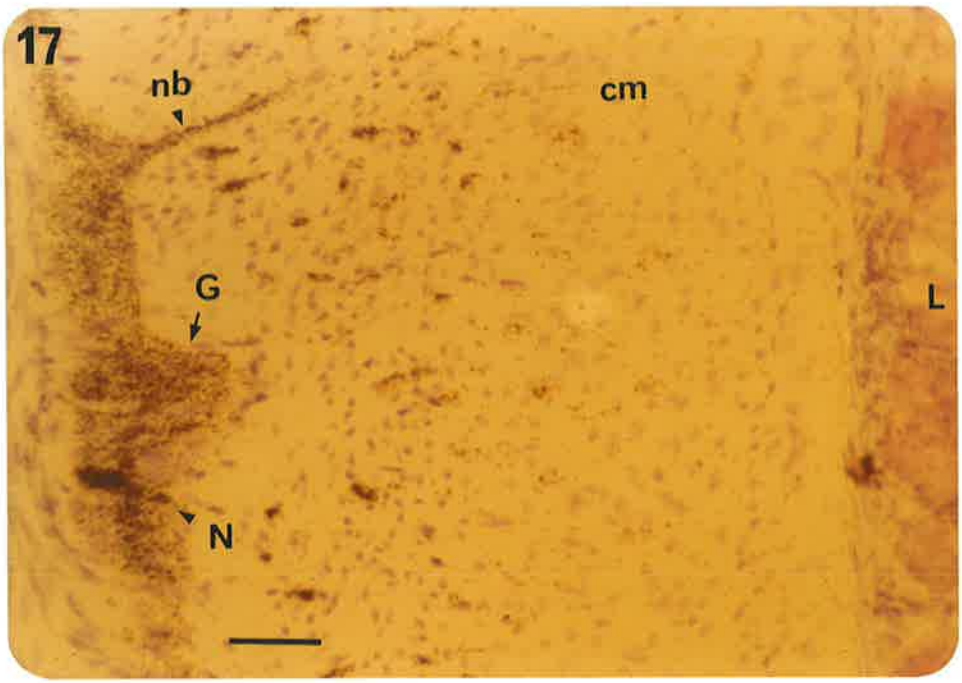
Light microscopic autoradiographs of guinea-pig intestine treated with [^{3}H]-GABA (5 x 10^{-9} M) in the presence of β-alanine 10^{-3} M. Transverse sections (20 μm) were prepared in paraffin and counter stained with cresyl violet.

FIG. 16. Ileum.

FIG. 17. Distal colon.

The layers of the intestine wall, serosa (s), longitudinal muscle (m), myenteric plexus (mp), circular muscle (cm), submucosa (sm), lumen (L) are identified by the cellular stain. Silver grains associated with [^{3}H] uptake are present over the myenteric plexus and circular muscle only. Ganglionic neurones (N) can be identified some of which show [^{3}H]-GABA labelling. Scale bar represents 50 μm.


16
cm
L
lm
G
mp


17
nb
cm
G
L
N

diffuse cover that was more obvious over the ganglia and primary meshworks and could be distinguished from the cresyl violet counterstain.

Incubation of the tissue with [^{3}H] β-alanine in the presence of L-DABA 10^{-3} M, which was observed to markedly reduce the uptake of [^{3}H]-GABA, did not alter the distribution of [^{3}H] β-alanine in the plexus, in keeping with the apparent specificity of L-DABA for neuronal high affinity uptake sites and an exclusive glial uptake for [^{3}H] β-alanine.

Autoradiographic localization of ^{3}H-GABA in sections of colon and ileum

In light microscopic autoradiographs of transverse paraffin sections from the guinea-pig ileum and distal colon, labelled with ^{3}H-GABA, there was an extensive distribution of label within the ganglia, and, in addition, a widespread distribution of ^{3}H-GABA uptake sites in the meshworks of the plexus, and particularly in the circular muscle layer. By contrast, accumulations of silver grains associated with ^{3}H-GABA uptake were almost completely absent from the longitudinal muscle, submucosa, muscularis mucosae or villi as seen in counterstained preparations (Figs. 16, 17).

A diffuse cover of silver grains occurred over the ganglia, together with dense accumulations of silver grains clearly over intraganglionic cells including their dendrites (Figs. 18, 19, 20). In many instances regions devoid of silver grains were outlined and resembled unlabelled cells near those labelled in the same section (Fig. 21).

A conspicuous feature was the accumulation of silver grains over structures, presumably fibre bundles, extending from the ganglia and the interconnecting primary meshwork into the circular muscle layer

Autoradiographs showing uptake of [^{3}H]-GABA (5 x 10^{-9} M) in the presence of β-alanine (10^{-3} M).

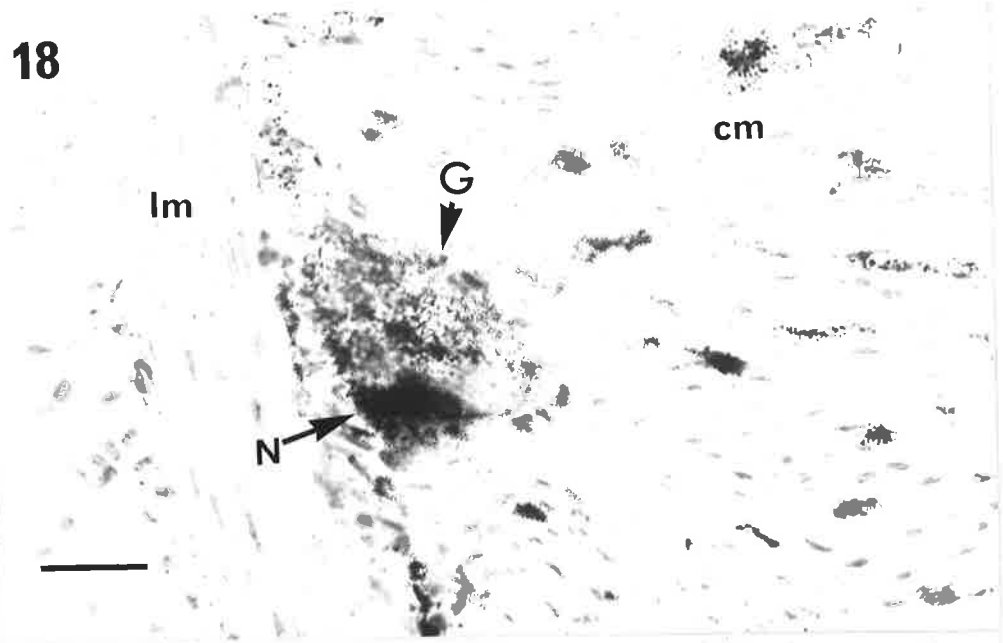
FIG. 18. Transverse paraffin sections prepared from the guinea-pig ileum. Counter stained with cresyl violet.

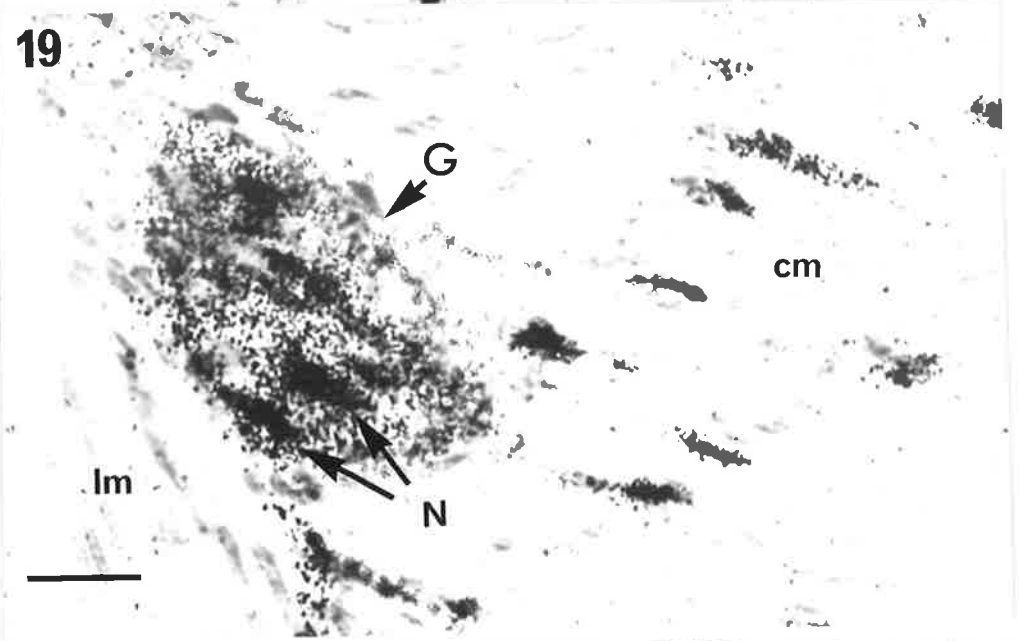
FIG. 19. Transverse paraffin sections prepared from the guinea-pig distal colon. Counter stained with cresyl violet.

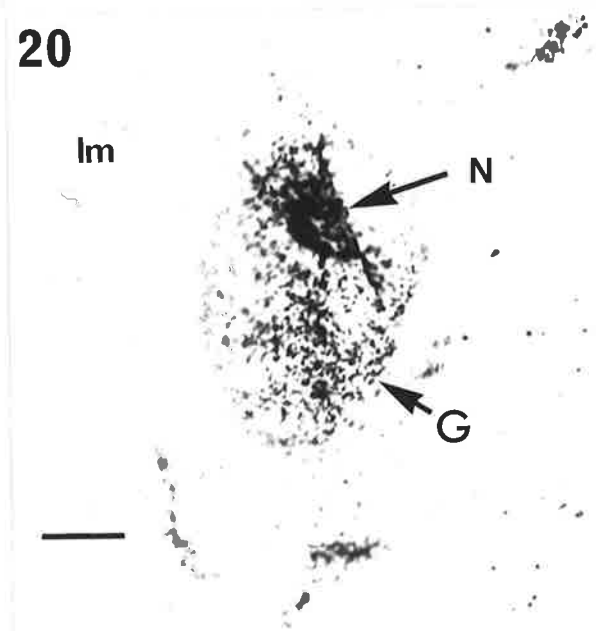
Cresyl violet stained cells characteristic of the different muscle and nerve layers and lumen can be seen. Silver grains are present only over the myenteric plexus and circular muscle. In the ganglia (G) labelled neurones (N) can be identified and on occasion their emergent processes observed. Scale bars represents 50 μm.

FIG. 20. Transverse paraffin sections prepared from the guinea-pig ileum. A neurone (N) intensely labelled with [^{3}H]-GABA is visible in the ganglion (G). Emergent processes can be easily distinguished. No counter stain. Scale bar represents 50 μm.

FIG. 21. Transverse paraffin section prepared from the guinea-pig ileum. Counter stained with cresyl violet. In a ganglion unlabelled cells (U) can be seen lying near neurone (N) intensely labelled with [^{3}H]-GABA. Scale bar represents 50 μm.


18
cm
G
lm
N


19
G
cm
lm
N

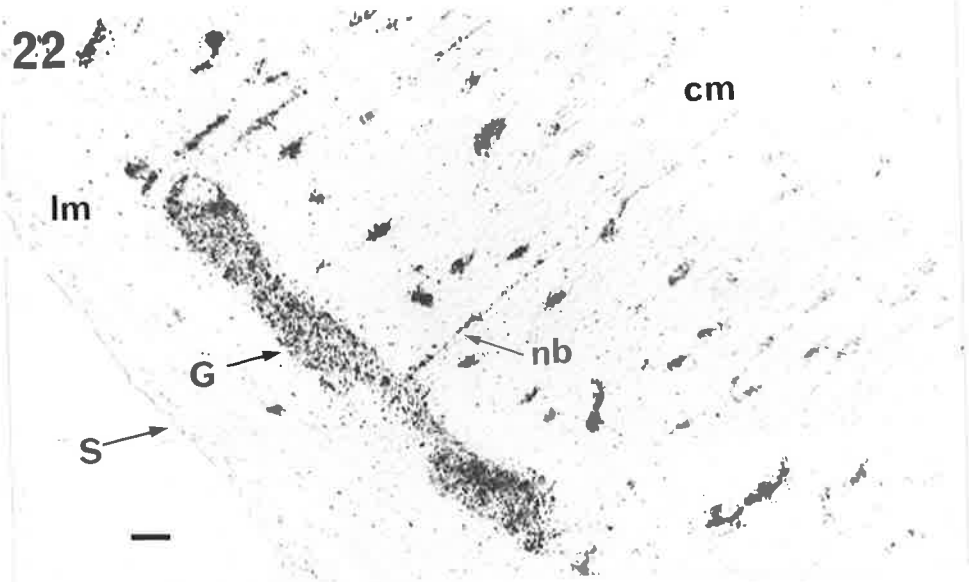

20
lm
N
G

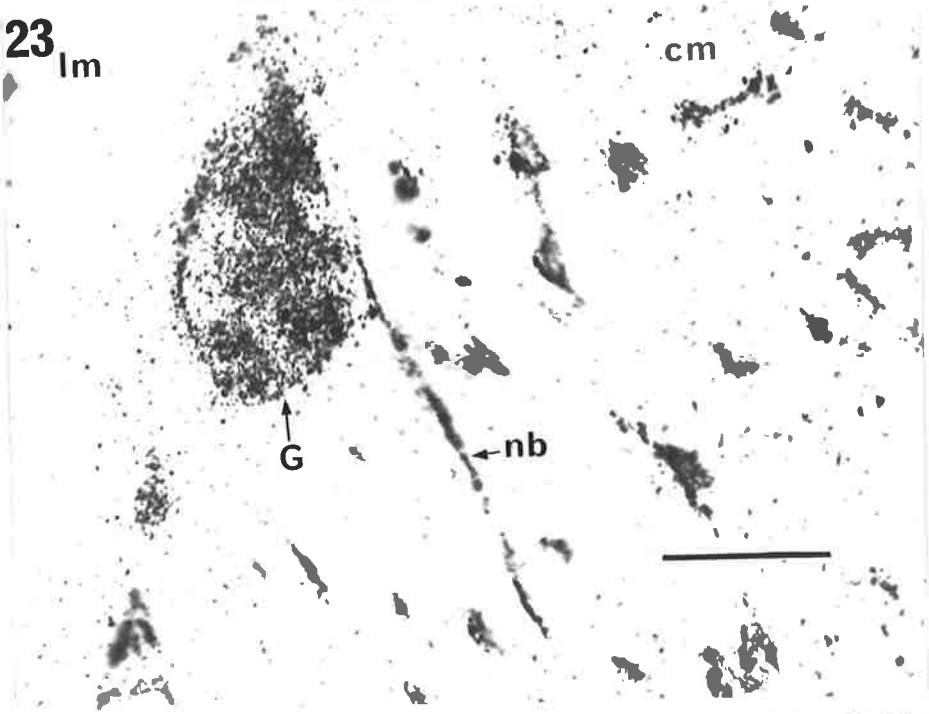

21
G

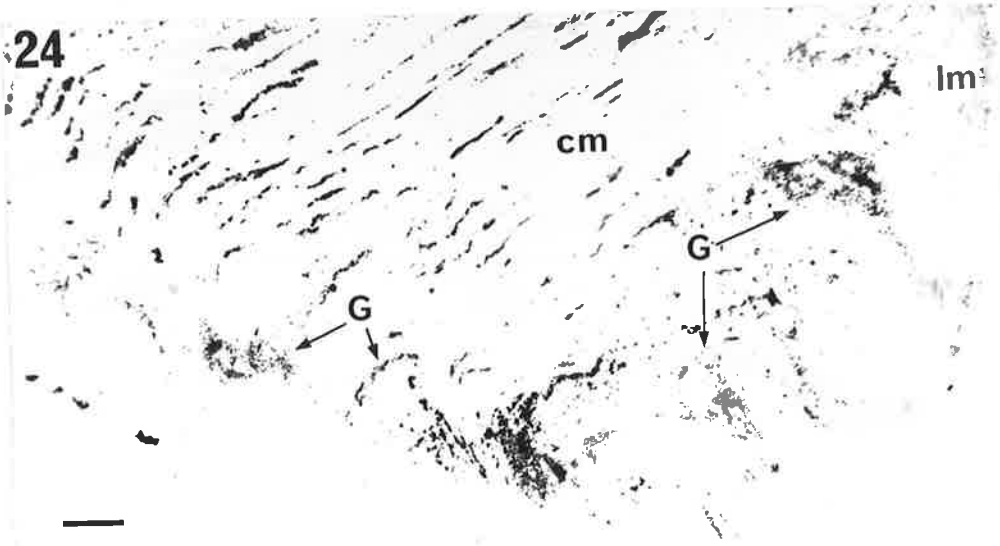
FIGS. 22 and 23.

Light microscopic autoradiographs of transverse paraffin sections (20 μm) prepared from the guinea-pig ileum. Accumulations of silver grains occur over the ganglia (G) and associated nerve bundles (nb). Dense clusters of silver grains also occur over the ciruclar muscle (cm). The serosa (s) and longitudinal muscle (lm) are free of [^{3}H]-GABA labelling. Scale bars respresent 50 μm.

FIG. 24. Transverse paraffin sections of the guinea-pig distal colon treated for [^{3}H]-GABA autoradiography. Sections were cut obliquely across the width of the colon wall. Silver grains occur over distinct tracts running parallel to the circular muscle fibres stretching across the layer between the ganglia (G). The tissue was counter stained with neutral red. Scale bars represent 50 μm.


22
cm
lm
nb
G
S


23
lm
cm
G
nb


24
lm
cm
G
G

(Figs. 22, 23). These trails of silver grains were found only over the outer half of the circular muscle layer, there being no label present in the innermost part of this layer or the submucosal plexus. There were also many punctate aggregations of silver grains scattered irregularly within the same region (Fig. 24). In transverse sections these started abruptly for the most part some 50-100 μm from the myenteric plexus layer, apparently representing transversely cut labelled fibre bundles. This notion was reinforced by the observation of many trails of silver grains running parallel to the circular muscle fibres in sections cut obliquely across the width of the colon wall (Fig. 25).

Discussion

Since β-emissions have little penetrating power it is perhaps surprising that so many [^{3}H]-labelled structures could be detected in the myenteric plexus by autoradiography based on dissected laminar preparations dipped in sensitive emulsion. However, the basal lamina enveloping the ganglia is sufficiently thin and possibly may even be broken in places by the dissection, so that the emulsion can be affected by β-particles arising from tritium labelled structures within ganglia and smaller division of the plexus. Moreover, unlike the situation in sympathetic and spinal ganglia, the non-neuronal elements of the plexus by no means completely ensheath the myenteric neurones, particularly where neuronal processes arise, so that many nerve cell bodies, dendrites and axons are bare or in immediate contact with basal lamina (Schofield, 1968; Gabella, 1971, 1972; Cook and Burnstock, 1976a). Consequently the conditions for autoradiography in dissected myenteric plexus laminar preparations, must be more favourable than might at first seem possible. Therefore it is most likely that

the dense accumulations of silver grains associated with [^{3}H] amino acid uptake in the myenteric plexus represent labelling of superficial neurones and their processes in the ganglia and meshworks where, in many instances, considerable detail of dendrites and other processes could be seen. This is confirmed by the similar labelling of neurones found in autoradiographs from paraffin sections of the intestinal wall, where there can be no question of limitation in the penetrating power of the β-emissions.

In all the autoradiographs following incubation with any of the tritiated amino acids, there was a diffuse cover of silver grains over the plexus, particularly the ganglia and the primary and secondary plexuses, but not the smooth muscle itself. There was little difference in the appearance of the diffuse silver grain cover in preparations labelled with ^{3}H-GABA alone or in the presence of β-alanine, a specific glial cell uptake blocker for GABA (Iversen and Kelly, 1975), which suggests that this labelling was not associated with glial cells, unless the substrate specificities of myenteric glial cells differ from those in the central nervous system. There is some controversy concerning glial elements of the myenteric plexus. Gabella (1969, 1979) describes glial cells, but Cook and Burnstock (1976b) maintain that these should be called instead Schwann cells, reserving the term 'glial cell' for the central nervous system. Be that as it may, it should be pointed out that blood vessels overlay, but do not penetrate the plexus (Gabella, 1979; Jacobs, 1977) consequently the variety of glial types would be restricted since astrocytic contacts on blood vessels would be missing, possibly with obligodendroglia represented by Schwann cells. Many of the cell bodies and processes of neurones in the ganglia are bare of 'glia' and although satellite nuclei outnumber neurones several fold (Gabella, 1979), there was no dense [^{3}H] β-alanine labelling with silver grains outlining glial cell bodies as is seen with [^{3}H] β-alanine radioautographs in sensory ganglia and central

nervous system (Schon and Kelly, 1975; Hosli and Hosli, 1980). Evidently the diffuse cover of silver grains over the plexus seen in this study, represents amino acid uptake by some non-neuronal tissue of the plexus, or may well be in part due to labelling of nerve terminals near, or receptors on, neurones receiving a GABA-ergic input within the ganglia.

Neurones with high affinity [^{3}H]-GABA uptake mechanisms are to be expected in the myenteric plexus if there is, as has been suggested (Krantis *et al.*, 1980; Krantis and Kerr, 1981a) GABA-ergic control of peristaltic activity. In the present study the use of selective inhibitors of high affinity uptake of [^{3}H]-GABA into neural and glial elements showed that the high affinity uptake sites were mainly within neuronal elements of all three meshworks of the plexus.

Reciprocal connexions are made between the submucous plexus and the ganglia of Auerbach's plexus. These connexions penetrate across the width of the circular muscle layer and give rise to a tertiary plexus innervating that layer (Gabella, 1972; Jessen *et al.*, 1980). The stout branches leaving Auerbach's plexus, between the longitudinal and circular muscle layers, were labelled by ^{3}H-GABA, but only over the outer half to two-thirds of the circular muscle layer. Silver trails from labelled tertiary bundles could be traced into the circular layer, and labelling in the tertiary plexus was also very evident in the laminar preparations.

This dense labelling of the tertiary plexus with [^{3}H]-GABA was a most unexpected finding in view of the evidence (Krantis *et al.*, 1980) that GABA acts upon the cholinergic motor neurones, and the non-cholinergic non-adrenergic inhibitory neurones of the plexus, but not on the smooth muscle of the intestine. From what is known of the functional organisation of the plexus it might be expected that GABA-ergic neurones would be confined

to ganglia or the primary plexus rather than in the secondary or, more particularly, the tertiary plexus where very dense ^{3}H-GABA labelling was found. Yet the latter labelling occurred over typical GABA-uptake sites, in that it was prevented by both ACHC, L-DABA and nipecotic acid, all of which are effective inhibitors of neuronal uptake of GABA (Schon and Kelly, 1975; Johnston, 1976). However, GABA receptors have also been described on mammalian peripheral axons (Brown and Marsh, 1975) as have receptors of the 'Baclofen' type (Bowery *et al.*, 1981), and the heavy labelling of the tertiary plexus would be explained if high affinity GABA uptake sites were associated with either of these axonal receptor-types. Furthermore, if, as shown by Bowery *et al.* (1979) and by Ong (1981) from this laboratory, activity of the myenteric plexus is subject to modulation (presumably prejunctional) by the novel $GABA_B$ (Baclofen) type of GABA receptor, then a GABA-ergic innervation responsible for such modulation might be expected to accompany the motor fibres of the tertiary plexus and would possess uptake sites, possibly some of those here seen as heavy ^{3}H-GABA labelling of the tertiary plexus, particularly where found coursing through the circular muscle layer in parallel with the muscle fibres, characteristic of nervous ramifications within the circular muscle. Enkephalins also inhibit electrically evoked contractions of the smooth muscle in the guinea-pig ileum (Van Nueten *et al.*, 1977; Waterfield *et al.*, 1977), by presynaptic actions at nerve terminals of motor nerves releasing acetylcholine (North *et al.*, 1979; Bowery *et al.*, 1981; Ong, 1981. The distribution of enkephalin immunoreactive fibres within the circular muscle as seen in sections of the guinea-pig ileum (Schultzberg *et al.*, 1980) bore a striking resemblance to the autoradiographs in this study although the presence of ^{3}H-GABA labelled fibres to fibre bundles innervating in most part only the outer half of the muscle is unexplained. A further possibility is that the labelled fibres may be in part GABA-ergic afferent fibres. If so, these represent sensory endings or hitherto

undescribed nerve cell soma located to the outer half of the circular muscle layer, where the label was found, and not sensory fibres from the submucous plexus or the plexus musculaire profond of Cajal (1911) (deep muscular plexus, Schofield, 1968) previously suggested by Gabella (1974) to be a source of afferent input to Auerbach's plexus, since ^{3}H-GABA is absent from these layers.

Although neuronal processes, presumably axons, were labelled in ganglia and the meshworks of the plexus by ^{3}H-GABA, surprisingly few fibres labelled with ^{3}H proline or ^{3}H leucine. No doubt the 20 minute incubation time with these particular tritiated amino acids was too short for any significant slow transport to occur. Slow transport of incorporated amino acid proceeds at about 200 μm/hr (Cowan *et al.*, 1972) so that anterograde transport for only some 70 μm would be seen after 20 minutes incubation. Neither of these amino acids effectively label by retrograde transport, nor do they significantly enter fibres of passage (Cowan *et al.*, 1972). Thus, at most, one would expect to see cell bodies and short, labelled processes as found here in the myenteric plexus.

Uptake of ^{3}H proline and ^{3}H leucine into cell bodies is seen in the central nervous system within 5 minutes of application of the label, and within 30 minutes cell bodies and a few dendrites are strongly labelled (Droz and Leblond, 1963). The kinetics of the uptake of these amino acids into neurones of the plexus was not investigated, but a variety of cells were visible after only 20 minutes incubation followed by immediate fixation and subsequent treatment for autoradiography. Most of the labelled cell bodies were in the ganglia, but many dense accumulations were also found not only within the primary and secondary fasciculi of the meshwork, but also at intersections of the tertiary plexus. Although the interstital cells of Cajal are now thought to be non-neuronal (Gabella, 1972) the

autoradiographs of laminar preparations contained many examples of dense accumulations of silver grains at intersections of the tertiary plexus. The resulting pictures bear a striking resemblance to the plexus and interstitial cells illustrated in Cajal figures 572, 573. More of these structures were labelled with [^{3}H] proline and ^{3}H leucine than with ^{3}H-GABA, but a significant number did label with the latter; their disposition and triangular shapes, illustrated in our figures 7, 8, 13, may be compared with the interstitial cells in the Cajal figures. No labelling of these structures was seen with ^{3}H-GABA if neuronal uptake blockers of GABA were present, which strongly suggests that they represent labelled neurones.

Cell bodies have already been noticed in the primary and secondary plexuses using the Nitro BT stain (Gabella, 1969, 1979). Of particular interest were the intensely labelled structures within the primary fasciculi of ileal segments incubated with the tritiated amino acids. Elongate extra-ganglionic neurones have been reported in the primary fasciculi (Takeo and Sugai, 1974), however, cells that were morphologically similar to those hitherto described only in ganglia were also observed within the primary fasciculi (compare Figs. 4 and 5 with Fig. 29 of Schofield, 1968). It is concluded that all three divisions of the meshwork within the plexus contain cells that are very likely neurones since they no longer labelled with ^{3}H-GABA after treatment with inhibitors of neuronal high-affinity GABA uptake.

There is a lack of agreement concerning the classification of the variety of cell types found in the ganglia of Auerbach's plexus. Cajal (1911) whilst describing various different cells nevertheless recognised only two fundamental types, those with short and those with long processes. Gunn (1968) reverted to Dogiel's classification with three subdivisions,

whilst, more recently, Schofield (1968) has recognised only two types: Type I, multipolar with short dendritic arborizations, and Type II, generally multipolar with elongate processes (but often lacking dendrites). Examples of all these could be seen in laminar preparations stained by the Nitro-BT method and in the autoradiographs using [^{3}H] proline and [^{3}H] leucine, e.g., compare Fig. 4 and Schofield (1968) Fig. 29. By contrast for the main part only two morphological varients were found to label ^{3}H-GABA. One of these was unipolar with the axon clearly labelled, as in Figures 9, 10 and 11 (compare Cajal Figure 569), the other, more common, had an offset nucleus in an elongate cell body with a brushlike basal dendritic arborization, as in Figure 12, c.f. Cajal (1911) (Figure 569). Evidently ^{3}H-GABA labelled only a fraction of the total cell population capable of being shown by autoradiography was with ^{3}H luecine or ^{3}H proline. The significance of these ^{3}H-GABA labelled cell types is not clear, however, there is great difficulty in correlating the structure and function of neurons on the basis of morphological considerations along, as can be seen in the claims of motor or association functions for Dogiel Type I cells, and sensory (Schofield, 1968), or cholinergic motor, for Type II cells (Hill, 1927; Ambache and Freeman, 1968).

Since more than one type of nerve cell possessed a GABA-uptake mechanism, it is possible that GABA subserves serveral functions in the myenteric plexus. In particular, GABA-ergic neurones could be involved in nerve pathways mediating reflex peristalsis since GABA has pharmacological actions at the inhibitory and excitatory motor nerves underlying this activity. Such a possibility is supported by the recent demonstration that GABA antagonism, markedly alters peristalsis in the guinea-pig colon as measured by faecal pellet movements (Ong, 1981). Together with biochemical and autoradiographic evidence (Jessen *et al.*, 1979; Hosoya *et al.*, 1981) for the localization of GABA and its synthesising enzyme (GAD) to mammalian myenteric

neurones, this evidence suggests a neurotransmitter role for GABA in the myenteric plexus.

CHAPTER IV

MORPHOLOGY OF MYENTERIC NEURONES

Introduction

Enteric neurones display varying susceptibility to a number of staining reactions including cholinesterase, silver impregnation, methylene blue and Nissl stains, all of which have been utilized in the histochemical and histological investigations of the morphology of the mammalian enteric nervous system (see Schofield, 1968). The Nissl methods do not stain cell processes (particularly axons), and although silver and methylene blue methods outline cell bodies and their processes, they are relatively complex and capricious in their use. Recently, the histochemical demonstration of a major group of oxidation-reduction enzymes, the pyridine-linked dehydrogenases which require either nicotinamide adenine nucleotide (NAD) (DPN) or its phosphorylated derivative NADPH (TPN) as co-enzymes, has been used for the investigation of the morphology of a number of animal tissues (see Pearse, 1960).

The technique (Pearse, 1960) is based on the direct transfer of electrons and H^+ from the co-enzyme substrate to the dehydrogenase for transfer to the electron acceptor (tetrazolium salt) reducing it to an intensely coloured insoluble formazan deposit, thus staining the cell blue.

More recently this technique has been applied to the morpholigical investigation of the myenteric plexus of rat caecum (Dupont *et al.*, 1965), rat intestine (Gabella, 1967), as well as guinea-pig and human rectum (Gabella, 1969). In these studies, the reduced form of the co-enzyme (NADH) and a higher potential tetrazolium salt (nitro BT, Pearse, 1960) were used, resulting in a more intensely blue deposit of diformazan. The authors describe the intra mitochondrial deposition of diformazan as only an indicator of the localization of the appropriate diaphorase (Pearse, 1960), however the term 'diaphorase' may be artifactual since this enzyme has been

identified to be a 'lipyl dehydrogenase' (White *et al.*, 1968).

The ability of this technique to distinguish nerve cells from non-neuronal elements is due to the very intense pyridine-linked enzyme activity within nerve cells, which results in their staining in a shorter time than is required for non-neuronal cells.

In the present study, a rapid, convenient modification of Gabella's technique has been used, with nicotinamide adenine dinucleotide phosphate (reduced form) NADPH as the co-enzyme substrate. This allows better observation of not only the soma but also of emergent processes within stretch mounts of myenteric plexus of the guinea-pig ileum.

Method

Segments of freshly excised guinea-pig proximal ileum 10 cm long were placed into Krebs solution (36°C), gassed with a mixture of 95% O_2 and 5% CO_2. While still bathed in Krebs solution, a glass rod 6 mm in diameter was positioned through the lumen of individual ileal segments and the longitudinal muscle layer with attached myenteric plexus was carefully stripped away in one continuous sheet. Portions of this 'whole-mount preparation' (stretch preparation, Gabella, 1979) were treated by a modification of the NADH-diaphorase (dehydrogenase) histochemical method of Gabella (1969).

The whole mount preparation of longitudinal muscle strip with interposed myenteric plexus was stretched out onto a slide with the plexus uppermost. The slide was then placed onto dry ice and allowed to freeze.

over. This was usually complete within 1 min. The length of the freezing period appeared to be critical, with best results always obtained from preparations allowed only the minimum time to freeze over. The frozen tissue was then carefully detached from the slide and placed into a vial containing the reaction mixture, and protected from the light by wrapping in aluminium foil. The reaction mixture was prepared by mixing equal volumes of Nitro BT 91 mg/ml) and Nicotinamide adenine dinucleotide phosphate (reduced form) NADPH (1.4 mg/ml) dissolved in 0.9% normal saline. These solutions were freshly prepared in separate light proof containers, and mixed together only immediately prior to the addition of the frozen tissue.

Tissues were allowed to incubate in the staining solution with gentle agitation at room temperature, for periods of between 10-20 min. The degree of staining could be determined by visual observation. When the tissue was judged sufficiently dark in colour, it was removed, carefully washed, placed on a slide with the myenteric plexus uppermost, and fixed with 4% glutaraldehyde for 5 min. Following fixation the tissue was washed, dehydrated and mounted for light microscopic observation.

A variation in the time of fixation and/or concentration of fixative did not appear to affect the localization of reaction product. However there was an obvious alteration in the intensity and clarity of the staining reaction. Material fixed for a short time with low concentration of fixative seemed to show improved definition and intensity of staining over that fixed for a longer time. This 'maturation' period lasted approximately 10 days after which there was no further noticeable alteration in the quality of the staining.

Results

The interconnected ganglia of the primary plexus (Pp) were clearly visible in these whole-mount preparations of the myenteric plexus stained with the modified NADPH - dehydrogenase histochemical technique (Fig. 1). Many nerve cells could be seen within the ganglia, and the soma of these cells showed varying degrees of staining from very intense to lightly stained. Large opaque nuclei were often visible. In those neurones intensely stained by this histochemical technique, clear definition of their soma and processes (which could on occasion be traced some distance from their origin) was usually obtained, although it was not often possible to distinguish between axon and dendrites, a limitation common to many histological procedures. But in many instances it was possible to identify several morphologically different types of neurones described in a number of studies as Type I, II and III neurones of Dogiel (1895, 1899) and variations of these types (Gunn, 1959, 1968; Gabella, 1979); Fig. 2. No obvious pattern of localization of these cells was apparent.

Dogiel described Type I cells as having numerous stout processes sometimes arranged in a cogwheel appearance. These cells usually possess a single slender axon which leaves the soma and passes from the ganglion into one of the fasciculi. An example of a Type I cell stained with this Nitro-BT method can be seen in Fig. 3. This neurone bore a strong resemblance to a Type I cell stained by a modified Bielschowsky method in Schofield (1968).

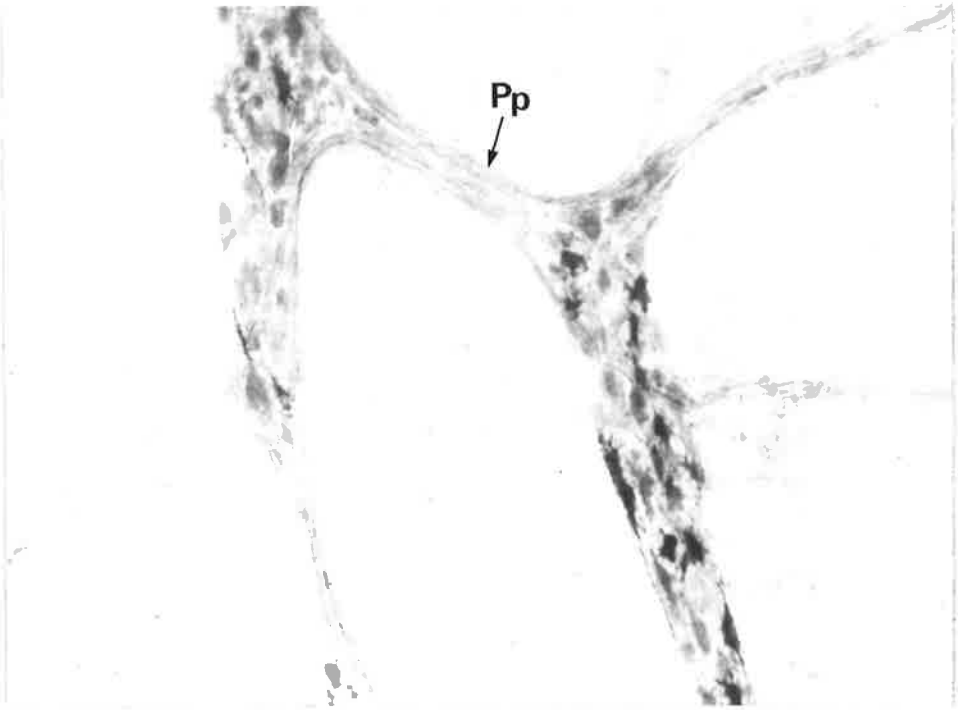
Multipolar cells with fewer processes than the Type I cells, but with characteristic fine processes of Type II cells were often observed (Fig. 4).

Within many ganglia various cell types could be observed in close

EXPLANATION OF FIGURES

All figures are light micrographs of the guinea-pig ileal myenteric plexus stained by the modified NADPH-dehydrogenase method. Scale bars represent 50 μm.

FIGURE 1. The ganglia containing many nerve cells, and the interconnecting fasiculi of the primary plexus (Pp) are clearly visible. Nerve cells display variable staining intensities. Nerve fibres can be seen coursing through the fasiculi.


Pp

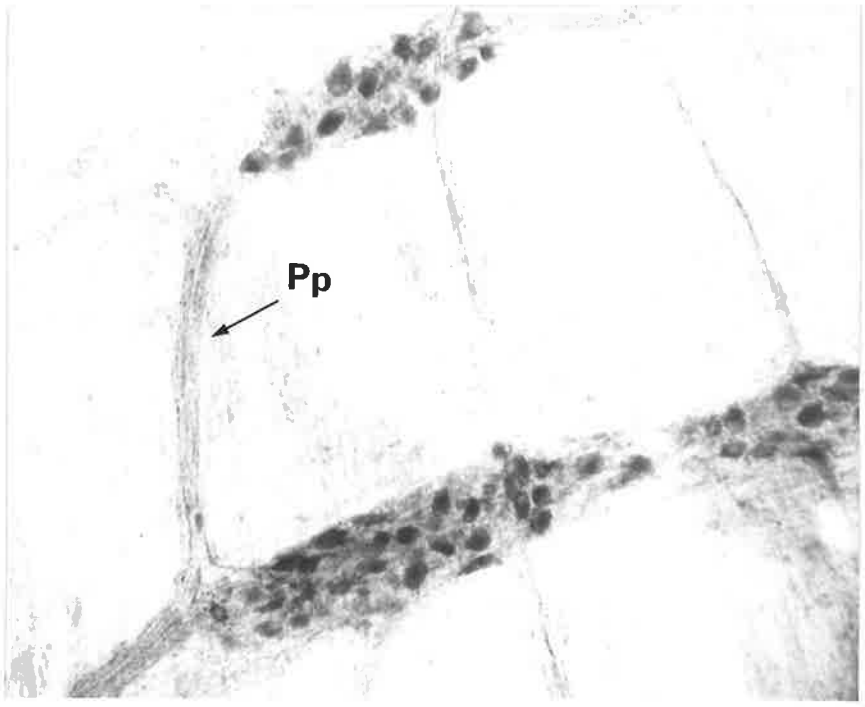

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FIGURE 2. Myenteric neurones of varying types are easily identifiable within ganglia (G). Emergent processes can be traced some distance from their origin.

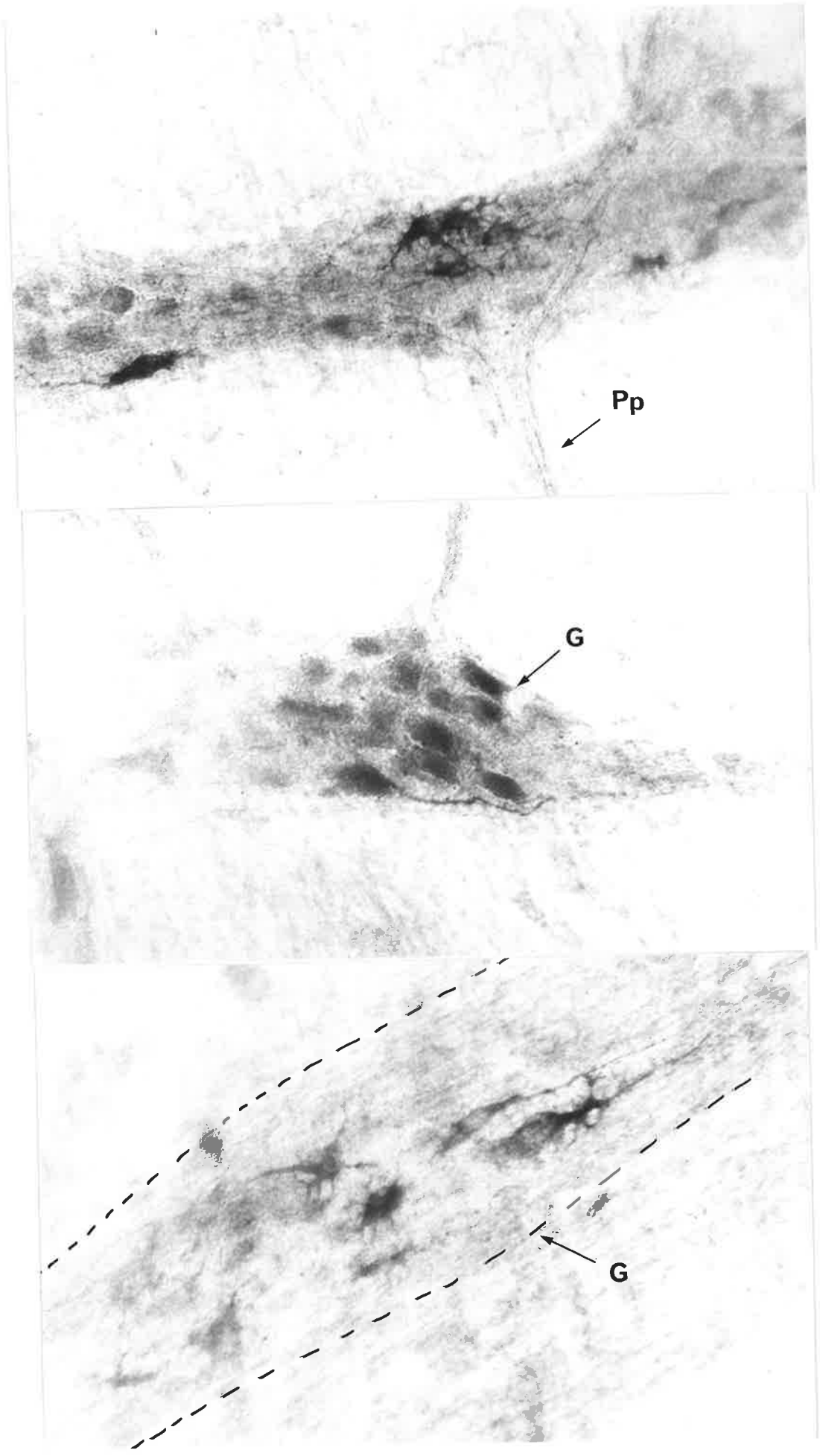
Pp
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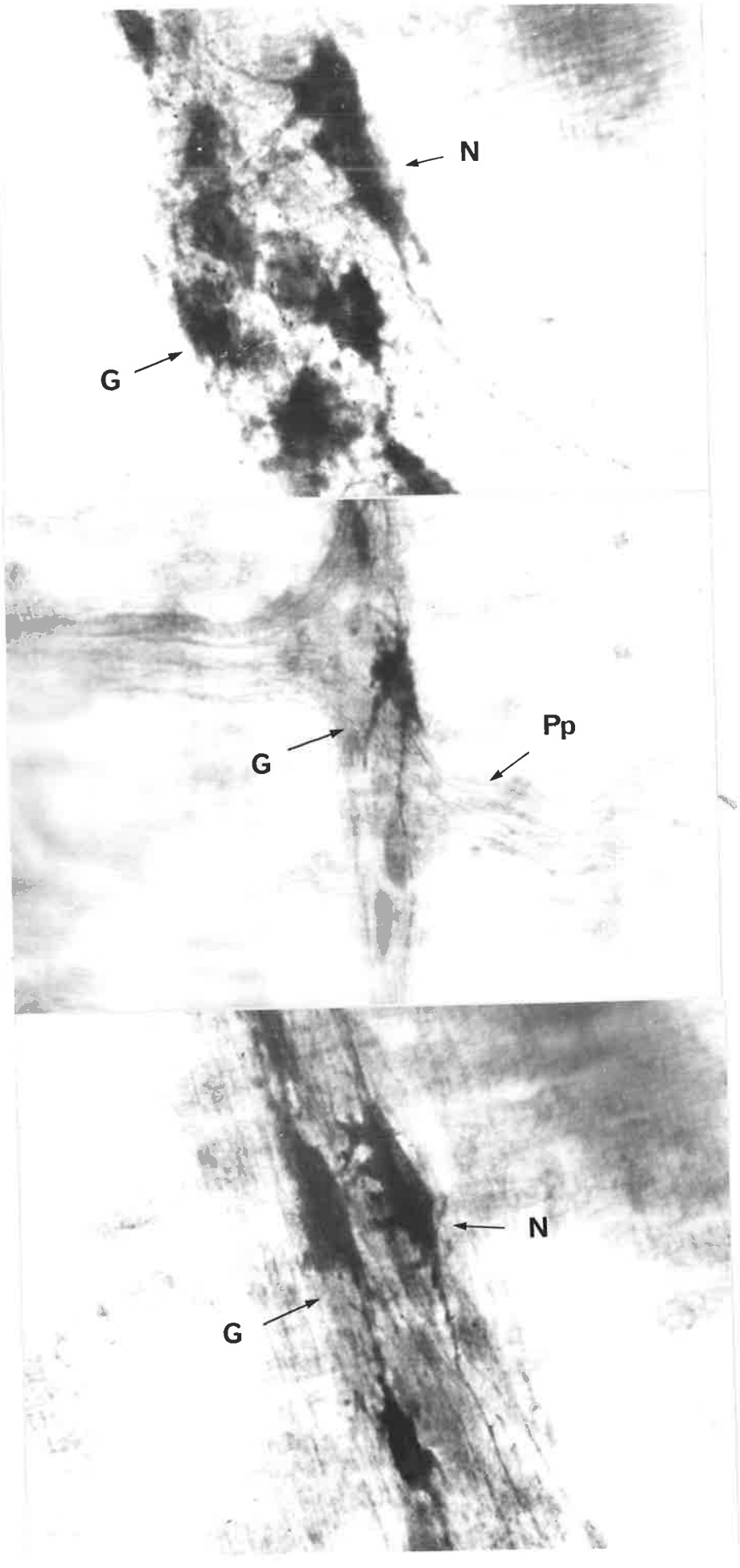
FIGURE 3. Dogiel type I nerve cells (N), with soma and emergent processes intensely stained.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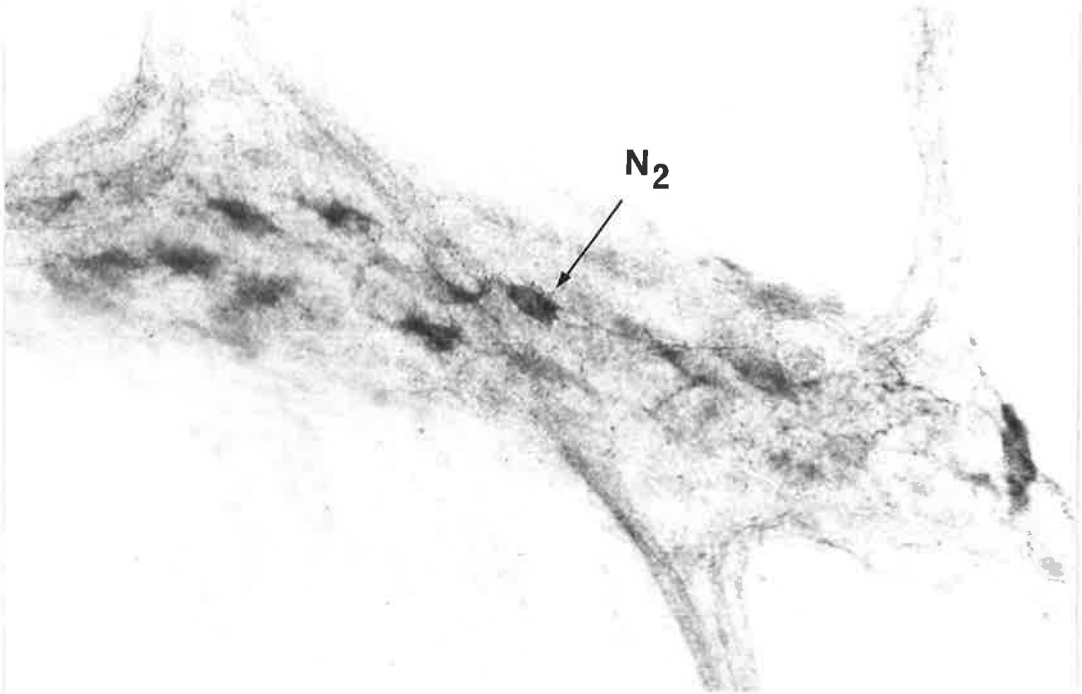
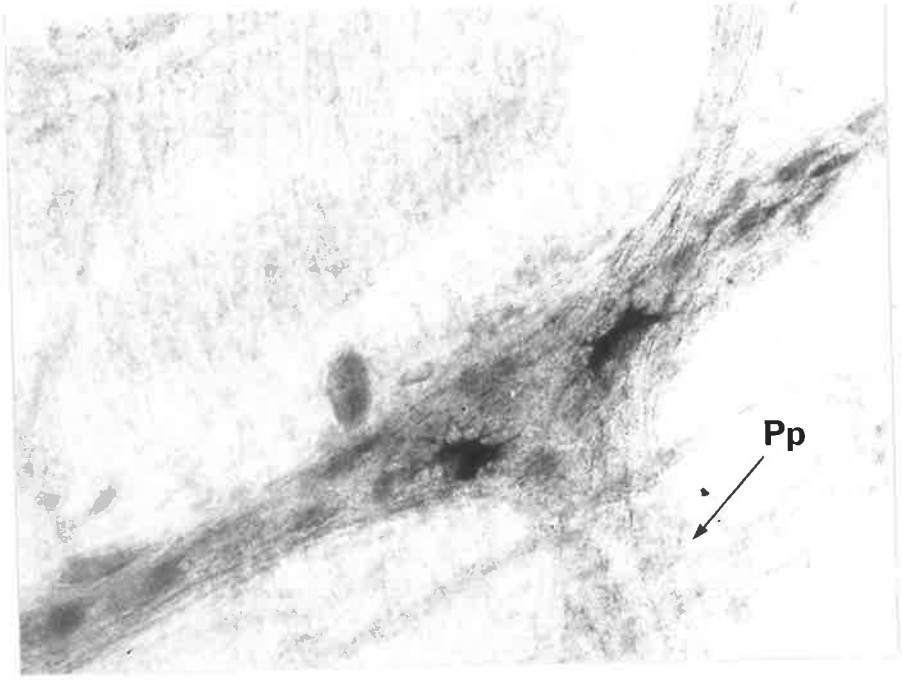


FIGURE 4. A ganglia containing many clearly defined nerve cells. The characteristic long emergent processes of a Dogiel type II neurone (N_2) are readily discernible.

FIGURE 5. Intensely stained nerve fibres can be seen in the fasiculi coursing between ganglia. In some instances enpassant fibres within ganglia can be identified.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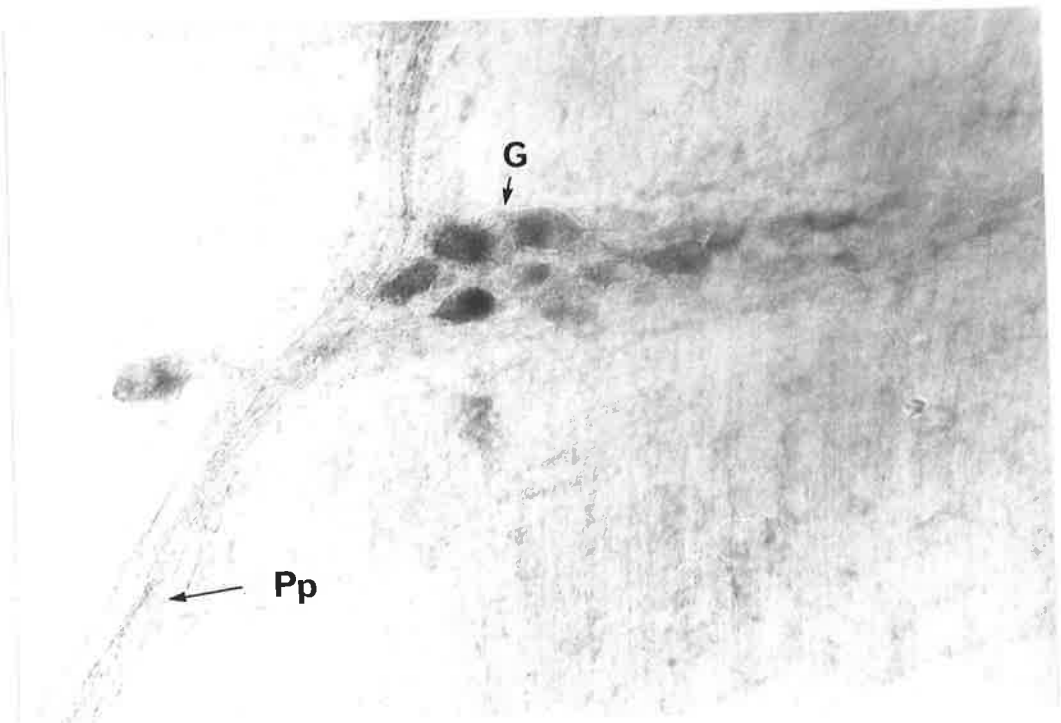
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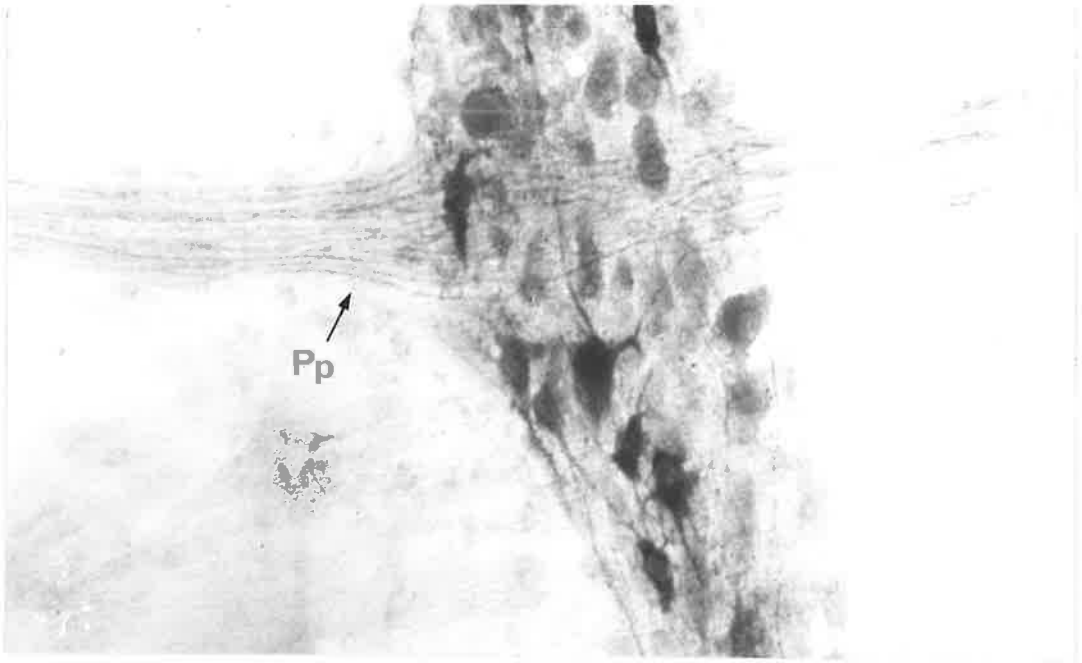
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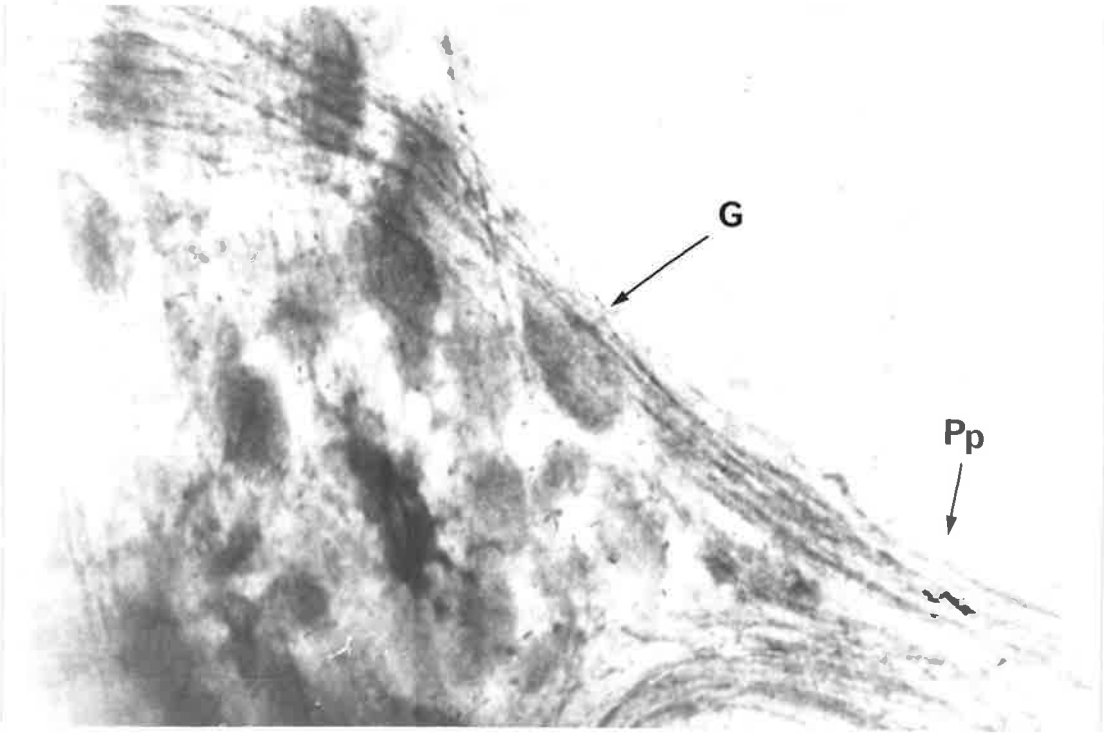
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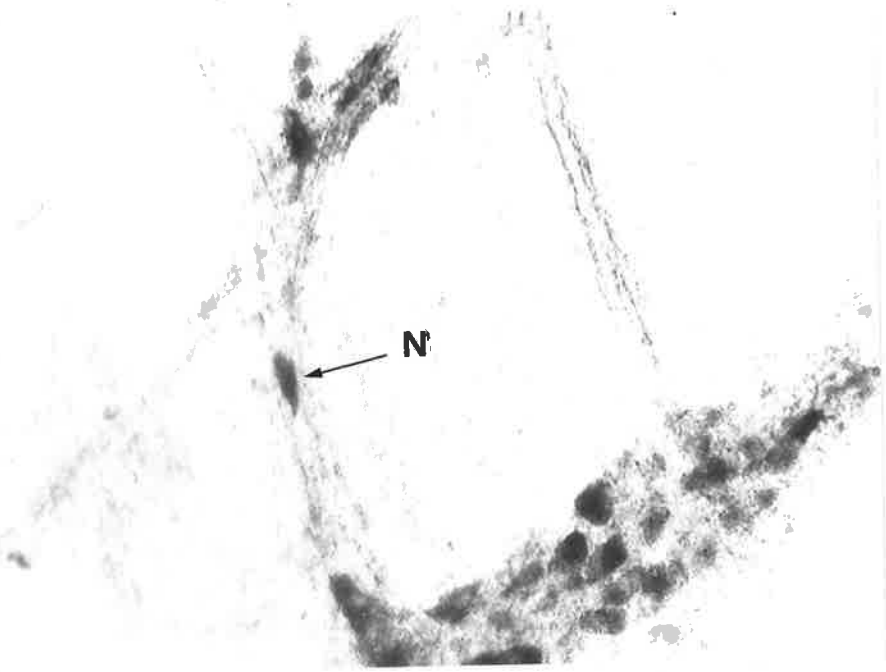


FIGURE 6. An extraganglionic neurone (N) located to a fasiculi of the primary plexus (Pp).

proximity to each other. In Fig. 4, an elongate neurone distinguished by short emergent processes aborised too either end of the soma can be identified lying near the Type II neurone.

In addition to being able to trace the processes of individual neurones some distance from their origin, it was possible to discern enpassant fibres coursing through the ganglia and along the interconnecting nerve bundles (fasciculi) of the primary meshwork (Fig. 5).

Elements of the secondary (Ps) and tertiary (Pt) meshworks of the plexus were not stained by this method, however extraganglionic neurones (Takeo and Sugai, 1974; Gabella, 1979) in the interconnecting fasciculi of the primary (Pp) meshwork were sometimes stained (Fig. 6).

Discussion

The NADH-diaphorase (dehydrogenase) histochemical technique used by Gabella (1967, 1969, 1972, 1979) afforded demonstration of nerve cell soma but with only little deliniation of the emergent processes. Conversely the staining associated with the NADPH-dehydrogenase activity observed in this study enabled better observation of the morphology of the nerve cells. It is well known that nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP), despite their similarity in molecular structure, rarely serve as interchangeable hydrogen (electron) acceptors/donors to the same pyridine linked enzymes (Lehninger, 1972). For the most part NAD-dependent reactions are associated with steps in cellular respiration, therefore the diformazan stain formed under these circumstances would be confined to the mitochondria (Pearse, 1960) located to the soma and terminal

region of the nerve cell, resulting in little or no staining of the nerve fibres. The NADP-dependent reactions provide one link by which electrons can be transferred from catabolic to biosynthetic pathways (Lehninger, 1972; Pearse, 1960) and any diformazan deposits associated with these reactions would occur mainly in the soluble cytoplasm affording better staining of the neurone soma and its processes.

The myenteric plexus and individual ganglionic neurones, as stained by this NADPH-dehydrogenase histochemical technique, bore a striking resemblance to neuronal elements of the ileal myenteric plexus stained by other methods (see Schofield, 1968; Gabella, 1979). The numbers of intensely stained nerve cells varied within individual ganglia and between preparations, therefore it was not possible to establish the relative numbers of morphologically different cell types. Furthermore, there appeared to be no distribution pattern for particular cell types, which is in contrast to the evidence from other studies (see Schofield, 1968).

For example the Dogiel Type I cell is proposed to be located at the periphery of the ganglion (Dogiel, 1895, 1899), but here were found in all locations within the ganglia. Many lighter stained cells were also present and these may represent a class of nerve cells not particularly sensitive to this technique. This could reflect a lower enzymatic activity for these cells, or inadequate cytoplasmic levels of the electron acceptor or the co-enzyme.

Endogenous co-enzyme levels are normally too low to allow histochemical dehydrogenase reactions to occur, and many factors operate under histochemical conditions to reduce the amount of available co-enzyme (Pearse, 1960) therefore it is necessary for co-enzyme levels to be increased and maintained at high levels throughout the incubation. The occurrence of

many intensely stained cells indicates the reaction mixture levels of the co-enzyme and electron acceptor to be appropriate and suggest instead differential penetration by the reactants into the nerve cells, presumably due to the short 'freeze' time used.

There is a vast literature on the morphology of enteric neurones (see Schofield, 1968; Gabella, 1976), characterised by a wide divergence of views as to the nature of these nerve cells. It may indeed be impossible to classify neurones into distinct catergories since it now appears that enteric neurones comprise a wide spectrum of morphological types. Nonetheless, the NADPH-dehydrogenase histochemical technique affords researchers a rapid and simple method for identifying various nerve cell types within the intramural nerve networks of the mammalian intestine.

CHAPTER V

UPTAKE AND RELEASE OF [^{3}H] GABA BY MYENTERIC NERVES

Introduction

The myenteric plexus of guinea-pig, cat and human intestine has been shown to contain the GABA synthesising enzyme glutamic acid decarboxylase (GAD) as well as endogenous GABA in low concentration (Jessen *et al.*, 1979; Hosoya *et al.*, 1981; Miki *et al.*, 1981). Also, neurones of the guinea-pig myenteric plexus are selectively labelled by [^{3}H]-GABA under conditions favouring high affinity uptake, as shown by autoradiography (Jessen *et al.*, 1979; Krantis and Kerr, 1981) which together with the pharmacological actions of GABA in the intestine (Krantis *et al.*, 1980; Krantis and Kerr, 1981; Ong, 1981), is strongly indicative of a transmitter role for GABA in the intestine. However, even the demonstration of a high affinity uptake system for GABA is at best only supportive evidence for the identification of GABA as a transmitter, for, even if radiolabelled GABA is accumulated by neuronal elements, the question still remains as to whether it enters a rapidly releasable neurotransmitter pool (Orrego, 1979). Stronger and more direct evidence in the enteric nervous system would be provided by the demonstration of an evoked release of GABA from myenteric plexus preparations, which is considered to be one of the primary and fundamental criteria for neurotransmitter identification (see Orrego, 1979). This criterion has been investigated for several amino acids in tissue slices or synaptosome preparations preloaded with radiolabelled amino acids, and the spontaneous and evoked efflux of radioactivity monitored (Orrego, 1979). Although investigation of the evoked release of [^{3}H]-GABA from the myenteric plexus is novel, other such preparations have been used to study the evoked release of radiolabelled substances including [^{3}H] adenosine 5'-hydroxytryptamine (Su *et al.*, 1971), [^{3}H] 5-hydroxytryptamine (Schultz and Cartwright, 1974), and [^{3}H] acetylcholine (Kilbinger and Wessler, 1980).

In general, neurally-evoked release of amino acid transmitters is calcium dependent (Fagg and Lane, 1979; Rubin, 1980) and the demonstration of a calcium dependent release of a substance is supportive of a transmitter role, although there are complications depending on the mode of stimulation and conditions under which the efflux study is conducted (Szerb, 1979). Nevertheless calcium dependent, electrically evoked release of [^{3}H] GABA from brain slices has several times been demonstrated (Srinivasan *et al.*, 1969; Katz *et al.*, 1969; Szerb, 1979), which prompted this attempt at a corresponding study in the enteric nervous system. A problem associated with interpretation of data for such evoked release of amino acids is the need to distinguish which tissue elements accumulate and release the radiolabelled amino acid. There is now considerable evidence that glial cells are an important site for amino acid uptake (Fagg and Lane, 1979) and glial cells may be depolarized, by high concentrations of potassium or by electrical stimulation, to release non-transmitter amino acids (Kuffler and Nicholls, 1966; Katz *et al.*, 1969; Watson, 1974; Orrego *et al.*, 1976). Furthermore, uptake and release of radiolabelled GABA has been demonstrated in sympathetic ganglia (Young *et al.*, 1973; Bowery and Brown, 1976) where GABA is not considered to be a transmitter but is accumulated by glia. However, the fact that [^{3}H]-GABA is also taken-up into glia by a high affinity uptake system is a complication that can largely be overcome by the use of β-alanine which inhibits any GABA uptake into glial cells (Schon and Kelly, 1975) thus ensuring that the greatest proportion of [^{3}H]-GABA released by electrical stimulation will be of neuronal origin. That a neural source is involved in the release of a putative transmitter from the tissue can be confirmed by preventing neural activity with a neurotoxin and by preventing uptake of transmitters with specific inhibitors of neuronal high affinity uptake. For GABA such neuronal uptake inhibitors are L,2-4-diaminobutyric acid L-DABA) and cis-3-aminocyclohexane-carboxylic acid (ACHC) (Iversen and Kelly, 1975; Bowery *et al.*, 1976), whilst nipecotic

acid inhibits both glial and neuronal GABA-uptake (Schousboe *et al.*, 1979). In addressing the problem as to whether GABA is a transmitter in the enteric nervous system I have now employed the criteria of uptake inhibition, calcium dependence, and neurotoxin sensitivity of release, in order to show that [^{3}H]-GABA can be accumulated by isolated longitudinal muscle-myenteric plexus preparations of both guinea-pig ileum and colon and subsequently released by appropriate electrical stimulation.

Methods

Guinea-pigs of either sex weighing 250-400 grams were stunned by a blow to the head and bled. Segments of ileum and of the proximal portion of the distal colon were removed and placed in Krebs (the composition as described in Chapter II). The solution was bubbled continuously with a gas mixture of 95% O_2 and 5% CO_2, and maintained at 36°C. Lengths of longitudinal muscle coat (3 cm) with attached myenteric plexus (laminar preparations) were then dissected from the segments by the method described in Chapter II and kept in the Krebs solution to which had been added amino-oxyacetic acid (AOAA), 2×10^{-5} M, to inhibit GABA metabolism (Wallach, 1961) and β-alanine, 10^{-3} M, to prevent GABA-uptake by glial cells (Schon and Kelly, 1975). This medium was used in all subsequent manipulations.

Individual preparations measuring approximately 5 mm in width and 25 mm in length when stretched out, were preincubated in glass incubation chambers for 10 min in 350 µl of the perfusion medium after which [^{3}H]-GABA was added to give a final concentration of 5×10^{-9} GABA, and incubation was continued for a further 20 min. The tissues were then

removed, blotted of excess incubating medium and suspended between platinum stimulating electrodes in a glass perfusion chamber containing 2 ml of the perfusion medium maintained at 36°C. The tissue was washed repeatedly (total 40 ml) over an equilibration period of 55 min, to establish a basal efflux of [^{3}H]-GABA, after which superfusion was commenced and 2 ml fractions collected at 30 sec intervals. During this superfusion phase electrical stimulation (Grass S48 Stimulator) was applied for 2 min periods, using 60 V and 3 ms duration pulse at various frequencies (5 to 100 Hz).

To test the influence of Ca^{2+} on the stimulated efflux of tritium, the Krebs solution was substituted by a Ca^{2+}-free perfusion medium containing 0.1 mM ethyleneglycol-bis-(β-amino-ethyl ether) N, N'-tetra-acetic acid (EGTA) and buffered with Tris (3 mM), the remaining constitutents being as in the Krebs solution, this solution also contained AOAA and β-alanine.

Superfusate fractions were collected and added to 10 ml of a Triton/ toluene scintillator, and the radioactivity measured by liquid scintillation spectrometry using a 10 minute counting period in a Packard tri-card liquid scintillation spectrometer model 2002. Counting efficiency (using an external standard) was determined at 36 ± 1, n = 9. Radioactivity has been expressed as c.p.m. rather than d.p.m. since variations in counting efficiency between samples was negligible.

Identification of [^{3}H]-GABA released by stimulation

Thin layer chromatography (TLC) on cellulose was used to identify [^{3}H]-GABA in the perfusate. Perfusate samples, taken every 30 sec, were collected over two minute periods and pooled. The first collection was made during basal efflux, just prior to stimulation, and a second was

made during the stimulation period. Each pooled sample was concentrated 10 fold by vortex evaporation and the GABA content adjusted to ≅ 4 mg/ml by the addition of unlabelled GABA, as was an aliquot of standard [^{3}H]-GABA. Chromatograms were run using the system n-butanol:pyridine:H_2O (1:1:1 by volume). The dried chromatogram was developed with ninhydrin over a GABA reference to identify its final position on the plate. The remainder of the TLC plate was then divided appropriately into 1 cm strips from origin to solvent front and the cellulose powder of each strip was scraped into a scintillation vial containing 1 ml of H_2O. The samples were counted in 10 ml of a Triton/toluene scintillator. Regions yielding tritium counts could then be related to GABA on the ninhydrin treated segments.

Compounds used were: β-alanine, amino-oxyacetic acid (AOAA), L,2-4-diaminobutyric acid (L-DABA), nipecotic acid, tetrodotoxin (TTX), (all Sigma), and veratridine (Sandoz). GABA (Calbiochem, Sigma) and [^{3}H]-GABA (66 Ci mmol^{-1}; 2,3-[^{3}H]-GABA) (Radiochemical Centre, Amersham). Scintillation fluid: 33 gs PPO and 1 gm Dimethyl Popop dissolved in a Triton 100/toluene mixture (1:2 parts/volume).

Results

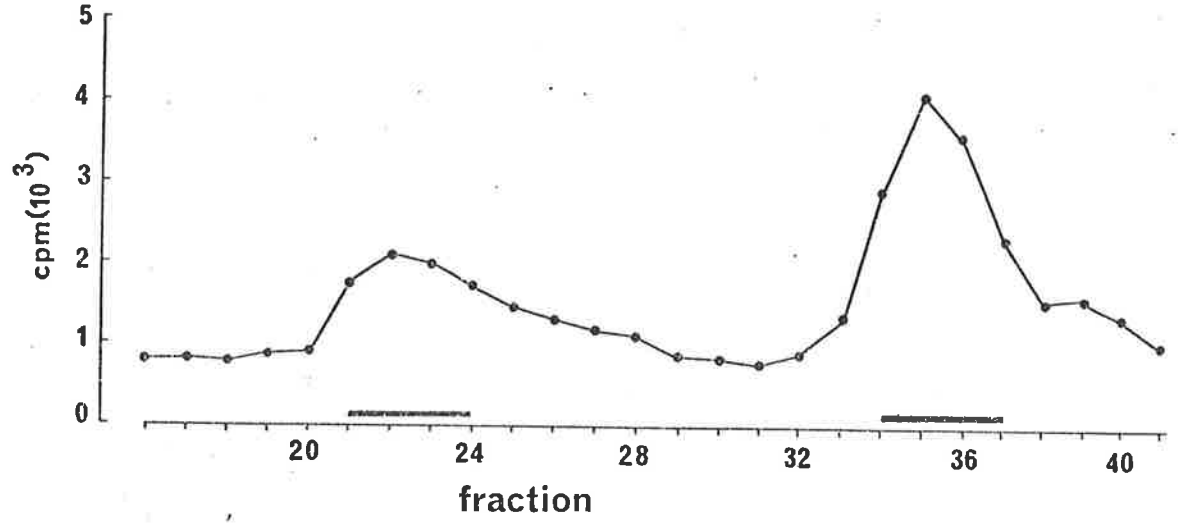
Uptake and release of [^{3}H]-GABA

The majority of experiments were conducted on preparations from the ileum. As a preliminary, longitudinal muscle-myenteric plexus preparations were incubated for various periods of time with [^{3}H]-GABA, 5 x 10^{-9} M, in the presence of AOAA and β-alanine; adequate uptake occurred within 20 min

FIG. 1 (a) The frequency dependent evoked release of [^{3}H]-GABA from guinea-pig ileal longitudinal muscle-myenteric plexus preparation. Each point represents the radioactivity of a 2 ml fraction collected during a 30 sec period. Abscissa: bars represent electrical stimulation (2 min) at 10 Hz, 20 Hz (3 msec duration, 60 V) respectively. AOAA 2 x 10^{-5} M and β-alanine 10^{-3} M were present throughout the experiment.

FIG. 1 (b) ^{3}H-GABA efflux during superfusion and electrical stimulation of longitudinal muscle-myenteric plexus preparations. ■ guinea-pig distal colon; ● guinea-pig ileum. Each point represents the radioactivity in a 2 ml fraction collected during a 30 sec period. Abscissa: bar represents 2 min electrical stimulation period (20 Hz, 3 msec duration, 60 V). AOAA 2 x 10^{-5} M and β-alanine 10^{-3} M were present throughout the experiment.

a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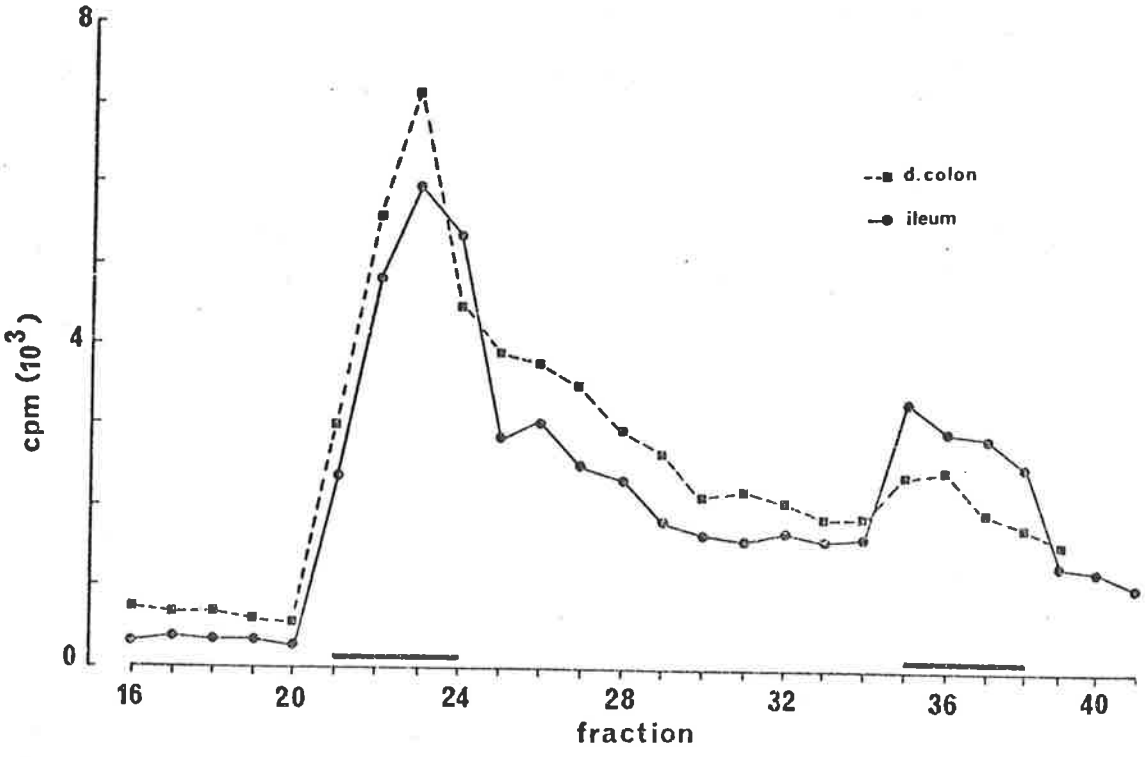
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FIG. 2. The effect of tetrodotoxin (TTX) on the stimulated efflux of [^{3}H]-GABA from guinea-pig ileal longitudinal muscle-myenteric plexus preparations. Adjacent segments of ileum were incubated under the same conditions and then arranged (at random) in a perfusion bath under the following conditions and order. ● normal perfusion medium; O normal medium with TTX 10^{-7} M; ■ normal medium; □ normal medium with TTX 10^{-7} M. Each point represents the radioactivity in a 2 ml fraction collected over a 30 sec period. Abscissa: bar represents a 2 min electrical stimulation period (20 Hz, 3 msec duration, 60 V). AOAA 2 x 10^{-5} M and β-alanine 10^{-3} M were present throughout the experiment.

FIG. 3. The effect of high affinity uptake inhibition on the basal and stimulated efflux of [^{3}H]-GABA from guinea-pig longitudinal muscle-myenteric plexus preparations. ● normal incubation and perfusion medium ■▲with L-DABA 10^{-3} present. O with nipecotic acid 10^{-3} M present. Each point represents the radioactivity in a 2 ml fraction collected during a 30 sec period. Abscissa: bar represents a 2 min electrical stimulation period (20 Hz, 3 msec duration, 60 V). AOAA 2 x 10^{-5} M and β-alanine 10^{-3} M were present throughout the experiment.

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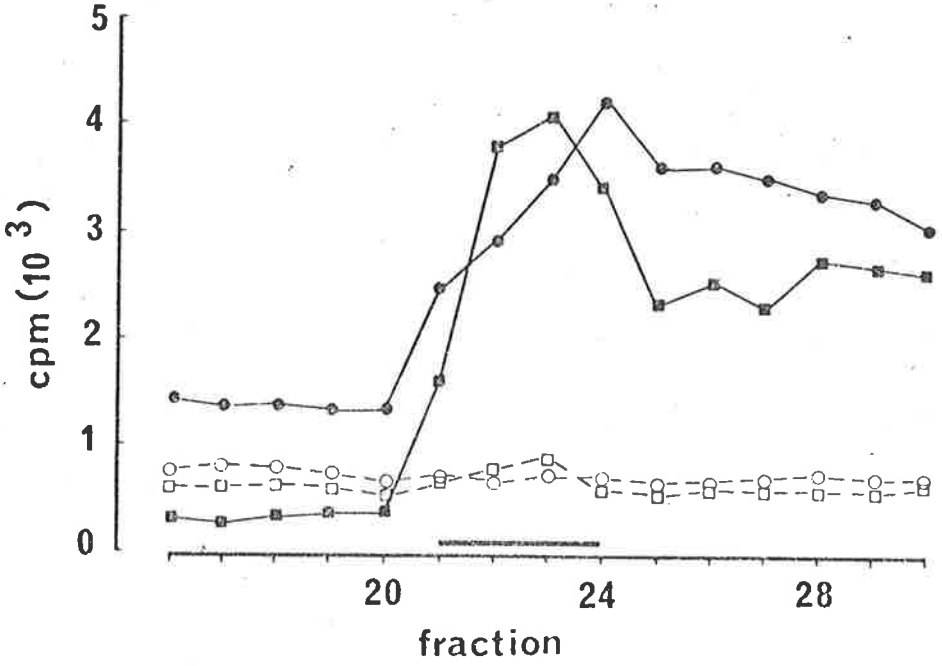
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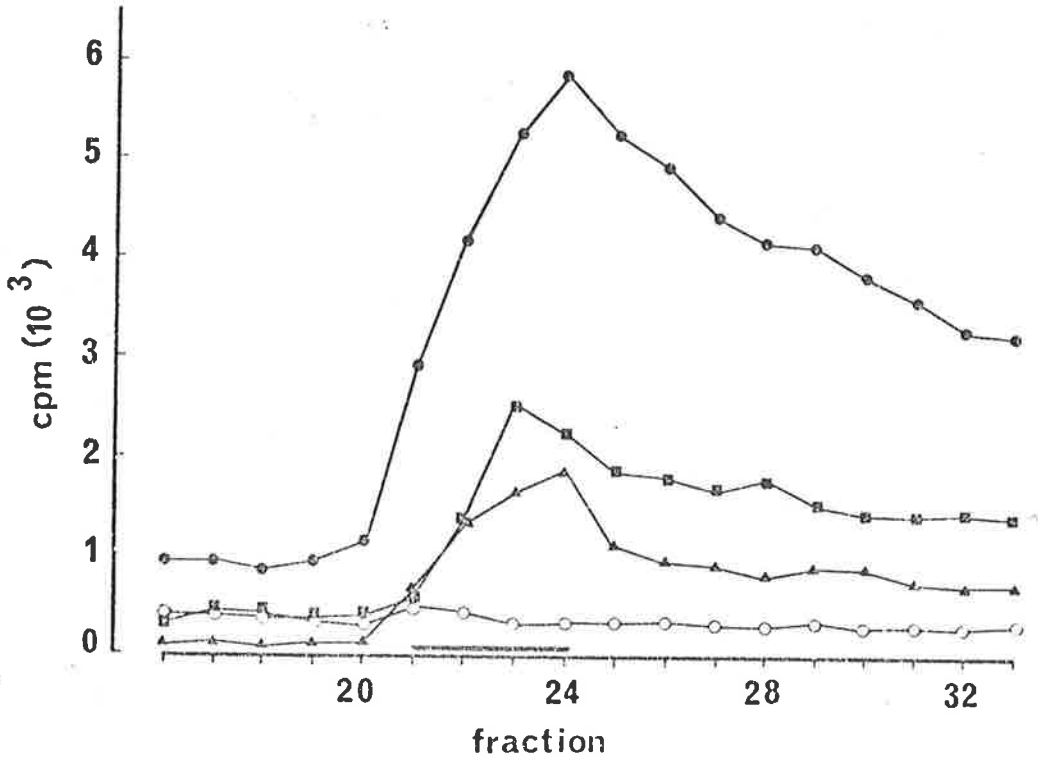
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of incubation, which was adopted as the standard time for loading the tissue with [^{3}H]-GABA. The efflux of tritium from the perfused preparation was followed over the washing period. There were two components in the unstimulated efflux of tritium: an initial, rapid washout-phase with high tritium content lasting some 30-40 minutes, followed by a slower relatively constant efflux level, here termed the basal efflux. Once this basal efflux was achieved, generally within one hour, electrical field stimulation of the preparation induced an immediate increased release of tritium (Fig. 1). The stimulated efflux of tritium reached a maximum within two minutes of stimulated at 20 Hz. These conditions were found to be optimal and used in all subsequent manipulations. On cessation of electrical stimulation the release of radioactivity again declined towards the basal efflux level. Succeeding periods of stimulation at 20 Hz were always less effective in provoking further tritium release.

Preparations from the distal colon treated in the same way displayed similar efflux characteristics (Fig. 1), but were not investigated further.

Neither KCl, 56 mM, nor veratridrine, 5 x 10^{-5} M were very effective in stimulating [^{3}H]-GABA efflux from the myenteric plexus, beyond and approximate doubling of counts with KCl depolarization and an almost undetectable increase with veratridine stimulation. Consequently we used electrical stimulation as the preferred method for evoking [^{3}H]-GABA release from the plexus preparations.

Tetrodotoxin (TTX) (10^{-6} M) which would block all neural activity when added to the perfusion medium, was effective in vitrually abolishing the stimulated release of [^{3}H]-GABA from ileal preparations (Fig. 2). These tests were run on duplicate, separate, tissues with and without TTX, since difficulties were encountered in washing-out the TTX rapidly enough to

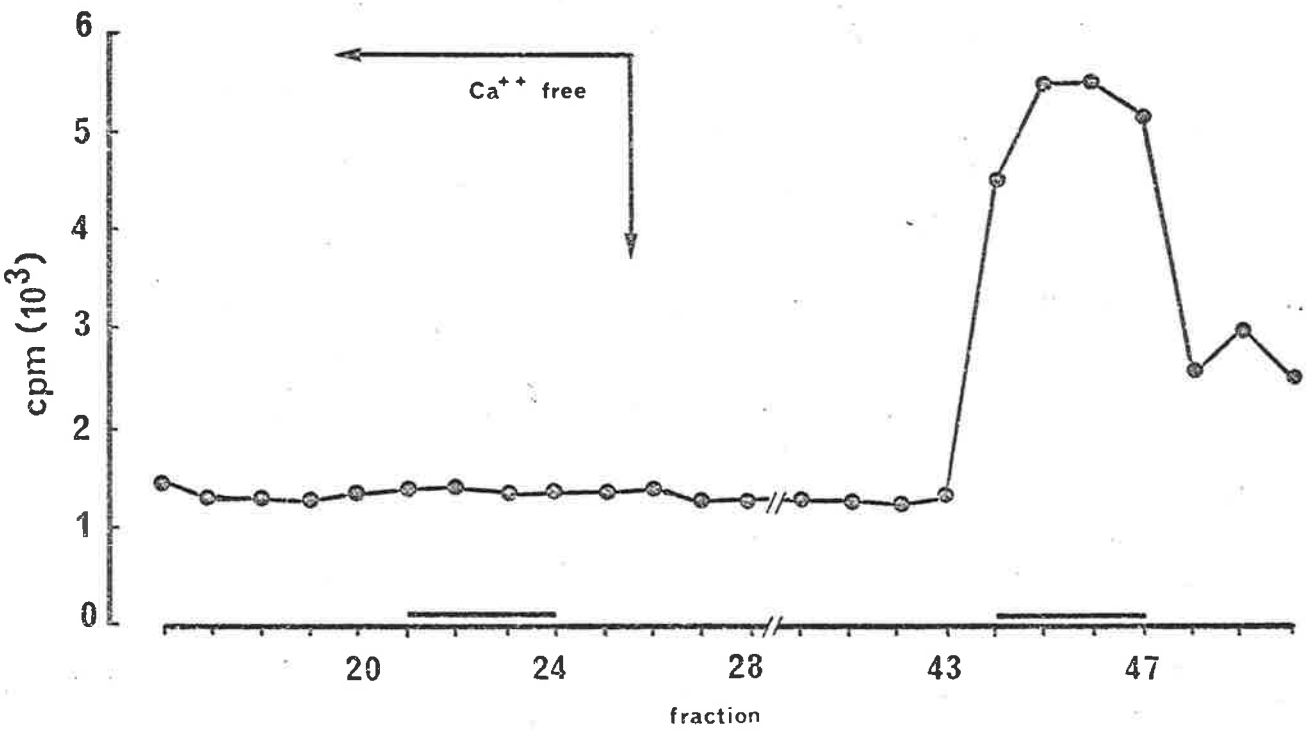

FIG. 4. Basal and electrical stimulation evoked efflux of ^{3}H-GABA from guinea-pig ileal longitudinal muscle-myenteric plexus preparation in calcium-free medium (with EGTA 3 mM) and in normal perfusing medium. Each point represents the radioactivity of a 2 ml fraction released during a 30 sec collection period. Abscissa: bars indicate 2 min electrical stimulation period (20 Hz, 3 msec duration, 60 V).

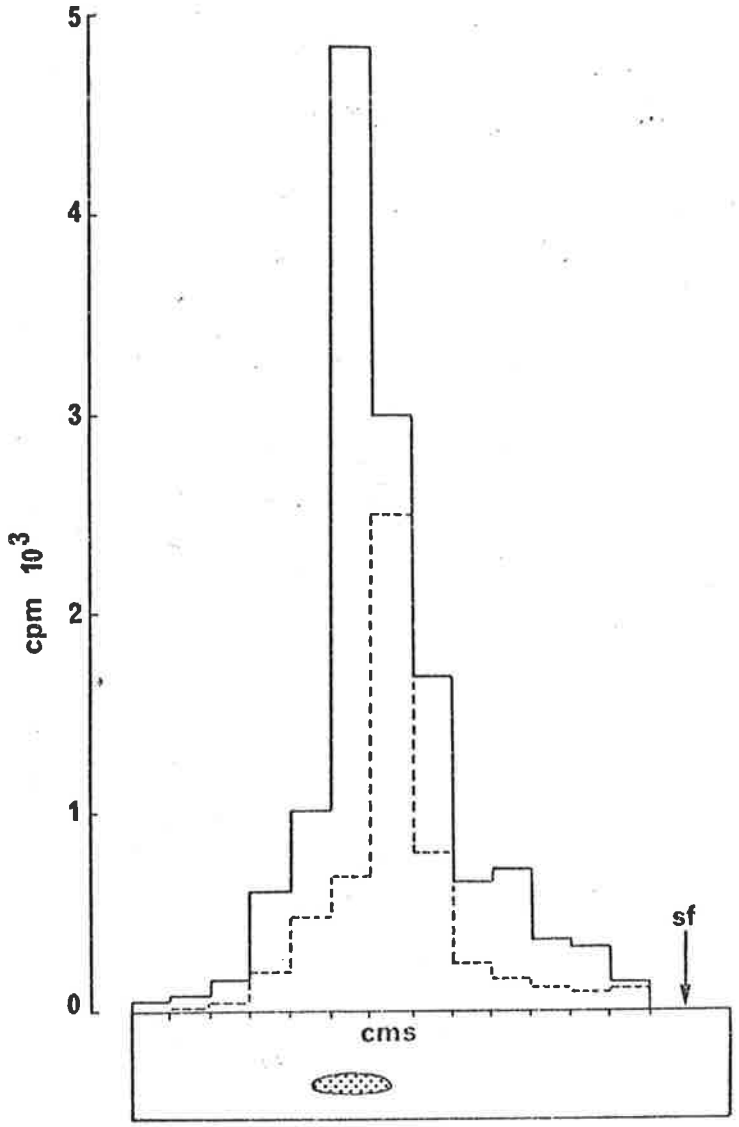

FIG. 5. Chromatographic identification of ^{3}H-GABA using cellulose thin layer chromatography. The histograms represent the radioactivity found in 1 cm strips of the chromatograms. Solid line: ^{3}H-GABA standard. Broken line: radioactivity found in the efflux during a 2 min stimulation period. Beneath the abcissa is a representation of a section of the chromatogram developed with ninhydrin. Localization of the GABA standard is shown as a shaded area. The solvent front (sf) is indicated by an arrow.

permit demonstration of stimulated [^{3}H]-GABA efflux from the washed TTX treated tissues.

When the ileal tissue was treated with the neuronal GABA-uptake inhibitors nipecotic acid (10^{-3} M) or L-DABA (10^{-3} M), before and during loading with [^{3}H]-GABA, the subsequent electrically evoked efflux of tritium was reduced, with L-DABA being less effective than nipecotic acid (Fig. 3).

Calcium dependence of stimulated ^{3}H-GABA release

In a further series of experiments, the ileal tissue was preloaded with [^{3}H]-GABA and then perfused with Ca^{2+}-free medium containing EGTA, 0.1 mM. Spontaneous efflux of tritium was unchanged under these conditions, but electrical stimulation no longer evoked any additional efflux. Upon replacing the perfusion medium with that containing a normal Ca^{2+} concentration, electrical stimulation again evoked an appreciable efflux of tritium (Fig. 4).

Identification of ^{3}H-GABA released by electrical stimulation

On TLC plates, the major portion of radioactivity released by electrical stimulation co-chromatographed with standard GABA and [^{3}H]-GABA, all seen to have a R_F value of 0.45 (Fig. 5). The tritium associated with the basal efflux, taken just prior to stimulation, ran with a similar R_F, but amounted only to some 5% of the peak stimulated output (not shown). There was no other major peak of tritium presented in chromatograms from either the basal or stimulated efflux.

Discussion

Spontaneous output of tritium fell throughout each experiment, first rapidly then more slowly. Carry-over of [^{3}H]-GABA from the incubating medium, as well as washout from non-neural sources, would account for the bulk of the initial, rapid, high-level efflux from the tissues during the early washing period. Despite the presence of AOAA in the media some of this tritium probably consisted of both [^{3}H]-GABA and metabolites including tritiated water (Gardner and Richards, 1981). The possibility can be discounted that significant [^{3}H]-GABA could be released from some non-neural source as a result of smooth muscle contractions during the stimulation period, for the efflux was greatly reduced by inhibiting neural [^{3}H]-GABA uptake. It is also unlikely that release of [^{3}H]-GABA from glial cells could contribute significantly to the observed efflux, whether spontaneous or stimulated, since β-alanine which was present throughout and is a more effective inhibitor of GABA-uptake than glial (Bowery *et al.*, 1976; Schousbe *et al.*, 1969), yet only the stimulated efflux was modified by this inhibitor.

Autoradiographic evidence showed that most of the [^{3}H]-GABA accumulated by ileal longitudinal-muscle myenteric-plexus preparations was incorporated into neurones of the plexus under conditions of high affinity uptake (Krantis and Kerr, 1981). It can therefore be assumed that similar accumulation of [^{3}H]-GABA had occurred in the plexus of the ileum and colon in the present study. In the autoradiographs there was clear evidence of cell bodies labelled within ganglia of Auerbach's plexus together with profuse labelling of processes in the primary, secondary and tertiary meshwork of the plexus (Krantis and Kerr, 1981). Furthermore, L-DABA and nipecotic acid which are inhibitors of neuronal GABA uptake, prevented this labelling in the autoradiographs (see Chapter III) and

greatly reduced the stimulated efflux of [^{3}H]-GABA relative to that from untreated tissues. Such labelled neurones and processes are, then, the most likely source of the [^{3}H]-GABA release from the plexus by electrical stimulation in this study.

AOAA was present throughout these experiments in order to prevent metabolism of [^{3}H]-GABA after uptake (Wallach, 1961). However, it has been suggested that AOAA, in the concentrations customarily employed for this purpose, is not entirely effective in blocking GABA catabolism (Gardner and Richards, 1981). Nevertheless the bulk of the radioactivity measured during electrical stimulation of the tissue was [^{3}H]-GABA that co-chromatographed with standard GABA. This neural [^{3}H]-GABA was readily mobilized but could be partially depleted by stimulation at 20 Hz for as little as two minutes, no doubt representing exhaustion of a limited releasable store. Both tetrodotoxin and calcium-free perfusion were able to stop the stimulated efflux of [^{3}H]-GABA, again confirming its neural origin. Frequencies of stimulation about 20 Hz were less effective in evoking [^{3}H]-GABA release, which would suggest the existence of GABA autoreceptors capable of depressing output of transmitter by a prejunctional inhibitory feedback mechanism. Alternatively the release may have been depressed by some other, co-released, transmitter.

Somewhat surprisingly, it was found that neither veratridine nor KCl were as effective as electrical stimulation in provoking [^{3}H]-GABA efflux from ileal plexus preparations, although both have been utilized in the studying of such efflux from other tissues (Szerb, 1979; Minchin, 1979; Srinivarson *et al.*, 1969). Whilst veratridine stimulation distinguishes neuronal from glial sources of transmitter efflux (Bowery and Neal, 1978) the more usual criterion is used as a stimulus. On the contrary, veratridine causes an increased output of transmitter in the absence of Ca^{2+} (Minchin,

1979; Szerb, 1979). Since we did not explore the influence of Ca^{2+} on this efflux in the intestine we have no explanation for the relatively small [^{3}H]-GABA efflux evoked by veratridine or KCl in the present study, particularly since KCl might be expected to release [^{3}H]-GABA from both glial and neuronal sources (Bowery and Neal, 1978).

There are conflicting reports concerning calcium dependence of transmitter output when using electrical stimulation. Although Szerb (1979) showed such dependence, Srinivasan *et al.* (1969) found a slightly increased [^{3}H]-GABA efflux from electrically stimulated brain slices in the presence of low Ca^{2+} without chelating agent, which is to be expected since there would likely be an electrically-hyperexcitable phase during progressive depletion of membrane Ca^{2+} under these conditions. In the present study this was avoided by the combined use of EGTA and no Ca^{2+} in the medium, thus more rapidly depleting Ca^{2+} from the membranes. There is the possibility that such treatment, particularly if prolonged, might damage the tissue and prevent transmitter output, but evidently this did not occur for there was a prompt return of the stimulated ^{3}H-GABA efflux upon restoring the Ca^{2+} content in the perfusion medium to normal. Electrical stimulation thus remains a valid method for eliciting putative transmitter output from the functional myenteric plexus, as has already been shown in the ileum for cholinergic transmission using [^{3}H]-choline (Szerb, 1976; Kilbinger and Wessler, 1980) and for [^{3}H]-5-hydroxytryptamine (Schulz and Cartwright, 1974; Jonakait *et al.*, 1979) as well as for [^{3}H]-adenosine triphosphate from taenia coli fo the guinea-pig (Su *et al.*, 1971). [^{3}H]-GABA released from the myenteric plexus is of neural origin, the release being Ca^{2+} dependent and tetrodotoxin sensitive, indicative of GABA being a transmitter in the guinea-pig enteric nervous system.

CHAPTER VI

THE EFFECTS OF GABA ANTAGONISM ON INTESTINAL MOTILITY

Introduction

The extensive ramifications of GABA-ergic neuronal elements within the myenteric plexus and their innervation of the circular muscle layer suggests that GABA released from such neurones could be involved in some physiological function of the guinea-pig intestine. Several functions are carried out by this organ including secretion, absorption, digestive mixing (segmentation) and propulsive activity (peristalsis). The pharmacological actions of applied GABA result in the stimulation of the final motor nerves mediating the respective inhibitory and excitatory reflexes underlying peristalsis, indicating that GABA-ergic mechanisms may very well be involved in the regulation of gastrointestinal motility. There have, however, so far been few investigations of any GABA-ergic neuronal involvement in peristalsis beyond the observation that applied GABA immediately depresses peristalsis in the isolated mammalian ileum (Hobbiger, 1958a, b; Inouye *et al.*, 1960). Although this effect does not in itself directly implicate any GABA-ergic mechanism in the control of peristalsis such an involvement may very well occur.

Investigation of peristalsis and its underlying mechanisms is difficult since the intrinsic nervous control of the gastrointestinal tract is very complex and not completely understood (see Chapter I). Bayliss and Starling (1899) gave the first detailed account of nerve-mediated 'reflexes' underlying the passage of substances through the intestine, which they called 'peristalsis'. From their experiments on anaesthetized dogs, rabbits and cats, Bayliss and Starling (1899, 1900, 1901) showed local stimulation of the intestine wall to elicit 'reflexes', a wave of contraction, (excitation) above and relxation in front of the point of stimulation and waves of contractions which travel in an aboral direction. Furthermore, they surmised that these effects were coordinated and involved local neuronal

mechanisms residing within the Auerbach's (myenteric) plexus. The initial observations of Bayliss and Starling were elaborated upon by Trendelenburg (1917) who devised the now classical experimental preparations whereby peristaltic movements in the intestine of the guinea-pig or rabbit were elicited by elevation of intraluminal pressure, which in turn caused radial stretch. Sensory receptors located in the wall of the intestine subsequently detect the distension and trigger contraction or relaxation of the respective intestinal regions. Trendelenburg distinguished two phases of peristalsis; a preparatory and emptying phase. The preparatory phase, which results in the relaxation of the intestine is not itself able to coordinate the onward movement of intestinal contents, rather, it abolishes activity in the region immediately in front of the contents, and onward movement results from contraction of the intestine wall behind the contents. The involvement of cholinergic motorneurones in the 'excitatory reflex' was determined by Bayliss and Starling (1899) and Trendelenburg (1917), and since confirmed by more recent studies (Hirst and McKirdy, 1974; Costa and Furness, 1976). Atropine and hyoscine blocked propulsion of the intestinal contents by disrupting the excitatory reflex however the inhibitory reflex remained intact.

The existence of cholinergic interneurones within the neural pathways for 'peristalsis' was first reported by Feldburg and Lin (1949) and Paton and Zaimis (1949). They showed the emptying phase to be inhibited by the nicotinic ganglion blocking agents, tubocurarine and hexamethonium. Using methods different from the classical Trendelenburg method for eliciting 'peristalsis', Hukuhara *et al.* (1958) proved that local mechanical or chemical stimulation of the intestinal mucosa of denervated, dog small intestine induced peristaltic movements which were abolished by hexamethonium. Furthermore, Crema *et al.* (1970) and Frigo and Lecchini (1970) showed propulsive movements of cat or guinea-pig colon induced by

a solid bolus to be abolished by hexamethonium. The inhibitory reflex has also been shown to be polysynaptic, involving cholinergic interneurones which in turn stimulate the intrinsic inhibitory motor neurones leading to the inhibition elicited by this pathway (Kosterlitz, 1968). The transmitter released at the neuro-effector junctions for this inhibitory reflex, is still unknown. (See 'intrinsic innervation', Chapter I).

According to Kosterlitz *et al.* (1956) the 'emptying phase' described by Bayliss and Starling (1899) does not involve a coordination of contractions within the two muscle layers of the intestine wall. Rather, contractions of either muscle is dependent upon the degree of radial stretch, with a greater distention being required to trigger contractions of the circular muscle. The contractions of the longitudinal and circular muscle layers usually follow each other. However, high concentrations of acetylcholine, carbamylcholine, or histamine block the longitudinal muscle, without affecting the circular muscle Kosterlitz (1968) and Hukuhara and Fukuda (1965) suggested that shortening of the long muscle was a consequence of the dilation of the circular muscle layer, however Kosterlitz and Watt (1965) pointed out that during the intervening period between circular muscle contracting the longitudinal muscle remains 'extended', which suggests this explanation is inadequate. Electrophysiological evidence (Kottegoda, 1970), shows that in transmurally stimulated segments of intestine the circular muscle are inhibited whilst the longitudinal muscle was excited and vice versa, suggesting a reciprocal rather than sequential excitation and inhibition of both muscle coats during peristalsis. However, as Hirst (1979) pointed out, transmural stimulation does not allow any observation of temporal separation between excitation and inhibition. Thus these results cannot be interpretated. Whether contractions of the longitudinal muscle during the preparatory phase is due to some neural activation is also unclear. Kosterlitz (1968) described the contractions

to be abolished by TTX, atropine and morphine, however, Hirst *et al.* (1975) when recording several centimetres from the point of distension found no electrophysiological correlates for neural activity during longitudinal muscle contraction in isolated segments of intestine. Interestingly, Feldburg and Lin (1949) found local anaesthetics did not block contractions of the long muscle during the preparatory phase.

Krantis *et al.* (1980) have shown that GABA induces relaxations of the guinea-pig ileal and colon longitudinal muscle coat which are blocked by TTX indicating that GABA has no direct effect upon the muscle. Adrenergic blockade of longitudinal muscle strips devoid of any attached nerve plexus did not prevent the GABA-induced responses, suggesting they were mediated by the non-adrenergic, non-cholinergic inhibitory neurones, intrinsic to the intestine. Furthermore, when recording mechanical activity from both muscle coats together, the action of GABA was simultaneous and the same. These findings suggest an intrinsic inhibitory innervation of the guinea-pig longitudinal muscle. According to Taxi (1965) and Gabella (1972) the longitudinal muscle does not receive any direct nervous innervation. However the close proximity (nanometres) of vesiculated axonal processes on the surface of the myenteric plexus to the muscle would allow the innervation of the muscle layer by diffusion of a neurotransmitter substance released from these neuronal processes. The findings of Krantis *et al.* (1980) equally disprove the idea of reciprocal or sequential relationships between the two muscle coats although it is possible that some integrative facility within the myenteric plexus could give rise to physiological events which could lead to coordination of the muscle coats in these ways.

The exact site of the sensory receptors mediating the coupling between radial stretch and muscular activity is still unknown. Bulbring *et al.* (1958)

showed that removal of the mucous membrane abolishes the reflex contraction of guinea-pig ileal circular muscle. Furthermore, they demonstrated the mucous membrane to be innervated by neurones arising from the ganglia of the submucous plexus, corroborated by the morphological description of Schofield (1968) for nervous innervation of the intestine wall. However, Ginzel (1959a, b) showed that coagulation of the mucous membrane by silver nitrate does not abolish the peristaltic reflex, and although an ileal segment sheds most of its mucous membrane whilst in an organ bath preparation, the reflex response to distension remains (Diament, 1961). This apparent difference in results could be explained on the basis of the different stimuli used to elicit the respective reflex contractions, or by sensory receptors being also located within the remaining layer of the intestine wall. Interestingly Frigo and Lecchini (1970) showed propulsion of a bolus did not occur in intestinal segments devoid of the submucosal and mucosal layers, and Hirst (1975) showed removal of the submucous plexus disrupts the radial stretch induced descending excitation but not the inhibition. However, according to Costa and Furness (1976) applying a localised stretch to the intestine wall by means of a weight elicits a relaxation anal (descending inhibition) and contraction oral (ascending excitation) to the point of distension which are unaffected by the removal of the submucous plexus and mucosa.

The controversy surrounding the importance of the three intrinsic nerve pathways; descending inhibitory, descending excitatory and ascending excitatory, in the peristaltic reflex may merely reflect the nature of the respective sensory receptors and their location within the wall of the intestine. Therefore although peristalsis can readily be demonstrated in isolated preparations of the intestine and the local responses recorded, analysis is made difficult by the complicated nature of the underlying nerve pathways which are not fully elucidated and show differing susceptibility

to specific modes of stimulation.

If GABA-ergic mechanisms are involved in the control of peristalsis, it would best be demonstrated by GABA antagonism in the myenteric plexus using GABA tachyphylaxis, or bicuculline. Peristalsis as initiated by faecal pellets would approximate as nearly as possible to the natural situation. Therefore modification of peristalsis by such antagonism as measured by faecal pellet propulsion in the isolated guinea-pig distal colon was used in the following study.

Materials and Methods

Guinea-pigs of either sex weighing 250-400 g were stunned by a blow to the head and bled. Segments of the distal colon were removed and placed in Krebs solution of the following composition [mM]: Na^+ 151.0; K^+ 4.7; Mg^{2+} 0.6; Ca^{2+} 2.8; Cl^- 143.7; $H_2PO_4^-$ 1.3; SO_4^{2-} 0.6; glucose 7.7. The solution was bubbled with a gas mixture of 95% O_2 and 5% CO_2 and maintained at 36°C.

Two experimental arrangements were used to examine the effects of GABA antagonism on intestinal motility. Freshly excised segments of distal colon containing faecal pellets were placed into warmed Krebs solution and the subsequent number of pellets expelled over 2 min intervals was recorded in the presence and absence of GABA antagonists. In a further series of experiments similar segments of distal colon, empty of faecal pellets, were arranged as described by Costa and Furness (1976) for recording the passage of faecal pellets along isolated segments of intestine. Pellets of faeces, previously dried and coated with polyurethane, were placed into the lumen at the oral end of

the segment at 10 min intervals, and their passage along a measured portion of the segment was timed. Activity of circular muscle during peristalsis was recorded by Grass FT03C transducers, using 2 g/cm springs connected by clips to the serosa of the colon wall.

Drug volumes were never more than 1% of the bath volume. Drugs used were: bicuculline (K & K Ltd.); gamma-aminobutyric acid (GABA) (Sigma), 5-hydroxytryptamine (Sigma).

Results

As previously described (Krantis *et al.*, 1980), upon the addition of GABA 3.10^{-5} g/ml to the bath an immediate transient relaxation of the colon was observed (Fig. 1). After equilibrium in the presence of this concentration of GABA, a further 3.10^{-5} g/ml of GABA was added to the bath without effect, confirming that desensitization of GABA had occurred. Motility, as measured by the rate of faecal pellet expulsion from the freshly excised colon, was significantly reduced by such desensitization to GABA 3×10^{-5} g/ml or by treatment with bicuculline 5×10^{-6} g/ml, 3.7×10^{-5} g/ml (Fig. 2). On occasion, pellet expulsion entirely ceased during GABA tachyphylaxis. The reduction in the rate of pellet expulsion was readily reversed by washing with Krebs solution.

The mean propulsive speed of the coated faecal pellets as timed along isolated segments of distal colon lay in the range 0.5-1.88 mm/sec, comparable to that observed by Costa and Furness (1976), and was reduced on average by some 60% following the establishment of GABA-tachyphylaxis. Again, in some instances the passage of the pellet completely stopped

FIG. 1. Recording of circular muscle activity from two points in a segment of distal colon during the addition of GABA. Recording O is from a point 20 mm oral to the recording point A. GABA 3×10^{-5} g/ml when first administered induced a relaxation of the distal colon. A second concentration of GABA 3×10^{-5} g/ml, was added to the bathing solution without washing out the previously applied GABA. The development of tachyphylaxis to applied GABA is evident from the lack of effect of the challenge concentration of GABA.

FIG. 2. The effect of GABA antagonism on pellet expulsion from the guinea-pig distal colon. Ordinate, percentage of control expulsion rate. Abcissa, histogram columns (mean and s.e. of 4-6 preparations) (C) control; (B) in the presence of bicuculline 5×10^{-6} g/ml; (B_1) bicuculline 3.7×10^{-5} g/ml; (G) in the presence of a desensitizing concentration of GABA, 3×10^{-5} g/ml.

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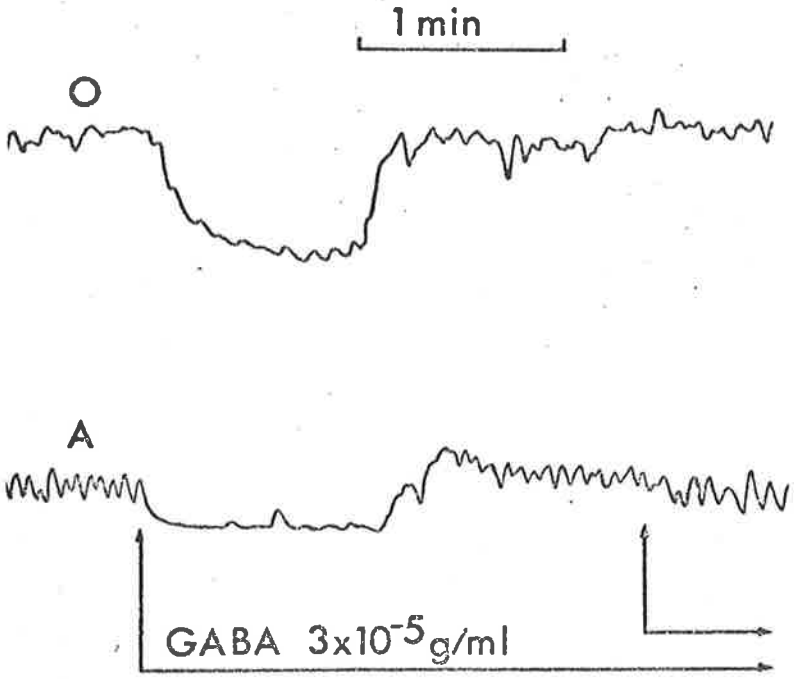
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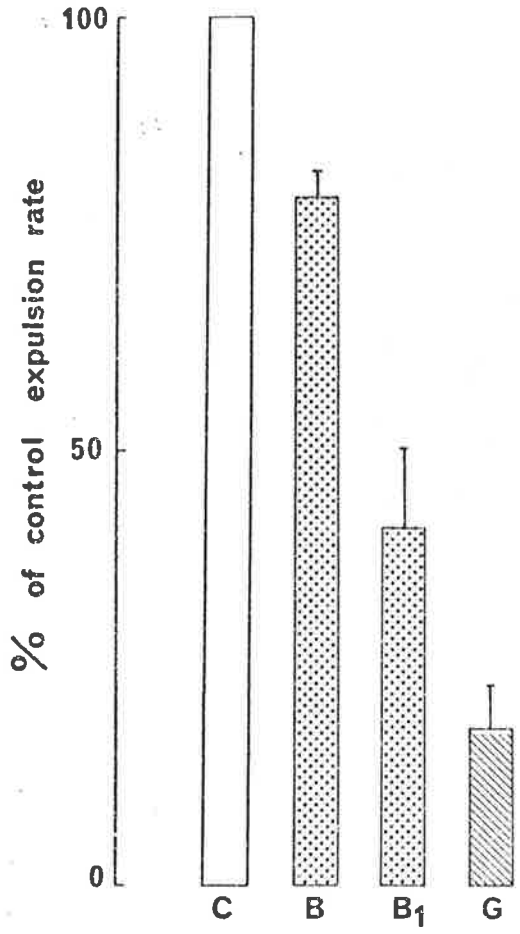
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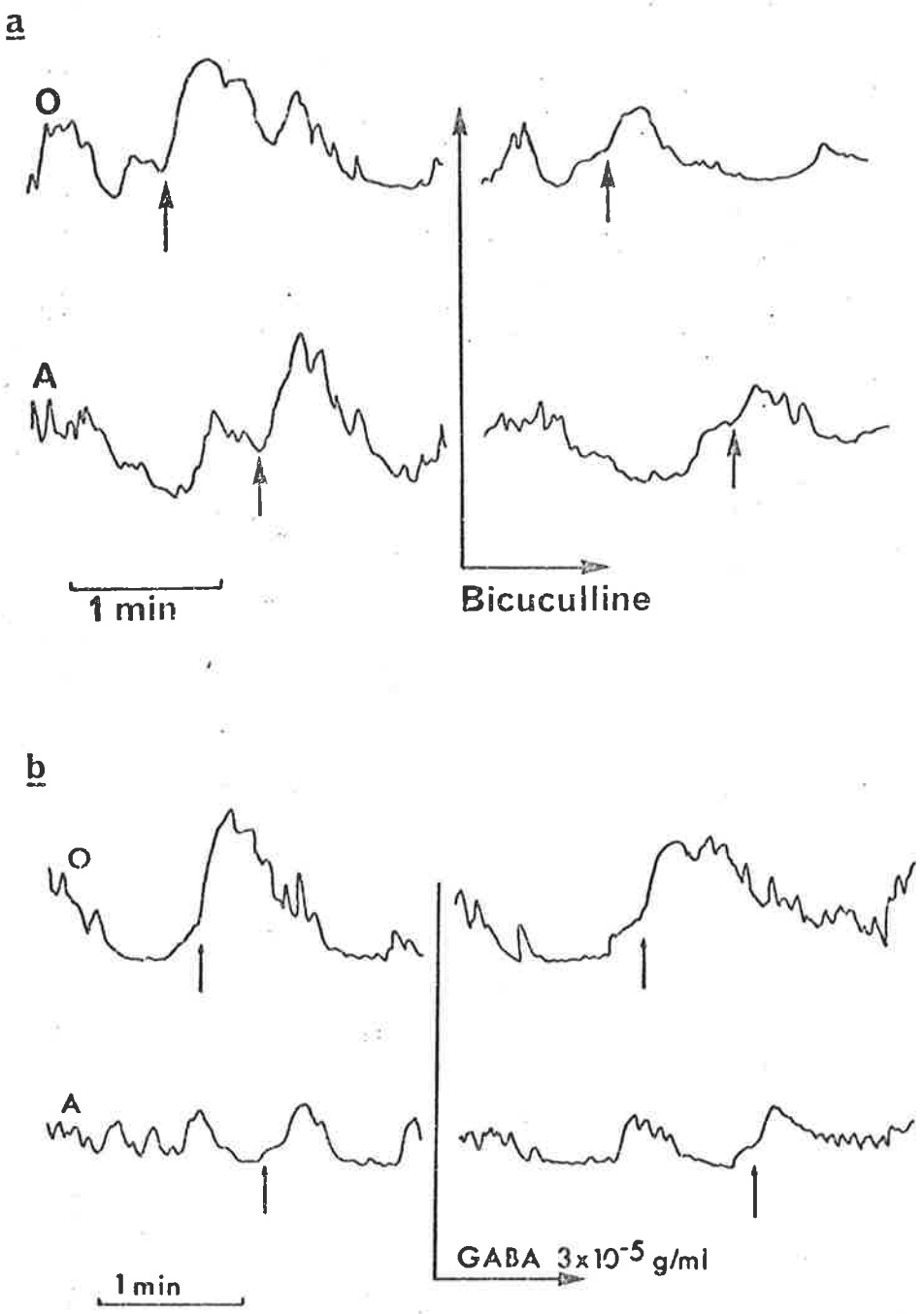


FIG. 3. Recording of circular muscle activity from two points in a segment of distal colon during the passage of a pellet. Recording O is from a point 20 mm oral to the recording point A. The arrows indicate the times at which the pellet passed each recording point. (a) in the absence and presence of bicuculline 3.7×10^{-5} g/ml. (b) GABA 3×10^{-5} g/ml.

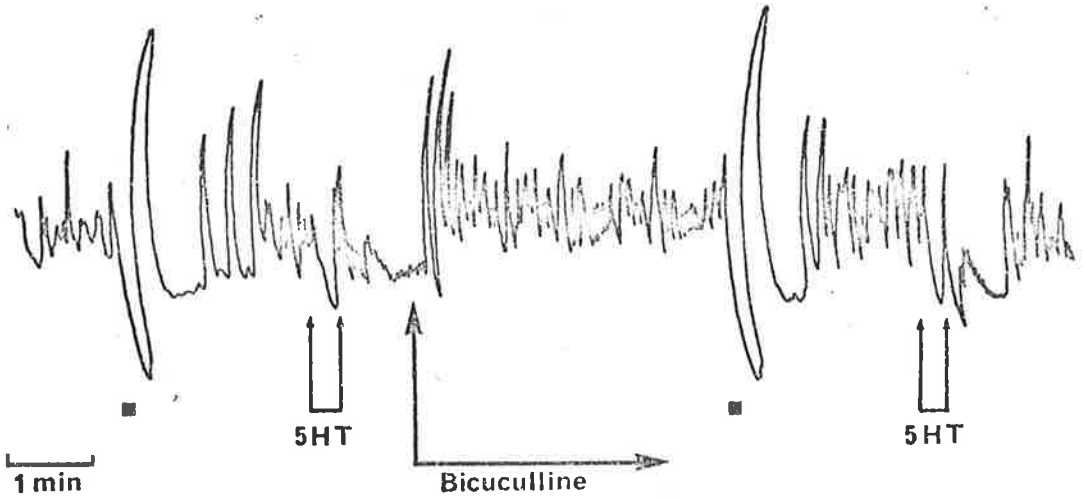


FIG. 4. The effects of bicuculline 5 x 10^{-6} g/ml on relaxations of the guinea-pig distal colon. Electrical stimulation (5 Hz, 0.5 msec duration, 60 V) and 5HT 2 x 10^{-5} g/ml induced relaxations as recorded in the longitudinal muscle were unaffected by bicuculline.

during GABA tachyphylaxis. Repeated washing of the tissue with normal Krebs solution reversed the effect of tachyphylaxis, and the slowing of propulsion was readily reproducible upon re-instituting GABA-tachyphylaxis in the same tissue.

Bicuculline 3.7 x 10^{-5} g/ml, also reduced the mean propulsive speed of the coated pellets by some 50%, and this action, too, was readily reversed following washout from the bath. The reduction in propulsive speed was dose-dependent and reproducible on the same tissue. The passage of the pellet was never stopped in the presence of bicuculline coupling.

During the onset of GABA antagonism following addition of bicuculline to the bath phasic activity, recorded from the circular muscle, was briefly dampened, but the overall 'tone' of the circular muscle was not significantly altered and normal activity was restored within a few minutes. In other preparations where GABA tachyphylaxis was employed, all recorded mechanical activity in the circular muscle first ceased, often with a transient relaxation immediately following addition of GABA 3 x 10^{-5} g/ml to the bath, and then slowly recovered during the onset of tachyphylaxis. The subsequent challenge dose of 3 x 10^{-5} g/ml was without effect. Circular muscle activity during peristalsis was modified both by GABA tachyphylaxis and by application of bicuculline, there being most often a reduction in the amplitude of the contraction of circular muscle behind the pellet, following its passage beneath the recording point (Fig. 3). Only occasionally was there any significant alteration of the relaxation preceeding the passage of the pellet. In a separate experiment segments of distal colon were arranged as described in Chapter II (Methods) so that the effects of electrical and pharmacological stimulation could be analyzed. In the presence of atropine, 2 x 10^{-7} g/ml, transmural stimulation (5 Hz, 0.5 ms, 60 V) and 5HT, 2 x 10^{-5} g/ml induced relaxations of the distal

colon which were unaffected by bicuculline 5 x 10^{-6} g/ml (Fig. 4).

Discussion

Evidently GABA-ergic mechanisms are in some way concerned with maintaining normal peristalsis, since it has now been shown that the rate of propulsion of faecal pellets in the colon is slowed, and the amplitude of the contraction behind the pellet is most often reduced during GABA antagonism by tachyphylaxis or bicuculline. The reduction in propulsive activity by bicuculline does not appear to be due to any possible actions on cholinergic components of peristalsis for bicuculline, at the concentrations used in this study, does not alter acetylcholine induced responses in the colon or ileum (see Chapter II). Furthermore, tetrodotoxin blocks all GABA actions in the intestine (Krantis *et al.*, 1980), so that the reduced motility with GABA-antagonism cannot be due to any direct motor or inhibitory actions of GABA on the smooth muscle of the intestine. Rather, applied GABA, acting through bicuculline-sensitive receptors, stimulates intrinsic neurones of the myenteric plexus (Krantis *et al.*, 1980; Krantis and Kerr, 1981b), and evidently antagonism of the actions of these neurones is responsible for the alterations in motility reported here. There is thus the strong possibility that GABA-ergic interneurones or sensory neurones are involved in normal peristalsis.

Peristalsis is usually described in terms of an ascending excitation and a descending inhibition resulting from a local stimulus, the Law of the Intestine (Bayliss and Starling, 1899). Of these, there is general agreement concerning the descending inhibition, but there remains some controversy as to the nature of the 'excitation' phase contributing to

descending excitation localised to the submucous mucosal layers (Hirst, 1979) and that for the ascending excitation most probably localised to the myenteric plexus (Costa and Furness, 1976).

According to Krantis *et al.* (1980) GABA antagonism, by GABA tachyphylaxis or bicuculline, does not alter the ascending excitatory or the descending inhibitory reflexes elicited by a localised stretch of the intestinal wall, and yet the contraction of the circular muscle behind the pellet was reduced by GABA antagonism in the present study. In this regard, it should be emphasized as Frigo and Lecchini (1970) have already pointed out that a faecal pellet is a different and evidently more physiological stimulus for eliciting peristalsis than is localised stretch of the kind used by Costa and Furness (1976), as well as by Krantis *et al.* (1980). It thus appears that radial stretch of the intestine wall by a pellet or intramural balloon preferentially elicits a reflex excitation of the intestine (Kosterlitz, 1968; Frigo and Lecchini, 1970) by a different neuronal pathway to that stimulated by localised stretch of the intestine wall.

A comparable depression of the descending inhibition was seldom seen in this study, although the GABA receptors on the inhibitory neurones would suggest that it could occur. Failure to observe any such depression may reflect an inappropriate experimental design for its demonstration in the present experiments. Therefore from the present results it appears that the contraction of the colon immediately behind the faecal pellet, elicited by radial stretch of the intestinal wall, involves some GABA-ergic relay, probably interneuronal, the antagonism of which is sufficient to interfere with peristalsis and slow or stop pellet propulsion.

It is generally accepted that the pathways mediating excitatory and inhibitory peristaltic reflexes involve cholinergic interneurones acting

through nicotinic-cholinergic receptors (Akubue, 1966; Burnstock *et al.*, 1966; Kosterlitz, 1968) yet the stimulating effects of GABA on the intestine are not blocked by the nicotinic antagonist pentolinium (Krantis *et al.*, 1980), so that GABA actions in the intestine can not be relayed through cholinergic interneurones.

Hobbiger (1958b) and Inouye *et al.* (1960) have shown that GABA antagonizes the contractile responses of the guinea-pig ileum to nicotine and 5-hydroxytryptamine stimulation of the cholinergic motor neurones. However, it seems unlikely that interactions between GABA and 5HT could explain the present results which are based on GABA antagonsim since it was found that 5HT actions in the guinea-pig distal colon are unaltered by GABA antagonism of the kind shown here to slow peristalsis.

Prejunctional GABA ($GABA_B$) receptors that are bicuculline insensitive and stimulated by Baclofen have been identified in the guinea-pig intestine (Bowery *et al.*, 1981; Ong, 1981). These receptors mediate a reduction in cholinergic nerve activity of the ileum and colon and it is possible that activation of these receptors may contribute to the GABA-ergic control of peristalsis. These receptors cannot be involved in the slowing of peristalsis by bicuculline. However, such an involvement cannot be excluded for the effect of applied GABA since GABA would act at both $GABA_A$ (bicuculline sensitive) and $GABA_B$ receptor populations. Indeed antagonism by desensitization to GABA, in contrast to bicuculline, completely stopped pellet propulsion (similar to the findings of Ong (1981) who also observed that antagonism of $GABA_A$ or $GABA_B$ receptors slowed pellet propulsion in the distal colon, and that these effects were additive resulting in a cessation of propulsive activity).

Many possible transmitters, or modulators, are now being proposed for

the mammalian enteric nervous system, among which we would include GABA as an excitatory transmitter in the guinea-pig intestine where it evidently plays a key role in the control of peristalsis, through interactions at an excitatory and an inhibitory receptor population.

CHAPTER VII

MYENTERIC PURINERGIC NERVES

Introduction

The term 'purinergic' was first used by Burnstock (1971) to describe a third autonomic nerve component which is neither adrenergic nor cholinergic but is thought by him to release as its principal active substance a purine nucleotide. The existence of a third class of autonomic nerves was suggested as early as 1898, when Langley reported that vagal stimulation produced relaxation of the stomach. It was subsequently shown that this 'relaxation' could best be revealed following cholinergic blockade (McSwiney and Robson, 1929). Since adrenergic blockade did not prevent the inhibitory response of the guinea-pig stomach to vagal stimulation (Greef *et al.*, 1962) whilst electrically induced hyperpolarizations or inhibitory junction potentials (IJP's) in guinea-pig taenia coli smooth muscle cells persisted in the presence of both atropine and gaunethidine (Burnstock *et al.*, 1963a, b; 1964), it was proposed that the responses were due to the release of a non-cholinergic non-adrenergic substance. In addition, these responses were prevented by low concentrations of the neurotoxin tetrodotoxin (TTX) (Bulbring and Tomita, 1967), or cold storage of the tissue at 4°C (Kuriyama *et al.*, 1967), which destroys nerves but leaves smooth muscle viable, suggesting a neuronal source for this non-adrenergic non-cholinergic substance. In addition, the effects of ganglion stimulants and blockers on isolated mammalian gut segments (Burnstock *et al.*, 1966), indicate that the neurones involved are intrinsic to the gut, with their soma located to the Auerbach's plexus (myenteric plexus). More recent studies (reviewed in Burnstock, 1972) have since confirmed this hypothesis.

There is now considerable evidence for the function of these non-adrenergic non-cholinergic inhibitory nerves in the mammalian gastro-intestinal tract (see Burnstock, 1972), including mediation of the reflex relaxation of sphincters (oesophogastric, internal anal); receptive

relaxation of the stomach facilitating increased stomach size with food intake, the descending inhibitory component of peristalsis and the mesenteric vasodilator reflex. Nerve-mediated inhibitory responses that involve neither cholinergic nor adrenergic neurones have also been described in the mammalian urinary tract, lung, eye and other organs (Appazeller, 1976; Burnstock and Bell, 1974; Burnstock, 1972, 1975, 1978). However, their roles remain to be determined.

Although various substances have been explored as possible candidates for the non-adrenergic non-cholinergic neurotransmitter in the ENS, none of the following are likely: catecholamines, cyclic-AMP, histamine (HA), 5-hydroxytryptamine (5HT), vasoactive intestinal peptide (VIP), glycine, gutamic acid, gamma-aminobutyric acid (GABA), prostaglandins, bradykinin (BK) and substance P (see Burnstock, 1975 and Chapter I).

According to Burnstock (1975), only ATP comes nearest to fulfilling the criteria for this transmitter. A neurotransmitter role for ATP was earlier suggested at central and peripheral endings of sensory fibres (Holton and Holton, 1954), but never confirmed. Recently adenosine-5'-triphosphate (ATP) has been implicated in neuronal activity, working through mechanisms other than its established bioenergetic actions. Nucleotides are found in all subcellular fractions of mammalian central neurones (Abood *et al.*, 1968). Furthermore, adenine nucleotides are released after electrical stimulation of superfused brain tissue (Pull and McIlwain, 1972) and from synaptosomes prepared from cerebral cortical tissues following electrical or potassium stimulation (Kuroda and McIlwain, 1974). But no pronounced actions of iontophoretically applied ATP are evident at central neurones (Curtis *et al.*, 1961; Krnjevic and Phillis, 1963) and it is therefore far from certain that ATP is the transmitter for any of these neurones. Indeed it is known that ATP forms part of the

noradrenaline (NA) chromogranin complex for NA storage in the adrenal medulla, and sympathetic nerve terminals, and it is also present with acetycholine where it is thought to be involved in maintaining intracellular osmotic pressure (Ribeiro, 1978).

On the other hand, adenine nucleotides do have postjunctional effects at smooth muscle of the mammalian gastrointestinal trace (Burnstock, 1972, 1978) as well as presynaptic actions at the motor axon terminal (Ginsborg and Hirst, 1972; Ribiero and Walker, 1975) and at both adrenergic (Hedqvist and Fredholm, 1976; Enero and Saidman, 1977) and cholinergic (Vizi and Knoll, 1976) nerve terminals localised in the gastrointestinal wall.

The adenine nucleotides, more specifically ATP, have been explored as the transmitter of the intrinsic non-adrenergic, non-cholinergic inhibitory nerves of the gastrointestinal wall (Furness, 1969a, b; Bianchi *et al.*, 1968). ATP when applied to gastrointestinal preparations acts directly upon the smooth muscle (Burnstock *et al.*, 1970) to cause hyperpolarization of the muscle cells (Axelsson and Holmberg, 1969; Malchikova and Poskonova, 1971). These actions of ATP are characteristic of the transmitter released by the non-adrenergic non-cholinergic inhibitory nerves (see Burnstock, 1972). Adenosine diphosphate (ADP) and adenosine also have similar actions, but ATP is the most potent.

The initial evidence for ATP or a related nucleotide as a possible inhibitory neurotransmitter came from the studies by Burnstock *et al.* (1970) and Satchell and Burnstock (1971) who observed elevated nucleoside levels in the venous efflux during stimulation of non-adrenergic non-cholinergic inhibitory fibres to the stomach in both toads and guinea-pigs. More direct evidence for the release of ATP from these nerves comes from a

study by Su *et al.* (1971), where chemical and electrical stimulation of enteric neurones caused a marked increased in the release of (^{3}H) from taenia coli incubated with (^{3}H) adenosine, which was blocked by TTX but not by gaunethidine. However the authors point out that they could not be certain whether the neurones that release [^{3}H] adenosine were non-adrenergic non-cholinergic inhibitory neurones.

In contrast to its inhibitory action, ATP also has direct excitatory actions on the smooth muscle of a number of mammalian organs (Burnstock, 1972) including the guinea-pig small intestine. These excitatory effects were generally more prevalent in preparations with low tone (e.g. rabbit stomach and guinea-pig ileum) and tissues often showed biphasic effects (brief contraction followed by relaxation) with applied ATP. In the guinea-pig colon the inhibitory mode predominates, whereas the ileum responds with relaxation at lower concentrations but with contraction at higher concentrations of ATP. Evidence has been presented that adenosine 5'-triphosphate (ATP) is an excitatory transmitter in the guinea-pig (Burnstock *et al.*, 1978), rabbit (Downie and Dean, 1977) and rat bladder (Brown *et al.*, 1979). However at present it is unclear whether the excitatory action of ATP in the intestine is in anyway related to a separate class of non-cholinergic excitatory "purinergic" neurones, since many of the effects are depressed by prostaglandin synthesis inhibitors (Brown and Burnstock, 1981). However a dual action for ATP on smooth muscle would not be an uncommon phenomenon, being established as normal functional modes for both acetylcholine (ACh) and noradrenaline (NA) (Ahlquist, 1948). The existence of two distinct populations of postsynaptic receptors for ATP has been investigated in the guinea-pig urinary bladder and taenai coli (Burnstock *et al.*, 1972a, b), yet no conclusions could be drawn from their observations.

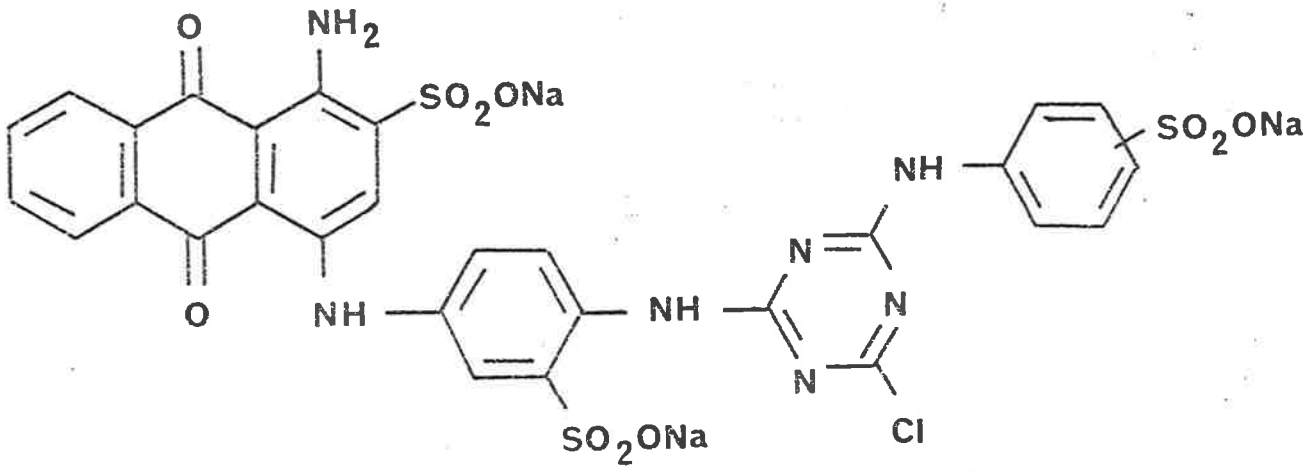
The ability of ATP to desensitize tissue (tachyphylaxis) (Holman and

Hughes, 1965) has been used to determine the involvement of ATP in the non-cholinergic excitatory and non-cholinergic non-adrenergic inhibitory responses of numerous preparations (Burnstock, 1972, 1978; Ohga and Taneike, 1977; Bartlett *et al.*, 1979), but with considerable disparity in the results amongst studies using similar or different tissues. In addition to the failure of some smooth muscle to develop tachyphylaxis to ATP, the phenomenon of desensitization does not allow researchers to distinguish specific sites of drug actions. Specific antagonists for the respective postsynaptic actions of ATP are presently not available, although several compounds have been used as such agents. These include high concentrations of quinidine (Burnstock *et al.*, 1970), imidazoline (Rikimaru *et al.*, 1971b; Tomita and Watanabe, 1973; Satchell *et al.*, 1973), methylxanthines (Ally and Nakatsu, 1976; Okwuasaba *et al.*, 1977) and the isatogens (see Burnstock, 1972), but only the latter is in any way specific, and at that, non-competitive. Indeed the methylxanthines are now considered to be only relatively specific against adenosine actions in reducing transmitter output (Burnstock, 1979). The non-specific and wide ranging pharmacological actions together with the considerable loss of smooth muscle tone that vitiates the use of these substances, has not allowed any reliable determination of the nature of the sites at which ATP acts.

The lack of a specific ATP receptor antagonist has prevented any definitive assessment of the possible role of purine nucleotides as transmitters in a number of tissues proposed to receive 'purinergic' nerve innervation.

Recently, Bohme *et al.* (1972) described a class of anthraquinone-sulphonic acid derivatives which can be looked upon as ATP analogs, where the 1-amino and 3' sulphonic groups are close in position to the γ-phosphate and 6-amino of the purine in the anti-conformation. These

Fig. 1



Cibacron blue 3GA

compounds have a specific affinity for the "dinucleotide fold", and this capacity to act as an affinity 'label' for ATP prompted the investigation of one of these derivatives (CB3G-A) Reactive Blue 2 (C.I. 61211) (Fig. 1) as an antagonist of ATP actions in the guinea-pig intestine. Furthermore an investigation of the involvement of ATP in the non-cholinergic non-adrenergic relaxation of the distal colon and in the non-cholinergic excitation of the ileum was carried out.

Methods

Guinea-pigs of either sex weighing 200-400 grams were stunned by a blow to the head and bled. Portions of the proximal part of the ileum or distal colon were quickly removed and placed into warmed modified Krebs solution (37°) (see Chapter II, methods) gassed with a mixture of 95% O_2 and 5% CO_2. All subsequent treatments were carried out in a similar solution.

Segments 4 cm in length, were carefully denervated of their mesenteric attachments and prepared as a conventional isolated smooth muscle preparation. Individual tissues were arranged inside a glass organ bath (10 ml) with one end attached to the bottom of the bath and the other end attached by a cotton thread to a rotary or force transducer. Changes in the length of the intestinal segments were recorded using a Grass Polygraph (Model 5D). Individual tissues were allowed to equilibrate for 40-60 min prior to the addition of the test agonist. Where an antagonist was added during the course of the experiment an appropriate equilibration time was allowed before adding the agonist.

Electrical Stimulation

Transmural stimulation of the intrinsic nerves was by a ring electrode consisting of two platinum wires 3 mm apart, positioned so that the tissue within the ring was in contact with the exposed platinum wires. Pulses were delivered from a Grass 548 stimulator using maximal voltage. The parameters for the different nerve stimulations are as follows: Non-adrenergic inhibitory nerves: 2 - 10 Hz, 0.5 msec duration, 10 sec stimulation period. Non-cholinergic excitatory nerves: 10 - 50 Hz, 0.2 msec duration, 10 sec stimulation period. Cholinergic excitatory nerves (twitch): 0.1 Hz, 0.1 msec duration, of varying train length. Where it was necessary to eliminate cholinergic events, atropine 2×10^{-7} g/ml was kept in the bath to block muscarinic receptors.

Drugs

The volume of drug added was never more than 1% of the total bath volume. Drugs used were: adenosine; adenosine 5'-triphosphate, aminophylline, atropine sulphate, bradykinin, cibacron blue 3G-A, gamma-aminobutyric acid (GABA), histamine, noradrenaline bitatrate, papaverine, tetrodotoxin (TTX).

Analysis

Responses are expressed as mm response, or % of maximum control response. Statistical significance was established using Student's t-test for paired and unpaired samples. Evaluation of dose response effects was by a Clark Plot (Clark, 1928). Lines of best fit were estimated by

regression analysis (least squares method). Regression line slopes of the Clark plot were compared using the method described in Chapter II.

Results

Responses of the Distal Colon to ATP

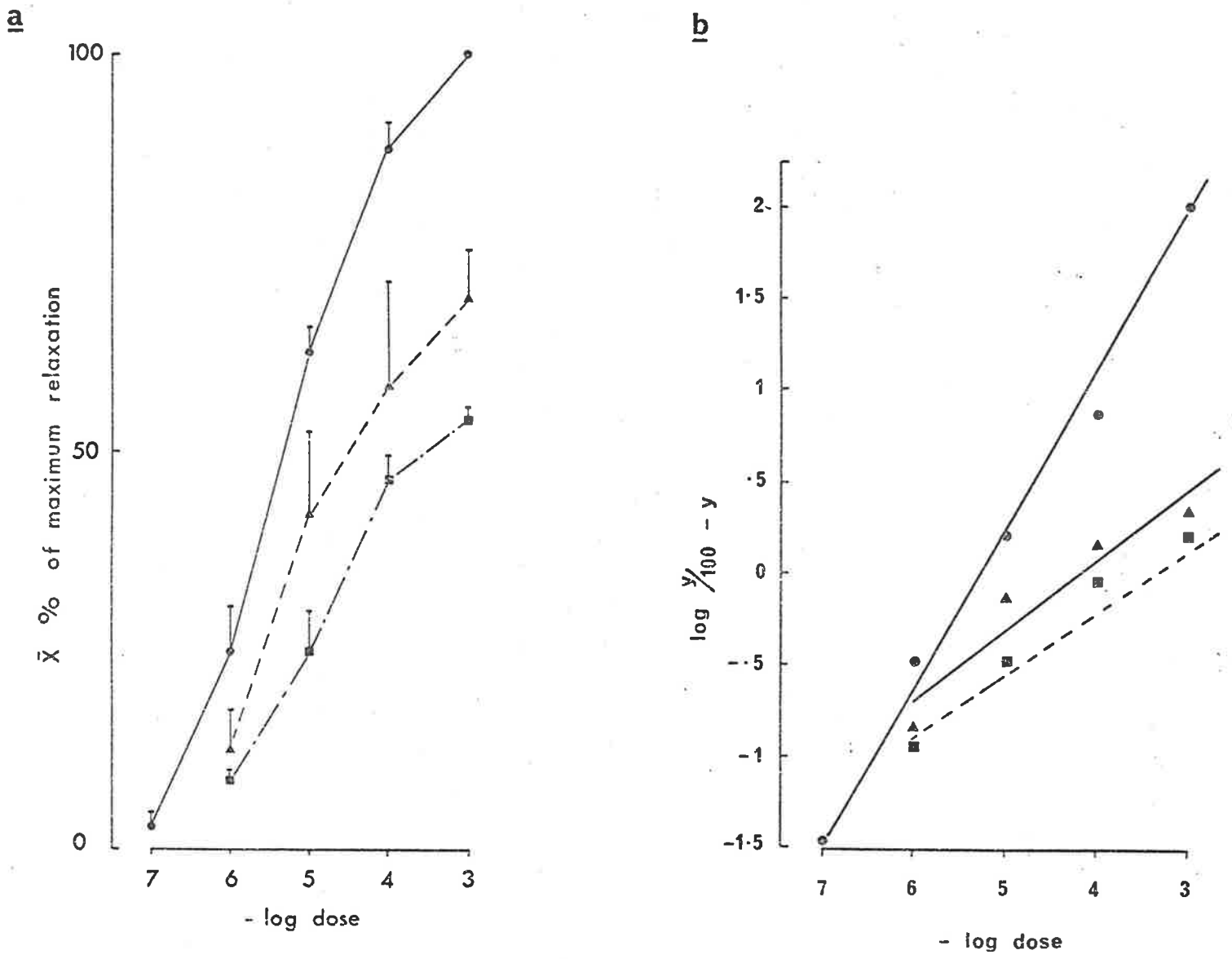
Since it was desired to investigate the relaxation *per se*, the experiments described here were carried out with atropine (2×10^{-7} g/ml) present in the bathing solution throughout. ATP produced concentration-dependent relaxations of isolated segments of distal colon. The relaxation was maximum within 20 secs and the response was sustained for the duration of ATP contact (30 sec). Where ATP was left in the bathing medium for longer than 30 sec the tone of the tissue usually returned to its resting value over a period of 8-10 min. ATP induced relaxations were unaltered in the presence of tetrodotoxin (TTX) 10^{-7} g/ml. The threshold concentration for the ATP induced relaxation was about 10^{-7} g/ml and maximum relaxation occurred with 10^{-3} g/ml (Fig. 2a).

A number of preparations showed marked tachyphylaxis to ATP (Mihich *et al.*, 1954; Kim *et al.*, 1968; Burnstock *et al.*, 1970). In this study, attempts to desensitize the tissue to ATP failed to show any tachyphylaxis which allowed the use of cumulative dose-response curves for the actions of ATP in the distal colon. This experimental design shortened the exposure time for the colon segments to the antagonist CB3G-A, thereby minimising the possibility of loss of tone, that has plagued the use of other antagonists in such studies.

FIGURE 2.

(a) The effect of CB3G-A on the concentration response curves for the relaxation elicited by ATP in the guinea-pig distal colon. ● responses in normal solution, ▲ responses to ATP 20 min after the addition of CB3G-A 1.2×10^{-5} g/ml to the bathing solution, ■ in the presence of CB3G-A 1.5×10^{-5} g/ml. Each point is the mean and standard error of responses in 4-8 preparations. Atropine 2×10^{-7} g/ml was present in the bath throughout the experiment.

(b) Clark plot of the ATP concentration response curve ● control responses, ▲ responses in the presence of CB3G-A 1.2×10^{-5} g/ml ■ 1.5×10^{-5} g/ml.


a
100
50
0
X̄ % of maximum relaxation
7
6
5
4
3
- log dose
b
2
1·5
1
·5
0
-·5
-1
-1·5
log y/100 - y
7
6
5
4
3
- log dose

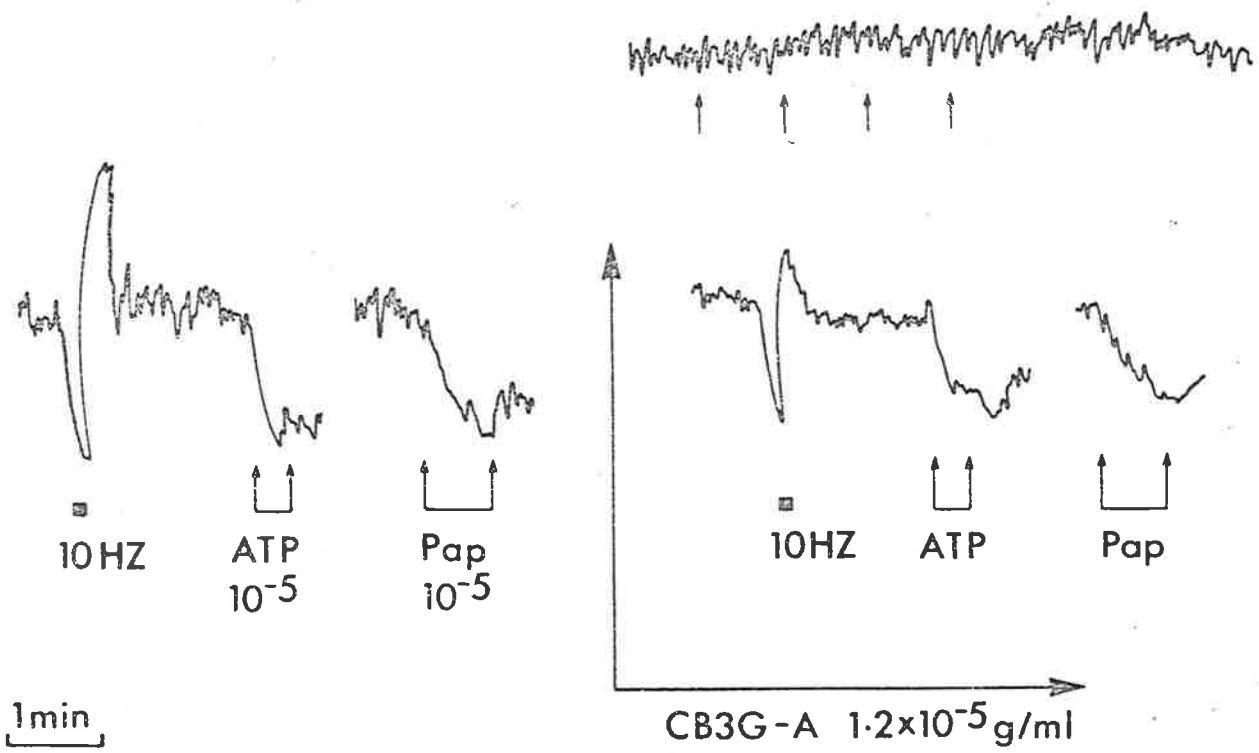


FIGURE 3. The effect of CB3G-A on relaxations of the guinea-pig distal colon to stimulation of the non-adrenergic inhibitory nerves. Electrical stimulation: 10 Hz, 0.2 msec duration, 60 V. ATP, 10^{-5} g/ml. Papaverine 10^{-5} g/ml. CB3G-A 1.2×10^{-5} g/ml was added in increments of 3×10^{-6} g/ml (indicated by arrows in uppertrace). Atropine 2×10^{-7} g/ml was present in the bathing solution throughout the experiment.

In general there is difficulty associated with investigation of distal colon relaxation, since most putative antagonists of the relaxation cause considerable loss of smooth muscle tone (McFarland *et al.*, 1971; Spedding *et al.*, 1975). A rapid drop in 'tone' during the administration of CB3G-A up to 5 x 10^{-5} g/ml could be avoided by raising the concentration of CB3G-A in increments of 3 x 10^{-6} g/ml added every 1-5 min, depending on the individual preparation. This avoided the necessity to 're-tone' the tissue and thereby limited the number of drugs present in the bathing solution. An equilibration period up to 10 min was usually allowed in the presence of CB3G-A before commencing the addition of ATP.

The effect of two concentrations (1.2 and 1.5 x 10^{-5} g/ml) of CB3G-A on the cumulative dose-response curve for ATP are shown in Fig. 2a. Both concentrations of the antagonist brought about a non-parallel shift of the curve to the right, and depression of the maximum response to ATP indicating the antagonism by CB3G-A to be of the non-competitive type. The relaxation elicited by ATP in the presence of atropine (2 x 10^{-7} g/ml) was similar to that for transmural stimulation at a low frequency (10 Hz) (Fig. 3) which according to a number of studies (Furness and Costa, 1972; Bennett *et al.*, 1966; Burnstock *et al.*, 1970) is an effective stimulus for the enteric inhibitory nerves. Although CB3G-A 1.2 x 10^{-5} g/ml reduced by 40% the ATP (10^{-5} g/ml) induced relaxation, it was without effect against the relaxation due to electrical stimulation, or the smooth muscle relaxant papaverine (10^{-5} g/ml). The so called 'rebound' contraction which is observed following cessation of electrical stimulation (Burnstock *et al.*, 1970) was also reduced by more than 50%.

At the two concentrations 1.2 and 1.5 x 10^{-5} g/ml of CB3G-A which caused significant shifts in the ATP dose-response curves, the voltage-response curve for the electrically induced relaxation was not significantly

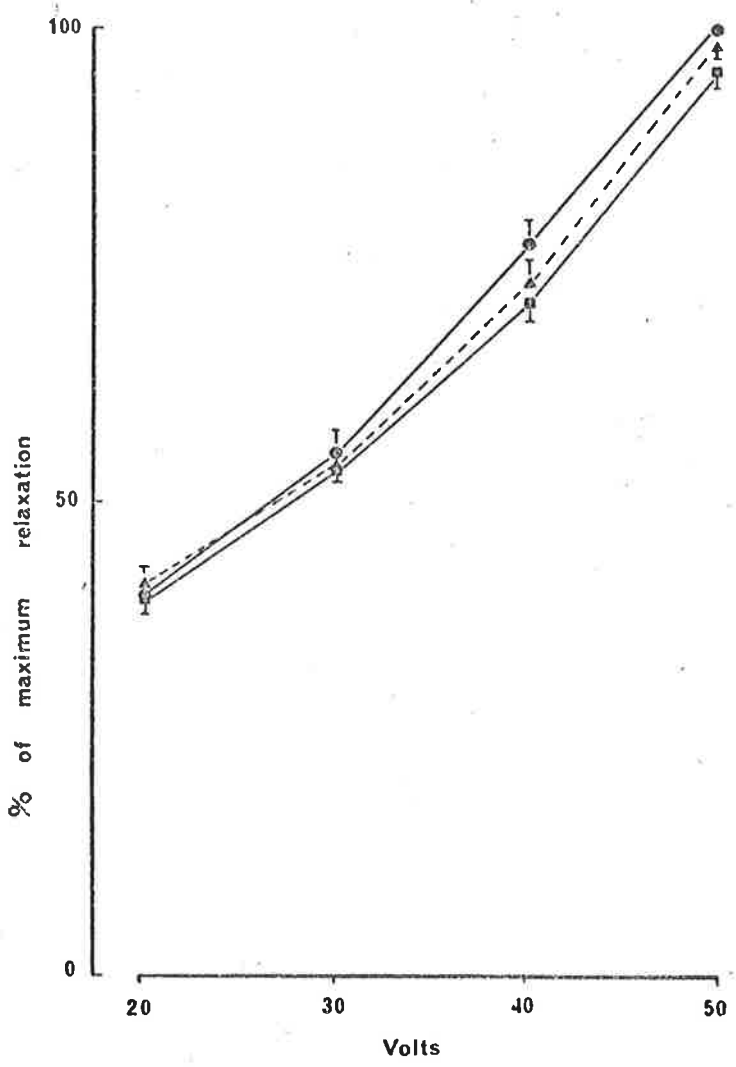


FIGURE 4. The effect of CB3G-A on the voltage response curve for the electrically induced relaxation of the guinea-pig distal colon. ● control responses, ▲ in the presence of CB3G-A 1.2×10^{-5} g/ml, ■ CB3G-A 1.5×10^{-5} g/ml. Electrical stimulation: 10 Hz, 0.5 msec duration. Each point is the mean and standard error of responses from 5 preparations. Atropine 2×10^{-7} g/ml was present in the bathing solution throughout the experiment.

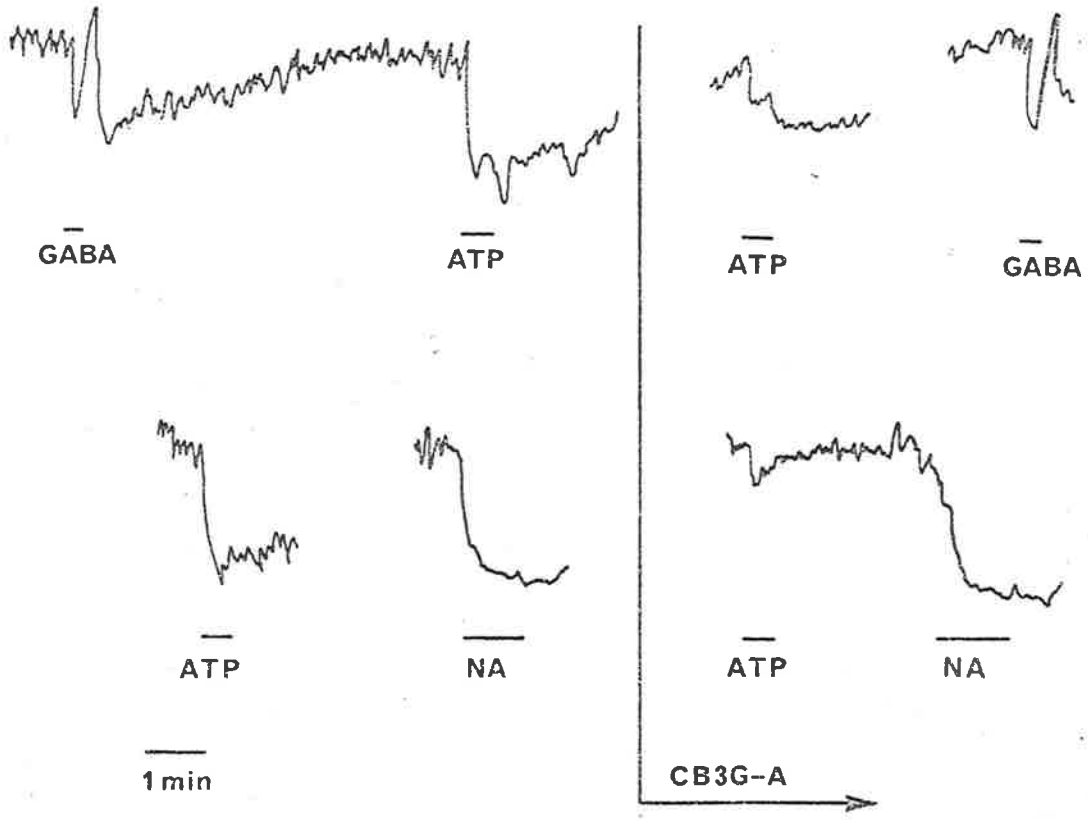


FIGURE 5. The effect of CB3G-A 1.2 x 10^{-5} g/ml on relaxations of the guinea-pig distal colon to various substances: GABA 10^{-5} g/ml, ATP 5 x 10^{-5} g/ml and noradrenaline (NA) 10^{-8} g/ml. CB3G-A was added 20 min before the responses were retested. Atropine 2 x 10^{-7} g/ml was present in the bathing solution throughout the experiment.

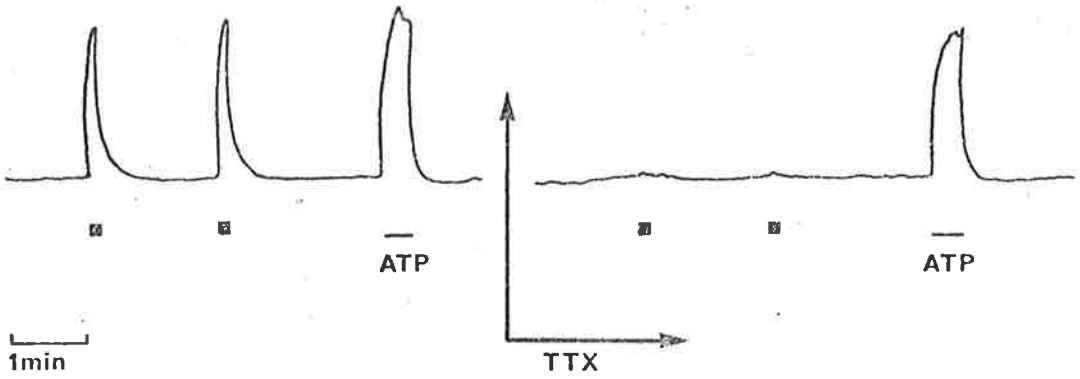


FIGURE 6a. The effect of tetrodotoxin (TTX) on non-cholinergic contractions of the guinea-pig ileum. ▪ 30 Hz, 0.2 msec duration 60 V. ATP, 2×10^{-5} g/ml. TTX 10^{-7} g/ml was added 15 min before the responses were retested. Atropine 2×10^{-7} g/ml was present in the bath throughout the experiment.

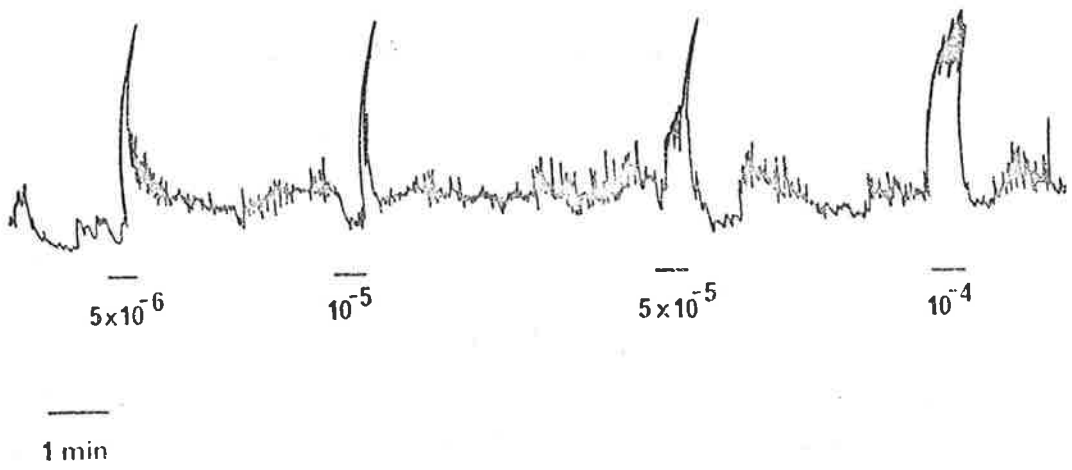


FIGURE 6b. Concentration dependent actions of ATP on the guinea-pig ileum. The drug concentrations shown are in g/ml. Atropine 2×10^{-7} g/ml was present in the bathing solution throughout the experiment.

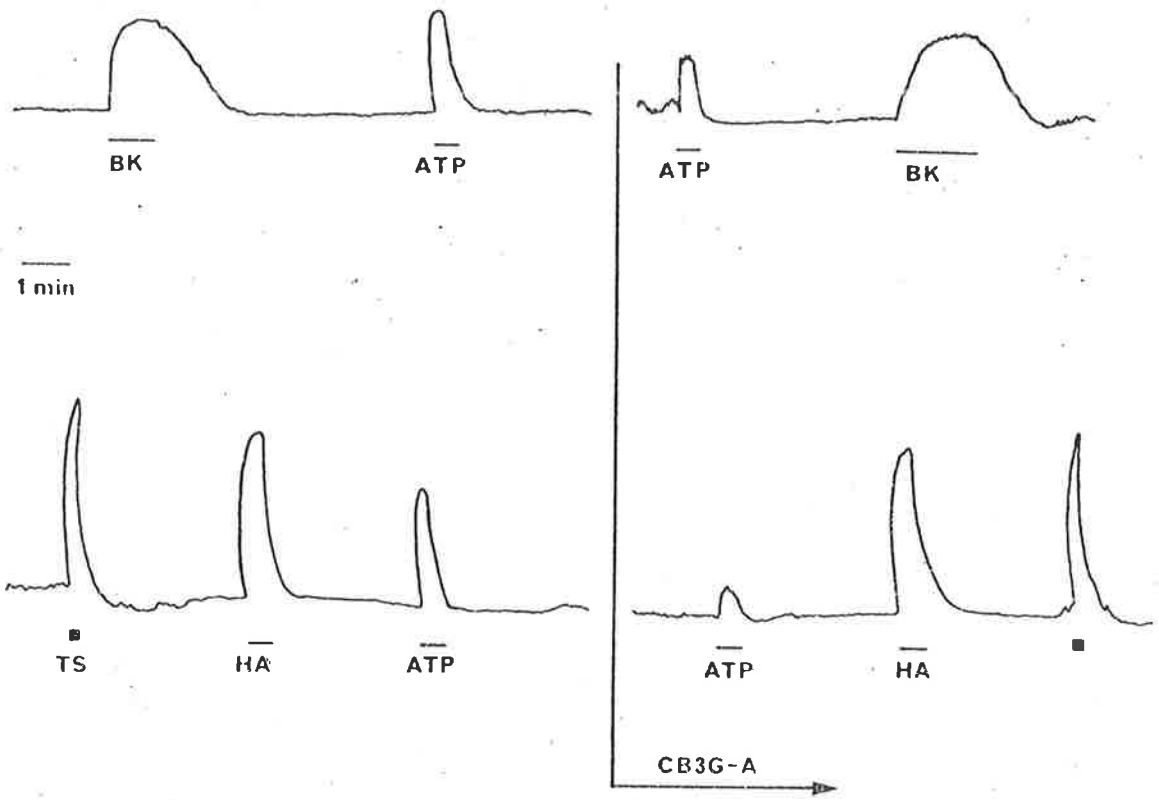
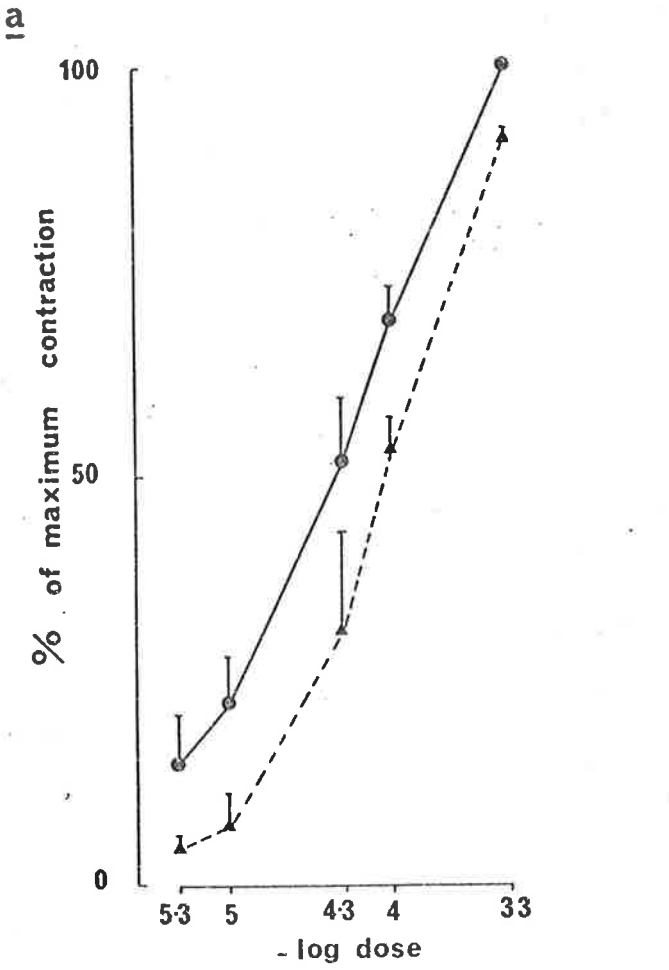


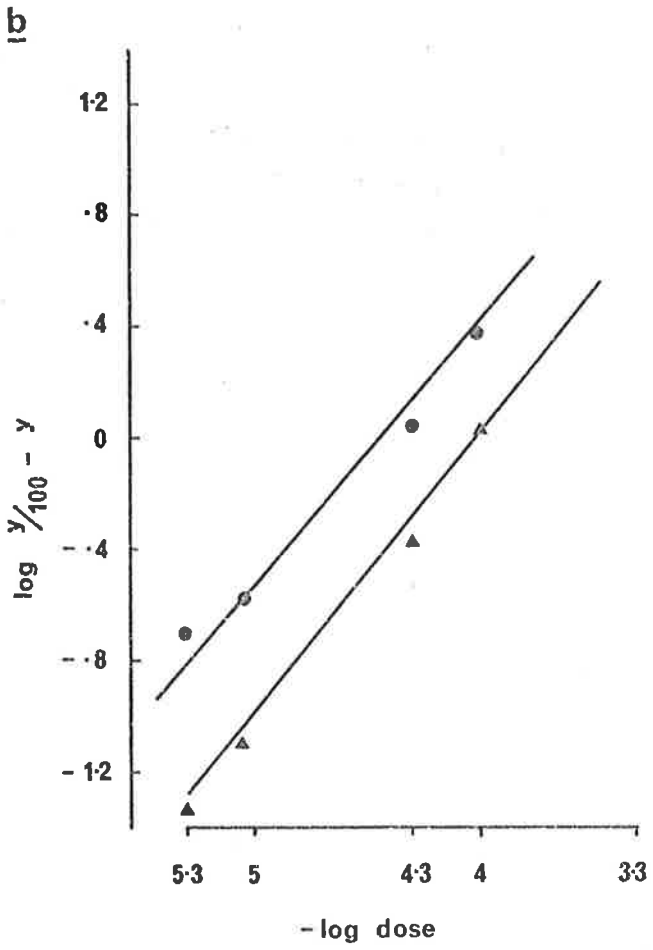
FIGURE 7. Non-cholinergic contractions of the guinea-pig ileum in the presence and absence of CB3G-A 9 x 10^{-7} g/ml. Electrical stimulation (TS) 30 Hz, 0.2 msec duration, 60 V. Histamine (HA) 3 x 10^{-7} g/ml. Bradykinin (BK) 5 x 10^{-9} g/ml. Adenosine-5'-triphosphate (ATP) 10^{-5} g/ml. CB3G-A was added to the bathing solution 15 min before the responses were retested. Atropine 2 x 10^{-7} g/ml was present in the bathing solution throughout the experiment.

FIGURE 8.

(a) The effect of CB3G-A on the concentration response curve for the ATP induced contractions of the guinea-pig ileum. ● responses in normal solution, ▲ responses to ATP 20 min after the addition of CB3G-A 9×10^{-7} g/ml to the bathing solution. Each point is the mean and standard error of responses in 6-8 preparations. Atropine 2×10^{-7} g/ml was present in the bath throughout the experiment.

(b) Clark plot of the ATP concentration response curve ● control respones, ▲ responses in the presence of CB3G-A 9×10^{-7} g/ml.


a
100
50
0
% of maximum contraction
5·3
5
4·3
4
3·3
- log dose


b
1·2
·8
·4
0
- ·4
- ·8
- 1·2
log y/100 - y
5·3
5
4·3
4
3·3
- log dose

affected (Fig. 4). Noradrenaline (NA) (receptors for which are located to the longitudinal muscle of the colon) and GABA-induced relaxations by stimulation of intrinsic inhibitory neurones (Krantis *et al.*, 1980) of the distal colon were unaffected by CB3G-A, 1.2×10^{-5} g/ml (Fig. 5).

Responses of the Ileum to ATP

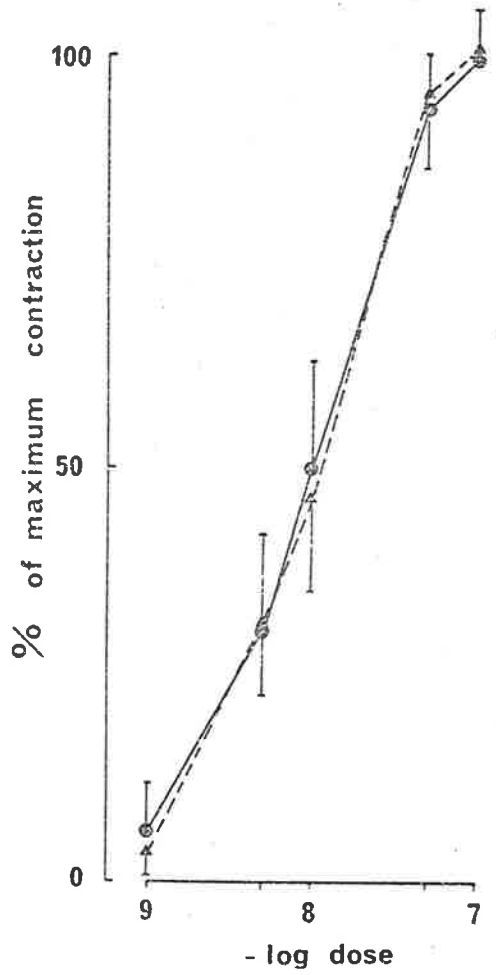
Responses of the ileum to ATP, in the presence or absence of atropine 2×10^{-7} g/ml, were found to vary depending on the 'tone' of the preparation (see Cocks and Burnstock, 1979; Campbell, 1966; Burnstock *et al.*, 1970). In ileal segments of 'medium tone', in which either relaxation or contraction could be easily observed, the type of response elicited by ATP varied with the concentration used (Fig. 6a). At low concentrations only relaxations occurred; biphasic responses were elicited at concentrations around 5×10^{-5} g/ml, whilst only contractions were evident at higher concentrations of ATP. These responses were the result of ATP actions directly upon the muscle since the responses were unaltered in the presence of TTX 10^{-7} g/ml (Fig. 6b).

In the presence of atropine (2×10^{-7} g/ml), ileal segments of 'low tone' were contracted by ATP (5×10^{-6} - 5×10^{-4} g/ml). At a concentration lower than that used to antagonise relaxations of the distal colon to ATP, CB3G-A 9×10^{-7} g/ml reduced the contraction of the ileum to ATP but did not significantly affect the response to transmural stimulation (30 Hz) or to the action of either bradykinin (BK) 5×10^{-9} g/ml or histamine (HA) 3×10^{-7} g/ml both of which are proposed to contract the ileal longitudinal muscle directly (Daniel, 1968; Bolton, 1979) (Fig. 7). The concentration-response curve for ATP (Fig. 8a) but not that for BK or HA (Figs. 9a, b) was shifted to the right. Although this action of CB3G-A was specific

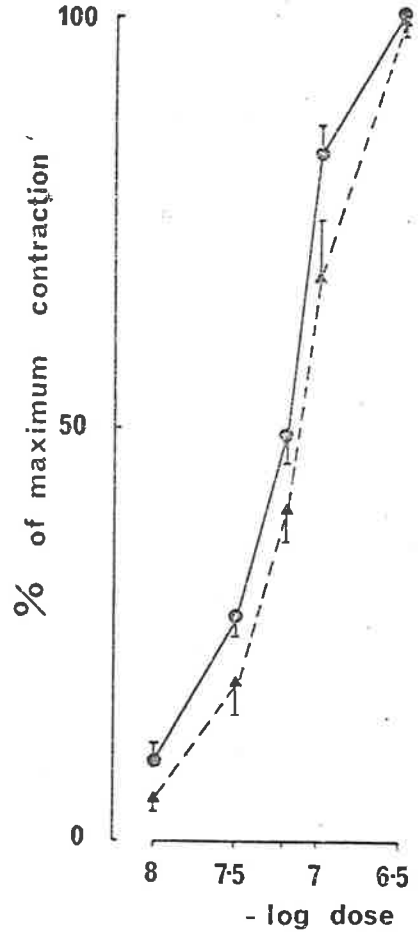
FIGURE 9a and b.

The effect of CB3G-A on the concentration response curves for contractions of the guinea-pig ileum by histamine (HA) (a) and bradykinin (BK) (b). ● responses in normal solution, ▲ responses 20 min after the addition of CB3G-A 9 x 10^{-7} g/ml to the bathing solution. Each point is the mean and standard error of responses in 6 preparations.

a


100
50
0
% of maximum contraction
9
8
7
- log dose

b


100
50
0
% of maximum contraction
8
7·5
7
6·5
- log dose

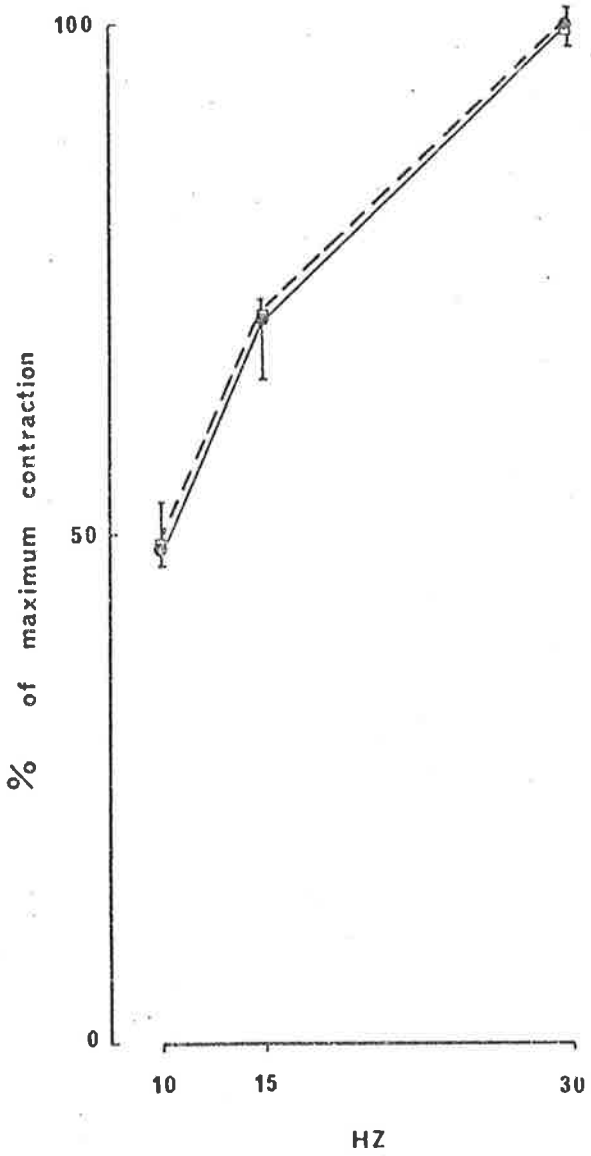


FIGURE 10. The frequency response curve for the non-cholinergic excitatory nerves in the presence and absence of CB3G-A. Electrical stimulation: 0.2 msec duration, 60 V. ● control responses, □ in the presence of CB3G-A 9×10^{-7} g/ml. Each point is the mean and standard error of responses from 6-10 preparations. Atropine 2×10^{-7} g/ml was in the bathing solution throughout the experiment.

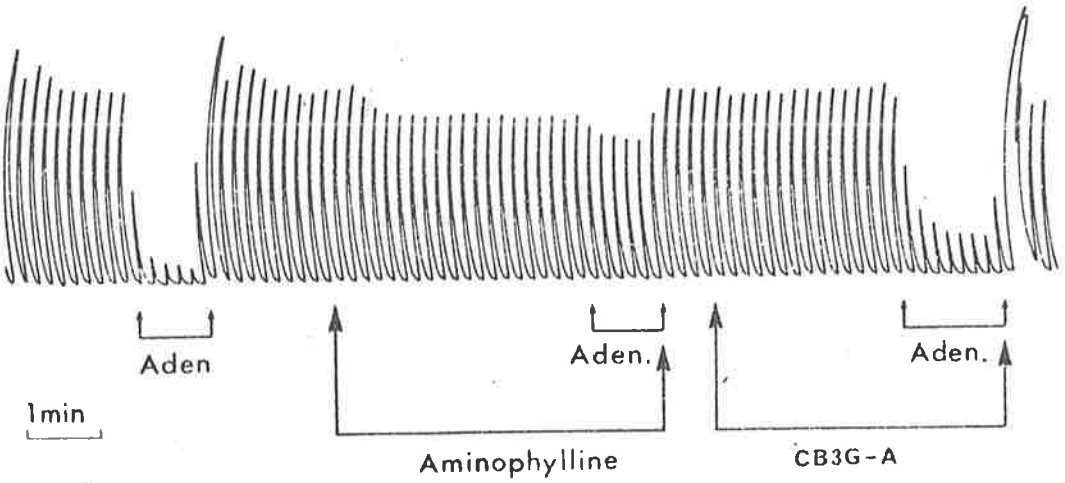


FIGURE 11. The affect aminophylline 7 x 10^{-5} g/ml and CB3G-A 9 x 10^{-7} g/ml on the inhibition of the cholinergic twitch response elicited by electrical stimulation of the guinea-pig ileum. Electrical stimulation: 0.1 Hz, 1 msec duration, supramaximal voltage. Adenosine was added at a final concentration of 10^{-6} g/ml.

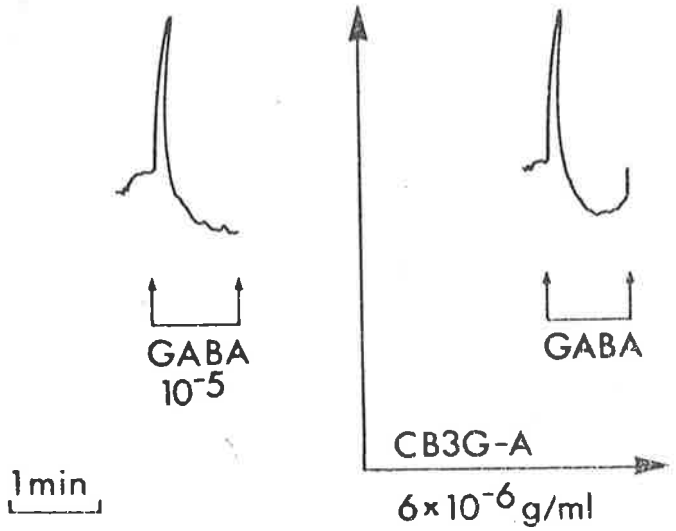


FIGURE 12. The effect of CB3G-A on the biphasic response of the guinea-pig ileum to GABA. CB3G-A 6 x 10^{-6} g/ml was added 15 min before retesting the response to GABA 10^{-5} g/ml.

for ATP induced responses, the non-parallel shift in the Clark plot (Fig. 8b) (significant, d.f. = 6, $P>0.05$) indicates this antagonism to be of the non-competitive type. At concentrations greater than 9×10^{-7} g/ml the action of CB3G-A became non-specific. The lack of antagonism by CB3G-A 9×10^{-7} g/ml of contractions induced by transmural stimulation of the non-cholinergic non-adrenergic excitatory nerves, suggests that ATP is not involved in the response mediated by these nerves. This is further supported by the lack of effect of CB3G-A on the frequency-response curve for the relaxation induced by electrical stimulation of these nerves (Fig. 10) despite the fact that the dose-response curve for ATP is shifted to the right by CB3G-A at the same concentration.

ATP and related substances modify adrenergic and cholinergic neurotransmission by inhibiting release of the respective neurotransmitter (Burnstock, 1972; Bartlett *et al.*, 1979; Hedqvist and Fredholm, 1976; Sawynok and Jhamandas, 1976) with an order of potency of, adenosine>AMP>ADP>ATP.

In order to determine whether CB3G-A is an antagonist of these prejunctional purine actions, the effect of CB3G-A 9×10^{-7} g/ml was compared with that of aminophylline 7×10^{-5} g/ml (a specific antagonist of adenosine, Bartlett *et al.*, 1979) in counteracting the effects of adenosine upon the electrically induced cholinergic twitch contractions in the ileum (Fig. 11). Adenosine 5×10^{-7} g/ml reversibly inhibited the transmurally stimulated contractions, and the inhibition by adenosine was prevented by aminophylline. However, in the presence of CB3G-A the adenosine-induced reduction in the responses to transmural stimulation was barely altered, and CB3G-A had no effect on the control responses alone. Following washouts of aminophylline and CB3G-A, the ileal contractions quickly recovered to their control amplitude.

GABA has been shown to stimulate the intrinsic cholinergic excitatory and non-cholinergic non-adrenergic inhibitory neurones of the ileum (Krantis *et al.*, 1980). Fig. 12 shows that this action of GABA (10^{-5} g/ml) on a segment of guinea-pig ileum was unaltered by CB3G-A (6×10^{-6} g/ml).

Discussion

Distal Colon

The results of the present study have demonstrated inhibitory actions of ATP on the guinea-pig distal colon, consistent with reports that this compound relaxes smooth muscle in a number of preparations (Burnstock, 1972, 1975). These relaxations were not due to ATP, or a related nucleotide, acting indirectly through adrenergic or non-cholinergic non-adrenergic inhibitory nerves, since TTX abolished the inhibitory responses to intramural nerve stimulation and CB3G-A antagonised ATP actions without affecting noradrenaline (NA) induced relaxations. Although there was parallelism between the effects of exogenously applied ATP and stimulation of non-adrenergic inhibitory neurones in the distal colon, only the ATP induced relaxations were antagonised by CB3G-A.

A variety of compounds have been shown to antagonise the actions of ATP and related nucleotides on gut muscle and other tissues, including quinidine (Burnstock *et al.*, 1970), imidazolines (Satchell *et al.*, 1973; Rikimaru *et al.*, 1971), 2-2'-pyridylisatgen (Spedding *et al.*, 1975; Rattan and Goyal, 1980) and methylxanthines (Okwuasaba *et al.*, 1977). However, at the concentrations used, all of the putative ATP antagonists so far tested have been found to be non-specific (Burnstock, 1975; Spedding *et al.*,

1975; Rikimaru *et al.*, 1971; Dean and Downie, 1978; Nickerson, 1970). The effects of the anthraquinone sulphonic acid derivative CB3G-A indicate that this substance (within a narrow concentration range), is a specific, although non-competitive, antagonist of ATP actions in the guinea-pig large intestine.

In addition to its ability to relax certain smooth muscle, ATP has been shown to cause the contraction of smooth muscle in a number of mammalian tissue (Burnstock *et al.*, 1972; Downie and Dean, 1977; Brown *et al.*, 1979; Burnstock *et al.*, 1978; Moritoki *et al.*, 1979; Westfall *et al.*, 1978). The ability of ATP to reversibly contract ileal preparations of low 'tone' in this study, in the presence of atropine and TTX, suggests a direct action of ATP upon the muscle through a non-cholinergic mechanism, and not through cholinergic neurones as is the case in the canine intestine (Northway and Burks, 1980) and rat ileum (Sakai *et al.*, 1979). Since the ileum is supplied by non-cholinergic non-adrenergic inhibitory and non-cholinergic excitatory nerves (Furness and Costa, 1973), there is the possibility that ATP could be the contractile substance mediating non-cholinergic excitatory transmission.

Ileum

Evidence advanced by other studies to suggest that ATP is the transmitter of these excitatory nerves includes: the blockade of responses to both ATP and transmural nerve stimulation by a number of agents used in the investigation of purinergic transmission (Satchell *et al.*, 1973; Burnstock *et al.*, 1972; Weetman and Turner, 1974) and the parallel depression of both ATP and transmural nerve stimulation induced responses by the development of tachyphylaxis to ATP (Burnstock *et al.*, 1972). Attempts to desensitize ileal preparations of this study to ATP were with mixed results and therefore it was not possible to draw any conclusions using this method. However, CB3G-A which specifically anatognised

contractions due to ATP was without effect on non-cholinergic responses to transmural stimulation, which is not consistent with ATP being the transmitter for excitatory responses within the guinea-pig ileum. Indeed there is now good evidence to suggest that the transmitter for these nerves is substance P (Furness *et al.*, 1980).

The specificity of CB3G-A for ATP actions, evident from its lack of effect toward contractions of the ileum induced by bradykinin (BK) and histamine (HA), suggests a unique site and mode of action for ATP and this antagonist in the small intestine. In fact a related compound, Procion Red HE3B antagonises only contractile responses to ATP (Kennedy *et al.*, 1981). A heterogeneous population of nucleotide receptors have been proposed for the mammalian gut (Burnstock *et al.*, 1970; Bartlett *et al.*, 1979) distinguishable by their resultant actions and their differing affinities for the various adenine nucleotides. These receptors include those already mentioned, which are more sensitive to adenosine and involved in prejunctional modulation of adrenergic and cholinergic nerve activity, and those located on the smooth muscle and characterised by their greater susceptibility to stimulation by ATP. In addition to the differentiation of these receptors in terms of potency orders for their purine agonists, the methylxanthines have been shown to be specific antagonists at the purinergic receptors (P_1) mediating adenosine prejunctional actions (Burnstock, 1978; Bartlett *et al.*, 1979). In this study, CB3G-A was without effect upon the cholinergic nerve mediated contractions, and did not prevent the methylxanthine sensitive adenosine inhibition of the cholinergic twitch response, indicating that CB3G-A does not antagonise the P_1 receptor or the mode of action of adenosine in the guinea-pig ileum.

According to Burnstock (1979), ATP acts on smooth muscle through specific receptors. However, the effects of ATP at the mammalian smooth

muscle are of two types; relaxation and/or contraction (see Burnstock, 1972). Similar dual effects of ATP on the ileum were observed in this study. The ready reversal of the antagonism by CB3G-A, following its washout from the bathing medium, indicates that this compound does not exert its effects by irreversible binding to ATP receptors.

Prostaglandins can exert contractile or relaxant effects, or modulate the contraction of smooth muscle (Bolton, 1979) and recently it has been found that ATP interacts with prostaglandins (Dean and Downie, 1978; Burnstock *et al.*, 1978; Moritoki *et al.*, 1979). The rebound contractions of smooth muscle following cessation of electrical stimulation of the intramural non-adrenergic inhibitory nerves or applied ATP may also involve prostaglandins (Burnstock *et al.*, 1975; Cocks and Burnstock, 1979). The findings that CB3G-A markedly reduced the 'rebound contraction', but not the relaxation of the colon following electrical stimulation of the inhibitory nerves, suggests the action of CB3G-A may be through its interference with an ATP stimulated prostaglandin formation in smooth muscle. Procion Red HE3B is rather specifically directed against this effect (Kennedy *et al.*, 1981). There is a further possibility, that CB3G-A interferes with the stimulus response coupling in some other way. It has been shown that CB3G-A and related compounds bind specifically to a number of enzymes containing the dinucleotide fold (Bohme *et al.*, 1972; Wilson, 1976) and therefore the action of CB3G-A may be with some enzyme involved in the smooth muscle responses to applied ATP.

In conclusion, CB3G-A (within a narrow concentration range) appears to be a useful antagonist for ATP actions in the guinea-pig large and small intestine. However its usefulness for the investigation of ATP actions on mammalian smooth muscle may depend upon the tissue under study, since Choo (1981) reports that CB3G-A has different effects towards ATP

actions on the rat and the guinea-pig bladder. The pharmacologic evidence presented here indicates that ATP has multiple effects on intestinal smooth muscle, but is unlikely to be the primary transmitter of either the non-adrenergic inhibitory or non-cholinergic excitatory nerves of the guinea-pig intestine. Nevertheless, the ability of ATP to mimick responses to stimulation of these nerves in this, and other studies, raises some interesting questions as to its functional role in these tissues. In view of the considerable evidence that ATP is localised at, and released from, nerve endings, including those of peripheral cholinergic and adrenergic nerves (Berneis, 1970; Dowdall *et al.*, 1974; Su *et al.*, 1971; Silinsky and Hubbard, 1973; Hedqvist and Fredholm, 1976) where it probably reaches effective contractions (Ribiero and Walker, 1973; Ginsborg and Hirst, 1972; Jawynok and Jhamandas, 1976; Wakade and Wakade, 1978) it is possible that ATP may have a synergistic, feedback, regulatory role in transmitter release following its degradation to AMP or adenosine.

CHAPTER VIII

GENERAL DISCUSSION AND CONCLUSION

GENERAL DISCUSSION AND CONCLUSION

The amino acid γ-aminobutyric acid (GABA) occurs throughout the brain and spinal cord of vertebrates where it is recognised as an inhibitory neurotransmitter (Curtis and Johnston, 1974). There is also evidence that GABA may have a transmitter function in autonomic ganglia (Obata, 1976). The aim of this thesis was to explore the hypothesis that GABA is a neurotransmitter of enteric neurones.

That GABA may have a neurotransmitter function in the guinea-pig intestine appears likely since a number of the necessary criteria as defined by Werman (1966) and Orrego (1979) have now been satisfied. In Chapter II the pharmacology of GABA actions in isolated preparations of the guinea-pig ileum is described. According to the classical criteria, interaction of the suspected neurotransmitter at postsynaptic sites should mimick the effects of the natural neurotransmitter. Furthermore, pharmacological agents which modify the operation of the natural neurotransmitter should similarly affect the action of the putative neurotransmitter. In the ileum, GABA caused stimulation of intrinsic inhibitory and excitatory motor neurones by interactions at specific bicuculline sensitive ($GABA_A$) receptors. Investigation of the ionic mechanisms for GABA induced contractions show these $GABA_A$ receptors are coupled with chloride (Cl^-) ionophores. Other studies (Bowery *et al.*, 1981; Ong, 1981) show GABA also depresses enteric excitatory neurones through interactions at baclofen sensitive $GABA_B$ receptors. These receptors which do not operate through Cl^- channels are proposed to be 'pre-junctional' and their activation, reduces transmitter output presumably due to an altered Ca^{2+} permeability (Bowery *et al.*, 1981).

A common pharmacology is shared between applied GABA and the endogenously released GABA as seen with the use of ethylenediamine (EDA) (Kerr and Ong, 1981). Bicuculline which blocks $GABA_A$ mediated stimulation of cholinergic neurones, reduced the EDA evoked cholinergic contraction of the guinea-pig ileum. Furthermore, applied EDA also caused a depression of the electrically evoked twitch response, presumably by the action of endogenous GABA (released by EDA stimulation of GABA-ergic neurones, Forster *et al.*, 1981) at $GABA_B$ receptors since this depression was blocked by desensitization of the ileum to baclofen.

The neuronal elements involved in this GABA-ergic transmission should contain the necessary precursors and synthetic enzymes for the synthesis of the proposed neurotransmitter (i.e., GABA). In addition, the putative neurotransmitter should be present in nerve terminals. There is now considerable biochemical evidence that GABA and its synthesizing enzyme glutamic acid decarboxylase (GAD) are present in myenteric neurones of the guinea-pig (Jessen *et al.*, 1979), cat (Hosoya *et al.*, 1981) and human (Micki *et al.*, 1981) intestine. However, specific localization of these substances to nerve terminals has not yet been confirmed. Such a study utilising electronmicroscopy would be particularly useful.

According to Werman (1966), Fagg and Lane (1979) and Orrego (1979), the demonstration of a synaptic release of the putative transmitter, in an amount proportional to the magnitude of stimulation applied, is of considerable importance for transmitter identification. This process must also show a true calcium dependency. In addition there must exist a mechanism whereby the proposed neurotransmitter is inactivated following its action at the postsynaptic site. However, due to the lack of sensitive assay procedures for a wide range of amino acids including GABA, and the technical difficulties involved in their use, many studies have used

exogenously applied radiolabelled substances to determine the uptake, localization and release of putative transmitter substances. Where it is established to be a neurotransmitter, GABA is removed from the synaptic cleft by a high affinity transport mechanism present in both neurones and glia (Iversen and Kelly, 1975). GABA taken up by this transport mechanism is eventually returned to the presynaptic neurotransmitter pool. Evidence for such a transport mechanism in the myenteric plexus of the guinea-pig was presented in Chapter III. High affinity [^{3}H]-GABA uptake sites are present throughout the meshworks of this nerve plexus, however, in contrast to the CNS, extrasynaptic uptake (e.g., glia) of [^{3}H]-GABA does not appear to be a major site for removal of [^{3}H]-GABA from myenteric synapses. Myenteric neurones accumulating [^{3}H]-GABA in this way could also be induced to release the radiolabelled amino acid following electrical stimulation, as described in Chapter V. [^{3}H]-GABA is released in a 'transmitter-like' manner, being prevented by treatment with tetrodotoxin (TTX), neuronal high affinity uptake inhibitors and calcium free medium. Recently, Taniyama *et al.* (1981) reported comparable uptake and release of [^{3}H]-GABA by the cat colon myenteric plexus with remarkable coincidence of the stimulus frequency used to elicit maximum evoked efflux of [^{3}H]-GABA.

Since GABA satisfies all of the primary criteria as described by Werman (1966) and Orrego (1979) to identify it as a neurotransmitter, it seems reasonable to conclude that GABA is a neurotransmitter of myenteric neurones in the guinea-pig intestine. Although other regions of the guinea-pig gastrointestinal tract have not been examined, there is a good likelihood that GABA is also a neurotransmitter in these, as well as in the gastrointestinal tract of other mammalian species including the rat, cat and human.

In the mammalian myenteric plexus 35% of the neurones have been

shown histochemically to be either amine handling or positive for VIP, somatostatin, enkephalin and substance P (Furness and Costa, 1980). Cholinergic neurones are the only intrinsic neurones for which both the transmitter and to some extent functions are known, however, specific histochemical methods for the localization of cholinergic neurones are still unproven and as yet have not been applied to the intestine. Many of the as yet unidentified neurones may, therefore, be cholinergic since acetylcholine is a transmitter of motor and interneurones, but there is still room for more neuronal types to be discovered. Amongst these we could include GABA-ergic neurones. Unfortunately, autoradiography does not allow quantitative analysis of cells accumulating the radiolabelled substances therefore it is not possible to estimate the proportion of myenteric neurones that are GABA-ergic. Preliminary experiments just completed in this laboratory confirm the presence of these GABA-ergic myenteric neurones. [^{3}H]-cis-3-aminocyclohexanecarboylic acid (ACHC) and [^{3}H] L,2-4-diaminobutyric acid (L-DABA) which are specifically accumulated by GABA-ergic neurones were localised to cells of myenteric ganglia.

Although the enteric nervous system (ENS) is relatively complex, it is still simpler than the brain and as such presents a simple and readily accessible model for the study of neurotransmission, particularly as it survives and functions well *in vitro* for long periods. In this regard it has proved invaluable for the isolation of the enkephalins (see Kosterlitz, 1979), receptors for which are present in the mammalian gastrointestinal tract. The chemical specificity of GABA-receptors *in vitro* have not been fully defined. Dose response curves for GABA, GABA-agonists and antagonists are difficult to measure in both peripheral and central nervous preparations (De Feudis, 1977). However, the ENS may afford a preparation more amenable to pharmacological study, where

as shown in this study it was possible to determine the relative potency of the competitive GABA antagonist bicuculline. Studies on GABA-ergic activities of GABA-analogues could be carried out using the mammalian intestine, including determination of the conformational requirements of $GABA_A$-agonists and the nature of the baclofen action. Indeed, recent studies by J. Ong in this laboratory (see Ong, 1981; Kerr and Ong, 1981) have confirmed the specificity of various GABA-analogues including, muscimol and 3-amino-1, propanesulphonic acid for the myenteric GABA receptors.

Postsynaptic membrane macromolecules involved in the action of transmitters can be assayed *in vitro* using radioactive ligand binding techniques (Peck *et al.*, 1976). Receptor sites for GABA have been identified by binding of radioactive GABA itself (Olsen *et al.*, 1978), the GABA-agonist muscimol (Beaumont *et al.*, 1978), and the GABA-antagonist bicuculline-methiodide (Mohler and Okada, 1977). Such receptor binding techniques would provide a more complete characterization of the enteric GABA receptor types.

It has been suggested that benzodiazepines and barbiturates exert the effects by facilitating GABA-ergic transmission (Guidotti, 1978, 1980), however, the exact nature of there actions is still controversial. Whether or not these substances exert an effect in the mammalian intestine needs to be determined. Recently Doble and Turnbull (1981) described a lack of effect of benzodiazepines on bicuculline insensitive ($GABA_B$) receptors in the field stimulated guinea-pig vas deferens.

As shown in Chapter VII ATP does not appear to be the transmitter of intrinsic excitatory and inhibitory neurones in the guinea-pig large and small intestine. However, investigations of the transmitter mediating

non-cholinergic non-adrenergic inhibition may be aided by GABA stimulation of these neurones, and the presence of $GABA_B$ receptors to the excitatory but not the inhibitory neurones (J. Ong personal communication). A number of questions relating to this arrangement of GABA receptors within the myenteric plexus remain to be answered. In particular, what role do GABA-ergic neurones and related receptors have in intestinal motility which is most obviously affected by GABA-antagonism? Nonetheless GABA is most certainly a neurotransmitter in the guinea-pig intestine where it exerts its effects at two populations of GABA-receptors.

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