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Purinergic Nerves*

G. BURNSTOCK

Department of Zoology, University of Melbourne, Parkville, 3052, Victoria, Australia

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I. Introduction

CLASSICALLY the autonomic nervous system consists of two components, cholinergic and adrenergic nerves. The object of this article is to review the evidence for a third nerve component in the autonomic system, which is neither adrenergic nor cholinergic. Since the evidence presented in this review indicates that the principal active substance released from these nerves, at least in the gut, is a purine nucleotide, they have been called tentatively "purinergic" (120). This term has been suggested for the same reasons put forward by Dale (165) when introducing the terms "adrenergic" and "cholinergic" nerves, namely "to assist clear thinking, without committing us to precise chemical identifications, which may be long in coming", and because their description as "non-adrenergic, non-cholinergic" nerves is clumsy and negative.

Hints of the existence of autonomic nerves other than those of the two classical components can be found in the early literature. In 1898, Langley (364) noted that stimulation of the vagus nerves could produce relaxation of the stomach. This relaxation was best revealed after blockade of the excitatory action of cholinergic fibres with atropine (379, 400). Relaxation was also demonstrated more easily in stomachs with high tonus (271, 375, 380), or when the vagus nerve was stimulated with high voltage or long duration pulses (546). In this early work, it was usually assumed that the inhibitory responses were due to nerves of sympathetic origin running in the vagal trunks. Later, it was shown that inhibition was due largely, if not entirely, to fibres of parasympathetic origin, since relaxation of the stomach produced by stimulation of autonomic centres in the brain was mediated by the vagus nerve itself (197, 198, 284, 493). Relaxation of cat stomach and mouse intestine produced by nicotine after exposure to atropine or botulinus toxin was taken to indicate the presence of inhibitory postganglionic neurones in the wall of the gut, but these too were regarded as adrenergic (10, 13).

The first hints that some of the inhibitory fibres to the vertebrate stomach were not adrenergic appeared when adrenergic neurone blocking drugs were used. The inhibitory response of the guinea-pig stomach to vagus nerve stimulation was not prevented by these drugs (259) except by high concentrations of bretylium sufficient to block transmission in ganglia (see Campbell, 141). Similarly, Paton and Vane (434) showed that relaxations in response to transmural stimulation of intramural nerves in the stomachs of cats, mice and guinea-pigs were resistant to blockade by xylocholine. However, the authors favoured the explanation that the inhibitory responses were due to adrenergic nerves that persisted under these conditions because the nerves were being stimulated peripherally to the site of action of the adrenergic neurone blocking drug.

Attention was focused on these nerves in the early 1960's when transmural stimulation of the guinea-pig taenia coli with single pulses of short duration was shown to produce large hyperpolarisations or inhibitory junction potentials in smooth muscle cells, which were associated with relaxation, and which persisted in the presence of both atropine and guanethidine (122–124). That these re-

sponses were nerve-mediated was established since they were abolished by low concentrations of tetrodotoxin (109, 357) or by storage of the tissue at 4°C for more than 100 hr (357). Inhibitory junction potentials have also been recorded in smooth muscle cells of the stomach during stimulation of the vagus nerves (42, 64, 65). Details of the electrophysiology of transmission from non-adrenergic inhibitory nerves to smooth muscle cells in different regions of the gut in several species of mammals and birds are described in section IV.

Evidence that these inhibitory responses are *not* due to adrenergic nerves is now conclusive. Relaxation of intestine produced by stimulation of perivascular sympathetic nerves is prevented by low concentrations of *alpha-* and *beta*-adrenoceptor antagonists or by adrenergic neurone blocking drugs, without affecting the inhibitory responses to transmural stimulation (78, 106, 125, 173, 174, 297, 342, 465). Inhibitory junction potentials and relaxations in response to transmural stimulation are unimpaired in the guinea-pig colon after degeneration of sympathetic adrenergic nerves (225; see fig. 2). Relaxation of the guinea-pig taenia coli in response to transmural stimulation or nicotine persists in organ cultures (462) and in anterior eye chamber transplants (129) after all adrenergic nerves have disappeared. In addition, transmission from intrinsic inhibitory neurones has been demonstrated in avian gizzard (55) and mammalian anal sphincter (240a), which are contracted by catecholamines.

More recent studies of the nervous control of the stomach (4, 42, 108, 141, 321–323, 395–397, 424), and of the effect of ganglion stimulants and blockers on isolated gut segments (125, 249) provide strong evidence that the cell bodies of non-adrenergic inhibitory nerves are located in Auerbach's plexus. In the stomach and distal rectum, the non-adrenergic inhibitory neurones are controlled by preganglionic, parasympathetic nerves running in the vagus and pelvic trunks respectively, but in the colon they do not appear to have extrinsic connections (see sections III and VI for detailed evidence).

Studies of the chemical nature of the transmitter released from non-cholinergic, non-adrenergic nerves supplying the gut have been carried out (126, 483, 520). Evidence that the neurotransmitter is adenosine triphosphate (ATP) is presented in detail in section II and discussed in relation to earlier suggestions of ATP release during antidromic stimulation of sensory nerves (299, 300).

Subsequent sections consider in more detail various aspects of purinergic nerves, including their ultrastructure (132, 467), the electrophysiology of transmission, and their possible distribution in different organs and species. The pharmacology of purine nucleotides and nucleosides is reviewed and the possibility that the non-cholinergic, non-adrenergic *excitatory* nerves shown to supply some organs (*e.g.*, urinary bladder, intestine) may also be purinergic is considered. A model of the synthesis, storage, release and inactivation of ATP during purinergic nerve transmission is presented as a basis for future work.

II. Identification of the Transmitter Substance

Several criteria need to be satisfied before a substance can be established as a neurotransmitter (see for example Eccles, 191): 1) the substance and the enzymes

necessary for its formation must be present in the neurone; 2) the substance must be released from the terminal axon when the nerve is activated; 3) the effect of the transmitter released on nerve stimulation must be mimicked by the exogenous application of the substance to the effector; 4) a mechanism for inactivation of the substance must be present, whether it involves enzyme action or uptake or both; 5) drugs which reduce or potentiate nerve mediated responses should similarly affect the responses to the exogenously applied substance.

Evidence has been put forward that adenosine triphosphate or a related nucleotide is the transmitter released by non-adrenergic inhibitory nerves in the gut (126, 135a, 482, 483, 484, 520). The authors showed that, in broad outline, all the above criteria for ATP to be established as the transmitter substance released by non-adrenergic inhibitory nerves were fulfilled. This evidence will be considered under each of these criteria in this section.

A variety of substances other than ATP have been explored as the possible transmitter released from non-adrenergic, non-cholinergic inhibitory nerves in the gut, including catecholamines, 5-hydroxytryptamine, cyclic AMP, histamine, prostaglandins, various amino acids such as alanine, arginine, histidine, glycine, glutamic acid and α-amino butyric acid, and the polypeptides bradykinin and substance P (14, 16, 78, 108, 125, 126, 285, 465, 500, 563). However, these substances were rejected as contenders by most workers on the grounds that they were either inactive or did not mimic the nerve mediated responses, that specific blocking drugs for these substances did not affect the nerve-mediated response, or that their action was by stimulation of nerves and not by direct action on smooth muscle. The suggestion has been made that prostaglandin E_1 may be the inhibitory transmitter in the circular muscle coat (346), based largely on the observation that it was released from the rat stomach during transmural stimulation (52), and that it inhibits the contractile response of the circular muscle to coaxial stimulation. However, prostaglandin E_1 cannot be the transmitter released from non-adrenergic inhibitory nerves supplying the longitudinal muscle since both prostaglandin E_1 and E_2 produce contraction (345). The possibility that there is a physiological or pathological role in gut motility for prostaglandins released from tissues other than nerves has also been discussed (49, 51, 438).

A. Formation and storage of ATP

Both ATP and the enzyme systems which synthesize ATP occur ubiquitously in cells, so that it is not contentious that non-adrenergic inhibitory nerves too are able to produce and store ATP. This is not significant evidence in itself for ATP as a neurotransmitter; the important question is whether ATP is stored in terminal axons in such a way that it can be released during nerve stimulation. Recent studies with radioactive tracers indicate that this is likely (520). Strips of taenia coli were shown to take up large amounts of tritium-labeled adenosine, adenosine monophosphate (AMP), adenosine diphosphate (ADP) and ATP (inosine or adenine were taken up to a much smaller extent). Adenosine was rapidly converted into and retained largely as ^{3}H-ATP (about 25% was accountable as ^{3}H-AMP, 10% as ^{3}H-inosine, but there was little or no ^{3}H-ADP). More of the labeled ATP was located in nerves than in muscle or other tissue components.

This was demonstrated by analysing the radioactivity in frozen sections cut serially at different levels through preparations of the caecal wall and overlying taenia muscle layer (Bevan, Su and Burnstock, unpublished observations); peaks of activity were located in Auerbach's plexus and in the outer, serosal region of the taenia where the neural component consists largely of terminal varicose fibres (62). Further evidence comes from studies of the radioactivity in preparations of chicken gizzard exposed to ^{3}H-adenosine, dissected in such a way to consist predominantly of either smooth muscle, connective tissue or nervous components; about 6 times more label was located in nerves than in the other tissues (Wright and Lynch, personal communication). That much of the labeled ATP is accessible to release during nerve stimulation is explained in the following section (II B).

Evidence will be presented that purinergic nerve terminals are characterised by a predominance of large vesicles with a characteristic form, designated here as large opaque vesicles (LOV), in order to distinguish them from the large granular vesicles (LGV) found in adrenergic and cholinergic nerves (see section IIIB). Isolation of this vesicle fraction by differential centrifugation and its chemical identification will be necessary [*cf.* Lagercrantz (360) for adrenergic nerve vesicles composition] before vesicle-bound storage of ATP in purinergic nerves can be confirmed.

B. Release of purine nucleotides

Stimulation of non-adrenergic inhibitory fibres to the stomach in both toads and guinea-pigs *via* the vagus nerves increases the venous efflux of adenosine and inosine (126, 483). Adenosine and inosine collected under the conditions of these experiments are likely to be the breakdown products of adenine nucleotides, since ATP introduced into the same perfusing system is quickly broken down into comparable proportions of adenosine and inosine (483).

That the release of nucleosides is due to stimulation of non-adrenergic inhibitory fibres in the vagus nerves rather than of cholinergic fibres was demonstrated by the use of the toad stomach preparation. In this preparation, all the preganglionic parasympathetic fibres in the vagus nerves make synaptic connection with non-adrenergic inhibitory postganglionic neurones in the wall of the stomach, while the postganglionic cholinergic fibres running in the vagus nerves are of sympathetic origin (142). Stimulation of the vagal roots (or of the vagosympathetic trunks) resulted in increased nucleoside efflux, but stimulation of the cervical sympathetic branch to the vagus nerves did not (126).

The possibility must be considered that the purine nucleotides or nucleosides released from nerves are not neurotransmitter substances but appear for other reasons. For example, it has been suggested that ATP is released from nerve membranes during propagation of an action potential (3, 351, 426). However, the amount of nucleosides collected during stimulation of non-adrenergic inhibitory nerves has been calculated to be at least 1000-fold greater than that released as a direct result of the process of axon membrane activation during impulse propagation (126, 483).

It is unlikely that the nucleoside release during non-adrenergic nerve stimula-

tion to the gut is derived from red blood cells trapped in the tissue vasculature, since the tissue was perfused with oxygenated nutrient medium *via* the coeliac artery for 20 min to remove all traces of blood before samples were collected. Nor is it likely to be largely due to release from muscle, since stimulation of portions of Auerbach's plexus from turkey gizzard dissected free of the underlying muscle resulted in efflux of purine nucleotides, in this case mostly AMP (126). The avian gizzard has been shown to be heavily innervated by non-adrenergic inhibitory nerves located in Auerbach's plexus, which contains both nerve cell bodies and varicose fibres (63, 64). Additional evidence that most of the nucleosides are not released from smooth muscle cells themselves comes from experiments with tritium-labeled adenyl compounds described below.

Holton (299) reported that ATP was released in the rabbit ear in concentrations sufficient to produce vasodilatation during stimulation of the great auricular nerve. She suggested that the ATP was liberated from sensory nerve endings after antidromic stimulation (see also 166, 280). However, this seems to be an unlikely explanation for the release of nucleotides from stomach preparations, since the fibres in the vagal trunk mediating gastric inhibition are preganglionic, and therefore efferent, in both guinea-pigs (108, 141, 424) and toads (142) and no effects on motility attributable to antidromic stimulation of vagal sensory fibres have been observed. Furthermore, it has been shown that the non-adrenergic inhibitory response to stimulation of the vagal nerves supplying the rabbit stomach was abolished after degeneration of the efferent, but not the afferent, component, achieved by vagal section above the nodose ganglion 8 days previously (B. Cragg, personal communication). In view of these results and of the reports of efferent fibres in the dorsal root outflow (31, 143, 275, 276, 369, 494, 532), it would be interesting to examine the possibility that there are postganglionic non-adrenergic, non-cholinergic fibres in the dorsal roots which supply parts of the vasculature and intestine (see section VI).

The possibility that ATP merely accompanies the release of the "real" transmitter substance from non-adrenergic inhibitory nerves, rather than being itself the neurotransmitter, cannot be excluded at this stage. However, inhibitory activity was found in only one region of chromatograms of gastric perfusates and this was solely attributable to the purine nucleotides and nucleosides found in this region. ATP has been shown to be released together with catecholamines from adrenal medullary vesicles and whole glands (185), but Stjärne and his colleagues (517, 518) were unable to find evidence of ATP release concomittantly with noradrenaline (NA) from sympathetic nerves of the cat spleen and they concluded that there were basic differences in the mechanisms of hormone secretion and neurotransmitter release. Relatively large amounts of ATP are known to be present in adrenergic nerves (202, 491, 516) and to be involved in both uptake and release of NA in isolated adrenergic nerve granules (203). However, in a recent review, De Potter (176) concluded that, when allowance is made for mitochondrial contamination of the fraction enriched in NA, "the ATP concentrations, relative to catecholamines, in the two particles (*i.e.*, splenic nerve storage particles and adrenal chromaffin granules) are markedly different, much

less being in the splenic nerve particles. Perhaps this result means that ATP does not play such an important role in the binding of NA within the storage particles of sympathetic nerves as has generally been believed." Nevertheless, since evidence for the release of neurotransmitter from adrenergic nerve terminals by exocytosis gains support (504), it is possible that some ATP will be detected in association with release of NA, chromogranin A and dopamine β-hydroxylase in future experiments. Adrenergic nerves in the guinea-pig gut, rabbit aorta and pulmonary ear arteries, after exposure to ^{3}H-adenosine, have been shown to release tritium-labeled compounds; however, unlike non-adrenergic, inhibitory nerves, release of labeled nucleotide (as well as NA) was prevented by guanethidine (520). No nucleotides or nucleosides were collected from the perfusate after selective stimulation of cholinergic nerves to the stomach (126).

More direct support for release of ATP from non-adrenergic inhibitory nerves to the gut comes from recent studies with radioactive tracers (520). Spontaneous relaxation or that produced either by intramural nerve stimulation or by nicotine in the presence of drugs which block the responses to cholinergic and adrenergic nerves (125) is accompanied by a marked increase in release of tritium-labeled material from taenia coli incubated in ^{3}H-adenosine. This release is blocked by tetrodotoxin (as is the mechanical response), suggesting that it is mediated by nerves. That release is not from muscle during changes in tone has been demonstrated, since NA and histamine, which produce relaxation and contraction of the taenia respectively, failed to cause tritium release. The situation is complicated by the release of label from adrenergic nerves, which are also able to take up ^{3}H-adenosine (520). However, the release of tritium resulting from transmural stimulation is not likely to be from adrenergic nerves, since the experiments were carried out in the presence of guanethidine which was shown to prevent the release of tritium-labeled nucleotides as well as NA.

Spontaneous inhibitory activity of two kinds is often observed in gut preparations. The first kind, the relaxant phase of pendular contractions, is probably myogenic, since it persists in the presence of tetrodotoxin (226). However, the second type of activity, consisting of large relaxations sometimes lasting more than 30 sec which occur at fairly regular intervals, may be due to spontaneous rhythmic discharges from non-adrenergic inhibitory neurones, since they are accompanied by a marked increase in release of tritium-labeled ATP from tissues previously soaked in ^{3}H-adenosine (Su, Bevan and Burnstock, unpublished observations). It is interesting that bursts of electrical activity which appear at comparable intervals have been recorded from single ganglion cells in Auerbach's plexus (574, 580; Prosser, personal communication), and trains of spontaneous hyperpolarisations resembling inhibitory junction potentials have been recorded in the longitudinal muscle coat of the carp stomach (315).

C. Direct actions of purine nucleotides and nucleosides on smooth muscle

The actions of purine nucleotides and nucleosides on smooth muscle other than those of the gut will be dealt with in sections V and VI.

The inhibitory action of the purine nucleoside, adenosine, on smooth muscle,

including that of the intestine of cats and rabbits, was reported as early as 1929 in a study of the pharmacology of nucleic acid derivatives (187). Since then, inhibitory actions of purine nucleotides and nucleosides on the gut of a variety of mammals, birds and amphibians have been described (21–23, 32, 94, 126, 135a, 186, 212, 244, 312, 514, 564).

Evidence is presented in section IV that the transmitter released by non-adrenergic inhibitory nerves produces transient hyperpolarisations or inhibitory junction potentials (IJP's). The transmitter appears to act by producing a specific increase in the potassium conductance of the postjunctional muscle membrane. It is therefore interesting to note that ATP causes hyperpolarisation of smooth muscle cells of the taenia coli (21, 312, 383a) and further that the hyperpolarisation produced by the direct action of ATP is reduced by high concentrations of K^+ (22). However, not much weight can be placed on the similarities of non-adrenergic inhibitory nerve and ATP action on the membrane potential until it is possible to make accurate estimates of the reversal potential for the muscle membrane under the influence of nerve-released transmitter and ATP respectively.

The possibility that ATP might cause relaxation *indirectly* by initiating action potentials in non-adrenergic inhibitory nerves was negated by the finding that tetrodotoxin abolished the inhibitory responses of atropinised intestinal muscle to stimulation of either perivascular or intramural nerves without affecting the relaxation produced by ATP (94, 126, 229). However, the possibility remains that ATP may cause release of an inhibitory transmitter by displacing it from transmitter stores in the terminals without initiating propagated impulses, in an analogous way to tyramine release of NA from adrenergic terminals.

The most potent inhibitory purine compounds on gut are ATP and ADP, which are about equipotent, with a threshold concentration for relaxation of the taenia coli of about 10^{-7} M (21, 126). Typically, the relaxation produced by ATP or ADP reaches a maximum and the muscle regains normal tone on washout more rapidly than is seen in relaxations induced by catecholamines; very often recovery begins before washout (126). In this respect the effects of ATP more closely mimic the inhibitory response of the taenia to non-adrenergic nerve stimulation than to adrenergic stimulation (125). The transient action of adenine nucleotides on smooth muscle may be explained in terms of their rapid deamination in tissue to inosine derivatives which are pharmacologically inactive (21, 151). Burnstock *et al.* (126) found that AMP and adenosine had about $1/100$ the potency of ATP in causing relaxation of the taenia coli. No effect was found with the following related compounds even with concentrations as high as 10^{-3} M: the purine base, adenine; the deaminated nucleoside, inosine, and its mononucleotide, IMP; the pyrimidine nucleotide, uridine, and its mononucleotide UMP. However, the related guanosine mononucleotide, GMP, causes relaxations in concentrations of about 3×10^{-5} M (21, 126). It is unlikely that cyclic AMP is the transmitter released from enteric nerves, since the sensitivity of the gut to this compound is relatively low (368). Thus it would appear that amongst this group of compounds, ATP and ADP are the best contenders for the transmitter

substance in view of their high inhibitory potencies. It now seems that, of the two, ATP is the most likely transmitter, since ^{3}H-adenosine taken up into the taenia coli is rapidly converted and stored largely as ^{3}H-ATP, with only traces of ^{3}H-ADP detectable (520).

The direct action of adenine compounds on smooth muscle (like the responses to non-adrenergic, non-cholinergic nerves) is not affected by drugs that block or potentiate cholinergic transmission (187, 229) or by adrenergic neurone-blocking drugs (126). *Alpha-* and *beta*-adrenoceptor blocking agents also fail to abolish the action of ATP on mammalian gut (157).

While the predominant response of mammalian gut to ATP is relaxation, excitatory responses of two kinds are sometimes revealed. This was described in a study (126) of the effects of ATP (in the presence of hyoscine) on 12 different gut preparations shown to contain non-adrenergic, non-cholinergic nerves, including guinea-pig stomach circular muscle, ileum and descending colon (141, 224, 297), rabbit stomach circular muscle, ileum and descending colon (173, 224, 297, 434), rat gastric fundus, ileum and colon (278, 298) and toad stomach (142) One kind consisted of contractions after washout of ATP, and is probably equivalent to the phenomenon of "rebound excitation" that follows non-adrenergic inhibitory responses (54, 123, 140, 226, 229, 564) rather than a direct response to ATP. The second type of excitatory action occurred while the nucleotide was still present in the organ bath and is probably a direct excitatory effect of ATP (126). Occasionally a brief contraction preceded relaxation; in some preparations, especially those in which the tone was commonly low (*e.g.*, guinea-pig ileum and rabbit stomach), contractions tended to occur only when high concentrations of ATP were applied. Similar dual effects of ATP have also been reported on other gut segments. For example, adenyl compounds have been reported to contract (99) or relax (383) amphibian stomach, and to excite (105, 314, 410) or inhibit (14, 32, 206, 406) the guinea-pig ileum. The possibility that these effects are related to the presence of excitatory purinergic nerves will be discussed in section V B.

D. Inactivation of ATP

The relatively rapid recovery of smooth muscle after application of ATP or stimulation of non-adrenergic inhibitory nerves and the absence of long-lasting action despite continued stimulation (see section IV A), indicate an efficient inactivation mechanism.

By analogy with other neuroeffector systems, if the non-adrenergic inhibitory nerves act on the gut by releasing ATP, the action of ATP must be terminated by uptake into nerves or smooth muscles and/or by breakdown of ATP by enzymes into compounds with greatly reduced potency.

When ATP was added to a perfusion fluid recycled through the vasculature of the stomach, very little ATP was recovered, but the perfusate contained substantially increased amounts of adenosine and inosine as well as some ADP and AMP (126). Dephosphorylation of ATP to AMP or adenosine would decrease inhibitory potency to $\frac{1}{100}$, and further breakdown by deamination to inosine

would decrease potency by more than $\frac{1}{10,000}$. No direct evidence for the breakdown of ATP by enzymic activity is available, but the gut is known to contain 5′-nucleotidase and adenosine deaminase (565). Mg-activated ATPase has also been located in micropinocytotic vesicles in smooth muscle membranes closely adjacent (200 Å) to non-adrenergic, non-cholinergic nerve profiles (see section III C).

Extracellular breakdown of ATP released from nerves would be analogous to the mechanism of inactivation of acetylcholine (ACh) released from cholinergic nerves by acetylcholinesterase. However, it is well known that localisation of ATPase is intracellular as well as extracellular (see section III C). Thus, by analogy with the mechanism of enzymic breakdown of NA released from adrenergic nerves, where monoamine oxidase (MAO) is located intracellularly in nerve terminals and both MAO and catechol-O-methyltransferase (COMT) in smooth muscle cells, the possibility must be considered that some or all the enzymes capable of breaking down ATP released from non-adrenergic inhibitory nerves are intracellular. However, there is no direct evidence available to support this possibility, and it seems unlikely in view of the evidence presented below that adenosine, but not nucleotides, can be taken up by the nerves.

Radioactive tracer studies have shown that tritium-labeled adenosine, ADP, AMP and ATP, but not inosine or adenine are taken up by gut preparations (520). It seems likely that nucleotides are broken down to adenosine before being taken up, in view of the evidence that adenosine, but not nucleotides, can readily pass through cell membranes (370, 568, 570). This has been tested in recent experiments in our laboratory (Lynch, personal communication) which have shown that when the adenosine moiety of ATP is labeled with tritium and the phosphate moiety labeled with ^{32}P in either the first or third position, the rate of uptake of ^{3}H into taenia coli is considerably greater than that of ^{32}P. This supports the view that the adenine nucleotides are broken down to adenosine before uptake occurs. However, kinetic studies will need to be carried out before this is verified. Since uptake of both ^{3}H-adenosine and ^{3}H-ATP occur indistinguishably within half a minute of exposure of the preparation to these labeled compounds, breakdown of ATP to adenosine appears to be a rapid process. This is in contrast to the results of uptake studies on red blood cells, where onset of uptake of ^{3}H-ADP takes about 2 min longer than for ^{3}H-adenosine (90).

Inactivation of transmitter released from adrenergic and cholinergic nerves is achieved essentially by mechanisms whereby the breakdown products of neurotransmitter or the transmitter itself are taken up by the nerves and re-incorporated (after resynthesis in the case of cholinergic nerves) into physiological stores (25, 103; fig. 3 a, b). While there is no direct evidence at this stage for this type of mechanism of biological economy in purinergic nerves, some support comes from the observation that the overflow of tritium-labeled compounds from taenia coli incubated in ^{3}H-adenosine on stimulation of non-adrenergic inhibitory nerves at 5 pulses/sec [which produces a maximal inhibitory response for this nerve, (126)] is low, whereas there is high overflow of tritium-labeled compounds with stimulation at 30 pulses/sec (520). This suggests that adenosine resulting from

extraneuronal breakdown of nerve-released ATP may be taken up again by nerves under physiological conditions but that, under the non-physiological conditions of high frequency stimulation, much of the additional adenosine produced is degraded further to inosine. Further support for this view comes from the potentiating effects of the adenosine-uptake inhibitor dipyridamole (discussed below).

E. Antagonism and potentiation of responses to nerve stimulation and ATP

While the pharmacology of non-adrenergic, non-cholinergic nerve transmission is considered in more detail in section V, the parallel action of some agents in modifying the responses to stimulation of non-adrenergic inhibitory nerves and directly applied ATP will be briefly described here, since this represents important supporting evidence for ATP as the neurotransmitter substance.

Tachyphylaxis to ATP. When tachyphylaxis to ATP is produced in the rabbit ileum (333, 403), there is a consistent depression of the inhibitory responses to non-adrenergic inhibitory nerve stimulation, but not to adrenergic nerve stimulation (126).

Quinidine. Quinidine ($1–5 \times 10^{-5}$ g/ml) reduced or abolished the relaxation of the taenia coli produced by noradrenaline and by perivascular adrenergic nerve stimulation (126). When the concentration of quinidine was raised to 2×10^{-4} g/ml, the inhibitory responses to both ATP and non-adrenergic inhibitory nerve stimulation were abolished. Still higher concentrations of quinidine were required before the tone and activity of the taenia were impaired. Quinidine has also been shown to antagonise the inhibitory action of ATP on the rabbit ileum (94). The effects of quinidine must be interpreted with caution, because of the lack of specificity. Nevertheless, quinidine is one of the few substances which has been shown to antagonise non-adrenergic inhibitory nerve action.

Dipyridamole. Low concentrations of dipyridamole (10^{-6} to 5×10^{-8} g/ml), a drug most commonly used as a coronary vasodilator (138, 273, 419) have recently been shown to potentiate the responses of the guinea-pig taenia (in terms of both amplitude and duration) to both non-adrenergic inhibitory nerve stimulation and ATP, while, if anything, reducing the responses to adrenergic nerve stimulation and NA (482). This drug has been shown to inhibit the uptake of adenosine into the heart (301, 338) as well as into the taenia coli preparations (482). The way that dipyridamole potentiates the responses is discussed more fruitfully in relation to the model for storage, release and inactivation of transmitter proposed in section V A.

F. Summary

Evidence that ATP is the transmitter substance released from non-cholinergic, non-adrenergic inhibitory nerves includes;

1) ATP and the enzymes necessary for its formation are present in non-adrenergic, non-cholinergic nerves. Tritiated-adenosine is taken up by the nerves, transformed into, and stored as ATP (but not ADP) in such a way that it is available for release during nerve stimulation.

2) ATP and its breakdown products (AMP, adenosine and inosine) are released into the perfusate during stimulation of non-cholinergic, non-adrenergic inhibitory nerves. Tritium-labeled compound is released during stimulation of these nerves in tissues previously exposed to ^{3}H-adenosine.

3) The response of smooth muscle to ATP closely mimics the response to nerve stimulation. Both are characterised by rapid onset of action, and this effect is transient, being maintained for no more than 20 to 30 sec; both produce hyperpolarisation of the smooth muscle membrane. ATP and ADP are the most active adenyl compounds, AMP and adenosine are about 100 times less active, while inosine and adenine are pharmacologically inactive.

4) Enzymes capable of breaking down ATP to less active derivatives, including ATPase, 5′-nucleotidase and adenosine deaminase, are present in tissues innervated by non-cholinergic, non-adrenergic nerves.

5) When tachyphylaxis is induced by repeated administration of ATP, the inhibitory responses to non-adrenergic nerve stimulation are abolished, while the inhibitory responses to periarterial adrenergic nerve stimulation persist. Quinidine blocks and dipyridamole potentiates the responses to both non-adrenergic nerve stimulation and ATP.

III. Structure of Purinergic Nerves

A. Location of purinergic neurones

In the gut, the cell bodies of purinergic neurones are probably localised in Auerbach's plexus. Terminal axons of these neurones in the taenia coli extend as far as the serosal surface and penetrate bundles of smooth muscle as well as running in the larger extracellular spaces (62). Electrophysiological studies have demonstrated that smooth muscle cells of both the circular and longitudinal muscle coats of the guinea-pig and rabbit colon are supplied by terminal axons of purinergic neurones (224, 503a). The diameters of axon profiles in nerve bundles in the muscle coats range from 0.3 μ to 1.5 μ, suggesting that the terminal regions of purinergic nerves are varicose as has been shown for other autonomic axons (119, 377a). The spatial spread of IJP's in the taenia coli led Bülbring and Tomita (109) to conclude that the terminal axons of non-adrenergic inhibitory nerves were probably several millimetres long.

Purinergic neurones in the stomach are under the control of preganglionic vagal parasympathetic fibres, but in the colon they appear to have intramural connections with cholinergic neurones and are not controlled by either pelvic parasympathetic or periarterial sympathetic extrinsic nerves. Both pre- and postganglionic fibres in the pelvic nerves provide extrinsic non-adrenergic inhibitory control of the distal rectum. (see section VI A).

Neurones in Auerbach's plexus of mammals have been classified histologically into two or sometimes three distinct types (17, 182, 183, 265, 286, 488). With methylene blue staining, type I cells are blue, but the more granular type II cells are violet; both cell types are confined to the plexus proper (286). Neurone cell bodies in the gut wall have also been distinguished histochemically on the basis of monoamine oxidase activity (234). Many ganglion cells in mammalian

enteric plexuses stain intensely for acetylcholinesterase (AChE), but some do not (265, 319, 335, 336, 526). Since no adrenergic neurone cell bodies have been seen with the fluorescence histochemical method in the mammalian gut, apart from those in the guinea-pig proximal colon (153, 232), and since sensory neurones are apparently largely confined to Meissner's plexus (265, 488), it seems possible that many of the neurones in Auerbach's plexus which do not stain for AChE are purinergic. There is no specific staining method available yet for identification of purinergic nerves, but autoradiographic studies following uptake of tritium-labeled adenosine may provide a means of differentiating the different nerve types both in whole mount preparations of Auerbach's plexus and in enteric neurones grown in tissue culture.

In addition to purinergic neurones in the gut wall, fibres which are neither adrenergic nor cholinergic have been shown to supply a variety of organs (see section VI for details). Some of these nerves are excitatory (for example, those to the urinary bladder and segments of the gut in lower vertebrates), while others are inhibitory (for example, those to the lung, and parts of the vascular system). Many of them are postganglionic; for example, some reach the amphibian intestine in the sympathetic periarterial nerves and appear to originate in the dorsal root ganglia; others (*e.g.*, those to the bladder and distal rectum) have been located in the pelvic outflow; while some appear to reach the stomach and oesophagus in the vagal outflow. However, it is not yet known whether any, some, or all these nerves are purinergic or whether they release yet further neurotransmitters. Exploration of the origin and distribution of these nerves is in its infancy, so that no attempt to categorise them at this stage will be made.

B. Fine structure of terminals

It is now generally accepted that autonomic nerve profiles which contain predominantly agranular vesicles are cholinergic, while those containing predominantly small (300–500 Å) granular vesicles are adrenergic, at least in mammals (119, 135, 196, 293; fig. 1a). In addition both cholinergic and adrenergic profiles contain a small number (about 3–4 %) of large (600–1500 Å) granular vesicles (86) which have also been called type I granular vesicles (261) or neurosecretory vesicles (526). It is known that the large granular vesicles, like the small granular vesicles, in adrenergic nerves are capable of synthesis, uptake and storage of catecholamines (35, 66, 81, 176, 210, 231, 242, 293, 538). In contrast, the large granular vesicles in cholinergic nerves do not take up catecholamines and their function is not yet known (132, 155).

A third type of profile, which has been characterised by large numbers of mitochondria, usually with only two cristae, has been identified recently in smooth muscle systems (fig. 1d). These profiles probably represent sensory nerves (128, 269, 317); serial sectioning of these nerves in the anterior cerebral arteries of the rat showed that they could be traced back to myelinated fibres (132).

In addition to the three types of profile for cholinergic, adrenergic and sensory nerves, a fourth nerve profile in association with smooth muscle has been described. This is characterized by a predominance of large granular vesicles, which

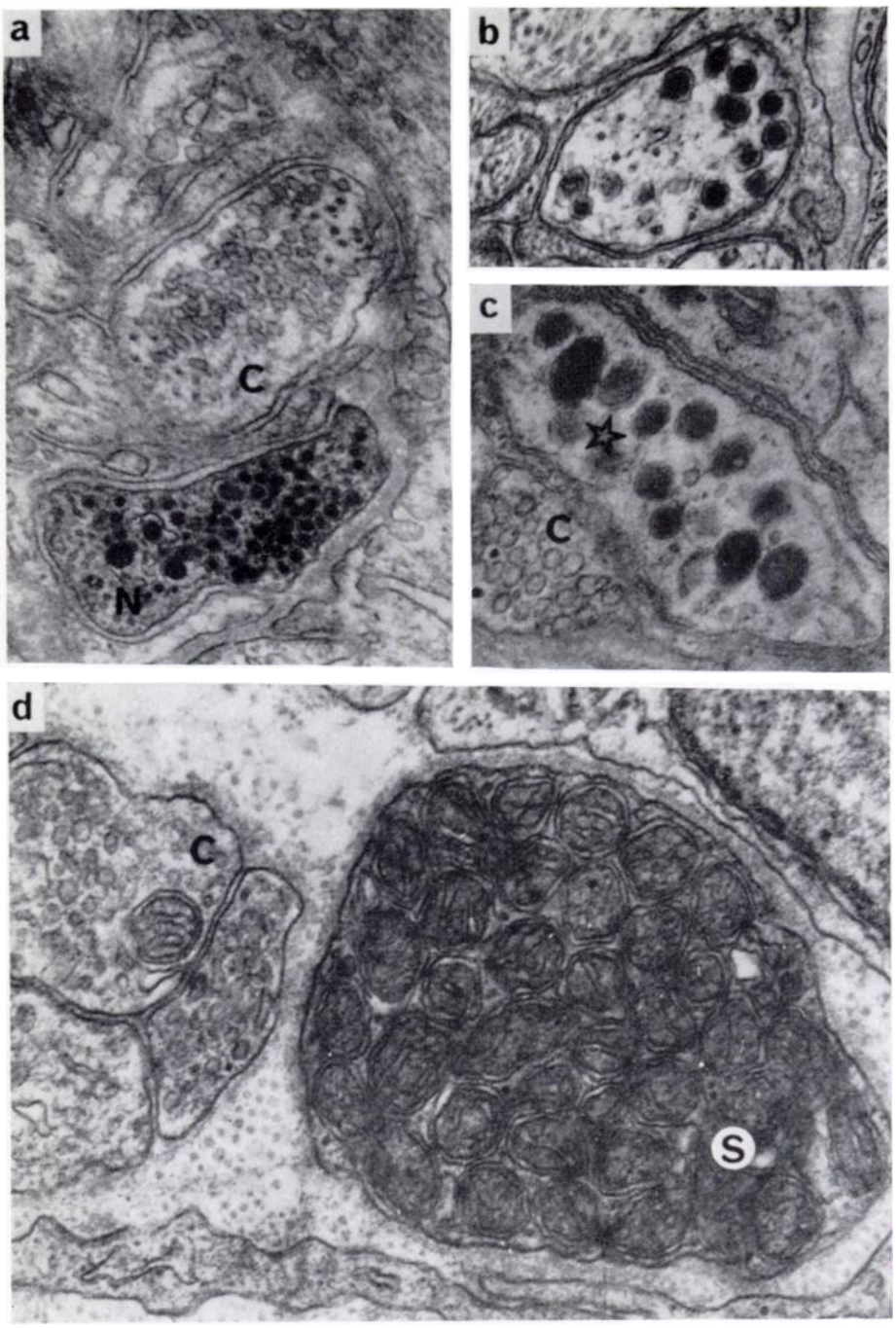

FIG. 1

differ from those seen in small numbers in adrenergic or cholinergic nerves in that they are bigger (800–2000 Å) and do not have a prominent electron-transparent halo between the vesicle membrane and its granular core (see fig. 1c, 132). This type of nerve profile sometimes also contains some small oblate or spherical profiles with electron transparent cores, as well as mitochondria (467). Baumgarten *et al.* (36), in their careful study of the mammalian large intestine designated these profiles as "p-type" fibres because of the similarity of the large granular vesicles to those of polypeptide-storing neurosecretory nerves (485). Many nerve profiles of this kind have been seen in the gut of a number of vertebrate species (36, 62, 69, 132, 270, 411, 436, 437, 469, 470, 499, 523, 526, 528) including human foetal gut (349), and in amphibian lung (467). Occasional profiles of this type have also been noted in other organs, including arterioles in the rat jejunum and mesentery (180). In order to distinguish these vesicles from the large granular vesicle (LGV) seen in adrenergic and cholinergic nerves, the term *large opaque vesicle* (*LOV*) has been introduced.

The strongest evidence that nerve profiles containing large opaque vesicles represent purinergic nerves comes from studies of the amphibian lung and mammalian gut. It has been shown recently that when the adrenergic nerves supplying the toad lung are destroyed with 6-hydroxydopamine (6-OHDA), both the purinergic inhibitory response to vagal stimulation (see section VI B) and the profiles containing a predominance of large opaque vesicles remain unchanged (467). These results are supported by the observation that treatment with two catecholamine-depleting drugs, (metaraminol and reserpine), while strongly reducing the amount of catecholamine detectable in the lung by histochemical and chemical assay techniques, did not deplete the cores of large opaque vesicles. Adrenergic and p-type nerve profiles in the large intestine of man, monkey and guinea-pig, were also shown to be differentially affected by 5- and 6-OHDA treatment (35). IJP's have been recorded in the intestine of the guinea-pig, after adrenergic denervation (225) and profiles containing a predominance of large opaque vesicles also persist (Burnstock, unpublished observations). Large opaque vesicles found predominantly in axon profiles in the mouse colon were unaffected by reserpine, although this treatment was sufficient to remove the granular cores

FIG. 1. Intra-axonal structure of different autonomic nerves: a. Cholinergic (C) and adrenergic (N) nerve profiles in the circular muscle coat of the vas deferens of a rat treated for 30 min with 5-hydroxydopamine (5-OHDA) (50 mg/kg). Note that *both* small and large granular vesicles in the adrenergic profile have taken up the drug, but not the agranular or large granular vesicles in the cholinergic profile. Osmium fixation. × 38,000. b. Adrenergic axon profile in Auerbach's plexus of chicken gizzard. In this case large granular vesicles (LGV) (which take up 5- and 6-OHDA) are predominant. Note the electrontransparent halo between the intravesicular granule and the limiting vesicle membrane. Osmium fixation. × 42,000. c. Non-adrenergic, non-cholinergic axon profile, ★ in large intestine of toad, treated for 45 min with 6-OHDA (100 mg/kg). The large opaque vesicles (LOV) (characterised by granulation *throughout* the vesicle) contained in these nerves do not take up 6-OHDA. Note also the cholinergic nerve profile, C. Glutaraldehyde-osmium fixation. × 48,000. d. Nerve profile, S, probably representing the terminal portion of a sensory nerve fibre in the finch ureter. Note the aggregation of small mitochondria, absence of vesicles and Schwann cell investment. The other axons, C, are probably cholinergic. Osmium fixation. × 48,000. [From Burnstock and Iwayama (132)].

from small granular vesicles in enteric adrenergic nerve profiles (499). Reserpine has also been shown to deplete the large granular vesicles in adrenergic nerves of sympathetic (242) and central (262) nervous systems, although these vesicles appear to be more resistant to degranulation by amine-depleting drugs than small granular vesicles (66, 537).

That the nerves containing large opaque vesicles are purinergic has been given further support from a study of the fine structure of nerve profiles in Auerbach's plexus of the avian gizzard (67, 69, 132). Cholinergic, adrenergic and purinergic nerves have been demonstrated physiologically in this tissue (64) and, in addition to profiles with largely agranular vesicles, two different types of profile containing predominantly large vesicles have been described. The large granular vesicles in one type of profile are characterised by a size range of 900 to 1200 Å with a dense granular core and a prominent halo between granules and vesicle membrane (fig. 1b); these vesicles are associated with catecholamines and take up 6-OHDA. The second type of profile contains vesicles, which are larger (1000–2000 Å) and do not have a prominent halo between granule and vesicle membrane; they are closely comparable to the large opaque vesicles described for purinergic nerves in the lung and intestine and are unaffected by 6-OHDA.

The evidence presented above that profiles characterised by a predominance of large opaque vesicles represent purinergic nerves needs confirmation by more direct evidence from specific histochemical or electronmicroscopic-autoradiographic methods. Recognition of purinergic nerves by their ultrastructure will help in the determination of their distribution in the body, especially when they are present in small numbers and therefore not easily recognised from pharmacological studies.

It is not known yet whether the purinergic neuromuscular junction has any special distinguishing features. All autonomic neuromuscular junctions are complicated by the presence of muscle effector bundles with low resistance pathways (represented by "nexuses") allowing electrotonic spread of activity between neighbouring smooth muscle cells, and also release of neurotransmitter "en passage" from extensive terminal varicose nerve fibres (132). In addition, the relationship of individual nerve varicosities to adjacent smooth muscle membranes varies considerably in different tissues (119). For example, in the vas deferens and iris, adrenergic nerve and muscle membranes are often separated by as little as 150 Å and specialisation of the postjunctional muscle membrane in the form of subsynaptic cysternae is common (458, 459, 540). The situation in the gut is different. Axon and muscle membranes seldom approach closer than 1000 Å in the longitudinal muscle coat, including the taenia coli (62, 363, 456, 469, 526), although close neuromuscular contacts are often seen in the circular muscle coat, with separation of nerve and muscle membranes of as little as 150 Å (101, 411, 470, 529). In the toad rectum, aggregations of micropinocytotic vesicles in the muscle membrane apposing the nerve terminals that contain a predominance of large opaque vesicles are common and many represent some postsynaptic specialisation (132). The significance of this feature in relation to the localisation of ATPase will be discussed below.

C. Histochemical localisation of adenosine triphosphatase and 5-nucleotidase and distribution of adenosine deaminase

The distribution of the enzymes which can degrade ATP and its derivatives is of interest because of the possibility that ATP is the transmitter released from purinergic nerves. The role of these enzymes in purinergic transmission is considered in the model proposed in section V A. Many enzymes with a wide variation in specificity can act to hydrolyse ATP. These include nonspecific alkaline phosphatases, polyphosphatases, nucleoside triphosphatases and specific adenosine triphosphatases.

Intracellular, as well as extracellular, localisation of these enzymes should be considered in relation to the transmitter inactivation mechanism (see section II D). Under certain conditions ATPase has been localised intracellularly in skeletal and cardiac muscle (362, 422, 508, 509). However, most of the histochemical studies described below did not reveal high levels in intracellular sites. This seems likely to be a reflection of the staining methods available rather than a true picture, since biochemical studies on homogenates of liver (331), kidney (361) and skeletal muscle (332) have demonstrated ATPase activity in mitochondrial, myosin and microsomal fractions. ATPase has also been shown in vesicles isolated from the adrenal medulla (29) and adrenergic nerves (360).

Adenosine triphosphatases. Adenosine triphosphatases (ATPases) are known to be associated with a variety of physiological processes in which ATP is involved in energy transfer, including muscle contraction, active transport, motility, synthesis, secretion *etc.*, but it was stressed in a recent review that the traditional concept of ATP as an energy source may not be its only role (28).

Although an extensive literature now exists describing ATPase localisation in different tissues, any general correlation of the published work at this stage is complicated by variations in experimental conditions, species variation, and lack of information about the functional significance of Mg-activated ATPases, nucleoside triphosphatases, alkaline phosphatases and polyphosphatases (28, 87, 89, 324, 502). The specificity of histochemical staining methods for ATPases has also been questioned (91, 254, 390, 405, 420, 432, 472) and this must be borne in mind when considering the reports of ATPase activity localised histochemically in a number of autonomically innervated tissues (30, 70a, 89a, 216, 217, 382, 414, 431, 432). There seems to be some general agreement that ATPase activity in intestine and in some blood vessels differs from its activity in other tissues in that the staining is more intense and is not easily inhibited by the ATPase inhibitor, *para*-chloromercuribenzoate (pCMB). It has been suggested that high polyphosphatase activity in these tissues may account for the lack of sensitivity of ATPase to pCMB inhibition (216, 432).

In an ultrastructural study of the localisation of nucleoside phosphatases in the rat small intestine, Rostgaard and Barrnett (474) confirmed the findings of Freiman *et al.* (216) that the muscularis mucosae and the inner part of the circular muscle layer show more intense activity than the rest of the muscle layers. The enzyme they described acts on nucleoside di- and triphosphates, and is resistant to osmium fixation. A similar enzyme has been described in the smooth

muscle cells of the toad bladder (34). The reaction product was localised in the micropinocytotic vesicles of the smooth muscle membrane (474). There was little evidence of intracellular deposition with this method. With mouse bladder smooth muscle, Lane (362) confirmed these results but, by altering the incubation conditions, he was also able to show deposition in the agranular endoplasmic reticulum. Nucleoside phosphatase activity has also been demonstrated in micropinocytotic vesicles of capillaries in the rat heart (388, 389). ATPase has been described on the membranes of unmyelinated nerves in rat intestine (474), brain (159, 536), retina (391) and peripheral nerves (421, 503).

A study has been carried out recently of the distribution of ATPase in purinergically and non-purinergically innervated smooth muscle preparations (576). At the light microscopic level, neither the cholinergic nor purinergic nerves supplying the smooth muscle of the chick gizzard showed any ATPase activity. However, adrenergic nerves in the guinea-pig iris and vas deferens showed intense ATPase activity which, at the electronmicroscopic level, was clearly associated with the Schwann cell membranes enveloping unmyelinated nerves, but not with the axonal membranes. In gut preparations of toad and chick, which are supplied by non-adrenergic, non-cholinergic nerves, the smooth muscle membrane stains heavily for ATPase. This is particularly so in the circular muscle coat, where, unlike the longitudinal coat, there are many close (150 to 200 Å) neuromuscular junctions [see Burnstock, (119)]. At the electronmicroscopic level, ATPase has been shown to be localised in micropinocytotic vesicles in the smooth muscle membrane (89a, 362, 474, 576). This might be significant in view of the abundance of micropinocytotic vesicles which are characteristic of the smooth muscle membrane adjacent to axons containing large opaque vesicles at close neuromuscular junctions in the circular muscle of the colon and rectum (132, 411).

5′-Nucleotidase. 5′-Nucleotidase was first described by Reis (452), who demonstrated the hydrolysis of adenosine 5′-phosphate and inosine 5′-phosphate over a wide pH range, with an optimum at pH 7.8. The enzyme is highly specific for the 5′ position and hydrolyses all pyrimidine and purine nucleotides to orthophosphoric acid and the nucleoside. In general, the histochemical demonstration of this enzyme has escaped the controversy that has surrounded the localisation of ATPase (253, 378, 414, 442, 554).

The levels of 5′-nucleotidase vary greatly in different species (89a, 453, 454). In the rat it is present in almost all tissues, while in the pigeon it is only found in the lungs; levels of 5′-nucleotidase in the rabbit are generally low. The enzyme is abundant in the medial layer of arteries and arterioles. In a study of 5′-nucleotidase distribution in human tissues, Freiman *et al.* (216) found high activity in most smooth muscles, but not in cardiac or skeletal muscle, confirming earlier reports (46, 378). However, Reis (452) found high levels of 5′-nucleotidase in frog and rat hearts, but much lower levels in the hearts of other species including dog, man, calf, pig, rabbit, fowl, turkey and pigeon. Dephosphorylation of AMP in the heart by 5′-nucleotidase has been demonstrated *in vivo* (36). Striking variations in 5′-nucleotidase activity were found in the smooth muscle of the dog

(216). No activity was seen in the oesophagus, stomach, muscularis mucosa, the inner zone of circular muscle throughout the gut, uterus, prostate and the great vessels. However, activity was strong in the longitudinal muscle of the intestine and in the outer layer of the circular muscle. Polyphosphatase activity was also weak in the circular muscle coat and strong in blood vessels. A wide and puzzling variation in 5′-nucleotidase activity has been observed in the small blood vessels of dog and man (89a, 216) and also in rat (378).

Adenosine deaminase. Adenosine deaminase is found in most tissues (151, 374, 408), where it acts on adenosine at physiological pH to produce inosine, which is pharmacologically inert. High levels have been found in the liver, spleen, small intestine, testis and adrenal. Conway and Cooke (151) found the highest concentrations of adenosine deaminase in the vermiform appendix, duodenum and jejunum of the rabbit, but there are likely to be species as well as organ differences in the distribution of this enzyme. It is also present in kidney, some other smooth muscles and in cardiac muscle, but skeletal muscle contains AMP deaminase.

D. Summary

1) The cell bodies of purinergic neurones are localised in the gut wall, probably in Auerbach's plexus. Terminal axons of these neurones supply the smooth muscle of both longitudinal and circular muscle coats.

2) The terminal regions of purinergic nerves appear to be varicose as has been shown for other autonomic nerves, with diameters varying between 0.3 μ at intervaricosities to about 1.5 μ at varicosities.

3) According to calculations based on electrophysiological studies, the purinergic terminal axons extend for several millimeters.

4) Purinergic inhibitory neurones in the stomach and distal rectum are under the control of preganglionic parasympathetic nerve fibres in the vagus and pelvic nerves respectively, but in the colon they appear to have intramural connections with cholinergic neurones and are not controlled by either parasympathetic or periarterial sympathetic extrinsic nerves.

5) Some postganglionic non-adrenergic, non-cholinergic excitatory nerves to the bladder and inhibitory nerves to the distal rectum are located in the pelvic outflow, while others reach the oesophagus and stomach in the vagal outflow. Postganglionic non-adrenergic, non-cholinergic fibres which supply parts of the vasculature and regions of the alimentary canal (particularly in lower vertebrates) are postganglionic, run in the sympathetic nerves and some appear to originate in the dorsal root ganglia.

6) Purinergic nerve profiles appear to be characterised by a predominance of large vesicles that differ from the large granular vesicles (LGV) seen in small numbers in adrenergic and cholinergic nerves in that they are bigger (800–2000 Å) and do not have a prominent electron-transparent halo between the vesicle membrane and its granular core; they have been termed *large opaque vesicles* (LOV). Thus purinergic nerve profiles can be distinguished from profiles of cholinergic nerves, which contain a predominance of small (300–500 Å) agranular

vesicles (AV), adrenergic nerves, which contain a predominance of small (300–500 Å) granular vesicles (SGV) and afferent nerves, which are characterised by large numbers of mitochondria.

7) Profiles of nerves which contain a predominance of large opaque vesicles have been seen in the gut of many mammalian and non-mammalian species and in amphibian lung, where non-adrenergic, non-cholinergic nerves have been demonstrated physiologically.

8) Nerve profiles containing a predominance of large opaque vesicles remain after destruction of adrenergic nerves in the amphibian lung, bird stomach and mammalian large intestine by 6-hydroxydopamine; purinergic nerve responses also remain unchanged under these conditions.

9) Mg-activated adenosine triphosphatase (Mg-ATPase) has been localised histochemically in relation to autonomic nerve bundles, particularly those containing adrenergic fibres. At the electronmicroscopic level ATPase is associated with the membranes of Schwann cells apposing unmyelinated axon membranes.

10) In purinergically innervated gut preparations, ATPase staining is heavy on muscle membranes and at the electronmicroscopic level this can be seen to be located in micropinocytotic vesicles. This might be significant in view of the abundance of micropinocytotic vesicles which are characteristic of the smooth muscle membrane at close neuromuscular junctions in the circular muscle of the large intestine where the nerves contain LOV.

11) 5′-Nucleotidase has been localised in the media of many, but not all, arteries and arterioles and in most visceral smooth muscles, particularly in the longitudinal and outer circular muscle layers of the gut, but not in skeletal muscle or in the cardiac muscle of most species.

12) Adenosine deaminase is found in most tissues with particularly high levels in small intestine. It degrades adenosine to inosine, which is pharmacologically inactive.

IV. Electrophysiology of Purinergic Transmission

Most studies of the electrophysiology of purinergic nerve transmission have been carried out on the taenia coli of the guinea-pig (see Bennett and Burnstock, 57; Holman, 296), but inhibitory junction potentials have also been recorded in the longitudinal muscle of the guinea-pig jejunum (285, 357), in longitudinal and circular muscle of the guinea-pig and rabbit colon (224, 225, 503a), in chicken and pigeon gizzard (63, 64, 65), in guinea-pig stomach (42, 43, 356) and in pig and sheep intestine (Furness, unpublished observations).

A. Inhibitory junction potentials

Stimulation of intramural purinergic nerves with *single pulses* of short duration (<0.3 msec) produces transient hyperpolarisations or inhibitory junction potentials (IJP's) of up to 25 mV in single smooth muscle cells of the gut (43, 60, 63, 64, 109, 122–124, 225, 226, 285, 356, 357, 521, 553). IJP's have also been recorded in the stomach in response to stimulation of vagal nerves (42, 64, 65). IJP's are unaffected by adrenergic neurone blocking agents or sympathetic denervation (see fig. 2), but are abolished by tetrodotoxin (42, 43, 60, 109, 225,

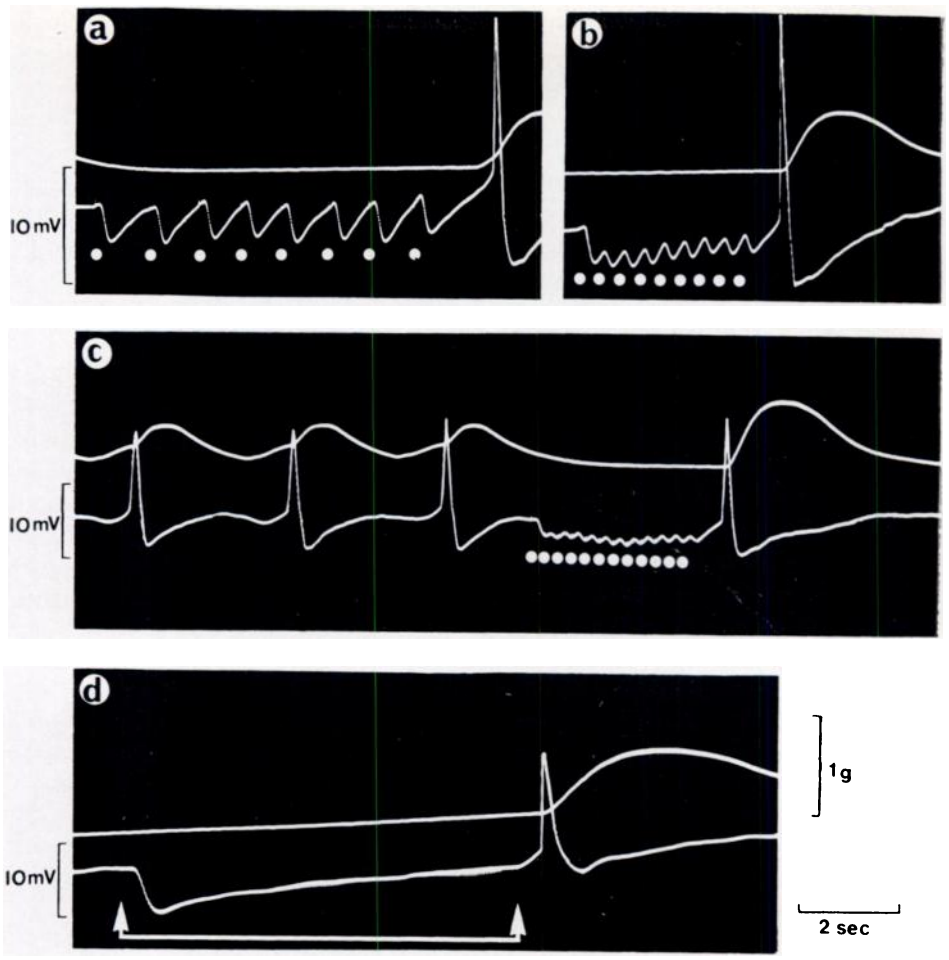

FIG. 2. Inhibitory junction potentials (IJP's) recorded in smooth muscle of the atropinised guinea-pig taenia coli in response to transmural stimulation of the intramural nerves remaining following degeneration of the adrenergic nerves by treatment of the animal with 6-hydroxydopamine (250 mg/kg ip. for 2 successive days) 7 days previously. Upper trace, mechanical record. Lower trace, changes in membrane potential recorded with a sucrose-gap method (136). The junction potentials recorded with this method are qualitatively, but not quantitatively, similar to those recorded with intracellular microelectrodes. a. Responses to low frequency stimulation (1/sec). Note the long latency and slow time course of individual IJP's, and rebound excitation (spike and contraction) following cessation of stimulation. b. Response to a stimulation frequency of 3/sec. Note summation of individual IJP's and rebound contraction. c. Response of a spontaneously active preparation to stimulation at 4/sec. Note that the hyperpolarisation maintained during repetitive stimulation raises the membrane potential beyond the zone of spontaneous initiation of action potentials and leads to relaxation. Rebound contraction follows cessation of stimulation. d. Responses to high frequency stimulation (30/sec). Note that individual IJP's fuse and are represented by a maintained hyperpolarisation followed by depolarisation and rebound excitation on cessation of stimulation.

357). In contrast, stimulation of adrenergic nerves supplying the gut with single pulses does not elicit IJP's (59, 245, 429); hyperpolarisation and associated relaxations usually only occur in response to repetitive stimulation at frequencies of more than about 5 pulses/sec, and the hyperpolarisation rarely exceeds 10 to 20 mV.

Repetitive stimulation of purinergic nerves results in summation of individual IJP's and hyperpolarisation of up to 50 mV. There may be some facilitation of the first 2 or 3 IJP's in a train (224). The amplitude of IJP's in response to single stimuli can be graded with increasing strength of stimulation up to a maximum of about 25 mV, whereas the maximum amplitude reached during repetitive stimulation is about 50 mV. The time taken to reach maximum hyperpolarisation during repetitive stimulation (10 pulses/sec or more) is slightly shorter than that of the rise time of a single IJP; when maximum hyperpolarisation is reached, the response begins to decay in spite of continuing stimulation. At stimulation frequencies of 30/sec or more, the relaxation is rarely maintained for more than about 15 sec. Even at physiological frequencies of 5 to 10 pulses/sec (125), the response begins to weaken after more than 20 to 30 sec.

The latency of the response to postganglionic stimulation of the intramural purinergic nerves in the gut of various species is long (about 45–80 msec) relative to the latencies of adrenergic excitatory junction potentials recorded in non-intestinal smooth muscle (about 6–20 msec) and to those observed at the mammalian motor end plate (about 0.2 msec) (see table 1). This long latency of the IJP, however, appears to be comparable to that of cholinergic excitatory junction potentials (EJP's) recorded in response to postganglionic stimulation in the guinea-pig taenia coli (55) and in chick oesophagus (423), although in more recent publications (224, 285) latencies of EJP's in the guinea-pig colon and jejunum have been reported to be as short as 15 to 20 msec (see table 1). The latency figures quoted in table 1 represent the time between stimulation of terminal nerve fibres and the appearance of the junction potential. They do not represent synaptic delay, since they include the time taken for nerve conduction. In a study of synaptic delay in the mouse vas deferens, Furness (228) plotted latency against distance and the delay for zero distance was obtained by extrapolation. A minimum latency of 5.1 msec was recorded when microelectrodes were placed within 100 to 300 μm of the cathodal stimulating electrode, of which 0.1 to 0.7 msec was calculated to be due to the nerve conduction time. Thus it was concluded that there was a synaptic delay of 4 to 5 msec for this tissue, with a conduction velocity of 0.46 to 0.6 m/sec in the terminal regions of the adrenergic nerve fibres. In the taenia coli, measurements of the increase in latency of the IJP at increasing distances between stimulating and recording electrodes implied a conduction velocity of 10 to 20 cm/sec in the terminal regions of purinergic nerves (109). Whether the long delay time of the IJP and also the EJP is due largely to presynaptic or postsynaptic factors is not yet known (57, 131, 296). It seems unlikely to represent the time required for transmitter to diffuse from nerve-terminals to the effector sites [see Furness (228), who calculated a negligible delay of 10^{-7} sec for diffusion of transmitter across a

TABLE 1

Comparison of the latency and time course of junction potentials in response to stimulation of postganglionic nerves

	Preparation	Minimum* Latency	Rise Time	Total Duration	Reference
		msec	*msec*	*msec*	
Inhibitory junction potentials (purinergic)	Guinea-pig taenia coli	80	200–500	1000	60
		140	200–300		109
	Rabbit colon	80	150–250	800–1200	224
	Guinea-pig colon	45	150–250	800–1300	224
	Pigeon gizzard (anoxic)	70	150–250	500–1500	64
	Guinea-pig jejunum	50	120–280	1000	357
	Guinea-pig stomach	150†	200–300	1000–1800	42
Excitatory junction potentials (cholinergic)	Guinea-pig colon	15	150–200	400–700	226
	Guinea-pig taenia coli	100	100–200	500–800	54
	Chick oesophagus	90	150–250	700–950	423
	Pigeon gizzard (anoxic)	70		430–900	64
	Guinea-pig jejunum	20			285
	Guinea-pig stomach	150†	200	400–500	42
Excitatory junction potentials (adrenergic)	Guinea-pig vas deferens	20	15–20 (half rise time)	1000	130
		6	40	500	354
	Mouse vas deferens	10	10–20	100–250	296
		5	28–34		228
	Cat nictitating membrane	20	50–80	500	192
	Rabbit ear and mesenteric arteries	12	70–100	500–1000	512
	Guinea-pig uterine artery	20		900–1000	48
End-plate potential	Cat skeletal neuromuscular junction	0.2	0.6	2–3	97

* The figures tabled for autonomic junctions do not represent synaptic delay, since they include the time taken for nerve conduction (see text page 530).

† These figures for the latency of junction potentials in the guinea-pig stomach were recorded following preganglionic stimulation of the vagus nerves.

200 Å synaptic cleft in the vas deferens] even though the minimum separation of nerve and muscle in the taenia appears to be of the order of 1000 Å (62). Latency measurements are highly temperature dependent (228).

The time course of IJP appears to be comparable, or perhaps slightly longer, than that of cholinergic EJP's recorded in the gut, but considerably longer than that of adrenergic EJP's recorded in the vas deferens and very much longer than the end plate potential (see table 1). The rise time of the IJP is about 150 to 250 msec; the decay time to half maximum is 200 to 400 msec and is approximately exponential, while the total duration of the IJP is of the order of 1 sec. The long time course of the IJP has a functional basis; the maximum rate of spontaneously firing action potentials in the taenia coli is about 1/sec (115, 295,

325), so that an IJP lasting for about 1 sec would inhibit spike initiation and thus bring about relaxation.

The reasons for the long time course of the IJP, or for that matter all autonomic junction potentials, need further exploration. It seems unlikely, according to calculations made from measurements taken from serial electronmicroscopy of autonomic neuromuscular junctions, that the long time course is due to asynchronous release of transmitter from the varicosities as a result of slow conduction of the action potential along the terminal regions of the nerves, or to the time for diffusion of transmitter from the varicosities to the muscle cells (57, 61, 131, 228). Two further possibilities remain: that the time course of the junction potential is determined by the rate of inactivation of the neurotransmitter or that it is determined by the electrical properties of the smooth muscle effector bundle. Furness (228) argues against the first possibility for the vas deferens largely on the grounds that there is a striking decrease in decay time of the EJP when the potassium concentration is increased and that high potassium would, if anything, reduce the rate of uptake of NA released from the nerve (85). The explanation for the long time course of junction potentials most favoured at the present time is that it is determined largely by the passive electrical properties of smooth muscle (57, 229, 296). Tomita (533) has shown that an electrotonic potential set up in the smooth muscle cells of the vas deferens by large external electrodes decays with a time constant of 100 msec, which is of the same order as the decay constant for the EJP in this tissue.

When the membrane potential of a single smooth muscle cell in the taenia coli is increased or decreased by passing current across the membrane, the amplitude of the IJP in the majority of cells is not altered (62). This result implies a high degree of electrotonic coupling between neighbouring muscle cells and is consistent with the observation of many "nexuses" or areas of low resistance between muscle cells in the taenia (62, 469, 534, 535).

Spontaneous hyperpolarisations with about the same time course and amplitude as IJP's have been observed on rare occasions (59, 224, 315). They may represent the spontaneous release of packages of transmitter from terminal regions of nerves, but it is possible that they are the result of nerve impulses generated spontaneously in purinergic neurones in the myenteric ganglia (see section II B).

B. Rebound excitation

A characteristic feature of the responses to stimulation of purinergic nerves is the rebound excitation which follows the main inhibitory response (42, 54, 122, 140, 226, 229, 564). Rebound excitation usually occurs at the end of stimulation, but may also "break through" during a period of repetitive stimulation, especially at high frequency. The electrical activity associated with rebound contractions consists of depolarisation after cessation of the hyperpolarisation produced by purinergic inhibitory nerve stimulation (see fig. 2). The membrane potential usually depolarises beyond its original level, with the result that the frequency of spike discharge, and therefore contraction, is greater than that seen before

nerve stimulation. In preparations where there are no spontaneous spikes and associated contractions, stimulation of the inhibitory purinergic nerves still produces hyperpolarisation. Since this is followed by rebound depolarisation and subsequent initiation of spike activity, the result is to produce *contraction* rather than relaxation. The mechanism of rebound excitation is not known (57, 226, 229), but it is significant that it also occurs after hyperpolarisation induced by both external and intracellularly applied currents (226, 358, 533).

C. Postsynaptic action of transmitter

Studies have been made of the ionic basis of the IJP (56, 58, 285). The results of varying the ionic composition of the bathing solution suggested that the transmitter caused a specific increase in K^+ conductance, *i.e.*, the equilibrium potential for transmitter action was approximately the same as the K^+ equilibrium potential (E_K). No evidence was found for increase in anion conductance. Determinations of the increase in membrane conductance in response to purinergic nerve stimulation are complicated by the low resistance pathways between muscle cells. This makes observations of IJP amplitude in cells difficult to interpret, since changes in membrane potential induced by current flow from intracellular electrodes depend on the extent of coupling with neighbouring cells (62). The hyperpolarisation produced by catecholamines or sympathetic stimulation of the taenia coli is slower and smaller than that produced by purinergic nerve stimulation. There is evidence that noradrenaline increases both K^+ and Cl^- conductance of smooth muscle in addition to its metabolic action (355). This might explain the difference in degree of hyperpolarisation produced by stimulation of adrenergic and purinergic nerves, *i.e.*, the membrane potential might be at equilibrium somewhere between E_K and E_{Cl} under the inhibitory action of adrenergic nerves, but close to E_K during the action of purinergic nerves.

Low $(Ca)_o$ and high $(Mg)_o$ have been reported to have no effect on the IJP (285); this surprising observation needs further exploration.

D. Interaction of purinergic with cholinergic and adrenergic responses in single cells

In the absence of specific blocking agents, the response of most smooth muscle cells of the taenia coli to transmural stimulation appears to be a mixture of inhibitory and excitatory junction potentials. Only small groups of cells are encountered which receive a predominantly cholinergic excitatory innervation (55). Occasionally, cells are found which respond to transmural stimulation with a diphasic response consisting of depolarisation followed by hyperpolarisation. In the presence of atropine, IJP's in response to single pulses can be seen in consistent form and amplitude in the majority of cells (60).

Stimulation of the pelvic nerves in rabbit and guinea-pig evoke EJP's in most cells of both the longitudinal (224, 245, 246) and circular muscle coats (224) of the colon, but transmural stimulation produces EJP's in only about 5% of the cells, IJP's being recorded in the remainder (224). It seems likely therefore that cholinergic nerves innervate all the muscle cells, but that when transmural stimulating electrodes are used, the IJP dominates the response. This conclusion

has been supported by experiments with the guinea-pig colon in which the interaction of EJP's and IJP's in individual smooth muscle cells was studied during extrinsic stimulation of cholinergic nerves and transmural stimulation of purinergic nerves (224). It was shown that the IJP was capable of obliterating the EJP, but the reverse situation was never observed. This suggests that the conductance change underlying the IJP may be considerably greater than that of the EJP. The possibility of occlusion at a ganglionic level, as suggested by the hypothesis of Kottegoda (345), seems unlikely, since stimulation of intrinsic inhibitory nerves by transmural electrodes is largely postganglionic (224).

Studies of the interaction of nerve-mediated excitation and inhibition of single smooth muscle cells have also been carried out in the avian (chick and pigeon) gizzard (65) and in guinea-pig stomach (42). In contrast to the situation in the colon (224), the EJP appeared to dominate the IJP in most muscle cells of the guinea-pig stomach when both cholinergic and purinergic nerves were stimulated together (42). This difference may be accounted for in terms of the smaller amplitude of IJP's evoked by preganglionic nerve stimulation in the stomach, compared with the larger IJP's recorded in the colon resulting from transmural stimulation of many postganglionic purinergic nerves. Some cells in the guinea-pig stomach receive only vagal inhibitory innervation; other cells are innervated solely by cholinergic excitatory nerves (42). Purinergic nerves may selectively innervate pacemaker regions in the stomach, since spontaneous action potentials were blocked by stimulation of purinergic nerves even in cells where IJP's were not recorded. In the bird gizzard, transmural stimulation frequently evoked an EJP followed by an IJP in the same cell (65). Cells were encountered which responded to stimulation of the vagus nerve with an EJP and to perivascular nerve stimulation with an IJP; other cells showed the opposite responses. EJP's evoked in the same cell by both vagal and perivascular stimulation summed with each other. Some cells responded with IJP's to both vagal and perivascular stimulation.

E. Summary

1) Transient hyperpolarisations or inhibitory junction potentials (IJP's) have been recorded in single smooth muscle cells in both the circular and longitudinal muscle coats of the guinea-pig, rabbit, pig, sheep, chick and pigeon gut in response to stimulation of enteric nerves.

2) IJP's persist in the presence of atropine and adrenergic neurone-blocking agents or after degeneration of sympathetic adrenergic nerves, but are abolished when nerve conduction is blocked by tetrodotoxin.

3) Repetitive stimulation of purinergic nerves results in summation of individual IJP's and hyperpolarisations of up to 50 mV; there may be facilitation of the first 2 or 3 IJP's in a train.

4) A feature of purinergic transmission is the rapid decay of the response to repetitive nerve stimulation; the amplitude of response is rarely maintained for more than 20 to 30 sec even at physiological frequencies of 5 to 10 pulses/sec.

5) The latency of the IJP in response to stimulation of postganglionic purin-

ergic nerves is about 45 to 80 msec. This latency appears to be slightly longer than that recorded for cholinergic EJP's recorded in gut. It is long compared to the latency of adrenergic EJP's in non-intestinal smooth muscle (about 10 msec) and of end plate potentials at the mammalian skeletal neuromuscular junction (about 0.2 msec).

6) The terminal regions of purinergic nerves conduct impulses at 10 to 20 cm/sec.

7) The time course of IJP's recorded in smooth muscle of the gut has a rise time of 150 to 250 msec and a total duration of 800 to 1200 msec. This is comparable to the time course of cholinergic EJP's recorded in gut muscle, but long compared to adrenergic EJP's recorded in the vas deferens. The reasons for this long time course (and also for long synaptic delay) are discussed.

8) There is evidence for spread of IJP's between neighbouring smooth muscle cells through low resistance pathways which correspond morphologically to "nexuses."

9) Spontaneous IJP's occur, but mostly only in low frequency trains.

10) A characteristic feature of the response to stimulation of purinergic nerves is rebound depolarisation associated with spikes and contraction, following the hyperpolarisation produced during the main inhibitory response. Consequently in low tone preparations, particularly those with little or no spontaneous spike activity, stimulation of inhibitory purinergic nerves can result in long latency contraction rather than relaxation.

11) There is evidence that the transmitter released from purinergic nerves acts by producing a specific increase in K^+ conductance; this is in contrast to evidence for the action of noradrenaline released from adrenergic nerves which produces an increase in both K^+ and Cl^- conductance. Low Ca^{++} and high Mg^{++} appear to have little, if any, effect on the IJP.

12) Studies of the interaction of responses of single muscle cells to stimulation of enteric, intrinsic and extrinsic nerves suggest that most cells receive both cholinergic excitatory and purinergic inhibitory innervation. When both nerves are stimulated together postganglionically, the IJP usually dominates in the colon, but the reverse occurs in the stomach in response to preganglionic stimulation of the nerves. A few groups of cells appear to be supplied exclusively by excitatory cholinergic nerves; other cells appear to be innervated solely by purinergic inhibitory nerves. The inhibitory response of muscle cells to sympathetic nerve stimulation is weak even with repetitive stimulation and, in many regions of the gut, is probably largely the result of overflow of NA from directly innervated structures such as enteric neurones and blood vessels.

V. Pharmacology of Adenyl Compounds and Purinergic Transmission

The high sensitivity of various smooth muscles to adenyl compounds was recognised long before the existence of purinergic nerves was suggested. However, there have been few studies on drugs which affect responses to purine nucleotides that help in the search for compounds that will block or potentiate purinergic transmission.

A. Model of synthesis, storage, release and inactivation of ATP at the purinergic neuromuscular junction

On the basis of the information summarised in the earlier chapters and by analogy with the pathways known for cholinergic and adrenergic systems (fig. 3 A, B), a tentative model of the synthesis, storage, release and inactivation of ATP during purinergic nerve transmission is proposed in figure 3C. This is intended to provide a basis for discussing the action of a variety of different drugs and to act as a stimulus to further studies in the field.

In this section of the review, drugs will be considered which have been shown to antagonise the action of adenyl compounds and those which have been shown to potentiate their action on a variety of preparations, although these drugs consist of a remarkable collection of largely unrelated compounds. Reports of the actions of these drugs on the responses of smooth muscle to stimulation of purinergic nerves will be included, wherever they have been examined. Eventually drugs may be found which reduce or augment purinergic transmission by acting at specific stages such as: ATP synthesis (although, unless these drugs are selective for the transmission process, they are unlikely to be useful); ATP release; ATP breakdown by ATPase, 5′-nucleotidase and adenosine deaminase; postsynaptic ATP receptor action (including analogues and derivatives of ATP); and adenosine uptake.

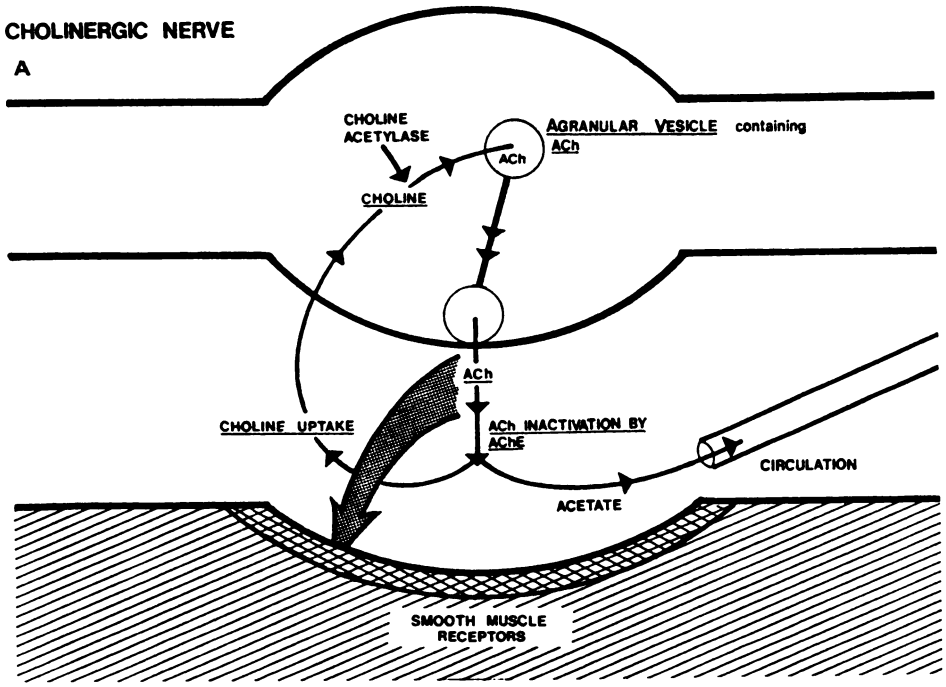

FIG. 3. Schematic representations of synthesis, storage, release and inactivation of autonomic neurotransmitters at: A, cholinergic; B, adrenergic; and C, purinergic neuromuscular junctions.

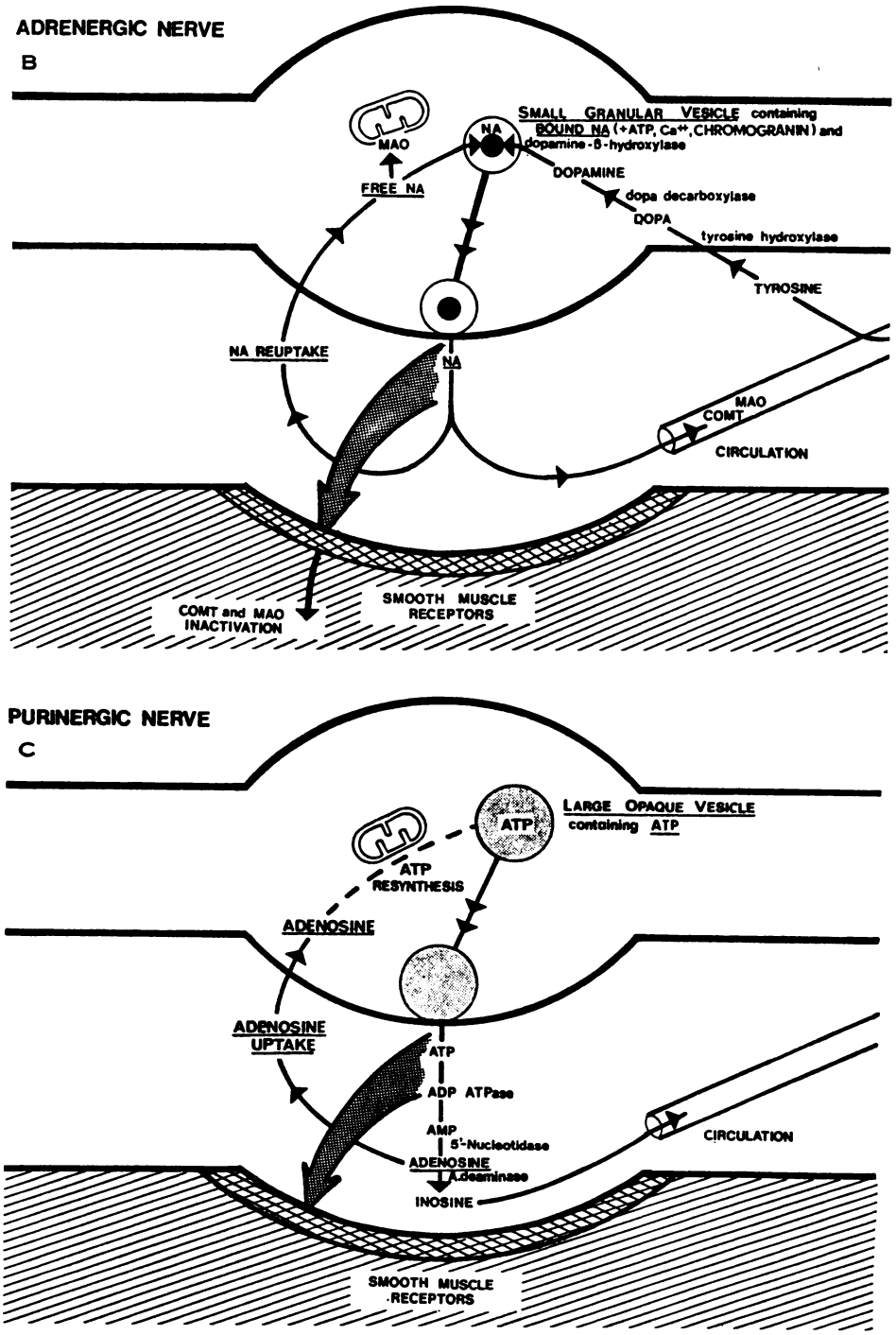
ADRENERGIC NERVE
B
MAO
NA
SMALL GRANULAR VESICLE containing
BOUND NA (+ATP, Ca++, CHROMOGRANIN) and
dopamine-β-hydroxylase
DOPAMINE
FREE NA
dopa decarboxylase
DOPA
tyrosine hydroxylase
TYROSINE
NA REUPTAKE
NA
MAO
COMT
CIRCULATION
COMT and MAO
INACTIVATION
SMOOTH MUSCLE
RECEPTORS
PURINERGIC NERVE
C
ATP
LARGE OPAQUE VESICLE
containing ATP
ATP
RESYNTHESIS
ADENOSINE
ADENOSINE
UPTAKE
ATP
ADP ATPase
AMP
5'-Nucleotidase
ADENOSINE
Adeaminase
INOSINE
CIRCULATION
SMOOTH MUSCLE
RECEPTORS

FIG. 3B AND 3C

Experiments with blockers or potentiators of a substance like ATP, which is widely involved in a number of vital cellular processes, including muscle contraction, must be interpreted with great care. In particular, even if a drug is shown to have parallel actions on the responses to purinergic nerve stimulation and to ATP, it is important to eliminate the possibility that the effect may be non-specific by also examining its affect on responses to unrelated excitatory and inhibitory substances such as histamine or noradrenaline. In spontaneously active gut preparations, care must also be taken that apparent changes in amplitude of a response in the presence of a blocking or potentiating drug are not secondary effects due to changes in tone.

B. Inhibitory and excitatory action of adenyl compounds

If the extrinsic or intramural autonomic nerves supplying a large variety of isolated smooth muscle preparations are stimulated in the presence of atropine and adrenergic neurone blocking agents, nerve responses are often revealed which may be inhibitory or excitatory or a mixture of both. In most preparations so far examined, these responses are mimicked by the direct action of ATP (16, 126, 127, 135a, 507): if the nerve-mediated response is relaxation (as it is in most mammalian gut segments), then ATP causes a mimicking relaxation; if the nerve-mediated response is contraction (as is the case in the intestine of lower vertebrates or in the urinary bladder), then ATP also causes contraction; if the nerve-mediated response is diphasic (usually relaxation followed by contraction), then ATP produces an identical diphasic response. In those preparations which are supplied by *both* non-adrenergic inhibitory and non-cholinergic excitatory nerves (*e.g.*, guinea-pig ileum, toad stomach), the responses to transmural stimulation and applied ATP do not always concur, but this may be explicable in terms of the geometry of electrode placement in relation to the two types of nerve fibres. This situation may be comparable to adrenergic excitatory and inhibitory control of adjacent regions of the gastrointestinal tract [see Furness and Burnstock (230)].

Atropine-resistant contractions of the longitudinal muscle of the intestine in response to nerve stimulation have been demonstrated in the guinea-pig (14, 50, 78, 178, 226, 229, 344) and cat (220–222). It has been shown that these contractions are not mediated by histamine, 5-HT, prostaglandins or catecholamines (15, 49). Some of the contractions appear to be due to rebound excitation following stimulation of non-adrenergic inhibitory nerves (229). The response of intestinal segments to ATP in fish, amphibians and reptiles is predominantly or exclusively excitatory, as are also the responses to nerve stimulation in the presence of adrenergic and cholinergic blocking agents (135a, 507).

In recent studies of the mammalian bladder, the excitatory response to non-cholinergic pelvic nerve stimulation has been shown to be mimicked by ATP more closely than by any other excitatory substances tested so far (16, 127, 190).

These observations raise the possibility that receptors mediating either relaxation or contraction may exist for ATP released from purinergic nerves. This

would not be surprising, since comparable dual effects are already established for the action of both cholinergic and adrenergic nerves on autonomic effectors (8). Receptors for catecholamines have been separated into two types, *alpha* and *beta*, which are distinguished by the relative potencies of adrenaline, noradrenaline and isoprenaline (8). These receptors may mediate excitation or inhibition, depending on the tissue, and in many tissues mediate antagonistic responses (9, 539). The possibility has been examined that two types of ATP receptors can be distinguished by the criteria developed for adrenoceptors. Thus the potencies of different members of the adenine nucleotide series (ATP, ADP, AMP and adenosine) were studied on a variety of tissues, including guinea-pig taenia coli and bladder and lizard and toad ileum (135a, 505, 507). In these preparations, regardless of whether the response was inhibitory or excitatory, ATP was usually the most potent, the order being ATP $\geq$ ADP $>$ AMP $\geq$ adenosine. Studies of the relative potencies of nucleotides and nucleosides on other tissues have generally given the same result. This has been shown for cardiac responses (260), vasodilatation of coronary vessels (260, 514, 571, 572), vasoconstriction of lung vessels (236), and responses of uterus (244, 556) and bronchiolar muscle (79). Thus, the different actions of adenine nucleotides in causing contraction or relaxation of smooth muscle do not appear to reflect different receptor types which are distinguishable on the basis of relative potencies as is the case for adrenoceptors. However, in view of the recent evidence for rapid uptake of adenosine, but not adenine nucleotides or inosine into purinergic nerves (section II D) it is possible that the true order of potency is being obscured in isolated organ experiments.

The possibility of distinguishing different muscle receptors for adenyl compounds on the basis of the differential action of blocking agents has also been explored (see table 2). In a careful study of the effect of adenyl compounds and their antagonists on the isolated uterus of guinea-pigs, kittens, rats and rabbits, Arulappu (20) concluded that two different receptors were involved: one receptor (related to the contractile action of adenosine) was blocked by the phenothiazines and the dibenzazepines; another receptor (related to the relaxing action

TABLE 2

*Excitatory and inhibitory actions of adenyl compounds: blocking action of quinidine and phenothiazine**

	Gut		Bladder		Uterus	
	ATP relaxation (mammals)	ATP contraction (lower vertebrates)	ATP contraction	Adenosine relaxation	Adenosine relaxation	Adenosine contraction
Quinidine	+†	+	+	−‡	+	−
Phenothiazine	−	−	−	∓	−	+

* [From Arulappu (20), Burnstock *et al.* (126, 127), and Sneddon *et al.* (507)].

† +, Block.

‡ −, No effect.

of adenosine) was blocked by quinidine. Quinidine has been shown to block the excitatory action of ATP on the gut of lower vertebrates (507) as well as the inhibitory action of ATP on mammalian gut preparations (126). In the bladder, the contraction produced by ATP or by non-cholinergic, non-adrenergic nerves was blocked by quinidine, but the inhibitory action of adenosine was not (127).

A few observations have been made about structure-action relationships among purine derivatives (see fig. 4). Activity is increased by attaching a ribose

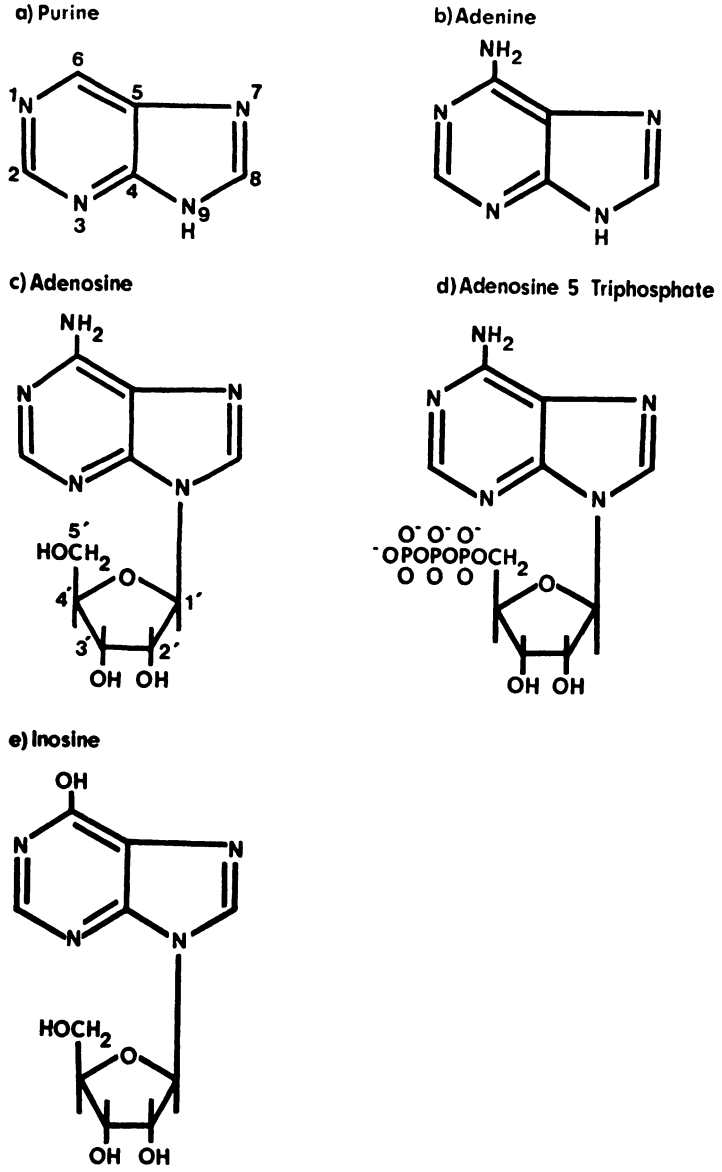

FIG. 4. Molecular structure of: a) purine; b) adenine; c) adenosine; d) adenosine 5-triphosphate; e) inosine.

group to the purine nucleus; adenine (fig. 4b) has $\frac{1}{10}$ to $\frac{1}{20}$ the potency of the adenine-ribose complex, adenosine (fig. 4c), in producing heart block in guinea-pigs (53). The ribose residue itself is not active (522). The presence of an amino group on the purine ring is necessary for the activity of these compounds (53, 320, 522); inosine (fig. 4e), the deaminated metabolite of adenosine, has little pharmacological action on smooth muscle (see section II C). The position of the amino group on the ring also affects activity; 2-amino purine riboside produces a positive chronotropic effect, while 6-amino purine riboside (adenosine) gives the opposite effect (320). A phosphate group attached to the D-ribose is not essential for the negative chronotropic effect of adenosine, but its position nevertheless modifies the nature of the effect (320). The addition of phosphate groups to adenosine increases its pharmacological activity (236; Satchell, personal communication). Boyd (95a) has recently pointed out that the separation of 4 to 6Å between the charged centres in the ATP molecule is similar to the separation of charged centres in the established neurotransmitters, noradrenaline and acetylcholine, as well as in 5-hydroxytryptamine, γ-aminobutyric acid and glycine. This similarity in molecular conformation, while not constituting proof of similarity of action, was taken as support for the hypothesis that nucleotides may serve as nerve transmitter substances. In the urinary bladder, adenyl compounds containing a pyrophosphate bond produced excitation only. However, sodium pyrophoshate alone had no effect on the bladder, indicating that both a purine compound and a pyrophosphate group are necessary for excitatory activity (127). In contrast, adenosine (but not inosine or guanosine) caused inhibition of the bladder.

Some studies have been carried out on the coronary dilator effects of analogues of adenosine and AMP (195). Alkylthio- and alkylamino-substitutions reduced the dilator activity of adenosine. 2-Methyl-thio and 2-methyl-amino adenosines were less potent than the corresponding ethyl analogues. A further increase in length of the alkylthio-side chain enhanced dilator activity, but branching of the propyl chain reduced this effect. The duration of coronary dilator activity was increased by these compounds to 5 to 15 times that of adenosine. Conversion of the adenosine analogues to their monophosphorylated derivatives generally reduced their dilator activity to about one third.

C. Drugs that antagonise the action of adenyl compounds

Mepacrine, quinine and quinidine. Raventos (448) was the first to observe that antimalarial drugs, in particular *quinine* and *mepacrine*, inhibit the action of adenosine on the guinea-pig heart. Both quinine and mepacrine greatly reduced the effect of adenosine and ATP on the heart-rate of the guinea-pig (260). Madinaveitia and Raventos (381), with a variety of antimalarial drugs, found that most of them inhibited the relaxation of the fowl rectal caecum produced by adenosine and reduced the duration of the auriculoventricular block produced by intravenous administration of adenosine in the anaesthetised guinea-pig. When quinine sulphate was injected intramuscularly or intravenously into cats, it decreased the effect of ATP not only on the heart, but also on the blood pressure (557). The respiratory response, however, was not affected either in the cat

or the rat. The actual mode of action of the antimalarials in inhibiting adenyl compounds was not satisfactorily explained by any of these workers. Quinine in concentrations up to 10^{-5} g/ml had no effect on the responses of the guinea-pig taenia coli to purinergic nerve stimulation, but neither did it block the direct action of ATP on this tissue (126).

Antagonism of the actions of adenosine by *quinidine* (a *dextro*-rotatory stereoisomer of quinine) on the cardiovascular system has been observed by several authors (260, 381, 557). Quinidine has also been shown to antagonise the inhibitory action of adenosine on the isolated guinea-pig and kitten uterus (20) and of ATP on the rabbit ileum (94). As discussed in more detail earlier (sections II E and V B), quinidine blocks responses to both ATP and non-cholinergic, non-adrenergic nerve stimulation in the gut (126, 507) and urinary bladder (127).

Phentolamine and imidazole. It was shown in a recent publication (464a) that high concentrations of imidazole (3.5×10^{-3} g/ml) and phentolamine (5×10^{-5} g/ml) blocked the relaxations of the guinea-pig taenia coli to exogenously applied ATP (1 to 5 μm), but did not block the maximal relaxations resulting from stimulation of non-adrenergic inhibitory nerves at a frequency of 5 to 10 pulses/sec. This result has been confirmed in our laboratory, although there was always some reduction of the nerve-mediated relaxations (Satchell and Dann, unpublished). However, further experiments revealed that the relaxation in response to lower frequencies of stimulation of non-adrenergic nerves (0.2 to 0.8 pulses/sec.) were markedly reduced by the same concentration of phentolamine (5×10^{-5} g/ml), and further that higher concentrations of ATP (greater than 5 μm) overcame the blocking action of phentolamine. It is likely that part, at least, of the blocking action of this concentration of phentolamine is non-specific, since the relaxations in response to nitroglycerine and amyl nitrite were usually also reduced. In view of these results, it appears that the action of phentolamine on the taenia coli cannot be taken as significant evidence either for or against the purinergic nerve hypothesis.

Caffeine and aminophylline. Caffeine and related compounds antagonise the auriculoventricular block produced by adenosine in the guinea-pig heart (530). Nichols and Walaszek (415) found that the fall in blood pressure in chickens, rabbits, cats and dogs caused by intravenous injection of adenosine, AMP, ADP and ATP was blocked by intravenous injection of caffeine. They also found that caffeine blocked the inhibitory effect of ATP on the rhythmic contractions of the isolated rabbit jejunum. De Gubareff and Sleator (175) showed that caffeine antagonised the powerful depressant effects of adenosine, AMP, ADP and ATP on contraction and action potentials of guinea-pig and human atrial muscle. They postulated that caffeine and adenosine derivatives have opposite effects on the binding of Ca^{++} at the critical site from which it is mobilised during activation.

Caffeine in concentrations up to 5×10^{-5} g/ml did not affect relaxations of the guinea-pig taenia coli in response to either ATP or purinergic nerve stimulation (126). Higher concentrations of caffeine lowered the tone of the preparation and still had no selective blocking action on ATP or nerve stimulation.

Aminophylline has been reported to decrease coronary dilatation produced by intravenous administration of adenosine (6, 83), but this drug has not yet been tested on purinergic transmission.

Dinitrophenol and related substances. A variety of drugs known to be uncouplers of oxidative phosphorylation and inhibitors of electron transport (310, 558) have been tested for their effects on ATP action and, in some cases, purinergic transmission (126, 277, 373). In general, they produce non-specific depression of smooth muscle and do not appear to be a promising group of substances in the search for specific blockers of purinergic transmission.

Noradrenaline. It was argued in a recent communication (480b), that a relatively greater reduction of the response to non-adrenergic inhibitory nerve stimulation compared to the response to exogenously applied ATP in the presence of NA constitutes evidence against the purinergic nerve hypothesis.

D. Drugs that potentiate the actions of the adenyl compounds

Adenosine uptake inhibitors. Dipyridamole (100) has been shown to potentiate the effects of adenosine and its derivatives on the guinea-pig heart and rabbit intestine (292, 514), the coronary arteries of dog and cat (138, 273, 419, 443, 489, 514), cat nictitating membrane (95) and renal vessels in dogs (138, 273). The mechanism underlying this potentiation has usually been explained in terms of blockade of intracellular uptake of adenosine (337, 339, 435), although interpretation of experiments on whole animals is complicated by the presence of red blood cells. *Papaverine*, like dipyridamole, inhibits the uptake of adenosine into red blood cells (339), but produced only slight potentiation of the effect of adenosine on the guinea-pig heart *in situ* (514). It has been shown recently that, whereas potentiation of adenosine action on the heart by dipyridamole and *hexobendine* (348) is due to competition with adenosine for the uptake process, the potentiation by *lidoflazine* (7) could not be explained either by block of adenosine uptake or by inhibition of adenosine deaminase (301). There are species differences in the action of these drugs. For example, the action of adenosine in reducing the heart rate of both guinea-pig and rats was potentiated by dipyridamole and hexobendine in the guinea-pig, but not in the rat (302, 338, 514). Dipyridamole has also been reported to act as an inhibitor of phosphodiesterase (495, 551) and of adenosine deaminase (514). However, in a recent study of guinea-pig hearts, neither dipyridamole nor lidoflazine were shown to inhibit adenosine deaminase (301).

The possibility that adenosine uptake may be part of the normal physiological inactivation mechanism for purinergic nerves has been discussed in section II D. Such a mechanism would be analogous to the uptake of choline in cholinergic transmission after degradation of released ACh by acetylcholinesterase (441) and with the uptake and reutilization of nerve-released NA in adrenergic transmission (84, 316). If this does occur, inhibitors of adenosine uptake would be expected to potentiate the responses to purinergic nerve stimulation by making more nucleotides and nucleosides available to postsynaptic receptors. As described earlier (section II E), dipyridamole and hexobendine potentiate the

effects of both ATP and purinergic nerve stimulation on the guinea-pig taenia coli without affecting the responses to sympathetic nerve stimulation or to NA (482).

ATPase inhibitors. Inhibitors of ATPase fall into two main categories; those which act on Na/K-activated ATPase and those which act on Mg-activated ATPase. In general, Na/K-activated ATPases are considered to be associated with Na and K transport in membranes. On the other hand, the role of Mg-activated ATPase is poorly understood; its distribution is discussed in detail in section III C.

It is of interest to examine the effect of ATPase inhibitors on relaxations produced by both purinergic nerves and ATP, in view of the likelihood that ATPases are involved in the degradation of ATP released from nerves. If this is the case, ATPase inhibitors would be expected to potentiate both responses. Unfortunately many of these drugs are potent inhibitors of many cellular activities and mostly depress all activity of smooth muscle preparations in a general way, except for thiourea which appears to potentiate both nerve-mediated and ATP-induced relaxations (Lederer, unpublished observations).

Low doses of ouabain, a cardiac glycoside which inhibits Na/K-activated ATPase (88, 501) potentiates the inhibitory effect of ATP on the guinea-pig heart (446). These authors suggested that this action of ouabain might be due to inhibition of adenosine deaminase in the heart. However, identical potentiation of the responses to adenosine and ACh by ouabain described in a later publication (445) provided strong evidence against this hypothesis. Ouabain was reported not to affect the inhibition produced by ATP in the guinea-pig taenia coli (21).

Inhibitors of adenosine deaminase. The actions of adenosine and the adenine nucleotides are usually short-lasting. One explanation for this might be that they are readily deaminated in blood and tissues to their corresponding inosine derivatives, which are pharmacologically inactive (151), but there may well be other factors involved, such as receptor or uptake mechanisms. By analogy with the role and sites of action of monoamine oxidase and catechol-O-methyl transferase in adrenergic transmission [see Iversen, (316)], it would clearly be unwise at this stage to make too many assumptions about the role of adenosine deaminase in purinergic transmission. Investigation of this role will be easier when more reliable adenosine deaminase inhibitors have been found. *8-Azaguanine* has been claimed to be an inhibitor of intestinal adenosine deaminase, but little potentiation of adenosine action on heart was detected (514).

Magnesium and calcium. The effects of Mg^{++} on the pharmacological actions of the adenyl compounds have been studied extensively (189, 260, 557). These workers found that high Mg^{++} prolonged the heart block produced by adenosine in the guinea-pig and rat. Mg^{++} has also been claimed to inhibit adenosine deaminase (80), but Stafford (513) found Mg^{++} had no effect on the rate of deamination of adenosine by bovine ventricle muscle deaminase and proposed that the potentiation of the adenyl compounds by Mg^{++} is due to the inhibition of dephosphorylation and not to deamination.

Magnesium has been shown to produce little consistent change in the response

of the gut to purinergic nerve stimulation (505), but in this case there may be presynaptic effects on release of transmitter as well as postsynaptic actions. Low Ca^{++} and high Mg^{++} were reported to have little effect on the IJP recorded in guinea-pig jejunum (285).

Since ATP is an efficient chelator of calcium (76), it has been suggested that in some cases the action of adenine nucleotides is not a direct action on the tissue, but is due to alteration of the calcium concentration by chelation or precipitation (207, 208). It has been suggested that the contractile effect of ATP on some smooth muscles may be due to its ability to complex magnesium present in the cell membrane, thereby favouring calcium entry and subsequently contraction (169).

ATP-induced contraction of the isolated perfused rat renal artery is dependent on the concentration of external Ca^{++}; increase of the Ca^{++}/Mg^{++} ratio in perfusion fluid enhances the contractile response to ATP, while reduction of Ca^{++} concentration markedly reduced the contractions induced by ATP, but not by Na (306).

E. Summary

1) A model of ATP synthesis, storage, release and inactivation is proposed as a basis for examination of the effect of drugs on purinergic transmission.

2) Inhibitory or excitatory responses to ATP and non-adrenergic, non-cholinergic nerve stimulation occur in different preparations. Evidence for distinguishing two types of muscle receptors for adenyl compounds is discussed.

3) Various drugs have been shown to antagonise or potentiate the direct action of adenyl compounds on smooth and heart muscle; these are considered in relation to purinergic transmission.

VI. Distribution and Evolution of Purinergic Nerves

The presence of purinergic nerves has been determined largely on the basis that nerve-mediated responses remain after stimulation of either extrinsic or intramural autonomic nerve fibres in the presence of cholinergic and both adrenergic neurone and adrenoceptor blocking agents. However, it does not follow that nerve fibres which are neither adrenergic nor cholinergic are all of the same type, releasing the same transmitter substance. Similarly, it would be unwise to assume from studies of neighbouring regions of one organ, such as the gut, that non-adrenergic, non-cholinergic inhibitory and excitatory responses are due to neurotransmitter released from one nerve type acting on different receptors. A comparative study of the anatomical origin of non-adrenergic, non-cholinergic fibres in different systems might help to clarify the situation. In some cases, information concerning the fine structural identification of nerves and the mimicking action of directly applied purine nucleotides gives additional evidence for the presence of purinergic nerves. It must be recognised that the direct response of tissues to purine nucleotides is not in itself sufficient evidence for the presence of purinergic nerves. For example, the non-innervated smooth muscle of the chick amnion is activated by ACh (37, 161, 204, 359), while that of the

umbilical artery is affected by noradrenaline (511, 525). Nevertheless, an account of the effects of adenine nucleotides on various autonomic effector tissues has been included, since it may encourage exploration for purinergic nerves in systems which appear to be particularly sensitive to these compounds. When specific drugs are found which will reliably block or potentiate purinergic responses it will be much easier to survey purinergic nerve participation in a wide range of organ systems.

A. Alimentary canal

Stomach. Evidence is now available to support the view that there is purinergic inhibitory nervous control of the stomach in fish (116, 117, 145, 315), amphibians (126, 142, 483) and birds (63–65, 68, 412, 484a), as well as in mammals (4, 5, 42, 108, 141, 218a, 259, 278, 321, 322, 323, 395, 396, 401, 425, 434). The cell bodies of these neurones appear to be located in Auerbach's plexus and many, if not all, are controlled by preganglionic parasympathetic nerves running in the vagus trunks. Concentrations of adrenoceptor or neurone blocking drugs which abolish the response to sympathetic (adrenergic) nerve stimulation do not block the inhibitory responses to vagal stimulation (42, 108, 141, 396) or reflex relaxation of the stomach (321, 322, 424, 425). The frequency of stimulation of vagal inhibitory fibres giving maximal responses is low (about 5–10 pulses/sec) compared to the stimulation frequencies (about 30 pulses/sec or more) required for maximal responses to sympathetic nerve stimulation (141). This is closely comparable to the earlier observations on the optimal stimulation frequencies of the two types of nerve fibres in the taenia coli (124, 125).

Preganglionic terminals on purinergic neurones appear to be cholinergic, since the inhibitory responses to stimulation of the vagus nerves (141, 259, 395, 396, 434) or resulting from vago-vagal reflexes (425) are blocked by nicotinic ganglion blocking agents. Bülbring and Gershon (108) have presented evidence to support the view that some at least of the vagal connections with postganglionic purinergic neurones in the stomach may be tryptaminergic. However, while Beani *et al.* (42) confirmed the existence of a small component of hexamethonium-resistant vagal inhibition, they were unable to repeat the experiments that were taken as evidence that this was due to the presence of tryptaminergic synapses in the vagal pathway (see also 411a). Furthermore Martinson (396) was unable to mimic the vagal relaxation of the stomach with 5HT, and fluorescence histochemical studies of the mammalian vagal nerve trunks failed to show more than a few fluorescent fibres which supply the stomach (409, 416). An alternative explanation would be the presence of a small component of postganglionic, nonadrenergic inhibitory fibres in the vagus nerves, which may or may not be purinergic.

In a recent review of the evolution of the vertebrate autonomic nervous system, Burnstock (118) showed that the vagal pathway to the stomach in lower vertebrates consists largely, if not entirely, of preganglionic cholinergic nerves forming synapses with purinergic inhibitory neurones in the gut wall. Antagonistic excitatory control of gastric motility is represented by both cholinergic and adrenergic nerve fibres of sympathetic origin. During the course of vertebrate

evolution, the purinergic inhibitory pathway in the vagus nerve has been retained; antagonistic excitatory control of gastric motility in mammals has been taken over by parasympathetic cholinergic neurones in the wall of the gut, while most of the sympathetic adrenergic nerves have come to form terminals about nerve cell bodies in Auerbach's plexus and are concerned with modulating intramural neurone activity (323).

Oesophagus. Stimulation of longitudinal and mucosal muscle strips from the oesophagus of the opossum revealed both cholinergic excitatory responses and rebound contraction mediated by non-adrenergic inhibitory nerves (372). A hyoscine-resistant contraction of the isolated oesophagus of the chicken in response to stimulation of the parasympathetic nerves has also been described (274). These responses were not blocked by bretylium or hexamethonium, but were abolished by nerve section; this implies that there is a non-cholinergic, non-adrenergic postganglionic component in the vagal pathway. Non-adrenergic, non-cholinergic nerves have been implicated in reflex relaxation of the oesophagogastric junction (150a).

Small intestine. Non-adrenergic inhibitory responses resembling those produced by purinergic nerves in other regions of the gut have been demonstrated in the small intestine of a variety of mammalian species, following the early recognition of intramural inhibitory neurones (10, 13, 38). These include: guinea-pig (224, 297, 342, 357, 506); mouse (297); rat (297, 298); rabbit (173, 174, 255, 297, 564); and cat (173, 174). No purinergic inhibitory responses have been demonstrated to date in the small intestine of lower vertebrates (135a, 148, 507). It is interesting in this respect that, in lower vertebrates, the vagus nerve does not extend so far down the gut as in mammals, where it is generally considered to reach the proximal colon (350, 404, 436). In teleost fish, it does not extend beyond the pyloric sphincter (117), in amphibians it is limited to the anterior duodenum (264), while in reptiles and birds it appears to extend into the ileum (417).

If the purinergic inhibitory nerves in the small intestine of mammals are controlled by preganglionic, parasympathetic fibres, it is still not clear what pathway the vagal fibres follow to reach different segments of the intestine. Stimulation of the vagus nerves in the presence of atropine produces relaxation of the intestine (38, 40, 111, 251), but there is evidence that the mediating nerves do not travel down within the gut wall, since inhibition in response to vagal stimulation persists in intestinal loops isolated from the stomach (38, 111). It seems unlikely that the parasympathetic fibres connecting with inhibitory purinergic neurones follow the perivascular plexuses, since the inhibitory responses of Finkleman preparations of small intestine to stimulation of perivascular nerves are abolished by adrenergic blocking agents (70, 172). However, Kewenter (330) found that the inhibitory responses of the cat small intestine to stimulation of the intrathoracic vagus only occurred when the sympathetic innervation of the intestine was intact. Further work will show whether these conflicting results are due to species variation or whether some more complex reflex pathways might be involved. Some of the reports of inhibitory responses of the intestine to vagus stimulation in non-atropinised animals may have been due to compensatory

release of catecholamine from the adrenal medulla after cardiac slowing and fall in blood pressure; even a slight rise in circulating catecholamines results in tension changes in gastrointestinal muscle [see Furness and Burnstock (230)].

Non-cholinergic, non-adrenergic *excitatory* nerves have also been shown to supply the small intestine of mammals, including rabbit (174, 252, 486), guinea-pig (14–16, 229, 250, 252, 343, 345, 407, 433), dog (38, 40, 258) and cat (174), amphibians (148, 507), reptiles (135a, 507), birds (63, 205) and cyclostome fish (Costa and Campbell, unpublished observations). Whether some, or all of these excitatory nerves are purinergic or whether they are nerves releasing yet another neurotransmitter substance has not yet been clearly resolved. "Atropine-resistance" in the rabbit must be treated cautiously because of the enzymic destruction of atropine observed in this species (11).

In lower vertebrates where non-cholinergic, non-adrenergic excitatory responses of the intestine are a prominent feature, there is some evidence that they may be mediated by purinergic nerves (507). The nerve-mediated contraction is closely mimicked by ATP and blocked by quinidine. The possibility that these nerves release catecholamines, 5-hydroxytryptamine, histamine, bradykinin or prostaglandin E_1 has been excluded (507). In mammals, the possibility that a prostaglandin is the transmitter released during atropine-resistant, nerve-mediated contractions of the intestine has been considered (65). However, patulin, a prostaglandin antagonist, does not block the nerve-mediated contractions of the guinea-pig ileum (14).

Unlike the inhibitory purinergic neurones, the cell bodies of non-cholinergic excitatory neurones do not appear to be in the wall of the small intestine in lower vertebrates; postganglionic fibres reach the gut *via* sympathetic periarterial nerves. Whether the non-cholinergic non-adrenergic responses to stimulation of periarterial nerves are due to fibres of sympathetic origin, to parasympathetic fibres invading the sympathetic trunks or possibly to antidromic stimulation of sensory fibres is not yet known. There are some clues from extensive early studies of the excitatory responses of the amphibian gut to stimulation of spinal autonomic nerves [see Campbell and Burnstock (145)]. It has been stated that there are no efferent fibres supplying the gut which run in the dorsal roots of the spinal nerves (163, 304, 367). However, there is now convincing evidence that the excitatory nerve fibres emerge from the cord almost exclusively in the dorsal roots (82, 275, 494, 515). The origin of these fibres is not known, but there is evidence that their cell bodies are in the dorsal root ganglia (82) and that cholinergic fibres leave the cord in the dorsal roots and form synapses with them (275, 276).

In mammals, there is evidence to suggest that some, at least, of the non-cholinergic excitatory responses are mediated by intramural neurones (14–16, 248, 343). However, responses can be elicited by extrinsic nerve stimulation. The non-adrenergic, non-cholinergic excitatory responses in the small intestine of mammals have been shown to be activated by stimulation of the vagus nerves (38, 40, 160, 258, 281, 318). However, it is possible that this is an indirect response resulting from stimulation of other nerves (40). Non-cholinergic excitation of the

longitudinal muscle of the distal ileum of the guinea-pig has been shown to be mediated by perivascular sympathetic nerves (407). In the guinea-pig ileum, two pharmacologically distinguishable non-cholinergic excitatory responses were defined, one being prominent at high frequencies of stimulation (50/sec; designated the A response by the authors) and the other being prominent at low frequencies (5/sec; designated the B response) (15, 16). Histamine was reported to be an antagonist of the type A response, and this seems specific, because contractions caused by bradykinin, 5-hydroxytryptamine, prostaglandin E_2, nicotine or dimethylphenylpiperazinium were unaffected. However, this action of histamine was abolished by nicotine. The type A response in guinea-pig ileum has been confirmed by Furness (229) who has presented further evidence that it is not cholinergic since it is antagonised by drugs which inhibit cholinesterases. The identification of the type A excitatory response is still uncertain. It seems unlikely to be due to excitatory purinergic nerves in this case and it is not yet clear whether it constitutes a physiological response. It seems likely that the type B excitatory response is a secondary (rebound) contraction resulting from stimulation of intramural inhibitory purinergic nerves.

Large intestine. Non-adrenergic *inhibitory* responses to transmural stimulation have been demonstrated in the large intestine of guinea-pig (41, 77, 78, 152, 224–226, 297), pig and chimpanzee (463), dog (366), rabbit (503a), mouse and rat (297, 506), Mongolian gerbil (249) and man (107, 157, 240, 464) as well as in the guinea-pig taenia coli [see Campbell (143)]. They have also been shown to supply the internal anal sphincter of monkey (449) and cat (240a), and possibly the rat anococcygeus muscle (246a).

The bulk of evidence supports the view that the sacral parasympathetic nerves do not mediate the purinergic inhibitory responses of the large intestine, with the exception of a short segment of the distal rectum. No inhibition was observed upon pelvic nerve stimulation of the main body of the colon in rabbit (39, 241, 246), cat (221, 311c) or guinea-pig (77, 444). In a careful study of the possible extrinsic connections of the intramural inhibitory neurones, Furness (224, 226) failed to record inhibitory junction potentials in smooth muscle cells of the guinea-pig colon in response to stimulation of the sacral parasympathetic nerves or periarterial sympathetic nerves. Thus it would appear that the purinergic neurones located in Auerbach's plexus of the colon are intramural, either established independently in the embryo or perhaps losing their association with extrinisic nerves at some later stage of development. The reduction in amplitude of purinergic inhibitory responses to transmural stimulation by ganglion-blocking agents (226) suggests that terminals of intramural cholinergic neurones are likely to be located on purinergic neurones, implying their involvement in enteric reflex activity. A tryptaminergic link for the activation of the intramural inhibitory neurones in the guinea-pig colon has also been proposed (78).

Non-adrenergic inhibition of a limited distal portion of the guinea-pig rectum in response to stimulation of the pelvic nerves has been demonstrated recently (Costa, personal communication); the non-adrenergic component in these nerves appears to be partly postganglionic and partly preganglionic.

The non-adrenergic inhibitory responses demonstrated in muscle taken from human large intestine (107, 157, 238–240), were not seen in aganglionic bowel taken from patients with Hirschsprung's disease (240).

Primary, non-cholinergic *contractions* to nerve stimulation were observed in the proximal colon (92, 93, 152, 229, 233) but were weak or absent in the distal colon of the guinea-pig (50, 226, 229). Ambache and Zar (16) examined hyoscine-resistant contractions of the distal colon of the guinea-pig. They found that contractions elicited at 50 pulses/sec were not significantly affected by histamine, unlike the type A responses described in the ileum, and it seems likely that they were secondary rebound contractions following the stimulation of intrinsic inhibitory nerves (226). However, non-cholinergic excitatory nerves appear to be present in the distal colon of cats (219, 221, 222) and dogs (251, 258). There is some evidence that in the cat and dog these excitatory nerves are controlled by fibres running in the pelvic nerves (219, 222, 251, 258), while in the guinea-pig they are activated by fibres in the perivascular nerves (152, 444). Non-cholinergic, non-adrenergic excitatory nerves have also been demonstrated in the large intestine of amphibians (135a, 507), reptiles (135a, 507) and birds (33).

B. Lung

There is good evidence for the presence of a purinergic inhibitory nerve supply to the interstitial musculature of the lung of amphibians (144, 467, 487, 575) and reptiles (71). Relaxation of the lung in response to stimulation of the vagus nerves is unaffected by adrenergic neurone and cholinergic blocking agents and persists after destruction of adrenergic nerves with 6-hydroxydopamine, as do nerve profiles containing a predominance of large opaque vesicles. ATP causes relaxation of the frog lung (402). Although there have been reports of bronchodilator responses to vagal stimulation in mammals (569), pharmacological and ultrastructural studies which would help decide whether or not the nerves involved are purinergic have not yet been carried out. Bennett and Drury (53) found that adenosine and AMP dilated the bronchioles.

It is interesting to note that Emmelin and Feldberg (199) found that intravenous injection of ATP produced profound changes in pulmonary ventilation in decerebrate cats, in some cases causing complete cessation of respiratory movements. They were of the opinion that this action was due to ATP acting on the respiratory centre, both directly as well as reflexly by impulses in the vagi probably originating in the lungs.

C. Urinogenital system

Urinary bladder. The pelvic nerves provide excitatory control of the urinary bladder and it was assumed for many years that this was mediated by classical cholinergic nerves, even though most of the response was resistant to blockade by atropine (365). It is also known that excitatory fibres in the hypogastric nerve supply (263) are not adrenergic, at least in the guinea-pig, and that the contractions are resistant to muscarinic receptor blockade (386, 387). "Atropine resistance" to the excitatory autonomic nerves supplying the bladder appears

to be a characteristic feature throughout the vertebrates, having been demonstrated in amphibians (134), reptiles (137) and marsupials (121), as well as placental mammals (11, 16, 127, 147, 150, 190, 193, 194, 263, 266, 282, 283, 311, 365, 542, 545). One explanation is that the ACh receptors at the neuromuscular junctions are inaccessible to atropine (147); another is that atropine is displaced from the receptors competitively by high local concentrations of ACh (311).

Another explanation for atropine resistance, favoured by most recent workers in the field, is that the majority of the excitatory nerves supplying the bladder are non-cholinergic (16, 127, 149, 190, 282). ATP (but not ACh) closely mimics the response to non-cholinergic excitatory nerve stimulation in terms of both its rate of onset and decline (16, 105, 399) and has been proposed as the transmitter released by these nerves (127, 190). As in the gut, the sensitivity of the bladder is greatest to ATP; ADP and AMP appear to have $\frac{1}{10}$ to $\frac{1}{200}$ of the potency respectively (16); cyclic AMP and S-adenosyl-methionine are inactive. Further evidence for a purinergic nerve supply to the bladder comes from experiments in which the excitatory responses of the guinea-pig bladder to both pelvic nerve stimulation and ATP were abolished by quinidine, without blocking the excitatory response to applied ACh (127).

When tachyphylaxis to contractions by ATP is produced in the guinea-pig bladder, there is usually some reduction of the excitatory responses to non-cholinergic nerve stimulation (127), although Ambache and Zar (16) did not find this to be the case under the conditions of their experiments. The different degree of reduction of responses to nerve stimulation after development of tachyphylaxis to ATP in the ileum and bladder may be explicable in terms of the differences in neuromuscular relationships in the two preparations. The minimum separation of nerve and muscle membranes in the longitudinal layer of the gut is rarely less than 1000 Å (62, 101, 363, 455, 457, 526, 528, 529, 577, 578) whereas there are many close (200 Å) neuromuscular contacts in the bladder (133, 135, 139, 411, 466, 528). The presence of narrow junctional clefts in the bladder may mean that ATP tachyphylaxis is more easily overcome by high concentrations of transmitter which accumulate in the junctional region after release from the nerves. In contrast, the purinergic receptors may be more widely dispersed along the muscle membrane in the gut, where the synaptic cleft is wide, so that transmitter released from nerves acts on an area of muscle similar to that reached by applied ATP.

Evidence that 5-hydroxytryptamine, bradykinin, histamine and catecholamine are not excitatory neurotransmitters in the bladder has been documented (16 127, 194, 266, 311).

Reproductive organs. Parasympathetic inhibitory fibres to the dog retractor penis, which are neither adrenergic nor cholinergic have been described and compared to those known in the gut (371). However, ATP does not appear to be the transmitter (371a).

Adenyl compounds cause contraction of the uterine smooth muscle of most species (20, 53, 112, 169, 179, 244, 556). ATP was most potent, AMP less so and adenosine least potent. IMP had about $\frac{1}{10}$ the potency of AMP, while adenine

and hypoxanthine were inactive (244). Barsoum and Gaddum (32) found that adenosine caused relaxation of the uteri of dogs, cats and rabbits, but in a more recent study, Arulappu (20) distinguished both excitatory and inhibitory receptors to adenosine in uterine muscle and showed selective block with phenothiazine and quinidine respectively (see table 2).

D. Cardiovascular system

Heart. Adenyl compounds have pronounced effects on the heart. There is no evidence yet for innervation by purinergic nerves and this seems unlikely in view of the long history of studies of heart innervation which include few reports of deviation from the classical picture. If they do supply the heart, they are likely to be fibres running in the vagal trunks to the sinus node, but in very small numbers compared to the vagal cholinergic component. Negative chronotropic and inotropic effects of adenyl compounds on isolated mammalian hearts have been described many times; they have been shown to affect the sinus more than atrioventricular conducting tissue in most species except guinea-pig and so cause a sinus bradycardia (53, 186, 187, 244, 260, 320, 337, 446, 498, 549, 550, 562). Negative chronotropic effects of adenyl compounds have also been demonstrated in the heart of frog (244, 430, 473, 547, 548, 549), and turtle (562).

A positive inotropic effect sometimes appears after the negative inotropic response of the heart to adenyl compounds (244, 260, 550). These workers attributed this effect either to direct action on the muscles or to a secondary reaction following dilatation of the coronary vessels. However, it may turn out to be explicable in terms of rebound excitation (see section IV B) which characteristically follows the inhibitory responses to both ATP and purinergic nerve stimulation of smooth muscle of the gut. Adenine nucleotides have positive inotropic effects on the hypodynamic frog heart (394, 430, 547, 548).

Some of the chronotropic effects on the heart seen *in situ* (80, 199, 260, 550) appear to be due to reflex action on the cardioinhibitory centre *via* cholinergic nerves, but in some species (*e.g.*, rat) this element is lacking, since atropine or section of the vagi had no effect on the response to adenyl compounds.

In most species, ATP was found to be the most potent of the adenyl compounds in its action on the heart (260). There is some indication (from a comparison of the effects of adenyl compounds, including ATP injected intravenously or into the sinus node artery) that injected ATP is broken down to AMP or adenosine in the heart (26, 320).

Blood vessels. It is recognised that the sympathetic nervous system has both dilator and constrictor components. While vasoconstriction is usually mediated by noradrenaline released from adrenergic nerves, the mechanism of vasodilatation is less well understood. Several different substances have been implicated in different parts of the vascular system: acetylcholine (543); histamine (45, 102, 168); noradrenaline which acts on *beta*-adrenoceptors (552); bradykinin (215, 290); prostaglandins (201, 303) and angiotensin (247). However, these mechanisms do not seem to explain adequately some reports of vasodilatation. Thus the possibility that purinergic nerves might also be involved will be considered.

Adenyl compounds cause dilatation of most blood vessels (268, 510), but they have been found to constrict vessels in the lung (199, 373, 451) and possibly spleen (393) and to contract the isolated renal arteries of the rat (306, 307) and helical strips of dog portal vein (555). In isolated strips of rabbit aorta, ATP has a biphasic effect, depending on the initial state of contraction (223). The doses required to produce vasodilatation *in vivo* are usually lower than the threshold doses which produce slowing of the heart (213, 392, 572).

Strong evidence has been presented recently for the existence of non-adrenergic, non-cholinergic inhibitory fibres to the *portal vein* of the rabbit (308, 309). The inhibitory responses induced by either electrical stimulation or nicotine are blocked by tetrodotoxin, and mimicked by the direct action of ATP. Thus, they appear to fit into the pattern already established for purinergic nerves in the gut, but the possibility that vasodilatation is the result of ATP released during antidromic stimulation of sensory nerves cannot be discounted (299).

There have been many reports of vasodilatation of *skin vessels*, either by reflex or in response to nerve stimulation, which is not wholly abolished by atropine (1, 27, 44, 102, 167, 468). Intra-arterial infusion of adenyl compounds in intact animals has led to increased flow rates and vascular dilatation of rabbit ear (53), cat hind limb (213), human finger and ear (171), and human hand and forearm (189, 257, 519). Similarly adenyl compounds cause a fall in perfusion pressure in isolated preparations of rabbit ear (53, 260, 300), dog forelimb (218) and hind leg (53, 273, 393). ATP causes constriction of isolated perfused segments of dog saphenous vein (544). When ATP is combined with magnesium, its vasodilator action is potentiated (189, 260), so much so, that Shepherd (496) remarked in his review that "if it is desired to increase the blood flow through the limb by intra-arterial infusion of drugs, Mg-ATP would be preferable to either acetylcholine or histamine."

Most workers agree that dilatation in response to adenyl compounds occurs predominantly in the smaller arterioles in muscle and skin, with some species differences in the relative sensitivities of the two vascular beds (268). The possibility of innervation of these vessels by purinergic nerve fibres has not been examined, but ATP has been found in the venous effluent from the rabbit ear during stimulation of the great auricular nerve (299).

Zimmerman (582) reported that stimulation of the lumbar chain in the presence of bretylium produced vasodilatation of the dog's paw. He suggested as did Beck (45) and Ryan and Brody (480), that histaminergic nerves might be involved in this response. Purine nucleotides cause vasodilatation of prevenous resistance vessels in the dog paw, and these have also been considered, amongst a variety of other substances, as possible mediators of the non-cholinergic, non-adrenergic dilator system which supplies them (27).

It is well known that *skeletal muscle* contraction is accompanied or followed by vasodilatation, which in fast muscles can commonly be great enough to cause an 8-fold increase in blood flow (289). A similar vasodilatation is common in stress situations and during fainting (496). The cause of this vasodilatation is not clear. Some appears to be mediated by cholinergic dilator fibres to the re-

sistance vessels (543), but most cannot be explained by either adrenergic or cholinergic innervation of the vessels (27, 327) and it is usually assumed that a dilator agent is associated with, and released during muscle contraction (268). Several agents have been considered as the cause of this vasodilatation (287, 291, 334) including adenine nucleotides, particularly ATP (96, 181a, 213a, 214, 287, 288, 313).

ATP- and adenosine-induced vasodilatation in the *coronary artery* has been studied extensively, because of its possible implication in coronary occlusion and relevance to the treatment of angina (18, 53, 73, 170, 187, 188, 211, 213, 244, 260, 273, 413, 477, 489, 514, 559, 560, 561, 571, 572). Most workers found ATP and ADP to be the most potent adenyl compounds on coronary vessel musculature; AMP, adenosine and cyclic AMP are from ¼ to ⅓ as potent as ATP, while adenine, hypoxanthene, guanine, cytosine, and uracil are either inactive or of very low potency (514, 555, 571, 572). This order of potency of the adenyl compounds is comparable to that observed in preparations of purinergically innervated gut. Uridine nucleotides may dilate (UTP, UDP) or constrict (UMP) the canine coronary bed (273). Both 5′-nucleotidase and adenosine deaminase have been found in the heart.

The view that purine nucleotides have a physiological role in the regulation of blood flow in the coronary arteries has been proposed (74, 75a, 461, 477). In the intact blood-perfused heart, transient occlusion of the left common coronary artery produces enough vasodilator activity in coronary sinus blood to lower resistance on perfusion into other organs. This lowering of resistance is similar to that produced by intra-arterial injection of adenosine or AMP (492). In the presence of the adenosine deaminase inhibitor 8-azaguanine, adenosine appears in the perfusates of anoxic isolated cat and guinea-pig hearts (328), and in the venous effluent of rabbit heart (460). The adenosine content of cardiac muscle tissue increases following or during coronary occlusion (427, 478). Adenosine, inosine, hypoxanthine and IMP appear in the myocardium during ischemia of the heart of rat (243) and rabbit (75). Finally, it has been demonstrated that adenosine appears in coronary sinus blood during reactive hyperemia in the blood-perfused dog heart, the assumption being that it originates in the cardiac muscle (476, 478). It is clear that extrinsic nerves supplying the heart are not responsible for such a regulatory process, but the possibility of intramural purinergic nerves being involved has not yet been investigated.

Vasodilatation of *mesenteric and intestinal vessels* is produced by adenyl compounds (162, 213, 267, 326, 392) in many cases with doses lower than those required to relax the intestine (213). No specific experiments have been carried out yet to see if there is a purinergic component in the innervation of these vessels. However, nerve profiles containing a predominance of large opaque vesicles have been described in arterioles in the jejunum and mesentery of the rat (180) and it has been known for many years that low frequency stimulation of splanchnic nerves reveals a vasodilator innervation of vessels in the small intestine and stomach (111, 164, 352, 353). It appears that the origin of these atropine-resistant vasodilator fibres is in the dorsal roots of lumbar nerves I to

IV and VI (352). Reflex vasodilatation of intestinal vessels by non-adrenergic, non-cholinergic nerves has also been reported (79a, 311b).

Following blockade of the adrenergic constrictor nerves to guinea-pig *uterine arteries*, a dilatation was revealed which was associated with hyperpolarisation and was resistant to hyoscine (47, 48). The transmitter responsible for this response remains obscure, but the pharmacological findings suggest that it is not histamine, serotonin or a catecholamine that stimulates *beta*-receptors (47).

The *cerebral vasculature* is relatively insensitive to intravenous injection of adenosine (138).

Adenyl compounds appear to cause both vasoconstriction of *lung vessels* (187, 199, 260, 373, 451) and vasodilatation (53, 475) especially after previous vasoconstriction with serotonin (479). The nature of the response is dose-dependent, at least in dog and cat, since Gaddum and Holtz (236) showed that ATP, adenosine and AMP caused vasodilatation in low doses, but vasoconstriction in higher doses. Hauge *et al.* (277) found that the initial response of the perfused rabbit lung to ATP injected into the pulmonary arterial tubing was vasodilatation, while after 10 min to 2 hr, this response changed to vasoconstriction.

Adenyl compounds have been reported to have variable and inconsistent effects on net blood flow through the *kidney* (53, 138, 187, 272, 275, 326, 329, 393, 428, 531). Studies of the effects of adenyl compounds on renal clearances have proved more informative (171, 305, 524) and it was concluded from the results that afferent glomerular arterioles constrict while efferent glomerular arterioles dilate. However, Tagawa and Vander (524) could find no arteriolar constriction with ATP. In whole perfused kidney, ATP and ADP were shown to cause vasodilatation, while adenosine and AMP induced vasoconstriction (273, 579). In contrast, the excised and perfused renal artery from the same animal did not respond to adenosine (579). Helical strips of renal arteries of the dog relax in response to ATP, adenosine and cyclic AMP (555). These findings suggest that different vessels, even in the same organ, may display different responses to adenyl compounds.

If adenine nucleotides have a physiological role in the regulation of blood flow through the renal vascular bed, ATP would be the logical candidate because it is the only one that consistently produces vasodilatation on intra-arterial administration (268, 492). AMP appears in the renal venous effluent after release of renal artery occlusion (256) and during both autoregulation and reactive hyperemia (480a, 492), and could represent the breakdown product of physiologically released ATP. This may be analogous to the finding of high levels of AMP in perfusates of Auerbach's plexus following stimulation of purinergic nerves (126). An additional finding that suggests that nucleotides participate in renal blood flow regulation is that spontaneous disappearance of autoregulation in the dog kidney can be restored with dipyridamole (428), an agent that potentiates the effects of adenine nucleotides (110) and purinergic nerve responses in the gut (482). The splanchnic nerve contains dilator fibres to kidney vessels (98, 352).

It has been known for many years that stimulation of the chorda tympani produces vasodilatation of the vessels of the *salivary gland*, which is not abolished

by concentrations of atropine that prevent salivary secretion (279). Perhaps the most popular contender for the vasodilator agent is bradykinin (290).

There is evidence that the parasympathetic fibres which control the vessels concerned with erection of the *penis* of dogs are neither adrenergic nor cholinergic (184); these vessels are sensitive to ATP.

E. Eye

Although there is no direct evidence for purinergic innervation of structures in the eye, some indirect evidence comes from a study of the reinnervation of ganglion-free transplants of guinea-pig taenia coli into the anterior chamber of the eye (129). As with other transplants of autonomically innervated smooth muscle into the anterior eye chamber (384, 385), the nerves in the transplant degenerate during the first day or two after transplantation. Nerves which normally supply the eye begin to reinnervate the transplant after about 1 week and by 2 to 4 weeks transmission is re-established.

A surprising result from analysis of the reinnervation of transplants of taenia coli was that, in addition to reinnervation by adrenergic and cholinergic nerves, there was physiological and pharmacological evidence for reinnervation by purinergic nerves (129). This result suggests that purinergic nerves are normally present, supplying some structures in the eye. It is conceivable that the non-adrenergic inhibition seen in the taenia transplants might be due to antidromic stimulation of ocular sensory fibres which have penetrated the transplant. These might release ATP as is reported in vascular smooth muscle (299) or prostaglandins as is reported for the trigeminal sensory fibres in the iris (12, 19, 72). However, it seems unlikely that the non-adrenergic response is due to sensory nerves growing into the taenia transplants, in view of the close similarity of the inhibitory responses in transplanted taenia to those recorded in control tissue where they have been shown not to be due to sensory nerves (125, 129). It is interesting in this respect that it has been suggested recently that the fall in intraocular pressure and hyperemia of the iris in the rabbit eye following degeneration of the adrenergic nerve supply, is due to the release of a non-adrenergic, non-cholinergic transmitter substance (538a) probably from nerves of parasympathetic origin (542a).

F. Summary

Evidence for the presence of purinergic inhibitory and excitatory nerves in a variety of tissues of the vertebrate visceral and cardiovascular system is discussed.

1) Purinergic inhibitory neurones are present in the stomach of fish, amphibians, reptiles and birds, but in mammals extend throughout the alimentary tract. In the stomach and distal rectum these neurones are controlled by preganglionic cholinergic fibres in the vagus and pelvic nerves respectively, but in the mammalian large intestine they do not appear to have extrinsic connections and are controlled by intramural cholinergic neurones. The extrinsic connections of purinergic inhibitory neurones in the mammalian small intestine are not yet fully understood.

2) Postganglionic, non-cholinergic excitatory nerves running in the periarterial sympathetic nerves are a prominent feature in the autonomic nervous control of the gut in lower vertebrates. In fish, amphibians and reptiles they reach all regions of the gut and may originate in the dorsal roots. ATP mimics the excitatory action of these nerves. In mammals they appear to be restricted to the colon (particularly the proximal region) and possibly the distal ileum; intramural non-cholinergic excitatory neurones are also present in the mammalian intestine. A small number of postganglionic, non-adrenergic inhibitory nerves appear to reach the oesophagus and stomach, and the distal rectum in the vagal and pelvic outflows respectively. Whether these nerves represent purinergic nerves or are nerves releasing another as yet unknown transmitter substance is considered.

3) There is evidence for purinergic inhibitory control of the lung of amphibians and reptiles. No search has been made yet for purinergic nerves in the mammalian lung.

4) The urinary bladders of fish, amphibians, reptiles, marsupials and placental mammals are supplied by excitatory nerves the effects of which are not blocked by atropine. There are indications that many of the nerves reaching the urinary bladder in the pelvic parasympathetic supply may be purinergic excitatory nerves rather than "atropine-resistant cholinergic fibres" as previously thought.

5) The possibility of purinergic nerve participation in control of parts of the cardiovascular system is discussed.

6) Indirect evidence for purinergic innervation in the *eye* largely from studies of the reinnervation of intestine in anterior eye chamber transplants is presented.

VII. Conclusions—Current Problems

It must be abundantly clear by now that knowledge of purinergic neurones is in its infancy. In contrast to the voluminous literature on the biochemistry, electrophysiology and pharmacology of transmission and morphology of adrenergic and cholinergic nerves, less than about 300 papers have appeared in relation to purinergic nerves. This figure includes earlier studies such as investigations of the direct action of ATP and related compounds on autonomic effector systems before purinergic nerves were postulated. Nevertheless, certain features of purinergic nerves are already clearly established. By drawing together what is known of these nerves in different disciplines and reinterpreting some earlier literature in the light of this knowledge, it is hoped that a stimulus will be provided for a concerted attack on the outstanding problems. Fortunately we have available, by analogy with the work already carried out on adrenergic and cholinergic systems, some clear guidelines for study (25). We have also the advantages of modern cellular methods of analysis not enjoyed by workers who first examined the classical autonomic components.

It may now be useful to summarise what is known about purinergic nerves, before suggesting some experimental approaches which might help to fill some of the many gaps in our knowledge of this system and clarify its physiological role.

1) Evidence that ATP is the transmitter released from non-adrenergic inhibi-

tory neurones in the gut includes the following: a) synthesis and storage of ATP in nerve terminals in such a way that is is available for release upon stimulation of the nerves; b) the release of endogenous purine nucleotides, and of tritium-labeled compounds during stimulation of purinergic nerves after exposure of nerves to ^{3}H-adenosine; c) the parallel effects of low concentrations of ATP and purinergic nerve stimulation; d) the presence in purinergically-innervated tissues of enzymes that degrade ATP, including ATPase, 5′-nucleotidase and adenosine deaminase; e) the demonstration that various drugs produce parallel blocking and potentiating actions on the responses to purinergic nerve stimulation and directly applied ATP.

2) The cell bodies of purinergic inhibitory neurones are located in Auerbach's plexus throughout the gut in mammals, but are limited to the stomach in lower vertebrates. In the stomach of all groups, they are controlled by preganglionic parasympathetic fibres running in the vagus nerves; in the mammalian large intestine they are controlled by intramural cholinergic nerves, but appear to be without extrinsic nerve connections, except in the distal rectum. Terminal varicosities of purinergic nerves appear to be characterised by a predominance of large vesicles. These have been termed here large opaque vesicles (LOV) in order to distinguish them from the large granular vesicles (LGV) found in small numbers in both adrenergic and cholinergic nerves, which are smaller and characterised by a prominent electron-transparent halo between the dense granule and vesicle membrane. ATPase is localised in micropinocytotic vesicles often aggregated in the smooth muscle membrane closely apposed to nerve profiles containing LOV.

3) Pre- or postganglionic stimulation of purinergic inhibitory nerves with single pulses produce inhibitory junction potentials (IJP's) in single smooth muscle cells of the gut. With repetitive stimulation IJP's sum and facilitate to produce an overall hyperpolarisation which inhibits spike activity and leads to relaxation.

4) Many autonomically innervated preparations are sensitive to adenyl compounds and it seems likely that smooth muscle has two receptors for ATP, mediating inhibitory or excitatory responses, depending on the organ system.

5) In addition to purinergic neurones in the gut wall, fibres which are neither adrenergic nor cholinergic have been shown to supply a variety of organs. Some of these nerves are excitatory (for example, those to the urinary bladder and segments of the gut in lower vertebrates), while others are inhibitory (for example, those to the lung, and parts of the vascular system). Many of them are postganglionic; for example, some reach the amphibian intestine in the sympathetic periarterial nerves and appear to originate in the dorsal root ganglia; others (*e.g.*, those to the bladder and distal rectum) have been located in the pelvic outflow; while some appear to reach the stomach and oesophagus in the vagal outflow. ATP mimics the nerve-mediated responses of most of these preparations; however, it is not yet known whether any, some, or all of these nerves are purinergic, or whether they release yet further neurotransmitters.

Some studies have been made of the physiological role of purinergic nerves

supplying the alimentary tract. Since the fluorescence histochemical method for localising monoamines has been applied to gut preparations, it has been recognised that adrenergic nerves do *not* form the major inhibitory pathway to the gut muscle as was accepted as the classical concept for many years. Rather, most adrenergic nerves form terminal networks about ganglion cells in the enteric plexuses (2, 35, 227, 235, 294, 319, 418, 450) and are concerned largely with modulation of local reflex activity (246, 322, 323, 340, 341). Thus, purinergic rather than adrenergic nerves are the main antagonistic inhibitory system to cholinergic excitatory nerves in the control of gut motility (fig. 5). Purinergic nerves are probably concerned in the phase of "descending inhibition" of peristalsis (40, 156, 298, 581), which is unaffected by sympathetic denervation (158). The powerful rebound contractions following the relaxation of the intestine produced by activation of purinergic nerves could provide an appropriate mechanism for assisting the passage of boluses further down the intestine during peristaltic propagation (311a). Purinergic nerves have also been implicated in the mechanism of "receptive relaxation" of the stomach (146a, 321, 322, 425), and reflex relaxation of the anal sphincter (240a) and of the oesophago-gastric junction (150a).

Perhaps the most outstanding gap in our knowledge of the purinergic system at this stage is the discovery of drugs which can selectively block or augment transmission. This gap will not be easy to fill, as pharmacologists who have studied drugs which modify adrenergic and cholinergic transmission processes will realise, and is likely to be particularly difficult since ATP is involved in so many cellular processes. It is hoped that the tentative model proposed in section V (fig. 3C) will provide a framework and stimulus to systematic studies in this field.

Since it is now known that enteric nerves take up ^{3}H-labeled adenosine, where it is rapidly converted and stored as labeled ATP (520), the possibility exists for localising ATP by autoradiographic methods. The procedure for combining microautoradiography and the histochemical fluorescence method for monoamines on the same section (398) should be particularly informative. It should also be possible to localise acetylcholinesterase in these sections (200), thus providing an opportunity to compare the distribution and relationships of adrenergic, purinergic and cholinergic neurones. The recent synthesis of a highly fluorescent adenosine triphosphate analogue, 1,N^{6}-ethanoadenosine triphosphate (492a), provides an exciting possibility for the development of a fluorescence histochemical method for the detection of purinergic nerves. At the electron-microscopic level, the possibility that ATP is contained in the large opaque vesicles which appear to be characteristic of purinergic nerves (section III B) can be tested with autoradiographic methods. This study, unlike those concerned with the relation of labeled NA and small granular vesicles (250–600 Å) (181, 527, 573), has the advantage that the smallest silver grain size available with current emulsions (about 850 Å) is capable of being completely contained by the large opaque vesicles (800–2000 Å), although the spread of particles in emulsion (481) will need to be taken into account. A further way of investigating the identity of the large opaque vesicles would be to apply gradient ultracentrifuga-

tion methods to separate a granular fraction from macerated tissues for biochemical analysis in a manner comparable to that described for separation of vesicles containing other neurotransmitter substances (177, 203, 440, 566, 567).

Another approach, currently being developed in our own laboratory, is to grow enteric neurones in tissue culture. Neurones of different types can be distin-

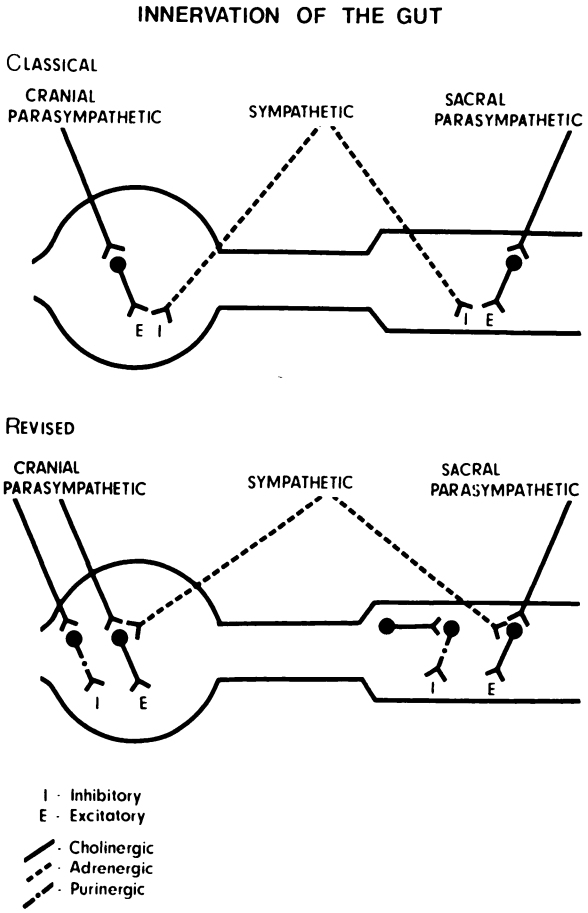

FIG. 5. Diagrammatic representation of the major components in the innervation of the gut, incorporating the results of recent studies of the distribution of both adrenergic and purinergic nerves (see text p. 559). The following qualifications to this simplified picture are necessary: 1) Early workers (146, 364) recognised the existance of some inhibitory fibres in the vagal outflow to the stomach, but they were usually assumed to be of sympathetic origin, so that, for the sake of simplicity, they have not been included in the diagram representing the classical situation. 2) Some adrenergic nerves are known to directly innervate the circular muscle coat of parts of the intestine of most vertebrates and of the stomach of some species (118, 154, 450). 3) The non-cholinergic, non-adrenergic excitatory innervation of regions of the gut (particularly in lower vergebrates) have not been included. Nor have the postganglionic, non-adrenergic inhibitory fibres in the vagal nerves to the stomach or in the pelvic nerves to the distal rectum. Extrinsic (pelvic nerve) control of some purinergic nerves in the distal rectum has also been omitted.

guished in these cultures and their differentiation into cholinergic, purinergic and sensory neurones is being investigated on the basis of histochemical, autoradiographic, transmission and stereoscanning electronmicroscopic analysis. If this is established, it should be possible to study the processes of synthesis, storage, release and uptake of ATP and related substances on individual purinergic neurones. A method of measuring the ATP content of cultivated neurones, using the luciferase technique described by Rasmussen and Nielsen (447), was recently reported (490). Studies of the embryonic origin and development of the different types of enteric neurones would also be valuable [see Andrew (17)].

It would be interesting to extend studies of the distribution of purinergic nerves not only within vertebrate visceral and cardiovascular systems, but also in glands (347), in the central nervous system (24, 104, 166, 199, 209, 237, 376, 377, 497) and in the more primitive nervous systems of invertebrates.

To end on a particularly speculative note, one might consider the possibility that a purine nucleotide represents the primitive neurotransmitter developed early in the evolution of nervous communication systems; that as the needs of animals became more sophisticated and it became a selective advantage to develop differential neurocontrol systems, new enzyme-forming systems were established in neurones, capable of producing other neurotransmitters. In this way various catecholamines, ACh, GABA, 5HT, glycine, glutamate and probably other transmitter substances as yet unidentified may have developed independently as neurotransmitters in different Phyla during the course of evolution. Whether ATP is the most primitive transmitter in this series of compounds or not, the question is, what is the mechanism whereby new neurotransmitter systems evolve? It seems most unlikely that a new nerve type with a structurally distinct form suddenly appeared during the course of evolution. It is more likely that there was a gradual evolutionary transition from one neurotransmitter to another within homologous neurones. If this is true, then one might begin to look more carefully over a wide range of animals for nerves showing gradations of transmitter mixtures, although, as Burn and Rand (113, 114) have already pointed out, this concept challenges the widely accepted view that each nerve releases only one transmitter (166, 471).

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Addendum

Since this review was submitted, experiments carried out in our laboratory (Costa and Burnstock, unpublished) give further support to the hypothesis that a purine compound is released from non-adrenergic inhibitory nerves. Following

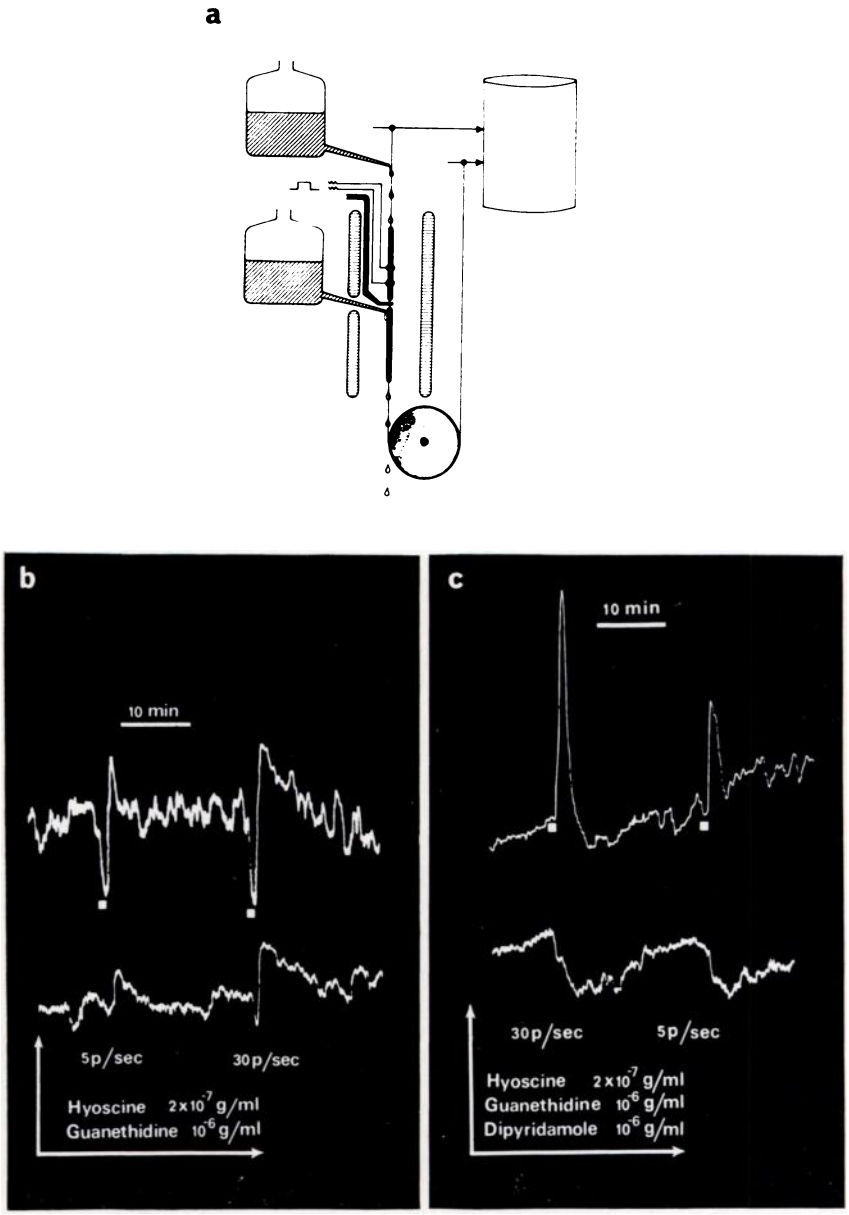

FIG. 6. a) Diagram of experimental set-up. b) Correlated changes in length of donor taenia preparation (upper trace) with recipient taenia preparation (lower trace) during transmural electrical stimulation (white squares) of the donor preparation. Note short-lasting relaxation of a recipient preparation after stimulation at 30/sec and smaller relaxation at 5/sec. Guanethidine (10^{-6} g/ml) and hyoscine (2×10^{-7} g/ml) present. c) Comparable records in the presence of dipyridamole 10^{-6} g/ml (as well as guanethidine and hyoscine). Note that larger and longer lasting responses to stimulation at both 5 and 30 pulses sec. occur in the recipient preparation (for explanation see text). The contractions seen in the donor preparation in this record are not primary contractions but rather "rebound contractions" following the primary non-adrenergic inhibitory response (see Section IV B).

the classical approach of Loewi (369a), two strips of taenia coli were arranged in series so that the perfusate of one preparation flowed over the second as described by Gaddum (235a). Hyoscine (2×10^{-7} g/ml) and guanethidine (10^{-6} g/ml) were in the perfusing solution. The upper preparation (donor) was stimulated transmurally, and the changes of length of the upper and lower taenia preparations were recorded isotonically on a smoked drum. In two out of twelve experiments, a short lasting relaxation of the lower preparation (recipient) was observed following stimulation of the donor preparation at 30 pulses/sec and in one experiment, the recipient preparation also gave a small relaxation at 5 pulses/sec. After addition of dipyridamole, which potentiates the responses of both purinergic nerves and exogenously applied ATP, probably by blocking the reuptake of adenosine into the nerves (482), the recipient preparation relaxed following stimulation of the donor preparation at both 5 pulses/sec and 30 pulses/sec in 10 out of 11 experiments. The relaxation was prolonged, as reported for the effect of dipyridamole on the responses to ATP and adenosine, but not on the responses to sympathetic nerve stimulation or NA (482). The results are illustrated in figure 6.

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