POSTER COMMUNICATIONS

A simple method for perfusion of the fourth ventricle of the rat: studies on GABA release

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One approach to the study of neurotransmitter function is to measure their release from brain tissue. Useful data have been obtained with *in vitro* preparations but there is less information available from *in vivo* systems. This is due partly to the complexity of the procedures involved. Thus we have developed a relatively simple method for perfusing the fourth ventricle of the anaesthetized rat, based on that used by Obata & Takeda (1969) in the cat. We describe the procedure and some preliminary results obtained with [^{14}C]-GABA release.

Male Sprague-Dawley rats (350–400 g) were anaesthetized with urethane (1.5 g/kg, i.p.) and fixed in a simple head-holder. The base of the cerebellum was exposed by removing part of the lamina of the first cervical vertebra and 1 mm of the base of the skull. The dura was pierced with a fine needle and a cannula, consisting of two fused stainless steel tubes (o.d. 0.8 mm, and one 2 mm longer than the other), was inserted into and parallel to the floor of the fourth ventricle, to a distance of 4–5 mm using a manipulator. Fluid at 37.5°C was pumped through the longer tube at a rate of 0.15 ml/min and withdrawn through the other, by means of a peristaltic pump. The preparation has been used for 6 h and control studies show that arterial blood pressure remains relatively constant (80–100mm Hg) and the E.E.G. shows a functional cerebral cortex.

In release studies the animals received aminooxyacetic acid (20 mg/kg, i.p.) 1 h before perfusion of the ventricle with artificial cerebrospinal fluid (ACSF) (Feldberg & Fleischhauer, 1960), containing 0.9×10^{-5} M [^{14}C]-GABA (specific activity 224 mCi/mmol), for 30 minutes. Normal ACSF was then substituted and samples collected every 10 min for liquid scintillation counting.

The initial high efflux of radioactivity (extracellular wash-out) declined within 90–100 min to a relatively constant level. Perfusion with iso-osmotic ACSF containing 50 mM potassium for 10 min, increased the efflux of radioactivity (2× basal, $n = 6$). Addition of β-alanine (5×10^{-4} M) to the ACSF, to block glial uptake of GABA, increased both the spontaneous and potassium-stimulated efflux, although only the latter appeared to be calcium-dependent. Most of the [^{14}C]-label remaining in the brain was found in the cerebellum and medulla and approximately 90% of the collected radioactivity was present as GABA. Pilot experiments indicate that direct electrical stimulation of the cerebellar cortex can also increase the efflux of GABA.

Thus we have developed a simple *in vivo* perfusion method which may also be used to study the release of endogenous GABA and other neuro-transmitters, and the effects of some centrally-acting drugs (e.g. anti-hypertensives).

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Effects of uptake inhibitors on the efflux and metabolism of [^{14}C]-GABA in the guinea-pig cerebral cortex *in vivo*

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A major route for the inactivation of GABA is believed to be by uptake into neurones and glial cells. Various compounds inhibit this uptake *in vitro* and although some are claimed to affect one process more than the other, little is known of the relative importance of the two processes *in vivo*. We have studied the effects of glial (β-alanine), neuronal (cis-1,3-aminocyclohexane carboxylic acid; ACHC), and mixed (nipecotic acid and L-2,4-diaminobutyric acid; DABA) uptake inhibitors (see Bowery, Jones & Neal, 1976) on the efflux and metabolism of [^{14}C]-GABA from the guinea-pig cerebral cortex.

Male Dunkin Hartley guinea-pigs (350–450 g) were anaesthetized with urethane (1.5 g/kg, i.p.) and the cortex exposed for superfusion through a small perspex cup (i.d. 5 mm) with artificial CSF (ACSF; Feldberg & Fleischhauer, 1960) at 0.03 ml/min; 37.5°C. The cortex was incubated with [^{14}C]-GABA (2 μCi/ml; 224 mCi/mmol) for 60 min and the efflux subsequently monitored in 15 min samples using liquid scintillation spectrometry. Amino oxyacetic acid (AOAA; 20 mg/kg, i.p.) was administered 1.5 h before loading. Uptake inhibitors were added to the ACSF when efflux had reached a steady baseline and their effects measured in sample 9 relative to the mean of control samples 7 and 8. GABA was separated from its metabolites by high voltage electrophoresis. β-Alanine (5×10^{-4} M), nipecotic acid (10^{-4} M) and DABA (5×10^{-4} M) were tested at 10×, and ACHC (6×10^{-3} M) at 100×, their IC_{50} concentrations *in vitro* (Bowery *et al.*, 1976; Iversen & Johnston, 1971). The results are shown in Table 1.

AOAA did not increase the total level of radioactivity but raised the proportion present as GABA from 27 ± 5% to 64 ± 7%. Uptake inhibitors only increased efflux in the presence of AOAA. β-Alanine was the most active compound producing a 369 ± 69% increase ($n = 4$), in GABA efflux compared with ACHC 153 ± 9% ($n = 4$), nipecotic acid 178 ± 16% ($n = 9$) and DABA 223 ± 72% ($n = 4$). ACHC and β-alanine produced a similar shift of the label from metabolites to GABA. If β-alanine specifically blocks the glial uptake system then this could be the more important route for inactivation of GABA *in vivo*.

M.J.C. is an MRC student.

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Table 1 The effects of β-alanine and ACHC on the distribution of [^{14}C]-label within superfusate samples of guinea-pig cerebral cortex

Condition		Mean Total Activity (dpm)	No. of observations	% of label present as: GABA	Glutamate	Glutamine	Aspartate	αKG	Mal/Cit.
Control:	No AOAA	6413 ± 836	5	23.5 ± 5.5	28.5 ± 9.3	21.2 ± 3.1	9.1 ± 2.7	7.5 ± 1.5	10.2 ± 1.3
	AOAA	6544 ± 557	8	60.2 ± 6.7	13.5 ± 5.1	19.4 ± 4.6	2.7 ± 0.5	1.5 ± 0.2	2.8 ± 0.5
β-Alanine:	No AOAA	6368 ± 999	5	51.6 ± 7.3	19.4 ± 6.9	17.8 ± 4.5	3.0 ± 1.1	3.1 ± 0.7	5.1 ± 0.6
	AOAA	30,691 ± 4548	4	75.8 ± 4.8	9.8 ± 5.7	11.9 ± 3.0	1.3 ± 0.4	0.6 ± 0.1	0.8 ± 0.4
ACHC:	AOAA	16,556 ± 596	4	78.7 ± 3.8	5.1 ± 2.2	13.9 ± 2.6	0.7 ± 0.2	0.5 ± 0.1	1.1 ± 0.6

All values in table and text are mean ± s.e. mean. Drug concns: β-alanine (5×10^{-4} M); ACHC (6×10^{-3} M).
α KG = α-ketoglutarate; Mal/Cit = total activity present as malate and citrate.

Does binding to the high and low affinity GABA binding sites represent binding to benzodiazepine receptor linked and non-benzodiazepine receptor linked GABA receptors?

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The displacement of sodium independent [^{3}H]-γ-aminobutyric acid (GABA) binding by various GABA agonists has been demonstrated by several groups of workers (Enna & Snyder 1975; Greenlee, Van Ness & Olsen 1978a; Krogsgaard-Larsen & Johnston, 1978). More recently, the presence in brain of an endogenous inhibitor of high affinity GABA binding (Costa, Guidotti & Toffano, 1978) which can be removed from synaptosomal membrane preparations by repeated washing (Greenlee, Van Ness & Olsen, 1978b) has been demonstrated. The present study compares [^{3}H]-GABA binding in unwashed and extensively washed synaptic membrane preparations and presents evidence of selectivity by certain GABA agonists for the low affinity GABA binding site.

Unwashed synaptic membranes from whole rat brain were prepared as described by Enna & Snyder (1975). Washed membranes were prepared initially as above but were then given additional treatment by freezing, thawing, resuspending in Tris-citrate buffer (0.05 M, pH 7.1) and centrifuging at 48,000 g for 20 minutes. This procedure was repeated four times. The binding assays were routinely performed at 37°C; preliminary experiments having established that the potency of agonists is approximately the same irrespective of whether binding studies are performed at 4°C or 37°C. [^{3}H]-GABA (10 nM) was used in all assays, non specific binding being defined as the radioligand bound in the presence of unlabelled GABA (100 μM).

The results obtained with a range of GABA agonists in both washed and unwashed preparations are presented in Table 1. It is apparent from these data that the potency of certain agonists, based upon the ratio IC_{50} unwashed/washed, was less affected by washing the membrane preparation, and presumably removing the endogenous ligand, than was that of others. This finding would suggest that the former category of compounds have varying degrees of selectivity for the low affinity binding site. Of these substances isoguvacine, (4,5-C) pyridin-3-ol and kojic amine have been shown not to enhance benzodiazepine (BZ) binding, while imidazole-acetic acid and 3-aminopropanesulphonic acid have been shown to be only weakly active. In contrast, GABA, muscimol and β-hydroxy GABA have been shown to markedly enhance BZ binding (Karobath, Placheta, Lippitsch & Krogsgaard-Larsen, 1979, Maurer 1979, Williams & Risely 1979).

Our findings suggest that the high and low affinity GABA binding sites demonstrated by Costa and others may respectively, be synonymous with the benzodiazepine receptor-linked and non-benzodiazepine receptor-linked GABA receptors which have been proposed by Karobath *et al.* (1979).

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Table 1 Inhibition of [^{3}H]-GABA binding in washed and unwashed synaptic membrane preparations

Drug	**IC_{50}(μM)* Washed	Unwashed	*IC_{50} (unwashed)/ IC_{50} (washed)*
GABA	0.087	0.75	8.6
Muscimol	0.007	0.14	20
β-Hydroxy GABA	1.4	9.5	7.0
Imidazole-acetic acid	0.9	5.0	5.6
Kojic amine	5.0	26	5.2
N-Methyl GABA	120	540	4.5
4,5,6,7-Tetrahydroisoxazolo-(4,5-C) pyridin-3-ol	0.28	1.2	4.3
Isoguvacine	0.19	0.28	1.5
3-Aminopropanesulphonic acid	0.37	0.5	1.4
Baclofen	>1000	>1000	—
γ-Hydroxybutyrate	>1000	>1000	—

* IC_{50}'s mean of at least two separate experiments.

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Kainic acid selectively destroys dopamine and beta-adrenergic receptor subtypes in rat striatum

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Recent studies using radioligand binding techniques have revealed heterogenous populations of many neurotransmitter receptors within the central nervous system. For example, sites having pharmacological properties of β_1- and β_2-adrenoceptors have been identified in cerebral tissue of many species (Nahorski, 1978), and recent studies have established that the neuroleptic [^{3}H]-spiperone labels both high and low affinity dopamine sites in rat striatum (Howlett & Nahorski, 1979). In the present communication we have used the neurotoxin kainic acid in an attempt to localise these sites within the striatum. Intrastriatal kainic acid injections selectively destroy intrinsic neurone cell bodies, sparing axons and terminals of extrinsic neurons, while glial cells undergo proliferation (see McGeer, Olney & McGeer, 1978).

Female hooded-Lister rats (200 ± 50 g) were anaesthetised (choral hydrate 350 mg/kg) and positioned in a stereotaxic apparatus. Kainic acid (5 nmoles in 1 µl of saline) was injected unilaterally into the striatum (AP 2.0, L 3.0, V-4.8), (Pellegrino & Cushman, 1967), at a rate of 0.25 µl/min. The contralateral side received an equivalent saline injection. The rats were killed 21–24 days later and corpus striata ipsi- and contralateral to the lesion were removed and membranes prepared for receptor binding assays. Success of the lesion was verified histologically.

Binding assays for β-adrenoceptors ([^{3}H]-dihydroalprenolol) and dopamine receptors ([^{3}H]-spiperone) were performed as previously described (Nahorski, 1978; Howlett & Nahorski, 1978). Selective identification of β_1- and β_2-adrenoceptors was achieved using the differential displacement of [^{3}H]-dihydroalprenolol with (±)-atenolol (3×10^{-6} M) and (−)-isoprenaline (2×10^{-4} M). High and low affinity dopamine sites were identified differentially using dopamine ($1 \times 1^{\wedge -6}$ M) and (+)-butaclamol (1×10^{-6} M).

Kainic acid lesions did not significantly alter high affinity (K_D 20 pM) [^{3}H]-spiperone binding sites, but the low affinity sites (K_D 225 pM) were markedly reduced by 43% (Bmax lesioned side = 324 ± 39 fmoles/mg protein; Bmax unlesioned side = 560 ± 46 fmoles/mg protein). [^{3}H]-Dihydroalprenolol binding to β_1-adrenoceptors in the corpus striata of the same animals was reduced by 30% (Bmax lesioned side = 42.1 ± 3.1 fmole/mg protein; Bmax unlesioned side = 60.48 ± 4.1 fmole/mg protein), whereas β_2 sites were not significantly influenced.

These results suggest that at least a proportion of β_1-adrenoceptors and low affinity dopamine sites may be located on nerve cell bodies within the striatum and, as such, provide some evidence for a different cellular localisation for β-adrenoceptor and dopamine receptor subtypes in this brain region.

The authors would like to thank the Wellcome Trust for financial support.

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Further observations on the effects of triiodothyronine (T3) administration to rats on brain 5-HT and dopamine systems

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Administration of thyroid hormone, both acutely and chronically, appears to alter central monoamine function, but no detailed attempts have been made to differentiate between acute and chronic effects. For example, clinically, repeated T3 administration potentiates the therapeutic effect of the tricyclic antidepressant imipramine (Wilson, Prange & Lara, 1974). In experimental animals acute administration of T3 potentiates L-DOPA induced behaviour in mice, and 6-hydroxydopamine-induced hypothermia in rats (Breese, Prange & Lipton, 1974). Furthermore, experimental hyperthyroidism in rats has been shown to alter many behavioural and neurochemical parameters of the central 5-HT, dopamine (DA), and noradrenaline systems (Rastogi & Singhal, 1976; Strömbom, Svensson, Jackson & Engström, 1977). The purpose of the present study, therefore, was to further investigate the effects of acute and chronic T3 administration on rat brain 5-hydroxytryptamine (5-HT) and dopamine (DA) systems.

Male rats were given T3 (100 μg/kg, L-triiodothyronine, sodium salt, Sigma Ltd.) S.C. in NaOH (0.02N) for either one or 10 days. Then approx. 24 h after the last dose the rats were tested for various neuropharmacological responses believed to be 5-HT or DA-mediated. The behavioural responses to increased 5-HT or DA function were studied by injecting tranylcypromine (TCP; 10 mg/kg i.p.; 0 min) followed by either L-tryptophan (TP) or L-DOPA (25 mg/kg i.p.; 30 min), (see Heal, Green, Boullin & Grahame-Smith, 1976). It was found that 24 h after either acute or chronic T3 administration, the hyperactivity responses to TCP + TP (measured on LKB Animex Activity Meters; sensitivity and tuning 30 μA) were markedly enhanced (350% and 220% increase above control activities respectively). There was no change in whole brain 5-HT or tryptophan accumulation measured at 75 min following TP injection in the rats treated chronically with T3. However, the rate of brain 5-HT synthesis (following an MAOI) was significantly greater in chronically T3-treated rats (0.28 ± 0.03 μg/g/h) than in controls (0.19 ± μg/g/h).

The effects of T3 administration on the rat brain DA system appear to be somewhat contradictory. Both acute and chronic T3 administration enhanced the hyperactivity response to TCP plus L-DOPA. Paradoxically, the accumulation of brain DA 30 min after L-DOPA in chronic T3-treated rats was less than in controls (28% reduction). Behavioural activity responses to the DA agonist apomorphine (1 mg/kg i.p.) were significantly attenuated (27% reduction) in chronically T3-treated rats (acute effects not tested). Striatal [^{3}H]-spiperone binding was unaltered in these animals. However, the cataleptogenic effect of haloperidol was significantly potentiated.

These results suggest that acute and chronic administration of T3 produce complex changes in both central 5-HT and DA systems. In the case of the DA system, clearly TCP + L-DOPA has different effects to apomorphine administration in chronically T3-treated rats. The attenuated apomorphine responses together with the lack of change in striatal [^{3}H]-spiperone binding, plus enhanced haloperidol-induced catalepsy, are suggestive of possible changes postsynaptic to striatal dopamine receptors.

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Blockade of neuronal responses to adenine derivatives by 4-aminopyridine

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Adenine derivatives such as adenosine and its nucleotides have powerful depressant actions on the firing rate of single neurones in the brain, when applied by microiontophoresis (Stone & Taylor, 1977). The mechanism of this effect is uncertain, but in many peripheral tissues such as smooth muscle and red blood cells adenosine triphosphate (ATP) in particular increases membrane potassium permeability, and such an action could underlie the depressant effect on central neurones. It is well known that 4-aminopyridine blocks membrane potassium channels, both calcium-dependent and voltage-dependent, whether applied extracellularly or intracellularly (Meves & Pichon, 1977). As the 4-aminopyridine molecule bears some structural similarities to adenosine, the effect of this compound was investigated on central neurone responses to adenines.

Male rats were anaesthetised with urethane (1.3 g/kg i.p.). Conventional techniques were used to record unit activity in the cerebral cortex, and to apply compounds by microiontophoresis from 7-barrelled micropipettes of tip diameters 8–10 μm. Purines were applied from 100 mM solutions, aminopyridine from 50 mM solution, pH 3.

Adenosine, adenosine monophosphate and ATP all depressed neuronal firing as described previously, when applied with iontophoretic currents of 10 to 140 nA. The iontophoresis of 4-aminopyridine, in contrast, had itself little effect on the firing rate of most cells (22) but increased the spontaneous activity of others (9).

When tested against any of the three adenine compounds 4-aminopyridine blocked their depressant effects on 24 of 26 cells, without affecting inhibitions produced by gamma-aminobutyric acid (GABA). This blockade was achieved with doses of 4-aminopyridine of 25 to 40 nA applied for 2–3 minutes. The use of larger currents often caused pipette blockage.

In order to reduce the possibility that presynaptic interactions may have contributed to these findings, the compounds were also tested against firing induced by pulses of glutamate, which is thought to increase sodium permeability at postsynaptic sites. Adenosine or ATP depression of these bursts was more variable than for spontaneous firing but could be blocked by 4-aminopyridine.

These results are consistent with at least two possible explanations:

(a) adenosine and ATP depress neurones by increasing the permeability to potassium which uses channels blocked by 4-aminopyridine. In this case the increase of potassium permeability must be different in some respect from that produced by GABA.

(b) 4-aminopyridine may be an antagonist at purine receptors on central neurones.

Supported by the Medical Research Council.

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A novel isotonic transducer suitable for use with isolated tissues

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A variety of electronic transducers has been designed for use with isolated tissues. These have included photoelectric, inductive and resistive types (Geddes & Baker, 1975), which are suitable for a wide number of applications. The type most commonly reported for use when assessing isotonic muscular contractions is a rotary potentiometric type which has a high voltage output for a relatively small extent of movement together with a high electrical stability. The major problem of this type of transducer is that it is very expensive. The relatively inexpensive device which will be demonstrated has been designed to equal or better the stability of existing forms of transducer and to produce a higher voltage output suitable for direct connection to a pen recorder.

The ultrasonic transducer utilises the finding that the amplitude of an ultrasonic signal which is reflected from a surface can be modified by the distance of the reflecting surface from the transmitter/receiver ultrasonic elements. In this device the reflecting surface is a modified fibreglass beam to which a tissue may be attached and change in length of the tissue results in amplitude changes in the reflected ultrasonic signal. Suitable electronic processing provides the output signal in volts and after attenuation can be made suitable for any type of pen recorder.

The transducer has been extensively tested in our laboratory and has been found suitable for use with a wide variety of tissues as will be demonstrated. The device is stable, extremely sensitive, reliable and may provide an inexpensive alternative to the usual type of isotonic transducer.

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The influence of cooling on the effects of noradrenaline, ATP and intramural inhibitory nerve activity in guinea pig taenia caeci

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Cooling ileal circular muscle reduces the amplitude but increases the half-amplitude duration of the inhibitory junctional potential (i.j.p.) evoked on stimulation of intramural neurones. In contrast, the electrotonic potential evoked by the use of large, extracellular polarising electrodes is little changed. Such observations led to the suggestion that the time course of the i.j.p. was not determined by the passive properties of the smooth muscle, and that the rate-limiting step in the inactivation of the inhibitory conductance change is a chemical reaction and not simply the result of transmitter diffusion (Lang, 1979).

Assuming that this suggestion is correct, and that the inactivation process is relatively efficient, cooling should be expected not only to prolong the effects of nerve activity but also to potentiate exogenous transmitter. In the present experiments we have compared the effects of cooling on responses of the taenia to inhibitory nerve stimulation and to ATP in the hopes that this might prove a test of the validity of the purinergic transmission hypothesis (Burnstock, Campbell, Satchell & Smythe, 1970). Segments of taenia were set up for organ bath or electrophysiological experiments as described by Small & Weston (1979), the Krebs' solution containing hyoscine (3×10^{-5} M) and guanethidine (10^{-5} M).

Cooling from 37.5°C to 27.5°C caused a small but significant potentiation of noradrenaline but did not influence the action of ATP. The primary relaxant responses to field stimulation remained unchanged in amplitude, but were often prolonged. Rebound contractions were significantly reduced at most stimulation frequencies.

Cooling reduced the apparent resting membrane potential of the muscle cells and their spontaneous spike frequency, thus confirming the earlier observations of Bulbring & Kuriyama (1963). The amplitude of the i.j.p. was reduced by cooling but its total duration was markedly increased.

Since cooling reduced the spontaneous spike frequency of the taenia, it is possible that the observed prolongation of the i.j.p. and nerve-mediated relaxation may in part be due to reduced muscle spontaneity. However, if cooling also prolonged transmitter action in this tissue by suppression of an efficient inactivation mechanism, then the failure of cooling to

potentiate exogenous ATP may argue against ATP's transmitter role.

The expert technical assistance of Miss A. Whitehead is gratefully acknowledged, as is support provided by the Smith, Kline and French Foundation and the Wellcome Trust.

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Purine action on guinea-pig trachealis muscle: an attempt to characterise the purinoceptor

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Burnstock (1978) proposed that P_1 purinoceptors were characterised by the relative order of agonist potency adenosine ≥ AMP ≥ ADP ≥ ATP with methylxanthines as selective antagonists whilst P_2 purinoceptors were characterised by the reverse order of agonist potency with 2-2'-pyridylisatogen as a selective antagonist. In the present study we have attempted to see whether such receptors are found in guinea-pig tranchealis muscle.

Tracheal strips (Coburn & Tomita, 1973) were mounted in Krebs' solution at 37.5°C for isotonic recording of relaxant responses. The effects of purines were studied by the construction of cumulative concentration/effect curves, effect being expressed as a percentage of the maximal relaxation evoked by noradrenaline.

ATP exhibited a complex concentration/effect relationship. Within the range $0.5–16 \times 10^{-6}$ M, ATP evoked small contractions followed by relaxation. The slope of the concentration/relaxation curve was relatively shallow in this region, and the relaxation tended to a maximum equivalent to 15% of the noradrenaline maximum.

In greater concentration ATP evoked relaxation only, the slope of the concentration/effect curve becoming identical to that of the corresponding curves for ADP, AMP, adenosine and β-γ-methylene-ATP. Comparison of the molar concentrations of these agents required to evoke a relaxation equivalent to 50% of a noradrenaline maximum revealed that they were equipotent.

Treatment of tissues with dipyridamole (5×10^{-7} M) potentiated ATP, ADP, AMP, adenosine and β-γ-methylene-ATP. In the presence of dipyridamole the purine derivatives were again found to be equipotent, thus confirming and extending the observations of Farmer & Farrar (1976).

The high concentrations of purines required to relax the tranchea made the actions of potential antagonists difficult to study. In tissues sensitised to purines by dipyridamole (2×10^{-6} M), theophylline (8×10^{-5} M) caused some loss of tracheal tone but slightly antagonised both adenosine and ATP without affecting their relative potencies.

2-2'-Pyridylisatogen ($10^{-5}–5 \times 10^{-5}$ M) did not prove to be a useful antagonist of purine action in the trachea since it caused a profound and irreversible loss of tracheal tone.

Three factors therefore precluded our detection of P_1 or P_2 receptors either in isolation or as a mixed population:

(a) the equipotency of the purine derivatives;

(b) the failure of theophylline to alter the potency of adenosine relative to ATP;

(c) the intrinsic muscle relaxant properties of 2–2' pyridylisatogen.

Of course the equipotency of the purine derivatives may represent their rapid metabolism to adenosine. In any event we must await the development of appropriate metabolic inhibitors and better purinoceptor blocking agents before the receptor characterisation can proceed further.

L.C. is in receipt of a CASE award.

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Effects of time and drug concentration on the induction of responsiveness to naloxone in guinea-pig ileum exposed to normorphine *in vitro*

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The guinea-pig ileum offers an integrated system of neurones for studying the induction and expression of opiate tolerance and dependence *in vitro* (Ehrenpreis, Light & Schonbuch, 1972; Hammond, Schneider & Collier, 1976; North & Karras, 1978; Villarreal, Martinez & Castro, 1977).

Ileal segments were incubated at 22 ± 2°C in modified Krebs solution (Hammond *et al.*, 1976) containing hexamethonium (70 μM) with or without normorphine. The aerated solution was automatically changed hourly. Test and control segments from the same ileum were then set up in pairs at 37°C, in fluids equivalent to those used for incubation. Thirty minutes later, the ileum was challenged with naloxone, and after washing, with acetylcholine (0.01 μM). Response was expressed as a ratio of the tensions produced by naloxone and acetylcholine.

Figure 1 shows the effect of duration of incubation in normorphine (1.0 μM) or in Krebs on responsiveness to naloxone (0.03 μM). A highly significant responsive-

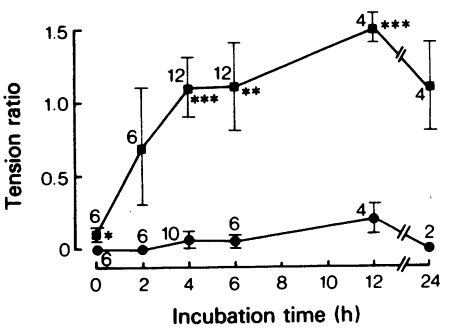

Figure 1 Effect of duration of incubation in normorphine on responsiveness to naloxone of segments of guinea-pig ileum. Ileal segments (ca. 10 cm), taken in pairs from the same guinea-pig, were incubated for 2–24 h in (■) normorphine (1.0 μM) or (●) in Krebs solution containing hexamethonium (70 μM). After incubation, or in the case of fresh tissue (Oh), immediately after being prepared, pairs of segments were set up for test at 37°C in fluid equivalent to that used for incubation, and, 0.5 h later, challenged with naloxone (30 nM). Responsiveness to naloxone (Tension ratio) is expressed as the ratio of the tension elicited by naloxone to that elicited by acetylcholine (0.01 μM). Each point is the mean ± s.e. mean of at least four experiments. Numbers beside each point show the actual number of tissues tested. For significance of differences (Student's *t*-test) between normorphine and Krebs incubation: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

ness was reached in 4 hours. The rise in responsiveness to naloxone on continued exposure to normorphine, coupled with the low response of control tissues, indicates the development of dependence, rather than an effect induced by stress before death (Bodycote & Chesher, 1979).

Naloxone (0.01–0.3 μM) elicited a dose-related contracture in tissues incubated for 24 h in normorphine (1 or 0.01 μM) or in Krebs (slopes, $P < 0.05$). The doses of naloxone (with 95% confidence limits) to elicit approximately half-maximal contracture were: 1 μM normorphine, 0.04 (<0.001–0.2) μM; 0.01 μM normorphine, 0.76 (0.4–1.3) μM; Krebs, 32 (6– > 1000) μM. The incubation concentration of normorphine necessary for naloxone (0.1 μM) to elicit a half-maximal contracture was 0.08 (0.004–0.3) μM.

Other experiments have shown that: (1) induction is specific and stereospecific; (2) the response to naloxone is stereospecific; and (3) incubation with an inhibitor, such as adrenaline, does not induce responsiveness to naloxone (Collier, Cuthbert & Francis, 1979). We have also shown that normorphine, applied before or after naloxone challenge, reverses the response to naloxone. These observations provide a critical method of measuring opiate dependence *in vitro*.

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Structural differences between typical anticholinergic substances and those used for Parkinson's syndrome

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One treatment for Parkinson's syndrome is the use of anticholinergic substances. I have noticed from studying crystal structures of such substances, for example benzetimide, trihexphenidyl, and procyclidine, that there are significant similarities and differences between the structures of these substances and those of typical anticholinergic substances affecting the peripheral autonomic postganglionic nervous system and, in the case of tertiary amines, the central nervous system.

Some similarities and differences can be determined from chemical structure alone. Both contain a tertiary or quaternary amine (only the tertiary amines enter the CNS), a phenyl ring, and usually a hydroxy and a large lipophilic group such as cyclohexyl bonded to the carbon atom to which the phenyl ring is bonded, forming an asymmetric center, usually R in both. The only obvious difference that can be seen from chemical structure alone is that while in pheripheral anticholinergic substances the number of bonded atoms between the basic nitrogen atom and the phenyl ring is usually 5, in anti-Parkinson's syndrome drugs it is universally 3.

Quantitative and conformational differences can be determined from three-dimensional structures analysed by x-ray diffraction. The major difference is that in ordinary anticholinergic substances the lone pair of electrons of a tertiary amine point in the same direction as the phenyl ring, and in anti-Parkinson's syndrome anticholinergics the lone pair points in the opposite direction. This is effected by moving the nitrogen atom from the same side of the chain linking the nitrogen atom to the phenyl ring to the opposite side. Thereby the mean nitrogen to phenyl distance is increased.

Electrochemical detection of 5-hydroxytryptamine and tryptamine

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Recently very sensitive and relatively inexpensive high performance liquid chromatographic (HPLC) techniques with electrochemical detection have been developed to assay brain catecholamines (Keller, Oke, Mefford & Adams, 1976) and 5-hydroxytrypamine (5-HT) (Sasa & Blank, 1977). Ponzio & Jonsson (1979) modified the original 5-HT assay for use with 1–20 mg samples and reported that tryptamine could be detected using the same potential (+0.5 V) as that used to oxidise 5-HT. The present study further investigates the electrochemical properties of 5-HT and tryptamine and their separation on an HPLC system using a cation exchange column.

The oxidation potentials of 5-HT creatinine sulphate, tryptamine HCl and L-tryptophan (Sigma) were determined in 10 ml 1×10^{-3} M solutions of each, prepared in citrate-acetate buffer (0.1 M, pH 5.2). Linear sweep voltammetry (CV-1A, Anachem Ltd.) was used with a semi-micro carbon paste working electrode and silver-silver chloride and platinum auxillary electrodes. The potential sweep was from 0.0 V to +1.2 V at 400 mV/s. At pH 5.2 5-HT oxidised at +0.45 V and tryptamine at +0.9 V (maximum oxidation). L-Tryptophan was oxidised at a similar potential to tryptamine.

HPLC separation of 5-HT and tryptamine was performed with a stainless steel column (25 cm × 2.1 cm i.d.) packed with a strong cation exchange resin (Vydac 30–44 μ particle size) eluted with citrate-acetate buffer (0.1 M, pH 5.2) at a flow rate of 0.4 or 0.6 ml/min metered by a high pressure pump. Oxidation of 5-HT and tryptamine was detected using a graphite paste electrode and a LC-4 detector (Anachem Ltd.) with the potential set between 0.5 V and +0.9 V. With the potential set at +0.5 V, +0.6 V or +0.8 V 5-HT was detected, with a retention time of 4.8 min (flow rate 0.6 mls/min), but not tryptamine. With the electrode potential set at +0.9 V both 5-HT and tryptamine were detected with the latter clearly separated from 5-HT having a retention time of 15.5 min. However with an equivalent concentration of both (50 pmol) in a mixed standard the tryptamine detection was approximately × 10 less sensitive than 5-HT which had a detection limit of about 0.1 pmol. L-Tryptophan, under the chromatographic conditions used, appeared in the solvent front. Two MAO inhibitors, tranylcypromine and nialamide, commonly used to increase brain 5-HT, were found to be electroactive (maximum oxidation potentials +1.0 and +0.76 V respectively). While nialamide appeared in the solvent front tranylcypromine had a retention time that overlapped that of tryptamine and was extracted from brain samples, together with 5-HT and tryptamine, using the method described by Ponzio *et al.* (1979).

Mice were injected with 0.9% saline or pargyline (75 mg/kg)—an MAO inhibitor which though electroactive does not interfere with the 5-HT assay—killed 2 h later, the brains and spinal cord were removed, 5-HT and tryptamine extracted as previously described (Ponzio *et al.*, 1979) and 20 μl of the final perchloric acid (0.1 M) extract injected onto the column. Pargyline increased brain (+78%, $n = 6$) 5-HT with the electrode potential set at +0.65 V but there was no apparent tryptamine peak in the samples with or without an added tryptamine internal standard. Similar results were obtained with the spinal cord.

The results confirm the previous report (Ponzio *et al.*, 1979) on the use of electrochemical detection in a highly sensitive 5-HT assay but show that tryptamine cannot be detected using a potential below +0.9 V and indicate the need to determine the electroactivity and separation characteristics of any drug used.

We thank Smith, Kline and French for a gift of tranylcypromine.

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Preyer's reflex and cortical evoked potential to assess audio-toxicity

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The method, based on the sound-induced movement of the external part of the ear (Preyer's reflex: PR), is known to provide a simple and reproducible assessment of the auditory toxicity of aminoglycoside antibiotics (Baird & Carter, 1978). Whether the results obtained with this technique can be correlated with electrophysiological findings is still equivocal. We have already conducted preliminary auditory function studies after the administration of kanamycin A to rats (Bourdois, Junghani, Perraud & Reinert, 1975). We have now investigated whether the results obtained with the PR in partially or totally deaf animals can be correlated with the cortical evoked potential (CEP) obtained from the auditive area on the rat.

A group of 8 male rats were given subcutaneous kanamycin A (400 mg/kg, single injection, daily) for 14 days according to the techniques of Harpur and d'Arcy (1975) and Chiba and Ando (1976); a second group of 8 males served as controls and received subcutaneous saline. The CEP was recorded by means of stainless steel screw electrodes inserted through the skull on the cortical surface, one week prior to the start of the experiment. The PR and the CEP were recorded on successive days before treatment, at the end of treatment, and 2 and 4 weeks later.

Before the treatment started, average thresholds were 58 dB (PR) and 74 dB (CEP) respectively at high frequency (13 kHz). By contrast, at high sound intensity (100 dB -PR- and 92 dB -CEP-) average thresholds in kHz were 1 (PR) and 2 (CEP) respectively. The differences are due to the fact that CEP is recorded with the rat placed in a plastic cage. Renal toxicity caused the death of 1 treated rat on day 11 and 3 rats on day 12. At the end of treatment, threshold values in the treated animals were comparable to controls except that the amplitude of the CEP was slightly decreased in two rats. Two weeks after the end of treatment, 3 rats showed elevated thresholds of CEP at both high frequencies and high sound intensity, although only 2 of these rats appeared to be deaf by PR. Four weeks after the end of treatment all three animals were deaf (PR). The remaining one rat showed no signs of deafness by either CEP or PR.

This preliminary experiment shows a parallelism between PR and CEP. The quantitative nature and possibly greater sensitivity of the CEP method is offset by the much greater relative labour involved.

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Studies on the mechanism of the contragestational effects of α-difluoromethyl ornithine, an irreversible inhibitor of L-ornithine decarboxylase

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L-Ornithine decarboxylase (ODC; E.C. 4.1.1.17) forms putrescine from L-ornithine and is the initial step in the biosynthesis of spermidine and spermine (Tabor & Tabor, 1976). An essential role for the enzyme in early murine embryogenesis has been suggested using the irreversible inhibitor of ODC, α-difluoromethylornithine (α-DFMO, RMI 71782), which, when administered during early gestation, arrested embryonic development (Fozard, Grove, Part & Prakash, 1979). The two objectives of the present study were to define the point during early gestation most vulnerable to the action of α-DFMO and to explore the effects of an inhibitor of putrescine catabolism on the contragestational response to α-DFMO.

Proven-fertile, CDA, HAM-ICR mice weighing 30–45 g were mated and the day of discovery of the vaginal plug was designated day 1 of pregnancy.

Gestational changes were assessed at autopsy on day 18 of pregnancy. Deciduomal ODC activity and polyamine concentrations were measured as previously described (Prakash, Schechter, Grove & Koch-Weser, 1978).

α-DFMO (200 mg/kg) was injected subcutaneously (s.c.) every 6 h on either day 5, 6, 7, 8, 9 or 10 of gestation. Autopsy on day 18 revealed no significant contragestational activity (decrease in viable foeti; increase in resorption nodules) as a result of treatment on days 5, 6, 9 or 10. Marked activity was however evident following treatment with α-DFMO on days 7 or 8 when the maximum increases in deciduomal ODC activity and putrescine concentrations are observed (Fozard *et al.*, 1979). Thus, animals treated on day 7 had a 75% reduction in foeti per mouse whilst drug administration on day 8 yielded only 4% of the expected number of foeti.

Aminoguanidine inhibits diamineoxidase (Pegg & McGill, 1978) the enzyme principally involved in putrescine catabolism in reproductive tissues (Guha & Jänne, 1976, Rojanskey, Neufeld & Chayen, 1979). Administration of aminoguanidine bicarbonate (Eastman), 25 mg/kg, s.c. at 10.00 and 12.00 on day 8 and every 6 h thereafter until 12.00 on day 9 had no effects on gestation. However, aminoguanidine pretreatment significantly attenuated the contragestational effects of α-DFMO administered on day 8. Thus, there were 9.6 ± 0.7 (mean ± s.e. mean, $n = 29$) foeti per saline-treated control mouse. The equivalent figure for the aminoguanidine group was 9.7 ± 1.0 ($n = 7$), for the α-DFMO group it was 0.4 ± 0.2 ($n = 15$) and for the aminoguanidine plus α-DFMO group it was 2.4 ± 0.9 ($n = 9$).

The results indicate a critical period during early murine gestation when intervention with an inhibitor of ODC is effectively contragestive. The phase is short ($\leqslant 2$ days) and corresponds exactly to the maximum increases in deciduomal ODC activity and putrescine concentration seen on days 7/8 of normal gestation. Moreover, putrescine, rather than ODC (Russell, Byus & Manen, 1976) seems the likely essential factor in early embryonic development. Thus, partial prevention of the contragestional effects of α-DFMO was achieved following administration of an inhibitor of putrescine catabolism.

Thanks are due to Mrs M. Nagy and Mrs M. Pionetti for conscientious and competent technical assistance.

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The action of benzocaine on voltage-clamped frog end-plates

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The effects on neuromuscular transmission of charged local anaesthetics, both permanently ionised (e.g. QX-222) and partially ionised (e.g. procaine), can be accounted for by blockage of end-plate ion channels. On the other hand, hydrophobic compounds, such as the *n*-alcohols, are believed to act less directly by an interaction with membrane lipids (see Colquhoun, 1978). We were therefore interested to see whether the action of benzocaine, a non-ionised local anaesthetic, is consistent with a channel block mechanism.

Experiments were performed on voltage-clamped frog end-plates at 8–14°C. Benzocaine (100–1000 μM) altered miniature end-plate currents (m.e.p.c.s) in a manner similar to the charged local anaesthetics. In

the absence of drug, m.e.p.c.s decayed as a single exponential of average time constant $\tau = 4.8$ ms at 9°C and −100 mV. After addition of benzocaine, m.e.p.c. decay was biphasic, consisting of an initial fast exponential component (τ_f) and a second smaller slow exponential phase (τ_s). Analysis of acetylcholine induced current fluctuations obtained in the presence of benzocaine revealed two similar components in the power spectra. In solutions containing 400 μM benzocaine, at 9°C and −100 mV, average values of τ_f and τ_s were 0.79 ms and 18.3 ms respectively. As the concentration of benzocaine was increased, τ_f became faster and τ_s became slower. The concentration dependence of τ_f and τ_s agreed well with a scheme in which benzocaine binds to open channels with a dissociation constant (K) of approx. 140 μM.

In the presence of benzocaine (500 μM), both the peak m.e.p.c. amplitude and the equilibrium response to low doses of bath applied acetylcholine were reduced by 50%. If benzocaine binds only to open channels, with $K = 140$ μM, then benzocaine (500 μM) should produce only very small inhibition of the equilibrium response to *low* concentrations of agonist (Adams, 1977). One explanation is that benzocaine might interact with closed as well as open channels, as originally proposed by Adams for the action of procaine.

However, two results are not consistent with the type of blockade of open and closed channels exhibited by the charged anaesthetics as the only mode of action of benzocaine. First, the extent of the inhibition of the equilibrium response to bath applied agonist increases more rapidly than expected with benzocaine concentration. In addition, the relative amplitudes of the fast and slow exponential components observed in the m.e.p.c. decay do not agree with those predicted by models of this kind. At the peak of the m.e.p.c., the fast component is too large by factors ranging from 1.2 to 4.0. A similar discrepancy was noted in the relative amplitudes of the fast and slow components obtained by fluctuation analysis.

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The uptake of choline into synaptosomes; non Michaelis-Menten kinetics demonstrated by a grouped least squares analysis

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Analysis of the kinetics of the uptake of choline into synaptosomes did not distinguish between a model including a high affinity carrier plus diffusion and one including high and low affinity carriers (Wheeler, 1979). However, an investigation of the contribution made by newly uptaken choline to the synthesis of ACh questioned the existence of a discrete high affinity transport mechanism specifically associated with cholinergic synaptosomes (Kessler & Marchbanks, 1979). We therefore investigated the fit of the initial velocities of choline uptake to the Michaelis-Menten equation, using the method of least squares described by Wilkinson (1961) and modified here for grouped values of uptake.

Fine adjustments of provisional estimates of K_m and V_{max} and the standard errors of the adjusted values were obtained (Table 1) by fitting the Michaelis-Menten function directly in the hyperbolic form, as described by Wilkinson. In this stage of the analysis, the significance of deviations from the Michaelis-Menten equation was assessed using the ratio (F, Table 1) of the deviation mean square to the residual mean square.

In all six independent experiments, deviations were significant ($P < 0.05$) unless the groups of velocities at choline concentrations greater than approx. 10 μM were omitted from the statistical analysis. Estimates of K_m and V_{max} obtained before omitting these groups indicated the deviations were to velocities larger than those predicted by the estimates of K_m and V_{max} obtained after the omissions (Table 1).

Uptake is thus consistent with a mechanism obeying Michaelis-Menten kinetics at choline concentrations between 0.1 and approx. 10 μM, final weighted mean K_m: 2.39 μM (s.e. mean 0.06, six independent experiments). Deviations from Michaelis-Menten kinetics at larger concentrations of choline may result from independent, low affinity transport or diffusion. An alternative model involving a single mechanism must account for the two-component kinetics of choline uptake.

Table 1 Least squares analysis of the kinetics of choline uptake into rat brain synaptosomes

Choline concentrations (μM): *Used for assays of uptake*	*Largest included in statistical analysis*	*Calculated F*	*P*	K_m, *s.e. mean* (μM)	V_{max}, *s.e. mean* pmol h^{-1} *mg tissue*$^{-1}$
(1) 0.13, 0.17, 0.25, 0.5, 1, 2, 5, 10, 20, 30, 40, 50	50	5.532	<0.001	7.70, 0.79	71.45, 2.40
	5	1.820	0.1–0.25	2.01, 0.09	37.92, 0.81
(2) 0.33, 0.4, 0.5, 0.67, 1, 2, 5, 10, 20, 30, 40	40	24.63	<0.001	11.26, 1.65	89.07, 5.52
	20	0.825	0.5–0.75	3.34, 0.20	50.28, 1.11
(3) 0.33, 0.4, 0.5, 0.67, 1, 2, 5, 10, 20, 40	40	56.47	<0.001	7.55, 0.83	57.51, 2.51
	5	1.147	0.25–0.5	2.44, 0.15	32.52, 1.04
(4) 0.33, 0.4, 0.5, 0.67, 1, 2, 5, 10, 20, 30, 40	40	7.987	<0.001	9.94, 1.87	56.98, 4.18
	20	1.637	0.1–0.25	3.13, 0.26	34.12, 1.00
(5) 0.1, 0.13, 0.17, 0.25, 0.5, 1, 2, 5, 10, 20, 30, 40	40	3.490	0.001–0.005	5.98, 0.73	57.23, 2.28
	10	0.564	>0.75	2.18, 0.13	36.24, 0.83
(6) 0.13, 0.17, 0.25, 0.5, 1, 2, 5, 10, 20, 30, 40, 50	50	5.455	<0.001	7.59, 0.69	60.40, 1.79
	20	1.047	0.25–0.50	3.63, 0.25	43.12, 1.10

Synaptosomes (P_2-fraction) from 20 mg-cerebral cortex (male Wistar rats, 100–150 g) were preincubated (10 min, 37°C), incubated (10 min, 37°C) with [^{3}H]choline (lμCi) at various specific radioactivities, in Tris-Krebs medium containing sodium (118 mM, Atterwill & Prince, 1978) then rapidly filtered (15 s, 0.65μ-Millipore filters) washed twice and analysed for radiolabel. Uptake was corrected for filter paper blanks and was linear with incubation time. Each P_2 preparation was used within 90 min of being finally prepared. Four replicate assays were made at each choline concentration. *P*: significance level of the deviations in a l-tailed F-test, degrees of freedom *k*-2, *n*-*k*; *k* = number of different choline concentrations included in the statistical analysis, *n* = corresponding total number of values of uptake included; *s.e. mean* = standard error of the mean calculated from the residual mean square associated with each analysis (Wilkinson, 1961).

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The effect of salmon calcitonin on acetic acid induced chronic gastric ulceration in the rat

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Calcitonin has been shown to inhibit acute models of gastric ulceration induced in the rat by a variety of stimuli, including indomethacin (Bates, Buckley & Strettle, 1979) and stress (Noda, Nozaki, Nishizawa, Okamoto, Morimoto, Morii & Wada, 1973; Bates & Barlet, 1974; Schwille, Steiner, Samberger & Schellerer, 1975; Jakesz, Hofbauer, Lehr & Schiessel, 1978). However, acute models of gastric ulceration do not necessarily reflect the potential action of an anti-ulcer drug to promote ulcer healing.

We have investigated the effects of salmon calcitonin on chronic gastric ulceration induced by acetic acid, a technique described by Okabe & Pfeiffer (1971).

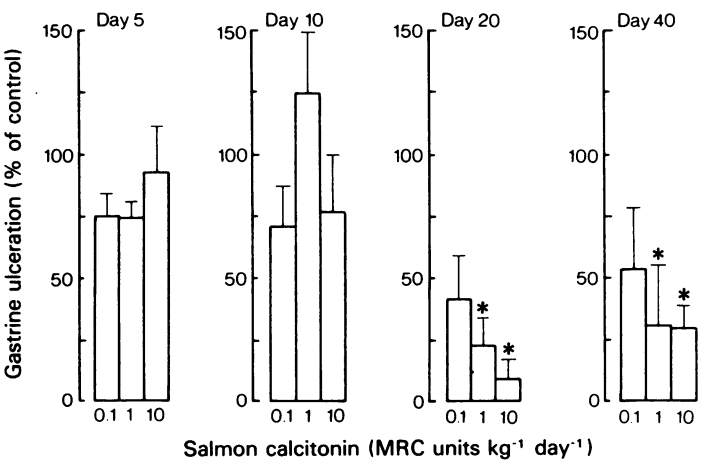

Figure 1 The effect of salmon calcitonin on acetic acid induced chronic gastric ulceration in the rat over varying time periods of calcitonin administration. The area of ulceration in calcitonin treated animals is expressed as a percentage of the area of ulceration in animals which received vehicle alone. Values are presented as means and s.e. mean. Each value is the mean of 6 determinations. Statistically significant differences are presented at the $P < 0.05$ level (*) (Mann-Whitney U-test).

Sprague–Dawley rats of either sex were anaesthetised (sodium pentobarbitone, 45 mg/kg) and ulcers were induced by the local application of glacial acetic acid in a 6 mm diameter plastic mould for 1 min to the serosal surface of the body of the stomach. From the day following acetic acid administration (day 1) either salmon calcitonin (0.1 MRC units/kg–10 MRC units/kg) or vehicle alone was administered s.c. once daily. The vehicle contained rat serum albumin (0.05 mg/ml) in sodium chloride solution (9 mg/ml). On days 5, 10, 20 and 40 three groups of rats were killed and the areas of gastric ulceration estimated.

The mean area of ulceration in control animals decreased from 27.0 ± 3.3 mm^2 (mean $\pm$ s.e. mean) at day 5 to 6.8 ± 1.3 mm^2 at day 10 and 4.9 ± 1.7 mm^2 at day 20. On day 40 the area of ulceration had increased to a value of 12.6 ± 4.0 mm^2. This pattern of decreased area of ulceration followed by a spontaneous increase in the area of ulceration has previously been described by Okabe & Pfeiffer (1971).

Salmon calcitonin did not have a statistically significant effect on the area of ulceration at day 5 or day 10 (Figure 1). The area of ulceration was reduced in a dose dependent manner at day 20 and at day 40 (Figure 1). A dose of 1 MRC unit/kg salmon calcitonin daily, decreased the areas of ulceration by 77% and 69% after 20 and 40 days respectively.

A comparison between this study and those of other workers (Okabe, Takata, Takeuchi, Naganuma & Takagi, 1976; Okabe, Takeuchi, Murata & Takagi, 1977) suggests that in the rat calcitonin may be more effective than carbenoxolone sodium and of comparable effectiveness to cimetidine in promoting the healing of acetic acid induced gastric ulcers.

The salmon calcitonin used in this study was kindly supplied by Drs. J.W. Bastian and J.P. Aldred, Armour Pharmaceutical Corp., Illinois, USA.

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A study of mechanisms involved in collagen and prostaglandin H_2-induced intravascular platelet aggregation in the rat

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Platelet adhesion and aggregation in the microvasculature following an intravenous injection of an aggregating agent can be quantified by monitoring the number of circulating platelets. The Technicon Auto-Counter has been modified (Smith & Freuler, 1973) and connected to an anaesthetized animal via a double cannula inserted into the carotid artery.

A fall in circulating platelet numbers can be induced by an intravenous injection or infusion of an aggregating agent. Adenosine diphosphate (ADP, 2.5–120 $\mu g\ kg^{-1}\ min^{-1}$) collagen (20–80 $\mu g/kg$), 5-hydroxytryptamine (5-HT, 10–80 $\mu g/kg$), prostaglandin H_2 (PGH_2, 0.5–2.0 $\mu g/kg$) and the synthetic analogue of PGH_2, U44069 (3.5–14.0 $\mu g/kg$) produced dose-dependent, reproducible falls in circulating platelet numbers in the rat but arachidonic acid, (AA, 0.5–2.0 mg/kg) did not produce consistent results.

Indomethacin (1–8 mg/kg) partially inhibited the collagen- and AA-induced fall in circulating platelet numbers. Adenosine ($0.25\ mg\ kg^{-1}\ min^{-1}$) infused for 10 min inhibited ADP-induced aggregation and

methysergide (10 μg/kg) inhibited 5-HT-induced aggregation. Collagen-induced platelet aggregation or the residual response following indomethacin treatment could not be inhibited by adenosine or methysergide.

1-*n*-Butylimidazole (50 mg/kg), an inhibitor of thromboxane synthetase (Blackwell, Flower, Russell-Smith, Salmon, Thorogood & Vane, 1978) did not inhibit *in vivo* platelet aggregation induced by collagen or AA but caused a slight inhibition of PGH_2-induced aggregation. Phthalazinol (1 mg/kg) a thromboxane A_2 (TxA_2) receptor antagonist (Shimamoto, Takashima, Kobayashi, Morita & Takahashi, 1976) had no effect on collagen- or AA-induced platelet aggregation.

These results show that the *in vivo* platelet aggregation caused by collagen and AA involves the synthesis of PGH_2 as the cyclo-oxygenase inhibitor indomethacin can partially inhibit this aggregation. As adenosine and methysergide did not affect the residual response to collagen following indomethacin treatment, it is concluded that ADP and 5-HT are not involved in the mediation of this response.

These experiments do not indicate that the conversion of PGH_2 to TxA_2 is an essential step in the mediation of collagen- and AA-induced aggregation in the rat although 1-*n*-butylimidazole did slightly inhibit PGH_2-induced aggregation.

G.G.D. is the holder of an SRC studentship.
We gratefully acknowledge the support of the Wellcome Trust.

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A simple method for generating thromboxane A_2

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The instability of thromboxane A_2 (TxA_2) dictates that it must be generated and used immediately if its biological activity is to be studied. Biosynthetic preparation of TxA_2 is cumbersome and complex, and therefore a simpler method of preparation would be valuable. Mechanical agitation of guinea-pig chopped lung releases rabbit aorta contracting substance (RCS) (Palmer, Piper & Vane, 1970) which is predominantly TxA_2 (Hamberg, Svensson & Samuelsson, 1975). We have found that it is possible to obtain graded, reproducible release of RCS by this method.

Rabbit aortic strip, guinea-pig lung strip, guinea-pig ileum and dog iris sphincter muscle were suspended for cascade superfusion with modified Krebs solution containing antagonists, at a rate of 5 ml/min (Coleman, Humphrey, Kennedy, Levy & Lumley, 1979). Guinea-pig lung was chopped manually into pieces about 2 mm cu and placed in a nylon mesh bag in a 10 ml heated (37°C) organ bath. Krebs solution, containing no antagonists, was dripped over the lung at a rate of 5 ml/min, and the effluent allowed to superfuse the assay tissues. In each experiment, cumulative concentration-effect curves to $PGF_{2\alpha}$ (0.001–10 μg/ml) were first obtained to determine tissue responsiveness. Preparations which did not respond were discarded. When chopped lung was stirred in an even, vigorous manner, with the plunger of a 1 ml disposable syringe for 1–30 s, a substance was released which contracted the aorta and lung strip, sensitivity of the two tissues being similar. Small contractions of guinea-pig ileum (17 out of 21 experiments) and dog iris (8 out of 19 experiments) were sometimes observed. Rabbit aorta and guinea-pig lung strip are highly sensitive to TxA_2; in contrast, guinea-pig ileum and dog iris are insensitive to TxA_2, but are sensitive to PGE_2 and $PGF_{2\alpha}$ respectively (Coleman *et al.*, 1979 and this meeting). Release of contractile activity was abolished or greatly reduced when indomethacin (2.8×10^{-6} mol/l) was included in the fluid superfusing the lung. Contractions of aorta and lung strip were greatly reduced when the thromboxane synthetase inhibitor imidazole (Moncada, Bunting, Mullane, Thorogood, Vane, Raz & Needleman, 1977) (4.4×10^{-3} mol/l) was included in the fluid superfusing the lung. In the presence of imidazole, responses of ileum and iris were potentiated, indicating that diversion of endoperoxide metabolism had occurred

(Nijkamp, Moncada, White & Vane, 1977). These results support the conclusion that RCS is predominantly TxA_2.

Contractile responses of the aorta and lung strip were related to the length of time for which the lung was stirred, and when repeated at 20 min intervals, at least 5 reproducible cumulative stimulus-effect curves could be obtained. We therefore suggest that this method represents a simple way of generating TxA_2 for pharmacological testing.

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Comparison of mianserin with desipramine and maprotiline on blood pressure responses to acetylcholine, histamine and 5-hydroxytryptamine in normotensive rats

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Mianserin, a novel antidepressant agent (Brogden, Heel, Speight & Avery, 1978), possesses both cardiac presynaptic and vascular post-synaptic α-adrenoceptor blocking properties and, like desipramine and maprotiline, inhibits the neuronal uptake mechanism for noradrenaline (as assessed by the cardiovascular responses to endogenous and exogenous noradrenaline in the pithed rat; Cavero, Gomeni, Lefèvre-Borg & Roach, 1980).

We have studied the effects of desipramine, maprotiline and mianserin on the blood pressure responses produced by acetylcholine, histamine and 5-hydroxytryptamine in rats.

Rats (C. River, Sprague–Dawley, weighing 220–280 g) were anaesthetized with sodium pentobarbitone (55 mg/kg, i.p.) and were then artificially respired. Blood pressure was measured from a cannulated carotid artery and drugs were injected into a femoral vein (Cavero *et al.*, 1979). The blood pressure lowering effects of i.v. acetylcholine (0.25–2.0 μg/kg) and histamine (2.5–20.0 μg/kg) were studied before and after i.v. administration of desipramine (0.1 mg/kg), maprotiline (0.5 mg/kg) and mianserin (0.1–3.0 mg/kg) in intact rats. I.v. atropine (0.1 mg/kg) and promethazine (0.1 mg/kg) were used as standard antagonists of muscarinic and histaminic receptors, respectively. The effects of i.v. mianserin (0.03–0.3 mg/kg) or methysergide (0.005 mg/kg) on the pressor responses to 5-hydroxytryptamine (5–40 μg/kg) were studied in pithed rats or in animals pretreated with syrosyngopine (1.0 and 5.0 mg/kg, s.c., one and 18 h before anaesthesia, respectively) in order to deplete peripheral stores of endogenous noradrenaline.

In the intact anaesthetized rat, desipramine, maprotiline and mianserin, unlike atropine, did not affect the blood pressure lowering effects of acetylcholine.

Mianserin and maprotiline but not desipramine inhibited the hypotensive effects of histamine and were approx. 3–5 times less potent than promethazine.

In pithed or syrosyngopine pretreated rats the blood pressure increases produced by 5-hydroxytryptamine were reversed to depressor responses after i.v. administration of mianserin (0.01–0.3 mg/kg) or methysergide (0.005 mg/kg). The latter effects were not inhibited by i.v. atropine (0.1 mg/kg), indomethacin (2.0 mg/kg), promethazine (1.0 mg/kg) or propranolol (1.0 mg/kg). Therefore, the hypotensive activity of 5-hydroxytryptamine was not mediated by the receptors blocked by each of these antagonists or by the formation and release of endogenous prostaglandin-like substances. However, this effect was antagonized by a high i.v. dose of mianserin (10 mg/kg) or methysergide (2.0 mg/kg).

Desipramine and maprotiline did not affect the 5-hydroxytryptamine pressor responses in pithed rats.

It is concluded that the three antidepressant agents, desipramine, maprotiline and mianserin are devoid of vascular muscarinic receptor blocking properties. Mianserin and maprotiline both inhibited vascular histaminergic receptors but only mianserin (0.01–0.1 mg/kg, i.v.) antagonized 5-hydroxytryptamine receptors, the stimulation of which produced pressor responses in pithed or syrosyngopine pretreated rats. Furthermore, the hypotensive effects elicited by 5-hydroxytryptamine in pithed or syrosyngopine rats pretreated with a small dose of mianserin were antagonized by a large dose of this antidepressant agent or methysergide suggesting that a different type of 5-hydroxytryptaminergic receptor from that mediating vasoconstriction may be responsible for the vasodepressor action of 5-hydroxytryptamine.

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Lack of feedback via presynaptic α-adrenoceptors by noradenaline released by a single pulse

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Noradrenaline released from sympathetic nerve terminals may act on presynaptic α-adrenoceptors to inhibit its subsequent release (Langer, 1977). Yohimbine, a presynaptic α-adrenoceptor antagonist, potentiates the twitch response and increases [^{3}H]-noradrenaline overflow from the mouse vas deferens following trains of pulses (Marshall, Nasmyth & Shepperson, 1977, 1978).

These results do not show whether the negative feedback process can regulate noradrenaline release during a single pulse. Therefore a method has been devised to measure the overflow of [^{3}H]-noradrenaline released by either one or two pulses from the mouse vas deferens. Modifications to the previous procedure (Marshall *et al.*, 1978) include the use of [^{3}H]-noradrenaline of higher specific activity and loading of [^{3}H]-noradrenaline in the presence of oestradiol (30 μM) to decrease extraneuronal and increase intraneuronal accumulation (Hughes, 1972).

Vasa were incubated in Krebs solution for 30 min with L-7,8-[^{3}H]-noradrenaline (0.59 μM, 35 Ci/mmol, Amersham). Six vasa were tied together, suspended in a 1.0 ml organ bath and washed for 90 minutes. Then the tissues were stimulated (2.0 ms, 64 V, Grass S48 stimulator) with 2 pulses at 2.0, 1.0, 0.2 and 0.1 Hz and finally with 1 pulse. There was a 10 min interval after each pair of pulses. The bath contents were collected 15 s after the single or second pulse and [^{3}H]-noradrenaline was separated from its metabolites (Graefe, Stefano & Langer, 1973).

The fractional release of [^{3}H]-noradrenaline for 1 pulse ($1.4 \pm 0.3 \times 10^{-5}$, mean ± s.e. mean) was less than that for 2 pulses at 0.2 Hz ($2.2 \pm 0.3 \times 10^{-5}$), 1.0 Hz ($2.3 \pm 0.4 \times 10^{-5}$) and 2.0 Hz ($2.6 \pm 0.2 \times 10^{-5}$) (*t*-test, $P < 0.05$). Yohimbine (100 nM) did not alter the fractional release of [^{3}H]-noradrenaline in response to a single pulse ($1.1 \pm 0.1 \times 10^{-5}$). This value was less than that for two pulses at frequencies of 0.1–2 Hz ($P < 0.01$). Yohimbine increased the noradrenaline overflow compared with controls whenever 2 pulses were given ($P < 0.05$).

The twitch response of a single vas deferens was elicited using either a single pulse or a pair of pulses (0.02, 0.1, 0.2, 1.0 and 2.0 Hz, 2.0 ms, 64 V). The response to a single pulse or to the first pulse in a pair was the same height and was unaltered by yohimbine, 10 or 100 nM. The height of the response to a second pulse was increased by yohimbine (10 or 100 nM) at 0.1, 0.2 and 1.0 Hz ($P < 0.01$).

These results show that the potentiation by yohimbine of the response to stimulation is associated with a drug induced rise in noradrenaline overflow. This suggests that feedback by endogenous noradrenaline occurs in a train of two pulses. Yohimbine did not affect either the overflow of noradrenaline or the post-junctional response to stimulation following a single electrical impulse. Therefore released noradrenaline does not appear to feed back onto presynaptic α-adrenoceptors to regulate its own release during one pulse.

We thank the Wellcome Trust for support for M.M.

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Cocaine and presynaptic α-adrenoceptor regulation of noradrenaline release in response to one and two pulses

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Blockade of the neuronal uptake for noradrenaline by cocaine usually potentiates responses to sympathetic nerve stimulation (Langer & Enero, 1974) and increases stimulation evoked noradrenaline overflow (Hughes, 1972). However, in the mouse vas deferens cocaine inhibits the twitch response to trains of electrical pulses (Marshall, Nasmyth & Shepperson, 1977). The effect of cocaine on the response of the vas deferens to one or two electrical pulses has been investigated.

Mouse isolated vasa deferentia were suspended in oxygenated magnesium-free Krebs solution at 37°C and stimulated (2.0 ms, 64 V) at 10 min intervals with a single pulse or two pulses (0.02, 0.1, 0.2, 1.0 and 2.0 Hz). Cocaine (100 nM and 10 μM) did not alter the height of a single twitch or the first twitch in a pair, but produced a concentration-dependent inhibition of the response to the second pulse (when expressed as a percentage of the first response) at 0.1, 0.2 and 1.0 Hz (t-test, $P < 0.001$). The inhibition produced by cocaine (10 μm) at 0.1, 0.2 and 1.0 Hz was reversed by the presynaptic α-adrenoceptor antagonist yohimbine (100 μm).

Mouse vasa deferentia were incubated in Krebs solution with L-7,8-[^{3}H]-noradrenaline, 0.59 μm (35 Ci/mmol, Amersham) (Markiewicz, Marshall & Nasmyth, 1980). Organ bath contents were analysed for noradrenaline separated from its metabolites after stimulation with one pulse (2.0 ms, 64 V) or two pulses (0.1, 0.2, 1.0 and 2.0 Hz). Cocaine (10 μm) increased the fractional release (FR) of [^{3}H]-noradrenaline after one pulse ($3.7 \pm 0.4 \times 10^{-5}$, mean ± s.e. mean, $P < 0.05$) compared with controls ($1.4 \pm 0.3 \times 10^{-5}$), and after two pulses ($P < 0.05$ at all frequencies). When yohimbine (100 nM) was present together with cocaine (10 μm) the FR of noradrenaline following one pulse ($4.6 \pm 1.1 \times 10^{-5}$) was similar to that in the presence of cocaine alone. However, the overall FR of noradrenaline for two pulses rose compared with cocaine (10 μm) alone ($P < 0.05$). The FR of noradrenaline in the presence of yohimbine (100 nM) was less for one and two pulses (0.1–2.0 Hz) than in the presence of cocaine (10 μm) and yohimbine (100 nM) ($P < 0.05$).

In conclusion, cocaine did not increase the size of the response to one pulse although it increased the fractional release of [^{3}H]-noradrenaline. With two pulses the fractional release of [^{3}H]-noradrenaline was also increased by cocaine although the twitch response following the second pulse was inhibited. These observations may reflect blockade of uptake by cocaine, increasing the proportion of released neurotransmitter which overflows into the organ bath and is measured as the 'fractional release'. In addition, the twitch inhibition following the second pulse may result from released noradrenaline persisting in the neuro-effector junction and inhibiting subsequent release via an action on pre-synaptic α-adrenoceptors. This hypothesis is supported by the reversal of the cocaine-induced twitch inhibition by yohimbine.

We thank the Wellcome Trust for support for K.G.

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Comparison of the α-adrenoceptors located on sympathetic and parasympathetic nerve terminals

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The stimulation of α-adrenoceptors located on sympathetic and parasympathetic nerve terminals produces inhibition of noradrenaline (Langer, 1974; Starke, Endo & Taube, 1975) and acetylcholine (Paton & Vizi, 1969; Wikberg, 1978; Drew, 1978) release respectively. On the basis of the selectivity of action of a number of α-adrenoceptor agonists and antagonists it has been proposed that the prejunctional α-adrenoceptors located on sympathetic nerve endings differ from the post-junctional α-adrenoceptors (Starke *et al.*, 1975; Drew, 1976; Cambridge, Davey & Massingham, 1977; Doxey, Smith & Walker, 1977). Langer (1974) has suggested that the α-adrenoceptors may be classified as α_1-(post-junctional) and α_2-(prejunctional) subtypes. However, Drew (1978) concluded that the pre-junctional α-adrenoceptors on parasympathetic nerve terminals were of the α_2-subtype, having shown them to be pharmacologically identical to those located on the sympathetic nerve terminals. We have therefore compared the selectivity of several different α-adrenoceptor antagonists on the α-adrenoceptors on sympathetic (rabbit pulmonary artery) and para-sympathetic (guinea-pig ileum) nerve endings.

The rabbit pulmonary artery preparation, previously labelled with [^{3}H]-noradrenaline (as described previously by Cambridge *et al.*, 1977) was used to measure the degree of α-adrenoceptor antagonism at pre- and post-junctional α-adrenoceptors, by the increase in transmitter release. ([^{3}H]-overflow) and reduction in contraction, respectively, after transmural stimulation, with each compound.

The guinea-pig ileum preparation (Paton, 1955, Drew, 1978, Wikberg, 1978) was used to measure the α-adrenoceptor activity of each compound at the α-adrenoceptors located on parasympathetic nerve terminals. This was achieved by measuring the degree of antagonism of the clonidine induced inhibition of the twitch response to transmural stimulation.

β-Adrenoceptors were blocked by the addition of propranolol (40 nM) to the Krebs' solution. In addition desipramine (0.6 μM) and normetanephrine (10 μM) were also added to the Krebs' solution to block uptake 1 and 2 in the pulmonary artery.

In the pulmonary artery, yohimbine, piperoxan and dibozane were selective antagonists at the prejunctional α-adrenoceptors. Piperoxan and dibozane caused some dose-related reduction in contraction, and therefore had some post-junctional α-antagonist action, but these effects occurred at relatively high concentrations than those which caused an increase in transmitter release. Phentolamine and WB-4101 (2-(2',6'-dimethoxy)phenoxyethylamine-methyl benzo dioxan) increased transmitter release and reduced contraction, and essentially lacked selectivity for either site. In contrast, prazosin, UK-33274 (a quinazoline analogue of prazosin) and labetalol were selective antagonists of the post-junctional α-adrenoceptors, and even at high concentration caused little or no increase in transmitter release.

In the ileum, dibozane, phentolamine, piperoxan, WB-4101 and yohimbine were competitive antagonists of the response to clonidine. These compounds produced dose-related parallel shifts to the right of the response to clonidine. In contrast prazosin, UK-33274 and labetalol were without effect in this preparation.

In summary, α-antagonists which prevent the α-adrenoceptor mediated modulation of sympathetic transmission, also prevent the α-adrenoceptor mediated modulation of parasympathetic transmission. The α-adrenoceptor at both types of nerve terminal appears to be the same, and supports the suggestion that neuronal α-adrenoceptors are of one type (i.e. α_2). Selective α_1-antagonists like prazosin, UK-33274 and labetalol will therefore not affect the integrity of the α-adrenoceptor mediated modulation of transmission at either sympathetic or parasympathetic nerve-terminals.

Thanks are due to J.A. Law and Mrs. S.T. Boughton for expert technical assistance.

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