EFFECTS OF THYROLIBERIN ON MAMMALIAN NEUROTRANSMITTER BIOCHEMISTRY

by

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Abbreviations

The abbreviations used in this thesis are those recommended in the Biochemical Journal publication "Policy of the Journal and Instructions to Authors" (revised 1978), except for:

ACh	acetylcholine
Ch	choline
DA	dopamine
NA	noradrenaline
TRH	thyroliberin

A key to the one-letter amino acid code is given on Page 5

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Summary

Thyroliberin (TRH) has been shown in a number of laboratories to have behavioural effects in animals unconnected with its endocrine function. Pharmacological studies have suggested the involvement of dopaminergic neurones in some of the observed effects and cholinergic neurones in others. Many of the behavioural effects of TRH in rodents appear to originate in the nucleus accumbens and septum brain regions. In this thesis the effects of TRH, one of its metabolites (cyclo HisPro) and a TRH-analogue (CG3703) on the release of dopamine and acetylcholine from rat brain tissue preparations were investigated.

TRH (10^{-6}-10^{-3}M) stimulated the release of (^{3}H)-dopamine from tissue-cube and P2 preparations of rat nucleus accumbens and striatum in a manner sensitive to the pH (pH 6.5 $>$ pH7.4) and composition of the incubation medium.

Cyclo-HisPro (1mM) stimulated the release of (^{3}H)-dopamine from P2 preparations of nucleus accumbens and striatum at pH 6.5 but appeared to be no more potent than TRH in its effects.

CG3703 ($10^{-6}10^{-3}$M) stimulated the release of (^{3}H)-dopamine from tissue cubes of rat nucleus accumbens at pH 6.5 but not at pH 7.4.

Neither TRH nor its analogues (0.1mM) had any consistent effect on the release of (^{3}H)-acetylcholine or (^{3}H)-choline from tissue cubes of nucleus accumbens or septum at pH 7.4.

50mM-KCl in all cases stimulated the release of (^{3}H)-dopamine from tissue preparations of nucleus accumbens and striatum at pH 6.5 and 7.4 and of both (^{3}H)-acetylcholine and (^{3}H)-choline from nucleus accumbens and septum.

These results suggest that the stimulation of release of dopamine by TRH arise from a direct interaction of the peptide with dopaminergic neurones, not by a modulation of dopamine release caused by TRH affecting release of acetylcholine. The increased potency of cycloHisPro and CG3703 relative to TRH in behavioural paradigms may result from their resistance to degradation.

I INTRODUCTION

1.1 Discovery and Identification of TRH

Thyrotropin-Releasing Hormone (TRH, Thyroliberin) is a tripeptide with the structure shown in Figure 1. This peptide was first isolated and purified from extracts of sheep hypothalamus (Burgus etal, 1969; Guillemin, 1978). It was the comparison between the mass - and infra-red-spectra of isolated TRH with the spectra of other peptides of known structure which led to the determination of the structure of this peptide (Fleischer etal, 1970). It was later shown that TRH isolated from porcine hypothalamus was identical to that of ovine origin. (Schally etal, 1969; Nair etal 1970).

Although the end-groups of TRH (pyroglutamate at the N-terminal and prolineamide at the C-terminal) are structures not commonly found in peptides and proteins, they are not unique to this hormone and occur in other peptides which have been isolated from the brain, as shown in Table 1. Table 1 lists some of the peptides found in mammalian brain which have been shown to affect neuronal activity and have therefore been grouped together functionally as neuroactive peptides (Hughes, 1978; Iversen, 1979; Burgen etal, 1980). This list is not exhaustive since newly-discovered peptides are constantly being added to the group (Tatemoto etal, 1982; Unsworth etal, 1982).

Chemically synthesised TRH has been shown to have hypophysiotropic effects indistinguishable from the natural product (Fleischer etal 1970). This introduction describes what is presently known of the biochemistry and neuropharmacology of TRH in hypothalamic and extrahypothalamic mammalian brain.

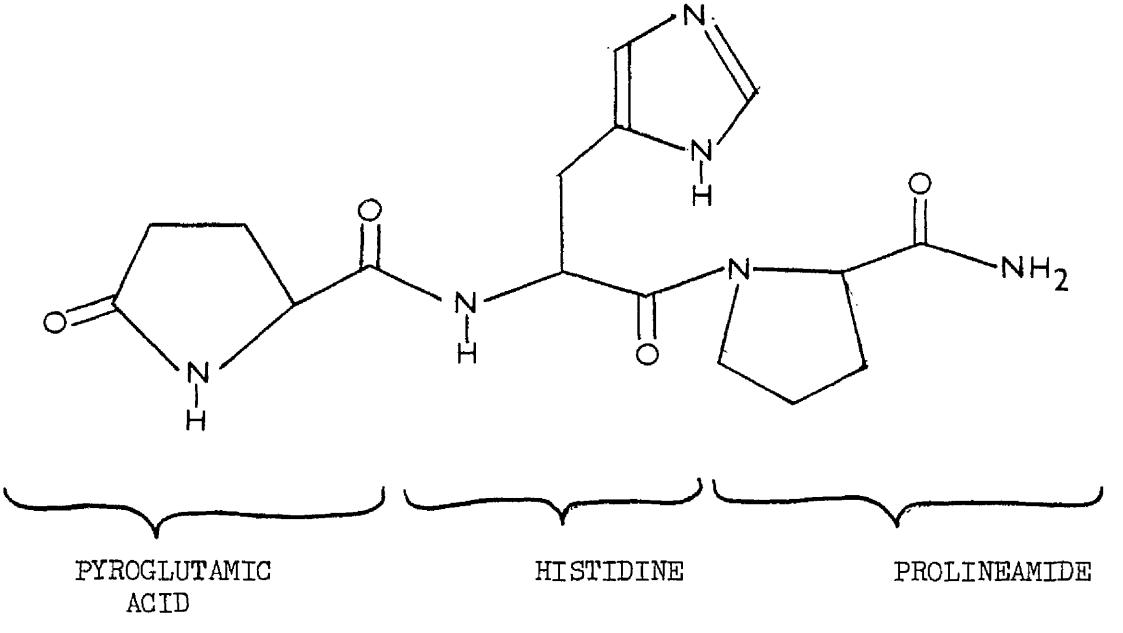


Figure 1: Structure of TRH

Table 1 : Primary Structures of Known Neuroactive Peptides

Peptide	Structure
Met-enkephalin	XGGFM
Leu-enkephalin	XGGFL
β-endorphin	XGGFMTSEKSQTPLVTLFKNAIVQNAHKKGQ
Substance P	RPKPQQFFGLM-NH_2
Angiotensin II	DRVXIHPF-NH_2
Corticotropin (ACTH)	SXSMEHFRXGKPVGKKRRPVKVXPDGAEAELAEAFPLEF
Lulilerin (LHRH)	pEHWSXGLRPG-NH_2
Oxytocin	CXIQNCPLG-NH_2 S-S
Vasopressin (ADH)	CXFQNCPRG-NH_2 S-S
Thyroliberin (TRH)	pEHP-NH_2
Melanostatin (MIF)	PLG-NH_2
Vasoactive Intestinal Polypeptide (VIP)	HSDAVFTDNXTRLRKQMAVKKXLNSILN-NH_2
Bombesin	pEQRLGNQWAVGHLM-NH_2
Cholecystokinin-like peptide	NXMGWMDF-NH_2
Carnosine	AH
Neurotensin	pELXENKPRRPXIL
Somatostatin	AGCKNFFWKTFTSC S-S

Table 2 : Key to Amino Acid Symbols Used in Table 1

Symbol	Meaning
A	Alanine
R	Arginine
N	Asparagine
D	Aspartic Acid
C	Cysteine
Q	Glutamine
E	Glutamic Acid
G	Glycine
H	Histidine
I	Isoleucine
L	Leucine
K	Lysine
M	Methionine
F	Phenylalanine
P	Proline
S	Serine
T	Threonine
W	Tryptophan
X	Tyrosine
V	Valine
pE	pyroGlutamic Acid
$-NH_2$	amide C-terminal
-S-S-	disulphide bridge

1.2 Hypothalamic TRH

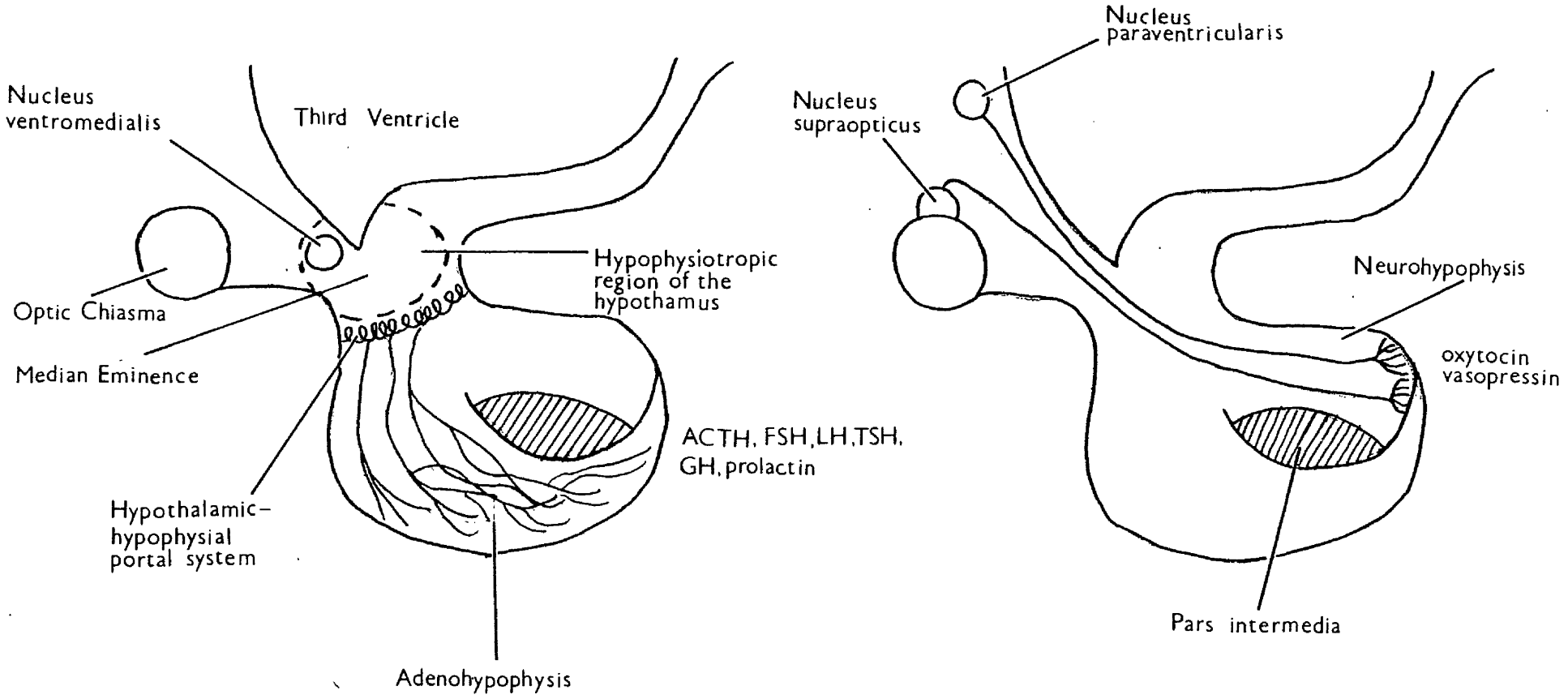
The highest concentrations of TRH in the brain are found within the hypothalamus, in particular in the median eminence and the nucleus ventromedialis pars medialis. (Brownstein etal 1974; Jackson and Reichlin 1974). These loci presumably contain perikarya of cells which form part of the parvocellular system, which secretes hormones into the hypothalamic capillary bed of the hypothalamic-hypophysial portal system. (Maddrell and Nordmann 1979). See Figure 2.

Little is known of the metabolism of TRH in these neurosecretory cells but, assuming that these cells are similar to other characterised neuro-secretory cells in the hypothalamus, the following may be an outline of the processes involved:

1.2.1 Biosynthesis

TRH biosynthesis takes place in the perikarya of the secretory cells and the peptide, either as a precursor or in its mature form is "packaged" in membrane-surrounded vesicles or secretory granules. Although the precise synthetic route is as yet uncertain, formation of TRH-like immunoreactivity in homogenates of hypothalamus has been observed in the presence of protein synthesis inhibitors (Reichlin et al 1976). This discovery rules out synthesis of TRH as a discrete peptide via transcription and translation of a single short gene but does not preclude continuing synthesis by enzymic processing of an already-translated precursor peptide in a manner similar to vasopressin synthesis (Hughes 1978), nor biosynthesis by the action of a "TRH synthetase", analogous to the route of glutathione synthesis (Mitnick and Reichlin 1972).

Figure 2 : Diagrammatic representation of the pituitary gland with its hypothalamic connections



Once synthesised, the pre-secretory form of TRH must enter the lumen of the smooth endoplasmic reticulum, to be transported to the Golgi apparatus and packaged into secretory granules. Processing for the production of the mature active peptide may occur at one or more of these steps (Maddrell and Nordmann 1979). Secretory granules or vesicles, formed within the perikaryon must be transported along the axon to the site of release. Such transport is shown to occur by ligation or sectioning of the axons of neurosecretory cells, which results in an increased hormone concentration on the side of the cut or tie proximal to the perikaryon. Studies with the electron microscope have suggested the involvement of microtubules in the transport of secretory granules.

1.2.2 Release

TRH release into the hypothalamic-hypophysial portal system appears to be dependent on the presence of Ca^{2+} ions but the exact mechanism has not yet been elucidated. Entry into the portal system lumen may take place through thinned or "fenestrated" patches of epithelium as is the case for vasopressin and oxytocin in the neurohypophysis. It has, however, not yet been shown that such fenestrations occur in portal capillaries. There is evidence that TRH is transported out of the median eminence and into the ventricular system. Tanycytes in the interface between the two systems have been implicated in this transport (Reichlin et al, 1976). Slow diffusion of TRH out of the cerebrospinal fluid into the portal system blood may maintain the basal level of TSH release from the pituitary gland. TRH release from the hypothalamus appears to be under the control of monoamines. Noradrenaline and dopamine have been found to

stimulate the release of TRH from mouse hypothalami in-vitro while serotonin inhibited the release, cholinergic agonists and antagonists having no effect (Grimm and Reichlin, 1973). Edwardson et al (1980) found no effects of prolactin nor growth hormone on TRH release suggesting that TRH secretion is controlled at a higher level than "short loop" feedback.

1.2.3 Hypophysiotropic Function

TRH stimulates the release of Thyroid-Stimulating Hormone (TSH, Thyrotropin), prolactin and, to a small extent, growth hormone from the adenohypophysis (anterior lobe of the pituitary). The TRH-stimulated release of these pituitary hormones is inhibited by Growth Hormone Release Inhibiting Factor (GHRIF, Somatostatin). Jackson and Reichlin, 1974; Reichlin et al, 1976; Vale et al 1977; Morley, 1979).

Schally et al (1968) reviewed the early work done on TRH extracted from bovine or porcine hypothalamus. TRH from either source increased plasma TSH concentrations in rats in-vivo and stimulated TSH release from rat pituitaries in-vitro. Stimulation by porcine TRH of TSH release from rat anterior pituitaries in-vitro was found to be dose dependent (0.03 - 1.08 ng TRH).

In mice in-vivo and in mouse pituitaries in-vitro, the release of TSH in response to TRH was inhibited by the thyroid hormones L-thyroxine and T_3 (tri-iodothyronine). Inhibitors of protein synthesis, e.g. puromycin, or of transcription, e.g. actinomycin D, prevent this thyroid hormone effect. This suggests that L-thyroxine and T_3 do not interact directly with TRH but rather on protein or RNA synthesis, perhaps leading to the production of a TSH-release-inhibiting factor.

Fleischer etal (1970) reported a dose-dependent increase in plasma TSH concentrations when TRH (250 - 750 ng) was injected intravenously (i.v.) into normal human subjects. In subjects with pituitary disease (hypopituitarism) and in subjects receiving L-thyroxine treatment for goitre there was little or no increase in plasma TSH concentrations following TRH treatment (500 - 750 ng, i.v.). It was suggested that TRH treatment could be used as a diagnostic test for pituitary dysfunction.

Gautvik etal (1980) reported that TRH-stimulated prolactin release from pituitary GH_3 tumour cells in culture was dependent on the presence of extracellular Ca^{2+} ions.

Tan and Tashjian (1981) studied the effects of TRH on $^{45}Ca^{2+}$ fluxes in prolactin secreting GH_4C_1 pituitary cells in culture and proposed that the peptide caused an initial release of Ca^{2+} from sites of adsorption on the external surface of the plasma membrane. Concomitant with this was a TRH-activation of specific calcium channels, causing an influx of Ca^{2+} ions which was, by some presently unknown mechanism, transduced into the secretion of prolactin.

In a small proportion of normal humans, TRH appears to produce a slight elevation in growth hormone secretion (Fleischer etal, 1970). In a variety of disease states in man, including acromegaly, diabetes mellitus and chronic liver disease, administration of TRH causes a reduction in growth hormone secretion and may be used as a diagnostic test to detect early abnormalities in growth hormone control which may lead to the onset of these conditions. (Scanlon and Hall, 1981).

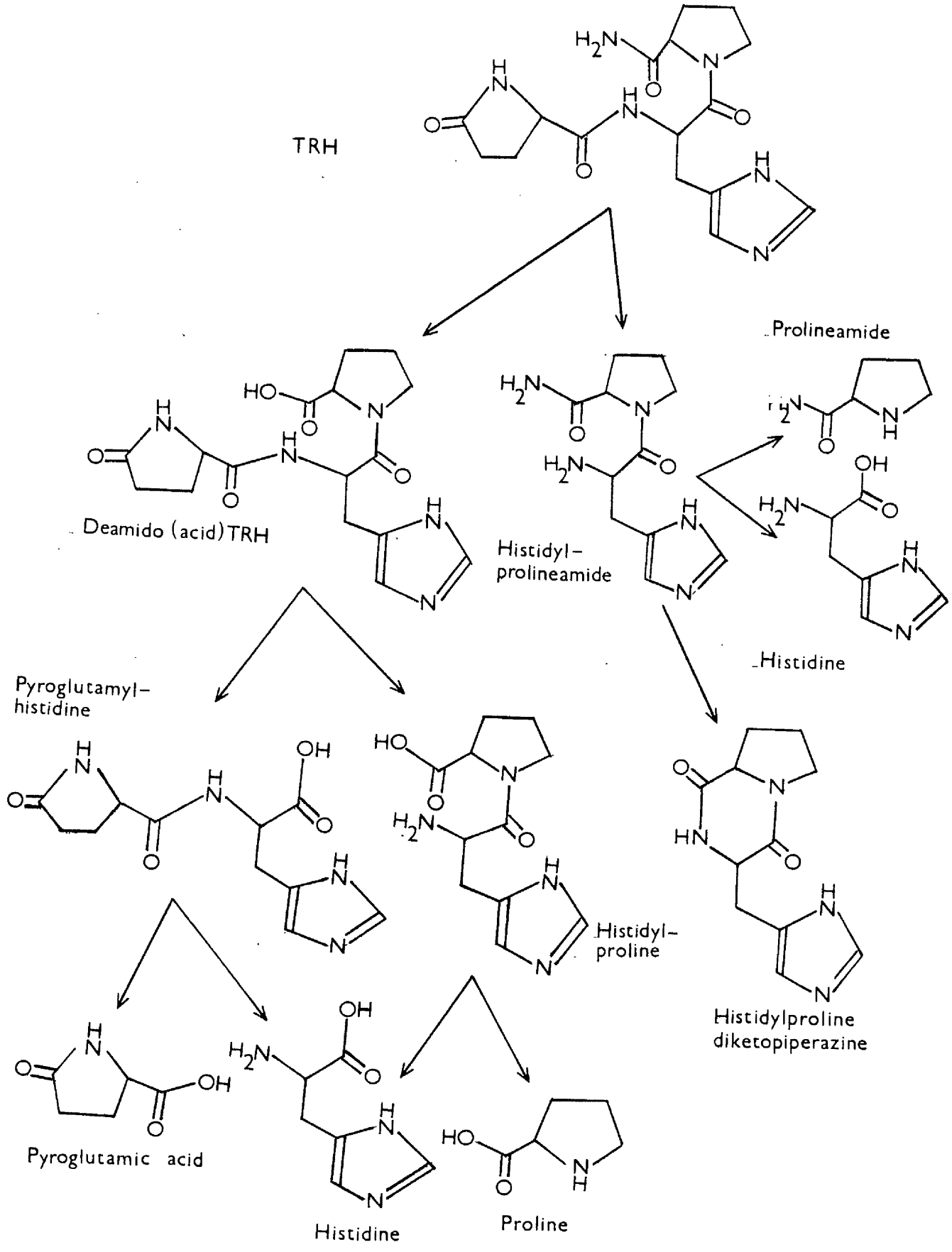
1.2.4 Degradation

The clearence of TRH from rat plasma and brain appears to be first order with respect to tissue concentration (Reichlin etal 1976) and TRH-degrading enzymes have been identified in hypothalamic and extrahypothalamic tissues (Prasad and Peterkofsky, 1976).
The initial steps in the degradation of TRH appear to be deamidation of the C-terminal prolineamide moiety by "TRH deamidase" and hydrolysis of the pyroglutamate N-terminal by pyroglutamyl aminopeptidase (Prasad etal, 1977; Busby etal 1982).
TRH deamidase has been classified as a 'post-proline cleaving enzyme' (Knisatschek and Bauer, 1979) or a prolyl endopeptidase, E.C.3.4.22.16. (Busby etal 1982) by virtue of its catalysing the hydrolysis of the proline amide residue. This enzyme is inhibited by peptides and peptide amides containing proline, e.g. Substance P and Angiotensin II, in a specific manner and by the antibiotic bacitracin nonspecifically. Deamidase activity has an optimum pH of about 7.4 and, on tissue fractionation, appears to be cytoplasmic in distribution (Griffiths etal 1980).
The pyroglutamyl peptidase involved in TRH degradation in porcine serum has been characterised and in contrast to other known pyroglutamyl peptidases, hydrolyses the pyroglutamyl-histidine bond of TRH but does not hydrolyse pyroglutamyl β-naphthylamide, which is a substrate for the other enzymes. Moreover, the TRH degrading enzyme was inhibited by EDTA and dithiothreitol whereas the other enzymes were activated by these agents. (Bauer and Nowak, 1979). These findings suggest that a specific enzyme for TRH degradation is present in porcine serum. Griffiths et al (1980)

found peptidase activity in the particulate fraction of centrifuged tissue homogenates whereas Busby etal (1982) found activity entirely in the soluble fraction. Both groups found that the enzyme had a pH optimum of 8-8.4.

The metabolites of the amidase and peptidase reactions may undergo further catabolism as shown in Figure 3. Matsui et al (1978) isolated and characterised an imidopeptidase for the hydrolysis of histidylprolineamide and suggested that inhibition of this enzyme and TRH deamidase by TSH and other peptide hormones, e.g. ACTH, directed TRH metabolism towards the production of histidylproline diketopiperazine (cyclo-His-Pro), which has been suggested to be an active form of TRH. (Prasad et al 1977). No enzyme catalysing the production of cyclo-His-Pro has yet been identified. This step may in fact be non-enzymic since the histidine amino group is sterically favoured for nucleophilic attack on the proline carbonyl group. Cyclo-His-Pro appears to undergo no further catabolism.

Figure 3 : Degradative pathways of TRH



1.3 Extrahypothalamic TRH

1.3.1 Distribution

Although the highest concentration of TRH is found in the hypothalamus, substantial amounts, albeit at lower concentrations, are present in extrahypothalamic and extraneural mammalian tissues. Table 3 shows the distribution of TRH in the rat. (Brownstein et al, 1974; Jackson and Reichlin, 1974; Winokur and Utiger, 1974; Oliver et al, 1974; Morley, 1979; Burgen et al 1980). The peptide has even been identified in invertebrates such as snails, which do not possess a pituitary gland, suggesting that the hypophysiotropic function of TRH may be a relatively recent use to which it has been put. (Maddrell and Nordmann, 1979). About 70% of the total TRH content of rat brain is present in extrahypothalamic tissues (Jackson and Reichlin, 1974). The concentration of extrahypothalamic TRH appears to be unaffected by lesions of the hypothalamus, suggesting that it is synthesised in-situ. (Jackson and Reichlin 1977).

The concentrations of TRH in hypothalamic and extrahypothalamic loci are not fixed but exhibit circadian variations, being lowest during the period of darkness. Other peptides, e.g. substance P and LH-RH, also undergo circadian concentration changes but these peptides are at their highest concentrations during the period of darkness (Kerdelhue, 1981). The reasons for the circadian variation in the levels of TRH and the other peptides are not known.

There has been some controversy as to whether immunoreactive TRH found in extrahypothalamic mammalian tissues is identical with the tripeptide isolated from the hypothalamus.

Table 3 : Distribution of immunoreative TRH in hypothalamic and extrahypothalamic tissues of the rat

Tissue	TRH concentration (pg/mg tissue ± S.E.) n = 10
Hypothalamus	255 ± 20
Thalamus	43 ± 3
Brain Stem	12 ± 0.53
Cerebrum	4.7 ± 0.17
Cerebellum	1.2 ± 0.06
Anterior Pituitary	2.2 ± 0.45
Posterior Pituitary	70 ± 16.4
Liver	0.06 ± 0.01
Kidney	0.09 ± 0.01
Pancreas	3.4 *
Duodenum	1.6 * S.E. not given
Colon	2.1 *
Plasma	0.09 ± 0.01

Values from Oliver (1974) except * from Morley (1979).

Youngblood et al (1978, 1979) compared the chromatographic properties, on TLC and GLC, of TRH-like immunoreactive substances from rat pancreas, eye and extra-hypothalamic brain, bovine and sheep pineals and human serum, urine and placenta with the properties of synthetic TRH and TRH extracted from frog skin. These workers found that very little of the purified extract from the tissues studied, which in all cases was rich in TRH-like immunoreactivity, co-chromatographed with authentic TRH. These findings cast serious doubt on whether TRH should be assigned any extrahypothalamic function at all. Other workers, however, tried to repeat the studies of Youngblood et al and found immunoreactive TRH to be chromatographically identical with synthetic pyroglu-his-pro-NH_2. Kreider et al (1979) used gel filtration and TLC to assess the authenticity of immunoreactive TRH from rat hypothalamus, frontal cortex, extrahypothalamic and whole brain. Spindel and Wurtman (1980) used TLC and reverse phase, ion pair HPLC on extracts of rat brain, spinal cord and pancreas. Both the latter groups suggested that the results obtained by Youngblood et al were artifacts of the multiple extraction procedures used to purify the TRH before chromatography and RIA. The latter findings suggest that, in rat at least, immunoreactive TRH found in extrahypothalamic tissues is authentic TRH.

1.3.2 TRH binding studies

One of the conditions which must be fulfilled by a chemical found in nervous tissue before it may be classified as a neurotransmitter is the presence of specific binding sites for it in the tissue. Burt and Snyder (1975) found both low and high affinity TRH binding in rat brain membrane preparations, the high affinity component (Kd $\simeq$ 50nM) resembling that found in pituitary membranes and the low affinity component (Kd $\simeq$ 5μM) being similar to binding in liver membranes.

Burt (1979) further localised the regions of high affinity TRH binding in rat brain, finding the highest concentration of binding sites localised in the nucleus accumbens and septum. Burt and Taylor (1980), and Taylor and Burt (1981) found that high affinity TRH binding sites in sheep brain resembled those in sheep pituitary ($K_d \simeq$ 40 nM) and that these sites were at the highest concentration in nucleus accumbens and septum. In a recent paper, Taylor and Burt (1982) surveyed the regional distribution of binding of $[^3H]$-(3-methylhistidyl) TRH to "TRH receptors" in the brains of rat, guinea-pig, rabbit, cat, dog, sheep, cattle and pig. In the rat, guinea-pig, cat and dog the concentrations of binding sites in the anterior pituitary were: 24.8 ± 4.6, 21.4 ± 5.2, 3.3 ± 0.6 and 2.8 ± 1.0 f mol/mg protein; those of the nucleus accumbens were: 11.3 ± 1.1, 16.8 ± 5.5, 7.2 ± 0.6 and 8.6 ± 4.6 f mol/mg protein; those of the dorsolateral septum were: 9.1 ± 0.6, 8.2 ± 0.7, 8.6 ± 2.0 and 7.6 ± 0.8 f mol/mg protein; and those of the deep amygdala were 27.1 ± 3.2, 54.0 ± 8.9, 11.1 ± 1.7 and 18.0 ± 3.4 f mol/mg protein respectively. In these four species the concentration of binding sites in the deep amygdala exceeds that in the nucleus accumbens and septum and, except for the rat, also exceeds that of the anterior pituitary. The highest anterior pituitary binding was found in sheep (62.4 ± 9.1 f mol/mg protein) and the highest binding in nucleus accumbens and septum were found in rabbit (34.9 ± 9.7 and 26.2 ± 4.0 f mol/mg protein respectively).

The results of this comprehensive study suggest that the effects of TRH which appear to originate in the nucleus accumbens and septum might be more fruitfully studied in the rabbit than in the rat. The discovery of a hitherto unsuspected TRH-sensitive region of mammalian brain opens another avenue of research and further complicates the neuropharmacology of the peptide.

1.3.3 Behavioural Effects

The behavioural effects of TRH on experimental animals fall into two broad categories: the stimulation of locomotor activity and the antagonism of narcosis. These effects are thought to have distinct aetiologies and will be dealt with separately.

1.3.3a) Locomotor Stimulation

The DOPA potentiation test is an in-vivo pharmacological screening test involving the administration of L-3,4-dihydroxyphenylalanine (L-DOPA) and a monoamine oxidase inhibitor (MAOI) to an experimental animal, followed by the drug being screened. L-DOPA-plus-MAOI leads to elevated motor activity in mice, probably as a consequence of increased catecholamine concentrations in central aminergic synapses. TRH was found to enhance this effect (Plotnikoff et al 1972). TRH-enhanced locomotor stimulation was found in both intact and hypophysectomised animals following oral or intraperitoneal administration of the peptide (0.1 mg/kg body weight). The effects of TRH were found to be dose dependent up to 0.8 mg TRH/kg body weight and were found when TRH was given up to eight hours after L-DOPA-plus-MAOI. Similar results were obtained using hypophysectomised animals, showing that these effects were unrelated to endocrine function.

Cohn et al (1975) found that TRH (50 µg/animal), when injected intracerebroventricularly into rats, produced tight head-to-tail circling motions when the animals were pre-treated, by unilateral injections into the right striatum, with the dopamine agonist apomorphine or with reserpine, which depletes vesicular stores of dopamine. Apomorphine pre-treated rats rotated clockwise while reserpine pre-treated rats rotated anti-clockwise, for a period of 30 minutes. These effects were found to be identical to those observed in similarly-pretreated rats given (+)-amphetamine (150 µg/rat).

Manberg et al (1979) found the behavioural effects of TRH to be similar to those of (+)-amphetamine in some respects but not in others, for example, both drugs induced hyperthermia in rats but only (+)-amphetamine produced stereotyped behaviour.

1.3.3a1 Biochemical Basis of Locomotor Stimulation

The results obtained from behavioural studies suggest the involvement of catecholamines in the effects of TRH on locomotor activity in experimental animals. The nature of the interaction of TRH with catecholaminergic neurones has been studied in a number of laboratories.

Keller et al (1974) found that TRH (10 mg/kg body weight) increased the content of the noradrenaline metabolite 3-methoxy-4-hydroxyphenyl-ethyleneglycol (MOPEG) in rat cortex, hypothalamus, mesencephalon-plus-medulla oblongata and "rest of brain" in both intact and thyroidectomised animals. The thyroid hormone tri-iodothyronine (T_3) (20 µg/kg body weight) produced a less pronounced increase in MOPEG in intact rats in all brain regions except "rest of brain", where the effect was comparable with that of TRH. Since TRH did not induce hypothermia, which would increase noradrenaline turnover, nor inhibit the clearance of other amine metabolites, it was suggested that the increase in MOPEG produced by the peptide was due to increased noradrenaline release and subsequent metabolism. Horst and Spirt (1974) found that TRH (10 mg/kg body weight, intraperitoneally (i.p.)) stimulated the release and turnover of dopamine in rat striatum and of noradrenaline in rat hypothalamus but not in brain stem. Chronic TRH administration over four days was not found to diminish catecholamine content, suggesting that biosynthesis was also stimulated.

In contrast with the foregoing findings, Reigle et al (1974) found no effect of acute or chronic administration of TRH on the metabolism of noradrenaline, serotonin nor dopamine in rat brain. In this study, however, no separation was made between MOPEG and 3-methoxy-4-hydroxymandelic acid (VMA) so changes in MOPEG content may not have been detected.

Mora et al (1979) found that conditioned behaviour in rats was strengthened by TRH and that this effect was antagonised by the tyrosine hydroxylase inhibitor α-methyltyrosine and by the dopamine β-hydroxylase inhibitor disulfiram. The conditioning was restored by L-DOPA.

Rips et al (1979) found that dopaminergic agonists amphetamine (2 mg/kg i.p.), L-DOPA (200 mg/kg i.p.) and p-chloroamphetamine (5 mg/kg i.p.) produced hypothermia in mice when given alone and potentiated significantly the hyperthermia produced by TRH (40 mg/kg i.p.). All of these drugs have agonistic effects on noradrenergic as well as dopaminergic pathways. The dopamine receptor agonist apomorphine (1 mg/kg i.p.) given alone produced hypothermia. When TRH and apomorphine were given together the effect was hyperthermia similar to that produced by TRH alone. The antagonists haloperidol (0.5 mg/kg i.p.), pimozide (4 mg/kg i.p.), chlorpromazine (2 mg/kg i.p.) and α-methyltyrosine (128 mg/kg i.p.) reduced but did not abolish TRH-induced hyperthermia whereas all of these drugs reversed the hyperthermia produced by amphetamine (8 mg/kg i.p.). Amphetamine-plus-TRH-induced hyperthermia was resistant to these antagonists.

The hypothermic effects of reserpine (1.5 mg/kg i.p.) were reversed 15 minutes after TRH administration and 30 minutes after amphetamine administration. The hyperthermia produced by TRH-plus-amphetamine was greater in reserpine treated rats than in non-treated rats.

The results of these biochemical and pharmacological studies on TRH suggest that the amphetamine-like stimulatory actions of the peptide may be mediated through dopaminergic and/or noradrenergic systems.

1.3.3a2 Possible sites of action of TRH in locomotor stimulation

Two catecholamine-rich regions of rat brain have been studied in relation to the biochemical effects of TRH. These regions are the striatum and the nucleus accumbens, which are part of the nigro-striatal and mesolimbic dopaminergic systems, respectively. Figure 4 shows the major dopaminergic tracts of the rat brain. (Cooper etal, 1976).

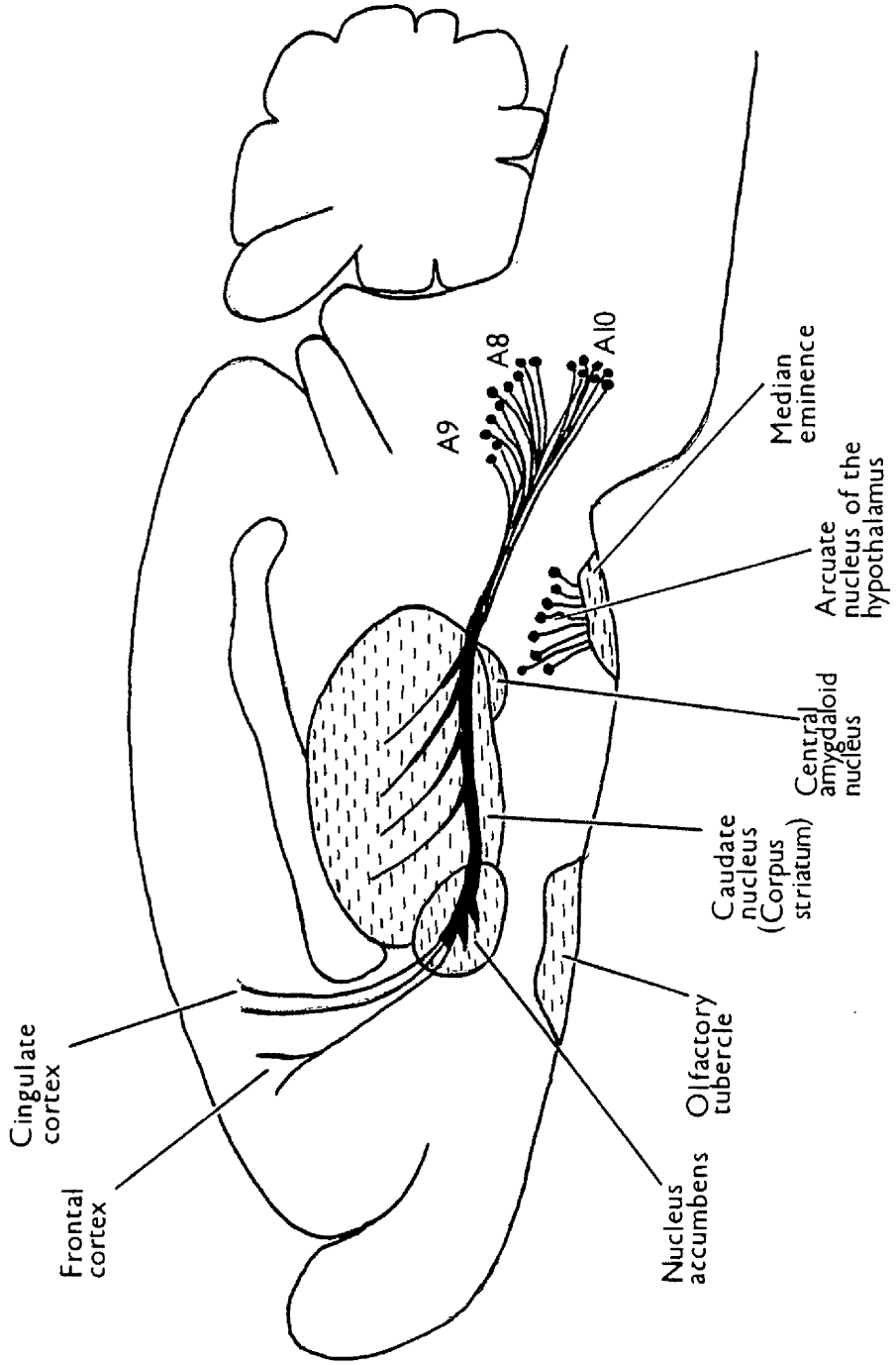
Miyamoto and Nagawa (1979) and Miyamoto etal (1978) showed that the mesolimbic system, particularly the nucleus accumbens, was sensitive to TRH. 5 mg TRH/kg body weight i.p. or 10 µg TRH injected bilaterally into the nucleus accumbens produced strong locomotor stimulation. Bilateral injection of 10 µg TRH into the caudate nucleus of the striatal system had no effect.

Heal and Green (1979) found that TRH-induced increases in locomotor activity in rats were abolished by bilateral injection of 6-hydroxydopamine (8µg) into the nucleus accumbens. 6-hydroxydopamine destroys catecholaminergic neurones. Intraperitoneal injection of the MAOI tranylcypromine (5 mg/kg body weight), followed by dopamine (5 µg) injected bilaterally into the nucleus accumbens produced an increased locomotor response in 6-hydroxydopamine treated rats.

Injection of TRH (20 mg/kg i.p.) into rats with unilateral lesions of the striatal system did not induce circling behaviour.

Injection of TSH (20 mg/kg i.p.) did not produce any behavioural changes.

Figure 4 : Major dopaminergic tracts of rat brain



These findings suggest that TRH exerts its influence on locomotor activity by causing or modulating the release of dopamine in the nucleus accumbens but not in the striatum and that there is no endocrine component to these effects.

In contrast, Costall et al (1979) did not observe any locomotor stimulation following bilateral injection of TRH (20 μg) into the mesolimbic system, striatum, midbrain, cortex nor lateral ventricles. Direct injection of TRH into the nucleus accumbens failed to enhance amphetamine-induced locomotor stimulation or reduce the depression produced by the dopamine receptor antagonist haloperidol. Moreover, TRH was found to induce stereotypic behaviour in rats, at variance with work outlined above. The discrepancy between reports on work using very similar techniques is difficult to reconcile and illustrates the uncertainties involved in in-vivo studies. In-vitro studies have consistently implicated the nucleus accumbens as a site of action of TRH.

The work of Burt (1978), Burt and Snyder (1975), Burt and Taylor (1980) and Taylor and Burt (1981, 1982) has shown the presence of high-affinity TRH-binding sites in the nucleus accumbens. The highest concentration of binding sites for TRH in the rat, however, appears to be in the deep amygdala region although this region does not seem particularly sensitive to TRH in behavioural studies. (Taylor and Burt, 1982).

The presence of binding sites alone does not necessarily define the presence of receptors unless binding is accompanied by a physiological response. Kerwin and Pycock (1979) showed that TRH (25-100 μM) stimulated the release of dopamine from the nucleus accumbens but not from the striatum. TRH did not affect the uptake of [^{3}H]-

dopamine into tissue slices of nucleus accumbens and neither basal nor dopamine-stimulated adenylate cyclase activity was affected. The binding of $[^3H]$ -spiperone, which binds to dopamine receptors, in membrane preparations of nucleus accumbens was also unaffected by TRH. TRH would therefore seem to affect primarily the release process.

Sharp et al (1981) found that TRH (10^{-4}M) stimulated the release of endogenous noradrenaline and dopamine from tissue slices of rat nucleus accumbens and septum.

1.3.3b Antagonism of narcosis

Breese et al (1974) found that intraperitoneal injection of TRH into mice prior to injection of ethanol reduced the duration of ethanol-induced sleep but there appeared to be no clear dose-dependence to the effect. Sleeping time was not affected by acid-TRH, its constituent amino acids, amphetamine, TSH nor thyroid hormones. This suggests that the effect is specific to TRH and involves brain mechanisms separate from those involved in the DOPA-potentiating effects of the hormone. TRH was later shown to antagonise not only ethanol-induced narcosis and hypothermia but also the depressive effects of pentobarbital and other centrally acting drugs (Breese et al 1975; Cott et al 1976).

Injections of TRH into the hippocampus of hibernating ground squirrels have been found to wake the animals and elevate their body temperature even when administered during the period of deepest sleep (Stanton et al 1980). This effect was accompanied by a short latent period. Shortening of latency was dose dependent (0.1 - 1000 ng peptide) and was not mimicked by acid-TRH.

Early studies (Prange et al, 1972; Prange and Wilson, 1972; Kastin et al, 1972) on depressed human subjects suggested that TRH had an antidepressant effect but later workers did not confirm this hypothesis (Dimitrihoudi et al, 1974; Morley, 1979).

1.3.3b1 Biochemical Basis of Antagonism of Narcosis

Breese etal (1975) and Cott etal (1976) found that TRH antagonism of ethanol and barbiturate narcosis was reduced and in some cases abolished by the muscarinic receptor antagonist atropine. This suggests that the peptide exerts its antinarcotic effects via cholinergic neurones. It was, however, found that the cholinergic agonist carbachol also reduced ethanol sleeping time in mice but appeared to antagonise TRH so that both drugs given together had

no effect on sleeping time. This suggests that TRH may not interact directly with cholinergic neurones.

Cott et al (1976) found that there was no significant difference between the concentration of ethanol in the brains of TRH-treated and untreated rats. This suggests that the antagonism of ethanol narcosis by TRH cannot be attributed to an increase in ethanol metabolism in the treated animals.

Malthe-Sørenssen et al (1978) found that intraventricular injection of TRH, somatostatin, neurotensin and angiotensin II all decreased the acetylcholine (ACh) content of rat brain parietal cortex but not that of the frontal cortex. TRH increased ACh turnover in parietal cortex but had no effect on hippocampus, pons-medulla nor diencephalon. None of the synthetic peptides L-prolylglycine, poly-L-proline and poly-L-glutamate had any effect on ACh turnover in any brain region tested, suggestingthat TRH had specific actions.

1.3.3b2 Possible sites of action of TRH antagonism of narcosis

Kalivas and Horita (1980) using antagonism of pentobarbital narcosis as the parameter, identified TRH-sensitive areas in the rat brain by injecting picomolar concentrations of the peptide through stereotaxically implanted cannulae. On this basis, TRH-sensitive sites seemed to be clustered in the lateral and medial septum, while the mesolimbic and striatal areas were found to be insensitive.

It has more recently been found that the antagonism of pentobarbital narcosis by TRH is blocked by lesions in the septum and fimbria of the septohippocampal system but not by lesions of the dorsal hippocampus (Kalivas, Simasko and Horita, 1981). Electrolytic lesioning of the septum blocked TRH-antagonism of narcosis, but lesioning by kainic acid, which destroys cell bodies, did not. This suggests that nerve fibres passing through the septum and fimbria, originating and terminating in presently unknown locations are involved in these TRH effects.

There is some evidence to suggest that the effects of TRH on body temperature may be peripheral in origin. Boschi and Rips (1981) found that intraperitoneal (i.p.) injections of TRH (40 mg TRH/kg body weight) into mice reversed the hypothermia induced by intracerebroventricular (i.c.v.) injection of noradrenaline (NA, 20 µg/mouse). The same dose of TRH (i.p.) was found to produce hyperthermia which was increased by i.p. NA (1 mg NA/kg body weight). I.c.v. injections of TRH did not produce hyperthermia. This suggests that noradrenergic systems, perhaps including peripheral ones, may be involved in the hyperthermia and reversal of hypothermia caused by TRH.

1.3.4 Interaction between TRH and neurotransmitters other than catecholamines and acetylcholine

Biochemical and pharmacological studies on TRH and its analogues have indicated that the peptide may interact with putative neurotransmitters other than catecholamines and acetylcholine to exert its behavioural effects.

Bennett and Edwardson (1977) observed that TRH enhanced K^+-induced release of glycine and, to a lesser extent, glutamate, aspartate and γ-aminobutyrate (GABA) from synaptosomes of sheep brain-stem and rat cortex. Basal release of amino acids was unaffected. Neither somatostatin nor LH-RH mimicked these effects, suggesting that TRH had a specific neuromodulatory effect.

Cott and Engel (1977) found that the GABA agonists γ-hydroxybutyric acid (GHBA), baclophen and aminooxyacetic acid (AOAA) inhibited TRH effects on ethanol narcosis and locomotor stimulation in mice. These results suggest that the behavioural effects of TRH may be mediated through an inhibition of GABA systems. Björkman et al (1981) found that the effects of TRH on mice treated with reserpine, tremor and reversal of hypothermia, was inhibited by GHBA and baclophen but not by the more specific GABA agonists γ-acetylenic GABA and sodium valproate. GHBA and baclophen, while producing effects similar to GABAergic stimulation, do not act on bicuculine-sensitive GABA receptors and are therefore thought not to be specific GABA agonists. Moreover, the GABA antagonist picrotoxin neither potentiated nor inhibited the TRH effects. These findings refute the hypothesis that TRH exerts its behavioural effects via GABAergic systems.

The same workers also found that the effects of TRH on reserpine-treated mice were unaffected by antagonists of cholinergic, serotonergic, adrenergic, dopaminergic and histaminergic receptors, which leaves the true origin of these effects uncertain.

The TRH analogue pyro-2-aminoadipyl-histidyl-thiazolidine-4-carboxamide (MK-771; see Figure 5, page 37) is almost equipotent with TRH in its hypophysiotropic effects but is of the order of 10-times as potent in eliciting a wide range of TRH-like behavioural effects, including antagonism of ethanol, pentobarbital and chlorpromazine narcoses and enhancement of motor activity in mice. (Yarbrough, 1979).

Rastogi et al (1981) studied the effects of MK-771 on rat brain dopaminergic and serotonergic systems. It was found that 15 mg MK-771/kg body weight increased dopamine synthesis in striatum, hypothalamus and pons-medulla and dopamine turnover in striatum and olfactory tubercle. 5-HT synthesis and turnover were increased by the same peptide concentration in hippocampus, hypothalamus, mid-brain and pons-medulla. Mid-brain tyrosine hydroxylase and tryptophan hydroxylase activities were enhanced. The increased potency of this synthetic peptide in the CNS may reveal effects of TRH too small to be observed with the natural peptide.

1.3.5 Interaction of TRH with other neuroactive peptides

In addition to those effects of TRH which appear to act directly on some aspect of neural function, the peptide also appears to interact with other neuroactive peptides, modifying their effects on nervous tissue. Observations of such interactions give an insight into possible fine-control mechanisms of the central nervous system.

The endogenous tridecapeptide neurotensin (NT) produces a hypothermic response in rats, potentiates neuroleptic and ethanol sleeping time and hypothermia, causes muscle relaxation, analgesia and decreases the active avoidance response in the "shuttle-box" behavioural paradigm. All of the above effects are antagonised by TRH (Nemeroff et al, 1980; Burgess et al, 1981).

The hypothermic response to NT is blocked by pretreatment of the animals with 6-hydroxydopamine, which depletes brain catecholamines, but is not blocked by antagonists of cholinergic, opiate, serotonergic nor noradrenergic systems. The ineffectiveness of noradrenergic antagonists suggests the involvement of dopaminergic neurones.

NT-induced hypothermia is augmented by the dopamine receptor antagonist haloperidol and by selective depletions of brain dopamine using desmethylimipramine (Nemeroff et al 1980). The basic mechanism(s) underlying the antagonistic effects of TRH on NT are presently unknown but may involve dopaminergic neurones.

The opiate β-endorphin produced a number of behavioural effects in rats, including reduction in motor activity, hypothermia, increased salivation and antinociception. TRH inhibits all of these effects except antinociception whereas all of the effects of β-endorphin are inhibited by the opiate receptor antagonist naloxone. This suggests that the TRH effects are not mediated directly via opiate receptors (Taché et al, 1977; Holaday et al, 1978).

β-endorphin inhibits the release of noradrenaline from rat cerebral cortex (Arbilla and Langer, 1977) and of dopamine from rat striatal slices (Loh[illegible] et al 1976). Since TRH is reported to affect the turnover and release of catecholamines (see Section 3.3), TRH may cause inhibition of opiate systems indirectly, by its effects on dopaminergic or noradrenergic neurones.

β-endorphin stimulates the release of prolactin and growth hormone from rat pituitary in vivo. TRH has a mixed effect on this response, elevating prolactin levels and reducing growth hormone levels in the plasma of β-endorphin-treated rats. (Taché et al 1977). The opiate is inactive as a releasing factor in mouse pituitary incubates in-vitro, suggesting that its actions may be of central nervous system origin.

1.3.6 Effects of TRH on neuronal activity

Glucose utilisation may be used as a measure of neuronal activity and can be followed by giving rats injections of $[^{14}C]$-2-deoxyglucose, which is taken up into cells by the glucose "carrier" but is not metabolised. The tissue being studied may then be sectioned and autoradiographed to visualise regions of active glucose utilisation. Nagai et al (1980) used this method to study the effects of TRH on glucose uptake in the brains of conscious and pentobarbital-anaesthetised rats. In conscious rats, glucose utilisation was found to be widespread and very active. TRH alone caused a generalised mild depression of utilisation. Pentobarbital caused severe widespread depression, which was antagonised by TRH in numerous loci, the most sensitive areas appearing to be the septum and the nucleus accumbens. This finding presumably reflects the sensitivities of these areas for TRH without necessarily proving that they are both involved in the behavioural effects of the peptide in anaesthetised animals.

Iontophoretic studies on the effects of TRH on neuronal activity have shown both excitatory and inhibitory effects. TRH has been shown to inhibit spontaneous or glutamate-evoked electrical activity in neurones of rat cerebellar and cerebral cortex, ventromedial hypothalamus (Renaud and Martin, 1974; Renaud, Martin and Brazeau, 1974) and rostral hypothalamus (Dyer and Dyball 1974).

Yarbrough (1976) found TRH to potentiate the excitatory effects of acetylcholine on rat cerebral cortex neurones.

1.3.7 TRH and cyclic nucleotides

The influence of TRH on intracellular levels of cyclic AMP (adenosine 3',5'-monophosphate) and cyclic GMP (guanosine 3',5'-monophosphate) in mammalian brain varies between brain regions studied.

Gautvik et al (1980) discovered that TRH elevated levels of cyclic AMP in cultured rat GH3 pituitary tumour cells.

Tsang and Martin (1976) and Tsang et al (1980) found that TRH depressed noradrenaline-induced increases in cyclic AMP in rat and human pineal glands but did not affect basal cyclic AMP levels. TRH was effective in this respect at 0.1 μg/ml whereas LH-RH, somatostatin and "DDD-TRH" (d-pyro-glutamyl-d-histidyl-d-prolineamide, the enantiomer of natural TRH) were required at much higher concentrations to reduce significantly noradrenaline-induced increases in cyclic AMP levels in this tissue.

Mailman et al (1978, 1979) found that TRH increased basal levels of cyclic GMP in the cerebellum of normal rats and rats treated with a range of anaesthetics, including ethanol and phenobarbital. No change in cyclic AMP levels were observed. The receptor antagonists atropine, atropine methylnitrate, chlorpromazine and morphine had no effect on TRH-induced increases in cyclic GMP, indicating that this effect, in cerebellum at least, was not related to activation of cholinergic, dopaminergic nor opiate receptors. Acid-TRH had no effect on cyclic GMP levels, suggesting that a specific TRH effect was being observed.

Green et al (1976) found that neither TRH nor the protein synthesis inhibitor cycloheximide affected dopamine-sensitive adenylate cyclase activity in rat caudate nucleus.

1.3.8 Effects of TRH metabolites

The metabolic products of TRH degradation which have been used in behavioural and biochemical studies are deamido-TRH (acid-TRH) and histidylproline diketopiperazine (cyclo-His-Pro). See Figure 3. These compounds are the major products of TRH degradation in rat serum and brain tissue homogenates and have been studied to determine whether they represent "active forms" of TRH.

Acid-TRH has been found to be inactive as a hypophysiotropic factor. (Reichlin et al 1976). It also lacks any effect on cyclic nucleotide levels (Mailman et al 1978, 1979) and does not antagonise ethanol narcosis (Prasad et al 1977) under conditions where TRH is active in these respects. Therefore acid-TRH appears to be a deactivated form of the parent peptide.

Prasad et al (1977) found that histidylproline diketopiperazine, when injected intraventricularly into rats at a dose of 1 μmol peptide/kg body weight reduced ethanol sleeping time to 50% that of the control duration, whereas 70 μ mol TRH/kg body weight was required to produce the same effect. However, in the same study, the dipeptide did not mimic TRH-antagonism of pentobarbital narcosis.

Bhargava (1980) found that both TRH and cyclo-His-Pro antagonised the hypothermia induced by delta-9-tetrahydrocannabinol when injected intracerebrally into mice at doses of 1 or 10 μg peptide/mouse. Only TRH had this effect when the route of administration was intraperitoneal injection.

Griffiths et al (1981) found that cyclo-His-Pro completely antagonised TRH-induced locomotor stimulation but did not antagonise d-amphetamine-induced hyperactivity. The dipeptide alone caused some stereotyped movements when injected directly into the nucleus accumbens.

Reports of cyclo-His-Pro effects on release of prolactin vary. Bauer et al (1978) found that cyclo-His-Pro inhibited both basal and TRH-stimulated release of prolactin from pituitary GH3 tumour cells. Lamberts and Visser (1981) found that cyclo-His-Pro only weakly inhibited basal prolactin release from normal cultured pituitary cells and had no effect on TRH stimulation nor on dopamine inhibition of prolactin release in the same system.

The findings of these studies suggest that conversion to cyclo-His-Pro may produce or modulate some of the effects of TRH observed in rats but do not suggest that cyclo-His-Pro is the universally active form of TRH.

1.3.9 TRH analogues

A number of structural analogues to TRH are commercially available and some of these are shown in Figure 5. These synthetic peptides have been found to be potent in mimicking the effects of TRH in neuropharmacological tests and behavioural paradigms (Flohé et al 1981; Heal et al 1981).

It has been suggested that the increased potency of these analogues is due to their resistance to TRH-degrading enzymes and hence increased biological half-lives. (Brewster etal 1980, 1981; Cowan and Vaught 1981; Dettmar et al 1981). Griffiths et al (1981) identified the metabolic products of some of the TRH analogues incubated with rat brain subcellular fractions. The order of resistance appeared to be:

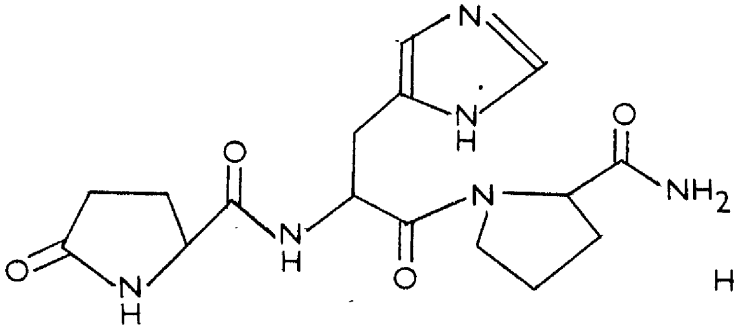
$$RX77368 > CG3509 > PGHPA > CG3703 \leq TRH,$$

an order which appears to be reflected to some extent in the potencies of the peptides since the same workers found RX77368 and CG3509 to have the greatest biological activity.

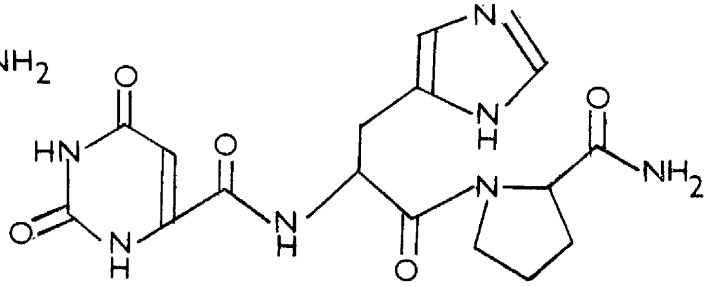
Flohé et al (1981) agreed with the finding that CG3509 had increased stability and biological activity over that of TRH but suggested that, from these points of view, CG3703 was the best analogue with which to investigate the effects of TRH in the central nervous system.

Hennies and Flohé (1982) found that CG3703 reduced the noradrenaline content of mouse brain in a dose-dependent manner (0.01-1.0 mg/kg body weight, i.p.) in mice treated with the tyrosine hydroxylase inhibitor α-methyltyrosine. TRH was about 80X less effective than CG3703. These findings suggest that CG3703 enhances the turnover of noradrenaline in mouse brain, an effect of TRH which may go unseen due to the natural peptide's rapid degradation in brain tissue. CG3703 did not affect the levels, rate of biosynthesis nor rate of utilisation

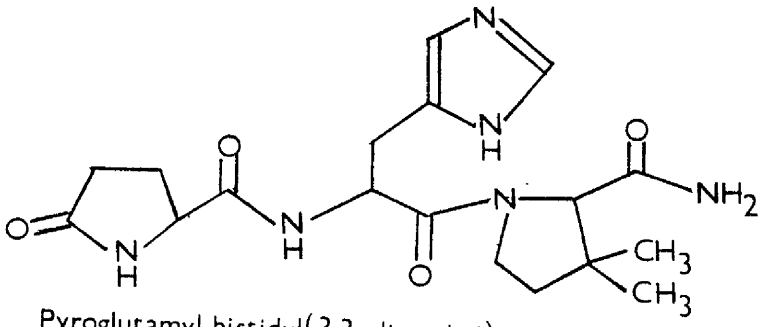
Figure 5 : TRH and some of its analogues



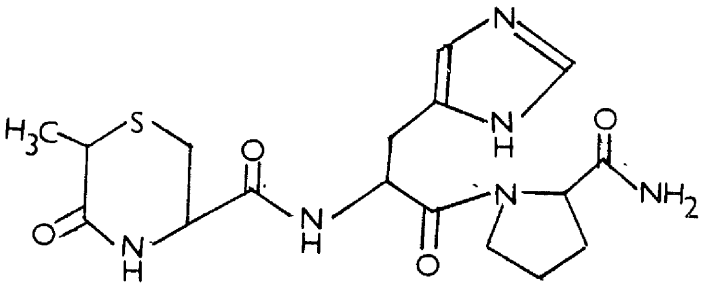
TRH



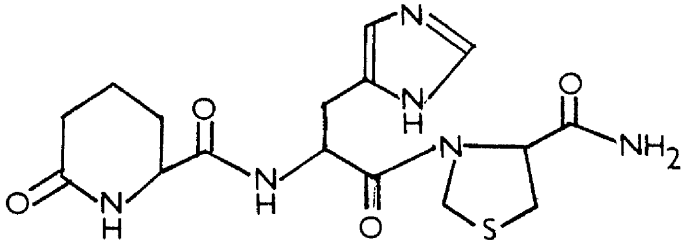
Orotyl histidyl prolineamide (CG3509)



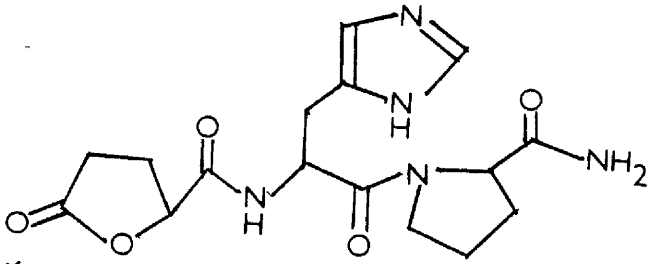
Pyroglutamyl histidyl(3,3-dimethyl)prolineamide (RX77368)



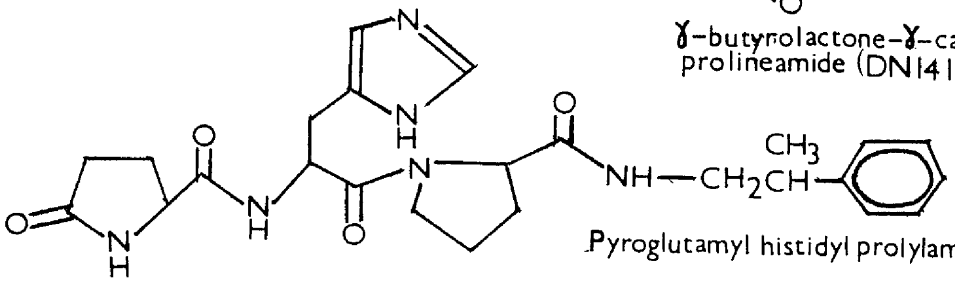
6-methyl-5-oxothiomorpholinyl-3-carbonyl histidyl prolineamide (CG3703)



Pyro-2-aminoadipyl histidyl thiazolidine 4-carboxamide (MK771)



γ-butyrolactone-γ-carbonylhistidyl prolineamide (DN1417)



Pyroglutamyl histidyl prolylamphetamine (PGHPA)

of serotonin in rat brain. It was therefore suggested that the "wet dog shakes" induced in rats by TRH were due to the peptide's actions on dopaminergic rather than serotonergic systems. This finding agrees with those of Hennies et al (1981) who found that "wet dog shakes" were antagonised by haloperidol, pimozide, chlorpromazine and propranolol more effectively than by serotonin receptor antagonists methy-sergide and cyproheptadine.

The analogue MK-771 appears to be equipotent with TRH in releasing TSH in rats but is more potent than TRH in inducing "wet dog shakes" in rats. (Cowan and Vaught, 1981). This analogue may be found a clinical use in treating narcolepsy since it has amphetamine-like stimulant effects but in behavioural tests on rats there appears to be no incentive for the animals to self-administer the drug, possibly indicating a reduced risk of addiction to this compound (Yarbrough, 1979).

The analogue DN-1417 has less endocrine activity than TRH but has been found to be more potent than TRH in depolarising the ventral root of rat spinal cord and in increasing the mono-synaptic reflex. (Ono and Fukuda, 1982). This analogue did not affect the membrane potential of the dorsal root nor increase the dorsal root reflex. The effects on the ventral root were not inhibited by chlorpromazine, haloperidol, atropine, cyproheptadine, baclophen, tetrodotoxin nor by pre-treatment with reserpine. These results suggest that both TRH and DN-1417 directly depolarise motoneurone membranes.

Fukuda et al (1980) chemically synthesised three classes of TRH analogues and tested them for endocrine and central nervous system activity. The analogues were classified by their N-terminals, being γ-butyrolactone-γ-carbonyl (Blc) (c.f. DN-1417),

2-ketopiperidine-6-carbonyl (Kpc) (≡ pyro-2-aminoadipyl, c.f. MK-771) and 3-oxoperhydro-1,4-thiazine-5-carbonyl (Otc) (≡ 5-oxothiomorpholinyl-3-carbonyl, cf. CG3703), and had prolineamide C-terminals substituted with various aliphatic and aromatic groups.

The analogues most effective in antagonism of pentobarbital sleep and potentiation of apomorphine-induced circling behaviour, Blc-his-pro-NH_2, Blc-3-methyl his-pro-NH_2, Otc-his-pro-NH_2 and Otc-his-pro-NH-nbutyl were tested for TSH-releasing activity. The Blc peptides were less active than TRH in releasing TSH from mouse pituitary (relative potencies w.r.t. TRH : 0.024 and 0.196 respectively) whereas the Otc peptides were slightly more active than TRH (relative potencies w.r.t. TRH : 1.58 and 1.19 respectively). It was suggested that the analogue Blc-his-pro-NH_2 (DN-1417) should be used to model central nervous system effects of TRH since its CNS effects were approximately 3-6 x more potent than TRH and its endocrine effects approximately 40x less potent.

Concluding Remarks

The discovery and identification of a factor from the hypothalamus which stimulated the release of TSH from the pituitary was a step towards the further understanding of control of pituitary and endocrine functions; the discovery that the same factor could affect animal behaviour and its identification in extrahypothalamic tissues introduced the possibility of another level of control of function of the central nervous system.

As work on TRH has progressed in numerous laboratories, the more it seems that this peptide may be performing different functions in different locations, ruling out the possibility of finding a unifying hypothesis for its actions. Despite these complications, the overall behavioural effects of TRH appear to be those of activation and reversal of depressed states and the use of degradation resistant analogues may further elucidate the *in vivo* function(s) of the natural hormone.

It has been suggested (Morley, 1979) that, since the wide range of effects of TRH and its wide distribution are now more fully appreciated, the name Thyrotropin-Releasing Hormone may itself no longer be adequate for this peptide and should be changed to Ubiquitous Regulatory Factor (U.R.F.) or, with deference to its discoverers, Guillemin Schally Factor (G.S.F.).

1.5 Introduction to Research Project

This project was initiated to study the biochemical effects of TRH on *in vitro* preparations of rat brain, with respect to neurotransmitter biochemistry.

Earlier work in this laboratory (Shapiro, L.M., PhD thesis, 1978; Shapiro, Bowes and Vaughan, 1980) suggested possible effects of TRH on the dopamine metabolism of rat caudate nucleus. High concentrations of TRH (10^{-3}M) were required to produce changes in dopamine turnover and release.

Kerwin and Pycock (1979) showed stimulation of release of pre-loaded dopamine from rat nucleus accumbens by TRH at peptide concentrations of 25-100 μM, whereas the caudate nucleus was found to be insensitive. The former region was therefore chosen to study TRH-stimulation or modulation of release of dopamine from "tissue cube" or "synaptosome" preparations of tissue.

This line of research was followed for most of the project.

Later work studied the effects of TRH, histidylproline diketopiperazine and the analogue CG3703 on release of acetylcholine from nucleus accumbens and septum.

II MATERIALS AND METHODS

2.1 Materials

Analar reagents were used throughout

Radiochemicals	Specific Activity
7,8- [^{3}H] -dopamine HCl 250µCi/250µl	44 Ci/m mol
[^{3}H] - choline chloride 250µCi/250µl	60 Ci/m mol
[^{14}C] -acetylcholine chloride 50µCi/250µl	26.5mCi/m mol

from Amersham International PLC, Amersham, Bucks., U.K.

Enzyme inhibitors

Pargyline HCl	Sigma London Chemical Co.Ltd., Poole, Dorset, U.K.
Bacitracin	"
Physostigmine (eserine)	"

Drugs

Tyramine HCl	BDH Chemicals Ltd., Poole, Dorset, U.K.
TRH acetate	Serva, c/o Micro-Bio Labs, London, U.K. or
	Pierce, Pierce and Warriner Ltd., Cheshire, U.K. or
	Sigma London Co.Ltd.
TRH analogue CG3703	Gift from Dr.L.Flohé, Grünenthal GmbH, West Germany
Histidylproline diketopiperazine	Pierce, Pierce and Warriner Ltd.

Fine Chemicals used in HPLC and TLC

Noradrenaline HCl	BDH Chemicals Ltd
Dopamine HCl	BDH Chemicals Ltd
1-heptane sulphonate	Fisons Co.Ltd.
Octyl sulphonate	"
Partisil ODS-10	Whatman Inc.
Partisil Co : Pel	"
Cellulose TLC plates (without fluorescent indicator)	E.Merck, AG Darmstat FRG
Choline Chloride	Sigma London Ltd
Acetylcholine chloride	"
Chloroplatinic acid	Gift from Dr.Kamal Aziz, Department of Chemistry, Glasgow University

Other Chemicals

2-amino-2-hydroxymethylpropane-1,3-diol (TRIS)	Sigma London Ltd.
Imidazole HCl	"
Bovine serum albumin	"
Sodium pyruvate	"
Ascorbic acid	BDH Chemicals Ltd
Folin-Ciocalteau reagent	"
Sodium dodecyl sulphate	"
N-2-Hydroxyethylpiperazine-N -2-ethane sulphonic acid (HEPES)	"
Reduced nicotinamide adenine dinucleotide, disodium (NADH)	PL Biochemicals Inc., c/o International Enzymes Ltd., Windsor, Berks, U.K.

2.2 Buffered salines

The buffered saline used in the filtration assay and some of the perfusion assays was that of Dayton et al (1979) and consisted of: 5mM-N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid (HEPES), 5mM-KCl, 120mM-NaCl, 1mM-Mg SO_4, 1mM-glucose, 2.5mM-$CaCl_2$, 0.5mM-ascorbic acid and 0.1mM-pargyline.

The pH of the saline was adjusted to pH 7.4 or pH 6.5 by addition of concentrated HCl.

The TRIS-buffered saline used in the perfusion and centrifugation assays was similar to that of Phizakerley and Fixter (1973), based on Krebs (1950) and consisted of:

20mM-2-amino-2-hydroxymethylpropane-1,3-diol (TRIS), 120mM-NaCl, 3.5mMNaHCO_3, 5mM-KCl, 1mM-Mg SO_4, 1.2mM-KH_2PO_4, 11.5mM-glucose, 0.5mM-ascorbic acid, 0.1mM-pargyline and 2.5mM-$CaCl_2$. The pH of the saline was adjusted to 7.4 or 6.5 by the addition of concentrated HCl.

When 50mM-KCl was used the Na Cl concentration was decreased to 75 mM.

In some cases, as indicated in the Results section, the TRIS was replaced by 20mM-imidazole.

1-litre batches of buffered salines, without glucose or ascorbic acid, were stored at $4^{o}C$. Glucose and ascorbic acid were added before each assay in a final volume of 100 ml saline.

Solutions containing TRH and other peptides (10^{-6}-10^{-3}M) also contained 50 μg bacitracin/ml to reduce degradation of the peptide.

Solutions used in assays for acetylcholine also contained 0.1mM-physostigmine to inhibit acetylcholinesterase.

2.3 Tissue preparation

2.3.1 Dissection

Male Wistar rats (250 $\pm$ 50g) were killed by cervical dislocation and decapitated. Brains were removed and placed in a petri dish on a filter paper (Whatman No.1, W.R.Balston Ltd., London) moistened with ice-cold HEPES, TRIS or imidazole buffered saline to await dissection.

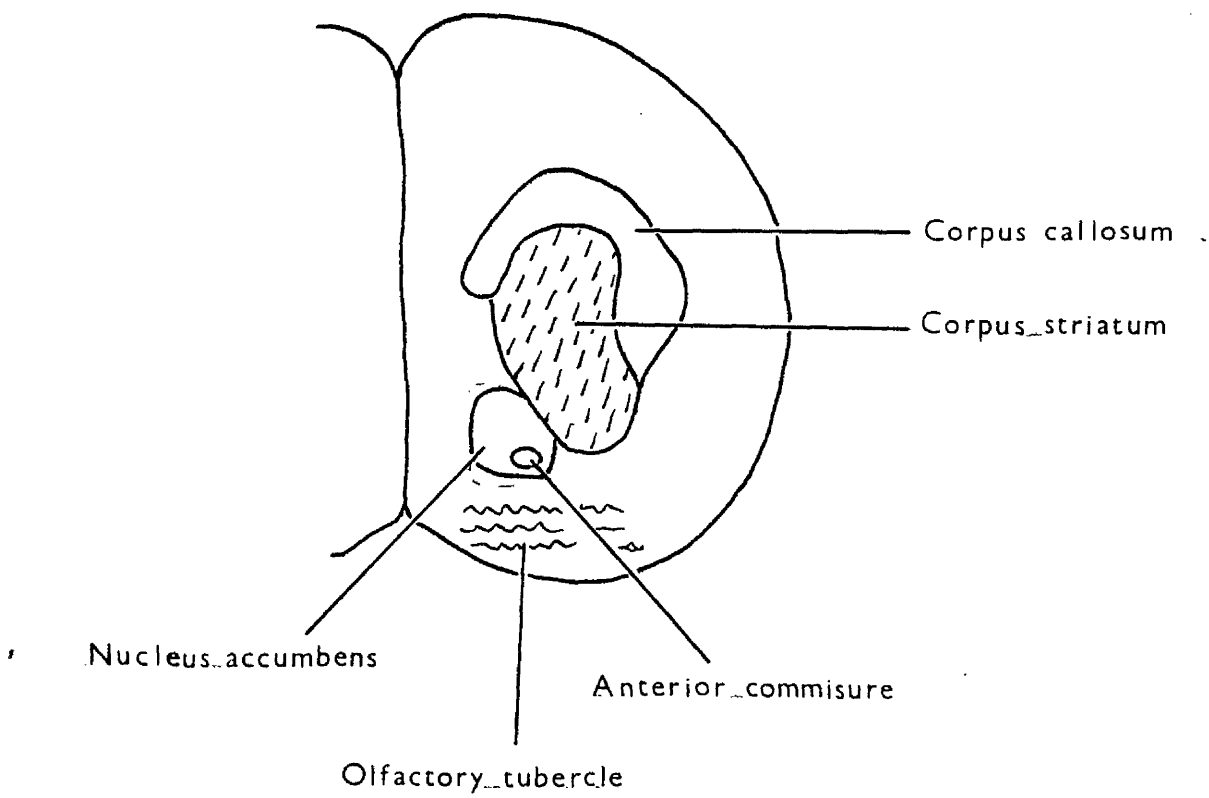
For the dissection of nuclei accumbens, a coronal section was made 2mm rostral to the optic chiasma and a second section at the optic chiasma. The slice of tissue produced was placed rostral aspect uppermost and the nuclei accumbens dissected by the method of Horn et al (1974) as shown in Figure 6.

Striata were dissected following the instructions of Glowinski and Iversen (1966) (Figure 7). Tissue which did not appear flecked or striated in colour was discarded.

For the dissection of septa a coronal section was made at the optic chiasma and a second section 3 mm caudal to the optic chiasma. The slice of tissue produced was placed rostral aspect uppermost and the septal region dissected by the method of Marsden (Dr.C.A.Marsden, Department of Physiology and Pharmacology, University of Nottingham Medical School : personal communication) as shown in Figure 8.

Hypothalami were dissected from the same section of brain as the striata, following the instructions of Glowinski and Iversen (1966 ; Figure 7).

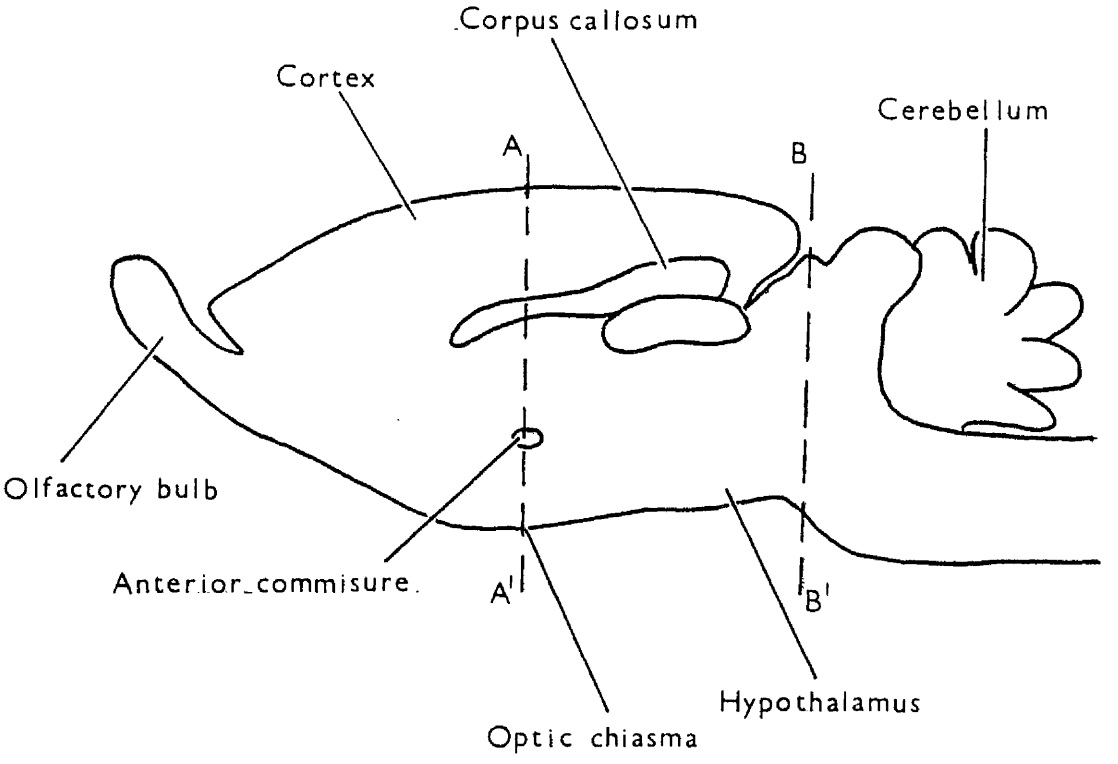
Figure 6 : Dissection of nucleus accumbens



Rostral aspect of coronal section 2 mm rostral to optic chiasma.

Half of brain is shown.

Figure 7 : Dissection of striatum and hypothalamus



a) Saggital section of brain, showing internal and external structures and positions of coronal sections (AA',BB') made in intact brain for the dissection of striatum and hypothalamus.

b) Rostral aspect of section AA'-BB'. Half of brain is shown

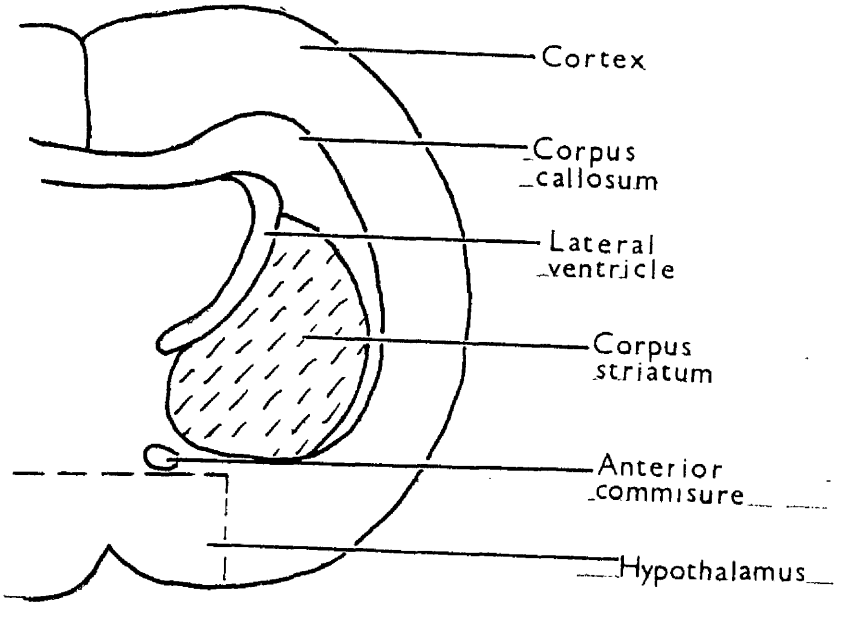
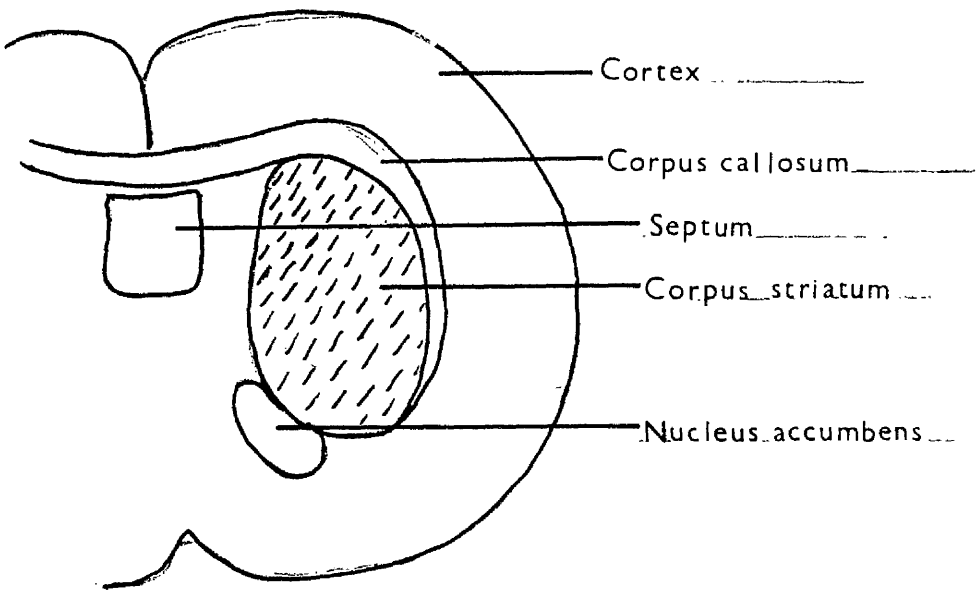


Figure 8 : Dissection of septum



Rostral aspect of 3 mm-thick coronal section immediately caudal to optic chiasma. Half of brain is shown.

2.3.2 Tissue cube preparation

Tissue from striatum, nucleus accumbens or septum was placed on filter paper (Whatman No.1), moistened with 5mM-HEPES-,20mM-TRIS- or 20mM imidazole-buffered saline, pH 7.4, containing 5mM-KCl,and chopped in two planes at 90° to one another using a tissue slicer set at 0.2mm (H.Mickle Ltd., based on the design of McIllwain and Buddle, 1953). The tissue fragments were suspended in 1-4ml of 5mM-HEPES-, 20mM-TRIS- or 20mM-imidazole buffered saline, pH 7.4, containing 5mM-KCl, for preloading with [^{3}H] -dopamine, or [^{3}H] -choline as outlined in the assay methods.

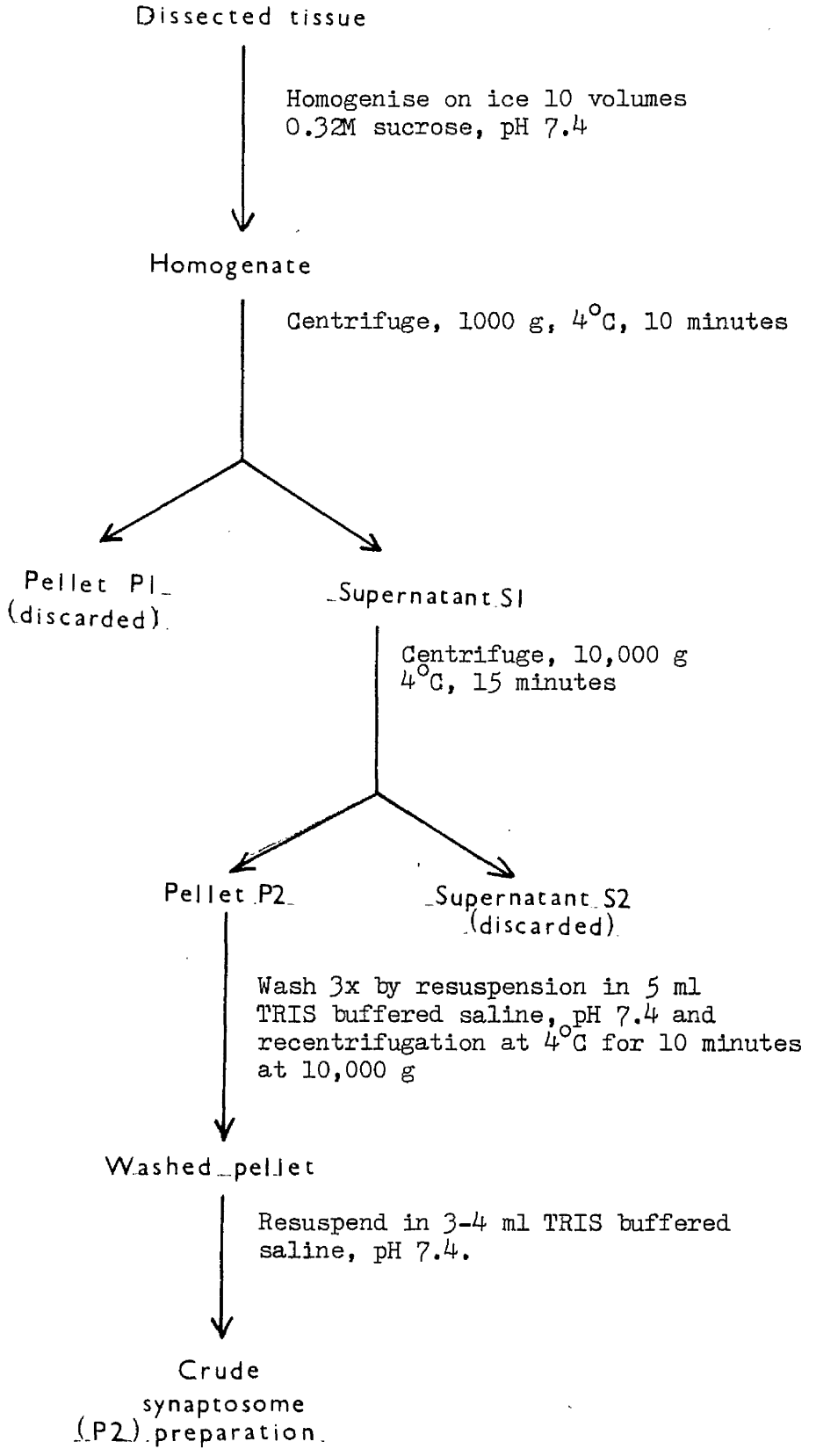
2.3.3. P2 preparation

Crude synaptosomes (P2 fractions) were prepared from striatal or nucleus accumbens tissue as shown in Figure 9, based on the method of Gray and Whittaker (1962). No further steps were taken to purify the nerve-ending fraction of this P2 preparation.
The integrity of the preparation was determined by the LDH assay (Section 2.4.1). The preparation was characterised as containing nerve ending particles by electron microscopy. Electron micrographs of P2 preparations were prepared by Mr.I.Montgomery, Physiology Department, Glasgow University.

2.3.4 Metabolic viability of tissue cubes of nucleus accumbens before and after pre-loading and washing

Tissue cubes of rat nucleus accumbens (20 - 40 mg wet weight) were placed in an oxygen electrode chamber (Rank Bros., Cambridge, U.K.) containing 2 ml 20 mM TRIS-buffered saline, pH 7.4 with 5 mM-KCl at 37 C, which had been allowed to equilibrate with the atmosphere. The rate of oxygen consumption was measured on a chart recorder (Rickadenki, Tohsin Electron Co.) for at least 2 minutes.

Figure 9 : Crude Synaptosome (P2) Preparation



100 μ moles of KCl was then added to the suspension of tissue cubes and the rate of oxygen consumption measured.

A second batch of tissue cubes (20-40 mg wet weight) were placed in 1 ml of 20 mM-TRIS buffered saline, pH 7.4, containing 5mM-KCl,/50 mg tissue at 37°C with shaking for 30 minutes. The tissue suspension was centrifuged at 10,000g for 10 minutes at 4°C in the MSE 18 centrifuge and resuspended and recentrifuged three times in 20mM-TRIS buffered saline pH 7.4 containing 5mM KCl. The final pellet was resuspended in 2 ml of the same saline and rates of respiration in the presence of 5 mM- and 50 mM - KCl determined using the oxygen electrode.

2.4 Assays

2.4.1 Lactate dehydrogenase assay (LDH ; E.C.1.1.1.27)

The integrity of the nerve ending particles in the crude P2 preparation was determined by measuring the proportion of occluded lactate dehydrogenase by the method of Johnson (1960).

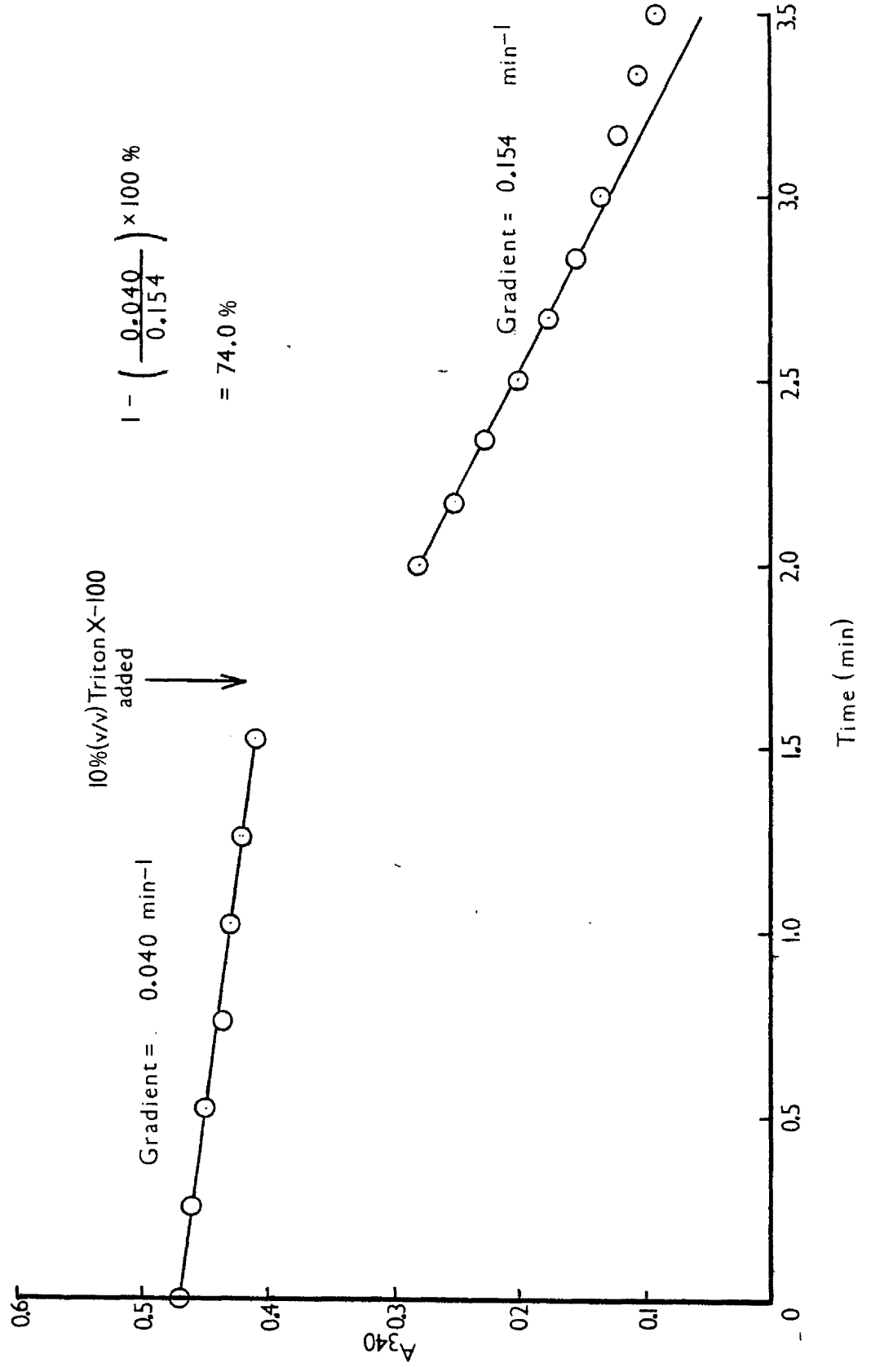
Free LDH activity was measured by adding 100 µl of P2 fraction (0.05 - 0.07 mg protein) to a cuvette in a Beckman Dual Beam spectrophotometer (Beckman Ltd., High Wycombe, Bucks., U.K.), which contained 2.9 ml NaH_2PO_4/Na_2HPO_4 buffer, pH 7.4 (final concentration 50 mM) containing sodium pyruvate (final concentration 0.33 mM) and disodium NADH (final concentration 80 µM). Absorbance (A_{340}) was measured every 15 seconds.

After a steady rate of oxidation had been reached 0.2 ml 10% (v/v) Triton X-100 was added to the cuvette to lyse vesicles for measurement of the total LDH activity in the P2 preparation. A_{340} was measured every 10 seconds (Figure 10).

The percentage of the total activity which was occluded by the nerve ending particles was calculated thus:

$$1 - \frac{(\text{free activity})}{(\text{total activity})} \times 100\% \quad \text{(Marchbanks, 1967)}$$

Figure 10 : Measurement of occluded lactate dehydrogenase

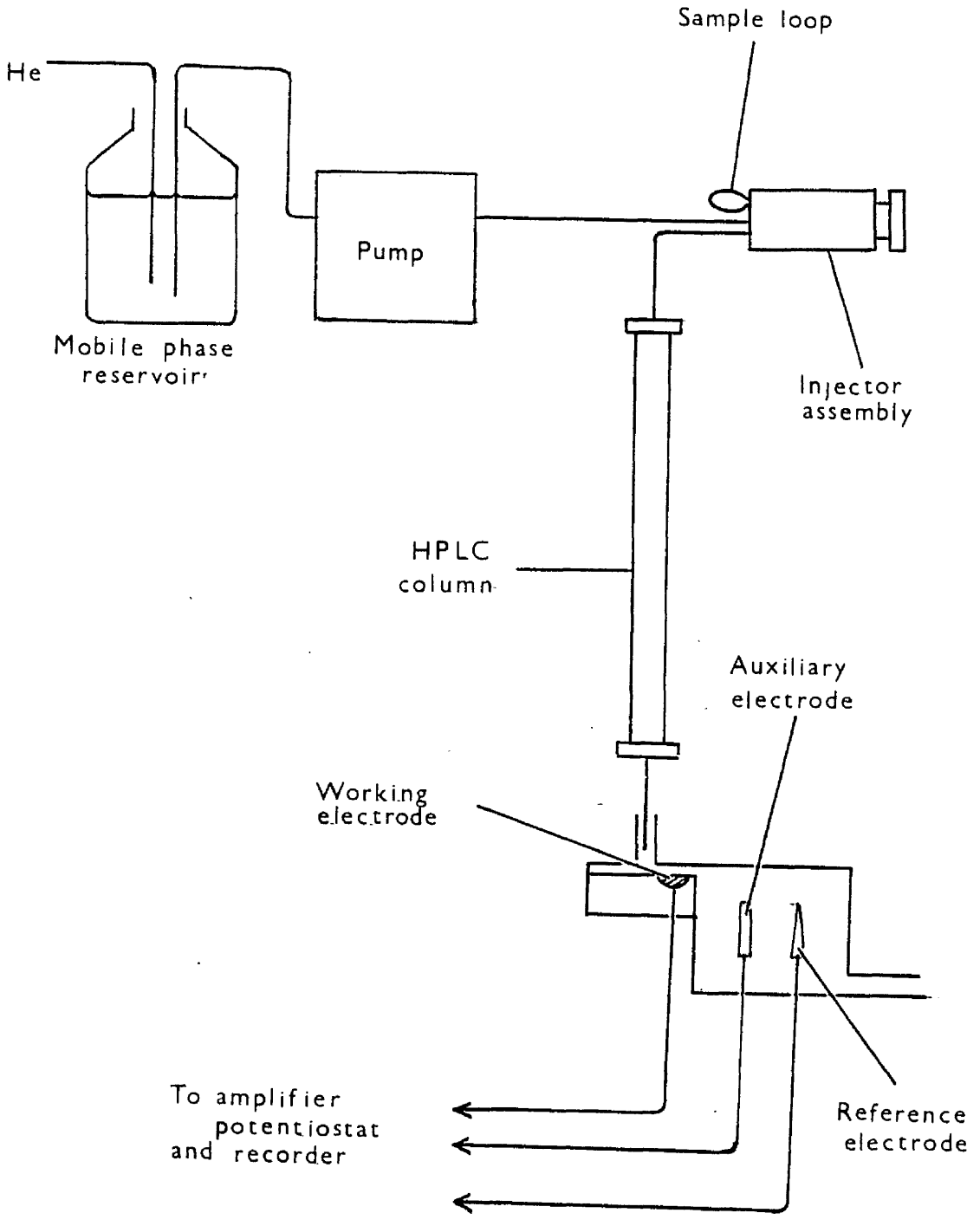


2.4.2 Assay of endogenous catecholamines from rat brain regions

Endogenous noradrenaline and dopamine were assayed by reversed phase (ion pair) high-performance liquid chromatography with electrochemical detection (HPLC-EC) using the apparatus shown in Figure 11.

Rat brain tissue (one hypothalamus, half-pair of striata or half-pair of nuclei accumbens) was weighed and homogenised in 200 µl 0.1 M - $HClO_4$ (containing 400 µl 1M-$NaHSO_3$/l) using a mini-drill (Expo Drills Ltd., London, U.K.) as an homogeniser with a teflon-tipped pestle, in microcentrifuge tubes (Eppendorf GmbH, Hamburg, F.R.G.). The tubes were centrifuged at 10,000 g for 2 minutes at 20°C in an Eppendorf 5412 bench centrifuge and the volume of liquid in each tube made up to 400 µl. Samples were stored at -20°C prior to assay. After thawing, aliquots of supernatant were injected onto a Partisil-10 ODS column (Whatman Ltd., 25 cm x 4.6 mm i.d.) via a six port rotary injection valve (Model 7120, Rheodyne Instruments Ltd., U.S.A.) fitted with a 100 µl sample loop. Separation of catecholamines was achieved using ion-pair, reversed phase chromatography (Knox and Jurand, 1976) with a mobile phase of 0.1M-KH_2PO_4 (pH 3.5), 0.2mM-sodium octyl sulphonate, 0.04 mM EDTA and 5-10% (V/V) methanol. The mobile phase was filtered through Sartorius filters (0.45 µm pore size) before use, degassed by helium and delivered at a constant flow rate of 1 ml/min using an LC3-XP pump (Pye Unicam Ltd., Cambridge, U.K.). At the start of the project, catecholamines were measured using a home made electrochemical detector cell and controller unit. The detector cell consisted of a carbon-paste working electrode (silicon-grease based carbon paste, CP-Si, Bio-Analytical Systems Inc), Ag/AgCl reference electrode and platinum auxiliary electrode. The operating potential of this system was 0.55V and the sensitivity of the controller

Figure 11 : HPLC-EC System



fixed at about 50nA/V.

Later assays for catecholamines were carried out using a TL-5 electrochemical detector cell (consisting of a glassy-carbon working electrode, Ag/AgCl reference electrode and a platinum auxiliary electrode) and a LC-4 Controller Unit (Bio-Analytical Systems Inc.). The operating potential was 0.55V and the current produced by oxidation of catecholamines was transduced by the LC-4 Controller Unit into a peak on a Philips PM 8251 single pen chart recorder (Philips Ltd.). The retention time of noradrenaline was about 6 minutes and that of dopamine about 12 minutes (Figure 12). Peak heights of samples were compared with the peak heights produced by standard solutions of noradrenaline and dopamine (4-20 ng/100 µl in 0.1M - $HClO_4$) and the unknown concentrations calculated from the calibration curve for each catecholamine (Figure 13). A linear relationship between catecholamine content and peak height was obtained up to at least 20 ng catecholamine.

Figure 12 : Elution of dopamine and noradrenaline from reversed-phase HPLC column

Sensitivity 50nA/V

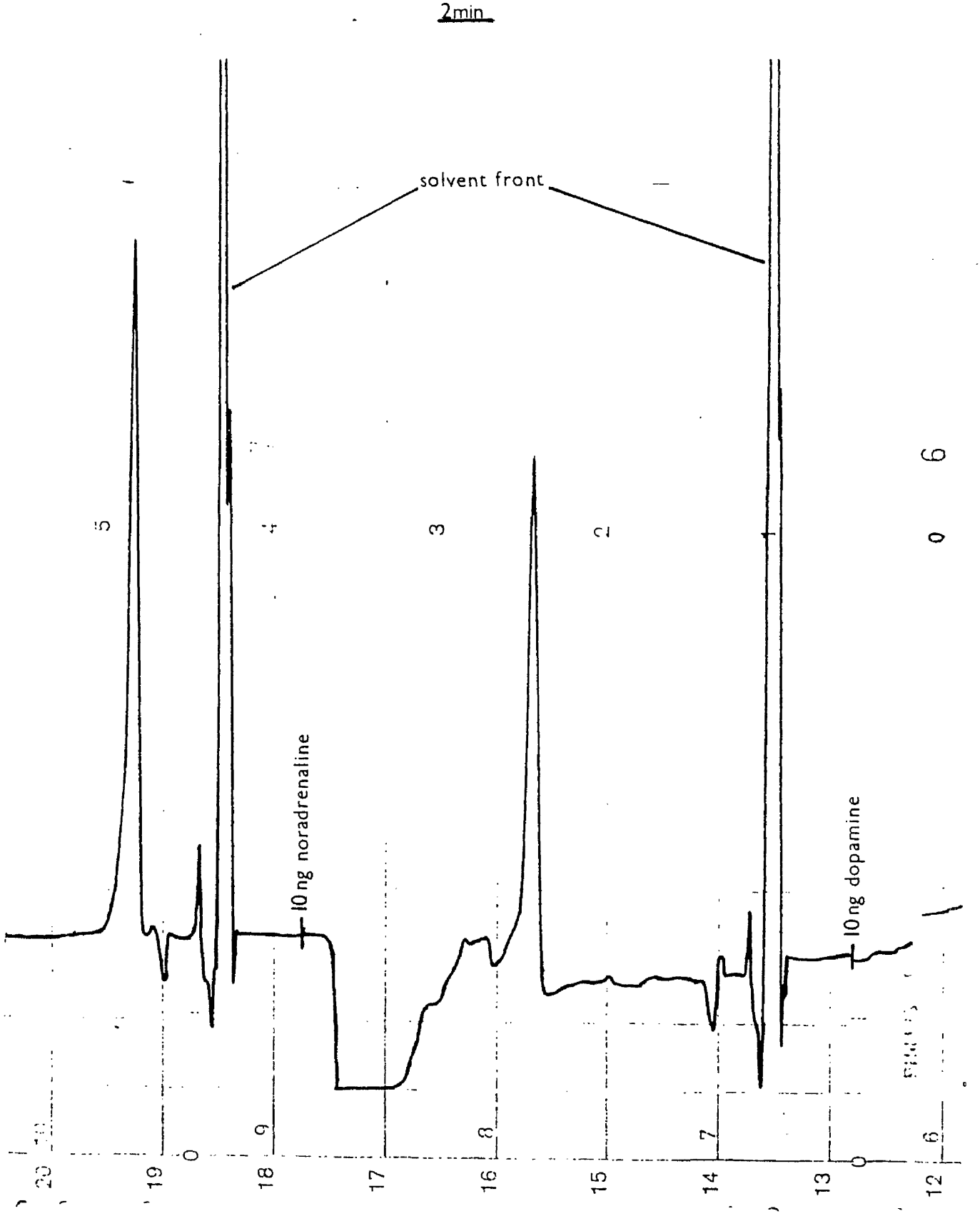
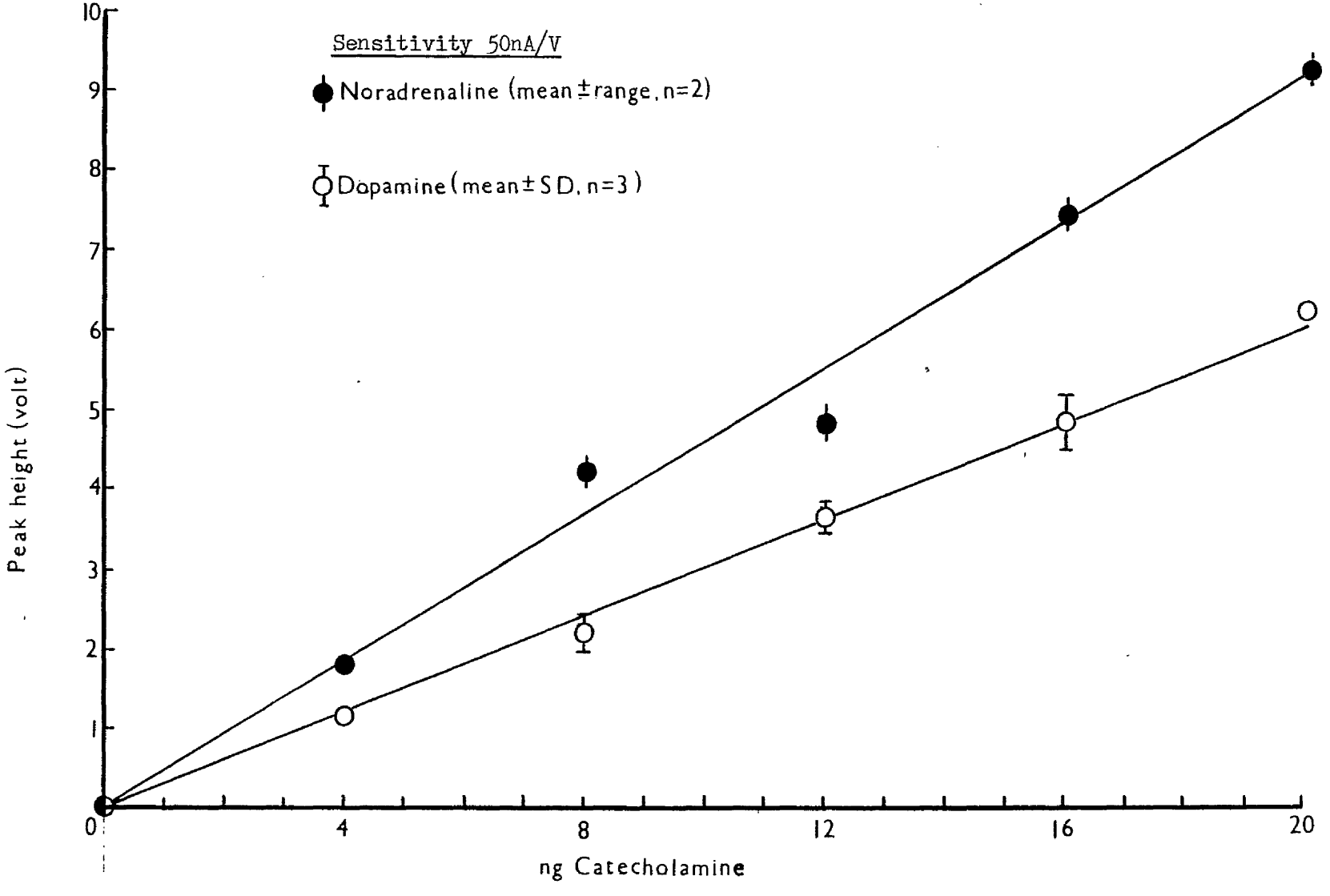


Figure 13 : Calibration Curve for Noradrenaline and Dopamine Detection by the Electrochemical Detector



2.4.3. Release of endogenous dopamine

Six pairs of rat nuclei accumbens (about 65 mg wet weight) were chopped into tissue cubes and suspended in 3.5 ml of 20 mM-TRIS buffered saline, pH 7.4, containing 5 mM-KCl . 500 µl of this suspension was added to 500 µl 20 mM-TRIS buffered saline, pH 7.4 in a glass bijou (control) and 2,500 µl of suspension was added to 500 µl TRIS buffered saline with 300 mM-KCl in a duplicate glass bijou (K^+). 500 ul aliquots were removed from the "K^+" bijou after 0, 5, 10, 20 and 30 minutes incubation at 37°C and a 500 µl aliquot was taken from the "control" bijou at 30 minutes. Between samples the tissue was kept suspended by sucking up and ejecting the suspension with the adjustable pipette used to take the samples.

The samples were placed in microcentrifuge tubes (Eppendorf) and immediately centrifuged at 10,000 g for 2 minutes at 20°C in the Eppendorf 5412 bench centrifuge. 400 µl of the supernatant was transferred to another microcentrifuge tube and the remaining supernatant removed by aspiration.

The pellets were homogenised in 100 µl 0.1M-$HClO_4$ (containing 400 µl 1M-$NaHSO_3$/l) using a mini-drill as homogeniser (Expo Drills). The volume of 0.1M-$HClO_4$ was made up to 500 µl and the tubes centrifuged at 10,000 g for 2 minutes at 20°C in the bench centrifuge (Eppendorf 5412). 100 µl aliquots of supernatant and pellet extract were assayed for dopamine using the HPLC-EC method.

Release was expressed as:

$$\frac{\text{ngDA in supernatant}}{\text{ngDA in supernatant + ngDA in pellet}} \times 100\%$$

$$= \frac{S}{S + P} \times 100\%$$

= Percentage release

Percentage release was plotted against time.

2.4.4 Release of exogenous $[^3H]$- dopamine

The release of $[^3H]$ - dopamine was followed in three ways:

The filtration assay involved incubating tissue in liquid filtration cartridges (Millipore S.A., Molsheim, France) for both uptake and release and terminating the reaction by using air to force the liquid through the nitrocellulose filter supporting the tissue.

In the perfusion assay the tissue, pre-loaded with $[^3H]$ - dopamine, was continuously bathed in incubation medium and samples of perfusate collected in scintillation vials.

In the centrifugation-terminated assay, release was allowed to take place in microcentrifuge tubes and was terminated by centrifugation. Supernatant was removed from each tube and radioactivity determined by liquid scintillation counting.

These different assay procedures were all applied to the tissue-cube preparation. Only the centrifugation-terminated assay was applied to the crude synaptosome preparation. The details of each assay are given below.

2.4.4.1 Filtration assay

200 µl aliquots of nucleus accumbens tissue cube suspension (5 pairs of nuclei accumbens/ml) in 5mM-HEPES buffered saline, pH 7.4 (containing 5mM-KCl and 10µg bacitracin) were placed on nitrocellulose filters (Schleicher and Schüll GmbH, Dassel, F.R.G., pore diameter 0.2 µm, filter diameter 13 mm) in plastic liquid filtration cartridges (Millipore).

The saline was forced through the filter by air and replaced by 200 µl of the same solution containing 2µCi 7,8- $[^3H]$ - dopamine (2.3×10^{-7}M dopamine). The tissue was incubated for 30 minutes at 20°C and uptake was terminated by using air to force the incubation medium through the filter. The tissue was washed three times with 5 ml of saline.

The tissue was incubated for 20 minutes at 20°C with 200 µl of 5 mM-HEPES buffered saline, pH 7.4 containing 5 mM-KCl and 50 µg bacitracin/ml ± 10^{-3}M TRH. Release of [^{3}H] - dopamine was terminated by using air to force the solution through the filter into scintillation vials, (Sterilin Ltd., Teddington, Middlesex, U.K.) and radioactivity determined by liquid scintillation spectrometry. Pellets of tissue were placed with the filters in scintillation vials for determination of radioactivity.

Release was expressed as:

$$\frac{\text{Counts per minuts (CPM) in supernatant}}{\text{CPM in supernatant + CPM in pellet}} \times 100\%$$

$$= \frac{S}{S+P} \times 100\%$$

2.4.4.2 Perfusion assay

A suspension of tissue cubes from four pairs of nuclei accumbens (40-50 mg wet weight) in 400 µl 20 mM-TRIS buffered saline, pH 7.4 containing 5mM-KCl and 20 ug bacitracin were incubated in a test-tube (Corex Ltd) with 4 µCi [^{3}H]-dopamine (2.3 x 10^{-7}M dopamine) at 37°C for 30 minutes with shaking.

Following incubation, 200 µl of suspension was placed in two liquid filtration cartridges (Millipore) with nitrocellulose filters (pore size 0.2 µm, Schleicher and Schüll) and the solution forced through the filters by air.

The cartridges were connected to a peristaltic pump (Technicon Ltd., London, U.K.) and immersed in a waterbath at 37°C. The tissue perfused at a rate of 0.5 ml/minute with 20 mM-TRIS buffered saline, pH 7.4 or pH 6.5 containing 5 mM-KCl and 50 µg bacitracin/ml at 37°C pre-gassed with 95% O_2, 5% CO_2.

Initially, 0.5 ml samples of perfusate were collected in scintillation vials and radioactivity determined until a steady basal release was obtained. The saline perfusing the tissue in one of the cartridges was then changed for 20 mM-TRIS buffered saline, pH 7.4 or 6.5, containing 50 µg bacitracin/ml and 50 mM-KCl, 50 mM-KCl-plus-1mM-tyramine or 0.5mM-TRH. Perfusion with this saline continued for the duration of the experiment and samples were collected every minute in scintillation vials for determination of radioactivity.

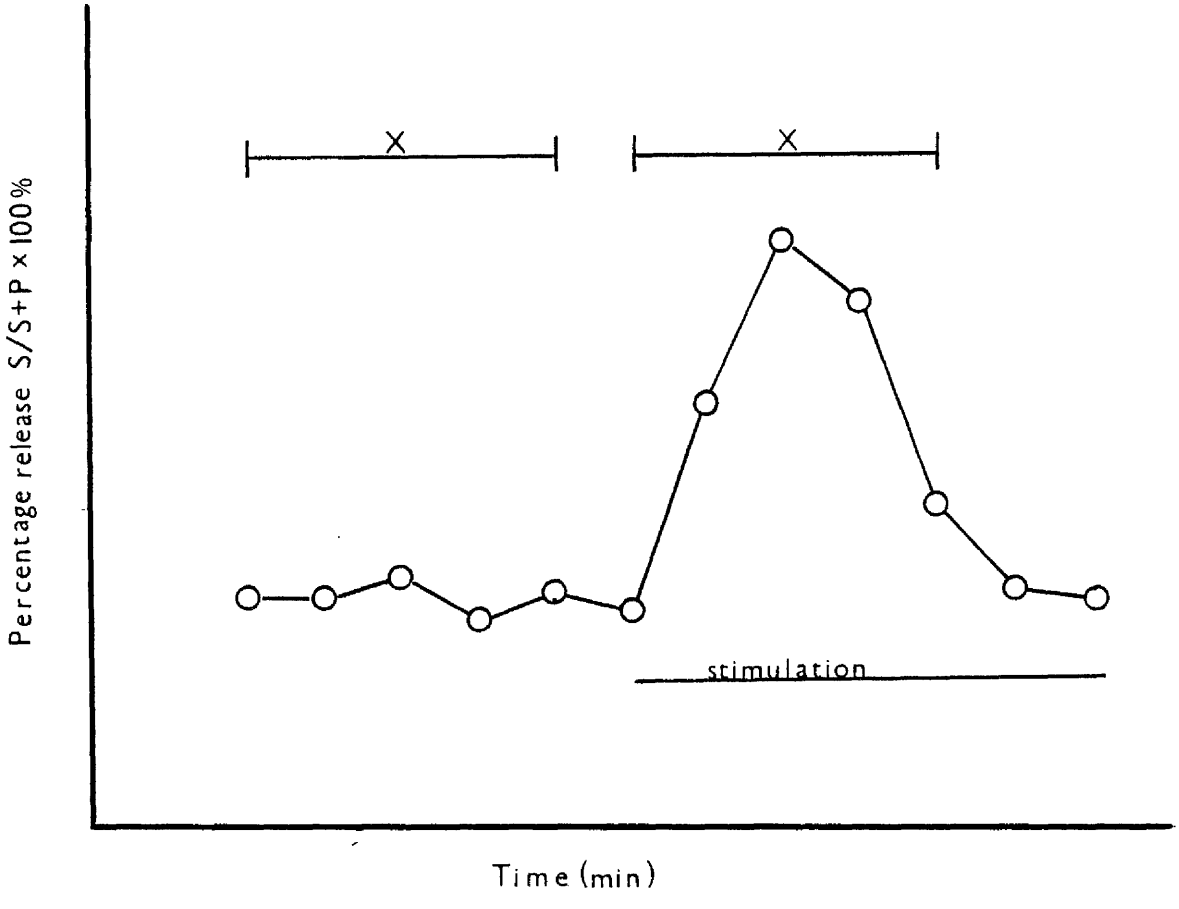
Tissue pellets on filters were placed in vials for determination of the radioactivity remaining in the tissue.

Release at each time-point was calculated by taking the CPM at that point to be CPM supernatant and the total of all the CPM in subsequent samples, plus the CPM in the final pellet, to be CPM pellet for that point.

Release was expressed as a percentage, as for the filtration assay. Stimulation of release due to treatment with 50 mM-KCl, 50 mM-KCl-plus-1mM tyramine or 0.5 mM-TRH was calculated by summing the percentage release over a period of basal release (SUM control) and over an equivalent period during treatment ($SUM_{stim.}$) (Figure 14) Percentage stimulation was calculated thus:

$$\frac{\text{SUM stim}}{\text{SUM control}} \times 100\%$$

Figure 14 : Calculation of percentage stimulation of release during perfusion assay (Example only)



SUMcontrol = sum of percentage release over period of x minutes before stimulation

SUMstim = sum of percentage release over equivalent period x minutes after the start of stimulation.

$$\text{Percentage stimulation} = \frac{\text{SUMstim}}{\text{SUMcontrol}} \times 100\%$$

2.4.4.3 Centrifugation - terminated assay

2.4.4.3a) Synaptosomes

200 µl of "S1" supernatant of nucleus accumbens or striatum were diluted 10-fold with 20 mM-TRIS buffered saline, pH 7.4, containing 5 mM-KCl and 50 µg bacitracin/ml, and incubated for 30 minutes with 4 µCi [^{3}H]-dopamine (4.5×10^{-8}M) at 37°C with shaking.

Uptake was terminated by centrifugation at 10,000 g for 15 minutes at 4°C in an MSE 18 centrifuge (Measuring and Scientific Equipment Ltd., London, U.K.). The pellet (P2 fraction) was resuspended in 5 ml 20 mM-TRIS buffered saline, pH 7.4, containing 5mM-KCl and 50 µg bacitracin/ml and recentrifuged at 10,000 g for 15 minutes at 4°C. The resulting pellet was washed three times by resuspension in 5 ml of the same saline followed by centrifugation at 10,000 g for 15 minutes at 4°C.

The washed pellet was resuspended in 3-4 ml of 20mM-TRIS buffered saline, pH 7.4 or 6.5, containing 5 mM-KCl and 50 µg bacitracin/ml at 0°C.

100 µl aliquots of this suspension (0.05-0.07 mg protein) were added to Eppendorf microcentrifuge tubes at 37°C containing 20 µl of the same saline, with 300 mM-KCl, 300 mM-KCl-plus-6 mM-tyramine or 6mM-TRH at pH 7.4 or 6.5.

Release was allowed to continue for up to 4 minutes and was terminated by immersion of the tubes in ice-water and addition of 500 µl ice-cold 20mM-TRIS buffered saline, pH 7.4 or 6.5 containing 5mM-KCl and 50 µg bacitracin/ml. The tubes were then centrifuged at 10,000 g for 2 minutes at 4°C in the Eppendorf bench centrifuge.

500 µl of supernatant were removed from each tube and added to scintillation vials (Sterilin Ltd). The remaining 100 µl were aspirated using an intravenous cannula (Portex Ltd., Hythe, U.K.).

Pellets were homogenised in 100 µl 0.1M-$HClO_4$ using a mini-drill (Expo Drills Ltd) with a teflon-tipped pestle as the homogeniser. The volume of 0.1M-$HClO_4$ was made up to 600 µl and the tubes centrifuged at 10,000 g for 2 minutes at 20°C in the Eppendorf bench centrifuge. 500 µl of the supernatant were removed to determine the radioactivity by scintillation spectrometry and the remaining 100 µl were aspirated using a cannula.

The pellets were dissolved overnight in 500 µl 2% SDS in 0.1M-NaOH and 100 µl aliquots of the solution taken for protein determination.

2.4.4.3 b) Tissue cubes

Tissue cubes of rat nucleus accumbens and striatum were incubated with 1 µCi [^{3}H]- dopamine/ml (2 pairs nuclei accumbens; 16-24 mg wet weight/ml or a half-pair striata; 20-30 mg wet weight/ml) in 20 mM-TRIS buffered saline, pH 7.4, containing 5mM-KCl and 50 µg bacitracin/ml, at 37°C with shaking for 30 minutes. Following incubation, uptake was terminated by centrifugation at 10,000 g for 10 minutes at 4°C in the MSE 18 centrifuge.

The tissue was washed three times by resuspension in 5 ml 20 mM-TRIS buffered saline, pH 7.4, containing 5mM-KCl and 50 µg bacitracin/ml and recentrifugation at 10,000 g for 10 minutes at 4°C in the MSE 18 centrifuge.

The tissue was resuspended in the same saline (600 µl/nucleus accumbens or 2.4 ml/striatum, final volume 9-12 ml) and dispensed in 600 µl aliquots into Eppendorf Microcentrifuge tubes at 0°C. The tubes were centrifuged at 10,000 g for 2 minutes at 20°C in the Eppendorf bench centrifuge. Following centrifugation the supernatant was aspirated from each tube except for the zero-time incubations, from which 500 µl of supernatant was transferred to scintillation vials.

To the other tubes was added 600 µl 20mM-TRIS buffered saline, pH 7.4 or pH 6.5, containing 5mM-KCl (control) or 50 mM-KCl in the absence and presence of either 0.1mM-TRH or 0.1mM-CG3703 and 50 µg bacitracin/ml, pre-warmed to 37°C.

The tubes were incubated for 5 minutes at 37°C with frequent vortexing to suspend the tissue.

Release of [^{3}H]-dopamine was terminated by immersion of the tubes in ice-water followed by centrifugation at 10,000 g for 2 minutes at 20°C in the Eppendorf bench centrifuge. 500 µl aliquots of supernatant were transferred to scintillation vials to determine the released radioactivity by scintillation spectrometry.

The pellets were treated to extract the [^{3}H]-dopamine remaining in the tissue as for the P2 preparation. The volume of 0.1M-$HClO_4$ was made up to 600 µl with 0.1 M-$HClO_4$ and the tubes centrifuged at 10,000 g for 2 minutes at 20°C. 500 µl aliquots of the supernatant were transferred to scintillation vials to determine the retained radioactivity by scintillation spectrometry.

The pellets were dissolved overnight in 500 µl 2% (w/v) SDS in 0.1MNaOH and 100 µl aliquots taken for protein determination.

2.4.4.4 Ca^{2+}-dependence of K^{+} stimulation of release of dopamine

a) Tissue cube preparation

Six rat striata were chopped individually (50-60 mg wet weight) into tissue cubes and incubated in 1 ml 20 mM-TRIS buffered saline, pH 7.4 containing 5mM-KCl, 1 μ Ci [^{3}H] -dopamine and either 2mM-EGTA or 0.5mM - 2.5 mM-$CaCl_2$ at 37°C with shaking for 30 minutes.
The procedure following uptake was the same as the routine assay for dopamine release terminated by centrifugation.

b) Synaptosome (P2) preparation

200 μl aliquots of S1 supernatant (1.7 mg protein) from 4 pairs of rat striata were diluted with 1.8 ml of 20mM-TRIS buffered saline, pH 7.4 containing 5mM-KCl, 4 μCi [^{3}H] -dopamine and either 2mM EGTA or 0.5mM-2.5mM-$CaCl_2$ and incubated at 37°C with shaking for 30 minutes. The procedure following uptake was the same as the routine assay for dopamine release terminated by centrifugation.

Tissue from each incubation was kept in the same $CaCl_2$ concentration throughout. 50mM-KCl was used to stimulate release. [^{3}H] -dopamine released was determined by liquid scintillation spectrometry. Percentage release was plotted against $CaCl_2$ concentration.

2.4.5 Acetylcholine release assay

Tissue cubes of nucleus accumbens or septum (90-120 mg wet weight) were incubated in 20mM-TRIS buffered saline pH 7.4 (50 mg tissue/ml) containing 5mM-KCl, 50 µg bacitracin/ml and 0.1mM-physostigimine with [^{3}H]-choline chloride (10 µCi/ml) in a 10 ml test tube (Corex Ltd) at 37°C with shaking for 30 minutes.

Termination of uptake and the washing procedures were the same as those used for the loading of tissue cubes with dopamine.

Conditions of release were the same as those used in the release of dopamine from tissue cubes and the assay was terminated by centrifugation at 10,000 g for 2 minutes at 20°C in the Eppendorf bench centrifuge. The proportions of acetylcholine and choline in the supernatant and pellet fractions were determined as follows:

100 µl aliquots of supernatant were placed in scintillation vials and the total radioactivity measured by liquid scintillation spectrometry

400 µl aliquots of the 500 µl remaining in each tube were transferred to other microcentrifuge tubes and freeze-dried overnight.

After freeze-drying the residue in the tubes was dissolved in 50 µl methanol containing 10mM-Ch and 10mM-ACh as markers. The proportion of radioactivity in 10 µl aliquots was determined following chromatography on cellulose TLC plates (Merck, without fluorescent indicator), which were developed for about 6 hours in butan-1-ol/water/ethanol/glacial acetic acid solvent (8 : 3 : 2 : 1 by volume). The plates were dried under warm air and sprayed with freshly prepared iodoplatinate reagent, made by mixing 20 volumes of 4.5% (w/v) KI in ethanol/water (1 : 1 v/v) with 1 volume of 5% (v/v) chloroplatinic acid, to locate the marker Ch and ACh.

The regions of the plate corresponding to marker Ch and ACh were transferred to scintillation vials for determination of radioactivity by liquid scintillation spectrometry. 3 ml of Toluene PPO/Triton X-100 scintillation fluid was added to each vial followed by 1.4 ml of distilled water to produce a gel. The vials were shaken vigorously to suspend the particles of cellulose in the gel.

The pellets were homogenised in 150 µl of methanol, containing 10mM-Ch 10mM-ACh as markers, using a mini-drill (Expo Drills Ltd) with a teflon-tipped pestle as homogeniser and were stored at -20°C for 30 minutes. The tubes were centrifuged at 10,000g for 2 minutes at 20°C and 100 µl of the supernatant taken for liquid scintillation spectrometry. The proportions of Ch and ACh in 10 µl aliquots of the pellet extract were determined following chromatography on cellulose TLC plates as described above.

The CPM in the aliquots of supernatant and pellet extract not subjected to chromatography were corrected to their original volumes thus:

$$\text{CPM}_{\text{Supernatant}}^{\text{TOTAL}} = \text{CPM}_{\text{Supernatant}}^{\text{ALIQUOT}} \times 6$$

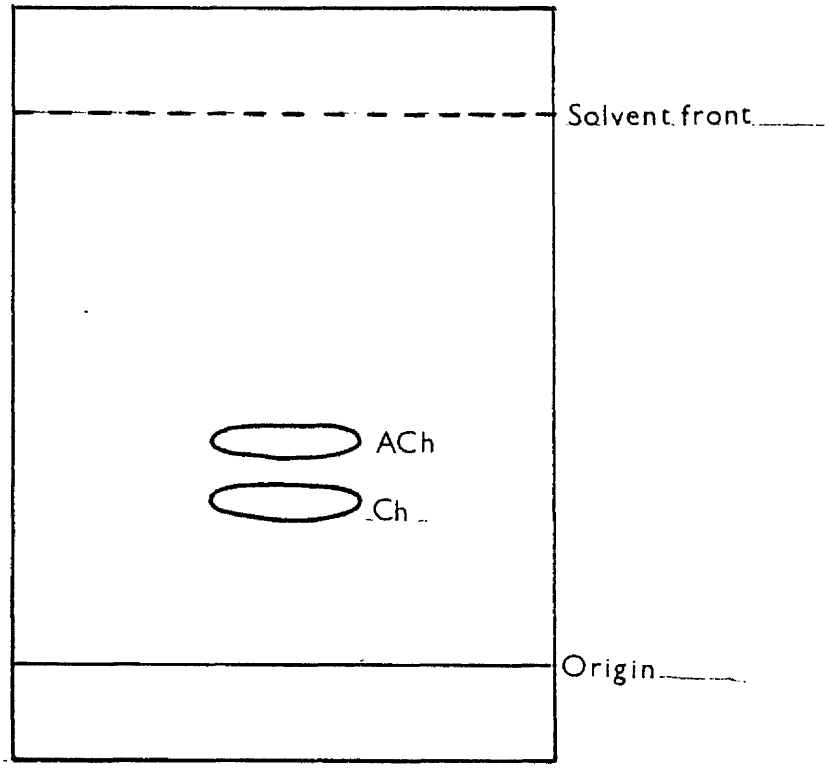
and

$$\text{CPM}_{\text{Pellet}}^{\text{TOTAL}} = \text{CPM}_{\text{Pellet}}^{\text{ALIQUOT}} \times 1.5$$

The CPM contributed to the $\text{CPM}^{\text{TOTAL}}$ in each fraction by Ch and ACh was determined using the proportions found on the TLC plates so that ACh release could be related to the total ACh content of the tissue and Ch release could be related to the total Ch content of the tissue.

Figure 15 shows a typical separation of Ch and ACh on the cellulose TLC plates in butan-1-ol/water/ethanol/glacial acetic acid solvent.

Figure 15 : Separation of Choline and Acetylcholine on Cellulose TLC plates



Solvent : Butan-1-ol/water/ethanol/glacial acetic acid (8:3:2:1, by volume)

Rf values	Choline (Ch)	0.33 ± 0.01 (n=8)
	Acetylcholine (ACh)	0.43 ± 0.02 (n=8)

2.5 Scintillation spectrometry (Turner, 1969)

Supernatant (400-500 µl) or pellet extract (100-500 µl) containing $[^3H]$-dopamine or $[^3H]$-choline or acetylcholine was placed in polythene insert-type scintillation vials (Sterilin Ltd., Teddington, Middlesex, U.K.). 3 ml of scintillation fluid, toluene/Triton X-100 (2 : 1 v/v) containing 0.8% (w/v) diphenyloxazole (PPO) was added and the vials capped and shaken to disperse the samples.

Radioactivity in each vial was determined using a liquid scintillation counter (Packard Tri-Carb 3255, Hewlett Packard Inc., Illinois, U.S.A. ; Beckman LS 6800, Beckman Instruments Ltd., High Wycombe, Bucks., U.K. ; or Intertechnique SL 4000 PG 4000, Intertechnique Ltd., Uxbridge, Middlesex, U.K.). Counts were recorded for 4 minutes with no subtraction of background and expressed as the mean of the counts per minuts (CPM). Counting efficiency was about 30% for ^3H and about 50% for ^{14}C (Table 4).

Table 4

Efficiency of counting of ^{3}H and ^{14}C

Isotope	μCi Counted	DPM	CPM	Efficiency CPM/DPM X 100%
^{3}H	10^{-2}	22200	6771	30.5
	10^{-2}	22200	6882	31.0
	10^{-2}	22200	7659	34.5
	10^{-2}	22200	7770	35.0
	10^{-2}	22200	7437	33.5
	10^{-2}	22200	7881	35.5
	10^{-2}	22200	6438	29.0
	10^{-2}	22200	5994	27.0
				Mean $\pm$ SD
				32.0 $\pm$ 3.1
^{14}C	2×10^{-3}	4440	2124	47.8
	2×10^{-3}	4440	2183	49.2
	2×10^{-3}	4440	2142	48.2
	2×10^{-3}	4440	2124	47.8
	2×10^{-4}	444	207	45.9
	2×10^{-4}	444	216	48.6
				Mean $\pm$ SD
				47.9 $\pm$ 1.1

2.6 Preparation of quench curve

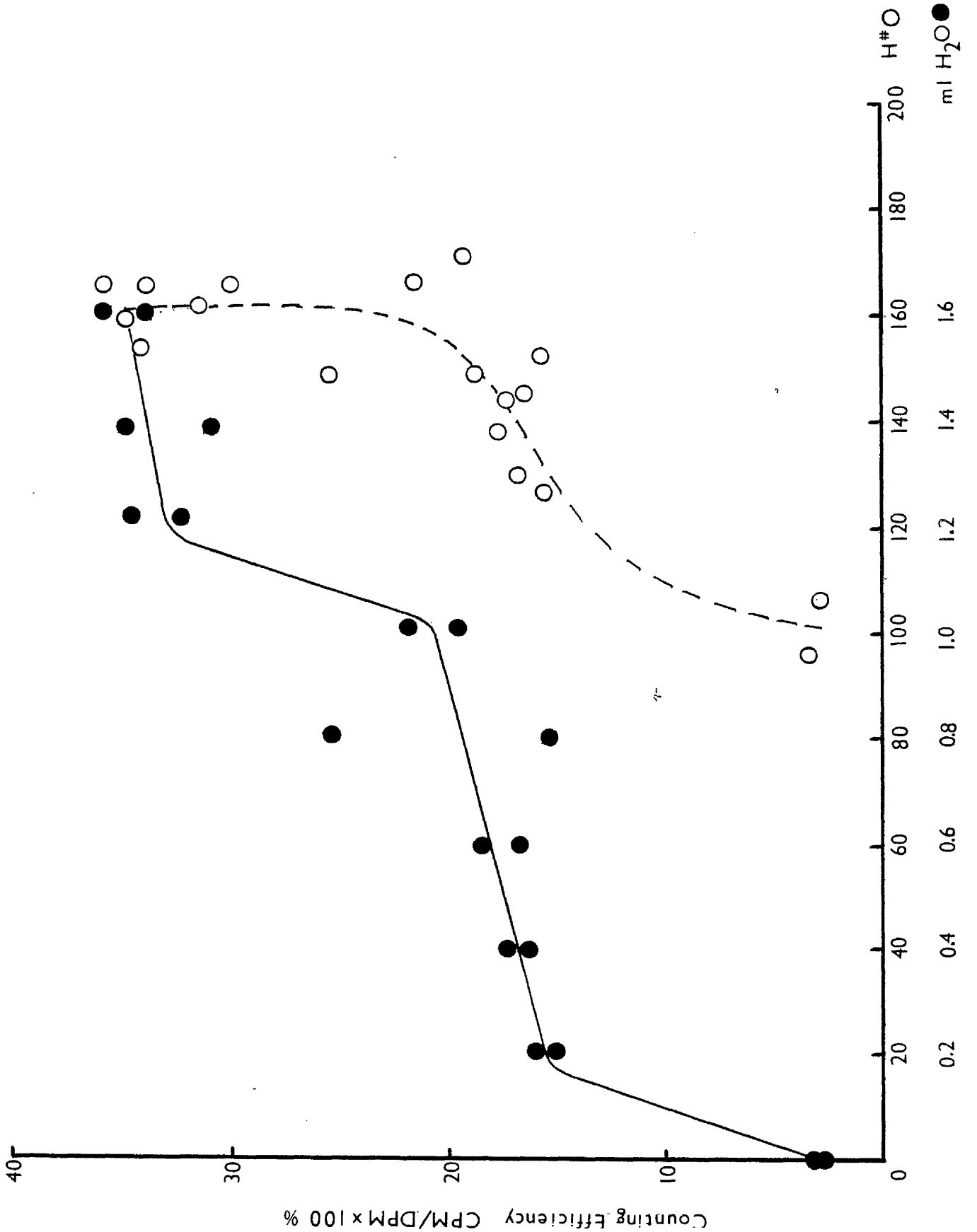
Cellulose TLC plates, without fluorescent indicator (Merck) were loaded with 18 x 50 µl samples of $[^3H]$-choline (2.5×10^4 DPM) in methanol containing choline (10mM) and acetylcholine (10mM) as markers. The plates were developed for about 6 hours in butan-1-ol/water/ethanol/glacial acetic acid solvent (8 : 3 : 2 : 1 by volume) and dried under warm air. The dry plates were sprayed with iodoplatinate reagent and the region of the chromatogram corresponding to Ch and ACh transferred into scintillation vials. 3 ml toluene-PPO/Triton X-100 (2 : 1 v/v) scintillation fluid was added to each vial with up to 1.6 ml distilled water. The vials were shaken vigorously to suspend the particles of cellulose and radioactivity determined by liquid scintillation spectrometry. Quenching was expressed as an H-number (H #), which is the difference between the maximum pulse height produced in each sample by the γ-emissions of an external standard (^{137}Cs) and the maximum pulse height, caused by the same external standard, in an unquenched sample, a value preprogrammed into the scintillation counter, on an arbitrary scale of 0 to 1000.

Counting efficiency was calculated for each sample as:

$$\frac{CPM}{DPM} \quad X \quad 100\%$$

Counting efficiency was plotted against H-number and ml H_2O as shown in Figure 16.

Figure 16 : Determination of efficiency of scintillation counting of $[^3H]$-choline on particles of cellulose suspended in a toluene-Triton X-100/water gel



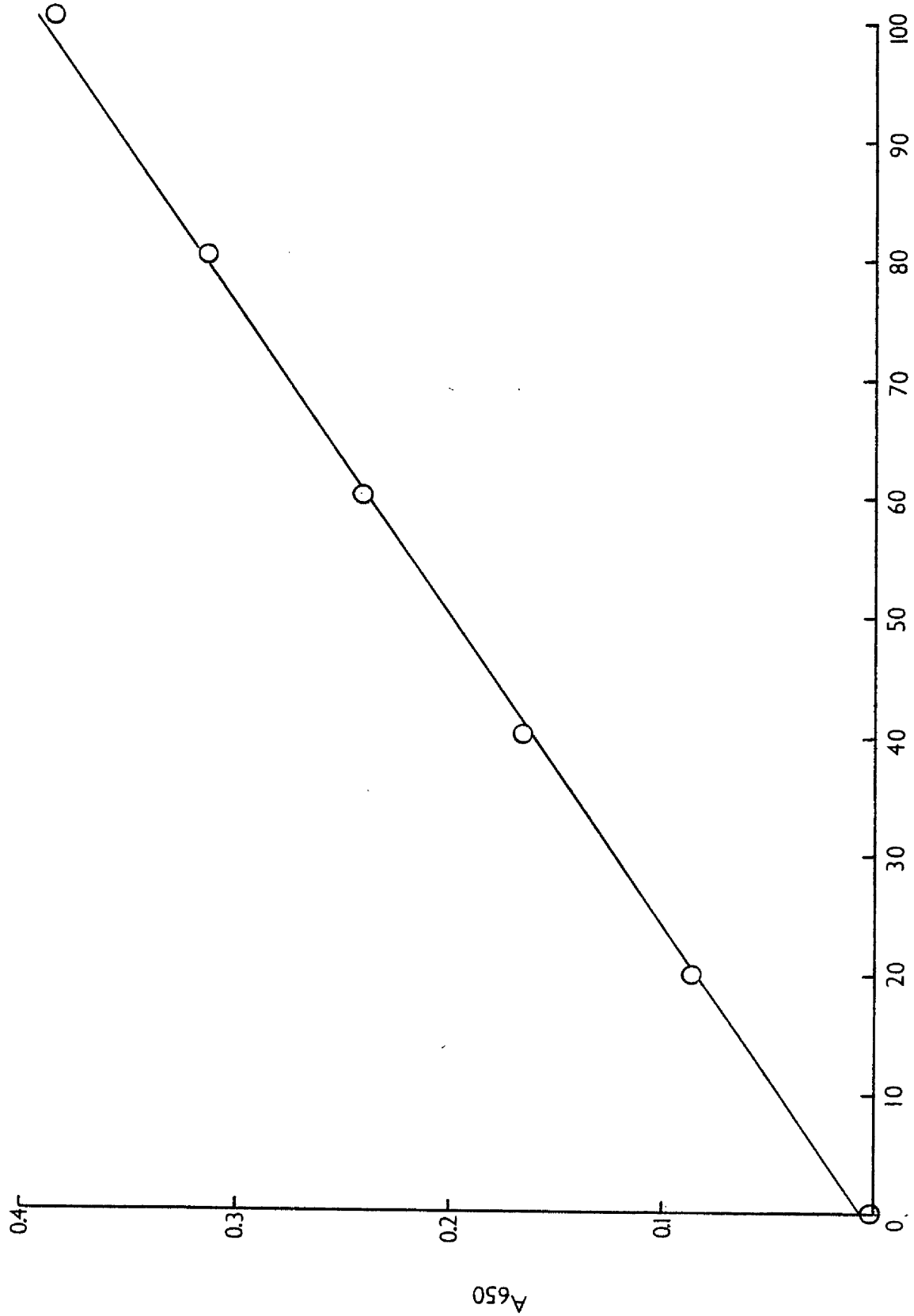
2.7 Protein estimation

Protein was estimated in tissue pellets by a modification of the method of Lowry et al (1951).

Pellets of tissue (4-6 mg wet weight) were dissolved in 500 µl of 2% (w/v) SDS in 0.1M NaOH overnight. 100 µl aliquots of solution were taken in duplicate for protein estimation and placed in test tubes. To each tube was added 3 ml of solution C (prepared by mixing 50 ml of 2% (w/v) anhydrous Na_2CO_3 and 0.2% (w/v) SDS in 0.1M NaOH (Solution A) with 1 ml of 0.5% $CuSO_4\ 5H_2O$ in 1% (w/v) trisodium citrate) (Solution B) and, after 10 minutes, 0.5 ml of Folin-Ciocalteau reagent, diluted 1 : 2 (v/v) with water.

The absorbance (A_{650}) of the samples was determined using a Beckman Dual Beam spectrophotometer (Beckman Ltd) after at least 30 minutes of colour development. Absorbance was read against a protein calibration curve (0-100 µg protein) using bovine serum albumin as a standard (Fig.17).

Figure 17 : Calibration Curve for Protein Estimation



2.8 Statistics

Statistical significance was determined by the Student t-test.

The factor t, the significance of deviation of a statistic from zero, was calculated by using the formulae:

$$S = \sqrt{\frac{(n_1 - 1)\ S_1^2 + (n_2 - 1)\ S_2^2}{(n_1 + n_2) - 2}}$$

and

$$t = \frac{\bar{x}_1 - \bar{x}_2}{s\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

where

S = mean of standard deviations of populations 1 and 2

S_1, S_2 = standard deviation of population 1, 2

n_1, n_2 = number of values in population 1, 2

x_1, x_2 = mean of values in population 1, 2

$(n_1 + n_2)-2$ = degrees of freedom

The probability of an effect happening by chance was found by entering the t-value into the t-distribution significance table (Pollard, 1978) at the required degree of freedom.

III RESULTS

Results

3.1 Characterisation of some regions of rat brain

Regions of rat brain corresponding to the nucleus accumbens, corpus striatum and hypothalamus were dissected as described in Materials and Methods. The wet weights of these regions, 12.29 $\pm$ 2.22 mg (n = 14) for the nucleus accumbens, 60.25 $\pm$ 13.80 mg (n = 18) for the corpus striatum and 67.80 $\pm$ 6.20 mg (n = 4) for the hypothalamus, were in agreement with the corresponding values found in the literature (Table 5).

Dopamine content of the nucleus accumbens, corpus striatum and hypothalamus respectively was: 6084.4 $\pm$ 896.9 ng/g (n = 8), 4499.2 $\pm$ 546.4 ng/g (n = 8) and 366.6 $\pm$ 84.0 ng/g (n = 4) and their noradrenaline content was 886.9 $\pm$ 457.9 ng/g (n = 8), 247.3 $\pm$ 64.6 ng/g (n = 8) and 2383.6 $\pm$ 96.0 ng/g (n = 4). These values are in agreement with the corresponding values found in the literature (Tables 6 and 7) and provide additional evidence that the correct brain regions were dissected.

3.2 Release of dopamine from in-vitro preparations of rat brain regions

3.2.1 Release of endogenous dopamine from tissue cubes of nucleus accumbens

Incubation of tissue cubes of rat nucleus accumbens in TRIS buffered saline, pH 7.4, containing 5mM-KCl, for 30 minutes at 37°C, caused an increase in the release of endogenous dopamine from 15.0% to 22.3%. Incubation in TRIS buffered saline, pH 7.4, containing 50mM-KCl, stimulated the release of dopamine from 15.0% to 30.0% in 10 minutes and to 38.3% in 30 minutes. (Figure 18).

This preliminary result showed that the release of dopamine during the linear portion of the time-course, in the presence of 50mM-KCl, was about 8 ng/500µl sample ($\simeq$ 1.6 ng/100 µl aliquot injected onto the HPLC column).

Table 5 : Weight of brain regions

Region	Weight (mg, mean ± SD)	Author
Nucleus	10.90 ± 0.40 n = 30	Wilk and Glick 1976
Accumbens	12.29 ± 2.22 n = 14	This study
	23.50 ± 1.10 n = 27	Horn et al 1974
Striatum	50.40 ± 2.30 n = 30	Wilk and Glick 1976
	60.25 ± 13.80 n = 18	This study
	102.0 ± 17.0 n = 20	Glowinski and Iversen 1966
Hypothalamus	67.80 ± 6.20 n = 4	This study
	110 ± 14 n =20	Glowinski and Iversen 1966

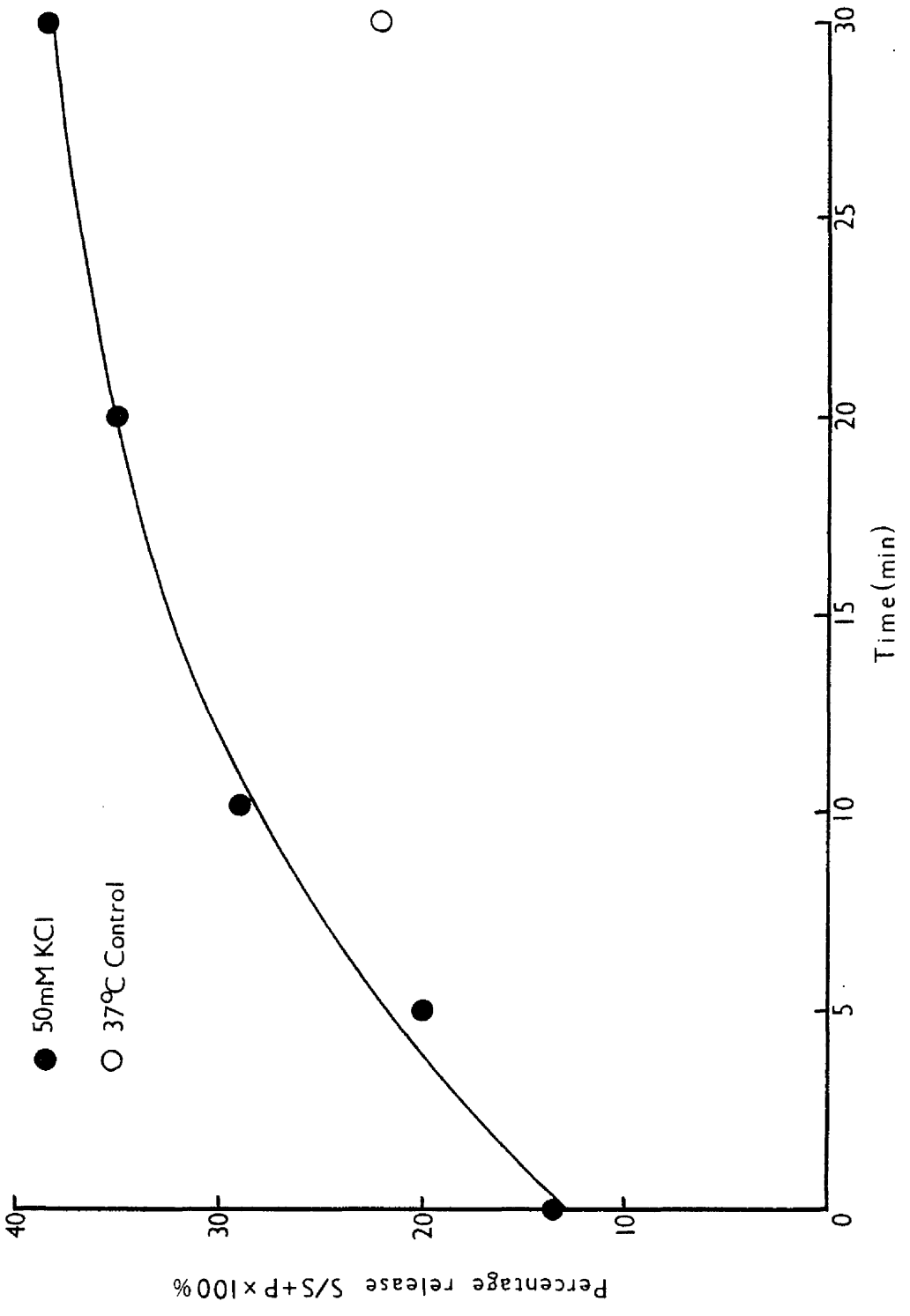
Table 6:DA Content of nucleus accumbens, striatum and hypothalamus

Region	ng DA/g wet wt. - SD			Author
Nucleus	4500	± 370	n = 3	Horn et al 1974
Accumbens	6084	± 896.9	n = 8	This study
	8420	± 519	n = 9	Oke et al 1978
Striatum	3800	± 360	n = 5	Metcalf 1974
	4230	± 50		Friedman et al 1973
	4499.2	± 546.4	n = 8	This study
	5470	± 1870	n =12	Gordon and Shellenberger 1973
	7112	± 1620	n =14	Shellenberger and Gordon 1971
	8070	± 920	n = 5	Coyle and Henry 1973
	11500	± 430	n =15	Shellenberger 1971
	15684	± 1419	n = 9	Oke et al 1978
	16782	± 386	n = 6	Holtzman 1974
Hypothalamus	260	± 20	n =15	Shellenberger 1971
	320	± 30	n = 6	Metcalf 1974
	366.6	± 84.0	n = 4	This study
	370	± 42	n = 9	Oke et al 1978
	580	± 100	n = 5	Coyle and Henry 1973

Table 7 : NA content of nucleus accumbens, striatum and hypothalamus

Region	ng NA/g wet wt. ± SD			Author
Nucleus accumbens	516	± 168	n = 9	Oke *et al* 1978
	886.9	± 457.9	n = 8	This study
Striatum	60	± 10	n =15	Metcalf 1974
	120	± 10	n = 15	Shellenberger 1971
	247.3	± 64.6	n = 8	This study
	250	± 30	n = 6	Glowinski and Iversen 1966
	300	± 90	n = 14	Shellenberger and Gordon 1971
	320	± 120	n = 12	Gordon and Shellenberger 1974
	350	± 200	n = 5	Coyle and Henry 1973
Hypothalamus	1070	± 110	n = 15	Shellenberger 1971
	1370	± 50	n = 60	Holtzman 1974
	1610	± 160	n = 9	Metcalf 1974
	1620	± 185	n = 9	Oke *et al* 1978
	1790	± 100	n = 6	Glowinski and Iversen 1966
	1840	± 20	n = 8	Friedman *et al* 1973
	1960	± 100	n = 5	Coyle and Henry 1973
	2383.6	± 96.0	n = 4	This study

Figure 18 : Time-course of release of endogenous dopamine from tissue cubes of rat nucleus accumbens in TRIS buffered saline, pH7.4 followed by sampling from a single incubation vessel.



This was too close to the limits of detection of the home-made electrochemical detector which was used at the beginning of this project to enable further work on release of endogenous dopamine to be carried out with confidence. It was therefore decided to concentrate on studying the release of (^{3}H)-dopamine from pre-loaded tissue preparations.

3.2.2 Metabolic viability of tissue cubes

The rate of oxygen uptake by tissue cubes of rat nucleus accumbens was not affected by the incubation and washing procedures used during the pre-loading of tissue with (^{3}H)-dopamine (Table 8). 50mM-KCl was found to stimulate (^{3}H)-dopamine release by 92% in the unwashed sample and 183% in the washed sample. The similarity between oxygen uptake before and after washing and the stimulation of O_2 uptake by 50 mM-KCl after washing suggests that the tissue was damaged very little by the pre-loading procedure.

The absolute values of O_2 uptake obtained are lower than those reported for rat cerebral cortex, tissue cubes by McIlwain and Buddle (1952), who found rates of uptake of about 1nmolO_2/min/mg tissue. The stimulation of O_2 uptake observed in the nucleus accumbens by 50 mM-KCl was about the same as that observed by McIlwain and Buddle on electrical stimulation of cerebral cortex tissue cubes.

3.2.3 Release of exogenous dopamine

a) Filtration assay

Previous work (Shapiro et al (1980)) suggested that TRH (10^{-3}M) stimulated the release of (^{3}H)-dopamine from P2 preparations of rat striatum. Thus, 10^{-3}M TRH was used in initial studies with tissue cubes of rat nucleus accumbens.

Table 8 : Effect of washing procedure on the metabolic rate of nucleus accumbens tissue cubes

	O_2 uptake n mol O_2/min/mg tissue			
	Basal		K^+ stimulated	
Wt. of tissue (mg)	Pre-wash	Post wash	Pre wash	Post wash
39.8	0.130		0.169	
37.2		0.139		0.448
38.0	0.146		0.289	
21.2		0.103		0.285
31.4	0.105		0.274	
34.4		0.107		0.250
Mean $\pm$ SD	0.127 ± 0.021	0.116 ± 0.020[a]*	0.244 ± 0.065	0.328 ± 0.105[b]*

* NS

a wrt pre-wash basal rate

b wrt pre-wash K^+ stimulated rate

TRH (10^{-3}M) had a variable effect on the release of (^{3}H)-dopamine. (Table 9).

The ratio: $\frac{\text{release in presence of TRH (T)}}{\text{release in absence of TRH (C)}}$

ranged from 0.84 to 1.55 between experiments.

Possible reasons for this variability are:

i) Inhomogeneity of the tissue preparations.

ii) Variations in the reuptake of released (^{3}H) dopamine during the assay.

iii) Variation in the extent of TRH degradation.

b) Perfusion assay

The perfusion assays were carried out in order to reduce possible variations in release caused by reuptake of the (^{3}H)-dopamine. The HEPES buffered saline was changed for 20 mM-TRIS buffered saline because Shapiro et al (1980) found a consistent effect of TRH in this saline.

Perfusion of rat nucleus accumbens tissue cubes with TRIS buffered saline, pH 7.4, containing 50mM-KCl, stimulated (^{3}H)-dopamine release. The peak produced was of shorter duration than the perfusion time with 50mM-KCl and the release returned to the baseline after 3 minutes (Figure 19, lower trace) 50mM-KCl stimulated (^{3}H)-dopamine release by about 50% (Table 10) stimulation of release was calculated as described in Materials and Methods.

Perfusion with 50mM-KCl-plus 1mM tyramine in TRIS buffered saline, pH 7.4 caused an immediate stimulation of (^{3}H)-dopamine release. The peak of release was about 4 x higher than that produced by 50 mM-KCl alone and took longer to return to the baseline although its decline began after the same period as for the 50 mM-KCl peak. (Figure 19, upper trace).

Table 9 : Effect of TRH (10^{-3}M) on the release of (^{3}H)-dopamine from tissue cubes of rat nucleus accumbens in HEPES buffered saline pH 7.4 using the filtration release assay.

Control			10^{-3}M TRH			
CPM in Supernatant	CPM in Pellet	$(S/S+P)\times 100\%$	CPM in Supernatant	CPM in Pellet	$(S/S+P)\times 100\%$	TRH / Control
5780	28243	16.99	3250	20875	13.47	
4512	33170	11.97	3499	18861	15.65	
5707	11940	32.34	5548	19001	22.60	
		20.43 ± 10.61			17.24 ± 4.77	0.84
51822	187943	21.61	55660	182202	23.40	
38048	286215	11.73	34326	241130	12.46	
57081	207021	21.61	68971	247413	21.80	
66489	184438	26.49	76093	172065	30.66	
		20.36 ± 6.20			22.08 ± 7.50	1.08
11965	30189	28.38	13152	24018	35.38	
16382	41874	28.12	14208	29060	32.84	
18157	35972	33.54	16425	33505	32.90	
		30.01 ± 3.06			33.71 ± 1.45	1.12
31239	163038	16.08	27236	96147	22.07	
24950	117416	17.56	33059	99546	24.93	
22934	165780	12.15	12574	40379	23.74	
		15.26 ± 2.79			23.58 ± 1.43 †	1.54
53970	175585	23.51	85315	173860	32.92	
47180	193053	19.64	69588	136915	33.70	
44007	165096	21.05	75645	115876	39.50	
79478	99362	44.44	71942	42774	62.71	
		27.16 ± 11.63			42.20 ± 13.98	1.55

† $p < 0.025$ w.r.t 37°C Control

Figure 19 : Effect of 50mMKCl and 50mMKCl-plus-1mMtyramine on the release of ^{3}H-dopamine from a tissue cube preparation of rat nucleus accumbens using the perfusion assay in TRIS buffered saline, pH7.4.

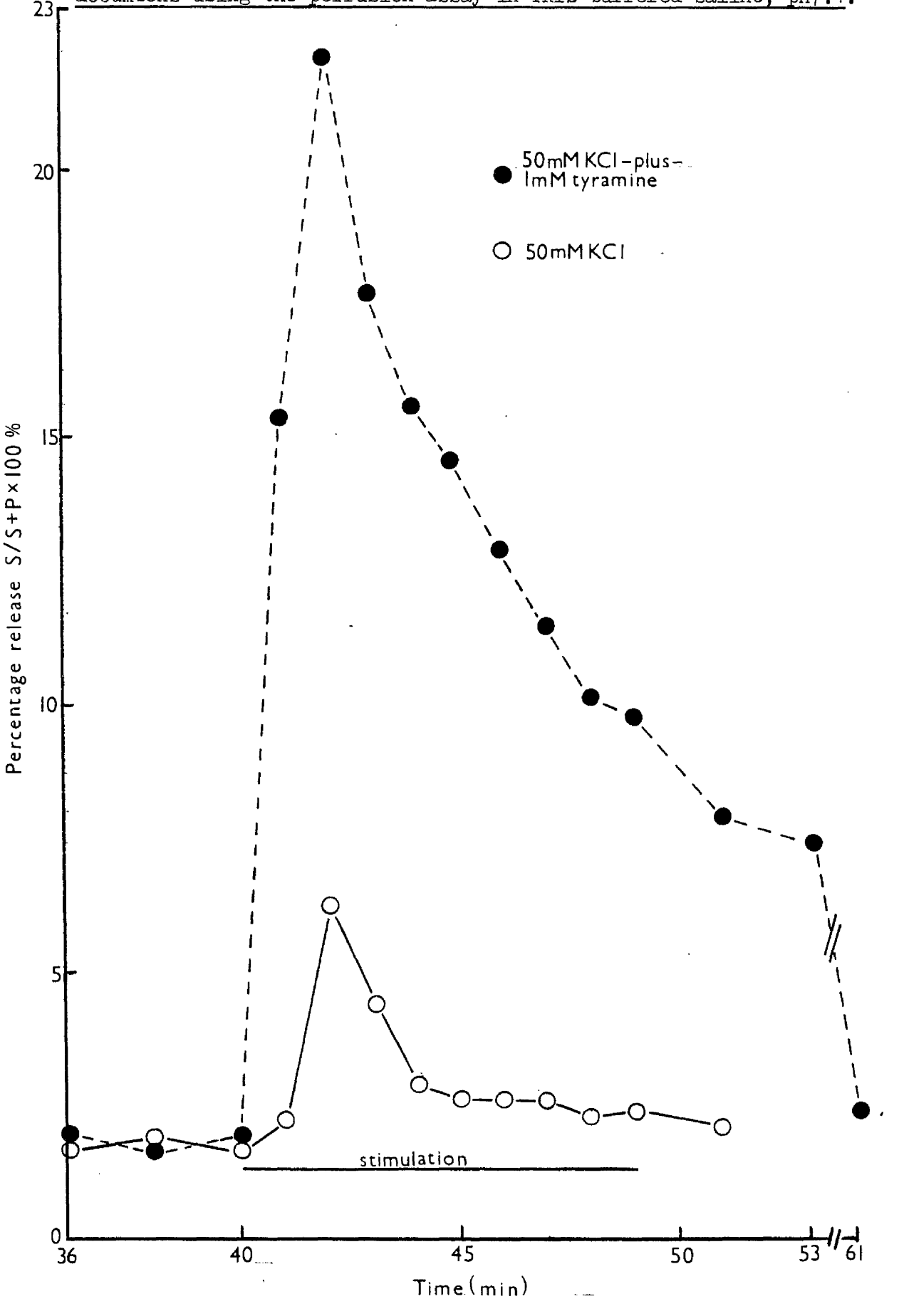


Table 10 : Stimulation of release of ^{3}H-dopamine from tissue cubes of rat nucleus accumbens by 50mM KCl in TRIS buffered saline, pH 7.4 as followed by the perfusion assay.

Summed Percentage Release $\frac{S}{S+P} \times 100\%$		Ratio
-K+ Baseline	+K+ Peak	Peak/Baseline
11.0	19.2	1.74
9.8	13.7	1.40
8.0	10.0	1.25
9.6	14.3	1.48
11.0	17.9	1.63
11.7	19.4	1.66
13.9	21.2	1.52
22.3	33.9	1.52
	Mean $\pm$ SD	1.52 ± 0.15

Perfusion with 50 mM-KCl-plus-1mM tyramine caused a consistent stimulation of release of between 300% and 800% basal release of (^{3}H)-dopamine (Table 11).

Kerwin and Pycock (1979) found a stimulation of (^{3}H)-dopamine release from tissue cubes of rat nucleus accumbens using 0.025mM-0.1mM TRH. In an attempt to produce a consistent stimulation, the mechanism of which could then be studied, 0.5 mM TRH was used in these assays.

No stimulation of (^{3}H)-dopamine release was observed in the presence of 0.5mM TRH at pH 7.4.

At pH 6.5, however, stimulation of (^{3}H)-dopamine release by 0.5 mM-TRH was observed (Figure 20).

Subsequent studies showed that 0.5mM TRH caused a consistent stimulation of (^{3}H)-dopamine release of about 20% at pH 6.5 (Table 12). This finding agreed with previous work by Shapiro et al (1980), who reported a greater stimulation of release of (^{3}H)-dopamine from striatal P2 preparations at pH 6.6 than at pH 7.2.

TRIS (2-amino-2 hydroxymethylpropane-1,3-diol) has a pKa of 8.6 so its buffering capacity below pH 7.0 is very low. It was therefore necessary to change the buffer system before studying the effect of TRH on (^{3}H)-dopamine release over a pH range of 6.0-7.4. It was not possible to use phosphate buffers owing to the requirement for Ca^{2+} ions in the perfusion medium so imidazole (pKa 7.0) and HEPES (pKa 7.55) were used.

When 20mM-imidazole, pH 7.4, replaced the TRIS, a slight inhibition, approximately 20%, of (^{3}H)-dopamine release was observed. At pH 6.5, apart from in one experiment where 75% stimulation of release was observed, no effect of TRH on (^{3}H)-dopamine was observed. (Table 13).

Table 11 : Stimulation of release of ^{3}H-dopamine from tissue cubes of rat nucleus accumbens by 50 mM KCl-plus-1mM-Tyramine in TRIS buffered saline, pH 7.4 as followed by perfusion assay.

Summed Percentage Release $\frac{S \times 100\%}{S+P}$		Ratio
Baseline	Peak	Peak/Baseline
21.5	58.3	2.71
12.6	100.9	8.00
6.4	20.4	3.18
	Mean$\pm$SD	4.63$\pm$2.90

Figure 20 : Effect of TRH(5×10^{-4}M) on the release of ^{3}H-dopamine from a tissue cube preparation of rat nucleus accumbens using the perfusion assay in TRIS buffered saline, pH7.4 and pH6.5.

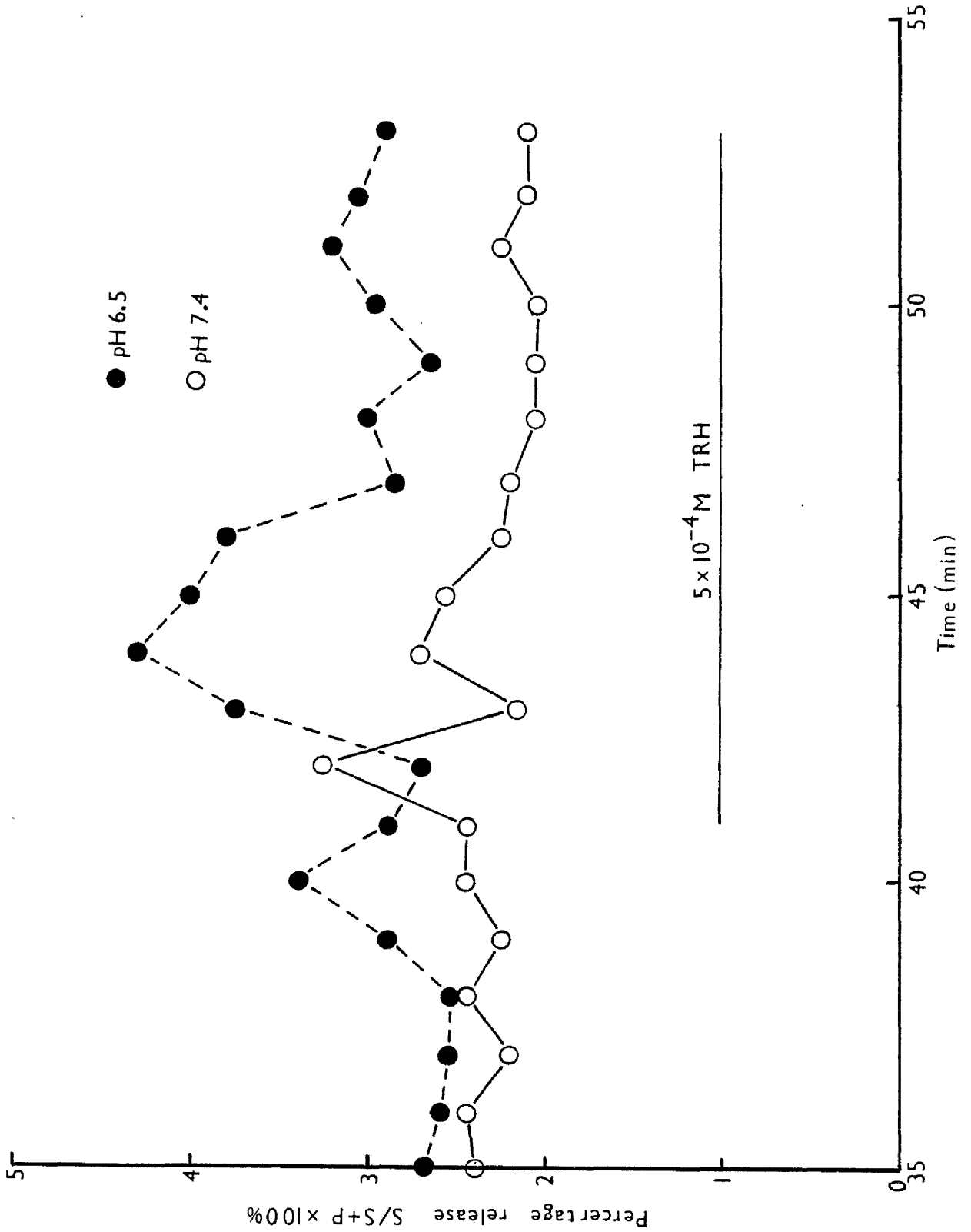


Table 12 : Effect of TRH (5×10^{-4}M) on release of ^{3}H-dopamine from tissue cubes of rat nucleus accumbens as followed by continuous perfusion in TRIS buffered saline at pH 6.5 and pH 7.4.

pH	Summed Percentage Release $\frac{S}{S+P} \times 100\%$		Ratio
	-TRH Baseline	+TRH Peak	Peak/Baseline
7.4	14.7	15.3	1.04
	14.0	15.1	1.07
	12.2	12.1	0.99
			1.03 ± 0.04 Mean-SD
6.5	16.5	21.3	1.29
	8.4	9.9	1.17
	9.0	10.7	1.19
	6.8	8.3	1.21
			1.21 ± 0.05 Mean-SD

pH 7.4 v pH 6.5 $\quad p < 0.005$

Table 13 : Effect of TRH (5×10^{-4} M) on release of ^{3}H-dopamine from tissue cubes of rat nucleus accumbens as followed by continuous perfusion in imidazole buffered saline at pH 6.5 and pH 7.4.

pH	Summed Percentage Release	$\frac{S}{S+P} \times 100\%$	Ratio
	-TRH Baseline	+TRH Peak	Peak/Baseline
7.4	11.5	9.6	0.83
	4.1	3.2	0.76
			0.79 ± 0.05 Mean $\pm$ Range
6.5	7.2	7.7	1.07
	14.4	12.4	0.86
	10.0	10.1	1.00
	6.2	6.0	0.97
	3.7	3.6	0.98
	8.5	15.0	1.75
			1.10 ± 0.32 Mean $\pm$ SD

Perfusion with 5mM-HEPES buffered saline, pH 6.5, containing 0.5mM-TRH produced variable results ranging from 22% inhibition to 32% stimulation of (^{3}H)-dopamine release (Table 14).

In conclusion, 0.5mM-TRH caused a small but consistent stimulation of release of (^{3}H)-dopamine from tissue cubes of nucleus accumbens at pH 6.5, but not at pH 7.4, in TRIS buffered saline. This effect could not be repeated in either imidazole or HEPES buffered salines, which were more effective buffers over the pH range of interest.

The perfusion assay had disadvantages in that replicate incubations could not be easily performed, the tissue was apt to clog the filters and basal release tended to be erratic, making stimulated release difficult to quantify accurately. To resolve these difficulties the assay procedure was changed to static incubation, terminated by centrifugation.

c) Centrifugation-terminated assay of release of (^{3}H)-dopamine from tissue cubes of rat nucleus accumbens

Tissue cubes of nucleus accumbens were incubated in TRIS buffered saline, pH 7.4, containing either 5mM-KCl or 50mM-KCl for 0-10 minutes and a time-course of release of (^{3}H)-dopamine under these conditions was obtained (Figure 21). The ratio:

$$\frac{(^3\text{H})\text{-DA release in 50mM-KCl}}{(^3\text{H})\text{-DA release in 5mM-KCl}}$$

was 1.77 at 5 minutes and 1.86 at 10 minutes. Thus 5 minutes was chosen as a suitable time for subsequent assays.

Tissue cubes of nucleus accumbens were incubated with a range of TRH concentrations (10^{-6}M-10^{-3}M) in TRIS buffered saline at pH 7.4 and 6.5. At pH 7.4 the results were variable and no clear concentration related effect of TRH was observed. At pH 6.5 a concentration-related stimulation of (^{3}H)-dopamine release (7-45%) was observed (Table 15).

Table 14 : Effect of TRH (5×10^{-4} M) on release of ^{3}H-dopamine from tissue cubes of rat nucleus accumbens as followed by continuous perfusion in HEPES buffered saline at pH 6.5.

Percentage Release $\frac{S}{S+P} \times 100\%$		Ratio
-TRH Baseline	+TRH Peak	Peak/Baseline
22.6	17.7	0.78
8.1	10.7	1.32
10.2	9.5	0.93
16.0	17.2	1.07
10.0	8.8	0.87
12.6	13.5	1.07
17.7	8.9	1.16
	Mean $\pm$ SD	1.03 ± 0.18

Figure 21 : Time-course of release of ^{3}H-dopamine from tissue cubes of rat nucleus accumbens in TRIS buffered saline, pH7.4, followed by the centrifugation terminated release assay.

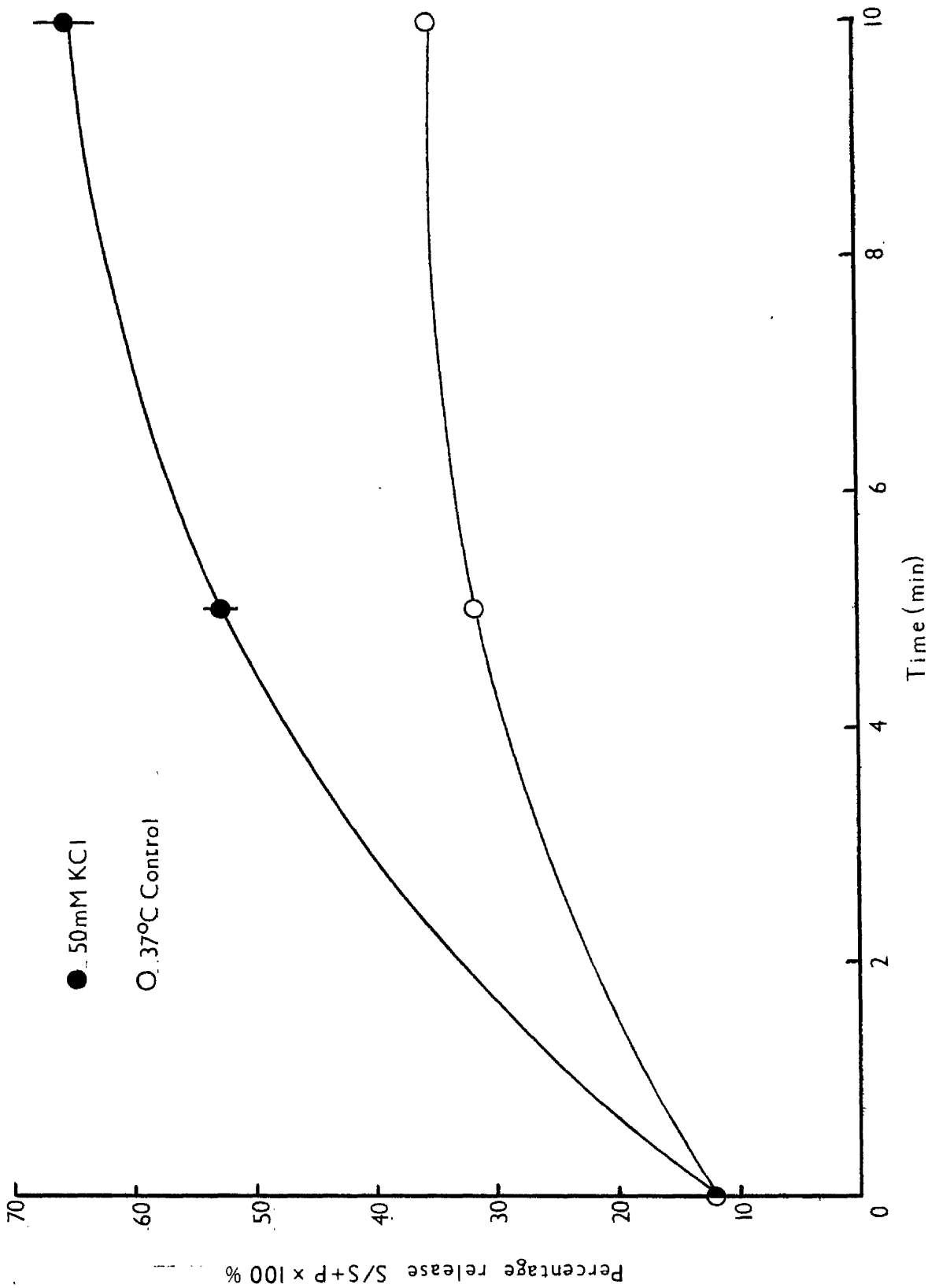


Table 15 : Effect of TRH (10^{-6}-10^{-3}M) on the release of (^{3}H)-dopamine from rat nucleus accumbens tissue cubes in TRIS buffered saline, pH 7.4 and pH 6.5 using the centrifugation-terminated assay.

Mean $^{S}/_{S+P}$ x 100% ± Range (n=2), change in mean, ratio of release										
pH	TRH Conc^{n}(M)	0°C Control	37°C Control	37°C Δ 0°C	TRH	TRH Δ 0°C	ΔTRH / ΔControl	50mM KCL	KCl Δ 0 °C	Δ KCL / Δ Control
7.4	10^{-6}	12.2±0.7	33.6±1.6	21.4	34.3±1.0	22.1	1.03	57.7±0.5	45.5	2.13
	10^{-5}				32.7±0.8	20.5	0.96			
	10^{-4}				35.0±0.3	22.8	1.06			
	10^{-3}				33.2±1.6	21.0	0.98			
	10^{-3}	13.9±0.7	25.8±0.3	11.9	30.6±0.8	16.7	1.40	61.5±0.4	47.6	4.00
6.5	10^{-6}	12.1±1.1	23.4±2.8	11.3	24.3±1.2	12.2	1.07	53.3±2.4	41.2	3.65
	10^{-5}				26.1±1.0	14.0	1.23			
	10^{-4}				27.6±0.9	15.5	1.37			
	10^{-3}				28.5±0.6	16.4	1.45			

The experiment was repeated using the TRH analogue CG3703 (6-methyl-5-oxothiomorpholinyl-3-carbonylhistidylprolineamide). At pH 7.4, CG 3703 (10^{-6}-10^{-3}M) did not cause any stimulation of (^{3}H)-dopamine release whereas at pH 6.5 up to 28% stimulation was observed as the concentration of CG3703 was increased to 10^{-3}M (Table 16).

In all cases 50mM-KCl caused a stimulation of release of (^{3}H)-dopamine release (76%-300%) showing that the preparations used were viable. The stimulation of release by 50mM KCl was found to be dependent on the presence of Ca^{2+}ions (Figure 22). Only a small Ca^{2+}-independent KCl-stimulation of release of (^{3}H)-dopamine was observed (KCl/37°C Control = 1.19) whereas in the presence of Ca^{2+}ions the KCl-stimulation was marked (KCl/37°C Control = 1.63). The finding suggests that 50mMKCl caused release of (^{3}H)-dopamine from within the tissue not by washing (^{3}H)-dopamine from sites of adsorption on the surface of the tissue.

d) Centrifugation-terminated assay of release of (^{3}H)-dopamine from P2 preparations of striatum and nucleus accumbens

Previous work (Shapiro et al, 1980) showed that TRH (1mM) stimulated the release of (^{3}H)-dopamine from striatal P2 preparations. It was decided to examine the effect of TRH on P2 preparations of nucleus accumbens in comparison with striatal P2 preparations. An advantage of using P2 preparations is that in washed or purified preparation there is a very little degradation of TRH (Parker et al, 1977; Shapiro et al, 1980; Biggins et al, 1981).

The presence of intact vesicles in the P2 preparations was determined by the presence of occluded lactate dehydrogenase (Table 17).

Table 16 : Effect of TRH analogue CG3703 (10^{-6}-10^{-3}M) on the release of (^{3}H)-dopamine from rat nucleus accumbens tissue cubes in TRIS buffered saline, pH 7.4 and pH 6.5 using the centrifugation terminated assay.

Mean $^{S}/_{S+P}$ x 100% ± Range (n=2), change in mean, ratio of release										
pH	CG3703 Conc^{n}(M)	0°C Control	37°C Control	37°C Δ 0°C	CG3703	CG3703 Δ 0°C	Δ CG3703 / Δ Control	50mM KCl	KCl Δ 0°C	Δ KCl / Δ Control
7.4	10^{-6}	6.1±0.2	35.9±0.7	29.8	37.5±2.0	31.4	1.05	58.7±0.7	52.6	1.76
	10^{-5}				35.5±2.8	29.4	0.99			
	10^{-4}				36.9±1.9	30.8	1.03			
	10^{-3}				36.6±1.7	30.5	1.02			
	10^{-3}	13.9±0.7	25.8±0.3	11.9	27.4±0.2	13.5	1.13	61.5±0.6	47.6	4.00
6.5	10^{-6}	13.3±0.7	22.9±1.6	9.6	22.7±1.8	9.4	0.98	50.2±0.6	36.9	3.84
	10^{-5}				24.4±1.5	11.1	1.15			
	10^{-4}				24.6±0.2	11.3	1.18			
	10^{-3}				25.6±1.1	12.3	1.28			

Figure 22 : Ca^{2+}-dependence of KCl stimulation of release of ^{3}H-dopamine from rat striatum tissue cube preparation in TRIS buffered saline, pH7.4.

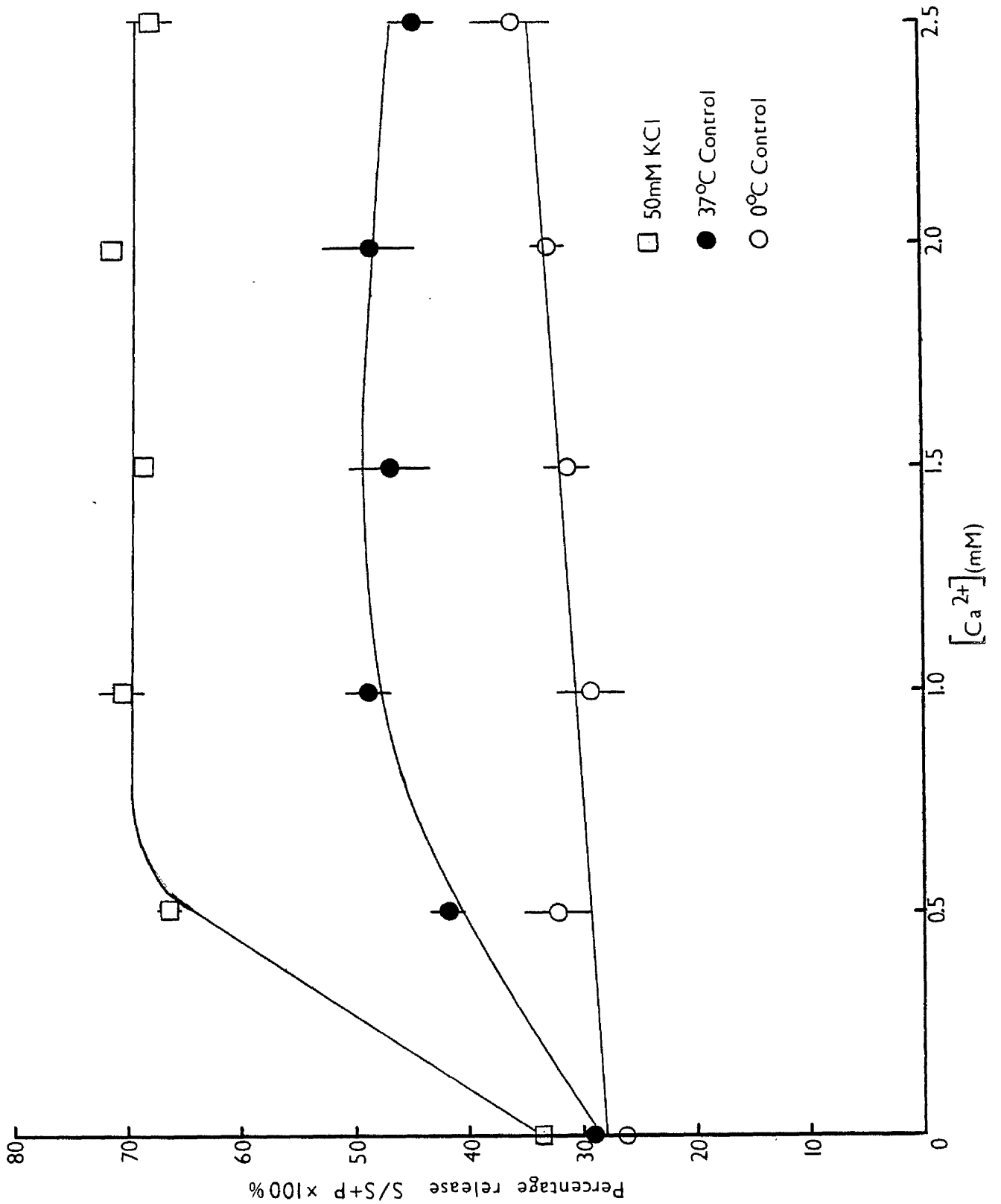


Table 17 : Percentage of total LDH activity occluded in P2 preparations

The value obtained agrees with the findings of Johnson and Whittaker (1962) who found that about 70% of the LDH activity recovered in the P2 fraction was localised in nerve ending particles and released on treatment with 10% (v/v) Triton X-100.

LDH activity (ΔA_{340}/minute)		
Free	Total	% Occluded
0.023	0.102	77.35
0.012	0.086	86.01
0.007	0.043	84.61
0.003	0.040	91.67
0.007	0.043	84.61
0.017	0.080	79.17
0.023	0.085	72.66
0.013	0.083	84.00
0.013	0.070	80.95
0.020	0.068	70.59
0.013	0.040	66.67
0.015	0.046	67.86
0.010	0.043	76.92
0.017	0.060	72.22
0.010	0.047	78.57
0.040	0.154	74.02
	Mean$\pm$SD	78.00$\pm$7.07

The presence of nerve-ending particles was determined by electron microscopy (Figures 23, 24 and 25).

The effect of 50mM-KCl and 1mM-tyramine on the release of (^{3}H)-dopamine was studied in P2 preparations of nucleus accumbens in TRIS buffered saline, pH 7.4. Release was terminated by centrifugation at 0-4^{o}C (Figure 26).

At 37^{o}C in the presence of 5mM-KCl release of (^{3}H)-dopamine increased linearly from 27% to 43% by 4 minutes. No time-dependent release could be detected at 0^{o}C, suggesting that the zero-time value of 27% represented residual extracellular (^{3}H)-dopamine. 50mM-KCl stimulated release by approximately 50% during the initial 2 minutes, following which no further stimulation of release was observed. 1mM-tyramine stimulated release approximately 150%, too rapidly to be resolved in the time taken to terminate the zero-time sample.

In view of the result with 50mM-KCl, subsequent assays with P2 preparations were incubated for 2 minutes.

TRH (1mM) had no consistent effect on the release of (^{3}H)-dopamine from P2 preparations of rat nucleus accumbens and striatum at pH 7.4 in TRIS buffered saline (Table 18).

In contrast, 50mM KCl stimulated release approximately 100% showing that the lack of response to TRH was unlikely to be the result of damage to the nerve-ending particles in the P2 preparation procedure.

The 50mM-KCl stimulation was found to be Ca^{2+}-dependent indicating (Fig.27) release of ^{3}H-dopamine, not washing of ^{3}H-dopamine from the surface of the tissue. A 41% Ca^{2+}-independent stimulation of release was observed.

At pH 6.5, TRH (1mM) and histidylproline diketopiperazine (1mM) stimulated the release of (^{3}H)-dopamine 20% and 26% respectively from nucleus accumbens P2 preparations (Table 19) and 39% and 41% respectively from striatal P2 preparations (Table 20).

Figure 23 : Electron micrograph of P2 fraction of nucleus accumbens showing the presence of synaptosomes containing synaptic vesicles and mitochondria (indicated by arrows, S). (X 7,500)

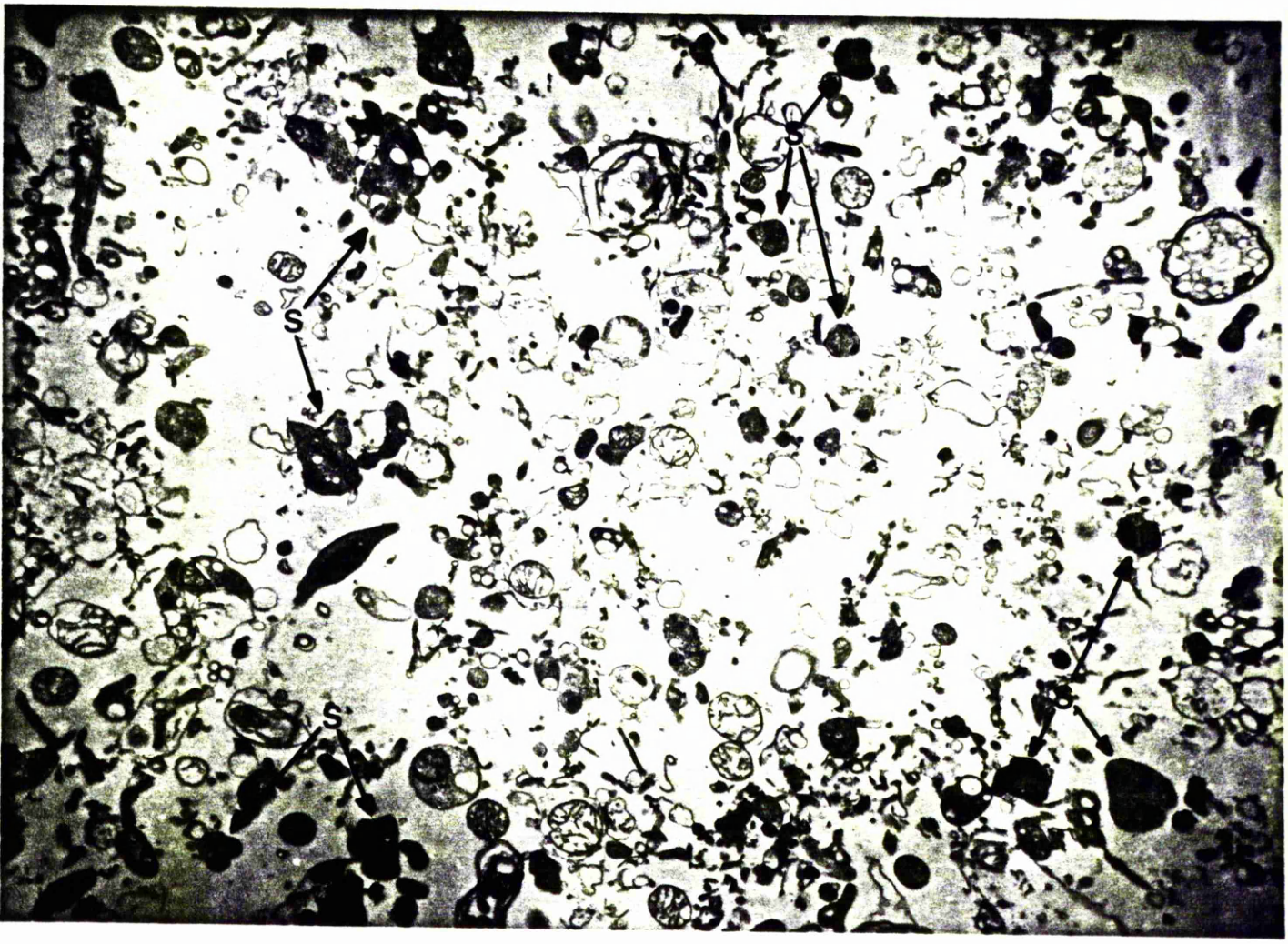

S
S
S

Figure 24 : Electron micrograph of a single synaptosome showing limiting membrane (LM), mitochondria (M) and synaptic vesicles (V)

(X 50,000)

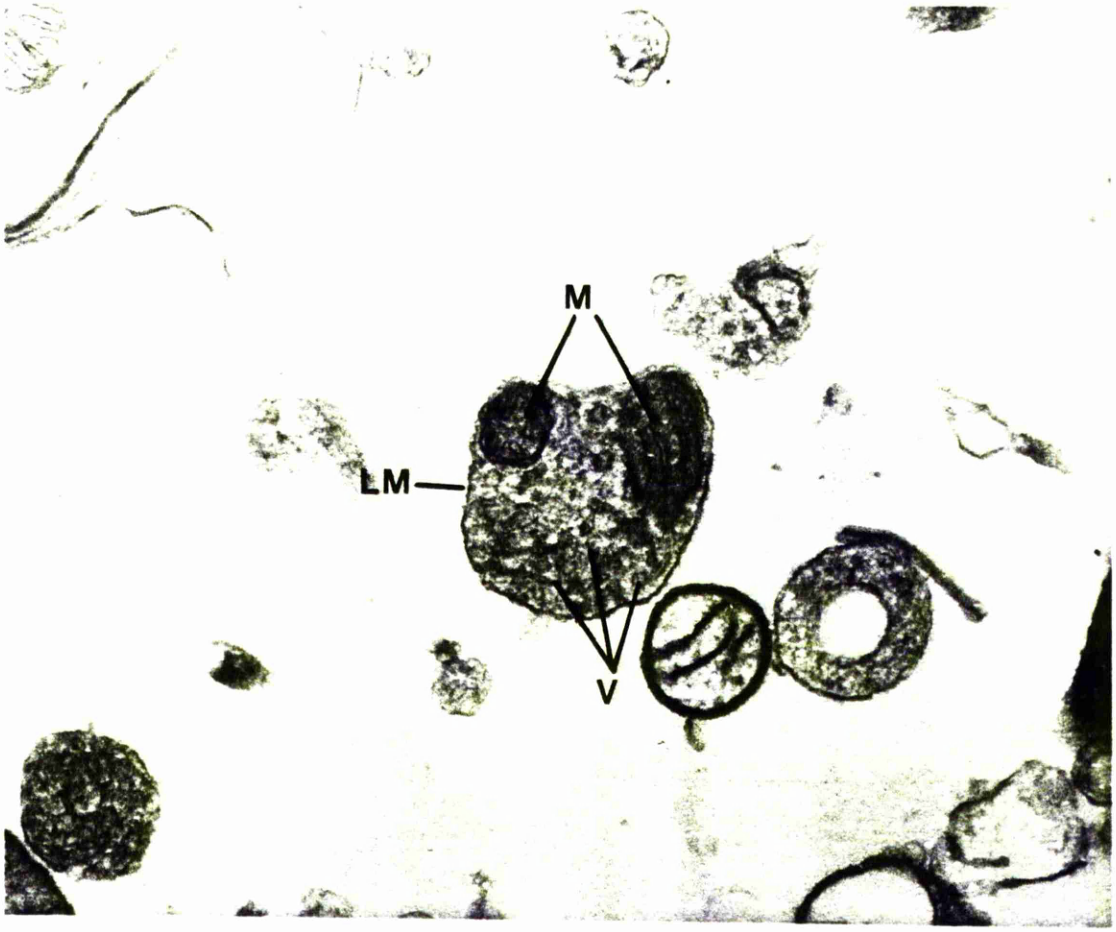

M
LM
V

Figure 25 : Electron micrograph of a single synaptosome showing the synaptic cleft (SC), the post-synaptic membrane (PSM) and associated post-synaptic density (PSD)
(X 50,000)

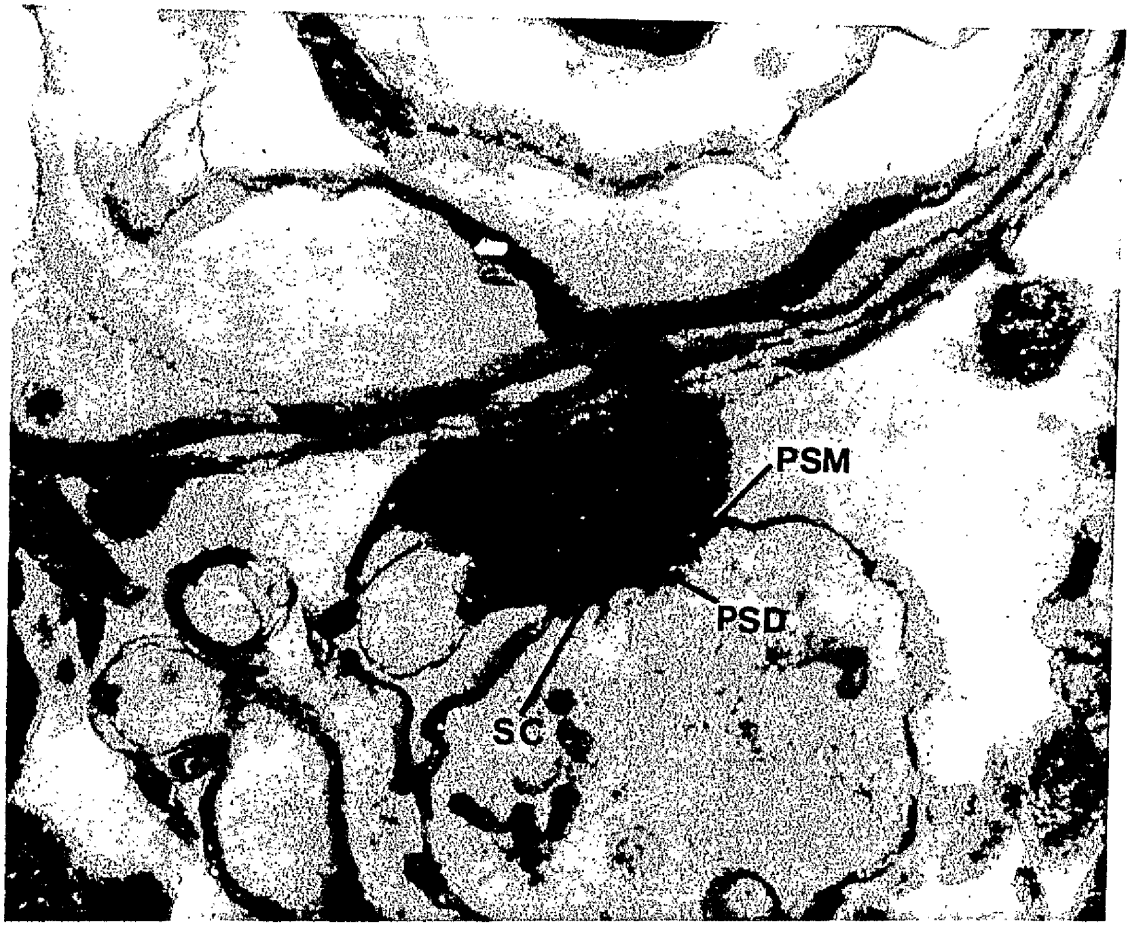

PSM
PSD
SC

Figure 26 : Time-course of release of ^{3}H-dopamine from a washed P2 preparation of rat nucleus accumbens in TRIS buffered saline pH 7.4, followed by the centrifugation terminated release assay.

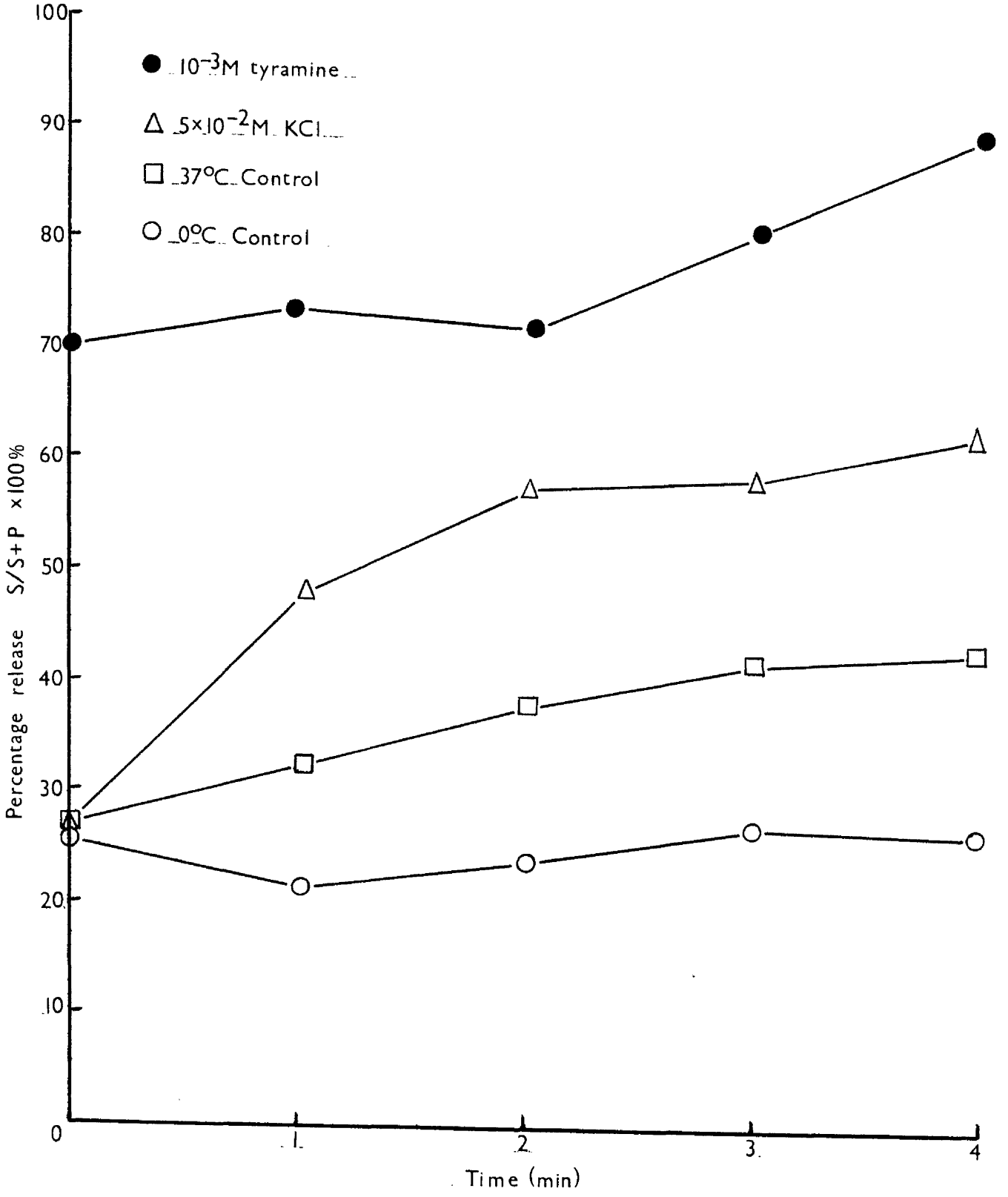


Table 18 : Effect of TRH (1 mM) on the release of (^{3}H)-dopamine from P2 preparations of rat nucleus accumbens and striatum at at pH 7.4 in TRIS buffered saline.

Mean $^S/_{S+P}$ * x 100% ± SD (n=3), change in mean, ratio of release									
Region	0°C Control	37°C Control	37°C $\Delta_{0°C}$	1mM TRH	TRH $\Delta_{0°C}$	$\frac{\Delta \text{TRH}}{\Delta \text{Control}}$	50mM KCl	KCl $\Delta_{0°C}$	$\frac{\Delta \text{KCl}}{\Delta \text{Control}}$
Nucleus	16.2±2.8	39.9±8.5	23.7	40.5±3.4	24.3	1.02	56.9±4.4	40.7	1.71
accumbens	12.9±1.3	32.7±4.8	19.8	30.5±2.4	17.6	0.89	57.1±2.6	44.2	2.23
	27.7±0.1	45.8±0.9	18.1	43.9±2.9	16.2	0.39			
	32.1±2.7	45.2±2.2	13.1	58.7±4.6*	26.6	2.03			
	34.4±6.7	62.2±7.0	27.8	65.5±2.6	31.1	1.12			
	18.7±4.4	42.2±1.2	23.5	43.5±7.6	24.8	1.05			
	20.9±0.9	42.5±0.9	21.6	43.0±0.9	22.1	1.02			
				Mean ± SD		1.14±0.40			1.97±0.36*
Striatum	21.5±1.2	50.4±0.5	28.9	51.3±1.2	29.8	1.03	65.9±1.6	44.4	1.54
	22.1±2.2	33.2±1.4	11.1	33.7±1.6	11.6	1.04	62.3±1.2	40.2	3.62
	40.8±0.8	63.2±0.5	22.4	62.5±0.3	21.7	0.97	71.3±1.2	30.5	1.36
	29.2±1.6	55.6±5.4	26.4	52.9±7.4	23.7	0.90			
				Mean ± SD		0.98±0.06			2.17±1.26

Figure 27 : Ca^{2+}-dependence of KCl stimulation of release of ^{3}H-dopamine from rat striatum P2 preparation in TRIS buffered saline, pH 7.4

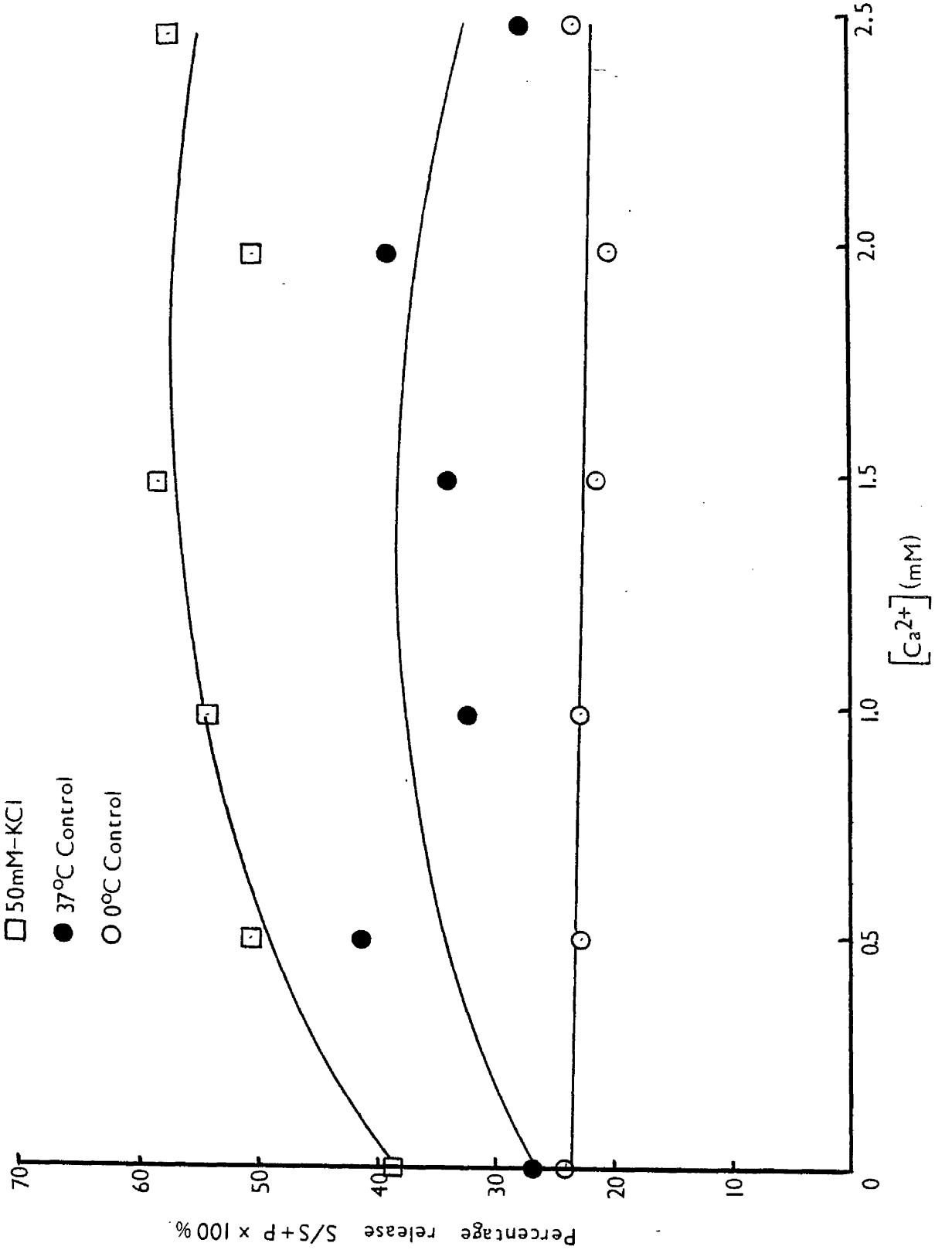


Table 19 : The effect of TRH (1mM) and histidylproline diketopiperazine (1mM) on the release of (^{3}H)-dopamine from rat nucleus accumbens P2 preparations at pH 6.5 in 20 mM TRIS-buffered saline.

Mean $^S/_{S+P}$ x 100% ± SD (n=3), change in mean, ratio of release											
0°C Control	37°C Control	37°C Δ 0°C	1mM TRH	TRH Δ 0°C	ΔTRH / ΔControl	1mM cHisPro	cHisPro Δ 0°C	ΔcHisPro / ΔControl	50mM KCl	KCl Δ 0°C	ΔKCl / ΔControl
33.1±2.6	44.8±11.4	11.7	47.6±2.9	14.5	1.24						
17.4±1.4	31.2±2.2	13.8	34.0±0.6	16.6	1.20	33.3±0.6	15.9	1.15	45.2±1.4‡	27.8	2.01
20.8±1.0	34.8±1.2	14.0	36.7±2.4	15.9	1.13						
15.1±0.6	26.4±1.6	11.3	30.2±1.5†	15.1	1.34	31.2±1.9†	16.1	1.42	44.0±1.7‡	28.9	2.56
16.5±0.5	28.4±1.2	11.9	29.5±1.1	13.0	1.09						
18.3±1.8	32.4±1.3	14.2	35.8±1.5†	17.5	1.23	35.6±1.7*	17.4	1.22	45.5±1.3‡	27.2	1.91
				Mean±SD	1.20±0.09		Mean±SD	1.26±0.14		Mean±SD	2.16±0.35

* $p < 0.05$
† $p < 0.025$
‡ $p < 0.005$

w r t 37°C Control

Table 20 : Comparison of the effects of TRH (1mM) and histidylproline diketopiperazine (1mM) on the release of (^{3}H)-dopamine from rat striatum P2 preparations at pH 6.5 in 20mM TRIS buffered saline.

Mean $^{S}/_{S+P}$ x 100% ± SD (n=3), change in mean, ratio of release											
0°C Control	37°C Control	37°C $\Delta_{0°C}$	1mM TRH	TRH $\Delta_{0°C}$	$\frac{\Delta TRH}{\Delta Control}$	cHisPro	cHisPro $\Delta_{0°C}$	$\frac{\Delta cHisPro}{\Delta Control}$	50mM KCl	KCl $\Delta_{0°C}$	$\frac{\Delta KCl}{\Delta Control}$
19.8±2.3	26.6±3.4	6.8	32.6±1.7 *	12.8	1.88	32.8±1.4 †	13.0	1.91	48.5±1.0 ‡	28.7	4.22
13.0±1.4	25.1±0.5	12.1	28.4±0.4 ‡	15.4	1.27	27.6±1.1 †	14.6	1.21	46.3±0.9 ‡	33.3	2.75
13.9±1.1	29.5±2.6	15.6	30.2±1.2	16.3	1.04	51.6±1.9	17.7	1.13	53.7±0.7 ‡	39.8	2.55
				Mean±SD	1.39±0.43		Mean±SD	1.41±0.43		Mean±SD	3.17±0.91

* p 0.05
† p 0.025
‡ p 0.005

w r t 37°C Control

3.2.4 Conclusion to the findings of the experiments on dopamine release

Irrespective of the assay procedure used, TRH, its metabolite histidylproline diketopiperazine (cyclo His Pro) and the analogue CG3703 appear to stimulate the release of (^{3}H)-dopamine from tissue preparations of both nucleus accumbens and striatum over a range of concentrations (10^{-6}-10^{-3}M TRH, 10^{-6}-10^{-3}M CG3703 and 10^{-3}M cyclo HisPro).

The stimulatory effects of these peptides are more clearly seen at pH 6.5 than at pH 7.4.

The largest stimulation of (^{3}H)-dopamine release, however, was only 45% greater than basal release, caused by 10^{-3}M TRH in tissue cubes of nucleus accumbens at pH 6.5. In view of the high concentration of peptide required to produce this relatively small effect it was decided not to pursue this effect any further and to investigate the effects of TRH on the release of acetylcholine.

3.3 Acetylcholine release studies

The nucleus accumbens and septum both contain high affinity TRH-binding sites (Burt and Snyder, 1975) and are sensitive to TRH in *in-vivo* (Miyamoto *et al*, 1978; Miyamoto and Nagawa, 1979; Heal and Green, 1979; Kalivas and Horita, 1980) and *in-vitro* assays (Kerwin and Pycock, 1979; Nagai *et al*, 1980; Sharp *et al*, 1981). Both regions are rich in cholinergic nerve terminals (Jacobowitz and Palkovits, 1974). Acetylcholine has been reported to stimulate dopamine release (Giorguieff *et al*, 1975; de Belleroche and Bradford, 1978) and TRH has been reported to stimulate acetylcholine turnover in rat brain tissue preparations (Malthe-Sørenssen *et al*, 1978). Thus a study on the effects of TRH on the release of acetylcholine from tissue cubes of rat nucleus accumbens and septum was carried out using the centrifugation terminated assay.

Sharp et al (1981) reported that 0.1mM TRH stimulated release of endogenous dopamine from tissue cubes of nucleus accumbens and septum at pH 7.4. If this stimulation were caused indirectly via cholinergic systems then these conditions should be suitable to demonstrate TRH effects on acetylcholine release. In addition, the effects of cyclo-HisPro (0.1mM) and CG3703 (0.1mM) on basal and K^+-stimulated acetylcholine release were also studied.

Tissue cubes of nucleus accumbens and septum were preloaded with (^{3}H)-choline as outlined in Materials and Methods. In the nucleus accumbens 0.703 $\pm$ 0.148% (mean $\pm$ SD) of the choline was converted to acetylcholine per mg tissue in 30 minutes compared with a 0.218 $\pm$ 0.040% conversion per mg tissue in 30 minutes for the septum (Table 21). In each case the standard deviation was about 20% of the mean, suggesting that these regions were dissected with equal accuracy.

In the preliminary experiments, the effect of 1mM-TRH and 50mM-KCl on the release of radioactivity from tissue pre-loaded with (^{3}H)-choline was studied. No steps were taken to determine the nature of the released radioactivity.

50mM-KCl stimulated ^{3}H release by 73 - 308%. 1mM-TRH stimulated release 0- 62% (Table 22).

One possible reason for the variability in the stimulation observed is that differing amounts of choline (Ch) might be released with the acetylcholine (ACh). It was therefore decided to separate the ACh and Ch in the supernatant and pellet fractions so that release of ACh could be calculated as a percentage of the ACh "pool" in the tissue. Both Ch and ACh were found in the supernatant and pellet fractions and a small amount of radioactivity remained associated with the origin. (Table 23).

Table 21 : Percentage conversion of pre-loaded (^{3}H)-choline to (^{3}H)-acetylcholine by tissue cubes of rat nucleus accumbens and septum.

The values presented are the percentage of the total counts in each experiment which were contributed by newly synthesised (^{3}H)-acetylcholine in tissue incubated for 30 minutes with 10 μ Ci (^{3}H)-choline/50 mg tissue.

Region	Total CPM	CPM in Choline	CPM in Acetylcholine	% Conversion of (^{3}H)-Ch to (^{3}H)-ACh per incubation	mg tissue	% Conversion per mg tissue in 30 mins
Nucleus accumbens	60679.2	25527.7	35151.5	57.93	79.6	0.728
	60100.2	28751.9	31348.3	52.16	62.7	0.832
	329893.8	120774.1	209119.7	63.39	69.2	0.916
	733013.2	311530.6	421482.6	57.50	102.5	0.561
	573926.1	271294.9	302631.2	52.73	85.8	0.614
	657072.1	300019.1	357053.0	54.34	96.2	0.565
Mean ± SD				5634 ± 4.20		0.703 ± 0.14
Septum	258295.9	158361.2	99934.7	38.69	141.7	0.273
	351878.0	215842.0	136036.0	38.66	148.1	0.261
	566481.1	389010.1	177471.0	35.04	206.1	0.170
	367450.3	272390.9	95059.4	25.87	120.9	0.214
	759823.1	555886.6	203936.5	26.84	129.7	0.207
	490000.2	351281.2	138719.0	28.31	152.2	0.186
Mean ± SD				32.23 ± 5.93		0.218 ± 0.04

Table 22 : Effects of TRH (10^{-3}M) on the release of radioactivity from tissue cubes of nucleus accumbens pre-loaded with (^{3}H)-choline at pH 7.4 in TRIS buffered saline using the centrifugation-terminated assay.

Mean $^{S}/_{S+P}$ x 100% ± SD (n=3), change in mean, ratio of release								
0°C Control	37°C Control	37°C Δ 0°C	10^{-3}M TRH	$\Delta^{TRH}_{0°C}$	$\frac{\Delta \text{ TRH}}{\Delta \text{ Control}}$	$5x10^{-2}$M KCl	KCl 0°C	$\frac{\Delta \text{ KCl}}{\Delta \text{ Control}}$
6.0±1.3	12.4±1.0	6.4	16.4±2.2 *	10.4	1.62	32.1±2.5 ‡	26.1	4.08
8.6±2.2	15.6±1.4	7.0	19.0±3.2	10.4	1.48	36.8±2.0 ‡	28.2	4.03
8.2±1.8	19.9±2.8	11.7	18.9±2.5	10.7	0.91	34.9±1.4 ‡	26.7	2.28
10.1±0.3	26.1±2.6	16.0	25.3±3.0	15.2	0.95	37.8±6.2 *	27.7	1.73
Mean ± SD					1.24±0.36			3.03±1.02

* p 0.05
‡ p 0.005
} w r t 37°C Control

Table 23 : Distribution of radioactivity between origin, choline and acetylcholine areas on cellulose TLC plate

	SUPERNATANT				PELLET			
	CPM IN FRACTION				CPM IN FRACTION			
Incubation	Origin	Choline	Acetylcholine	%ACh	Origin	Choline	Acetylcholine	%ACh
$0^{o}C$ Control(a)	19.5	33.6	29.9	36.0	47.5	220.0	384.5	58.9
(b)	24.9	19.9	18.7	29.4	56.5	181.8	320.5	57.3
(c)	13.4	19.5	15.7	32.3	35.3	319.3	354.5	50.0
$37^{o}C$ Control (a)	24.6	57.7	61.1	42.8	52.5	165.3	354.8	61.9
(b)	8.3	87.3	89.1	48.2	23.0	98.0	211.5	63.6
(c)	11.8	33.7	69.7	60.5	40.3	197.0	304.5	56.2
10^{-4}M TRH (a)	11.7	62.8	34.8	31.8	35.8	192.3	284.8	55.5
(b)	24.5	81.6	61.8	36.8	42.0	136.3	293.5	62.2
(c)	12.9	49.6	46.1	42.4	31.3	123.5	216.8	58.3
$5x10^{-2}$MKCl (a)	14.2	104.8	126.6	51.5	27.3	105.8	154.8	53.8
(b)	14.1	67.5	122.5	60.0	43.3	199.5	197.3	44.8
(c)	28.5	84.6	191.4	62.8	58.5	236.5	211.8	41.8

The percentage of ACh in the supernatant increased from about 30% in the 0°C control to about 60% in the 50 mM-KCl stimulated incubation. Total CPM in the Ch and ACh spots increased from about 40 in the 0°C control to about 200 in the 50 mM-KCl stimulated incubation. In the pellet fraction the percentage of ACh dropped from about 55% in the 0°C control to about 45% in the 50mM-KCl stimulated incubations, the absolute CPM in Ch and ACh falling from about 600 to about 400.

No appreciable hydrolysis of ACh to Ch occurred during the freeze-drying step in the separation of Ch from ACh (Table 24). Thus the Ch present in the supernatant fraction was not formed by hydrolysis of ACh during isolation.

The effects of 0.1mM-TRH, 0.1mM-histidylproline diketopiperazine and 0.1mM-CG3703 on the release of (^{3}H)-ACh and (^{3}H)-Ch from tissue cubes of rat nucleus accumbens and septum were studied in the presence of 5mM-KCl and 50 mM-KCl.

0.1mM-TRH had no consistent effect on either basal or 50mM-KCl-stimulated release of (^{3}H)-ACh (Table 25) or (^{3}H)-Ch (Table 26) from nucleus accumbens.

50mM-KCl stimulated (^{3}H)-ACh release from nucleus accumbens by up to 540% and (^{3}H)-Ch release by up to 260%.

0.1mM-TRH had no consistent effect on basal nor 50mM-KCl stimulated release of (^{3}H)-ACh (Table 27) nor (^{3}H)-Ch (Table 28) from tissue cubes of septum. 50mM-KCl stimulated the release of (^{3}H)-ACh by up to 1900% and release of (^{3}H)-Ch by up to 280%.

0.1mM-cycloHis-Pro had an inhibitory effect, up to 62%, on basal release of (^{3}H)-ACh from tissue cubes of septum in all cases except one,

Table 24 : Estimation of stability of acetylcholine on freeze-drying

	ORIGIN		CHOLINE		ACETYLCHOLINE		TOTAL	% Recovery at 50% Counting Efficiency
uCi ^{14}C/spot	CPM	% Total	CPM	% Total	CPM	% Total	CPM	
0.04	49.0	0.12	127.0	0.31	41047.5	99.57	41223.5	93.69
0.08	62.5	0.08	220.0	0.28	76716.6	99.64	76999.1	87.50
0.16	192.0	0.11	406.5	0.24	169131.5	99.65	169730.0	96.44
	Mean$^{+}_{-}$SD	$0.10^{+}_{-}0.02$		$0.28^{+}_{-}0.03$		$99.62^{+}_{-}0.04$		$92.54^{+}_{-}4.58$

Table 25 : Effect of TRH (0.1mM) on basal and 50mMKCl stimulated release of (^{3}H)-acetylcholine from tissue cubes of rat nucleus accumbens in TRIS buffered saline, pH 7.4.

Mean $^{S}/_{S+P}$ x 100% $\pm$ SD (n=3), change in mean, ratio of release											
0°C Control	37°C Control	37°C Δ 0°C	0.1mM TRH	TRH Δ 0°C	$\frac{\Delta \text{ TRH}}{\Delta \text{ Control}}$	50mM KCl	KCl Δ 0°C	$\frac{\Delta \text{ KCl}}{\Delta \text{ Control}}$	TRH+ KCl	TRH+KCl Δ 0°C	$\frac{\Delta \text{ KCl+T}}{\Delta \text{ KCl}}$
4.4±1.3	19.9±6.2	15.5	11.6±0.3	7.2	0.46	42.7±3.7 †	38.3	2.47	48.6±4.2	44.2	1.15
4.0±0.7	11.3±1.2	7.3	10.9±1.9	6.9	0.94	39.6±1.6 ‡	35.6	4.88	37.5±7.9	33.5	0.94
4.0±0.3	15.4±1.4	11.4	15.6±2.3	11.6	1.02	43.4±2.4 ‡	39.4	3.45	44.4±5.9	40.4	1.02
3.1±1.2	9.8±1.3	6.7	12.3±2.3	9.2	1.37	46.1±2.6 ‡	43.0	6.42	40.7±8.6	37.6	0.87
2.9±0.3	7.1±1.1	4.2	8.8±1.0	5.9	1.40	28.3±3.0 ‡	25.4	6.04	36.9±9.1	34.0	1.34
3.2±0.5	10.2±3.2	7.0	8.6±0.2	5.4	0.77	30.2±2.4 ‡	27.0	3.86	27.2±1.5	24.0	0.89
Mean±SD					0.99±0.36			4.52±1.54			1.03±0.

† $p < 0.025$

‡ $p < 0.005$

Table 26 : Effect of TRH (0.1mM) on basal and 50mM-KCl stimulated release of (^{3}H)-choline from tissue cubes of rat nucleus accumbens in TRIS buffered saline, pH 7.4.

Mean $^{S}/_{S+P}$ x 100% ± SD (n=3), change in mean, ratio of release											
0°C Control	37°C Control	37°C $\Delta_{0°C}$	0.1mM TRH	TRH $\Delta_{0°C}$	$\frac{\Delta \text{TRH}}{\Delta \text{Control}}$	50mM KCl	KCl $\Delta_{0°C}$	$\frac{\Delta \text{KCl}}{\Delta \text{Control}}$	TRH + KCl	KCl+TRH $\Delta_{0°C}$	$\frac{\Delta \text{KCl+TRH}}{\Delta \text{KCl}}$
6.8±1.3	20.9±8.9	14.1	24.3±4.0	17.1	1.24	32.8±9.9	26.0	1.84	31.4±4.9	24.6	0.9
5.1±1.3	17.0±2.1	11.9	16.2±3.2	11.1	0.93	^{a}‡ 25.7±0.6	20.6	1.73	^{b}† 30.6±1.4	25.5	1.2
6.7±2.6	18.9±0.5	12.2	16.2-2.9	9.5	0.78	^{a}† 29.2±4.2	22.5	1.84	26.2±1.6	19.5	0.8
5.0±1.1	12.1±0.8	7.1	14.5±3.2	9.5	1.34	^{a}‡ 30.4±1.8	25.4	3.58	^{b}* 22.8±4.2	17.8	0.7
5.6-0.3	10.9±0.8	5.3	12.0-0.7	6.4	1.21	^{a}† 18.3±1.6	12.7	2.40	16.2±0.5	10.6	0.8
6.7±1.2	14.9±5.9	8.2	12.8±1.8	6.1	0.74	23.4±4.3	16.7	2.04	23.6±1.2	16.9	1.0
Mean±SD					1.04±0.26			2.24±0.70			0.93±0.1

* $p < 0.05$ † $p < 0.025$ ‡ $p < 0.005$

a WRT 37°C Control

b WRT 50mM KCl

Table 27 : Effect of TRH (0.1mM) on basal and 50mMKCl stimulated release of (^{3}H)-acetylcholine from tissue cubes of rat septum in TRIS buffered saline pH 7.4.

Mean $^S/_{S+P}$ x 100% ± SD, (n = 3) change in mean, ratio of release											
0°C Control	37°C Control	37°C Δ 0°C	0.1mM TRH	TRH Δ 0°C	Δ TRH / Δ Control	50mM KCl	KCl Δ 0°C	Δ KCl / Δ Control	TRH+ KCl	KCl+TRH Δ 0°C	Δ KCl+TR / Δ KCl
7.1±3.3	11.6±4.1	4.5	12.7±2.4	5.6	1.24	27.4±2.0 ‡	20.3	4.51	28.0±1.2	20.9	1.03
1.8±0.3	6.3±2.9	4.5	6.6±0.5	4.8	1.07	15.6±6.9	13.8	3.07	9.7±4.8	7.9	0.52
5.6±0.5	12.0±3.3	6.4	10.7±1.4	5.1	0.80	22.4±1.8 †	16.8	2.62	20.0±0.1	14.4	0.86
8.9±1.7	10.2±2.7	1.3	18.6±13.6	9.7	7.46	25.7±1.4 ‡	16.8	12.92	30.9±8.9	22.0	1.31
6.0±2.1	10.8±1.1	4.8	7.3±2.7	1.3	0.27	28.0±5.3 †	22.0	4.58	25.2±3.4	19.2	0.87
7.3±0.7	8.3±2.1	1.0	10.1±1.7	3.2	3.20	27.6±5.4 ‡	20.3	20.30	30.1±10.1	22.8	1.12
Mean ± SD					2.34±2.69			8.00±7.11			0.95±0.27

† $p < 0.025$
‡ $p < 0.005$

Table 28 : Effect of TRH(0.1mM) on basal and 50mMKCl stimulated release of (^{3}H)-choline from tissue cubes of rat septum in TRIS buffered saline, pH 7.4.

Mean $^S/_{S+P}$ x 100% ± SD, (n = 3) change in mean, ratio of release											
0°C Control	37°C Control	37°C Δ 0°C	0.1mM TRH	TRH Δ 0°C	Δ TRH / Δ Control	50mM KCl	KCl Δ 0°C	Δ KCl / Δ Control	TRH+ KCl	KCl+TRH Δ 0°C	Δ KCl+TRH / Δ KCl
7.2±2.1	16.6±5.5	9.4	16.9±2.8	9.7	1.03	25.6±4.1	18.4	1.96	28.4±7.7	21.2	1.15
5.6±0.7	15.7±3.4	10.1	17.5±9.2	11.9	1.18	23.6±9.4	18.0	1.78	13.8±4.8	8.2	0.45
11.2±3.5	17.6±4.2	7.4	16.6±1.1	5.4	0.73	*a 25.4±2.1	14.2	1.92	*b 21.0±0.7	9.8	0.69
12.3±2.3	15.3±1.6	3.0	26.9±11.7	14.6	4.90	*a 23.7±2.2	11.4	3.80	26.1±5.2	13.8	1.21
11.5±1.5	15.9±7.4	4.4	14.0±5.3	3.5	0.79	28.9±9.2	17.4	3.95	19.3±3.1	8.8	0.51
10.5±1.1	16.9±0.5	6.4	16.3±0.9	5.8	0.90	† 21.3±1.7	10.8	1.69	22.1±5.1	11.6	1.08
Mean ± SD					1.59±1.63			2.52±1.06			0.85±0.34

* $p \leq 0.05$
† $p \leq 0.025$

a WRT 37°C Control
b WRT 50mM KCl

when a 61% stimulation of release was observed (Table 29). This dipeptide had no consistent effect on 50mM-KCl stimulated (^{3}H)-ACh release, nor on basal nor 50mMKCl-stimulated (^{3}H)-Ch release from tissue cubes of septum (Table 30).

0.1mM-CG3703 had no consistent effect on either basal or 50mM-KCl stimulated release of (^{3}H)-ACh (Table 31) or (^{3}H)-Ch (Table 32) from tissue cubes of nucleus accumbens.

0.1mM-CG3703 had no consistent effect on either basal or 50mM-KCl stimulated release of (^{3}H)-ACh (Table 33) or (^{3}H)Ch (Table 34) from tissue cubes of septum.

Table 29 : Effect of histidylproline diketopiperazine (cycloHisPro) on basal and 50 mM-KCl stimulated release of (^{3}H)-acetylcholine from tissue cubes of rat septum in TRIS buffered saline, pH 7.4.

Mean $^{S}/_{S+P}$ x 100% ± SD, (n = 3) change in mean, ratio of release											
0°C Control	37°C Control	$\Delta^{37°C}_{0°C}$	0.1mM cycloHisPro	cHisPro Δ 0	$\frac{\Delta \text{cHisPro}}{\Delta \text{Control}}$	50mM KCl	$\Delta^{KCl}_{0°C}$	$\frac{\Delta \text{KCl}}{\Delta \text{Control}}$	cHisPro+ KCl	KCl+ cHisPro Δ 0°C	$\frac{\Delta \text{KCl+cHisPro}}{\Delta \text{KCl}}$
7.1±3.3	11.6±4.1	4.5	8.8	1.7	0.38	27.4±2.0‡	20.3	4.51	26.2±1.7	19.1	0.94
1.8±0.3	6.3±2.9	4.5	5.2±0.9	3.4	0.75	15.6±6.9	13.8	3.07	12.1±3.2	10.3	0.75
8.9±1.7	10.2±2.7	1.3	11.0±1.7	2.1	1.61	25.7±1.4‡	16.8	12.92	27.2±5.0	18.3	1.09
6.0±2.1	10.8±1.1	4.8	8.6±2.0	2.6	0.54	28.0±5.3†	22.0	4.58	23.2±4.4	17.2	0.78
7.3±0.7	8.3±1.2	1.0	8.1±4.1	0.8	0.80	27.6±5.4‡	20.3	20.30	39.4±12.9	32.1	1.58
Mean ± SD					0.82±0.47			9.08±7.38			1.03±0.34

† $p \leq 0.025$
‡ $p \leq 0.005$

Table 30 : Effect of histidylproline diketopiperazine on basal and 50mM-KCl stimulated release of (^{3}H)-choline from tissue cubes of rat septum in TRIS buffered saline, pH 7.4.

Mean $^S/_{S+P}$ x 100% ± SD (n=3), change in mean, ratio of release											
0°C Control	37°C Control	Δ 37°C / 0°C	0.1mM cycloHisPro	cHisPro Δ 0°C	ΔcHisPro / Δ Control	50mM KCl	Δ KCl 0°C	Δ KCl / Δ Control	cHisPro+ KCl	KCl+cHisPro Δ 0°C	ΔKCl+cHisP / ΔKC
7.2±2.1	16.6±5.5	9.4	19.9	12.7	1.35	25.6±4.1	18.4	1.96	31.3±7.1	24.1	1.31
5.6±0.7	15.7±3.4	10.1	16.1±1.3	10.5	1.04	23.6±9.4	18.0	1.78	24.0±4.1	18.4	1.02
12.3±2.3	15.3±1.6	3.0	15.1±2.2	2.8	0.93	23.7±2.2 *	11.4	3.80	24.2±2.9	11.9	1.04
11.5±1.5	15.9±1.4	4.4	16.1±6.3	4.6	1.05	28.9±9.2	17.4	3.95	22.5±3.5	11.0	0.63
10.5±1.1	16.9±0.5	6.4	12.1±6.1	1.6	0.25	21.3±1.7 †	10.8	1.69	23.7±4.0	13.2	1.23
Mean ± SD					0.92±0.41			2.64±1.14			1.05±0.26

* $p < 0.05$

† $p < 0.025$

Table 32 : Effect of TRH analogue CG3703 (0.1mM) on basal and 50mM-KCl stimulated release of (^{3}H)-acetylcholine from tissue cubes of rat nucleus accumbens in TRIS buffered saline, pH 7.4.

Mean $^{S}/_{S+P}$ x 100% ± SD (n = 3), change in mean, ratio of release											
0°C Control	37°C Control	37°C Δ 0°C	0.1mM CG3703	CG3703 Δ 0°C	$\frac{\Delta \text{CG3703}}{\Delta \text{Control}}$	50mM KCl	KCl Δ 0°C	$\frac{\Delta \text{KCl}}{\Delta \text{Control}}$	CG3703 + KCl	KCl + CG3703 Δ 0°C	$\frac{\Delta \text{KCl+CG3703}}{\Delta \text{KCl}}$
3.1±1.2	9.8±1.3	6.7	13.8±2.4	10.7	1.59	46.1±2.6 ‡	43.0	6.45	46.1±3.5	43.0	1.00
2.9±0.3	7.1±1.1	4.2	7.9±1.6	5.0	1.19	28.3±3.0 ‡	25.4	6.04	34.5±3.9	31.6	1.24
3.2±0.5	10.2±3.2	7.0	7.5±1.0	4.3	0.61	30.2±2.4 ‡	27.0	3.86	26.5±2.0	23.3	0.86
Mean ± SD					1.13±0.49			5.45±1.39			1.03±0.19

‡ $p < 0.005$

Table 32 : Effect of TRH analogue CG3703 (0.1mM) on basal and 50mM-KCl stimulated release of (^{3}H)-choline from tissue cubes of rat nucleus accumbens in TRIS buffered saline, pH 7.4.

Mean $^{S}/_{S+P}$ x 100% ± SD (n = 3), change in mean, ratio of release											
0°C Control	37°C Control	Δ 37°C 0°C	0.1mM CG3703	CG3703 Δ 0°C	$\frac{\Delta \text{ CG3703}}{\Delta \text{ Control}}$	50mM KCl	KCl Δ 0°C	$\frac{\Delta \text{ KCl}}{\Delta \text{Control}}$	CG3703 KCl	KCl + CG3703 Δ 0°C	$\frac{\Delta \text{ KCl + CG3703}}{\Delta \text{ KCl}}$
5.0±1.1	12.1±0.8	7.1	14.7±3.8	9.7	1.37	30.4±1.8 ‡a	25.4	3.58	26.9±5.2	21.9	0.86
5.6±0.3	10.9±0.8	5.3	10.0±2.7	4.4	0.83	18.3±1.6 †a	12.7	2.40	25.6±3.1 *b	20.0	1.57
6.7±1.2	14.9±5.8	8.2	13.4±0.9	6.7	0.82	23.4±4.3	16.7	2.04	27.2±4.0	20.5	1.23
Mean ± SD					1.01±0.31			2.66±0.78			1.22±0.35

* $p \leq 0.05$
† $p \leq 0.025$
‡ $p \leq 0.005$

a WRT 37°C Control
b WRT 50mM KCl

Table 33 : Effect of TRH analogue CG3703 (0.1mM) on basal and 50mM-KCl stimulated release of (^{3}H)-acetylcholine from tissue cubes of rat septum in TRIS buffered saline, pH 7.4.

Mean $^{+S}/_{S+P}$ x 100% ± SD (n = 3), change in mean, ratio of release

0°C Control	37°C Control	37°C Δ 0°C	0.1mM CG3703	CG3703 Δ 0°C	ΔCG3703 / ΔControl	50mM KCl	KCl Δ 0°C	ΔKCl / ΔControl	CG3703 + KCl	KCl + CG3703	ΔKCl+CG3 / ΔKCl
7.1±3.3	11.6±4.1	4.5	12.0±3.4	4.9	1.09	27.4±2.0 ‡a	20.3	4.51	32.4±9.9	25.3	1.25
1.8±0.3	6.3±2.9	4.5	4.8±0.9	3.0	0.67	15.6±6.9	13.8	3.07	22.9±0.9	21.1	1.53
5.6±0.5	12.0±3.3	6.4	8.7±1.8	3.1	0.48	22.4±1.8 †a	16.8	2.62	20.4±4.9	14.8	0.88
8.9±1.7	10.2±2.7	1.3	14.5±1.6	5.6	4.3	25.7±1.4 ‡a	16.8	12.90	35.2±2.8 †b	26.3	1.56
6.0±2.1	10.8±1.1	4.8	8.1±5.3	2.1	0.44	28.0±5.3 †a	22.0	4.58	21.9±8.7	15.9	0.72
Mean ± SD					1.39±0.64			5.54±4.21			1.19±0.38

†p ≤ 0.025
‡p ≤ 0.005

a WRT 37°C Control
b WRT 50mM KCl

Table 34 : Effect of TRH analogue CG3703 (0.1mM) on basal and 50mM-KCl stimulated release of (^{3}H)-choline from tissue cubes of rat septum in TRIS buffered saline, pH 7.4.

Mean $^S/_{S+P}$ x 100% ± SD (n = 3), change in mean, ratio of release											
0°C Control	37°C Control	37°C Δ 0°C	0.1mM CG3703	CG3703 Δ 0°C	$\frac{\Delta CG3703}{\Delta Control}$	50mM KCl	Δ KCl 0°C	$\frac{\Delta KCl}{\Delta Control}$	CG3703 + KCl	KCl+CG3703 Δ 0°C	$\frac{\Delta KCl+CG37}{\Delta KC}$
7.2±2.1	16.6±5.5	9.4	23.3±6.0	16.1	1.71	25.6±4.1	18.4	1.96	32.0±8.0	24.8	1.35
5.6±0.7	15.7±3.4	10.1	15.0±0.4	9.4	0.93	23.6±9.4	18.0	1.78	26.7±9.7	21.1	1.17
11.2±3.4	17.6±4.2	7.4	16.0±2.7	4.8	0.65	*a 25.4±2.1	14.2	1.92	22.4±5.9	11.2	0.79
12.3±2.3	15.3±1.6	3.0	15.9±2.9	3.3	1.10	*a 23.7±2.2	11.4	3.80	*b 31.4±4.0	19.1	1.67
11.5±1.5	15.9±7.4	4.4	12.4±3.4	0.9	0.20	28.9±9.2	17.4	3.95	18.7±4.7	7.2	0.41
Mean ± SD					0.92±1.05			2.68±1.09			1.08±0.49

* $p < 0.05$

a WRT 37°C Control
b WRT 50mM KCl

3.3.1 Conclusion to the findings of the experiments on acetylcholine and choline release

Contrary to the work on acetylcholine release reported in the literature (Wonnacott and Marchbanks, 1976; Wonnacott, 1980), this study found that high extracellular KCl concentrations caused the release of both (^{3}H)-acetylcholine and (^{3}H)-choline from brain tissue preparations. The percentage release of (^{3}H)-choline relative to the (^{3}H)-choline pool in the tissue was about half that of (^{3}H)-acetylcholine relative to the (^{3}H)-acetylcholine pool under depolarising conditions caused by 50mM-KCl (Table 35). This value was more consistent in the nucleus accumbens than in the septum.

TRH and its analogues (0.1mM) had no significant effect on basal nor 50mM-KCl stimulated release of either (^{3}H)-acetylcholine or (^{3}H)-choline. The ratio release of (^{3}H)-acetylcholine/(^{3}H)-choline was unaffected by these compounds.

Table 35 : (^{3}H)-acetylcholine release relative to (^{3}H)-choline release in the presence of drugs and 50mM-KCl, from tissue cubes of rat nucleus accumbens and septum in TRIS buffered saline, pH 7.4.

	$\frac{\Delta \text{ Drug}}{\Delta \text{ Control}}$ ACh / $\frac{\Delta \text{ Drug}}{\Delta \text{ Control}}$ Ch				$\frac{\Delta \text{Drug+KCl}}{\Delta \text{ KCl}}$ ACh / $\frac{\Delta \text{ Drug+KCl}}{\Delta \text{ KCl}}$ Ch		
Region	0.1mM TRH	0.1mM CycloHisPro	0.1mM CG3703	50mM KCl	50mMKCl +0.1mMTRH	50mMKCl + 0.1mMCycloHisPro	50mM KCl + 0.1mM CG3703
Nucleus	0.37		1.16	1.34	1.21		1.16
accumbens	1.01		1.43	2.82	0.76		0.79
	1.31		0.74	1.87	1.17		0.68
	1.02			1.79	1.24		
	1.16			2.52	1.55		
	1.04			1.89	0.88		
Mean$^+$SD	0.98$^+$0.32		1.11$^+$0.35	2.04$^+$0.54	1.14$^+$0.29		0.88$^+$0.25
Septum	1.20	0.28	0.64	2.30	0.91	0.72	0.92
	0.91	0.72	0.72	1.72	1.15	0.73	1.31
	1.09		0.74	1.36	1.25		1.11
	1.52	1.73	3.91	3.40	1.08	1.05	0.93
	0.34	0.51	2.00	1.16	1.70	1.24	1.76
	3.55	3.20		12.01	1.04	1.28	
Mean$^+$SD	1.44$^+$1.11	1.29$^+$1.20	1.60$^+$1.41	3.65$^+$4.17	1.19$^+$0.27	1.00$^+$0.7	1.21$^+$0.34

IV DISCUSSION

4.1 Scope of thesis

The main aim of this thesis was to study the effect of TRH on the release of DA and ACh in order to provide further evidence of a role for TRH as a neurotransmitter or neuromodulator in the mammalian central nervous system (CNS). Before a chemical can be defined as a neurotransmitter, the following criteria must be fulfilled:

1. The chemical must be stored in the nerve endings from which it is released.
2. It must be released upon pre-synaptic stimulation and shown to be present in the extracellular fluid in the vicinity.
3. When applied post-synaptically it must mimic the action seen when the presynaptic system is stimulated.
4. Specific antagonists should be recognised which prevent the action of both the chemical itself and electrical stimulation.
5. Some mechanism must be present to limit the duration of action of the chemical at its post synaptic receptors.

These criteria were originally formulated from evidence for a neurotransmitter role for acetylcholine (Bachelard, 1974) and have been applied to other putative neurotransmitters.

Part of criterion (3) was examined for TRH in this thesis : the search for an effect of exogenously applied TRH on the release of other better classified neurotransmitters, to try and obtain biochemical support for the hypothesis based on behavioural studies that one action of TRH is to stimulate dopamine release and to investigate whether any effect observed was direct or via modulation by cholinergic mechanisms.

The effects of TRH and some of its analogues on the release of dopamine from rat nucleus accumbens and striatum and of acetylcholine from nucleus accumbens and septum were studied using different tissue preparations and release assays. The relative merits of the preparations and assays are discussed below, followed by discussion of the results obtained in their use.

4.2 Tissue Preparations

4.2a) General

In-vitro tissue preparations are important to the study of the biochemistry of the mammalian CNS because they allow the observation of the metabolism and release of specific neurotransmitters in specific brain regions, which could not be performed in-vivo or in intact brain in-vitro. Tissue cube or synaptosome (P2) preparations, for example, allow neurotransmitter release to be followed directly instead of by inference based on changes in metabolite levels in the cerebrospinal fluid, urine or plasma.

In-vitro preparations also allow the manipulation of neurotransmitter biochemistry so that regulating factors and the effects of the activity of one transmitter system on another can be determined.

Tissue cubes and P2 preparations were used in this study.

4.2b) Tissue cubes

Tissue cube preparations may be regarded as very crude homogenates in which the fragments of tissue still retain some structural integrity so that the relationship between the nerve endings and associated cells is largely unchanged. The chopping process increases the surface area of the tissue for the uptake of radiolabelled transmitters and precursors, allows a greater proportion of released transmitters to diffuse into the incubation medium and increases the accessibility of the receptor sites for drugs.

Tissue cubes of the nucleus accumbens were found to be largely undamaged by the 30 minute incubation and repeated centrifugation involved in the loading of the tissue with (^{3}H)-dopamine or (^{3}H)-choline, as indicated by the similarity between the rates of oxygen uptake before and after incubation and washing and the K^+-stimulation of oxygen uptake. (Table 8). The basal levels of oxygen uptake observed were about one eighth of those observed by McIlwain and Buddle (1952) in tissue cubes of rat cerebral cortex chopped at 0.2 x 0.2 mm. Although compared with these findings for this region the rate of oxygen uptake observed in the nucleus accumbens appears very low, a possible indication of tissue damage during chopping, the observation of a K^+-stimulation of similar magnitude before and after incubation and washing indicates that the preparation sustained little further damage in this process.

Addition of 50mM-KCl to the incubation medium stimulated the release of (^{3}H)-dopamine, (^{3}H)-acetylcholine and (^{3}H)-choline from tissue cubes of rat nucleus accumbens, striatum and septum.

A tissue cube preparation of striatum was tested for the Ca^{2+}-ion dependence of the KCl stimulation, (Figure 22), and was found to be sensitive to the presence of Ca^{2+}. High extracellular K^+ concentrations are thought to depolarise neuronal membranes in a manner similar to an action potential in which an influx of Ca^{2+} ions is necessary in the transduction of the depolarisation into the release of neurotransmitter from vesicular stores in the nerve ending. This process is termed "stimulus-secretion coupling". Although Ca^{2+} is involved in this process in-vivo, other divalent cations, e.g. Ba^{2+} and Sr^{2+} but not Mg^{2+}, have been found to support the release of some neurotransmitters in-vitro (Cotman et al, 1976). Mg^{2+} ions do not couple stimuli to secretion and inhibit the effects of Ca^{2+} and the other ions when present in similar concentrations, indicating that these ions compete

either for a cell-membrane ionophore or for a site in the "coupling mechanism" within the nerve ending. The actual mechanism by which the influx of Ca^{2+} into the nerve ending couples stimulus to secretion is as yet unresolved but a number of hypotheses have been proposed.

These include:

Ca^{2+} chelation by negatively charged lipids on the inner surface of the cell membrane and/or outer surface of the vesicular membranes, increasing the frequency of membrane fusion by reducing mutual repulsion;

Ca^{2+}-induced decrease in cytoplasmic viscosity leading to an increased frequency of collisions between the vesicles and the cell membrane; and

the involvement of actin and myosin-like proteins with Ca^{2+}-binding sites equivalent to troponin-C which, when activated, physically pull vesicles into fusion proximity with the cell membrane (Maddrell and Nordmann, 1979).

The effects of high extracellular K^+-ion concentrations in stimulating release may not be identical to those of an action potential or electrically-induced depolarisation. Arbilla and Langer (1978) reported that 40mM-K^+ stimulated (^3H)-dopamine release from slices of rat striatum by up to 40% in the absence of Ca^{2+} ions. In this study an approximately 19% stimulation of release was observed in the absence of Ca^{2+} (Figure 22). O'Fallon et al (1981) suggested that the Ca^{2+} independent component of release came from the cytoplasmic "pool" of neurotransmitter, mediated by K^+-inhibition of the Na^+,K^+-ATPase. These workers found that 20 μM-ouabain, a specific inhibitor of the Na^+,K^+-ATPase, also stimulated (^3H)-dopamine release from tissue cubes of rat striatum without a depolarisation effect.

50mM-K^+ on its own was only found to cause the release of a small fraction of the total releasable pool of (^3H)-dopamine, as confirmed by the effect of 1mM-tyramine (Figure 19), which displaces catecholamines from all storage pools within the tissue, of which the vesicular stores

are only a small part (Javoy and Glowinski, 1971).

The relative integrity of the tissue cube preparation, while allowing intercellular interactions to occur, is disadvantageous with respect to the penetration of drugs to receptor sites not on the surface of the fragments, and metabolism of drugs by enzymes present in the preparation. High concentrations of drug have to be used to overcome these problems. 10^{-4}-10^{-3}M-TRH was found to be necessary for noticeable stimulation of the release of (^{3}H)-dopamine. Sharp et al (1981) found that 10^{-4}M-TRH was required to stimulate release of endogenous dopamine from tissue cubes of nucleus accumbens ($\simeq$ 3%) and septum ($\simeq$ 33%).

To reduce the possibility of degradation of TRH by the tissue cube preparations, all the media used contained the peptide antibiotic Bacitracin.

McKelvy et al (1976) reported that Bacitracin (1.7×10^{-4}M) completely inhibited TRH peptidase activity in guinea-pig brain subcellular fractions. Knisatschek and Bauer (1979) and Bauer and Nowak (1979) found that 10mM-Bacitracin inhibited by 65% the TRH aminopeptidase from porcine serum whereas 10^{-5}M-Bacitracin inhibited by 55% the TRH amidase from bovine anterior pituitary. More recently, Busby et al (1982) reported that as much as 20mM-Bacitracin was required to inhibit pyroglutamate aminopeptidase and proline endopeptidase (TRH amidase) in rat brain homogenates. These workers suggest that the effects of Bacitracin are non-specific but do not explain why other workers have reported inhibition of TRH degradation over a range of lower concentrations of Bacitracin.

In this study 1.7×10^{-5}M-Bacitracin was added to all the media used because this was the concentration used by Kerwin and Pycock (1979), who reported stimulation of release of (^{3}H)-dopamine from tissue cubes of rat nucleus accumbens (up to 100%) with 25 - 100 μM-TRH.

The tissue-cube preparation, therefore was found to be viable with respect to the study of neurotransmitter release and the limitations in its use were taken into account.

4.2c) P2 preparations

P2 preparations may be regarded as refined tissue homogenates in which a sub-population of tissue fragments, the nerve-ending particles, are selectively concentrated. The method used for P2 preparation was the same as that used by Shapiro (1978). No density-gradient centrifugation was used to purify further the nerve-ending particles so the P2 suspension also contained fragments of myelin, non-synaptosomal vesicles and mitochondria. This was confirmed by electron micrography (Figures 23, 24 and 25), which also revealed the presence of relatively highly stained particles, containing subcellular vesicles and mitochondria, some associated with post-synaptic membranes. The high percentage ($>$ 70%) of the lactate dehydrogenase occluded by the preparations in all cases confirmed that the preparations contained a high proportion of sealed subcellular particles.

50mM-KCl stimulated the release of (^{3}H)-dopamine from P2 preparations. A P2 preparation of striatum was found to exhibit Ca^{2+} sensitivity to 50mM-KCl stimulated release of (^{3}H)-dopamine (Figure 27), suggesting that release by stimulus-secretion coupling was taking place, although the effect of Ca^{2+} was not as marked as that in the tissue cube preparation. A Ca^{2+}-independent K^+-stimulated release of about 41% was observed.

P2 preparations provide a simplified system for the study of neurotransmitter biochemistry because there are no diffusional barriers for drugs and released neurotransmitters can diffuse quickly into the incubation medium. An additional advantage is that washed or purified P2 preparations do not degrade TRH (Parker et al, 1977; Shapiro et al 1980; Biggins et al, 1981;) unless the preparation is subjected to lysis of vesicles and the release of cytoplasmic enzymes (Griffiths et al 1980).

One disadvantage is that, as synaptosomes are isolated from one another, these preparations only allow the study of direct drug-receptor interactions. Indirect effects of a drug on neurotransmitter biochemistry cannot be observed without a greater structural integrity. The concentration of TRH used (10^{-3}M) was the same as that used by Shapiro et al (1980) and the effects on (^{3}H)-dopamine release were similar to those observed in the tissue cube preparation, suggesting that lack of diffusion to receptors is not the limiting factor in the releasing effects of TRH.

In conclusion, although both tissue cube and P2-preparations were found to be viable with respect to uptake and release of (^{3}H)-dopamine, exhibiting stimulus-secretion coupled release, the tissue-cube preparation appeared to be the better of the two in these respects. Moreover, tissue cubes were more easily and rapidly prepared than were P2 preparations, with fewer preparatory stages being involved. In general, basal release from tissue cubes was lower than that from P2 preparations, reducing the background over which the small effects of TRH and its analogues had to be observed.

Tissue cubes appeared to be the more convenient preparation with which to study the effects of TRH on the release of neurotransmitters.

4.3 Release Assays

4.3a) Filtration

The filtration assay was similar to that used by Raiteri et al (1974) except that positive pressure instead of negative pressure was used to separate the incubation medium from the tissue. The apparatus used was very similar to that used by Haycock and Meligeni (1977). This type of assay provides a convenient method of pre-loading, washing and stimulating release without excessive physical manipulation of the tissue.

However, as this is a static incubation technique, problems arise when studying the release of catecholamines due to their rapid reuptake by the tissue.

To eliminate the problem of reuptake, and replace metabolised TRH, the perfusion assay was tried.

4.3b) Perfusion

This technique was essentially similar to those of Srinivasan et al (1969), McIlwain and Snyder (1970), Raiteri et al (1978), Kerwin and Pycock (1979), Kamal et al (1980) and O'Fallon et al (1981).

One advantage of perfusion is that it allows tissue to be conveniently washed without excessive manipulation.

With this technique time-courses of release may be followed easily and tissue preparations can be treated consecutively with different media, e.g., a single batch of tissue cubes could be treated with TRH, the TRH washed out and then treated with 50mM-KCl to assess the viability of that preparation.

In addition to the advantages of perfusion, the technique had a number of disadvantages, namely:

tissue cubes tended to clump together on the filters.

This led to irregularities of flow within the perfusion chamber, possible non-uniform exposure of the tissue to the TRH and therefore to erratic levels of basal release and stimulation; the technique was wasteful of reagents. Large volumes of TRH solutions had to be prepared for each assay; and

replicate incubations were not found to be easily performed on a single batch of tissue.

Upward perfusion was tried to eliminate the clumping of the tissue on the filters but the tissue cubes were found to either sink into the inlet tube or be forced into the outlet.

The static assay terminated by centrifugation was tried as an alternative to filtration and perfusion.

4.3c) Centrifugation terminated assay

The technique was similar to that used by Eitan and Herschkowitz (1977) and Shapiro et al (1980), except that it was applied to tissue-cube preparations as well as to P2 preparations.

Despite the fact that this is another static technique with the associated problems of reuptake of (^{3}H)-dopamine, there were a number of advantages of this technique, namely:

sufficient replication could be carried out to allow statistical evaluation of the results of a single experiment;

each assay was self-contained, i.e. the amount of radioactivity found in the supernatant in any tube could be directly related to that remaining in the tissue in that tube and absolute CPM released could be related to the quantity of tissue in each tube; and release could be terminated rapidly by cooling and immediate centrifugation.

The tissue was vortexed continually over the period of the assay in an attempt to increase the diffusion of released neurotransmitter into the incubation medium and so to reduce the extent of reuptake of (^{3}H)-dopamine. This assay was also used for the release of (^{3}H)-acetylcholine and (^{3}H)-choline, where reuptake would not interfere with the results obtained.

The centrifugation-terminated assay was the one most used in this study.

4.4 Discussion of Results

4.4a) Release of dopamine

1) Filtration

10^{-3}M TRH in the filtration assay produced a variable effect on the release of (^{3}H)-dopamine from nucleus accumbens tissue cubes, ranging from no stimulation to 55% stimulation relative to the controls.

This variation was probably due to a combination of the drawbacks of this assay and lack of uniform penetration of the TRH. These results prompted the change to the perfusion assay.

2)Perfusion

The medium for this assay was changed from the HEPES buffered saline of Dayton et al (1979), which was used in the filtration assay, to the TRIS buffered saline used previously by Shapiro (1978) and Shapiro et al (1980).

Shapiro (1978) reported that TRH (10^{-3}M) stimulated dopamine release from rat striatal P2 preparations to a greater extent at pH 6.5 compared with pH 7.4. This study supported this observation in tissue cubes of nucleus accumbens since 5×10^{-4}M TRH had little or no effect on the release of (^{3}H)-dopamine at pH 7.4 whereas at pH 6.5 this concentration of peptide caused a small (approximately 20%) stimulation of release. One explanation for this pH effect may be that the optimum pH for TRH inactivation by TRH amidase, isolated from rat hypothalamus, is pH 7.38 and that this activity falls off sharply on either side of the optimum so that at pH 6.5 it is only about 40% that at pH 7.38 (Griffiths et al, 1980).

A second possibility is the effect of pH on the ionisation of the histidine residue in the TRH molecule. At pH 7.4 this group is only 28% ionised whereas at pH6.5 it is 76% ionised. If the TRH receptor has a greater affinity for the ionised histidine residue than for the unionised residue then the effects of TRH will be seen more clearly at pH values below 7.0. Kerwin and Pycock (1979) observed TRH-stimulated (^{3}H)-dopamine release ($\simeq$ 100%) at pH 7.4 but did not look for a pH effect on this release.

At pH6.5 the buffering capacity of TRIS (pKa = 8.6) is very low so in an attempt to study the effects of pH on TRH-stimulated release and retain a good buffering capacity, the TRIS in the perfusing medium was changed for an equimolar concentration of imidazole (pKa = 7.0).
No stimulation of (^{3}H)-dopamine release by 5 x 10^{-4}M TRH was observed when TRIS was replaced by imidazole at either pH 6.5 or pH 7.4. In view of the similarity between histidine and the imidazole in the saline, it would seem possible that imidazole could act as an antagonist of TRH receptors, particularly since the ratio of imidazole to TRH was 400:1. The competition of imidazole with TRH was not pursued further because it was necessary to try and establish a definite effect of TRH on dopamine release before studying the influence of TRH-inhibitors on that effect.

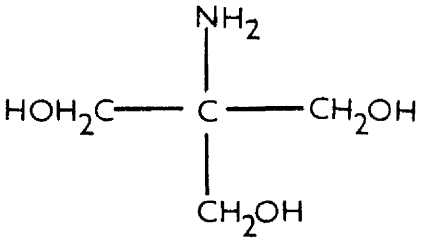
Variable results were obtained when the TRIS saline was replaced by HEPES saline, ranging from inhibition, through no effect, to stimulation of the releaseof (^{3}H)-dopamine by 5 x 10^{-4}M TRH. There is little similarity between HEPES and any part of the TRH molecule (Figure 28) so the reason(s) for the lack of consistency of TRH in this saline remains unclear. Since assays using HEPES buffered saline produced such variable results, the use of this saline was discontinued and TRIS buffered saline was used in all subsequent assays.

3)Centrifugation-terminated release in tissue cubes

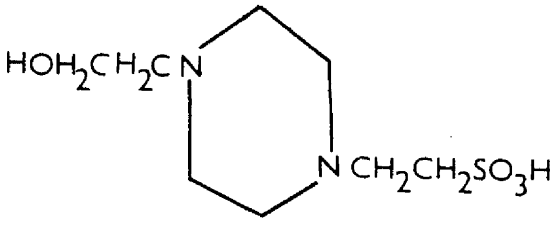
The results obtained using this technique were qualitatively similar to those obtained using the perfusion technique, viz: no clear effect of TRH (10^{-6}M - 10^{-3}M) on the release of (^{3}H)-dopamine at pH 7.4 but an apparent concentration dependent stimulation of release of up to 45% with 10^{-3}M TRH at pH 6.5.

The analogue CG3703 (10^{-6}-10^{-3}M) appeared to stimulate release in a concentration dependent manner at pH 6.5 but not at pH 7.4, but its effects were less marked than those of TRH (up to 28%).

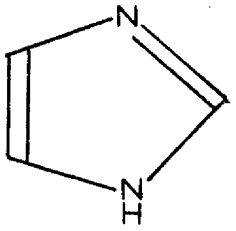
Figure 28 : Comparison of the structures of compounds used in buffered salines with the structure of TRH.



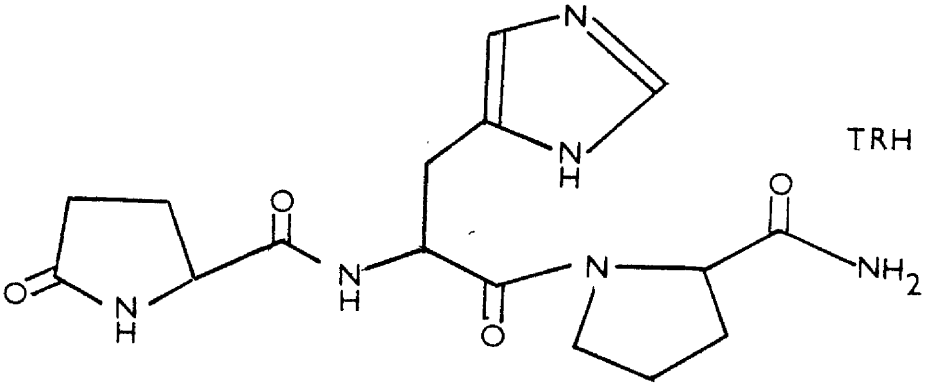
2-amino-2-hydroxymethylpropane-1,3-diol (TRIS)



N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid (HEPES)



Imidazole



TRH

This study provides no evidence that CG3703 is more potent than TRH in stimulating the release of (^{3}H)-dopamine but gives no indication of whether or not the effects of CG3703 outlive those of TRH due to an increased biological half-life, as Flohé et al (1981) advocate. However, this study does support the pharmacological studies of Hennies et al (1981), who suggested that CG3703 interacts with dopaminergic neurones.

4) Centrifugation terminated release in P2 preparations

This study supports the results of Shapiro et al (1980) in that 10^{-3}M TRH stimulated the release of (^{3}H)-dopamine from striatal P2 preparations to a greater extent at pH 6.5 compared with pH 7.4. Nevertheless, TRH had only at best a slight and variable stimulatory effect on (^{3}H)-dopamine release from striatal P2 preparations. (4-88%). In nucleus accumbens P2 preparations a more consistent stimulation of release by 10^{-3}M TRH was observed, at pH 6.5 (9-34%) but not at pH 7.4. This agreed with the results of the tissue cube experiments.
The metabolite of TRH, histidylproline diketopiperazine (cyclo His Pro, 10^{-3}M,), proposed by Prasad et al (1977) to be a more active form of TRH in some behavioural studies, also appeared to stimulate the release of (^{3}H)-dopamine from P2 preparations of nucleus accumbens (15-42%) and striatum (13-91%) at pH 6.5 but its effects were not markedly greater than those of TRH in the nucleus accumbens and were no less variable in the striatum. An effect of cyclo His Pro but not TRH in P2 preparations would have implied that TRH underwent processing in tissue cubes to the cyclic dipeptide, which then stimulated the release of dopamine. The results obtained indicate that this does not occur, or at least that if processing to cyclo His Pro does occur then TRH receptors will accept it and TRH equally well. The increased potency of cyclo His Pro over TRH which has been observed in-vivo may be consequence of the deactivation of TRH by deamidation, which the cyclic dipeptide does not undergo.

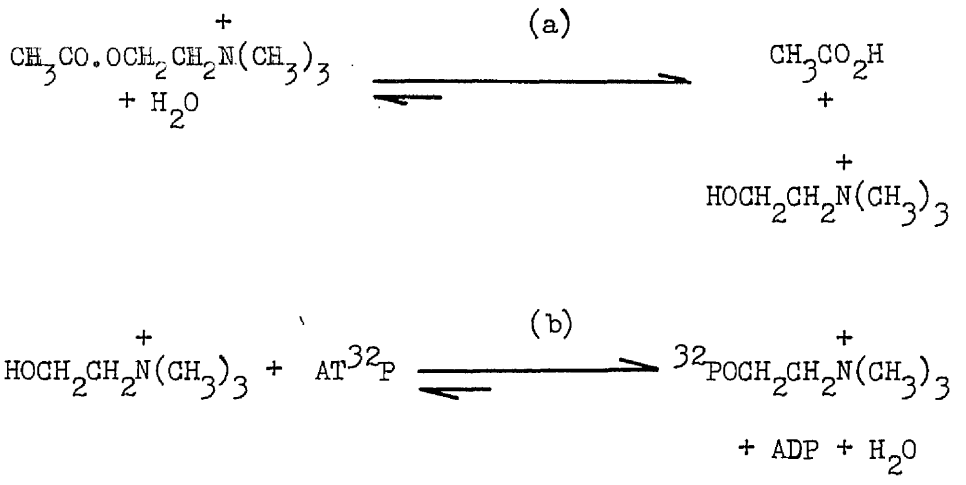
In general, the work on TRH-dopamine interactions presented in this thesis is in agreement with the findings of other workers (Kerwin and Pycock, 1979), that TRH and its analogues stimulate the release of (^{3}H)-dopamine from tissue preparations of rat nucleus accumbens. The findings also agree with those of Shapiro et al (1980) in that the effects observed are dependent in magnitude on pH. TRH was not observed to stimulate consistently the release of (^{3}H)-dopamine from P2 preparations of nucleus accumbens or striatum at pH 7.4 whereas Shapiro (1978) found a consistent stimulation of release at pH 7.2 (15-59%) and a larger stimulation at pH 6.6 (149%) from striatal P2 preparations.

The requirement for high concentrations of TRH ($\geq 10^{-4}$M) to produce a slight stimulation of (^{3}H)-dopamine release agrees with the findings of Sharp et al (1981), who found high concentrations of TRH to be necessary to stimulate release of endogenous dopamine.

In view of evidence implicating TRH in cholinergic processes (Section 1.3.3b 1) and the reported effects of acetylcholine on dopamine release (Langer, 1974; Criorguieff et al, 1975; de Belleroche and Bradford, 1978; de Belleroche and Gardiner, 1982), it seemed reasonable to study the effects of TRH and its analogues on acetylcholine release in the regions of rat brain best correlated to its behavioural effects, i.e. the nucleus accumbens and the septum.

4.4b) Release of acetylcholine

Acetylcholine (ACh) is not a convenient substance to assay directly and most methods apart from bioassay entail hydrolysis by acetylcholinesterase followed by further reactions yielding a substance which can be more easily determined, e.g.



(a) = acetylcholinesterase (E.C.3.1.1.7)

(b) = choline kinase (E.C.2.7.1.32)

Choline-^{32}P-phosphate is passed down a cation exchange column, which retains residual AT^{32}P, and is estimated by liquid scintillation counting (Cooper et al, 1978).

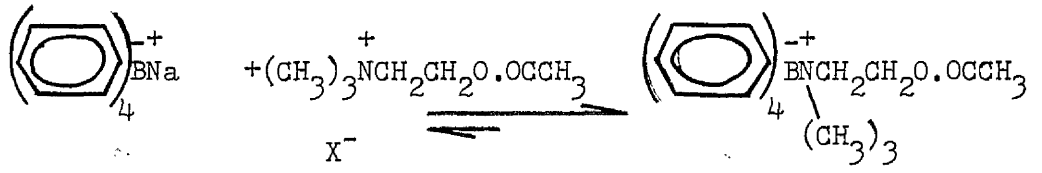
It is more convenient to measure directly the release of (^{3}H) or (^{14}C)-ACh although the relationship between the release of radiolabel as a percentage of the total radioactivity in the tissue and the release of endogenous ACh from the total releasable pool cannot be readily determined.

An additional complication with ACh is that tissue cannot be directly pre-loaded with (^{3}H)-ACh because choline (Ch), not ACh, is taken up by the tissue and is subsequently acetylated (Cooper et al 1978).

In order to assay ACh specifically, it must be separated from Ch. In release assays such as those used in this study a great dilution takes place when the neurotransmitter is released into the incubation medium so the ACh and Ch in the medium must first be concentrated.

Fonnum (1969) used a form of liquid ion exchange with a "resin" consisting of sodium tetraphenylboron in butylethylketone. The extraction of acetylcholine and choline followed the following scheme:

a)Formation of an organic-soluble complex:

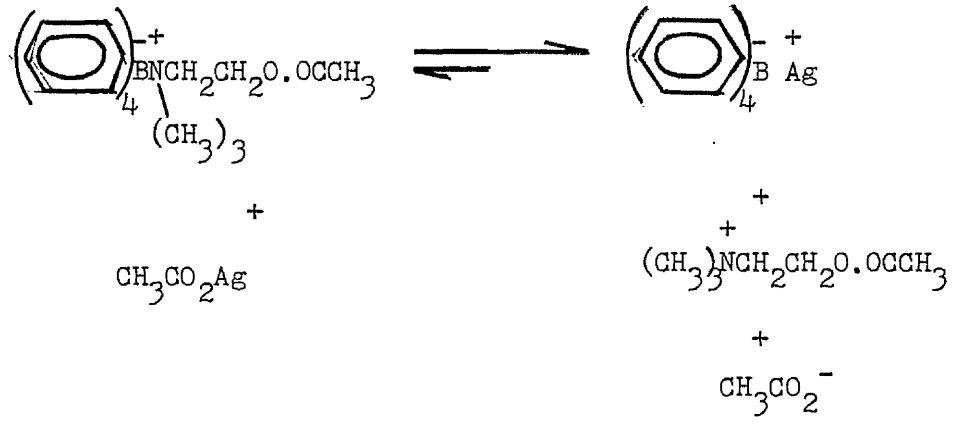


Organic Aqueous Organic

Aqueous

b)Separation of the organic and aqueous layers

c)Breaking of the organic complex with Ag^+ ions:



Marchbanks and Israel (1971) added a washing step with diethyl ether to remove contaminating tetraphenylboron and acidification to remove excess silver acetate.

d)Separation of water layer and evaporation to dryness in a desiccator.

The extracted acetylcholine was redissolved in a small volume of methanol containing choline and acetylcholine as markers for the separation on cellulose TLC.

This technique is sensitive to the organic solvent used. Fonnum (1969) found the best solvents to be vinylacetonitrile or butyl ethyl ketone/ acetonitrile (5 : 1 v/v), which extracted about 90% of the ACh from the aqueous phase.

The volatile and toxic solvents used in this technique require the extraction to be performed in the fume cupboard and care must be taken in separating the organic and aqueous layers to prevent an emulsion forming on the interface. (Marchbanks and Israel, 1971).

In this study, (^{3}H)ACh and (^{3}H)Ch in the medium were concentrated by freeze-drying and re-dissolved in a small volume of methanol containing ACh and Ch as markers for the separation on cellulose TLC plates. This process was simpler and non-toxic compared with the organic extraction, was not found to hydrolyse the ACh and also gave $\simeq$ 90% recovery of ACh (Table 24).

The R_F values obtained on cellulose TLC in butan-1-ol/water/ethanol/glacial acetic acid solvent (0.43 for ACh, 0.33 for Ch) were lower than those reported by Marchbanks and Israel (1971), who found 0.56 for ACh and 0.45 for Ch, but were found to be consistent between experiments (Figure 15).

Using the TLC separation, tissue cubes of nucleus accumbens and septum were shown to acetylate (^{3}H)-Ch to (^{3}H)-ACh. It was, however, found that 40-70% of the (^{3}H)-Ch in the tissue was left unacetylated. These percentages seem rather high considering that the concentration of Ch added (0.17 μM final concentration) was well below the Km of the high affinity choline uptake mechanism (Km = 1.2 μM; Yamamura and Snyder, 1972), which is tightly coupled to ACh synthesis (Cooper et al, 1978). Yamamura and Snyder (1972) reported an inverse relationship between the concentration of Ch incubated with cortical synaptosomes and conversion of Ch to ACh. Conversion was about 16% with 10^{-4}M Ch and about 70% with 5×10^{-7}M Ch after 15 minutes of incubation. This suggests that in this study substantial amounts of (^{3}H)-Ch were still adsorbed to the surface of the tissue even after successive washes.

The K^+-stimulation of release of (^{3}H)-Ch as well as (^{3}H)-ACh from tissue cubes of both nucleus accumbens and septum is at variance with reports on ACh release from both tissue slice (Richter and Marchbanks, 1971; Mulder et al, 1974) and P2 preparations (Wonnacott and Marchbanks, 1976; Murrin, 1977, Wonnacott, 1980, Marchbanks et al, 1981). The stimulation of (^{3}H)-Ch release observed in 50mM-KCl may therefore be due to displacement of the (^{3}H)-Ch adsorbed to the tissue by the increased K^+-ion concentration in the medium.

The K^+-induced stimulation of (^{3}H)-ACh release (200-500% over 5 minutes) was similar to that observed by other workers (Wonnacott (1980) : 188% in 20 minutes; Wonnacott and Marchbanks (1976) : 450%; Richter and Marchbanks (1971) : 560%; Mulder et al (1974) : 700% in 10 minutes).

Neither TRH (10^{-3}M) nor its analogues cyclo His Pro (10^{-3}M) and CG3703 (10^{-3}M) were found to have any significant effect on the release of (^{3}H)-Ach from tissue cubes of nucleus accumbens or septum under basal or depolarising conditions. The most consistent effect observed was that of mild inhibition by cyclo His Pro of basal (^{3}H)-ACh release from tissue cubes of septum in which most (4/5) of the preparations responded in this way. The third preparation, however, responded to 10^{-3}M cyclo His Pro with a stimulation of (^{3}H)-ACh release as large as the greatest inhibition observed.

This study provides no evidence that the effects of TRH in rats which appear to arise from some interactions with cholinergic neurones originate in either the nucleus accumbens or septum. The lesioning studies of Kalivas et al (1981) suggest that the sensitivity of the septum in behavioural paradigms may be due to effects of TRH on nerve fibres passing through that region, synapsing in some presently unidentified brain region.

Malthe-Sørenssen et al (1978) observed that TRH (28nmol, i.c.v.) reduced the ACh content and stimulated ACh turnover of rat parietal cortex, but had no effect on the ACh content or turnover of frontal cortex, striatum, hippocampus, diencephalon or brainstem. Rat parietal cortex contains high affinity TRH receptors in the same concentration as those in the septum (9.2 $\pm$ 0.9 f mol/mg protein for parietal cortex, 9.1 $\pm$ 0.6 f mol/mg protein for septum) (Taylor and Burt, 1982) so this region may be a contender for the site of the actions of TRH on cholinergic processes.

The recent finding that, in the rat, the amygdala has the highest concentration of high affinity TRH receptors of any brain region (Taylor and Burt, 1982) has opened the question of whether or not any of the behavioural effects of TRH may be mediated by this region. The amygdala, also, is rich in cholinergic nerve fibres (Jacobowitz and Palkovits, 1974).

4.5 Effects of peptides other than TRH on neurotransmitter biochemistry

The reported effects of TRH on neurotransmitter biochemistry must be discussed in context with those of other neuroactive peptides so that the importance of these effects may be kept in perspective. The effects of other peptides are outlined below:

4.5a) Endorphins

The effects of β-endorphin on in-vitro tissue preparations have been studied in a number of laboratories.

Loh et al (1976) reported that β - endorphin (0.6 - 3.0 μM) inhibited the release of (^{3}H)-dopamine from rat striatal slices by up to 99% ($p<0.02$). This effect was found to be 18-33x greater than that produced by 3.0 μM-morphine and was reversed by 3.0 μM-naloxone ($p<0.01$). The pentapeptide methionine-enkephalin (met^5-enkephalin), which has the same structure as the first five amino acids of β-endorphin, did not inhibit striatal dopamine release in concentrations as high as 100 μM.

Izumi et al (1977) reported that injections of β-endorphin (15 nmol i.c.v.) into rats reduced slightly (≃ 10%) the depletion of dopamine in the striatum following α-methyltyrosine administration, suggesting an inhibition of dopamine release.

Arbilla and Langer (1978) observed that the release of (^{3}H)-noradrenaline from tissue slices of rat cerebral cortex was inhibited by 50% by 0.6 μM β-endorphin ($p<0.001$). 10 μM-morphine was required to inhibit (^{3}H)-noradrenaline release by the same extent. The inhibitory effects of both β-endorphin and morphine were inhibited by 3 μM-naloxone ($p<0.005$). These workers found no effect of β-endorphin (2 μM) or morphine (30μM) on 20mM-KCl stimulated (^{3}H)-dopamine release from tissue slices of rat striatum.

Wilkes and Yen (1980) reported that β-endorphin (0.5 nmol) inhibited the release of dopamine (DA) and dihydroxyphenylacetic acid (DOPAC), by 68-71% and 49-55% ($p<0.05$) respectively, from rat medial basal hypothalamus. Naloxone (0.5 nmol) stimulated DA release by 85-132% and DOPAC release by 120-148% ($p<0.05$). When administered together (0.5 nmol), β-endorphin and naloxone had no effect on DA or DOPAC release.

Schoemaker and Nickolson (1980) reported that des-tyr^1-γ-endorphin (amino acids 2-17 of β-endorphin) (0.06 μM) depressed K^+-evoked release of (^{3}H)-dopamine from rat striatum, nucleus accumbens and frontal cortex by almost 20% ($p<0.02$), without affecting basal (^{3}H)-dopamine release.

These findings suggest that β-endorphin and its derivatives have potent inhibitory effects on the release of dopamine and noradrenaline which are probably elicited by presynaptic interactions with opiate receptors.

4.5b) Melanostatin (Melanocyte stimulating hormone release inhibiting factor, MIF

Friedman et al (1973) observed that MIF (1.0 and 4.0 mg/kg i.p.) administered chronically to rats for four days increased significantly (17% and 25% respectively, $p<0.01$) striatal dopamine content but not hypothalamic noradrenaline content, relative to that in animals given the saline vehicle alone.

Acute doses of up to 5 mg/kg i.p. had no effect on the levels of either catecholamine.

Shapiro et al (1980) found that MIF (10^{-3}M) stimulated the release of (^{3}H)-dopamine from P2 preparations of rat striatum by 14-48% at pH 7.2 ($p<0.05$) and by 85% at pH 6.6 ($p<0.05$) in the presence of 1.25 mM-$CaCl_2$. In the presence of 2mM-EDTA, 10^{-3}M MIF inhibited (^{3}H)-dopamine release 7% at pH 7.2 and 31-51% ($p<0.05$) at pH 6.6.

4.5c) Other peptides

Malthe-Sørenssen et al (1978) observed that i.c.v. injections of neurotensin (6 nmol), angiotensin II (4.8 nmol) and somatostatin (6.1 nmol) reduced the ACh content of rat parietal cortex 33%, 39% and 28% respectively ($p<0.01$) without affecting Ch content.

The same doses of neurotensin and somatostatin stimulated ACh turnover in the diencephalon by about 71% ($p<0.01$). Somatostatin (6.1 nmol i.c.v.) stimulated ACh turnover in the brainstem by 139% ($p<0.01$). Higher doses of somatostatin, (15-21 nmol i.c.v.) stimulated ACh turnover in the hippocampus by 108-140% ($p<0.005$).

The effects of other peptides on neurotransmitter biochemistry, especially the inhibitory effects of endorphins on catecholamine release, appear to be greater than the reported stimulatory effects of TRH and require much lower peptide concentrations in-vitro. Tache et al (1977), however, observed that the depression of motor activity in rats given β-endorphin (50 μg i.c.v.), about 40% ($p<0.05$) was completly reversed by TRH (10 μg i.c.v.), which stimulated motor

activity by about 125% relative to the control ($p<0.005$) in the presence of β-endorphin. In the absence of β-endorphin, TRH stimulated motor activity by about 100% relative to the control ($p<0.005$).

These findings suggest that the difference in potencies of TRH and β-endorphin in-vitro may not necessarily indicate that TRH has less intrinsic biological activity but that the tissue preparations presently in use may be in some way unsuitable to exhibit the effects of TRH.

4.6 Summary to Discussion

TRH appears to stimulate to a small extent the release of (^{3}H)-dopamine from tissue preparations of rat nucleus accumbens and striatum. This effect is more clearly seen at pH 6.5 than at pH 7.4 and is more consistent in the nucleus accumbens than in the striatum. The TRH analogues cyclo His Pro and CG3703 appear to have effects on the release of (^{3}H)-dopamine similar to those of TRH. The reported increased potency of the latter two peptides in behavioural paradigms may therefore be due to resistance to degradation rather than to increased biological activity.

The effects of TRH on dopamine release in the nucleus accumbens appear to be direct and not mediated via cholinergic effects on dopamine release since TRH stimulates (^{3}H)-dopamine release from P2 preparations, but not of ACh from tissue cubes.

TRH, cyclo His Pro and CG3703 have not been found to have any consistent effect on ACh or Ch release from tissue cubes of nucleus accumbens or septum. On this basis, the reported behavioural effects of TRH which appear to arise from interactions of the peptide with cholinergic processes do not appear to originate in the nucleus accumbens or septum per se, but may require the presence of neurones connecting these regions to presently unidentified sites of ACh release.

Interactions of TRH with other peptides, e.g. β-endorphin, which have been found to inhibit dopaminergic activity, may consitute a fine control mechanism in the CNS.

V REFERENCES

References

Arbilla, S. and Langer, S.Z. (1977) Nature (London) 271 559-560

Bachelard, H.S. (1974) "Brain Biochemistry" (Goodwin, T.W. and Ashworth, J.M., editors) Chapman and Hall, London

Bauer, K., Gräf, K.J., Faivre-Bauman, A., Beier, S., Tixier-Vidal, A., and Kleinkauf, H. (1978) Nature (London) 274 174-175

Bauer, K. and Nowak, P. (1979) European J.Biochem 99 239-246

Bennett, G.W., and Edwardson, J.A. (1977) J.Endocrinol 73 27P-28P

Bhargava, H.N. (1980) Life Sci 26 845-850

Biggins, J.A., Dodd, P.R., Edwardson, J.A., Hardy, J.A., McDermott, J.R. and Smith, A.I. (1981) Abstract, TRH Satellite Symposium 8th International Society for Neurochemistry Meeting, Nottingham p25.

Björkman, S., Lewander, T., Karlsson, J-A., Koskinen, L-O, and Zetterstrom, T. (1981) J.Pharm.Pharmacol 33 580-585

Boschi, G., and Rips, R. (1981) Abstract, TRH Satellite Symposium, 8th International Society for Neurochemistry Meeting, Nottingham p 36

Breese, G.R., Cott, J.M., Cooper, B.R., Prange Jr., A.J. and Lipton, M.A. (1974) Life Sci 14 1053-1063

Breese, G.R., Cott, J.M., Cooper, B.R., Prange Jr., A.J., Lipton, M.A. and Plotnikoff, N.P. (1975) J. Pharmacol. Exp.Ther. 193 (i) 11-22

Brewster, D., Dettmar, P.W., Lynn, A.G., Metcalf, G., Morgan, B.A. and Rance, M.J. (1980) European J. Pharmacol. 66 65-71

Brewster, D., Humphrey, M.J. and Wareing, M.V. (1981) Neuropeptides 1 (3) 153-165

Brownstein, M.J., Palkovits, M., Saavedra, J.M., Bassiri, R.M. and Utiger, R.D. (1974) Science 185 267-268

Burgen, A., Kosterlitz, H.W. and Iversen, L.L. (Editors) "Neuroactive Peptides", Proceedings of the Royal Society of London discussion meeting, London 1980.

Burgess, S.K., Luttinger, D., Hernandez, D., Nemeroff, C.B. and Prange Jr., A.J. Abstract, TRH Satellite Symposium, 8th International Society for Neurochemistry meeting, Nottingham, p.29.

Burgus, R., Dunn, T.E., Desiderio, D. and Guillemin, R. (1969) C.R. Acad. Sc. Paris 269 1870-1873

Burt, D.R. (1978) Neurosci. Abst. 4 406

Burt, D.R. and Snyder, S.H. (1975) Brian Res. 93 309-328

Burt, D.R. and Taylor, R.L. (1980) Endocrinology 106 (5) 1416-1423

Busby, W.H., Youngblood, W.W. and Kizer, J.S. (1982) Brain Res. 242 261-270

Cohn, M.L., Cohn, M. and Taylor, F.H. (1975) Brain Res. 96 134-137

Cooper, J.R., Bloom, F.E. and Roth, R.H. (Joint Authors) (1978) "The Biochemical Basis of Neuropharmacology" Third Edition, Oxford University Press, New York

Costall, B., Hui, S-C, Metcalf, G. and Naylor, R.J. (1979) European J. Pharmacol 53 143-150

Cotman, C.W., Haycock, J.W. and White, W.F. (1976) J. Physiol. 254 475-505

Cott, J.M., Breese, G.R., Cooper, B.R., Barlow, T.S. and Prange Jr., A.J. (1975) J. Pharmacol. Exp. Ther. 196 594-604

Cott, J.M. and Engel, J. (1977) Psychopharmacology 52 145-149

Cowan, A. and Vaught, J.L. (1981) Abstract, TRH Satellite Symposium, 8th International Society for Neurochemistry meeting, Nottingham, p.40.

Coyle, J.T. and Henry, D. (1973) J.Neurochem. 21 61-67

Dayton, M.A., Geier, G.E. and Wightman, R.M. (1979) Life Sci. 24 917-924

de Belleroche, J.S., and Bradford, H.F. (1978) Advances in Biochemical Psychopharmacology Vol.19. (P.J.Roberts, Editor) Rowen Press, New York

de Belleroche, J.S. and Gardiner, I.M. (1982) Br. J. Pharmac. 75 359-365

Dettmar, P.W., Lynn, A.G and Metcalf, G. (1981) Abstract, TRH Satellite Symposium, 8th International Society for Neurochemistry meeting, Nottingham. p.44.

Dimitrihoudi, M., Hanson-Norty, E. and Jenner, F.A. (1974) Lancet 1 456

Dyer, R.G. and Dyball, R.E.J. (1974) Nature (London) 252 486-488

Edwardson, J.A., Isaacs, J., Dodd, P.R., Decotte, P.M., Demeneze, C.E., Lloyd, G.E. and Dockray, G.J. (1980) "Biochemical and Cellular Aspects of Parvocellular Neurosecretion". In "Role of Peptides in Neuronal Function". J.L.Barker and T.G. Smith, Editors, Marcel Dekker, New York

Eitan, A. and Herschkowitz, M. (1979) European J. Pharmacol 45 323-327

Fleischer, N., Burgus, R., Vale, W., Dunn, T. and Guillemin, R. (1970) J.Clin. Endocrinol 31 109-112

Flohé, L., Bauer, K., Friderichs, E., Gunzler, W.A., Herrling, S., Otting, F. and Schwertner, E. (1981) Abstract, TRH Satellite Symposium, 8th International Society for Neurochemistry meeting, Nottingham, p.30.

Fonnum, F. (1969) Biochem J. 113 291-298

Friedman, E., Friedman, J. and Gershon, S. (1973) Science 182 831-832

Fukuda, N., Nishimura, O., Shikata, M., Hatanaka, C., Miyamoto, M., Saji, Y., Nakayama, R., Fujino, M. and Nagawa, Y (1980) Chem. Pharm. Bull. 28 (6) 1667-1672

Gautivik, K.M., Iversen, J-G. and Sand, O. (1980) Life Sci. 26 995-1005

Giorguieff, M.F., le Floc'h, M.L., Westfall, T.C., Glowinski, J. and Besson, M.J. (1976) Brain Res. 106 117-131

Glowinski, J. and Iversen, L.L. (1966) J. Neurochem 13 655-669

Gordon, J.H. and Shellenberger, M.K. (1974) Neuropharmacology 13 129-137

Gray, E.G. and Whittaker, V.P. (1962) J.Anat. 96 79-87

Green, A.R., Heal, D.J., Grahame-Smith, D.G. and Kelley, P.H. (1976) Neuropharmacology 15 591-599

Griffiths, E.C., Kelley, J.A., Klootwijk, W. and Visser, T.J. (1980) Molecular and Cellular Endocrinology 18 59-67

Griffiths, E.C, McDermott, J.R., Smith, A.I. and Edwardson, J.A. (1981) Abstract, TRH Satellite Symposium, 8th International Society for Neurochemistry meeting, Nottingham, p.51.

Griffiths, E.C., Slater, P. and Webster, V.A.D. (1981) ibid p.50

Grimm, Y. and Reichlin, S. (1973) Endocrinology 93 626-631

Guillemin, R. (1978) Science 202 390-402

Haycock, J.W. and Meligeni, J.A. (1977) Life Sci. 21 1837-1844

Heal, D.J. and Green, A.R. (1979) Neuropharmacology 18 23-31

Heal, D.J., Green, A.R. and Youdim, M.B.H. (1981) Abstract, TRH Satellite Symposium, 8th International Society for Neurochemistry meeting, Nottingham. p.27.

Hennies, H-H and Flohē, L. (1982) Biochem. Pharmacol. 31 2430-2432

Hennies, H-H., Friderichs, E. and Flohē, L. (1981) Abstract, TRH Satellite Symposium, 8th International Society for Neurochemistry meeting, Nottingham, p.52

Holaday, J.W., Tseng, L-F., Loh, H.H. and Li, C.H. (1978) Life Sci. 22 1537-1544

Holtzman, S.G. (1974) Biochem. Pharmacol. 23 3029-3035

Horn, A.S., Cuello, A.C. and Miller, R.J. (1974) J. Neurochem 22 265-270

Horst, W.D. and Spirt, N. (1974) Life Sci. 15 1073-1082

Hughes, J. (Editor) (1978) "Centrally Acting Peptides" The Macmillan Press, New York.

Iversen, L.L. (1979) Scientific American 241 118-129

Izumi, K., Motomatsu, T., Chretien, M., Butterworth, R.F., Lis, M., Seidah, N. and Barbeau, A. (1977) Life Sci. 20 1149-1156

Jackson, I.M.D. and Reichlin, S. (1974) Endocrinology 95 856-862

Jackson, I.M.D. and Reichlin, S. (1977) Nature (London) 267 853-854

Jacobowitz, D.M. and Palkovits, M. (1974) J.Comp. Neur. 157 13-28, 29-42

Javoy, F. and Glowinski, J. (1971) J. Neurochem. 18 1305-1311

Johnson, M.K. (1960) Biochem. J. 77 610-618

Johnson, M.K. and Whittaker, V.P. (1963) Biochem. J. 88 404-409

Kalivas, P.W. and Horita, A. (1980) J. Pharmacol. Exp. Ther. 212 203-210

Kalivas, P.W.,Simasko S.M. and Horita, A. (1981) Brain Res. 222 253-265

Kamal, L.A., Arbilla, S. and Langer, S.Z. (1981) J. Pharmacol. Exp. Ther. 216 592-598

Kastin, A.J. Ehrensing, R.H., Schalch, D.S. and Anderson, M.S. (1972) Lancet 2 740-742

Keller, H.H., Bartholini, G. and Pletscher, A. (1974) Nature (London) 248 528-529

Kerdelhue, B., Palkovits, M., Karteszi, M. and Reinberg, A. (1981) Brain Res. 206 405-413

Kerwin, R.W. and Pycock, C.J. (1979) Br. J. Pharmacol. 67 323-325

Knisatschek, H. and Bauer, K. (1979) J. Biol. Chem. 254 10936-10943

Knox, J.H. and Jurand, J. (1976) J.Chromatogr. 125 89-101

Krebs, H.A. (1950) Biochim. Biophys Acta 4 249-269

Kreider, M.S., Winokur, A. and Utiger, R.D. (1979) Brain Res. 171 161-165

Kubek, M.J. Lorincz, M.A. and Wilber, J.F. (1977) Brain Res. 126 196-200

Lamberts, S.W.J. and Visser, T.J. (1981) European J. Pharmacol. 71 337-341

Langer, S.Z. (1974) Biochem. Pharmacol. 23 1793-1800

Loh, H.H., Brase, D.A., Sampath-Khanna, S., Mar, J.B. and Way, E.L. (1976) Nature (London) 264 567-568

Lowry, O.H., Rosebrough, N.J. Farr, A.L. and Randall, J.R. (1951) J. Biol. Chem. 193 265-275

Maddrell, S.H.P. and Nordmann, J.J. (1979) "Neurosecretion" Blackie & Son

Mailman, R.B., Frye, G.D., Mueller, R.A. and Breese, G.R. (1978) Nature (London) 272 832-833

Mailman, R.B. Frye, G.D., Mueller, R.A. and Breese, G.R. (1979) J.Pharmacol, Exp. Ther. 208 169-175

Malthe-Sørenssen, D., Wood, P.L., Cheney, D.L. and Costa, E. (1978) J. Neurochem. 31 685-691

Manberg, P.J. Nemeroff, C.B. and Prange Jr., A.J. (1979) Progress in Neuro-Psychopharmacology 3 303-314

Marchbanks, R.M. (1967) Biochem. J. 104 148-157

Marchbanks, R.M. and Israel, M. (1971) J. Neurochem. 18 439-448

Marchbanks, R.M. Wonnacott, S. and Rubio, M.A. (1981) J. Neurochem 36 379-393

Matsui, T., Prasad, C. and Peterkofsky, A. (1979) J. Biol. Chem. 245 2439-2445

McIlwain, H. and Buddle, H.L. (1952) Biochem. J. 53 412-420

McIlwain, H. and Snyder, S.H. (1970) J. Neurochem. 17 521-530

McKelvy, J.F., Le Blanc, P., Landes, C., Perrie, S., Grimm-Jorgensen, Y. and Kordon, C. (1976) Biochem. Biophys. Res. Commun. 73 507-515

Metcalf, G. (1974) Anal. Biochem. 57 316-320

Mitnick, M. and Reichlin, S. (1972) Endocrinology 91 1145-1153

Miyamoto, M. and Nagawa, Y. (1977) European J. Pharmacol 44 143-152

Miyamoto, M., Narumi, S., Nagai, Y., Shima, T. and Nagawa, Y. (1978) Japan. J. Pharmacol 29 335-347

Mora, S., Nasello, A.G. and Fieschi, L. (1979) Pharmac. Biochem. Behav. 13 137-139

Morley, J.E. (1979) Life Sci. 25 1539-1550

Mulder, A.H., Yamamura, H.I., Kuhar, K.J. and Snyder, S.H. (1974) Brain Res. 70 372-376

Murrin, L.C., De Haven, R.N. and Kuhar, M.J. (1977) J. Neurochem 29 681-687

Nagai, Y., Narumi, S., Nagawa, Y., Sakurda, O., Ueno, H. and Ishii, S. (1980) J. Neurochem. 35 963-971

Nair, R.M.G., Barrett, J.F., Bowers, C.Y. and Schally, A.V. (1970) Biochemistry 9 1103-1106

Nemeroff, C.B., Bissette, G., Manberg, P.J., Osbahr III, A.J., Breese, G.R. and Prange Jr., A.J. (1980) Brain Res. 195 69-84

O'Fallon, J.V., Brosemer, R.W. and Harding, J.W. (1981)
J. Neurochem 36 369-378

Oke, A., Keller, K. and Adams, R.N. (1978) Brain Res. 148 245-250

Oliver, C., Eskay, R.L., Ben-Jonathan, N. and Porter, J.C. (1974)
Endocrinology 95 540-546

Ono, H. and Fukuda, H. (1982) Neuropharmacology 21 739-744

Parker, Jr., R.C., Neaves, W.B., Burnea, A. and Porter, J.C. (1977)
Endocrinology 101 66-75

Phizakerley, P.J.R. and Fixter, L.M. (1973) J. Neurochem. 20 123-134

Plotnikoff, N.P., Prange, Jr., A.J., Breese, G.R., Anderson, M.S. and
Wilson, I.C. (1972) Science 171 417-418

Pollard, J.H. (1977) "A handbook of numerical and statistical techniques"
Cambridge University Press

Prange, A.J., and Wilson, I.C. (1972) Psychopharmacologia 26 Suppl.82

Prange, A.J., Wilson, I.C., Lara, P.P., Alltop, L.B. and Breese, G.R. (1972)
Lancet II 999-1002

Prasad, C., Matsui, T. and Peterkofsky, A. (1977) Nature (London) 268 142-144

Prasad, C. and Peterkofsky, A. (1976) J. Biol. Chem. 254 3229-3234

Raiteri, M., Angelini, F. and Levi, G. (1974) European J. Pharmacol
25 411-414

Raiteri, M., Cerrito, F., Cervoni, A.M. and Levi, G. (1978)
J. Pharmacol. Exp. Ther. 208 195-202

Rastogi, R.B., Singhal, R.L. and Lapierre, Y.D. (1981)
European J. Pharmacol. 73 307-312

Reichlin, S., Saperstein, R., Jackson, I.M.D., Boyd III, A.E. and Patel, Y.
(1976) Ann. Rev. Physiol 38 389-424

Reigle, T.G., Avni, J., Platz, P., Schildkraut, J.J. and Plotnikoff, N.P.
(1974) Psychopharmocologia (Berlin) 37 1-6

Renaud, L.P. and Martin, J.B. (1975) Brain Res. 86 150-154

Renaud, L.P., Martin, J.B. and Brazeau, P. (1975) Nature (London)
255 233-235

Richter, J.A. and Marchbanks, R.M. (1971) J. Neurochem 18 691-703

Rips, R., Desiles, M. and Puech, A.J. (1979) Progress in Neuro-Psycho-
pharmacology 3 337-344

Scanlon, M.F. and Hall, R. (1981) Abstract, TRH Satellite Symposium,
8th International Society for Neurochemistry meeting, Nottingham. p.32

Schoemaker, H. and Nickolson, J. (1980) Life Sci. 27 1371-1376

Schally, A.V., Arimura, A., Bowers, C.Y., Kastin, A.J., Sawano, S. and Redding, T.W. (1968) Recent Progress in Hormone Research 24 497-588

Schally, A.V., Redding, T.W., Bowers, C.Y. and Barrett, J.F. (1969) J. Biol. Chem. 244 4077-4088

Shapiro, L.M. (1978) Ph. D. thesis, University of Glasgow

Shapiro, L.M., Bowes, G.M. and Vaughan, P.F.T. (1980) Life Sci. 27 2099-2108

Sharp, T., Marsden, C.A. and Bennett, G.W. (1981) Abstract, TRH Satellite Symposium, 8th International Society for Neurochemistry meeting, Nottingham. p.28.

Shellenberger, M.K. (1971) Neuropharmacology 10 347-357

Shellenberger, M.K. and Gordon, J.H. (1971) Anal. Biochem. 39 356-372

Spindel, E. and Wurtman, R.J. (1980) Brain Res. 201 279-288

Srinivasan, V., Neal, M.J. and Mitchell, J.F. (1969) J.Neurochem. 16 1235-1244

Stanton, T.L., Winokur, A. and Beckman, A.L. (1980) Brain Res. 181 470-475

Tache, Y., Lis, M. and Collu, R. (1977) Life Sci. 21 841-846

Tan, K-N. and Tashjian Jr., A.H. (1981) J.Biol Chem. 256 8994-9002

Tatemoto, K., Cariquist, M. and Mutt, V. (1982) Nature (London) 296 659-660

Taylor, R.L. and Burt, D.R. (1981) Brain Res. 218 207-217

Taylor, R.L. and Burt, D.R. (1982) J. Neurochem. 38 1649-1656

Tsang, D., Lal, S. and Finalyson, M.H. (1980) Brain Res. 188 278-281

Tsang, D. and Martin, J.B. (1976) Life Sci. 19 911-918

Turner, J.C. (1968) Int. J. Appl. Radiat. Isotopes 19 557-563

Unsworth, C.D., Hughes, J. and Morley, J.S. (1982) Nature (London) 296 519-522

Vale, W., Rivier, C. and Brown, M. (1977) An. Rev. Physiol 39 473-527

Wilk, S. and Glick, S.D. (1976) European J. Pharmacol 37 203-206

Wilkes, M.M. and Yen, S.S.C. (1980) Life Sci. 27 1387-1391

Winokur, A. and Utiger, R.D. (1974) Science 185 265-267

Wonnacott, S. (1980) J. Neurochem. 34 1567-1573

Wonnacott, S. and Marchbanks, R.M. (1976) Biochem J. 156 701-712

Yarbrough, G.G. (1976) Nature (London) 263 523-524

Yarbrough, G.G. (1979) Progress in Neurobiology 12 291-312

Yamamura, I.Y. and Snyder, S.H. (1972) Science 178 626-628

Youngblood, W.W., Humm, J. and Kizer, J.S. (1979) Brain Res. 163 101-110

Youngblood, W.W., Lipton, M.A. and Kizer, J.S. (1978) Brain Res. 151 99-116