EFFECT OF HYPOTHALAMIC HORMONES ON PRESYNAPTIC DOPAMINERGIC MECHANISMS

by

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A Thesis submitted to the
University of Glasgow
for the degree of
DOCTOR OF PHILOSOPHY

Department of Biochemistry, October, 1978.

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Acknowledgments

I would like to thank all those who have helped me in the course of my postgraduate work, especially:

Professor R.M.S. Smellie and Professor A.R. Williamson for making available the facilities of the Department;

Dr P.F.T. Vaughan for his constant supervision and interest;

Dr L.M. Fixter for much friendly advice;

The Science Research Council for granting me a Studentship;

My family for their continued support and encouragement;

Miss M.A. Ferenth for typing this manuscript.

Abbreviations

Abbreviations used in this thesis are those recommended in the Biochemical Journal Instructions to Authors (revised 1978) with the following additions.

DA	dopamine
DOBA	3,4-dihydroxybenzoic acid
DOPAC	3,4-dihydroxyphenylacetic acid
DOPET	3,4-dihydroxyphenylethanol
HVA	4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid)
MIH	melanocyte-stimulating hormone release-inhibiting hormone (melanostatin)
3MT	3-O-methyldopamine hydrochloride
NA	noradrenaline
TRH	thyrotropin-releasing hormone (thyroliberin)

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Summary

The hypothalamic hormones melanostatin (MIH) and thyroliberin (TRH) have been shown by others to have a direct action on adrenergic neurons, distinct from their role in regulating the release of pituitary hormones. This present thesis examines the effect of MIH and TRH on the biosynthesis, release and oxidative deamination of dopamine in rat brain tissue preparations.

Neither MIH (10^{-4}M) nor TRH (10^{-4}M) altered monoamine oxidase activity in tissue slices or sucrose homogenates. Autoradiographic analysis showed that there were two products of the oxidative deamination of dopamine by striatal homogenates, one of which was DOPAC. Roughly equal amounts of DOPAC and an unknown compound were formed. MIH and TRH did not alter the total amount of, or the distribution of label between, the two metabolites.

Neither MIH (10^{-4}M) nor TRH (10^{-4}M) had any effect on the rate of dopamine synthesis by striatal homogenates. Calcium chloride (0.85 mM) stimulated dopamine synthesis by striatal synaptosomes incubated in 18.4 μM tyrosine, but neither MIH (10^{-4}M) nor TRH (10^{-4}M) altered the rate of dopamine synthesis. Neither peptide significantly stimulated the rate of dopamine synthesis in the presence of 0.85 mM calcium chloride.

Neither MIH (10^{-4}M) nor TRH (10^{-4}M) altered the rate of dopamine synthesis by striatal synaptosomes in the presence of 1.70 mM calcium chloride at 1.07 μM tyrosine. Tyramine, a sympathomimetic amine, inhibited dopamine synthesis, 50%

inhibition occurring at 3.3×10^{-5}M.

Both tyramine (10^{-4}M) and amphetamine (10^{-4}M) stimulated dopamine synthesis in the presence of 2 mM EDTA but the magnitude of the drug-induced stimulation varied inversely with the basal rate of dopamine synthesis. TRH (10^{-4}M) did not stimulate dopamine synthesis under these conditions.

Amphetamine (10^{-4}M) stimulated release of dopamine from striatal synaptosomes in the presence of 1.25 mM calcium chloride and also in the presence of 2mM EDTA. MIH (10^{-3}M) and TRH (10^{-3}M) stimulated release of dopamine only in the presence of calcium chloride.

These results suggest that MIH and TRH release dopamine from vesicular stores by exocytosis while amphetamine releases dopamine from a cytoplasmic pool regulating tyrosine hydroxylase.

I INTRODUCTION

Introduction

The hypothalamic hormones are a group of low molecular weight peptide hormones synthesised by neurons in the hypothalamus and stored in nerve endings in the median eminence. They are transported by the portal vessels to the pituitary gland where they regulate the release of the anterior pituitary hormones (Table 1a). (Blackwell and Guillemin, 1973; Reichlin et al., 1976: Schally et al., 1973, 1977). The release of peptides from the hypothalamus is itself closely regulated. For example, neurotransmitters such as acetylcholine, dopamine and serotonin, and steroid hormones (Bennett et al., 1975a, b; Edwardson and Bennett, 1974) can modify the release of the hypothalamic hormones. For example, dopamine stimulated the release of luteinizing hormone - releasing hormone (Bennett et al., 1975a), while acetylcholine stimulated the release of corticotropin-releasing hormone (CRH) (Edwardson and Bennett, 1974). Dopamine, however, inhibited the release of CRH (Edwardson and Bennett, 1974), but had no effect on the release of thyrotropin-releasing hormone (TRH) (Bennett et al., 1975a).

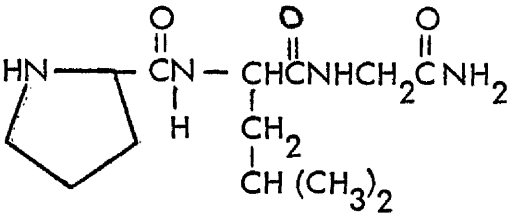
1.1 Role of Hypothalamic Hormones in Brain Function

The first indication that the hypothalamic hormones might have an extra-pituitary role came when it was found that some of the peptides, particularly melanocyte-stimulating hormone release-inhibiting hormone (MIH) and TRH (Fig. 1), had behavioural effects, some of which could also be detected in hypophysectomised rats. The idea of an extrapituitary role

Table 1a Hypothalamic Hormones controlling Release of Pituitary Hormones

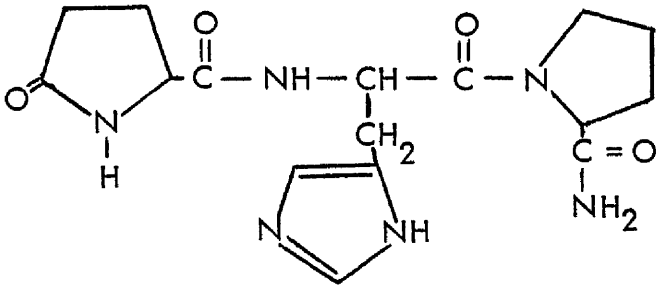
Abbreviation	Hypothalamic Hormone
CRH	Corticotropin (ACTH)-releasing hormone
TRH	Thyrotropin (TSH)-releasing hormone
LHRH	Luteinizing hormone (LH)-releasing hormone
FSHRH	Follicle-stimulating hormone (FSH)-releasing hormone
GHRH	Growth hormone (GH)-releasing hormone
GHRIH	Growth hormone (GH)-release-inhibiting hormone
PRH	Prolactin-releasing hormone
PRIH	Prolactin release-inhibiting hormone
MRH	Melanocyte-stimulating hormone (MSH)-releasing hormone
MIH	Melanocyte-stimulating hormone (MSH) release-inhibiting hormone

Where the identity of the physiological hypothalamic hormone is unclear, the term 'factor' is often used instead of hormone. eg MRF = MSH-releasing factor. MSH is liberated from the intermediate lobe of the pituitary. All other pituitary hormones listed are liberated from the anterior lobe.



L-prolyl-L-leucyl-glycinamide

MIH



L-pyroglutamyl-L-histidyl-L-proline amide

TRH

Fig. 1 Molecular Structures of MIH and TRH

was strengthened by the observation that as much as 80% of the total brain content of these hormones was found outside the hypothalamus (Table 1b).

Table 2 summarises some of the behavioural effects of the hypothalamic hormones and indicates their possible site of action. For example, the hypothalamic hormones may act as neurotransmitters themselves since they depress the activity of central neurons or may act as modulators of aminergic or cholinergic neurons. The evidence implicating the hypothalamic hormones in the control of dopaminergic and noradrenergic neurons is given below to provide a background for discussing the possible mode of action of the hypothalamic hormones on catecholaminergic neurons.

1.2 Catecholamines and Behaviour

The catecholamines dopamine, noradrenaline and adrenaline are neurotransmitters in the brain (Hebb, 1970). The highest concentration of dopamine is found in the basal ganglia (Bertler and Rosengren, 1959a, b), regions in which there is very little noradrenaline, while noradrenaline is concentrated in the hypothalamus (Bertler, 1961; Carlsson et al., 1958; Sano et al., 1959). Only small amounts of adrenaline are detectable in the central nervous system (Van der Gugten et al., 1976).

Both the adrenal medullary catecholamines and brain catecholamines have been implicated in a wide range of behavioural functions (Barchas et al., 1972; Friedhoff, 1975). Correlations have been reported between catecholaminergic

Table 1b Regional Distribution of TRH, LHRH and GHRIH in Rat Brain

TRH
LHRH } Data of Wilber et al (1976)

GHRIH Data of Brownstein et al (1975)

Brain region	TRH ng/g wet weight	TRH ng/region	LHRH ng/g wet weight	LHRH ng/region	GHRIH ng/g wet weight	GHRIH ng/region
Cortex	2.9	2.80	6.4	6.20	0.03	30.0
Brainstem	1.8	0.45	6.8	1.70	0.05	9.8
Cerebellum	4.7	1.40	32	9.50	0.02	4.5
Striatum	-	-	-	-	0.05	3.2
Midbrain	9.5	3.60	84	32.00	0.06	9.5
Hypothalamus	116	5.0	205	8.80	2.12	39.3
Pituitary	16	0.14	167	1.50	-	-
Thalamus	-	-	-	-	0.15	17.5
Olfactory bulb	-	-	-	-	0.02	1.0
Septum and preoptic area	-	-	-	-	0.64	24.7

Table 2 Behavioural Effects of Hypothalamic Hormones

Behavioural Effects	Hypothalamic Hormone	Possible Site of Action
L-dopa potentiation Plotnikoff and Kastin (1976)	TRH, MIH, LHRH, GHRIH	Dopaminergic neurons
Apomorphine potentiation Plotnikoff and Kastin (1974)	MIH	
Harmine antagonism Huidobro-Toro et al. (1975)	MIH	
Oxotremorine antagonism Plotnikoff et al. (1972a)	MIH	
Deserpidine antagonism Plotnikoff et al. (1973)	MIH	
Induction of rotational behaviour Cohn et al. (1975)	TRH	
Pentobarbital antagonism Breese et al. (1975) Schmidt (1977), Yarbrough (1978) Yarbrough and Singh (1978)	TRH	Cholinergic neurons
Induction of "barrel rotation" Cohn and Cohn (1975)	GHRIH	
Serotonin potentiation Plotnikoff and Kastin (1976)	TRH, LHRH	Serotoninergic neurons
Induction of shaking behaviour Wei et al. (1975)	TRH	Morphine receptors
Depressant action on central neurons Dyer and Dyball (1974) Renauld and Martin (1975) Renauld et al. (1975)	TRH, LHRH GHRIH	Peptidergic neurons
Induction of mating behaviour Moss and McCann (1973) Pfaff (1973)	LHRH	

function and emotion, mood, stress, depression, psychosis, drug craving,learning and memory. Current hypotheses on the role of catecholamines in behaviour have been developed from the effects of drugs in producing and relieving behavioural malfunction.

The following account describes specific aspects of behaviour in which the hypothalamic hormones, as well as cerebral dopamine and noradrenaline, may be involved.

1.2.1 Dopamine and Locomotory Activity

There is extensive evidence suggesting an involvement of dopamine in regulating locomotory activity. The most definite connection between a dopaminergic system and a specific locomotory abnormality is the role of striatal dopamine in the motor disturbances involved in Parkinson's disease.

1.2.1.1 Parkinson's Disease

The most obvious biochemical abnormality of the Parkinsonian syndrome is dopamine depletion from the nigrostriatal nerve terminals (Hornykiewicz, 1966). Thus drugs which reduce striatal dopamine, for example reserpine, or antagonise dopamine receptors, for example chlorpromazine, produce a hypokinetic state in animals similar to Parkinsonism in man. L-dopa (L-3, 4-dihydroxyphenylalanine), a precursor of dopamine which crosses the blood-brain barrier, reduces the symptoms of Parkinson's disease (Cotzias, 1969) and counteracts the effect of drugs reducing or blocking the effects of dopamine. The therapeutic action of L-dopa is unclear but presumably it must

increase striatal dopamine content. Thus drugs potentiating the effect of L-dopa may be of value in the treatment of Parkinson's disease.

Cotzias et al (1967) demonstrated that melanocyte-stimulating hormone (MSH) aggravates the symptoms of Parkinson's disease. This, together with the observation that MIH potentiates the behavioural effects of L-dopa (see below), presumably by stimulating the formation of dopamine, and antagonises oxotremorine induced tremors (Plotnikoff et al., 1972a) even in hypophysectomised rats, suggested that MIH might be of value in the treatment of Parkinson's disease (Kastin, 1967). Though MIH did ameliorate some of the symptoms of Parkinson's disease, it is not known precisely how MIH exerts its therapeutic effects (Barbeau, 1974; Kastin et al., 1976).

1.2.1.2 Rotational Behaviour

Rotation in Lesioned Rats

Rotation can be induced in rats lesioned unilaterally in the nigrostriatal tract by drugs influencing dopaminergic function of the basal ganglia (Glick et al., 1976).

After lesioning of the substantia nigra, the dopamine neurons and terminals degenerate but the dopamine receptors remain intact. The receptors are believed to become super-sensitive. Thus an imbalance between the nigrostriatal pathways on the two sides of the brain is created. Potentiation of this imbalance by dopaminergic agents is thought to induce rotation. Rotation occurs in the direction of the side of the brain in which less dopamine mediated activity occurs.

Thus presynaptic acting agents, for example amphetamine, affect the dopamine terminals only on the unlesioned side, with a resultant turning toward the lesion. In contrast, drugs which directly affect the receptors, for example apomorphine, will have a greater effect on the striatum of the lesioned hemisphere and produce a turning away from the lesioned side (Ungerstedt, 1971).

Rotation in Intact Rats

Rotational behaviour can also be induced in intact rats. Unilateral intrastriatal injection of dopamine or apomorphine produced turning away from the side of drug administration, an effect that can be blocked by prior bilateral intrastriatal injection of chlorpromazine. Moreover, unilateral injection of chlorpromazine alone produced turning toward the side of injection (Ungerstedt et al., 1969).

High doses of amphetamine (15 - 25 mg/kg) induced rotation in normal rats resembling that induced by lower doses (1 - 5 mg/kg) in lesioned rats (Jerussi and Glick, 1974). This suggested the presence of an intrinsic and normal bilateral imbalance in dopamine content of left and right nigrostriatal systems and that potentiation of this imbalance by amphetamine produced rotation.

In agreement with this suggestion, Glick et al. (1976) found that the dopamine content of left and right striata differed by 10 - 15%. After amphetamine administration, the dopamine content of left and right striata differed by approximately 25%. In response to amphetamine, rats consistently rotated away from the side containing the higher level of dopamine.

Intracerebroventricular injection of TRH (0.3 - 0.5 mg/kg) or amphetamine (1.0 - 1.5 mg/kg) induced rotational behaviour in non-lesioned rats pretreated with intraperitoneal injection of either apomorphine (4 mg/kg) or reserpine (4 mg/kg). Pretreatment with apomorphine led to clockwise rotation while pretreatment with reserpine led to counterclockwise rotation. The direction of rotation depended only on the drug used for pretreatment as the direction was the same whether the drug was injected into the left or right lateral ventricle. Intracerebroventricular administration of TRH (0.3 - 0.5 mg/kg) or amphetamine (1.0 - 1.5 mg/kg) or apomorphine (1.0 - 1.5 mg/kg) or reserpine (1.0 - 1.5 mg/kg) alone did not induce rotation (Cohn et al., 1975). Presumably pretreatment with apomorphine and reserpine produced an imbalance in dopamine content between left and right sides of the brain. Potentiation of this imbalance by TRH and amphetamine would induce rotational behaviour. This suggested that TRH, like amphetamine, could stimulate release of dopamine from presynaptic neurons.

1.2.1.3 L-dopa Potentiation Test

The L-dopa potentiation test (Everett, 1966), a test used for screening anti-depressant drugs, provides further evidence for the involvement of dopamine in locomotory behaviour.

In this test, mice are given pargyline, a monoamine oxidase inhibitor, orally and after 4 hours are injected intraperitoneally with L-dopa. The drug under study is given at selected times, for example, 1, 4, 8 or 24 hours before L-dopa. After L-dopa administration, the behavioural

response of groups of 4 mice to the drug are observed for 1 hour. A typical response consists of a series of behavioural responses including marked irritability and motor reactivity, for example, piloerection, increased salivation, jumping, squeaking, fighting. This response is rated slight (1), moderate (2) or marked (3) potentiation of L-dopa depending on the extent of the response. The hypothalamic hormones MIH, TRH, GHRIH and LHRH (Plotnikoff and Kastin, 1976) and the tricyclic antidepressant drugs imipramine, norimipramine and amitriptyline (Everett, 1966), all potentiate the behavioural effects of L-dopa. This suggested that the hypothalamic hormones might potentiate the effects of L-dopa by inhibition of reuptake of dopamine.

The potentiation of L-dopa by MIH and TRH was independent of the presence of a number of somatic organs including the pituitary gland (Plotnikoff et al., 1971, 1972a, b, 1974a, b, c). This indicated an extrapituitary effect of the hypothalamic hormones on the central nervous system. Further, animals pretreated with FLA-63 [bis-(1-methyl-4-homopiperazinyl-thiocarbonyl)-disulfide], a dopamine-β-hydroxylase inhibitor, before subjected to the L-dopa potentiation test still respond to TRH (Plotnikoff and Kastin, 1976) suggesting that TRH acts through dopaminergic, rather than noradrenergic, neurons.

1.2.2 Noradrenaline and Depression

The catecholamine hypothesis of affective disorders (Bunney and Davis, 1965; Schildkraut, 1965) proposed

that some, if not all depressions are associated with a decrease in catecholamines, particularly noradrenaline, at certain central synapses. Conversely, mania may be associated with an excess of such amines. At least two subgroups have been identified. In one group patients may have an alteration in noradrenergic systems, probably central, which is related to their depressive states as prior to treatment, low levels of 4-hydroxy-3-methoxyphenylethylene-glycol (MOPEG), a degradation product of noradrenaline, are found in the cerebrospinal fluid and urine of patients. In the second group serotonin systems may be altered as patients respond to amitriptyline (which inhibits reuptake of serotonin) but not to amphetamine (which inhibits reuptake of noradrenaline and dopamine). Further, these patients had normal urinary levels of MOPEG (Maas, 1975).

The actions of both the major classes of antidepressants, the monoamine oxidase inhibitors and the tricyclic anti-depressants, may be mediated by noradrenaline. For example, iproniazid, a monoamine oxidase inhibitor, increases the concentration of cerebral monoamines, while imipramine, a tricyclic antidepressant, inhibits reuptake of noradrenaline and serotonin. These drugs increase the availability of noradrenaline and serotonin at central synapses and this effect is thought to underlie their therapeutic action.

A revised catecholamine hypothesis of affective disorders (Ashcroft et al.,1972; Bunney et al., 1977) suggests that depression might reflect a state of pathologic

hyposensitivity of postsynaptic noradrenergic receptors in brain. Thus the therapeutic action of antidepressants might be related to postsynaptic rather than presynaptic mediated events and would involve an increase in receptor sensitivity.

Reserpine causes depression presumably by depleting amine storage vesicles in presynaptic neurons (Schildkraut, 1965). However, reserpine can also increase the sensitivity of the limbic noradrenergic receptor-coupled adenylate cyclase system to noradrenaline (Williams and Pirch, 1974). This would result in feedback inhibition of noradrenaline synthesis and a decrease of noradrenaline at functional synapses. The action of tricyclic antidepressants and monoamine oxidase inhibitors would in this case be to desensitise hypersensitive noradrenaline receptors and to reduce the feedback inhibition of the biosynthesis of noradrenaline (Sulser et al., 1978).

TRH has been claimed to be effective in the treatment of depression (Kastin et al., 1972; Prange et al., 1972) but there is much disagreement (Benkert et al., 1974; Dimitrikoudi et al., 1974; Hollister et al., 1974). TRH could exert its antidepressant effect by action on the presynaptic neuron, for example, by inhibiting monoamine oxidase or by desensitisation of hypersensitive postsynaptic noradrenergic receptors.

Conclusions

An account has been given of some examples of the behavioural effects of catecholamines in which the hypothalamic

hormones also play a role. Evidence has been presented that the behavioural effects of the hypothalamic hormones might be mediated by catecholamines but as yet there is no conclusive evidence.

The metabolism of catecholamines is now described and ways in which the metabolism of catecholamines might be regulated indicated to provide a background for discussing possible ways in which the hypothalamic hormones might regulate catecholamine metabolism and thus influence behaviour.

1.3.1 Metabolism of Catecholamines

The catecholamines are formed in brain from their amino acid precursor tyrosine according to the scheme shown in Fig. 2. This sequence of enzymic steps which was first demonstrated in the adrenal medulla (Blaschko, 1939) has now been confirmed in the brain (Mandel, et al., 1975).

Biosynthesis

Tyrosine is taken up from the bloodstream and concentrated in presynaptic nerve endings in the brain. In the cytoplasm of these nerve endings, tyrosine is converted to dopa by the enzyme tyrosine hydroxylase. Tyrosine hydroxylase, the rate limiting enzyme in the biosynthetic pathway, requires oxygen, ferrous iron and a reduced pteridine cofactor for activity (Nagatsu et al., 1964). The enzyme is subject to feedback inhibition by catechols, particularly dopamine (Kuczenski and and Mandell, 1972).

Dopa is rapidly converted to dopamine by dopa decarboxylase (Holtz et al., 1938), a pyridoxal phosphate requiring enzyme.

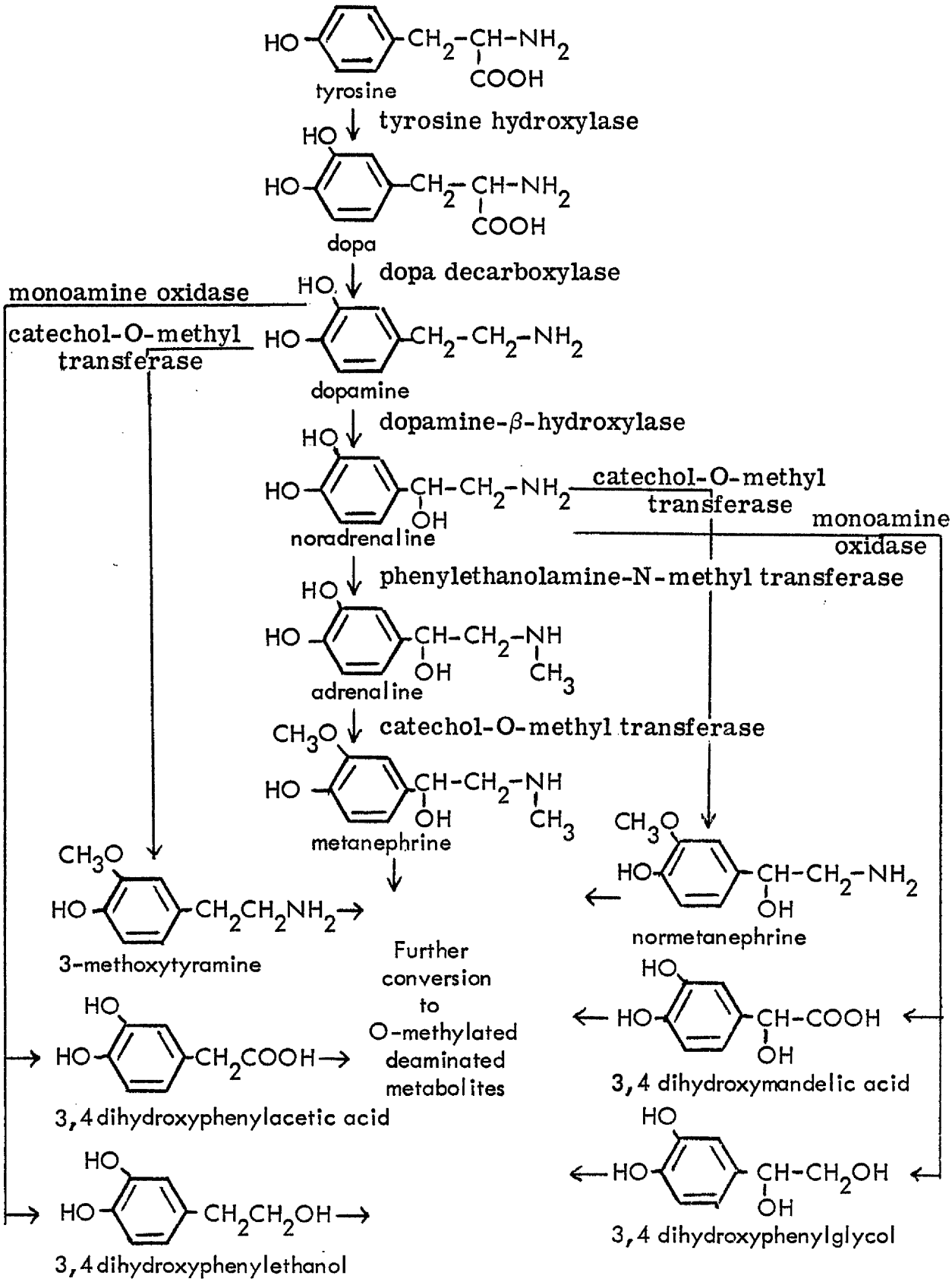


Fig. 2 Metabolism of Catecholamines

Dopa decarboxylase has been renamed aromatic amino acid decarboxylase in view of its lack of specificity (Lovenberg et al., 1962). Both hydroxylation and decarboxylation steps apparently occur within the cytoplasm of nerve terminals. Dopamine is converted to noradrenaline by dopamine-β-hydroxylase (Udenfriend and Creveling, 1959), a copper containing enzyme. This enzyme is localised in the same storage particles as those containing the bulk of noradrenaline. Phenylethanolamine-N-methyl transferase (Pohorecky et al., 1961; Saavedra et al., 1974), where present, is located in the cytoplasm and catalyses the conversion of noradrenaline to adrenaline.

Release

There are two mechanisms by which catecholamines can be released from sympathetic nerve terminals (Smith, 1973). The first, exocytosis, exhibits an absolute requirement for calcium. The second, displacement diffusion, is not calcium dependent. In the central nervous system, depolarisation-induced release of catecholamines (Arnold et al., 1977; Blaustein et al., 1972) is thought to occur by exocytosis. In contrast, release of catecholamines by tyramine (Patrick and Barchas, 1976) and amphetamine (Ziance et al., 1972) is thought to occur by displacement diffusion. Once in the synaptic cleft, the catecholamines can exert an effect on a post-synaptic cell.

Inactivation

After the catecholamines have exerted an effect on a postsynaptic cell, their action must be terminated. In the central nervous system catecholamines have their action

terminated chiefly by reuptake (Iversen, 1974).

1.3.2 Control of Catecholamine Metabolism

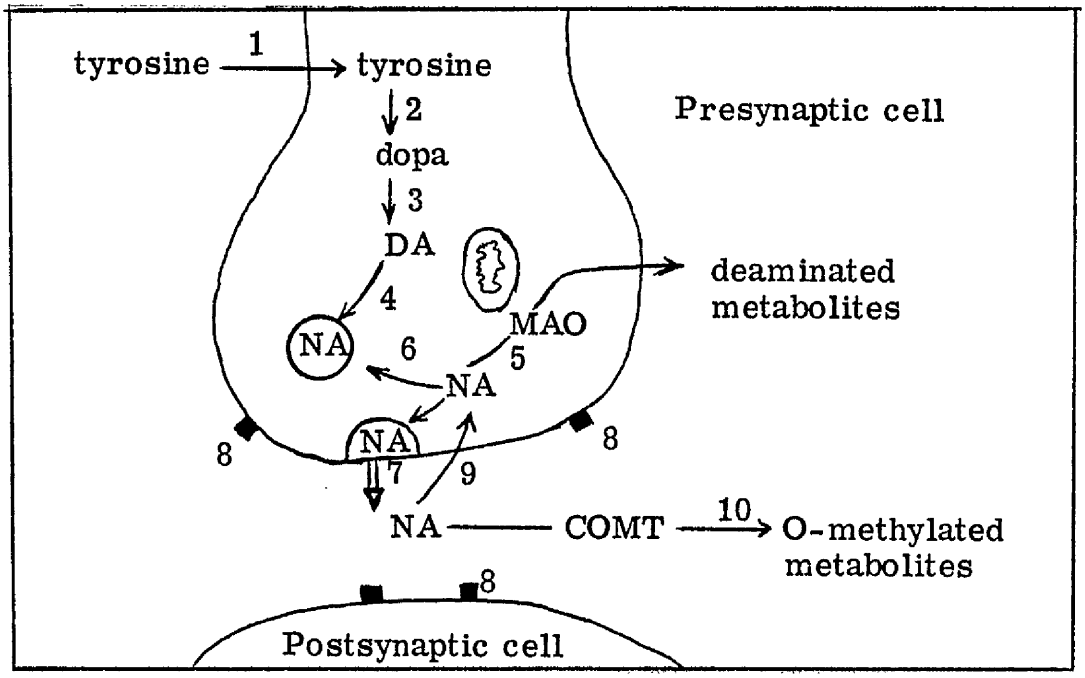
Fig. 3 represents a model of a central noradrenergic neuron and indicates the various sites at which drugs might interact with catecholamine metabolism. Some of these drugs are listed in Table 3, together with their site of action.

Interaction of drugs at some of these sites could increase the concentration of catecholamines at central synapses. An increase in catecholamine concentration could result from stimulation of catecholamine release, for example by amphetamine, or by inhibition of reuptake, for example by imipramine or by stimulation of catecholamine synthesis.

Tyrosine hydroxylase is the enzyme most susceptible to pharmacological manipulation as it is the rate limiting step in catecholamine synthesis. An increase in catecholamine synthesis could be achieved by direct action on the enzyme, for example, by altering its physical state (Kuczenski, 1975a). An increase in catecholamine synthesis could also be achieved indirectly, for example, by increasing release of dopamine from a pool inhibiting tyrosine hydroxylase (Kuczenski, 1975b) or by blockade of postsynaptic receptors (Fuller and Steinberg, 1976).

1.3.2.1 Possible Mode of Action of Hypothalamic Hormones on Catecholamine Metabolism

The behavioural effects of the hypothalamic hormones suggest that they are most likely the result of an increase in activity of central dopaminergic or noradrenergic neurons



Site 1	Precursor transport
Site 2	Tyrosine hydroxylase
Site 3	Dopa decarboxylase
Site 4	Dopamine-β-hydroxylase
Site 5	Monoamine oxidase (MAO)
Site 6	Storage
Site 7	Release
Site 8	Interaction with receptors
Site 9	Reuptake
Site 10	Catechol-O-methyltransferase (COMT)

Fig. 3 Model of Central Noradrenergic Neuron

Table 3 Some Drugs Interfering with Catecholamine Metabolism

Drug	Action on Catecholaminergic Neurons
α-methyl-p-tyrosine	Inhibits tyrosine hydroxylase
Tyramine	Stimulates release
Amphetamine	Stimulates release Inhibits reuptake Inhibits monoamine oxidase
Pargyline, Amitriptyline	Inhibit reuptake
Pyrogallol	Inhibits catechol-O-methyl transferase
Apomorphine	Stimulates dopamine receptors
Haloperidol, Chlorpromazine	Block dopamine receptors
Isoprenaline	Stimulates β-noradrenaline receptors
Phentolamine	Blocks α-noradrenaline receptors
Reserpine	Depletes storage vesicles
6-hydroxydopamine	Destroys catecholaminergic neurons

which could raise the concentration of dopamine or noradrenaline at central synapses. However, in general, it has been found that the hypothalamic hormones do not increase the endogenous levels of catecholamines (Kastin et al., 1975; Kostrzewa et al., 1974; Plotnikoff et al., 1974a, 1975). Thus an increase in synthesis and/or release or a decrease in degradation, of catecholamines is more likely.

Biosynthesis

Direct evidence in support of the idea that hypothalamic hormones stimulate biosynthesis of catecholamines is that MIH induced a dose-related increase in striatal dopamine synthesis in slices obtained from treated normal rats (Friedman et al., 1973) while TRH stimulated the conversion of [^{14}C] tyrosine to [^{14}C] noradrenaline in rat brain (Keller et al., 1974). There is indirect evidence that MIH and TRH stimulate catecholamine biosynthesis. TRH increased the conversion of cerebral noradrenaline to normetanephrine (Horst and Spirt, 1974) without altering the endogenous noradrenaline content, thus implying a concomitant increase in noradrenaline synthesis.

Release

Stimulation of release is also a possible site of action of MIH and TRH, as TRH stimulated release of [^{3}H] noradrenaline from hypothalamic synaptosomes and [^{3}H] dopamine from striatal synaptosomes (Horst and Spirt, 1974). Preliminary results suggested that MIH stimulated release of dopamine from striatal slices (Mulder and Smelik, 1975).

Further, TRH facilitated the decrease of brain noradrenaline

produced after administration of the tyrosine hydroxylase inhibitor α-methyl-p-tyrosine (Breese et al., 1974; Constantinidis et al., 1974; Keller et al., 1974). As no change in the level of noradrenaline was detected after administration of TRH alone (Constantinidis et al., 1974; Keller et al., 1974) it must be assumed that an increase in synthesis compensated for the increase in release and catabolism. This suggested that the turnover of noradrenaline was enhanced by TRH. MIH accelerated α-methyl-p-tyrosine induced disappearance of dopamine in rat brain, suggesting that MIH, like TRH, could stimulate release and turnover of catecholamines (Pugsley and Lippmann, 1977).

Inactivation

Inhibition of catecholamine reuptake is not considered a likely site of action of MIH and TRH as MIH (Kostrzewa et al., 1976) and TRH (Horst and Spirt, 1974) did not affect the uptake of [^{3}H] dopamine by striatal synaptosomes. TRH did not prevent uptake of [^{3}H] noradrenaline into hypothalamic synaptosomes (Horst and Spirt, 1974) or uptake of [^{3}H] dopamine or [^{3}H] noradrenaline into rat brain synaptosomes under the experimental conditions in which imipramine caused greater than 50% inhibition of reuptake (Tuomisto and Männistö, 1973).

If MIH and TRH do inhibit the degradation of dopamine and noradrenaline then the enzymes monoamine oxidase and catechol-O-methyl transferase are possible sites of action. However, TRH may not inhibit monoamine oxidase (Breese et al., 1974). Impairment of O-methylation may be a possible extra-hypophyseal mechanism of action of MIH (Carman, 1973) as MIH caused a

decrease in urinary HVA without a concomitant dimunition of urinary DOPAC (Sandler et al, 1973).

1.4 Comparison of the Effects of the Hypothalamic Hormones, MIH and TRH and Amphetamine

Evidence is accumulating, mainly from behavioural studies, that the effects of TRH and to a lesser extent MIH, are in many ways similar to those produced by amphetamine.

MIH induced stereotyped behaviour in cats similar to that seen after amphetamine administration (North et al., 1973). TRH induced rotational behaviour in non-lesioned rats like that induced by amphetamine (Cohn et al., 1975) and also produced amphetamine-like effects in mice (Malik, 1976) and rabbits (Horita and Carino, 1975). In the yohimbine screening test for anti-depressant drugs, TRH acted more like amphetamine than like a tricyclic drug (for example, imipramine) or a monoamine oxidase inhibitor (Hine et al., 1973). Clinically, TRH administration leads to a worsening in the state of schizophrenia, an effect that is also seen when amphetamine is given (Bigelow et al., 1975; Davis et al., 1975). Further, intravenous injection of TRH causes characteristic changes in the electroencephalogram of conscious patients resembling those produced by amphetamine and protriptyline (Itil, 1974).

Conclusions

Evidence suggests the hypothalamic hormones, like amphetamine, influence behaviour and that they may do this by stimulating the release and turnover of catecholamines. Thus the aim of this thesis is to study the relationship between the

behavioural effects of the hypothalamic hormones and catecholamine metabolism and to see if this leads to an interpretation of the behavioural effects. In addition the biochemical effects of the hypothalamic hormones, MIH and TRH, are compared with those of amphetamine to see if the common behavioural effects have a common biochemical basis.

1.5 Methods of Studying the Effects of Hypothalamic Hormones on Catecholamine Metabolism

Most studies investigating the effects of hypothalamic hormones on catecholamine metabolism have involved the intraperitoneal injection of MIH or TRH with subsequent measurement of some aspect of catecholamine metabolism, for example biosynthesis of catecholamines. On intraperitoneal administration of MIH or TRH it is unclear how much peptide can actually enter the brain as their ability to penetrate the blood-brain barrier may be poor (Dupont et al., 1975; Pelletier et al., 1975; Witter, 1975). Even if peptides do cross the blood-brain barrier it is uncertain which brain regions would be affected. Further, the half-life of exogenous peptides in plasma is very brief (May and Donabedian, 1973; Redding et al., 1973). For this reason it was decided, in this thesis, to add MIH and TRH to in vitro tissue systems.

As MIH and TRH may, like amphetamine, stimulate release and biosynthesis of dopamine or inhibit monoamine oxidase, it was decided to examine the effect of MIH and TRH on these three presynaptic dopaminergic mechanisms.

II MATERIALS AND METHODS

2.1 Materials

Analar reagents were used throughout.

Fine chemicals were obtained as follows.

Radiochemicals

[ethylamine-1-^{14}C] dopamine hydrochloride	specific activity 52 mCi/mmol
[ethylamine-1,2-^{3}H] dopamine hydrochloride	specific activity 10 Ci/mmol
L-[U-^{14}C] tyrosine	specific activity 486 mCi/mmol
L-[side chain-2,3-^{3}H] tyrosine	specific activity 22 Ci/mmol

from Radiochemical Centre, Amersham, Bucks, U.K.

[^{14}C] toluene	specific activity 5.26×10^{5} dpm/g
[^{3}H] toluene	specific activity 2.58×10^{6} dpm/g

from Packard Instrument Co. Inc., Caversham, Berks, U.K.

Reducing agents and inhibitors

Ascorbic acid	BDH Chemicals Ltd., Poole, Dorset, U.K.
Nicotinamide adenine dinucleotide reduced, disodium (NADH)	PL Biochemicals Inc. c/o International Enzymes Ltd. Windsor, Berks, U.K.
Pargyline hydrochloride	Abbott Labs., Queenborough, Kent, U.K.

Drugs

Tyramine	BDH Chemicals Ltd.,
Melanostatin	Pierce, Pierce and Warriner Ltd., Cheshire, U.K.
Thyroliberin	Pierce or Serva, c/o Micro-Bio Labs, London, U.K.
(+)-Amphetamine sulphate	generously supplied by Dr D. Pollock, Dept. of Pharmacology, University of Glasgow.

Standards for autoradiography

Tyrosine	BDH Chemicals Ltd.
Dopamine hydrochloride	
Noradrenaline hydrochloride	
3,4-dihydroxybenzoic acid	
3,4-dihydroxyphenylethanol	Regis Chemical Co. c/o Micro-Bio Labs.
3-O-methyldopamine hydrochloride	Aldrich Chemical Co., Wembley, Middlesex, U.K.
3,4-dihydroxyphenylacetic acid	
4-hydroxy-3,methoxyphenylacetic acid	

Other chemicals

Silica gel sheets	Camlab, Cambridge, U.K.
Aluminium oxide, neutral, Brockman Grade 1 (alumina)	BDH Chemicals Ltd.
N-tris (hydroxymethyl) methyl glycine (Tricine)	

Bovine plasma albumin	Armour Pharmaceutical Co. Eastbourne, Essex, U.K.
Sodium pyruvate	Sigma London Chemical Co. Ltd., Poole, Dorset, U.K.

2.2 Tissue Preparation

Brains of adult male Wistar rats (200 ± 25g) were dissected according to the method of Glowinski and Iversen (1966) into the following regions: cerebellum, medulla-pons, hypothalamus, midbrain, striatum, hippocampus, cortex (Fig. 4).

2.2.1 Tissue Slices

Tissue from cortex-striatum, medulla-pons and cerebellum was chopped to small fragments using a McIlwain tissue chopper (McIlwain and Buddle, 1953) set on 0.26 mm and suspended in 1 ml of phosphate-saline.

2.2.2 Sucrose Homogenates

Tissue from cortex, striatum, medulla-pons and cerebellum was homogenised in 4 vol of ice-cold 0.3 M sucrose using ten strokes of a hand operated glass-glass homogeniser (clearance 0.25 mm).

2.2.3 Crude Synaptosomes (P_2 fraction)

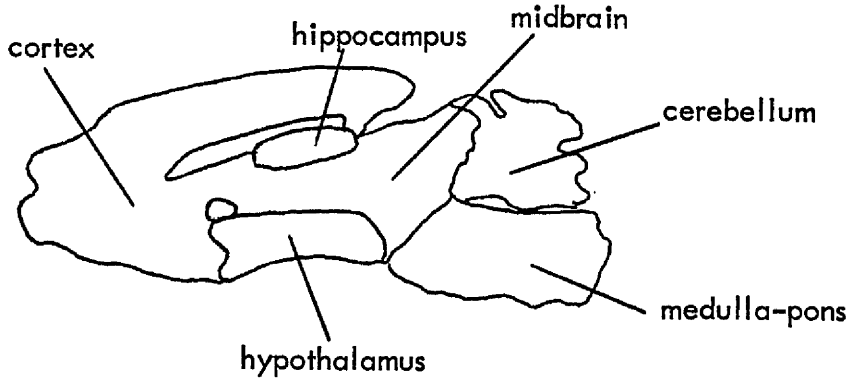
This preparation was made at 0 - 4°C according to the scheme outlined in Fig. 5.

Striatal tissue was homogenised in 10 vol of ice-cold 0.3 M sucrose, centrifuged at 1,000 g for 15 min and the supernatant (S_1) recentrifuged at 17,000 g for 20 min. The pellet formed was resuspended in 0.3 M sucrose to give the P_2 fraction (Gray and Whittaker, 1962).

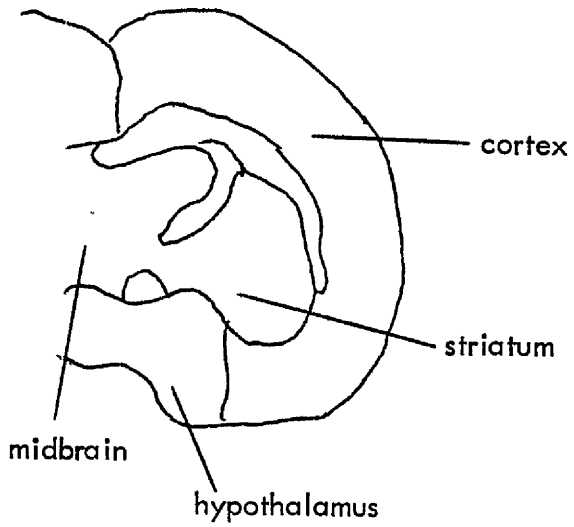
Electron micrographs of the P_2 fraction were prepared by Mr. I. Montgomery of the Electron Microscopy Unit, Department of Physiology, University of Glasgow.

Fig. 4 Regions of the Brain

(a) Sagittal section



b) Coronal section, half of brain is shown



Preparation of P_2 Fraction

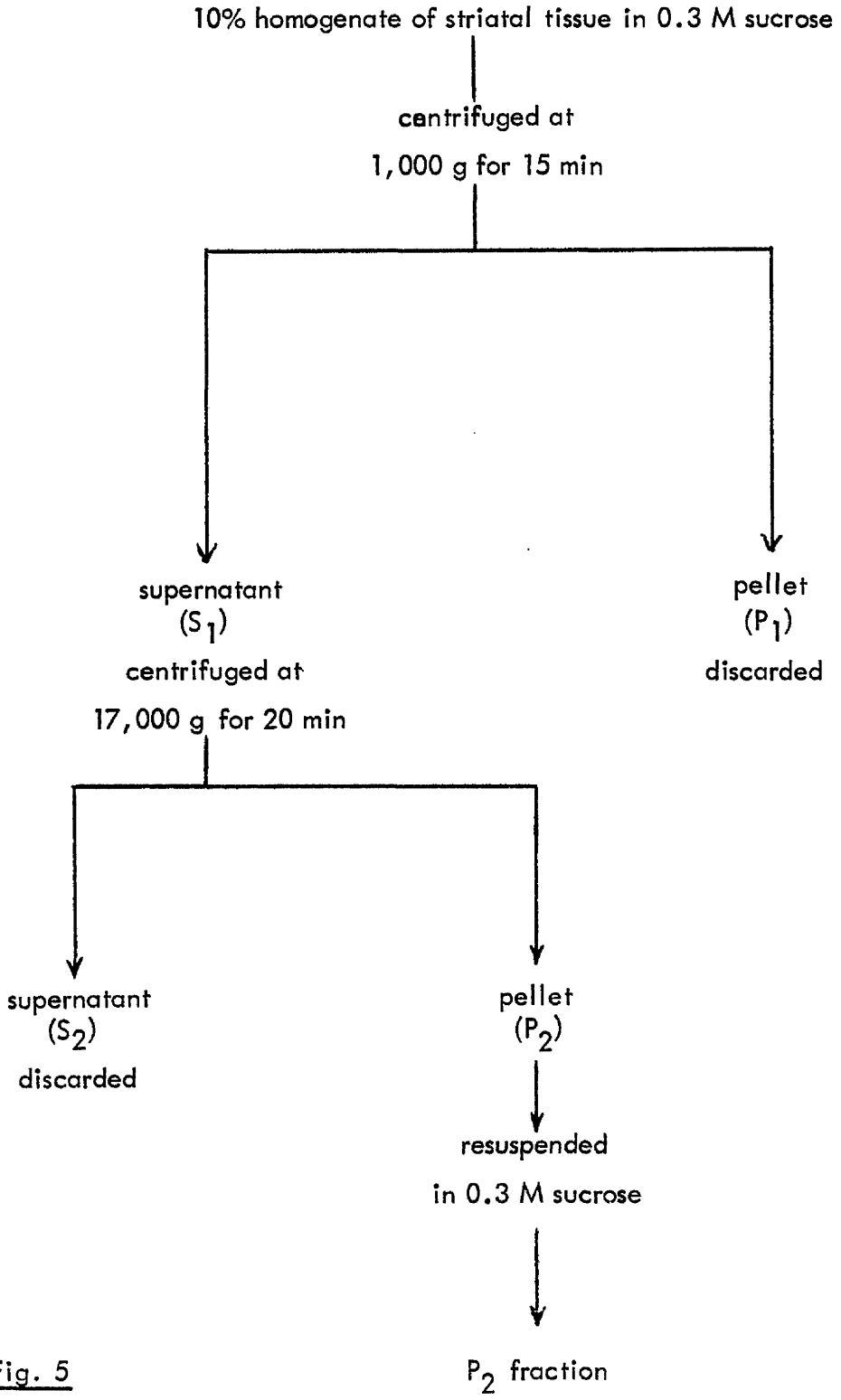


Fig. 5

2.2.4 Salines

Salines are based on Krebs (1950) in which constituent solutions are isotonic with 0.154 M NaCl.

1) Phosphate-saline contained 13.85 mM sodium phosphate buffer, pH 7.4, 116.1 mM NaCl, 3.55 mM $NaHCO_3$, 4.74 mM KCl, 1.18 mM $MgSO_4$, 1.18 mM KH_2PO_4 and 11.54 mM glucose. pH of saline was 7.4.

2) Tris-saline contained 21.32 mM Tris-HCl, pH 7.4, instead of sodium phosphate buffer (Phizackerley and Fixter, 1973). $CaCl_2$, where present, replaced an equal volume of isotonic NaCl. pH of saline was 7.3.

Salines containing ascorbic acid were prepared fresh daily. Addition of ascorbic acid lowered the pH of the saline to 7.2. Addition of EDTA lowered the pH to 6.6.

2.3 Assays

2.3.1 Fluorimetric Assay of Dopamine and Noradrenaline in Hypothalamus and Striatum

Dopamine and noradrenaline were determined by a modification of the method of Shellenberger and Gordon (1971).

Extraction and Isolation of Dopamine and Noradrenaline

Tissue samples (100 - 150 mg hypothalamus; 250 - 300 mg striatum) were homogenised in 3 ml of 0.4 M perchloric acid containing 1 g $Na_2S_2O_5$ and 1 g EDTA per 1. The homogenates were left to stand on ice for 10 min then centrifuged at 20,000 - 28,000 g for 15 min. The supernatant was removed and adjusted to 6 ml with 0.4 M perchloric acid. The pH was adjusted to 8.0 with Tricine solution (17.9 g Tricine

and 25 g disodium EDTA per 1 of 0.5 M NaOH). Dopamine and noradrenaline were adsorbed on to columns of alumina (300 mg) in pasteur pipettes plugged with glass wool. The columns were washed with 10 ml of distilled water then sucked dry before elution of dopamine and noradrenaline with 3 ml of 0.05 M perchloric acid. Recovery of dopamine was 63 ± 7% (n=6); of noradrenaline 79 ± 7% (n=15) (means ± S.D.).

Estimation of Dopamine and Noradrenaline

1 ml of 0.05 M perchloric acid eluate was adjusted to pH 6.5 with 1.5 ml of 0.1 M phosphate-EDTA solution (9 g disodium EDTA added to 1l of 0.1 M phosphate buffer and the pH adjusted to 7.0 with 5.0 M NaOH). 0.2 ml of iodine reagent (0.1 M, 2 g potassium iodide plus 0.5 g iodine in a final vol. of 40 ml) was added and the solution mixed immediately. After exactly 2 min, 0.5 ml of alkaline sodium sulphite solution (2.5%; 1 ml of a solution containing 250 mg/ml sodium sulphite diluted to 10 ml with 5.0 M NaOH) was added and the solution mixed again. After 2 min, 0.4 ml of glacial acetic acid was added and the solution heated in a boiling water bath for 3 min, cooled on ice for 5 min and the fluorescence due to noradrenaline read at room temperature in an Aminco Bowman spectrophotofluorometer (V.A. Howe and Co. Ltd., London, U.K.), slit no. 5, 800 V, sensitivity 100 (activation peak 375 nm; fluorescence at 485 nm).

Samples were heated at 100°C for an additional 5 min. After cooling, the dopamine fluorescence was read at 0 - 4°C (activation peak 320 nm; fluorescence at 370 nm). Tissue

blanks were developed by reversing the order of addition of the iodine and alkaline sulphite solutions.

Standard curves for estimation of dopamine and noradrenaline are shown in Figs. 6 and 7 respectively.

Samples containing 100 ng dopamine and 100 ng noradrenaline were included in each series of determinations and used to convert fluorescence (relative intensity) to ng dopamine or noradrenaline.

2.3.2 Biosynthesis of Dopamine

Brain tissue was incubated with radioactive tyrosine and the dopamine formed separated from tyrosine by alumina adsorption as described above.

0.1 ml of sucrose homogenate (2 mg protein) was preincubated for 10 min in 0.2 ml of 0.28 M phosphate buffer, pH 6.2 (McGeer et al., 1967) using air as the gas phase. The reaction was started by the addition of 1 μCi of L-[U-^{14}C] tyrosine.

0.1 ml of P_2 fraction (0.4-0.7 mg protein) was preincubated with 0.1 ml of Tris-saline for 5 min and the reaction started by the addition of 0.1 ml of Tris-saline containing 1 μCi of L-[side-chain-2,3-^{3}H] tyrosine.

All assays were terminated by the addition of an equal volume of ice-cold 0.8 M perchloric acid containing 2 g $Na_2S_2O_5$ and 1 g EDTA per 1. 200 μg dopamine was added as carrier and homogenates centrifuged at 20,000-28,000 g for 15 min. The supernatant was removed and adjusted to 5 ml with 0.4 M perchloric acid. The pH was adjusted to

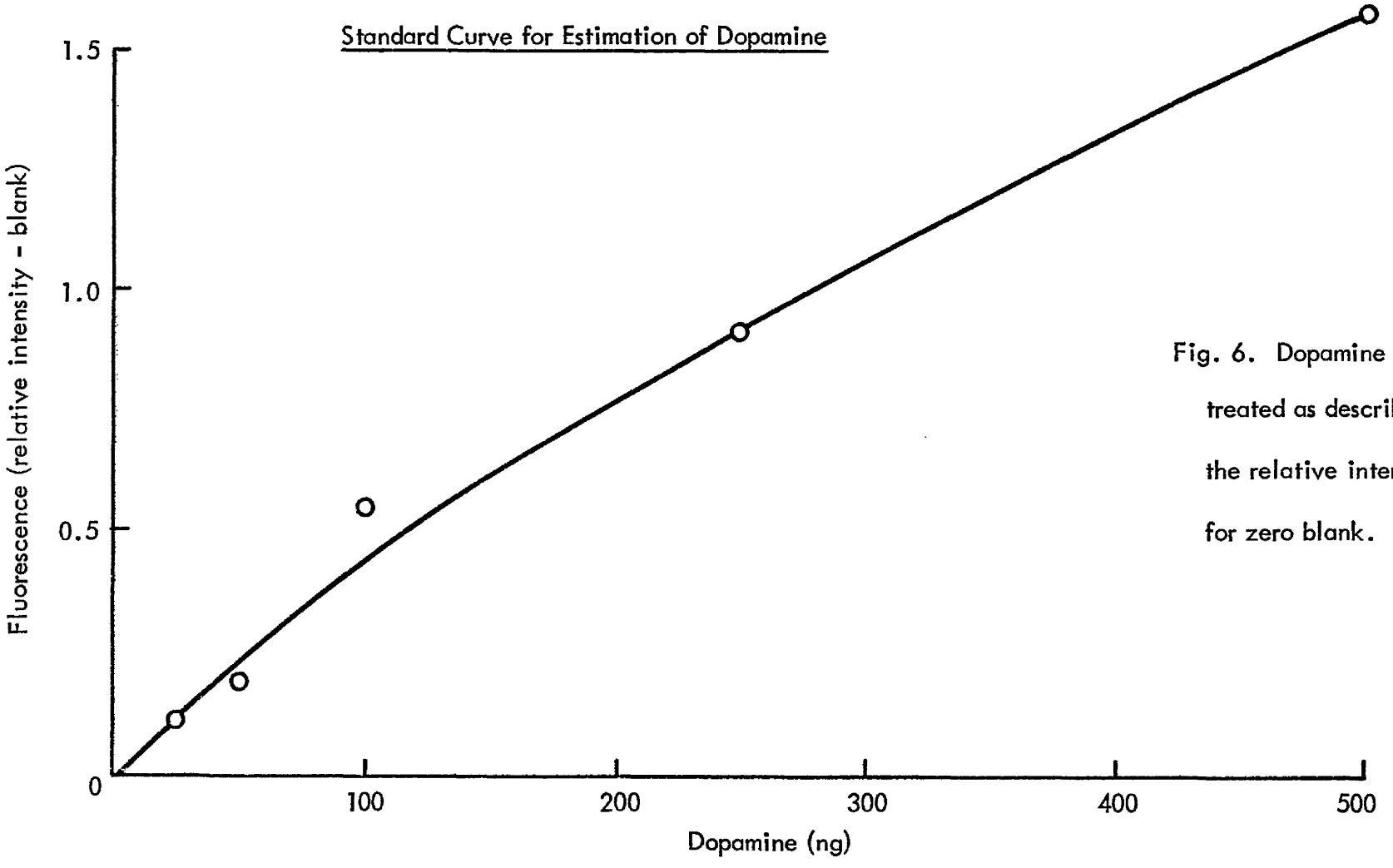


Fig. 6. Dopamine standards were treated as described (2.3.1) and the relative intensity corrected for zero blank.

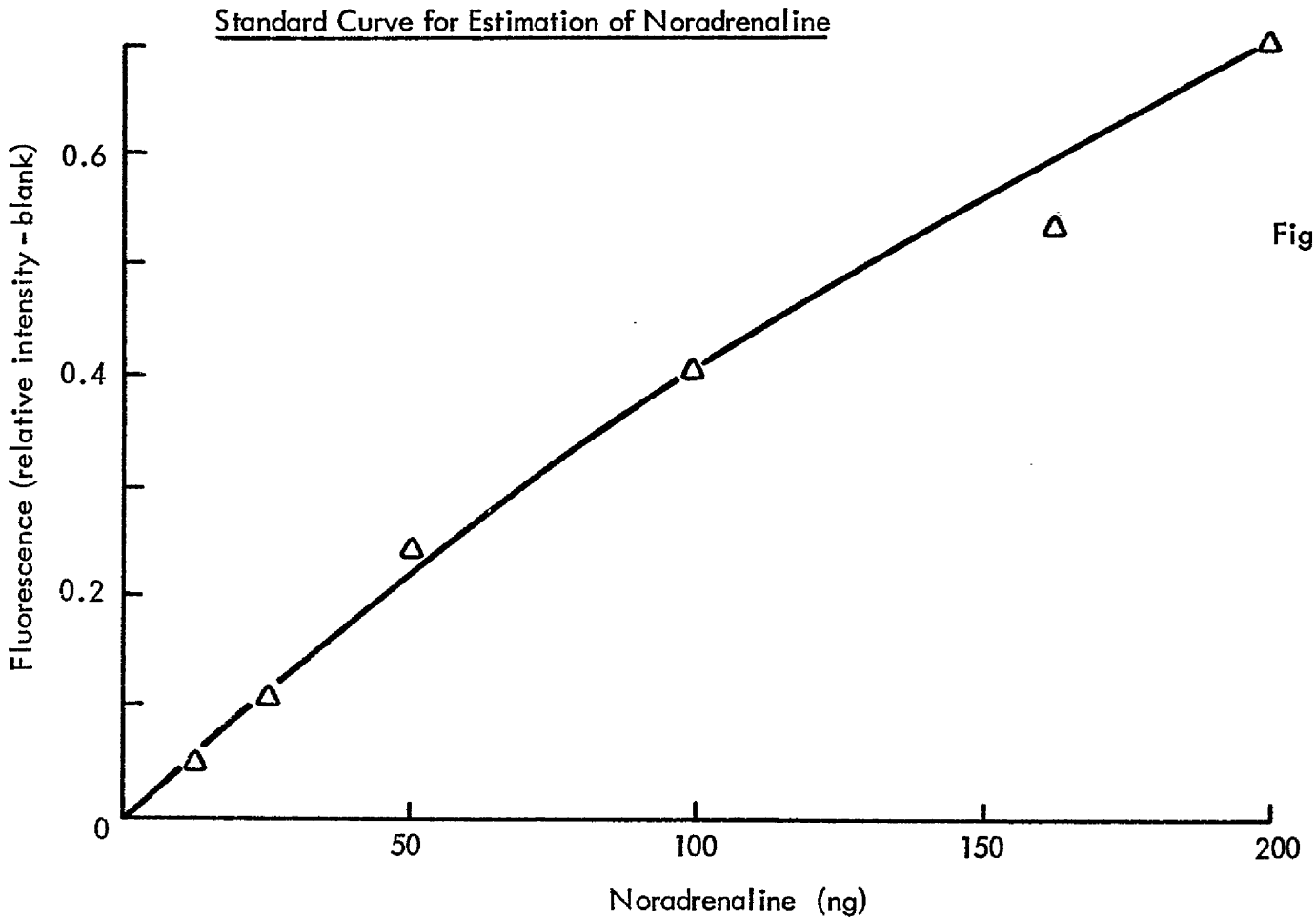


Fig. 7. Noradrenaline standards were treated as described (2.3.1) and the relative intensity corrected for zero blank.

8.0 with Tricine solution (17.9 g and 25 g disodium EDTA per 1 of 0.5 M NaOH). Catechols were adsorbed on to columns of alumina (300 mg) which were washed with 10 ml of distilled water and catechols eluted with 3 ml of 0.4 M HCl. Recovery of carrier dopamine was greater than 70%.

For estimation of [^{14}C] catechols, 10 ml of 'Triton' scintillant (Triton X-100, 1,000 ml; toluene 2,000 ml; PPO 15 g; POPOP 1.5 g) was added to 3 ml of the acid eluate to give a stable emulsion (Turner, 1968). Counting efficiency was greater than 45%. For [^{3}H] catechols, 10 ml of 'Triton' scintillant was added to 1 ml of column effluent for estimation by liquid scintillation counting. Counting efficiency was greater than 30%.

Isotope added to incubation medium was used as the reagent blank. Blank values were 1,500-1,800 cpm in experiments using [^{14}C] tyrosine as substrate. When [^{3}H] tyrosine was used as substrate, blanks were 500-1,000 cpm. In general, sample counts were at least 10 times blank values.

Counts were converted to pmol dopamine per min using the formula:-

$$\frac{\text{specific activity of tyrosine (nmol/}\mu\text{Ci) x dpm in eluate } \frac{\text{cpm}}{\text{efficiency (\%)}} \text{ x } 10^3}{\text{dpm/}\mu\text{Ci } (2.2 \text{ x } 10^6) \text{ x time of incubation (min) x recovery from alumina (\%)}}$$

Identification of Reaction Products

The identification of products formed was confirmed by autoradiography.

Acid eluates from alumina columns were dried down *in vacuo* and the residues taken up in 10 µl of acetone: 0.1 M HCl

(9 : 1 v/v) for chromatography in two dimensions on plastic backed silica gel sheets (10 cm x 10 cm) together with 2 µg each of the following standards:- tyrosine, dopamine, DOPAC and HVA. The following solvent systems were used:-

(1) 1st dimension - the organic phase of chloroform : acetic acid : H_2O (2 : 2 : 1 by volume) (Osborne et al., 1975)

(2) 2nd dimension - butan-1-ol : pyridine : acetic acid : H_2O (15 : 2 : 3 : 5 by volume) (Aures et al., 1968).

Standards were detected using diazotized sulphanilic acid (Sandler and Ruthven, 1969) and radioactive compounds located by autoradiography using Kodirex X-ray film (KD 54T).

A chromatographic map showing the separation of tyrosine from its metabolites is shown in Fig. 8.

2.3.3 Oxidative Deamination of Dopamine

Tissue slices and sucrose homogenates were incubated with radioactive dopamine and the monoamine oxidase products extracted with ethyl acetate using a modification of the method of McCaman et al. (1965).

Tissue slices (5.8-20 mg protein in 1 ml of phosphate-saline) and sucrose homogenates (0.1 ml(1.5-2.0 mg protein) in 0.2 ml of 0.28 M phosphate buffer, pH 6.2) were incubated for 30 min with 0.2 µCi of [ethylamine-1-^{14}C] dopamine hydrochloride. The reaction was terminated by the addition of HCl to give a final concentration of 0.4 M. Tissue samples were homogenised in 1 ml of 0.4 M HCl and cooled to -20°C for 30 min. Precipitated protein was removed by centrifugation at 3,000 rpm for 15 min (MSE bench centrifuge, Measuring and Scientific Equipment, London, U.K.). 2 ml of ethyl acetate was added

Chromatographic Map to Show the Separation of Tyrosine and its Metabolites after 2-Dimensional Chromatography.

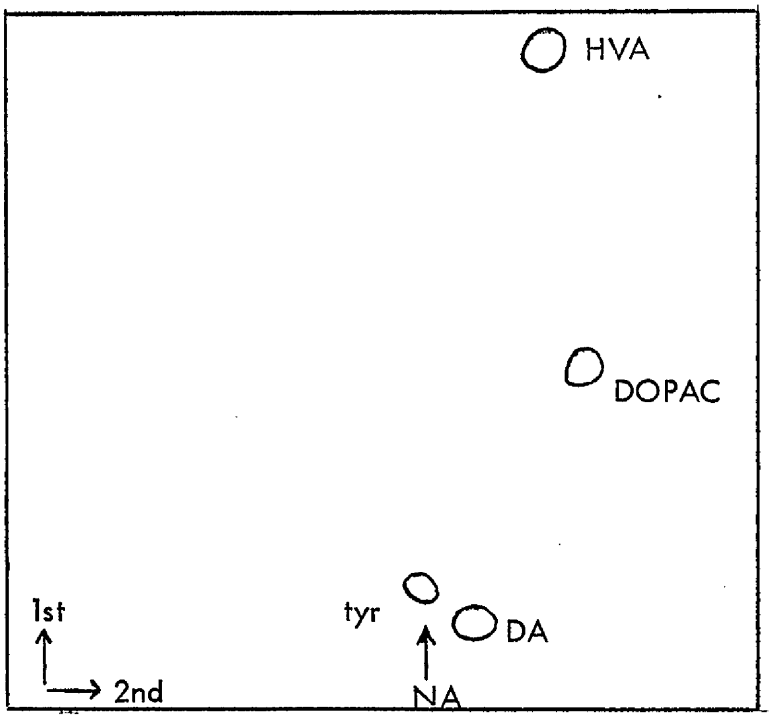


Fig. 8. Tyrosine, dopamine, DOPAC and HVA were separated on silica gel sheets using the following solvent systems as described (2.3.2).

1st dimension - the organic phase of chloroform : acetic acid : H_2O (2 : 2 : 1 by volume)

2nd dimension - butan-1-ol : pyridine : acetic acid : H_2O (15 : 2 : 3 : 5 by volume)

DA - Dopamine

DOPAC - 3,4-dihydroxyphenylacetic acid

HVA - 4-hydroxy-3-methoxy-phenylacetic acid

NA - noradrenaline

tyr - tyrosine

to each sample and the monoamine oxidase products extracted into the organic layer by vigorous mixing for 30 s. After centrifugation (MSE bench centrifuge, 1,000 rpm, 5 min) 1.5 ml of the organic layer was washed with 1 ml of 0.4 M HCl and the radioactivity in 1 ml estimated by liquid scintillation counting. Counting efficiency was greater than 70%.

Identification of Reaction Products

The identity of the products was checked by autoradiography as described for biosynthesis studies. Ethyl acetate layers were dried down in vacuo and the residues extracted 4 times with 10 µl of ethyl acetate. The extracts were chromatographed on silica gel sheets together with 2 µg each of dopamine, DOPAC, HVA, 3MT and DOPET using solvent system 1 (the organic phase of chloroform : acetic acid : H_2O (2 : 2 : 1 by volume)).

The radioactive spots were scraped off the chromatograms and dispersed in a stable emulsion formed by adding 0.9 ml of water to 3 ml of 'Triton' scintillant. The radioactivity was determined by liquid scintillation counting. The results are expressed as a percentage of counts recovered.

2.3.4 Release of Dopamine

Release of dopamine was measured using a modification of the method of Eitan and Hershkowitz (1977).

2.5 ml of S_1 fraction (11-16 mg protein) was diluted with 9 vol of Tris-saline containing 0.13 mM pargyline, 1.14 mM ascorbic acid (TSPA) 1.25 mM $CaCl_2$ and 10 µM EDTA and the diluted S_1 suspension preincubated at 37°C for 5 min. Uptake

was terminated by centrifugation at 10,000 g for 15 min at 4°C and the resulting pellet rinsed twice with 4 ml of ice-cold TSPA containing either 1.25 mM $CaCl_2$ (TSPAC) or 2 mM EDTA (TSPAE).

The pellet was resuspended with 2.5 ml TSPAC or TSPAE and 0.1 ml aliquots of the suspension were added to 2.9 ml TSPAC or TSPAE at 37°C containing the drug to be tested in its final concentration. Dopamine release was allowed to proceed for 2 min at 37°C with shaking. Release was terminated by the addition of 3 ml of ice-cold TSPAC or TSPAE and centrifugation at 10,000 g for 15 min at 4°C. The supernatant was collected and the pellet dissolved in 2 ml of 3% sodium dodecyl sulphate at 20°C for 30 min. The radioactivity in 1 ml of supernatant and pellet was estimated by liquid scintillation counting. Counting efficiency was greater than 30%.

[^{3}H] dopamine released is expressed as:-

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

The radioactivity released from 0.1 ml aliquots of P_2 fractions incubated at 0°C was used as a blank value and subtracted from all results.

2.3.5 Preparation of Standard Curves for Determination of Efficiency of Counting

250 µl of standard radioactive toluene solution ([^{3}H] toluene 2.58 x 10^6 dpm/g or [^{14}C] toluene 5.26 x 10^5 dpm/g; density of toluene 0.866) was weighed in a glass scintillation

vial then diluted to 250 ml with 'Triton' scintillant. 10 ml aliquots of the 'Triton' scintillant containing radioactive standard was dispensed into glass counting vials containing quenching agent. The vials were capped and mixed by vigorous hand shaking for 30 s then placed in the counter to cool for 30 min before counting. Samples were counted either by the sample channels ratio method using a Nuclear Chicago Isocap 300 (G.D. Searle, High Wycombe, Bucks.) or by the external standard ratio method using an Intertechnique SL4000 PG4000 (Uxbridge, Middlesex, U.K.). In general, samples were counted to give at least 5,000 cpm to ensure statistical accuracy.

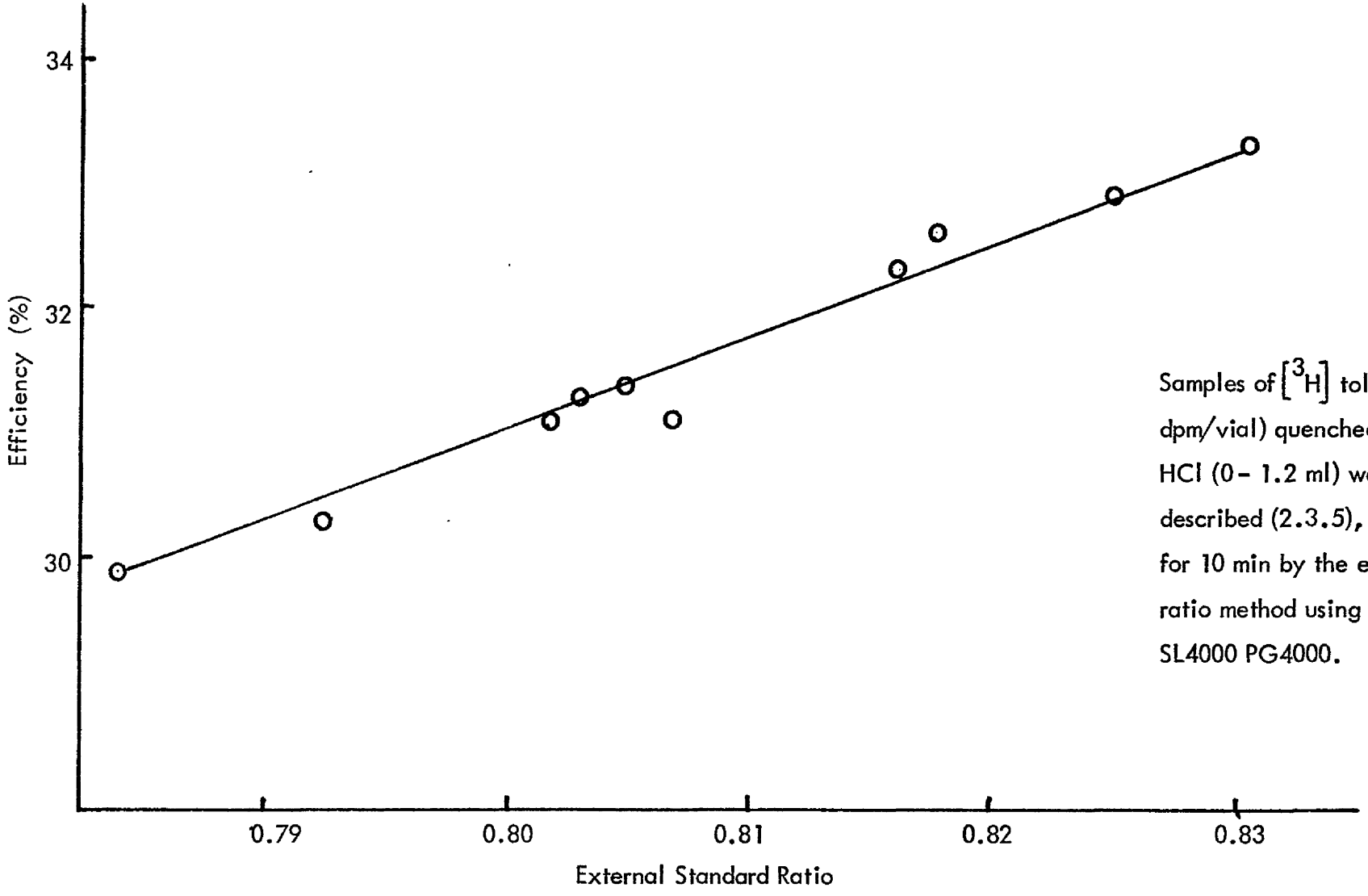
The efficiency of counting of tritiated samples quenched with 0.4 M HCl is shown in Fig. 9.

2.3.6 Lactate Dehydrogenase (LDH)

0.1 ml of P_2 fraction (0.04-0.07 mg protein) was added to 2.9 ml of 50 mM potassium phosphate buffer, pH 7.4 containing 0.33 mM sodium pyruvate and 80 µm NADH (final concentration/3 ml) in a cuvette in a Beckman DB spectrophotometer (Beckman Instruments Ltd., Glenrothes, Fife, U.K.), stirred and the change in A_{340} with time recorded for 1 min at 37°C to give the activity of free LDH. 0.2 ml of 10% Triton X-100 was added and the change in A_{340} was recorded for a further 1 min to measure the total LDH activity (Fig. 10). Occluded LDH is given as

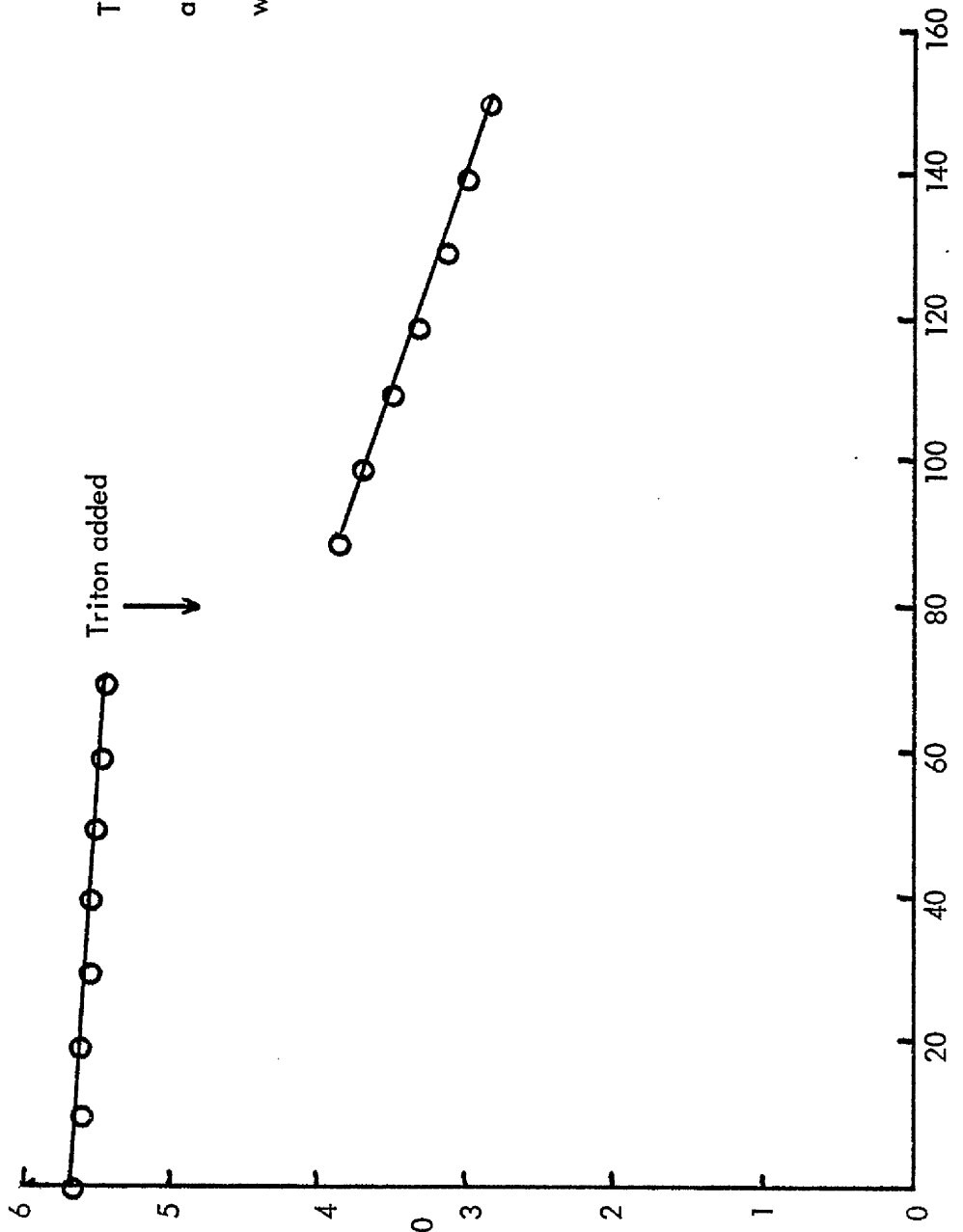
$$1 - \left(\frac{\text{free LDH}}{\text{total LDH}}\right) \times \frac{100}{1} \%$$ (Marchbanks, 1967).

Fig. 9. Determination of Efficiency of Counting of [^{3}H] toluene Quenched with 0.4 M HCl



Samples of [^{3}H] toluene (22,136 dpm/vial) quenched with 0.4 M HCl (0 - 1.2 ml) were prepared as described (2.3.5), and counted for 10 min by the external standard ratio method using an Intertechniqu SL4000 PG4000.

Fig. 10. Measurement of Occluded Lactate Dehydrogenase



The change in A_{340} with time before and after addition of Triton X-100 was measured as described (2.3.6).

2.3.7 Protein Estimation

Precipitated protein was dissolved in 1 ml of 0.1 M NaOH and aliquots assayed using the Bailey (1962) modification of the method of Lowry et al, (1951). Bovine plasma albumin was used as standard (Fig. 11).

2.4 Statistical Methods

2.4.1 Wilcoxon (or Mann-Whitney) Test

Statistical significance was determined using the Wilcoxon (or Mann-Whitney) test (Colquhoun, 1971), a non-parametric test which makes no assumption about the distribution of the observations or about the homogeneity of variances in the two samples.

Observations are ranked in ascending order and each observation replaced by its rank before performing the test. If the null hypothesis is true, the distribution of the sum of the ranks for the sample with n observations (R_1) can be shown to have mean μ_1 and standard deviation σ. The approximate standard normal deviate is calculated as

$$u = \frac{R_1 - \mu_1}{\sigma}$$

where μ_1 (mean of R_1) $= \frac{n(m + n + 1)}{2}$

σ (standard deviation of R_1) $= \sqrt{\frac{mn(m + n + 1)}{12}}$

n = sample with n observations

m = sample with m observations

The rarity of the result is judged from tables of the standard normal distribution. A value of p less than 0.05 was considered significant.

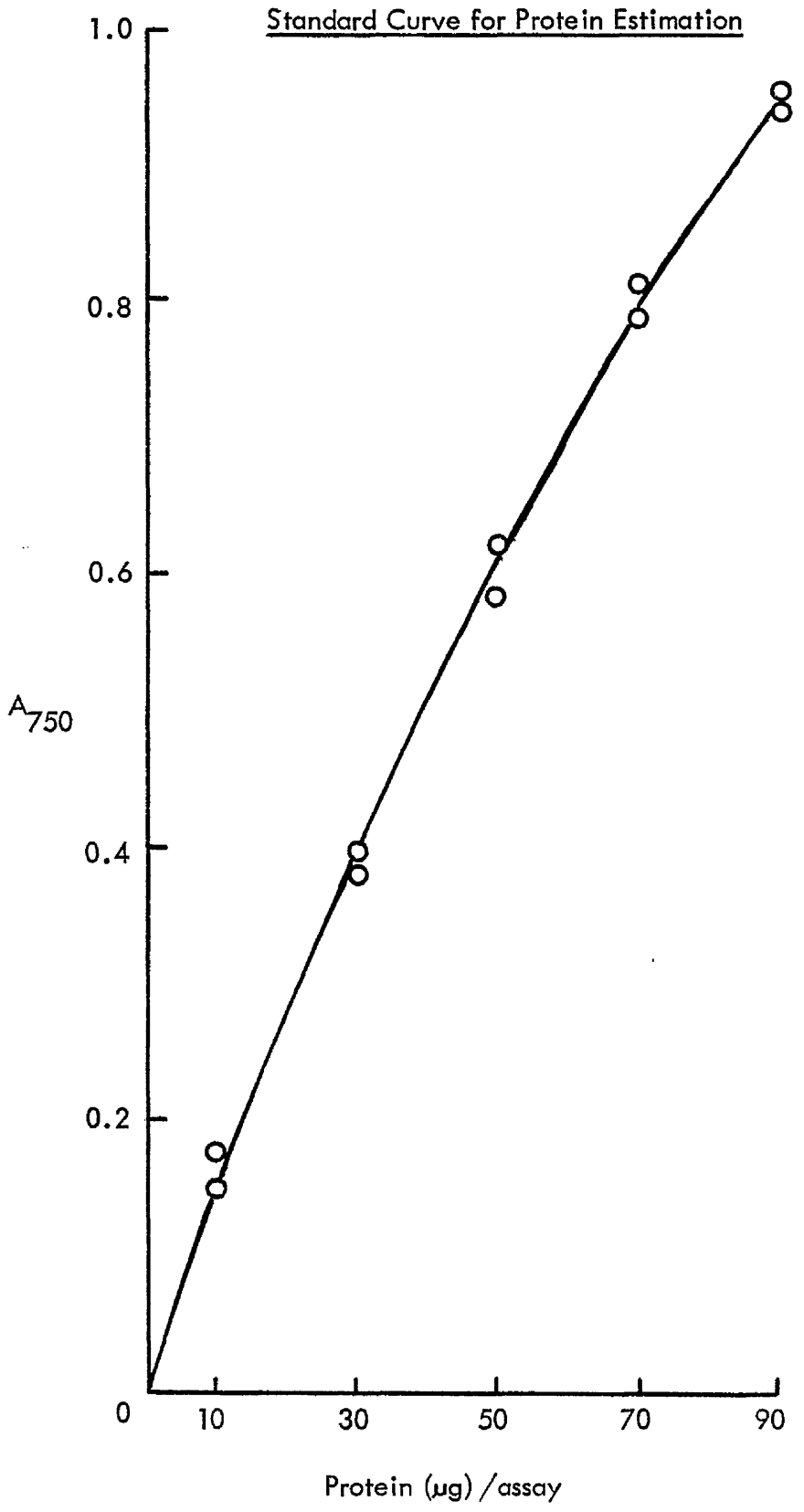


Fig. 11 BSA standards were treated as described (2.3.7) and A_{750} corrected for zero blank.

2.4.2 Linear Regression

Linear regression was used to determine whether or not there was a relationship between the two variables, dopamine synthesis (x) and drug-induced increase in dopamine synthesis (y) (Colquhoun, 1971).

The test assumes that the variable y is linearly dependant on the variable x, i.e. the points (x_i, y_i) lie on the line with equation $y = \alpha + \beta x$.

The points (x_i, y_i) do not actually lie on any single straight line. Random variation from this relationship described by a variance σ^2, is assumed to be in the variable y. The variable x is assumed not to vary randomly.

Estimates a and b of α and β are obtained as follows:-

a = intercept on y axis = $\bar{y} - b\bar{x}$

b = slope of line = $\frac{Cxy}{Cxx}$

where $\bar{y} = \frac{\Sigma y_i}{n}$ and $\bar{x} = \frac{\Sigma x_i}{n}$

$Cxy = \Sigma x_i y_i - n\bar{x}\bar{y}$

$Cxx = \Sigma x_i^2 - n\bar{x}^2$

n = number of observations.

σ^2, a variance of the points about the line $y = a + bx$ is estimated by

$$S^2 = \frac{1}{n-2}\left(Cyy - \frac{(Cxy)^2}{Cxx}\right) \quad \text{where} \quad Cyy = \Sigma y_i^2 - n\bar{y}^2 .$$

Testing the Slope in Regression

This parametric method tests the null hypothesis that the slope of the line $y = \alpha + \beta x$ is equal to zero, i.e.

there is no correlation between x and y.

The test statistic used to measure the deviation of β from zero is

$$t = b\sqrt{\frac{Cxx}{S^2}}$$

The value of t is obtained from tables of Student's t distribution with $n - 2$ degrees of freedom.

III RESULTS

RESULTS

3.1 Characterisation of Rat Brain Regions

The weight of the seven brain regions was determined and agreed with those obtained by others (Table 4). The hypothalamic catecholamine concentration (dopamine 0.28 ± 0.08 μg/g protein; noradrenaline 1.53 ± 0.47 μg/g protein; means ± S.D. of 7 preparations) and striatal catecholamine concentration (dopamine 1.89, 2.23 μg/g protein; noradrenaline 0.08, 0.26 μg/g protein), was determined and was in agreement with reported mammalian hypothalamic (Table 5) and striatal (Table 6) values. These results suggest that these regions have been correctly identified.

3.2 Oxidative Deamination of Dopamine

As Horst and Spirt (1974) showed that 10^{-4}M TRH stimulated release of dopamine from striatal synaptosomes, this concentration of peptide was used in these studies.

In general, neither MIH (10^{-4}) nor TRH (10^{-4}M) altered monoamine oxidase activity in tissue slices from cerebellum (Table 7), cortex plus striatum region (Table 8) or medulla-pons (Table 9). However, due to variable results and large standard deviations, it was not possible to decide whether or not peptides at this concentration had an effect on monoamine oxidase activity in tissue slices. Thus it was decided to use sucrose homogenates.

Chromatographic Studies

Chromatographic analysis showed that DOPAC and an unidentified dopamine metabolite with R_F value greater than

Table 4 Characterisation of Rat Brain Regions

Brain regions from the number of animals indicated were weighed. Results are expressed as a percentage of total brain weight and are means ± S.D.

A - Data of Glowinski and Iversen (1966)

B - Data of Holtzman (1974)

C - Data of Shellenberger and Gordon (1971)

Some portions of the cortex are lost.

Midbrain includes thalamus and hypothalamus.

Brain region	% of total brain weight			
	This work n = 5	A n = 20	B n = 60	C n = 14
Cortex	42.3 ± 2.1	43.9 ± 3.2	56.0 ± 4.2	22.4 ± 3.1
Medulla-pons	18.8 ± 1.2	13.8 ± 1.3	16.7 ± 1.5	18.8 ± 1.9
Cerebellum	13.9 ± 2.4	14.6 ± 1.2	-	24.8 ± 3.7
Striatum	11.6 ± 1.5	5.6 ± 0.9	4.8 ± 0.5	11.8 ± 2.8
Hippocampus	6.7 ± 0.8	8.2 ± 1.2	-	-
Midbrain	3.2 ± 0.6	8.0 ± 0.5	14.9 ± 1.9	22.3 ± 2.4
Hypothalamus	3.5 ± 0.4	6.0 ± 0.8	7.6 ± 0.8	-
Total weight of brain (mg)	1609	1832	1452	1258

Table 5 Hypothalamic Catecholamine Content

The hypothalamic catecholamine content expressed as ug/g are means ± S.D. with the number of determinations in parentheses.

Author	Dopamine ug/g ± S.D.	Noradrenaline ug/g ± S.D.
Coyle and Henry (1973)	0.58 ± 0.10 (5)	1.96 ± 0.10 (5)
Friedman et al (1973)	-	1.84 ± 0.02 (8)
Glowinski and Iversen (1966)	-	1.79 ± 0.10 (6)
Holtzman (1974)	-	1.37 ± 0.05 (60)
Metcalf (1974)	0.32 ± 0.03 (6)	1.61 ± 0.16 (9)
Shellenberger (1971)	0.26 ± 0.02 (15)	1.07 ± 0.11 (15)

Table 6 Striatal Catecholamine Content

The striatal catecholamine content expressed as ug/g are means ± S.D. with the number of determinations in parentheses.

Author	Dopamine ug/g ± S.D.	Noradrenaline ug/g ± S.D.
Coyle and Henry (1973)	8.07 ± 0.92 (5)	0.35 ± 0.2 (5)
Friedman et al (1973)	4.23 ± 0.05 (8)	-
Glowinski and Iversen (1966)	-	0.25 ± 0.03 (6)
Gordon and Shellenberger (1974)	5.47 ± 1.87 (12)	0.32 ± 0.12 (12)
Holtzman (1974)	16.78 ± 0.39 (60)	-
Metcalf (1974)	3.80 ± 0.36 (5)	0.06 ± 0.01 (5)
Shellenberger (1971)	11.50 ± 0.43 (15)	0.12 ± 0.01 (15)
Shellenberger and Gordon (1971)	7.11 ± 1.62 (14)	0.30 ± 0.09 (14)

Tables 7 - 9 Effect of MIH and TRH on Monoamine Oxidase Activity in Tissue Slices

Tissue slices (5.8 - 20 mg protein) were incubated for 30 min in 1 ml of phosphate-saline with 0.2 μCi of [^{14}C] dopamine (final concentration 3.85 μM) in the presence or absence of peptide as shown.

Results are expressed as pmol/min/mg protein and are means ± S.D. for the number of determinations in parentheses. Each experiment represents the results from a separate preparation of brain slices from 2-6 rats.

*significantly greater then control $p < 0.05$.

Table 7 Effect of MIH and TRH on Monoamine Oxidase Activity in Tissue Slices

Cerebellum

Additions	Expt 1 Monoamine Oxidase activity pmol/min/mg ± S.D.	Expt 1 % stimulation	Expt 2 Monoamine Oxidase activity pmol/min/mg ± S.D.	Expt 2 % stimulation
None	0.89 ± 0.16 (4)	-	2.19 ± 0.65 (4)	-
MIH 10^{-4}M	0.80 ± 0.16 (4)	-10	1.79 ± 0.65 (4)	-18
TRH 10^{-4}M	*1.60 ± 0.78 (4)	+80	1.80 ± 0.41 (4)	-18

Additions	Expt 3 Monoamine Oxidase activity pmol/min/mg ± S.D.	Expt 3 % stimulation	Expt 4 Monoamine Oxidase activity pmol/min/mg ± S.D.	Expt 4 % stimulation
None	2.99 ± 0.22 (4)	-	3.24 ± 0.47 (4)	-
MIH 10^{-4}M	3.00 ± 0.26 (4)	0	3.43 ± 0.65 (4)	+6
TRH 10^{-4}M	2.98 ± 0.71 (4)	0	3.87 ± 0.71 (4)	+19

Table 8

Cortex plus striatum

	Expt 1		Expt 2	
Additions	Monoamine Oxidase activity pmol/min/mg ± S.D.	% stimulation	Monoamine Oxidase activity pmol/min/mg ± S.D.	% stimulation
None	0.27 ± 0.05 (4)	-	2.82 ± 1.19 (4)	-
MIH 10^{-4}M	0.34 ± 0.08 (4)	+26	3.69 ± 0.76 (4)	+31
TRH 10^{-4}M	0.41 ± 0.16 (4)	+52	3.37 ± 0.04 (4)	+20

	Expt 3		Expt 4	
Additions	Monoamine Oxidase activity pmol/min/mg ± S.D.	% stimulation	Monoamine Oxidase activity pmol/min/mg ± S.D.	% stimulation
None	1.43 ± 0.19 (4)	-	1.12 ± 0.31 (4)	-
MIH 10^{-4}M	*1.99 ± 0.43 (4)	+39	1.00 ± 0.23 (4)	-11
TRH 10^{-4}M	*2.01 ± 0.31 (4)	+41	0.98 ± 0.16 (4)	-12

Table 9

Medulla-pons

	Expt 1		Expt 2	
Additions	Monoamine Oxidase activity pmol/min/mg ± S.D.	% stimulation	Monoamine Oxidase activity pmol/min/mg ± S.D.	% stimulation
None	4.73 ± 0.96 (4)	-	1.79 ± 0.14 (4)	-
MIH 10^{-4}M	4.65 ± 0.25 (3)	-2	2.17 ± 0.43 (4)	+21
TRH 10^{-4}M	6.19 ± 1.18 (4)	+31	2.08 ± 0.16 (4)	+16

that of DOPAC were the main products formed when striatal homogenates were incubated with [^{14}C] dopamine (Table 10). This unknown compound did not cochromatograph with the main dopamine metabolites HVA, DOPET or 3MT. However, the unknown compound has the same R_F value as DOBA, the decarboxylation product of DOPAC. Further studies are needed to identify the unknown compound.

Neither MIH nor TRH appeared to stimulate monoamine oxidase activity (Table 11) or alter the ratio of unknown compound to DOPAC formed. However, as less than 50% of counts added initially were recovered, it is not possible to place too much reliance on this result.

The effect of MIH and TRH on monoamine oxidase activity in four regions of the brain is shown in Table 12. The peptides did not alter the rate of deamination of dopamine in medulla-pons and cerebellum. As the lack of any effect might be due to degradation of peptide, a second addition of peptide was made after 15 min. A second addition of TRH stimulated monoamine oxidase activity in the cortex. The addition of TRH inhibited monoamine oxidase activity in the 'rest' of the brain. However, a second addition of TRH give an activity that was not significantly different from the control value.

3.3 Biosynthesis of Dopamine

Preliminary Studies

Chromatographic analysis showed that dopamine was the major product formed when striatal homogenates were incubated

Table 10 R_{DOPAC} values of Metabolites of Dopamine

Striatal homogenates (1.5-2.0 mg protein) were incubated in 0.28 M phosphate buffer for 30 min with 0.2 μCi of [^{14}C] dopamine (final concentration 12.8 μM). Ethyl acetate extracts were chromatographed on silica gel sheets together with the following standards: dopamine, DOPAC, HVA, 3MT and DOPET. The solvent system used was the organic phase of chloroform : acetic acid : H_2O (2 : 2 : 1 by volume). Results expressed as R_{DOPAC} values

$$= \frac{\text{distance migrated from origin}}{\text{distance migrated from origin by DOPAC}}$$

Dopamine Metabolite	R_{DOPAC} value
HVA	1.8 ± 0.1 (9)
DOBA	1.2 ± 0.0 (4)
Unknown compound	1.2 ± 0.1 (11)
DOPAC	1.0
DOPET	0.8 ± 0.1 (9)
3MT	0.2 ± 0.0 (8)
Dopamine	0

Table 11 Chromatographic Analysis of Products Formed by Striatal Sucrose Homogenates Incubated with [^{14}C] Dopamine

Sucrose homogenates (1.5-2.0 mg protein) were incubated in 0.28 M phosphate buffer for 30 min with 0.2 μCi of [^{14}C] dopamine (final concentration 12.8 μM) in the presence or absence of peptide as shown. The reaction was stopped by the addition of acetone : 0.1 M HCl (9 : 1 v/v). Protein was removed and samples dried down *in vacuo*. The residues were extracted with acetone : 0.1 M HCl (9 : 1 v/v) and chromatographed on silica gel sheets using the solvent system of the organic phase of chloroform : acetic acid : H_2O (2 : 2 : 1 volume). The radioactive spots were scraped off the chromatograms and radioactivity determined by liquid scintillation counting. Results are expressed as a percentage of counts recovered.

Sample	% counts	Total counts	%Unknown compound +DOPAC	Unknown compound / DOPAC
No peptide				
Unknown compound	7.7	35,101	13.8	1.3
DOPAC	6.1			
Dopamine	86.2			
Unknown compound	3.7	38,522	8.6	0.8
DOPAC	4.9			
Dopamine	91.4			
MIH 10^{-4}M				
Unknown compound	7.0	47,320	12.8	1.2
DOPAC	5.8			
Dopamine	87.2			
Unknown compound	4.8	28,897	9.9	0.9
DOPAC	5.2			
Dopamine	90.1			
TRH 10^{-4}M				
Unknown compound	5.1	44,298	9.8	1.1
DOPAC	4.7			
Dopamine	90.2			
Unknown compound	7.5	70,506	14.6	1.1
DOPAC	7.1			
Dopamine	85.4			

Table 12 Effect of MIH and TRH on Monoamine Oxidase Activity in Sucrose Homogenates

Sucrose homogenates (1.5-2.0 mg protein) were incubated in 0.28 M phosphate buffer for 30 min with 0.2 μCi of [^{14}C] dopamine (final concentration 12.8 μM) in the presence or absence of peptide as shown. Second additions of peptide were made after 15 min. 'Rest'-portion of brain remaining after the medulla-pons, cortex and cerebellum have been removed. Results from a single preparation are expressed as pmol/min/mg protein and are means ± S.D. for the number of determinations in parentheses.

* significantly greater than control $p < 0.05$

**significantly less than control $p < 0.05$

Additions	Monoamine oxidase activity pmol/min/mg ± S.D.			
	Medulla-pons	Cortex	Cerebellum	'Rest'
None	3.56±0.26(4)	2.97±0.15(4)	2.63±0.58(4)	3.65±0.19(4)
MIH 10^{-4}M x 1	3.74±0.22(4)	2.82±0.25(4)	2.71±0.43(4)	3.71±0.20(4)
MIH 10^{-4}M x 2	4.00±0.58(4)	3.14±0.25(4)	-	3.43±0.15(4)
TRH 10^{-4}M x 1	3.77±0.43(4)	3.24±0.26(3)	2.69±0.27(4)	**3.33±0.11(4)
TRH 10^{-4}M x 2	3.79±0.50(4)	*3.30±0.31(4)	-	3.56±0.10(4)

with [^{14}C] tyrosine. Trace amounts of DOPAC were formed (Fig. 12). No significant difference was seen in chromatographic maps when either MIH (10^{-4}M final concentration) or TRH (10^{-4}M final concentration) was added to the incubation.

The rate of dopamine synthesis by sucrose homogenates increases with time for at least 30 min (Fig. 13) and with protein to at least 1 mg (Fig. 14). As shown in Table 13, neither MIH (10^{-4}M final concentration) nor TRH (10^{-4}M final concentration) had any effect on the rate of conversion of [^{14}C] tyrosine to [^{14}C] dopamine.

These results suggested that the method was reproducible and that it was worthwhile to try to use a crude synaptosomal preparation to study the effect of MIH and TRH on the biosynthesis of dopamine. Further, this preparation may have less peptidase activity than sucrose homogenates as the S_1 fraction has been removed (Griffiths et al, 1975; 1976).

Studies with Striatal P_2 Fractions

Electron micrographs of the striatal P_2 fraction are shown in Figs. 15-17. In Fig. 15, particles, about 1 μm diameter, bound by a single membrane and containing mitochondria, synaptic vesicles and vacuoles, are visible. High-power electron micrographs of synaptosomes in the preparation are presented in Figs. 16 and 17. The limiting membrane, mitochondria, synaptic vesicles (about 40 nm diameter) and vacuoles are clearly visible (Fig. 16). The synaptic cleft and postsynaptic membrane are identifiable constituents of the synaptosome shown in Fig. 17.

The percentage of occluded lactate dehydrogenase of nine preparations was 85.4 ± 4% (mean ± S.D.) in agreement with

Chromatographic Analysis of Products Formed by Striatal Homogenates Incubated with [^{14}C] tyrosine

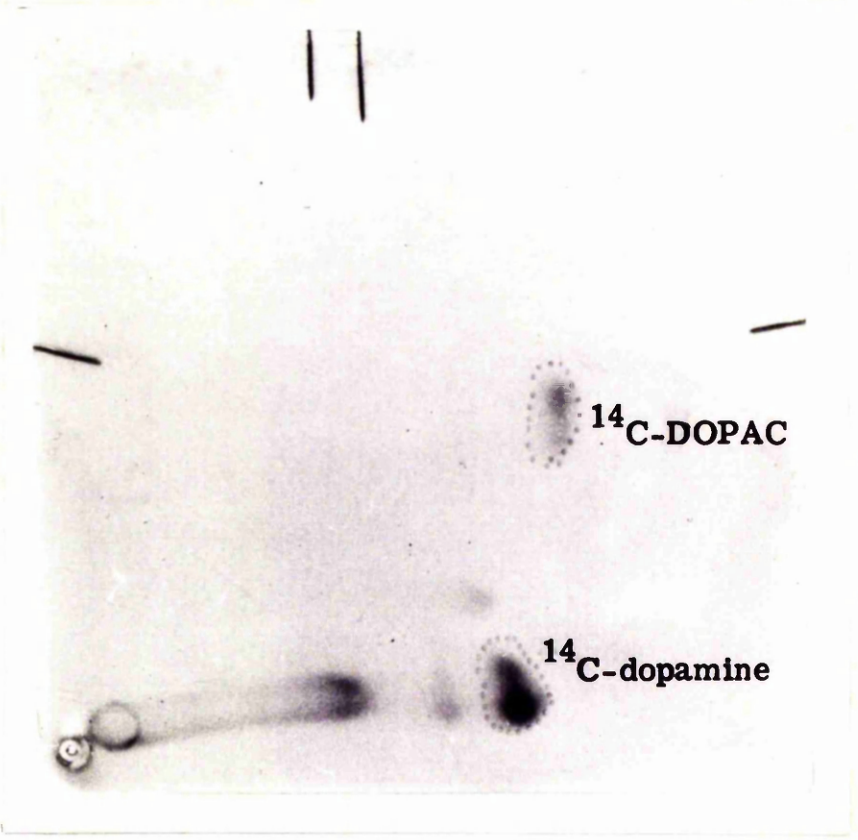


Fig 12

Sucrose homogenates (2 mg protein) were incubated in 0.28 M phosphate buffer for 30 min with 1 μCi of [^{14}C] tyrosine (6.86 μM). Eluates from alumina columns were dried down *in vacuo* and the residues taken up in acetone: 0.1 M HCl (9 : 1 v/v). Extracts were chomatographed in two dimensions on silica gel sheets together with the following standards:- tyrosine, dopamine, DOPAC and HVA. The solvent systems used were:-

1st dimension - the organic phase of chloroform:acetic acid: H_2O (2 : 2 : 1 by volume)

2nd dimension - butan-1-ol:pyridine:acetic acid:H_2O (15 : 2 : 3 : 5 by volume).

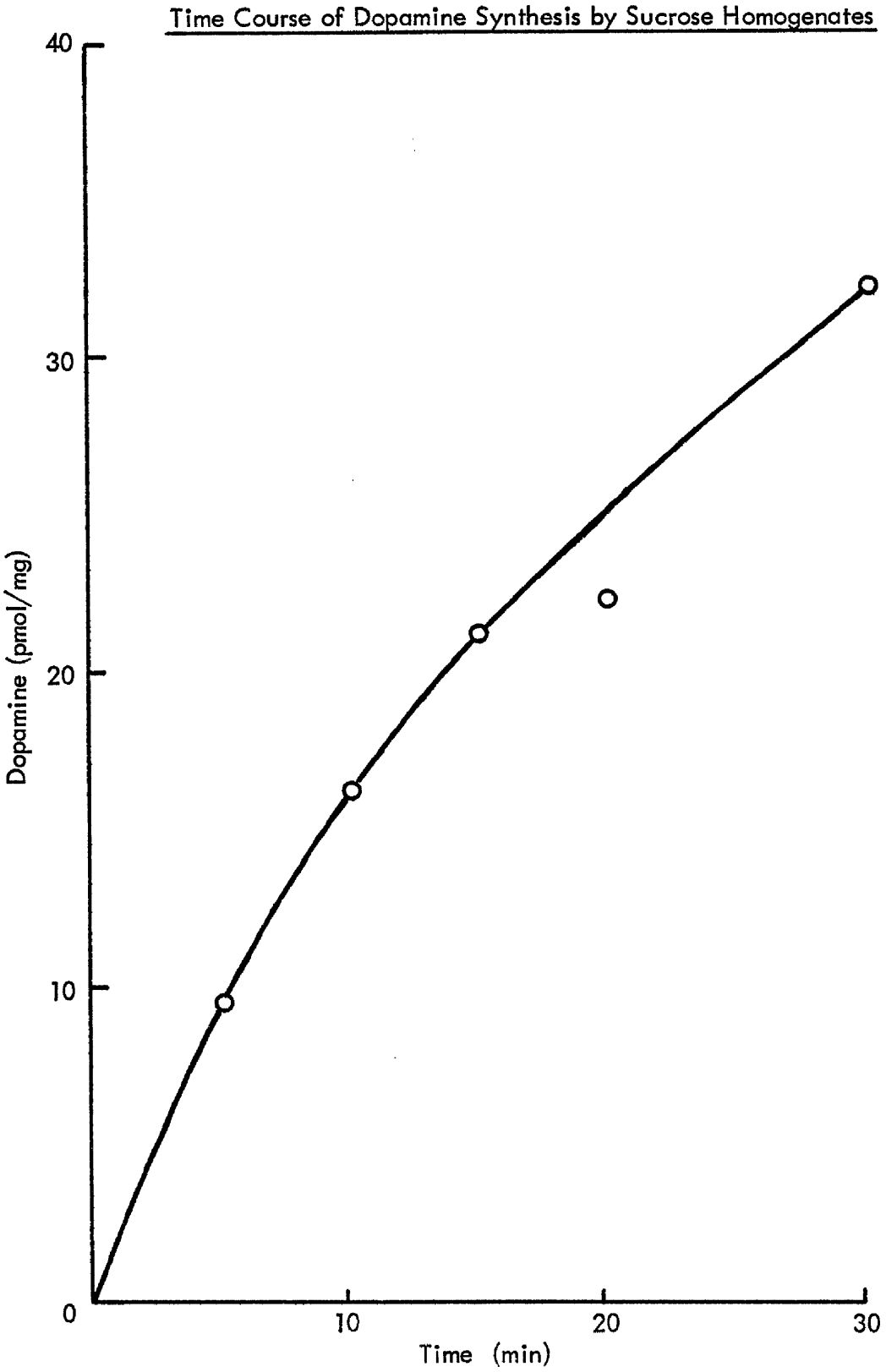


Fig. 13. Sucrose homogenates (2 mg protein) were incubated in 0.28 M phosphate buffer with 1 μCi of [^{14}C] tyrosine (final concentration 6.86 μM) for the times shown. Dopamine synthesis is expressed in pmol/mg.

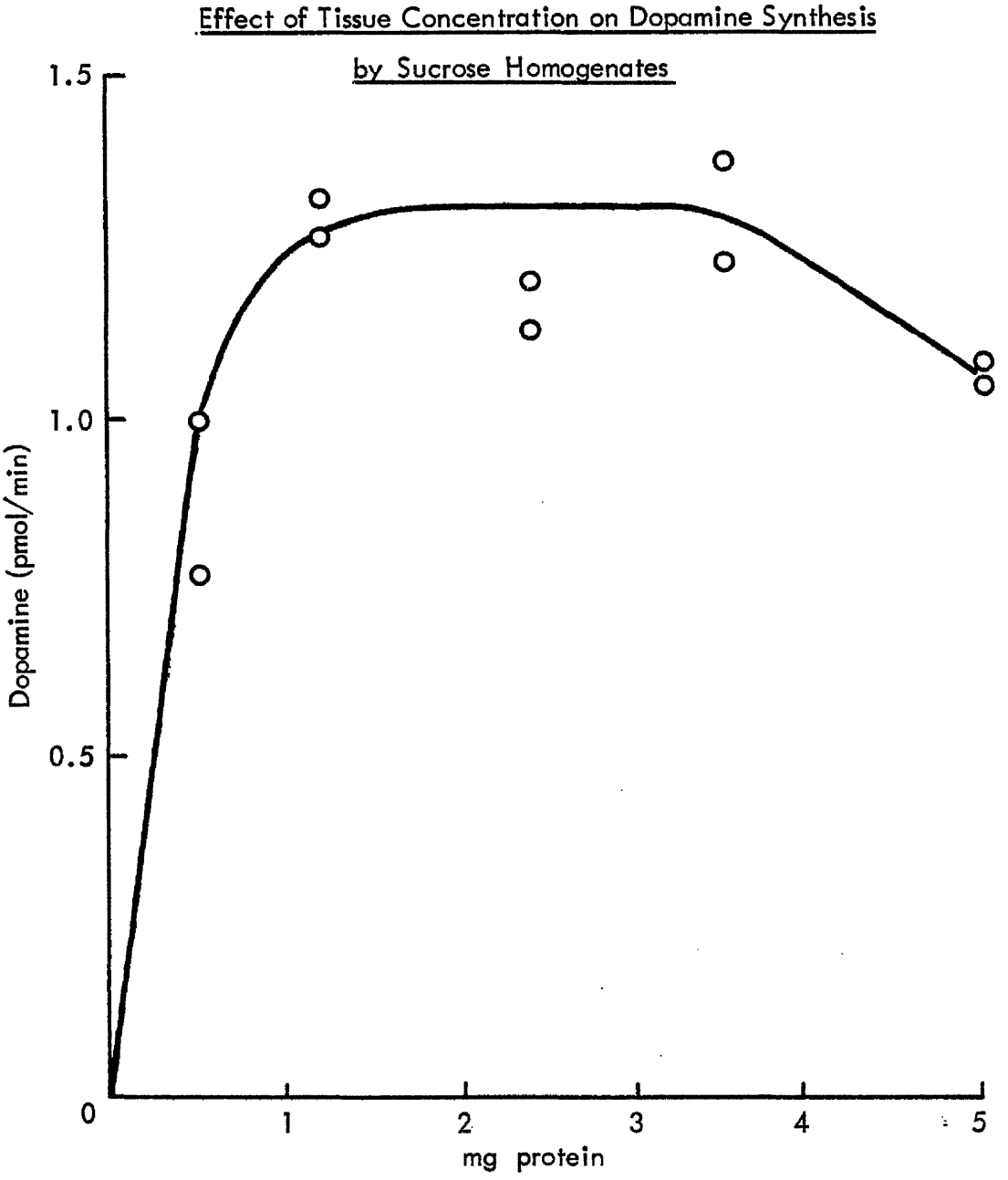


Fig. 14. Sucrose homogenates (0.5 - 5.0 mg protein) were incubated in 0.28 M phosphate buffer with 1 μCi of [^{14}C] tyrosine (final concentration 6.86 μM) for 30 min. Dopamine synthesis is expressed in pmol/min.

Table 13 Effect of MIH and TRH on Dopamine Synthesis by Sucrose Homogenates

0.1 ml of sucrose homogenate (2 mg protein) was incubated for 30 min with 1 μCi of [^{14}C] tyrosine (final concentration 6.86 μM) in 0.28 M phosphate buffer containing pargyline (final concentration 0.4 mM) in the presence or absence of peptide as shown.

Results are expressed as pmol/min/mg protein and are means ± S.D. of four determinations from a single homogenate.

Additions	Dopamine pmol/min/mg ± S.D.
None	1.02 ± 0.10 (4)
MIH 10^{-4}M	0.88 ± 0.08 (4)
TRH 10^{-4}M	0.99 ± 0.05 (4)

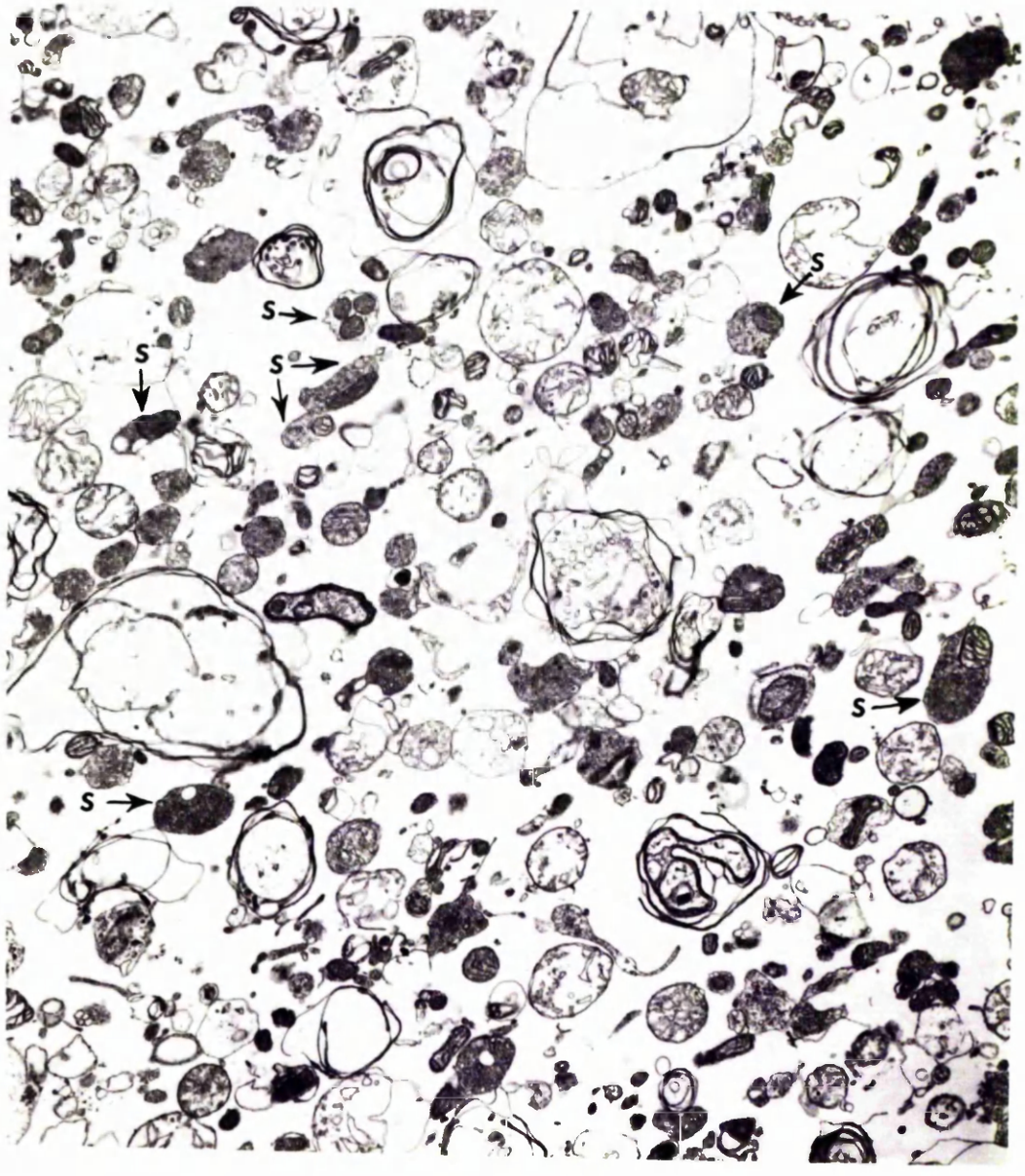


Fig. 15. Electron micrograph of the P_2 fraction. Several synaptosomal profiles (arrows identified by S) are seen containing mitochondria, synaptic vesicles and vacuoles.

(x 12,500)

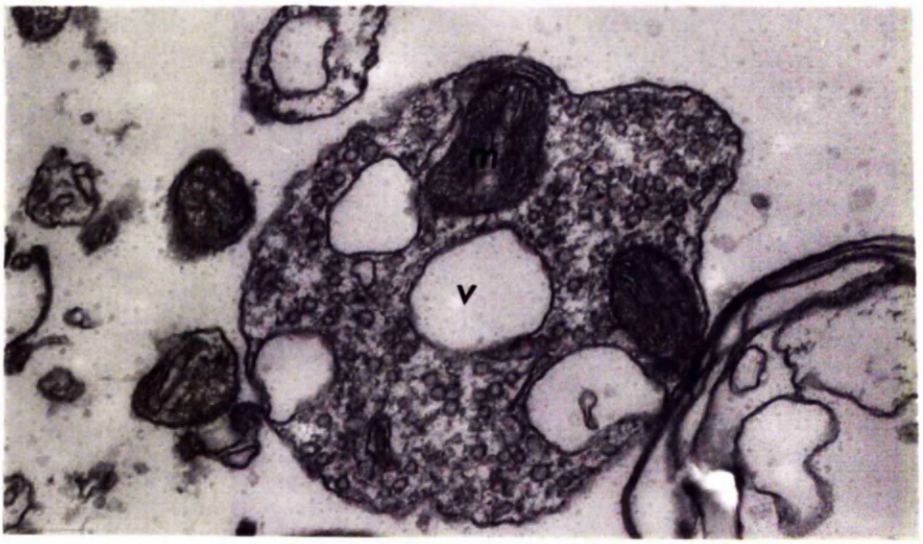


Fig. 16. Electron micrograph demonstrating the ultrastructure of a single synaptosome from the specimen shown in Fig. 15. The limiting membrane, four vacuoles (V), two mitochondria (m) and many synaptic vesicles are clearly visible.
(x 47,000)

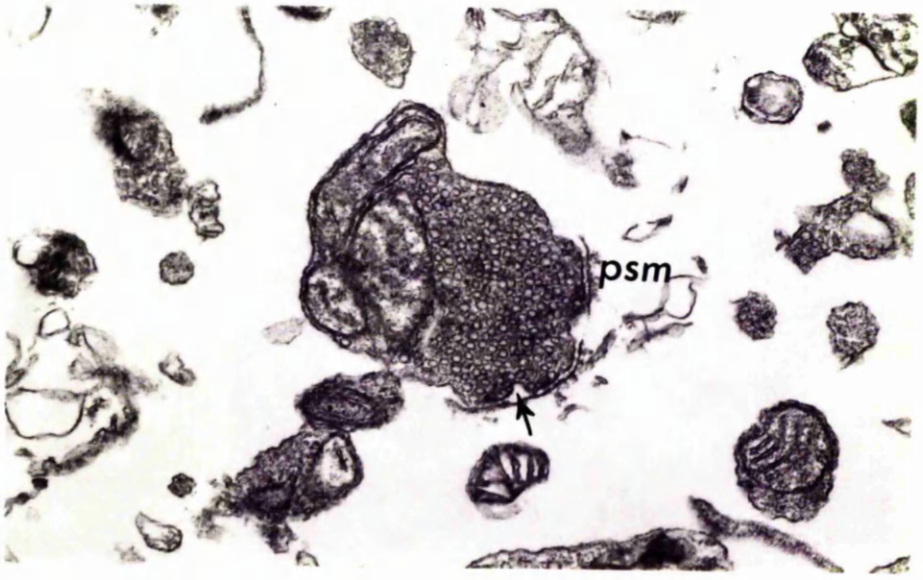


Fig. 17. Electron micrograph of a synaptosome showing the synaptic cleft (arrow) and post synaptic membrane (psm).
(x 60,000)

the value of 80% obtained by Heaton and Bachelard (1973) for cortical synaptosomes. Further, P_2 fractions formed dopamine from tyrosine without the need of exogenous cofactors. Tyrosine hydroxylase activities (0.4 - 1.8 pmol/min/mg) were in agreement with literature values (Table 14).

These results suggested that a useful preparation for studying dopamine synthesis had been obtained.

The rate of dopamine synthesis by the P_2 fraction increased with time for at least 30 min (Fig. 18) and with protein to at least 0.68 mg (Fig. 19). Incubations routinely were for 20 min and contained 0.40-0.65 mg protein.

As calcium stimulated tyrosine hydroxylase isolated from rat striatum (Gutman and Segal, 1973), the effect of calcium on dopamine synthesis was examined. Addition of 0.85 mM calcium chloride caused a 30% increase in the rate of dopamine synthesis by the P_2 fraction (Table 15). Neither MIH (10^{-4}M) nor TRH (10^{-4}M) altered the rate of dopamine synthesis. Further, neither peptide significantly stimulated the rate of dopamine synthesis in the presence of 0.85 mM calcium chloride (Table 15).

Modification of Dopamine Synthesis Assay

As only approximately 1,000 counts per sample were being recovered, the present assay required modification. Therefore, the amount of non-radioactive tyrosine added to the incubation was reduced, giving a final concentration of tyrosine of 1.07 µM instead of 18.4 µM. This modification caused a five fold increase in the number of counts converted to dopamine.

Effect of Calcium on Dopamine Synthesis at Reduced Concentrations of Tyrosine

Addition of calcium did not significantly increase the rate of dopamine synthesis at reduced concentrations of tyrosine

Table 14 Tyrosine Hydroxylase Activity in Striatal P_2 Fractions

Author	Tyrosine Hydroxylase Activity pmol/min/mg
Goldstein et al. (1976)	0.42 ± 0.04
Iversen et al.(1976)	0.105 S.D. not given
Kapatos and Zigmond (1977)	11.65 ± 0.04
Karobath (1971)	5.19 ± 3.60
Katz et al. (1976)	11.0 S.D. not given
Kuczenski (1975b)	0.55 ± 0.04
Patrick et al (1975)	0.17 ± 0.01

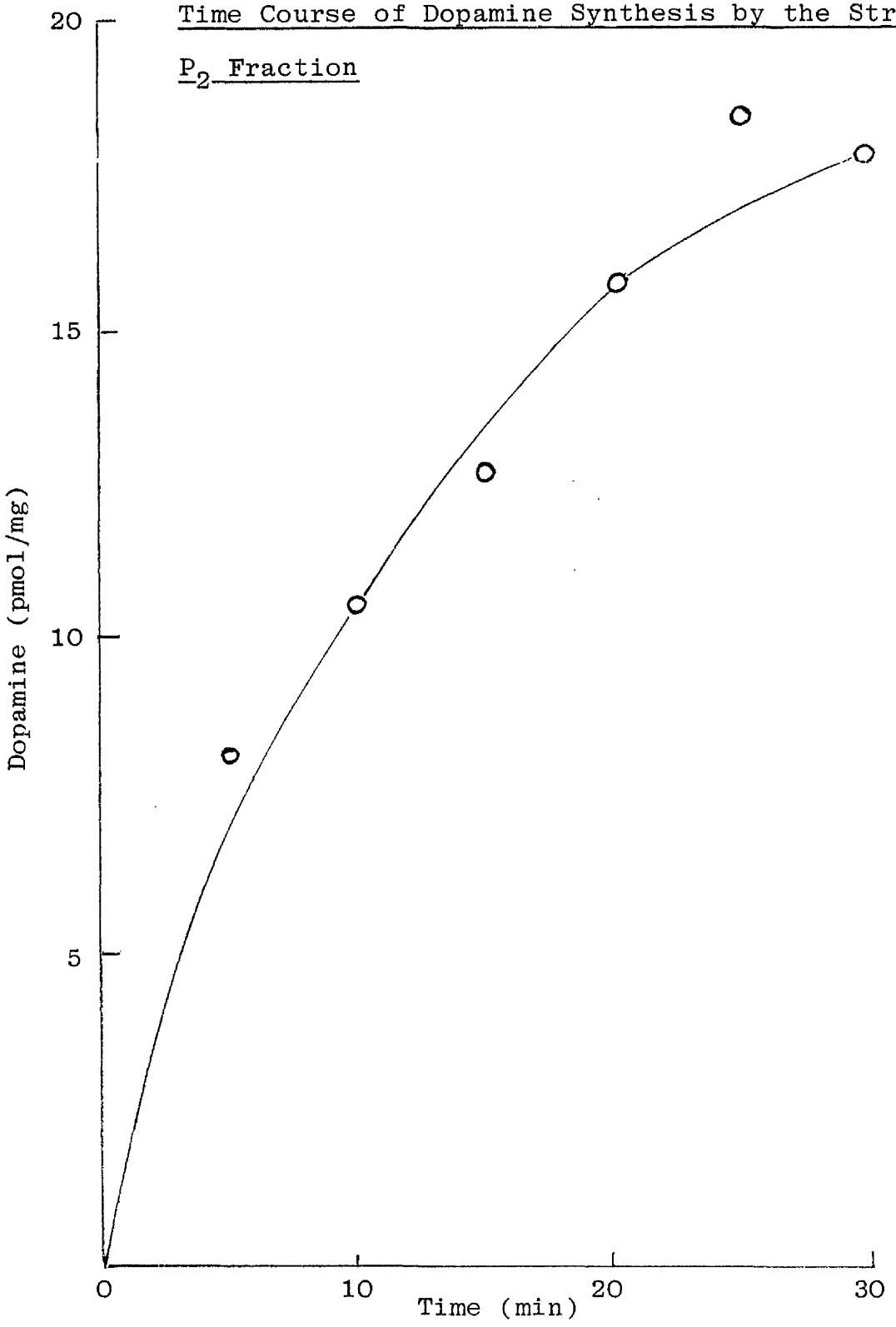


Fig. 18. 0.1 ml of striatal P_2 fraction (0.47 mg protein) was incubated in Tris-saline with 1 μCi of [^{3}H] tyrosine (final concentration 1.07 μM) for the times shown. Dopamine synthesis is expressed in pmol/mg protein. No significant difference in the time course was observed when synaptosomes were incubated with 18.4 μM tyrosine.

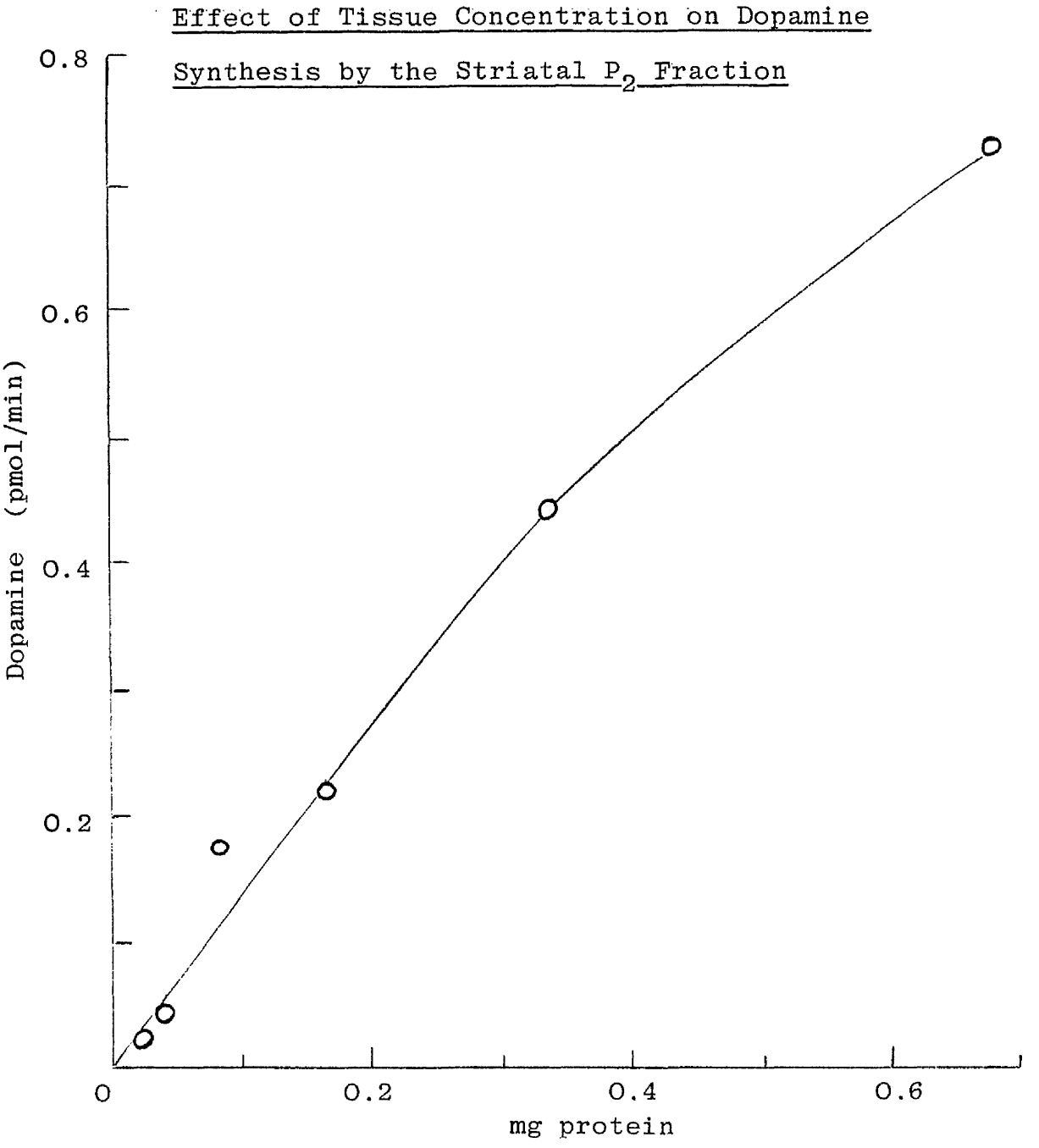


Fig. 19. 0.1 ml of striatal P_2 fraction (0.02-0.68 mg protein) was incubated in Tris-saline with 1 μCi of [^{3}H] tyrosine (final concentration 18.4 μM) for 20 min. Dopamine synthesis is expressed in pmol/min.

Table 15 Effect of MIH and TRH on Dopamine Synthesis by the Striatal P_2 Fraction

0.1 ml of a striatal P_2 fraction (0.40 mg protein) was incubated for 20 min in Tris-saline with 1 µCi of [^{3}H] tyrosine (final concentration 18.4 µM) in the presence or absence of peptide as shown.
Results are expressed as pmol/min/mg protein and are means ± S.D. of four determinations from one P_2 preparation.

*significantly greater than in the absence of 0.85 mM calcium chloride $p < 0.05$.

Additions	Dopamine pmol/min/mg ± S.D.	
	no calcium chloride	0.85 mM calcium chloride
None	0.62 ± 0.14 (4)	0.81 ± 0.11 (4)
MIH 10^{-4}M	0.63 ± 0.05 (4)	*0.82 ± 0.12 (4)
TRH 10^{-4}M	0.59 ± 0.16 (4)	*0.85 ± 0.08 (4)

(Table 16).

Effect of Peptides on Dopamine Synthesis at Reduced Concentrations of Tyrosine

Neither MIH (10^{-4}M) nor TRH (10^{-4}M) altered the rate of conversion of [^{3}H] tyrosine to [^{3}H] dopamine in the presence of 1.70 mM calcium chloride (Table 17).

MIH and TRH have been shown to have behavioural effects similar to those of amphetamine (Cohn et al, 1975; North et al, 1973). Thus it was of importance to determine whether or not amphetamine and tyramine, a drug with effects similar to those of amphetamine, could stimulate dopamine synthesis under these conditions, as a drug-induced stimulation of dopamine synthesis could increase the concentration of dopamine in the synaptic cleft and thus lead to the behavioural effects. Tyramine, however, inhibited dopamine synthesis, 50% inhibition occurring at 3.3 x 10^{-5}M (Table 18). The effect of amphetamine on dopamine synthesis under these conditions was not determined as its effect on catecholamine metabolism (release of catecholamines from peripheral and central nerve endings) is thought to be similar to that of tyramine (Raiteri and Levi, 1978).

Effect of Drugs on Dopamine Synthesis in the Presence of EDTA

Kuczenski (1975b) showed that tyramine and amphetamine could stimulate dopamine synthesis in synaptosomes incubated in phosphate buffered saline containing 2 mM EDTA. It was thus important to check that this stimulation could be achieved in Tris-saline containing 2 mM EDTA and to determine whether or not MIH and TRH could stimulate dopamine synthesis under these conditions.

The stimulatory effect of tyramine and amphetamine on dopamine synthesis was confirmed in Tris-saline containing

Table 16 Effect of Calcium on Dopamine Synthesis

0.1 ml of striatal P_2 fraction (0.54 mg protein) was incubated for 25 min with 1 µCi of [^{3}H] tyrosine (final concentration 1.07 µM) in Tris-saline containing calcium chloride as shown.

Results from a single preparation are expressed as pmol/min/mg protein and are means ± S.D. for the number of determinations in parentheses.

Additions	Dopamine pmol/min/mg ± S.D.
None	0.50 ± 0.04 (3)
Calcium chloride 1 mM	0.55 ± 0.01 (3)
Calcium chloride 2 mM	0.53 ± 0.02 (3)

Table 17 Effect of MIH and TRH on Dopamine Synthesis

0.1 ml of striatal P_2 fraction (0.61 mg protein) was incubated for 20 min with 1 μCi of [^{3}H] tyrosine (final concentration 1.07 μM) in Tris-saline containing 1.70 mM calcium chloride in the presence or absence of peptide as shown.

Results are expressed as pmol/min/mg protein and are means ± S.D. of four determinations from one P_2 preparation.

Additions	Dopamine pmol/min/mg ± S.D.
None	0.57 ± 0.01 (4)
MIH 10^{-4}M	0.54 ± 0.04 (4)
TRH 10^{-4}M	0.55 ± 0.08 (4)

Table 18 Effect of Tyramine on Dopamine Synthesis

0.1 ml of striatal P_2 fraction (0.7 mg protein) was preincubated for 10 min in Tris-saline containing 1.70 mM calcium chloride in the presence or absence of tyramine as shown, then incubated for 20 min with 1 μCi of [^{3}H] tyrosine (final concentration 1.07 μM).
Results from a single preparation are expressed as pmol/min/mg protein.

Tyramine (M)	None	3.3×10^{-7}	3.3×10^{-6}	6.7×10^{-6}	3.3×10^{-5}	3.3×10^{-4}
Dopamine pmol/min/mg	0.36	0.33	0.27	0.21	0.18	0.20

2 mM EDTA. Preliminary studies (Table 19, experiments 1-5) showed that maximum stimulation of dopamine synthesis by amphetamine and tyramine occurred over the range 10^{-5}-10^{-4}M and suggested that 10^{-4}M peptide could stimulate dopamine synthesis. In a further experiment (Table 19, experiment 6) both TRH (10^{-4}M) and amphetamine (10^{-4}M) independently stimulated dopamine synthesis but in two subsequent experiments (Table 19, experiments 7 and 8), TRH (10^{-4}M) did not influence dopamine when amphetamine (10^{-5}M and 10^{-4}M) stimulated dopamine synthesis. In the next experiment (Table 19, experiment 9) amphetamine (10^{-4}M) did not influence dopamine synthesis. TRH (10^{-4}M), however, inhibited dopamine synthesis under these conditions. TRH (10^{-4}M) did not interfere with amphetamine (10^{-4}) induced stimulation of dopamine synthesis (Table 19, experiments 6 and 7).

However, more detailed study revealed that the magnitude of the stimulation of dopamine synthesis by amphetamine (10^{-4}M) (Fig. 20) and tyramine (10^{-4}M) (Fig. 21) varied inversely with the basal rate of dopamine synthesis (0.55 $\pm$ 0.10 (4) - 1.79 $\pm$ 0.11 (4) pmol/min/mg protein: data from the 9 separate experiments in Table 19). A significant negative correlation ($p < 0.05$) between amphetamine (10^{-4}M) induced stimulation and the basal rate of dopamine synthesis was found (Fig. 20)

No such correlation could be found for the data with TRH (10^{-4}M) (Fig. 22). This suggested that the mechanism of action of this peptide was different from that of the sympathomimetic amines amphetamine and tyramine. The results

Table 19 Effect of Drugs on Dopamine Synthesis

0.1 ml of striatal P_2 fraction (0.4 - 0.7 mg protein) was preincubated for 10 min in Tris-saline containing 2 mM EDTA in the presence or absence of drug as shown then incubated for 20 min with 1 µCi of [^{3}H] tyrosine (final concentration 1.07 µM). Results are expressed as pmol/min/mg protein and are means ± S.D. for the number of determinations in parentheses. Each separate experiment represents data from a single P_2 preparation of 3 rats.

* significantly greater than control $P < 0.05$

** significantly less than control $P < 0.05$

Table 19 Effect of Drugs on Dopamine Synthesis

Dopamine pmol/min/mg ± S.D.

Additions		Expt 1	Expt 2	Expt 3	Expt 4	Expt 5
None		1.09; 1.53	0.99±0.06(4)	1.20; 1.26	1.25±0.09(3)	1.45 ± 0.15(3)
Tyramine	10^{-7}M	1.33	-	-	-	-
	10^{-6}M	1.44	-	-	-	-
	10^{-5}M	1.57	1.08±0.05(4)	-	-	-
	10^{-4}M	1.52	*1.25±0.14(4)	1.35; 1.41	-	-
	10^{-3}M	0.78	-	-	-	-
Amphetamine	10^{-7}M	1.26	-	-	-	-
	10^{-6}M	1.45	-	-	-	-
	10^{-5}M	1.65	-	-	-	-
	10^{-4}M	1.60	-	-	1.27±0.10(3)	1.45 ± 0.22(3)
	10^{-3}M	1.13	-	-	-	-
MIH	10^{-6}M	-	-	-	1.21±0.03(3)	-
	10^{-5}M	-	-	1.13	1.29±0.09(3)	-
	10^{-4}M	-	-	1.26	1.43±0.14(3)	-
	10^{-3}M	-	-	1.04	1.25±0.06(3)	-
	10^{-4}M no preincubation	-	-	1.07	-	-
TRH	10^{-6}M	-	-	-	-	1.28 ± 0.01(3)
	10^{-5}M	-	-	0.91	-	1.33 ± 0.08(3)
	10^{-4}M	-	-	1.01	-	1.60 ± 0.38(3)
	10^{-3}M	-	-	1.04	-	*1.74 ± 0.19(3)
	10^{-4}M no preincubation	-	-	0.88	-	-

Table 19 Effect of Drugs on Dopamine Synthesis (contd)

Dopamine pmol/min/mg ± S.D.

Additions	Expt 6	Expt 7	Expt 8	Expt 9
None	0.55±0.10(4)	0.69±0.07(4)	1.01±0.09(4)	1.79±0.11(4)
Amphetamine 10^{-5}M	-	-	*1.20±0.10(4)	-
10^{-4}M	*0.77±0.07(4)	*0.87±0.10(4)	*1.28±0.04(4)	1.82±0.11(4)
TRH 10^{-4}M	*0.71±0.08(4)	0.71±0.08(4)	1.04±0.05(4)	**1.36±0.04(4)
10^{-4}M	-	-	-	1.62±0.14(4)
no preincubation				
TRH 10^{-4}M+ Amphetamine 10^{-4}M	*0.79±0.03(4)	*0.87±0.07(4)	-	-

Fig. 20. This figure summarises data obtained from all experiments in Table 19. 0.1 ml of striatal P_2 fraction was incubated as described in the legend for Table 19 and the rate of dopamine synthesis (pmol/min/mg) determined. The drug-induced change in dopamine synthesis is given as the % stimulation. Each point represents data from a separate preparation of three rats. The relationshop between the basal rate of dopamine synthesis and the drug-induced change in dopamine synthesis was determined by linear regression analysis as described in methods (2.4.2.).

Fig. 20 Relationship between Amphetamine (10^{-4}M)-induced Increase in Dopamine Synthesis and Basal Rate of Dopamine Synthesis

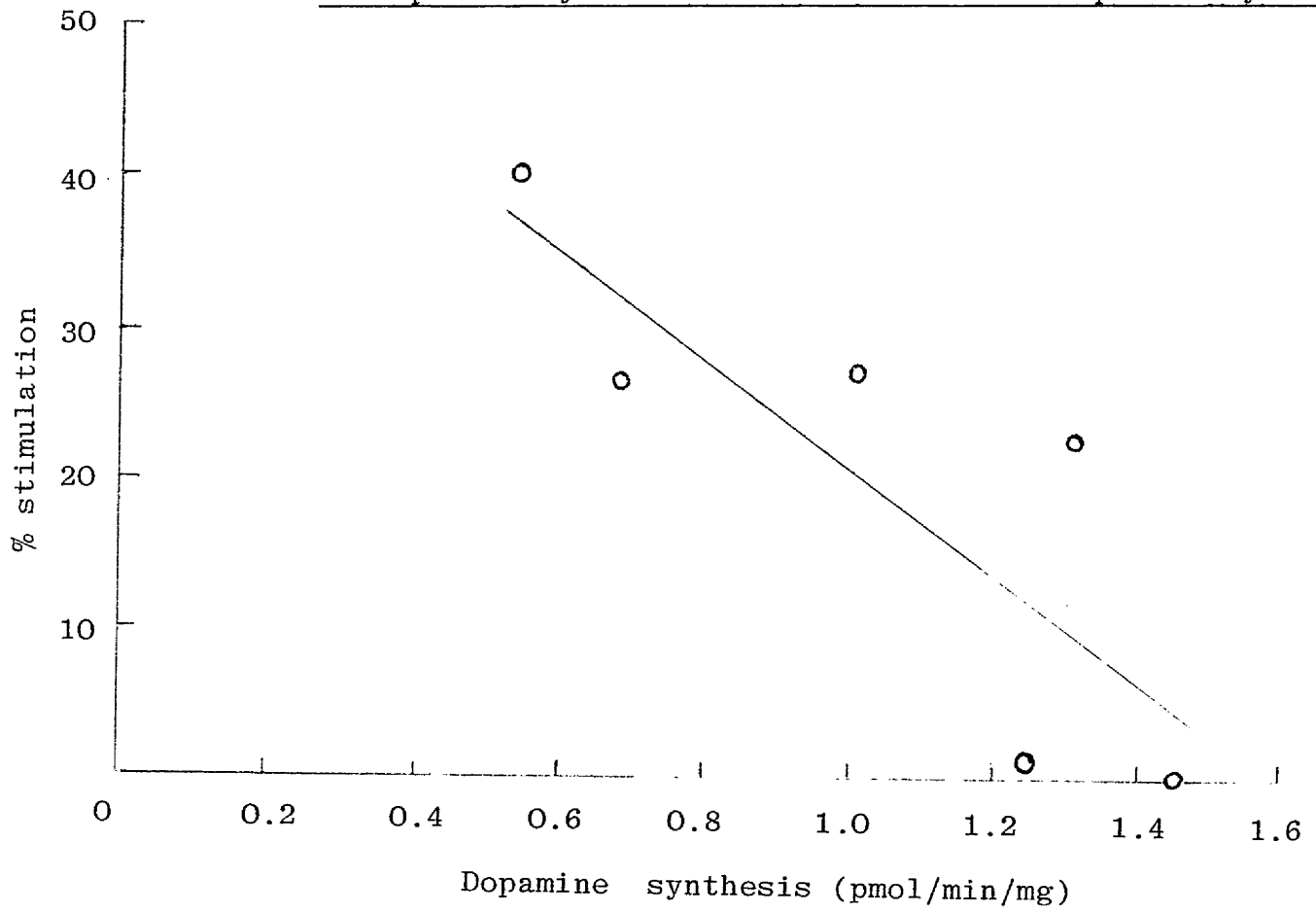


Fig. 21 Relationship between Tyramine (10^{-4}M)-induced Increase in Dopamine Synthesis and Basal Rate of Dopamine Synthesis

0.1 ml of striatal P_2 fraction was incubated and data plotted as described in the legend for Fig. 20.

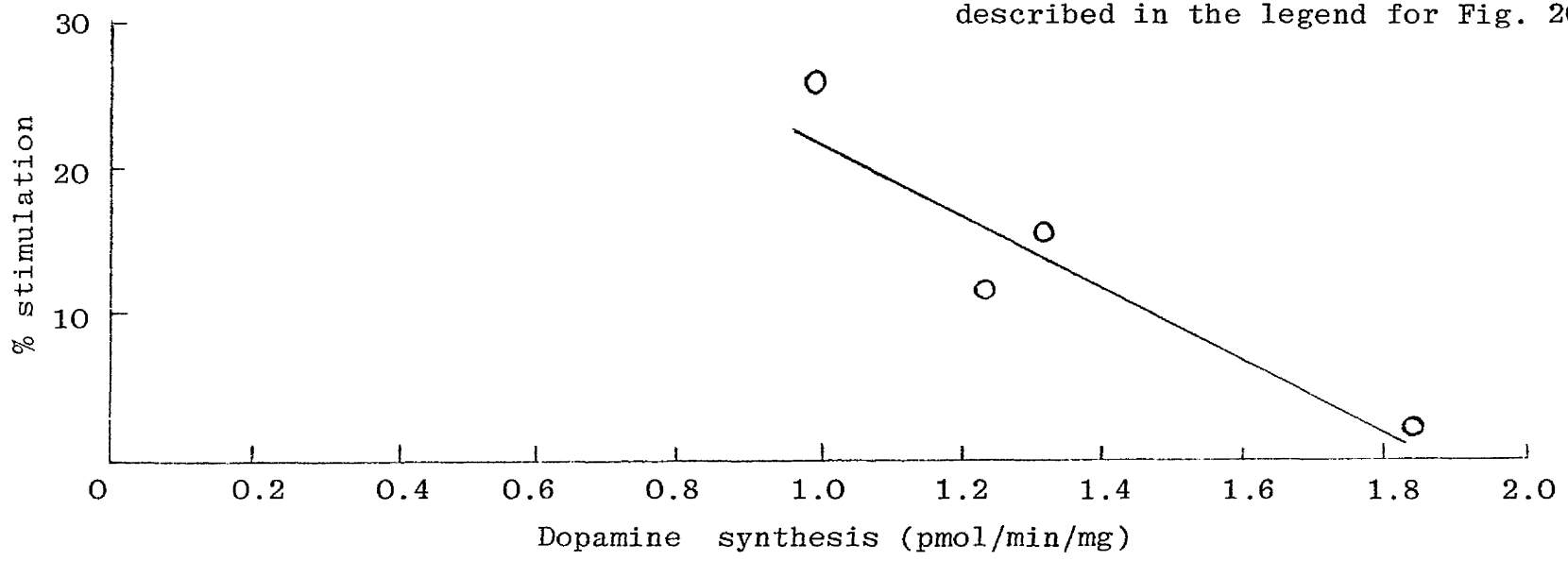
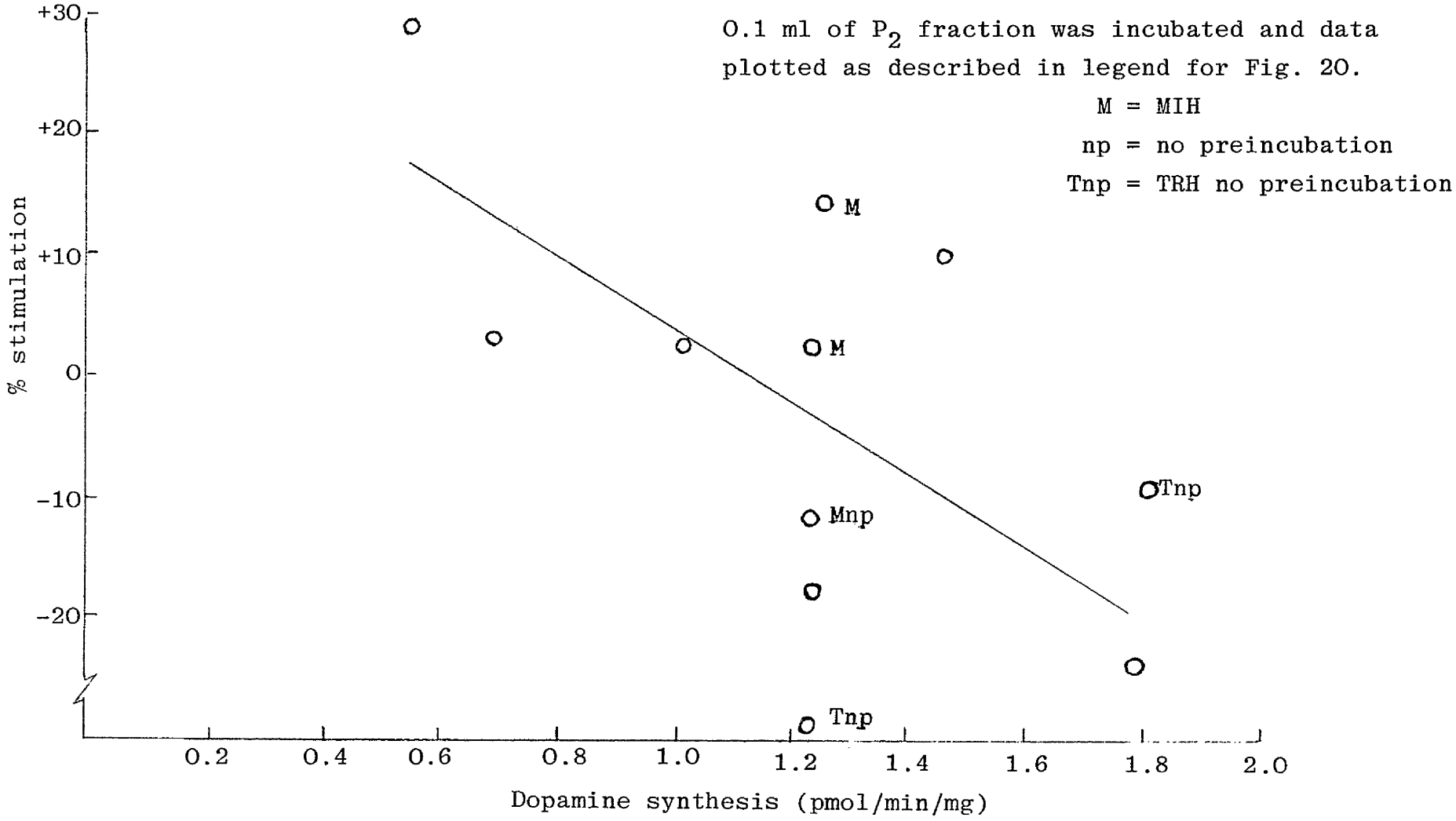


Fig. 22 Relationship between TRH (10^{-4}M)-induced Change in Dopamine Synthesis and Basal Rate of Dopamine Synthesis



of experiments in which synaptosomes were not preincubated with TRH (10^{-4}M) were not very different from those in which preincubation with TRH (10^{-4}M) occurred (Fig. 22). Thus it was unlikely that the difference in action between TRH and amphetamine and tyramine was due to degradation of peptide.

Possible Reason for Variation in Basal Dopamine Synthesis Rate

It has been suggested that there are two distinct storage pools of dopamine in dopaminergic nerve terminals (De Belleroche et al, 1976; Doteuchi et al, 1974; Javoy and Glowinski, 1971; Kuczenski, 1975b). According to the model of Kuczenski (1975b), these pools are a large storage pool and a smaller pool functional in the regulation of tyrosine hydroxylase. The activity of tyrosine hydroxylase may then reflect the size of this regulatory dopamine pool. If the concentration of dopamine in the inhibitory pool is high, then tyrosine hydroxylase activity will be low. Thus releasing agents, for example amphetamine and tyramine, may be able to stimulate dopamine synthesis by releasing tyrosine hydroxylase from feedback inhibition. Conversely, if the inhibitory pool is depleted, tyrosine hydroxylase activity will be high and unable to be increased further as releasing agents cannot act.

Attempt to Alter the Size of the Inhibitory Dopamine Pool

As the variation in dopamine synthesis may be due to the size of an inhibitory dopamine pool, an attempt was made to alter the dopamine content of the P_2 fraction (Table 20).

The P_2 fraction was incubated in Tris-saline containing 10^{-4}M dopamine to load the inhibitory pool. After centrifugation

Table 20 Effect of Preincubation with Dopamine on Dopamine Synthesis

0.5 ml of S_1 fraction was incubated for 20 min with 1 ml of Tris-saline containing 0.4 mM pargyline, 120 µM EDTA and 1.65 mM ascorbic acid in the presence or absence of dopamine. Preincubation was terminated by centrifugation at 17,000 g for 15 min. The P_2 fractions were resuspended and incubated as described in legend for Table 19. Results are expressed as pmol/min/mg protein and are means ± S.D. for three determinations from one P_2 preparation.

*significantly less than incubation in the absence of dopamine $p < 0.05$.

Additions	Dopamine pmol/min/mg ± S.D.
None	1.84 ± 0.08 (3)
Dopamine 10^{-4}M	*0.82 ± 0.07 (3)

and resuspension, dopamine synthesis was measured. An identical sample was first incubated in Tris-saline without dopamine to deplete the inhibitory dopamine pool. Then dopamine synthesis in the dopamine depleted P_2 fraction was measured.

In agreement with the above predictions, incubation with dopamine caused a greater than 50% inhibition of the conversion of [^{3}H] tyrosine to [^{3}H] dopamine (Table 20).

As the stimulation of tyrosine hydroxylase by amphetamine and tyramine occurs as a result of stimulation of release of dopamine (Kuczenski, 1975b) and the effect observed on tyrosine hydroxylase may depend on the size of an inhibitory dopamine pool (Kuczenski, 1975b), it was decided to examine the effect of amphetamine, MIH and TRH directly on release. Further, it was important to determine whether or not differences in the effects of these drugs on dopamine synthesis would be reflected in differences in dopamine release.

3.4 Release of Dopamine

Uptake of dopamine by striatal P_2 fractions increased approximately thirty fold over the range 2.5×10^{-9}M - 2.5×10^{-7}M dopamine (Table 21). Therefore 2×10^{-8}M dopamine was used to load P_2 fractions. Eitan and Hershkowitz (1977) reported that 25.6 pmoles of dopamine were taken up per mg of protein when S_1 fractions were loaded with 7×10^{-8}M dopamine. The data of Table 21 is in agreement with this value.

When the P_2 fraction was loaded with 2×10^{-8}M dopamine,

Table 21 Uptake of [^{3}H] Dopamine by Striatal P_2 Fractions

0.2 ml of S_1 fraction was incubated for 10 min in 3.8 ml of Tris-saline with the concentration of [^{3}H] dopamine shown. Results are expressed as pmol/mg protein and are means ± S.D. for the number of separate experiments in parentheses.

Dopamine concentration (M)	Uptake pmol/mg ± S.D.
2.5×10^{-9}	3.30 ± 0.27 (4)
2.0×10^{-8}	9.63 ± 3.64 (20)
2.5×10^{-8}	16.60 ± 4.86 (6)
2.0×10^{-7}	73.65; 76.00
2.5×10^{-7}	119.00 ± 5.89 (4)

the rate of release was linear for at least 2 min in the absence of amphetamine (Fig. 23). Amphetamine stimulated release over the time interval 0-5 min, the greatest stimulation being observed at 1 min. Therefore the effect of drugs on a 2 min release of dopamine was studied.

Eitan and Hershkowitz (1977) reported that 1.3 and 3.0 (data from 2 striatal P_2 fractions) pmol of dopamine were released per min per mg of protein in the presence of 1.3 mM calcium chloride. In this thesis 0.69 $\pm$ 0.43 (mean $\pm$ S.D. of 14 striatal P_2 fractions) and 0.62 $\pm$ 0.26 (mean $\pm$ S.D. of 11 striatal P_2 fractions) pmol of dopamine were released per min per mg of protein in the presence of 1.25 mM calcium chloride and 2 mM EDTA respectively. This suggested that a suitable preparation for studying dopamine release had been obtained.

In one experiment (Fig. 24), the P_2 fraction was loaded with 2 x 10^{-7}M dopamine. The time course of dopamine release in the presence of 2 mM EDTA or 1.25 mM calcium chloride over a period of 1 h is shown. Release of dopamine was observed to be biphasic, a quick release occurring followed by a slower release. Incubation at 0^{o}C for 1 h did not increase the blank value.

Effect of Drugs on Release of Dopamine

Preliminary studies indicated that amphetamine, MIH and TRH stimulated release of dopamine from striatal P_2 fractions in the presence of 1.25 mM calcium chloride. Maximum stimulation of release with amphetamine (400%) occurred at 10^{-4}M

Time Course of Release of Dopamine from the Striatal P_2 Fraction

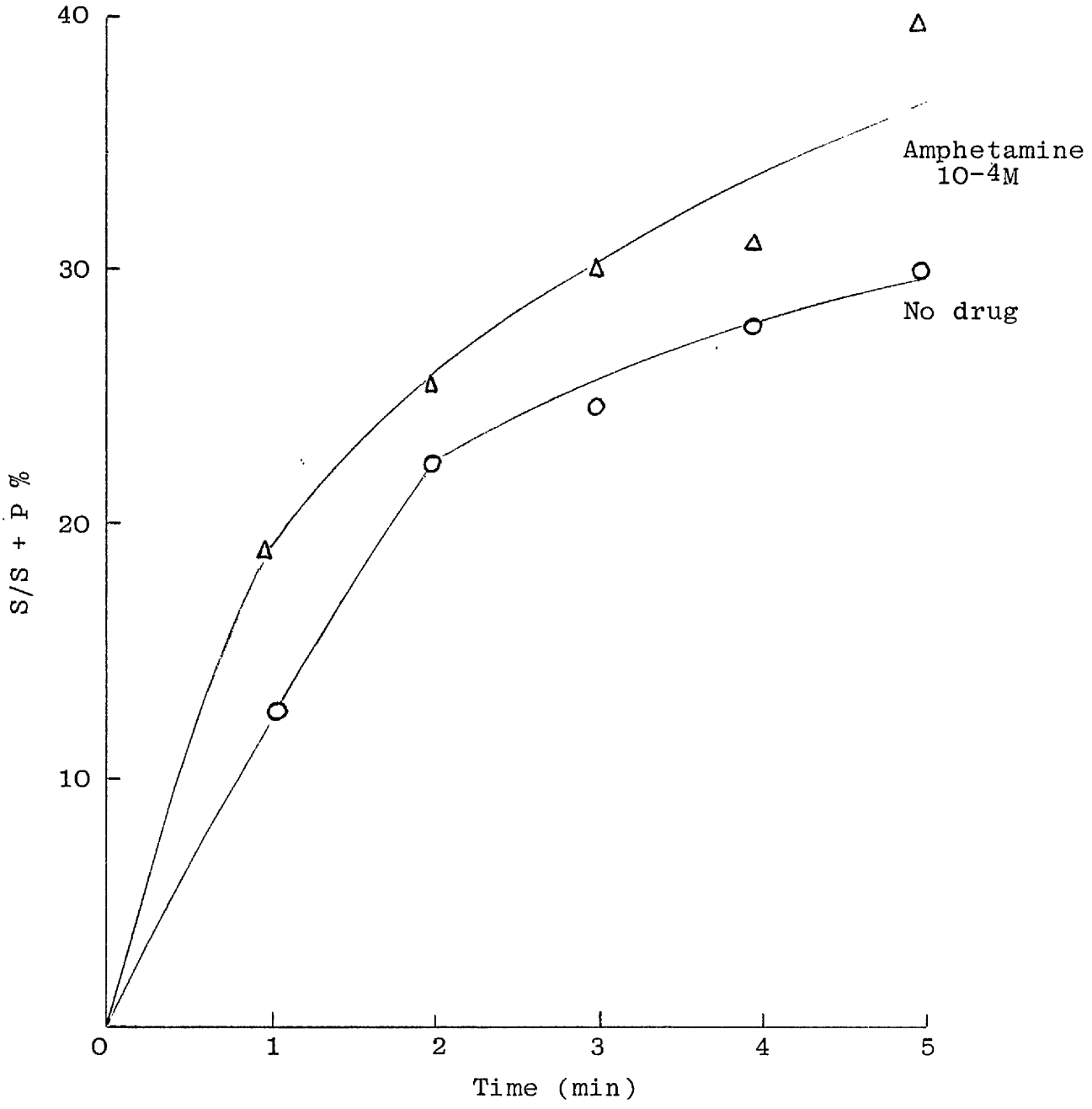


Fig. 23 The S_1 fraction was loaded for 10 min with [^{3}H] dopamine (final concentration 2 x 10^{-8}M). 0.1 ml of resuspended pellet was added to 2.9 ml of saline containing 1.25 mM calcium chloride in the presence or absence of amphetamine and release of dopamine allowed to proceed for 0-5 min.

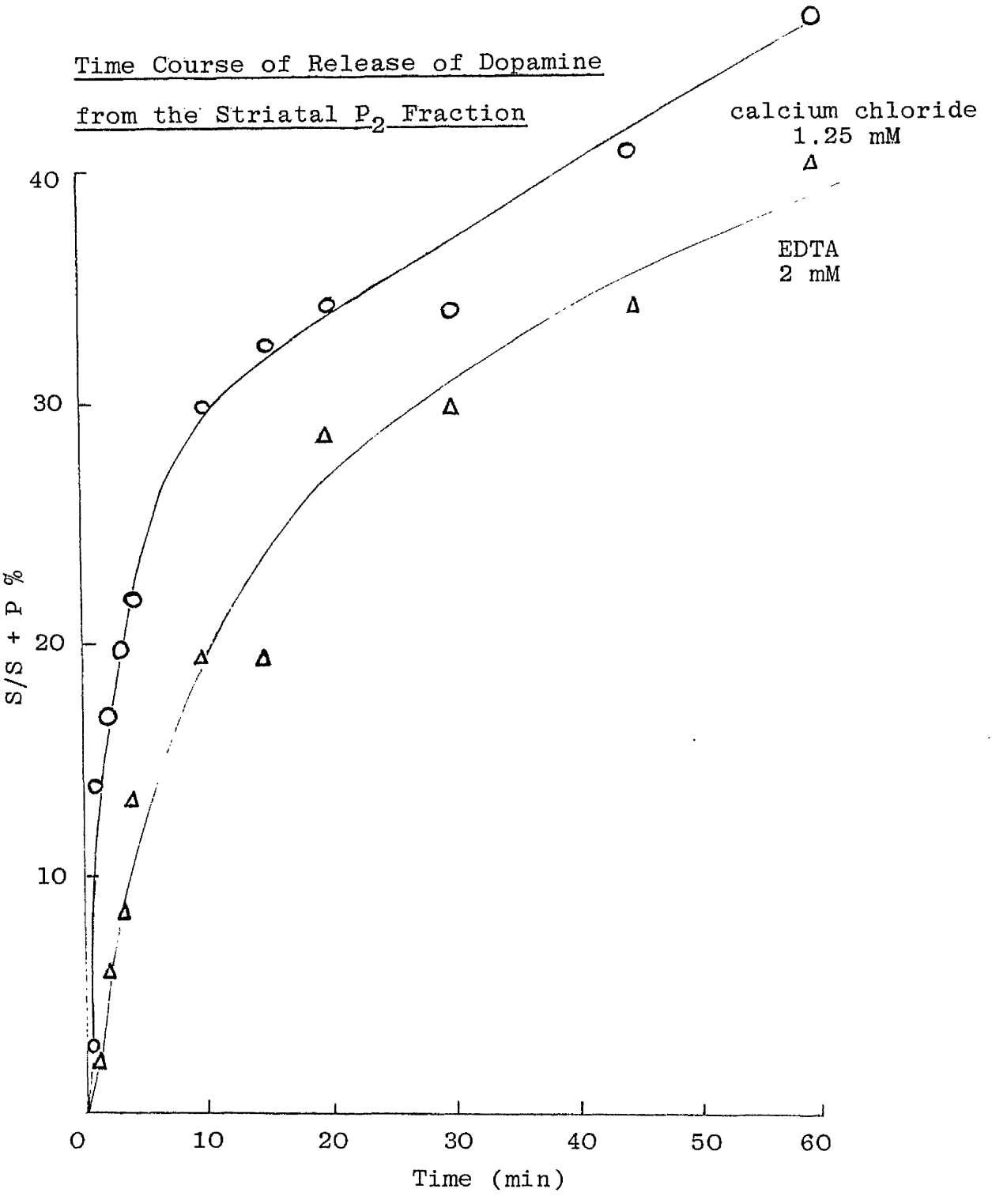


Fig. 24 The S_1 fraction was loaded for 20 min with [^{3}H] dopamine (final concentration 2 x 10^{-7}M). 0.1 ml of resuspended pellet was added to 2.9 ml of saline containing either 1.25 mM calcium chloride or 2 mM EDTA and release of dopamine allowed to proceed for 0-60 min.

(Table 22), while the greatest stimulation observed with the hypothalamic peptides, MIH (130%) (Table 23) and TRH (110%) (Table 24) was at 10^{-3}M. Thus these drug concentrations were used in all experiments.

The amount of dopamine taken up by P_2 fractions varied from preparation to preparation as did the ratio of S/S + P and the % stimulation. However, a drug-induced stimulation of release was consistently observed.

Influence of Calcium on Drug-Induced Release of Dopamine from Striatal P_2 Fractions

Kuczenski (1975b) examined the effect of amphetamine and tyramine on dopamine synthesis in the presence of EDTA at pH 6.6, while Patrick and Barchas (1976) examined the effect of tyramine on dopamine synthesis in the presence of calcium chloride at pH 7.2. Thus it was necessary to determine the effect of calcium on drug-induced release at both pH's.

At pH 7.2, amphetamine (10^{-4}M) stimulated release of dopamine in the presence of calcium chloride (118-129%) (Table 25, experiments 1 and 2) and also in the presence of EDTA (74%) (Table 28, experiment 4). MIH (10^{-3}M) (Table 26, experiments 1-4) and TRH (10^{-3}M) (Table 27, experiments 1-4) stimulated release of dopamine in the presence of calcium by 14-48% and 15-59% respectively, but the stimulation was less than that induced by amphetamine (118-129%). Unlike amphetamine neither MIH (10^{-3}M) (Table 29, experiment 4) nor TRH (10^{-3}M) (Table 30, experiment 5) stimulated release of dopamine in the presence of EDTA.

Table 22 Effect of Amphetamine on Release of Dopamine from the Striatal P_2 Fraction

The S_1 fraction was loaded for 10 min with [^{3}H] dopamine (final concentration 2 x 10^{-8}M). 0.2 ml of resuspended pellet was added to 2.8 ml of Tris-saline containing 1.25 mM calcium chloride in the presence or absence of amphetamine and release of dopamine allowed to proceed for 2 min. S/S + P values are corrected for a 0° blank of 37.6%.

Additions	cpm		S/S+P %	% Stimulation
	Supernatant	Supernatant (S) + Pellet (P)		
None	17,466.0	39,424.5	6.7	-
	13,108.5	27,873.0	9.4	
Amphetamine				
10^{-7}M	14,583.0	32,016.0	7.9	-2
10^{-6}M	16,206.0	27,725.0	20.9	+159
10^{-5}M	23,391.0	38,733.5	22.8	+183
10^{-4}M	35,625.0	45,568.5	40.6	+404
10^{-3}M	20,106.0	31,516.5	26.2	+225

Table 23 Effect of MIH on Release of Dopamine from the Striatal P_2 Fraction

The S_1 fraction was loaded for 10 min with $[^3H]$ dopamine (final concentration 2 x 10^{-8}M). 0.1 ml of resuspended pellet was added to 2.9 ml of Tris-saline containing 1.25 mM calcium chloride in the presence or absence of MIH and release of dopamine allowed to proceed for 2 min. S/S + P values are corrected for a 0^{o} blank of 45%.

Additions	cpm		S/S+P %	% Stimulation
	Supernatant	Supernatant (S) + Pellet (P)		
None	8,457.0	16,521.0	6.2	-
	7,737.0	14,619.0	7.9	
MIH 10^{-7}M	7,672.5	14,495.0	7.9	+12
10^{-6}M	8,263.5	15,986.5	6.7	- 5
10^{-5}M	7,545.0	13,302.5	11.7	+66
10^{-4}M	7,839.0	14,226.0	10.1	+43
10^{-3}M	7,782.0	12,712.5	16.2	+130

Table 24 Effect of TRH on Release of Dopamine from the Striatal P_2 Fraction

The S_1 fraction was loaded for 10 min with [^{3}H] dopamine (final concentration 2 x 10^{-8}M). 0.1 ml of resuspended pellet was added to 2.9 ml of Tris-saline containing 1.25 mM calcium chloride in the presence or absence of TRH and release of dopamine allowed to proceed for 2 min. S/S + P values are corrected for a 0° blank of 46%.

Additions	cpm		S/S+P %	% Stimulation
	Supernatant	Supernatant (S) + Pellet (P)		
None	7,147.5	12,956.5	8.8	-
	7,485.0	12,753.5	12.3	
TRH 10^{-7}M	8,020.5	14,448.0	9.1	-14
10^{-6}M	7.885.5	13,011.0	14.2	+35
10^{-5}M	8,155.5	13,388.5	14.5	+37
10^{-4}M	8,295.0	13,195.5	16.5	+56
10^{-3}M	8,721.0	12,752.0	22.0	+109

Tables 25-27 Effect of Drugs on Release of Dopamine from Striatal P_2 Fractions in the Presence of Calcium Chloride

S_1 fractions were loaded for 10 min with [^{3}H] dopamine (final concentration 2 x 10^{-8}M). 0.1 ml of resuspended pellet was added to 2.9 ml of Tris-saline (pH 6.6 or pH 7.2) containing 1.25 mM calcium chloride and release of dopamine allowed to proceed for 2 min. Results are means ± S.D. of four determinations from a single preparation.

* significantly greater than control $P < 0.05$.

Tables 28-30 Effect of Drugs on Release of Dopamine from Striatal P_2 Fractions in the Presence of EDTA

S_1 fractions were loaded for 10 min with [^{3}H] dopamine (final concentration 2 x 10^{-8}M). 0.1 ml of resuspended pellet was added to 2.9 ml of Tris-saline (pH 6.6 or pH 7.2) containing 2 mM EDTA and release of dopamine allowed to proceed for 2 min. Results are means ± S.D. of four determinations from a single preparation.

* significantly greater than control $P < 0.05$

** significantly less than control $P < 0.05$

Table 25 Effect of Amphetamine on Release of Dopamine from Striatal P_2 Fractions in the Presence of Calcium Chloride

Additions	[^{3}H] dopamine release S/S + P %		
	Expt 1	Expt 2	Expt 3
	pH 7.2	pH 7.2.	pH 6.6
None	15.3±0.9 (4)	17.2±2.5 (4)	9.1±2.0 (4)
Amphetamine 10^{-4}M	*33.5±0.5 (4)	*39.4±0.8 (4)	*33.4±1.0 (4)
% Stimulation	+118	+129	+267

Table 26 Effect of MIH on Release of Dopamine from Striatal P_2 Fractions in the Presence of Calcium Chloride

Additions	[^{3}H] dopamine release S/S + P %				
	Expt 1	Expt 2	Expt 3	Expt 4	Expt 5
	pH 7.2	pH 7.2	pH 7.2	pH 7.2	pH 6.6
None	5.9±1.1 (4)	15.8±3.6 (4)	14.7±1.8 (4)	14.9±1.3 (4)	9.1±2.0 (4)
MIH 10^{-3}M	*8.7±0.2 (4)	*20.5±2.2 (4)	*16.7±3.7 (4)	*22.0±2.5 (4)	*16.8±2.3 (4)
% Stimulation	+47	+30	+14	+48	+85

Table 27 Effect of TRH on Release of Dopamine from Striatal P_2 Fractions in the Presence of Calcium Chloride

Additions	[^{3}H] dopamine release S/S + P %				
	Expt 1	Expt 2	Expt 3	Expt 4	Expt 5
	pH 7.2	pH 7.2	pH 7.2	pH 7.2	pH 6.6
None	17.1±1.0 (4)	18.2±2.6 (4)	16.8±3.1 (4)	14.9±1.3	9.1±2.0 (4)
TRH 10^{-3}M	*21.6±1.6 (4)	22.2±5.2 (4)	19.3±6.2 (4)	*23.7±1.1 (4)	*22.7±0.6 (4)
% Stimulation	+26	+22	+15	+59	+149

Table 28 Effect of Amphetamine on Release of Dopamine from Striatal P_2 Fractions in the Presence of EDTA

Additions	[^{3}H] dopamine release S/S + P %			
	Expt 1	Expt 2	Expt 3	Expt 4
	pH 6.6	pH 6.6	pH 6.6	pH 7.2
None	4.1±1.5 (4)	6.2±1.0 (4)	9.3±1.6 (4)	21.5±0.8 (4)
Amphetamine 10^{-4}M	*16.9±1.2 (4)	*18.0±0.7 (4)	*25.0±0.3 (4)	*37.4±0.5 (4)
% Stimulation	+312	+190	+169	+74

Table 29 Effect of MIH on Release of Dopamine from Striatal P_2 Fractions in the Presence of EDTA

Additions	[^{3}H] dopamine release S/S + P %			
	Expt 1	Expt 2	Expt 3	Expt 4
	pH 6.6	pH 6.6	pH 6.6	pH 7.2
None	5.6±1.0 (4)	7.6±1.5 (4)	9.6±1.2 (4)	21.3±0.6 (4)
MIH 10^{-3}M	3.9±1.3 (4)	**4.1±0.5 (4)	**4.7±1.0 (4)	19.9±0.5 (4)
% Stimulation	-31	-46	-51	-7

Table 30 Effect of TRH on Release of Dopamine from Striatal P_2 Fractions in the Presence of EDTA

Additions	[^{3}H] dopamine release S/S + P %				
	Expt 1	Expt 2	Expt 3	Expt 4	Expt 5
	pH 6.6	pH 6.6	pH 6.6	pH 6.6	pH 7.2
None	11.8±1.0 (4)	7.6±0.3 (4)	11.3±2.6 (4)	9.1±1.0 (4)	21.3±0.6 (4)
TRH 10^{-3}M	11.7±1.9 (4)	*9.3±0.7 (4)	9.8±1.4 (4)	8.0±0.7 (4)	21.0±0.5 (4)
% Stimulation	-1	+22	-13	-13	-1

At pH 6.6, amphetamine (10^{-4}M) again stimulated release of dopamine in the presence of calcium chloride (267%) (Table 25, experiment 3) and also in the presence of EDTA (169-312%) (Table 28, experiments 1-3). MIH (10^{-3}M) stimulated release of dopamine in the presence of calcium chloride by 85% (Table 26, experiment 5). In the presence of EDTA, MIH (10^{-3}M) inhibited release of dopamine by 31-51% (Table 29, experiments 1-3). TRH (10^{-3}M) stimulated release of dopamine in the presence of calcium chloride by 149% (Table 27, experiment 5). TRH (10^{-3}M) did not influence release of dopamine in the presence of EDTA (Table 30, experiments 1-4).

IV DISCUSSION

Discussion

The work presented in this thesis was carried out to obtain biochemical support for the idea that the behavioural effects of the hypothalamic hormones, MIH and TRH, are a consequence of an increased amount of dopamine at central synapses (Cohn et al, 1975; Plotnikoff and Kastin, 1976). In vitro preparations were used to reduce the complications inherent in vivo studies, for example, the inability of peptide to penetrate the blood-brain barrier efficiently. The work will be discussed in two main sections. Firstly, a critical analysis of the preparation used and secondly, a discussion of the effects of MIH and TRH on presynaptic dopaminergic mechanisms to try to relate these to the reported behavioural effects of the hypothalamic peptides.

4.1.1 In vitro Preparations used to Study the Effect of Drugs on Dopaminergic Mechanisms

As the striatum has been implicated in the control of locomotory activity (Lloyd and Hornykiewicz, 1975), this region was chosen for study.

Tissue slices have proved a popular tool for many biochemical investigations (McIlwain, 1975), including the study of dopaminergic mechanisms (Arnold et al., 1977; Holmes and Rutledge, 1976; Uretsky and Snodgrass, 1977). This preparation is less complex than an intact organ but it still has considerable structural integrity. As most of the cells are intact, it is unnecessary to fortify the preparation with coenzymes or cofactors. Further, in

the absence of a normal blood supply, materials normally exchanged with blood are exchanged with the fluid which surrounds or flows over the tissue. Thus the problem of the inability of peptide to penetrate the blood-brain barrier is avoided. Cut surfaces are an advantage in that added substrates may penetrate but a disadvantage in that leakage of materials out of the slice may also occur. The structural integrity of tissue slices encouraged their use in this thesis. However, the variability of results obtained rendered the preparation unsuitable for studying presynaptic functions. Thus it was decided to use crude synaptosomal preparations.

Synaptosomes, pinched off nerve endings, may be a useful in vitro system for studying presynaptic function (Christiansen and Squires, 1974). They display many of the general biochemical and physiological characteristics associated with intact nervous tissue, for example, linear respiration, ATP production and maintainence of high potassium levels (De Belleroche and Bradford, 1973). Crude synaptosomal preparations (P_2 fraction; Gray and Whittaker, 1962), however, have been used by many workers for studies of synaptosomal properties, especially for studies of catecholamine metabolism (for example, Andrews et al., 1978; Bagchi and Smith, 1976; Cho et al., 1977; Christiansen and Squires, 1974; Coyle, 1972; Coyle and Snyder, 1969; Goldstein et al., 1976; Harris et al., 1975; Holz and Coyle, 1974; Iversen et al., 1976; Kapatos and Zigmond, 1977;

Karobath, 1971; Katz et al., 1976; Kuczenski, 1975b; Patrick and Barchas, 1974, 1976; Patrick et al., 1975; Seeman and Lee, 1974; Snyder and Coyle, 1969). Crude synaptosomal preparations contain osmotically sensitive sodium, potassium and occluded lactate dehydrogenase, the richest source being the synaptosomal fraction (Marchbanks, 1967). As the release of osmotically sensitive sodium, potassium and occluded lactate dehydrogenase had similar characteristics with respect to the degree of osmotic 'shock' necessary and the action of detergents (Marchbanks, 1967), the integrity of the synaptosomal membrane is often assessed by measuring the activity of lactate dehydrogenase under iso-osmotic conditions and then again after disruption of the synaptosome with triton X-100 (Heaton and Bachelard, 1973; Whittaker, 1969).

Most of the tyrosine hydroxylase activity in P_2 fractions was found to be associated with the synaptosomal fraction after sucrose density gradient centrifugation (Andrews et al., 1978; Coyle, 1972). Further, Kuczenski (1975b) reported that when crude synaptosomes were used to synthesise dopamine the results were identical to those obtained using purified synaptosomes. Similarly Coyle and Snyder (1969) reported that when S_1 fractions were incubated with [^{3}H] dopamine, [^{3}H] dopamine was taken up by synaptosomes and not by mitochondria. These observations suggest that it is valid to use crude synaptosomal preparations to study dopamine synthesis and release. However, as indicated by De Belleroche and Bradford (1973), caution is needed in the use of such a crude preparation,

as a third of the protein present in the fraction is contributed by mitochondria and another third by myelin fragments.

In this work, electron micrographs revealed particles of the correct size and shape and content as synaptosomes (Gray and Whittaker, 1962) and measurement of occluded lactate dehydrogenase indicated the integrity of the synaptosomal membrane. Further, the P_2 fraction synthesised dopamine and took up and released dopamine at rates comparable with literature values. This suggested that a useful preparation for studying dopaminergic mechanisms had been obtained.

4.1.2 Complications in the Study of the Biochemical Effects of Hypothalamic Hormones

The hypothalamic hormones are rapidly degraded by brain peptidases. Thus, if the peptide is degraded before it can act, no effect on dopaminergic mechanisms might be observed. Alternatively, if an effect is observed, this could be due to a degradation product of the peptide and not to the peptide itself. The peptidases are not confined to the hypothalamus but are also found in the areas of the central nervous system where the hypothalamic hormones have been detected and may have their actions (Griffiths et al., 1975, 1976).

The primary mechanisms involved in the degradation of TRH are removal of the pyroglutamyl moiety by pyroglutamyl peptidase or deamidation (Marks, 1978). Histidyl-proline

amide is unstable and can be converted to histidyl-proline diketopiperazine which has been reported to antagonise ethanol narcosis (Prasad et al., 1977). This suggested that TRH metabolites may play a part in its biological effects. Unlike TRH, MIH was not inactivated by deamidation. Inactivation was achieved by cleavage of Pro-Leu and Leu-Gly-NH_2 bonds. The order of peptidase action was not determined (Marks, 1978).

Subcellular fractionation of various brain areas (hypothalamus, thalamus, cortex, cerebellum) indicated that between 56 and 91 percent of peptidase activity was present in supernatant fractions (Griffiths et al., 1975, 1976). Further, the highest levels of peptidase was found in the 27,000 g supernatant of subcellular fractions of hamster hypothalami (Prasad and Peterkofsky, 1976). This suggested that sucrose homogenates and crude synaptosomes (S_1 fraction) would have high peptidase activity while purified synaptosomes would have none. In agreement with this suggestion Parker et al., (1977) found that TRH was degraded by crude synaptosomes (S_1 fraction) but not by synaptosomes purified by sucrose density gradient centrifugation.

It is improbable that degradation of MIH and TRH by brain peptidases is of importance in this work. Firstly, the P_2 preparation is unlikely to have high levels of peptidase as the S_2 fraction has been removed. Secondly, peptide was not preincubated with the preparation, thus decreasing the possibility of degradation of peptide. Thirdly, second additions of peptide did not alter the rate of the

oxidative deamination of dopamine. Thus the lack of any effect of MIH and TRH on the biosynthesis or oxidative deamination of dopamine was unlikely to be due to degradation of peptide. However, the possibility that degradation products of TRH, for example histidyl-proline diketopiperazine, exerted an effect on dopamine release cannot be excluded at present.

4.2 Effect of MIH and TRH on Presynaptic Dopaminergic Mechanisms

4.2.1. Effect of MIH and TRH on Inactivation of Dopamine

MIH and TRH had no effect on the oxidative deamination of dopamine, suggesting that inhibition of monoamine oxidase is unlikely to be the primary site of action of the hypothalamic hormones. This agrees with the work of Breese et al. (1974), who reported that TRH had no effect on the oxidative deamination of noradrenaline in mouse brain. However, Wirz-Justice and Lichtsteiner (1977) observed that while TRH inhibited monoamine oxidase in the median eminence of male rats, it stimulated monoamine oxidase in pro-oestrous female rats.

Autoradiographic analysis revealed that there were two products of the oxidative deamination of dopamine. One, DOPAC, is the major metabolite of dopamine in brain (Goldstein et al., 1959). The other had the same R_F value as DOBA, the decarboxylation product of DOPAC. However, any DOBA formed from [ethylamine-1-^{14}C] dopamine would not be radiolabelled.

De Belleroche et al. (1976) have previously reported the formation of DOBA in synaptosomal preparations from sheep brain. DOBA has also been isolated from urine after intravenous injection of dopamine (Alton and Goodall, 1969) or noradrenaline (Peskar et al., 1971). In this thesis, roughly equal amounts of the two dopamine metabolites were formed; MIH and TRH did not alter the total amount of the two metabolites formed or the distribution of counts between them.

Further, MIH and TRH have been reported not to inhibit reuptake of catecholamines (Breese et al., 1974; Horst and Spirt, 1974; Tuomisto and Männistö, 1973). Thus as MIH and TRH did not inhibit either the oxidative deamination or the reuptake of dopamine, the behavioural effects of the hypothalamic hormones are unlikely to be due to an inhibition of inactivation of catecholamines. Further, although it may be effective in the treatment of depression, (Kastin et al., 1972; Prange et al., 1972) TRH may not act like the major known antidepressants, namely the tricyclic drugs and the monoamine oxidase inhibitors.

4.2.2. Effect of Drugs on Release of Dopamine

Release of catecholamines from central nerve terminals has only been partly characterised. In comparison, much more is known about release of catecholamines from the peripheral nervous system and the adrenal medulla.

Stimulation of the preganglionic nerve fibres of the adrenal medulla by acetylcholine released noradrenaline

accompanied by ATP, dopamine-β-hydroxylase and chromogranin in the same ratio as they are found in isolated chromaffin granules. Thus it was suggested that noradrenaline was released from the adrenal medulla by exocytosis, that is, the contents of the noradrenergic vesicles were extruded directly to the cell exterior and were not released through a hole in the cell membrane. This process demonstrated an absolute requirement for calcium and was termed stimulus-secretion coupling (Douglas, 1968).

Release of noradrenaline from nerve terminals of adrenergically innervated peripheral organs can be demonstrated as the innervated end-organs can be isolated and the perfusate collected during stimulation analysed for the presence and quantity of neurotransmitter. When nerves to the spleen were stimulated, the ratio of noradrenaline to dopamine-β-hydroxylase released was greater than that found in vesicles isolated from the splenic nerve (Gewirtz and Kopin, 1970). This led to doubts about exocytosis as the mechanism of release of noradrenaline from sympathetic nerves.

Two mechanisms have been suggested to explain efflux of noradrenaline from sympathetic nerve terminals (Smith, 1973). The first, exocytosis, involves fusion of neurotransmitter containing storage vesicles with the plasma membrane resulting in extrusion of vesicle contents through an opening made in the membrane. This process exhibits an absolute requirement for calcium and is thought to be analogous to stimulus-secretion coupling in the adrenal medulla. The second type,

which is not calcium-dependent, and therefore distinct from calcium-dependent release of noradrenaline by nerve impulses, involved displacement of neurotransmitter from storage vesicles into the cytoplasm of the nerve terminals and passage of transmitter across the cell membrane into the extracellular space. A carrier located in the cell membrane would facilitate removal of neurotransmitter from the cell cytoplasm and protect it from deamination. The sympathomimetic amines, tyramine and amphetamine are thought to release noradrenaline from sympathetic nerve terminals in this way.

It is harder to demonstrate release of neurotransmitter from a given type of nerve ending in the central nervous system than in the peripheral nervous system (Cotman et al., 1976) as it is difficult to collect material released in vivo. Synaptosomes offer many advantages for studying the release of neurotransmitters in the brain (Blaustein et al., 1972; De Belleroche and Bradford, 1972a, b). For example, drugs can be applied without intervening diffusion barriers and neurotransmitter released can be collected quickly and directly. Depolarisation-induced release of noradrenaline (Blaustein et al., 1972) and of dopamine (Patrick and Barchs, 1976) from synaptosomes was found to be calcium dependent and thus met the requirement of stimulus-secretion coupling. For this reason calcium-dependent release has been taken as evidence for exocytosis (mechanism 1) in the central as well as the peripheral nervous system.

In this thesis, amphetamine (10^{-4}) stimulated release

of dopamine from striatal P_2 fractions in the presence of 2 mM EDTA at both pH 6.6 and pH 7.2. This suggested that release of dopamine by amphetamine can occur, in part, by a non-exocytotic process. Amphetamine may thus release catecholamines in the central, as well as the peripheral, nervous system by displacement-diffusion (mechanism 2).

Rutledge (1978) has described a model, analogous to the model proposed by Smith (1973) for release of noradrenaline from sympatheticneurons, to explain amphetamine-induced release of noradrenaline from isolated brain tissue (Fig. 25). Amphetamine and sodium are cotransported into the nerve ending and as a result the carrier is on the inside of the membrane. Amphetamine then displaces noradrenaline from storage sites and noradrenaline can now bind to the carrier and be transported out of the nerve ending. Stimulation of dopamine release could also occur in this way (Rutledge, 1978).

Uretsky and Snodgrass (1977) reported that though amphetamine (10^{-6}M) increased release of [^{3}H] catechols (dopa and dopamine) formed from [^{3}H] tyrosine by rat striatal slices in calcium-free medium the release produced by amphetamine was increased in medium containing 1.25, 2.5 and 10 mM calcium chloride. Further, De Belleroche et al. (1976) reported that amphetamine (1.19×10^{-4}M)-induced release of [^{14}C] dopamine formed from [^{14}C] dopa by striatal synaptosomes was significantly reduced in calcium-free medium containing 0.5 mM EGTA.

These reports suggested that stimulation of release by amphetamine could occur in two ways, one of which was dependent on calcium (presumably exocytosis) and one independent of

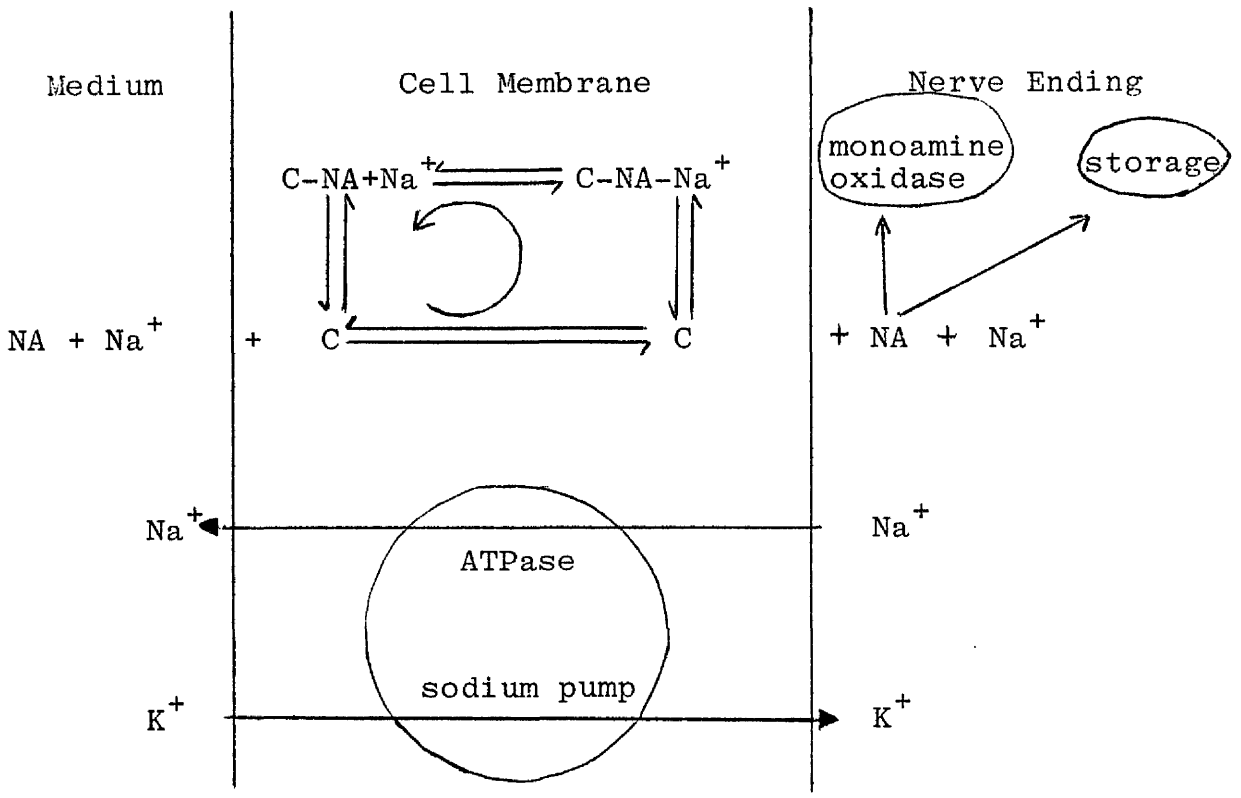


Fig. 25. Diagram of a Postulated Mechanism by which Noradrenaline is Released from Nerve Endings

Uptake of NA occurs when the external sodium and NA concentrations are high relative to the respective free intra-neuronal concentrations or when the carrier is facing the outside of the nerve ending. Efflux of NA is more likely when amphetamine is transported into the nerve ending. The carrier would now be on the inside of the membrane. Amphetamine would displace NA from binding sites thus making it available for binding to the carrier.

calcium (presumably displacement-diffusion). In the absence of calcium amphetamine could only stimulate release by displacement-diffusion while in the presence of calcium amphetamine could stimulate release by both displacement-diffusion and exocytosis. Thus the decrease in amphetamine-induced release of dopamine in the absence of calcium could be explained.

However, Arnold et al. (1977) reported that amphetamine (10^{-7}-10^{-3}M)-induced release of endogenous dopamine from slices of cerebral cortex did not depend on calcium. In contrast, potassium-induced release of endogenous dopamine was markedly calcium dependent. Thus amphetamine-induced release of endogenous as well as exogenous, dopamine occurs, at least in part, by a mechanism other than exocytosis. These authors also found that different proportions of endogenous and [^{3}H] (ie exogenous) dopamine were released by different concentrations of amphetamine. For example, amphetamine (10^{-7}-10^{-5}M)-released similar amounts of endogenous and exogenous dopamine but amphetamine (10^{-5}-10^{-3}M) released a greater percentage of endogenous than exogenous dopamine. This suggested that endogenous and exogenous dopamine were released from different pools. In conclusion, though it is difficult to compare the reported effects of calcium on amphetamine-induced release of dopamine as release may occur from different pools, the results obtained by the various authors, despite the different tissue preparation used, can still be explained in terms of the two release mechanisms, exocytosis and displacement-diffusion.

Tyramine (10^{-4}M) stimulated release of dopamine from striatal P_2 fractions in the presence of either 1 mM EDTA or 1 mM calcium chloride (Patrick and Barchas, 1976). Tyramine may thus, like amphetamine, release dopamine from central nervous tissue by displacement-diffusion. This supports the idea that tyramine and amphetamine have identical effects on central and peripheral nerve endings (Raiteri and Levi, 1978).

Stimulation of release of dopamine from striatal P_2 fractions by MIH and TRH occurred only in the presence of calcium chloride but stimulation of release of dopamine by amphetamine could occur also in the presence of EDTA. This suggested that release of dopamine by the hypothalamic hormones, MIH and TRH, and by amphetamine occurred in different ways.

As stimulation of release of dopamine required calcium, this suggested that MIH and TRH might release dopamine from storage vesicles by exocytosis (mechanism 1). This agrees with the work of Horst and Spirt (1974) who showed that TRH could not stimulate release of dopamine from striatal P_2 fractions, if the storage vesicles were depleted by reserpine. These authors, however, examined the effect of TRH on dopamine release after 1 h. In this thesis, when release was studied under the conditions of Horst and Spirt (1974), release was linear for 2 min. Thus the effect of drugs on a 2 min release of dopamine was studied. Similarly, Eitan and Hershkowitz (1977) only required a 3 min release of dopamine from striatal P_2 fractions to show a stimulatory effect of potassium chloride which is thought to release dopamine from storage vesicles.

Results presented in this thesis showing a stimulation of release of dopamine by MIH do not agree with the preliminary reports of Kostrzewa et al. (1976) indicating that MIH did not alter the release of dopamine from dopamine-loaded P_2 fractions. However, no details were given so it is not possible to compare their results with those in this thesis.

Greenberg et al. (1976) reported that when synaptosomes (S_1 fraction) were incubated with [^{3}H] MIH ([2,3-^{3}H] pro-leu-gly NH_2) uptake of peptide by synaptosomes was passive. However, as they did not identify the radiolabelled compound taken up, their conclusion may not be valid. But Parker et al. (1977) found that when synaptosomes (S_1 fraction) were incubated [^{3}H] TRH pglu-his-[2,3-^{3}H] pro NH_2) the radiolabelled compound taken up was [^{3}H] proline rather than [^{3}H] TRH. In contrast, when metabolism of [^{3}H] TRH was minimised by using a purified synaptosomal preparation, uptake of radioactivity by synaptosomes was abolished. These authors therefore concluded that TRH, unlike amphetamine, did not penetrate synaptosomes.

TRH and MIH may therefore act on the synaptosomal membrane to stimulate release of dopamine by a calcium-dependent mechanism analogous to the mechanism of action of peptide hormones such as glucagon and insulin (for reviews see Bitensky and Gorman, 1972; Czech, 1977). The action of these peptide hormones is thought to be mediated by increased cellular concentrations of calcium. In addition, glucagon can stimulate adenylate cyclase to produce cAMP which promotes the phosphorylation of membrane proteins, thus increasing the permeability of the membrane to calcium.

Both cAMP and calcium mediate the release of pituitary hormones by their hypothalamic regulating hormones (Guillemin et al., 1971; Schally et al., 1972). However, the work of Cohn et al. (1976) and Green et al., (1976) suggested that the central effects of TRH were not mediated by cAMP. Further, MIH did not alter the levels of cAMP in various regions of the rat brain (Christiensen et al., 1976). Thus cAMP is unlikely to play a role in the behavioural effects of MIH and TRH.

4.2.3. Compartmentation of Dopamine in the Striatum

The results of release experiments in this thesis suggested that dopamine exists in two pools in striatal synaptosomes. Features of dopamine storage will now be described to provide a model of release and biosynthesis in striatal synaptosomes.

De Belleroche et al. (1976) identified two compartments of striatal dopamine by differential labelling with isotopic precursors, tyrosine and dopa, and from specific radioactivity measurements. Tyrosine provided a source for the dopamine pool synthesised and released in response to potassium depolarisation whereas dopa did not enter this pool. The pool of dopamine formed from dopa was largely recovered from the incubation medium in the presence of amphetamine, though this pool was not released by depolarisation. This pool appeared to be prone to oxidation to DOPAC and HVA which are preferentially released from synaptosomes.

Kuczenski (1975b) obtained evidence for two dopamine pools in synaptosomes using a different approach. His reasoning was as follows:-

The ED_{50} for amphetamine-induced release of dopamine from striatal synaptosomes was near 5 x 10^{-5}M (Azzaro and Rutledge, 1973), while the ED_{50} for accelerated dopamine synthesis was almost 50-fold lower, near 1 x 10^{-6}M. Similarly, the ED_{50} for exchange of unlabelled dopamine in the medium with [^{3}H] dopamine in the synaptosomes was near 5 x 10^{-6}M, whereas the ED_{50} for inhibition of synaptosomal tyrosine hydroxylation by exogenous dopamine was near 5 x 10^{-7}M (Karobath, 1971). Thus it would appear that the inhibitory pool of dopamine is very small and does not achieve rapid equilibrium with the majority of exchangeable or amphetamine-releasable dopamine in vitro.

There is other evidence to suggest that dopamine may exist in two pools in the striatum. After intraperitoneal injection of α-methyl-p-tyrosine to rats, the dopamine concentration in the striatum declined biphasically (Doteuchi et al., 1974; Javoy and Glowinski, 1971). From this biphasic decline, Javoy and Glowinski (1971) inferred that there were two distinct storage forms of dopamine in dopaminergic nerve terminals, namely a functional pool of transmitter which contained about 26% of the total striatal dopamine and a main storage pool (74% of the total dopamine). The functional pool of dopamine turned over more rapidly than did the main storage pool. However, Doteuchi et al. (1974) suggested that the evidence for this interpretation was insufficient as α-methyl-p-tyrosine did not cause an immediate and complete inhibition of tyrosine hydroxylase.

The concentration of dopamine in striatal terminals has been calculated to be greater than 10^{-2}M (Andén et al., 1966). As the inhibitory constant for inhibition of striatal tyrosine hydroxylase by dopamine was reported to be 35 μM (Kuczenski and Mandell, 1972), less than 1% of total striatal dopamine would be sufficient to regulate tyrosine hydroxylase (Costa and Meek, 1974).

Newly synthesised striatal dopamine is thought to be released in preference to stored dopamine during nerve stimulation (Besson et al., 1969, 1971). In addition, the results of Weiner and his co-workers (Alousi and Weiner, 1966; Weiner and Rabadjya, 1968) with sympathetic nerves suggest that release of catecholamine with stimulation occurs with a concomitant decrease in the size of a small, chemically undetectable pool, which is functional in the regulation of tyrosine hydroxylase. Further, the behavioural effects of amphetamine are reserpine-resistant (Smith, 1963) and can be blocked by α-methyl-p-tyrosine (Weissman and Koe, 1965; Weissman et al., 1966). This also supports the suggestion of multiple pools of dopamine.

4.2.4 Regulation of Tyrosine Hydroxylase

There are a number of different mechanisms involved in the regulation of tyrosine hydroxylase, the rate-limiting step in the biosynthesis of catecholamines (Nagatsu et al., 1964). One of the most important factors which regulate tyrosine hydroxylase is feedback inhibition by catechols, particularly dopamine (Kuczenski and Mandell, 1972), which competes with the

enzyme for the pteridine cofactor (Nagatsu et al., 1971). Thus an increase in dopamine synthesis could be achieved by increasing release of dopamine from a pool inhibiting tyrosine hydroxylase (Kuczenski, 1975b) or by altering the physical state of the enzyme (Kuczenski, 1975a), thus making it less sensitive to feedback inhibition by dopamine (Roth et al., 1975).

According to Kuczenski (1975a), tyrosine hydroxylase in striatal nerve endings exists in two states, namely, a soluble, less active form and a membrane-bound, activated form. The soluble form can be converted to the active form by the addition of calcium. Activation of tyrosine hydroxylase is associated with a ten-fold increase in affinity for the synthetic cofactor 6,7-dimethyl-5,6,7,8-tetrahydropterin and a seven-fold increase in affinity for the substrate tyrosine. The inhibitory constants for inhibition by dopamine of the two forms of tyrosine hydroxylase were not given.

Tyrosine hydroxylase can be activated by enhanced neuronal activity. According to the model of Roth et al. (1975), an increase in impulse flow in dopaminergic neurons results in an increase in the frequency of depolarisation of the neuronal terminals with a consequent increase in the influx of calcium. Calcium then initiates a series of reactions which leads to activation of tyrosine hydroxylase via a cAMP-dependent protein kinase. Activation of tyrosine hydroxylase is mediated by a five-fold increase in affinity for the presumed natural cofactor tetrahydrobiopterin, a three-fold increase in affinity for the substrate tyrosine and six-fold decrease in affinity for dopamine.

However, dopaminergic unlike noradrenergic neurons paradoxically increase synthesis in response to a cessation of impulse flow (Roth et al., 1975). The most dramatic change is the greater than 700-fold increase in the inhibitory constant for dopamine. The kinetic alterations, however, unlike those produced by increased impulse flow, are completely reversed by addition of calcium to the incubation medium. Thus the increase in tyrosine hydroxylase activity which occurs in the striatum during cessation of impulse flow may occur as a result of a diminished influx of calcium ultimately resulting in an allosteric activation of tyrosine hydroxylase, thus making it less sensitive to feedback inhibition by dopamine (Roth et al, 1975). As indicated above, the role of calcium in the regulation of tyrosine hydroxylase is unclear.

Stimulation of dopamine receptors also reversed the increase in dopamine synthesis induced by blockade of impulse flow (Roth et al., 1975). This suggested that dopamine receptors may play a direct role in the control of dopamine biosynthesis (Morgenroth et al., 1976b).

Results presented in this thesis showed that 0.85 mM calcium stimulated dopamine synthesis by striatal synaptosomes by 30%. This agrees with the work of Gutman and Segal (1973) who reported that calcium (0.05 - 0.2 mM) could stimulate tyrosine hydroxylase isolated from four different regions of the rat brain including the striatum. However, Goldstein et al. (1970) reported that addition of calcium (2×10^{-3}M) resulted in a 60% inhibition of dopamine synthesis in striatal slices. In contrast, Morgenroth et al. (1976a) reported that addition

of the calcium chelator, EGTA (50 μM) caused a marked increase in the activity of striatal tyrosine hydroxylase present in high speed supernatants. Calcium (100 μM) had no effect on striatal tyrosine hydroxylase (Morgenroth et al., 1976a). But Lerner et al. (1977) reported that neither EGTA (50 μM) nor calcium (100 μM) stimulated soluble striatal tyrosine hydroxylase. Further, Patrick et al. (1975) found that the addition of various concentrations (0.1 - 10 mM) of calcium to rat brain striatal synaptosomes did not significantly alter the basal synthesis rate and McGeer et al. (1967) reported that 10^{-4}M calcium had no significant effect on tyrosine hydroxylase activity in rat brain homogenates.

Thus despite the different preparations used there is considerable disagreement as to the effect of calcium on tyrosine hydroxylase. The stimulation observed is not related to an increase in impulse flow (Roth et al., 1975) but may be due to conversion of tyrosine hydroxylase from a soluble to a membrane-bound form with concomitant activation of the enzyme (Kuczenski, 1975a).

4.2.5. Relationship Between Release and Synthesis of Dopamine

Kuczenski (1975b) showed that amphetamine and tyramine could stimulate dopamine synthesis by striatal P_2 fractions incubated in the presence of 2 mM EDTA. Further, neither drug altered the activity of tyrosine hydroxylase or dopa decarboxylase in the absence of synaptosomal integrity. He therefore concluded that stimulation of synthesis was a consequence of depletion of an inhibitory pool of dopamine in

contact with tyrosine hydroxylase. However, high concentrations of amphetamine (10^{-3}M) inhibited tyrosine hydroxylase, presumably by movement of dopamine into the inhibitory pool.

In agreement with Kuczenski (1975b), it has been shown in this thesis that both tyramine and amphetamine stimulated dopamine synthesis by striatal P_2 fractions incubated in the presence of 2 mM EDTA, maximum stimulation with tyramine and amphetamine occurring between 10^{-5}M and 10^{-4}M. Amphetamine (10^{-3}M) inhibited dopamine synthesis by 14% in agreement with the value of 18% obtained by Kuczenski (1975b). Tyramine (10^{-3}M) inhibited dopamine synthesis by 40% but Kuczenski (1975b) found that tyramine (10^{-3}M) stimulated dopamine synthesis by 5%. These results support the idea that tyramine and amphetamine stimulate dopamine synthesis in the same way (Kuczenski, 1975b).

Consistent with the idea of an inhibitory pool was the observation that the stimulation of dopamine synthesis obtained with tyramine and amphetamine was inversely related to the basal rate of dopamine synthesis. Thus tyramine and amphetamine would only stimulate dopamine synthesis if there was a significant level of dopamine in the inhibitory pool. This hypothesis could be tested by measuring the endogenous level of dopamine and tyrosine hydroxylase activity in the same synaptosomal preparation.

In contrast, tyramine (3.3×10^{-4}M) inhibited dopamine synthesis by 44% in the presence of 1.7 mM calcium chloride. This agrees with the work of Patrick and Barchas (1976) who

showed that 10^{-4}M tyramine inhibited dopamine synthesis by 48% in the presence of 1 mM calcium chloride. However, tyramine (10^{-4}M) also inhibited dopamine synthesis (by 50%) in the presence of 1 mM EGTA (Patrick and Barchas, 1976). But Kuczenski (1975b) observed that stimulation not inhibition of dopamine synthesis occurred in the presence of EDTA. This discrepancy cannot be explained at present. The results of Patrick and Barchas (1976) are consistent with release of dopamine from storage vesicles into the cytoplasmic pool and transport of dopamine through the synaptosomal membrane. Movement of dopamine into the inhibitory pool must occur in order that inhibition of dopamine synthesis be observed.

An alternative explanation is that dopamine might stimulate presynaptic dopamine receptors (autoreceptors) and thus inhibit dopamine synthesis (Starke et al., 1977) in the presence of EGTA but not in the presence of EDTA. However, stimulation of autoreceptors also inhibited dopamine release (Starke et al., 1977). This is in conflict with the results of Patrick and Barchas (1976) who observed stimulation of release in the presence of EGTA.

Depolarising agents, for example veratridine, stimulated release of dopamine from striatal P_2 fractions (Patrick et al., 1975; Patrick and Barchas, 1976) by a calcium-dependent mechanism. Stimulation of dopamine synthesis also occurred, as a result of diminished end-product inhibition of tyrosine hydroxylase. Thus release must occur from both inhibitory and vesicular pools.

Reserpine, which depletes amine storage vesicles, was found to inhibit dopamine synthesis in the presence of EDTA (2 mM) (Kuczenski, 1975b), and also in the presence of calcium chloride (1 mM) (Patrick and Barchas, 1974), an effect consistent with movement of vesicular dopamine into an inhibitory pool in contact with tyrosine hydroxylase. Thus releasing agents will have different effects on synthesis, depending on whether they release dopamine from a vesicular or an inhibitory pool.

The hypothalamic hormones MIH and TRH stimulated release of dopamine from striatal P_2 fractions but stimulation of release by MIH and TRH differed from that of amphetamine, the former being calcium-dependent, the latter independent of calcium. This suggested that MIH and TRH did not release dopamine from an inhibitory pool as did amphetamine and tyramine, but released dopamine from a different, possibly vesicular, pool. This difference in release was reflected in the synthesis studies. Amphetamine and tyramine stimulated dopamine synthesis in the presence of 2 mM EDTA, but no obvious stimulatory effect of TRH on dopamine synthesis was observed, though at high basal rates of dopamine synthesis TRH had an inhibitory effect. In agreement with Patrick and Barchas (1976) tyramine inhibited dopamine synthesis in the presence of 1.7 mM calcium chloride, but MIH and TRH did not alter the rate of dopamine synthesis under these conditions.

There is a parallel between the actions of TRH and methylphenidate, α-phenyl-2-piperidine acetic acid methyl ester.

Like TRH, methylphenidate can induce stereotyped behaviour and release dopamine from striatal P_2 fractions (Kuczenski and Segal, 1975). However, methylphenidate was less efficient than amphetamine in stimulating dopamine synthesis. The behavioural effects of methylphenidate, in contrast to those of amphetamine, were sensitive to reserpine but insensitive to α-methyl-p-tyrosine. This suggests that methylphenidate, like TRH (Horst and Spirt, 1974) released dopamine from reserpine-sensitive vesicular stores and not, like amphetamine, from a pool inhibiting tyrosine hydroxylase (Kuczenski, 1975b).

4.2.6. Relationship Between the Biochemical and Behavioural Effects of MIH and TRH

Table 31 summarises the effects of the sympathomimetic amines, tyramine and amphetamine, and the hypothalamic hormones, MIH and TRH, on release and synthesis of dopamine. The main conclusion is that MIH and TRH can, like amphetamine, stimulate release of dopamine from striatal synaptosomes in the presence of calcium chloride. Unlike amphetamine, MIH and TRH did not stimulate either the release or synthesis of dopamine in the presence of EDTA. MIH inhibited dopamine release, while TRH had no effect. Neither MIH nor TRH influenced dopamine synthesis. Tyramine inhibited synthesis of dopamine in the presence of calcium (Patrick et al, 1975); MIH and TRH had no effect. Another difference between the hypothalamic hormones and amphetamine is that MIH and TRH did

Table 31. Summary of Effects of Drugs on Release and Synthesis of Dopamine

Drug	Release		Biosynthesis	
	EDTA	Calcium chlordie	EDTA	Calcium chloride
Amphetamine	+	+	+	ND
Tyramine	ND	ND	+	-
MIH	-	+	O	O
TRH	O	+	O	O

ND not determined

O no effect

\+ stimulation

\- inhibition

not inhibit reuptake of catecholamines (Horst and Spirt, 1974; Kostrzewa et al., 1976; Tuomisto and Männistö, 1973) nor did they inhibit oxidative deamination of catecholamines (Breese et al., 1974; this work).

Amphetamine is thought to produce its behavioural effects chiefly by interaction with central dopaminergic neurons (Moore, 1977). The principal biochemical effects of amphetamine are stimulation of release of amine from nerve terminals (Azzaro and Rutledge, 1973; Besson et al., 1969) and inhibtion of neuronal uptake (Azzaro et al., 1974; Ross and Renyi, 1964). At higher concentrations of amphetamine (10^{-4}-10^{-2}M) inhibition of monomine oxidase can occur (Glowinski et al., 1966; Rutledge, 1970). Stimulation of dopamine synthesis, through release of tyrosine hydroxylase from feedback inhibition by dopamine, has also been reported (Kuczenski, 1975b).

As indicated above, there are differences at the biochemical level between the effects of the hypothalamic hormones, MIH and TRH, and amphetamine but one can still account for the behavioural effects of the hypothalamic hormones in which dopaminergic neurons may be the site of action. Both amphetamine and the hypothalamic hormones, MIH and TRH, stimulate release of dopamine, but they do it in different ways. Both result in an increase in concentration of dopamine in the synaptic cleft which would activate the post-synaptic receptor and presumably lead to the behavioural changes. Thus increased release of dopamine newly synthesised

from L-dopa could explain the potentiation of L-dopa by MIH and TRH (Plotnikoff and Kastin, 1976) and also the possible beneficial effects of MIH in Parkinson's disease (Barbeau, 1974; Kastin et al., 1976). Further TRH could, like amphetamine, potentiate any imbalance in dopamine content between left and right sides of the brain and thus induce rotational behaviour (Cohn et al., 1975).

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