THE EFFECTS OF CERTAIN DRUGS ON THE UPTAKE
AND RELEASE OF ^3H -NORADRENALINE IN RAT
WHOLE BRAIN HOMOGENATES

by

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ABSTRACT

Employing an in vitro method adapted from Snyder and Coyle (1969) and using rat whole brain homogenate, twelve drugs (cocaine, tyramine, four tricyclic antidepressants, and six antihistamines) were studied with respect to their effects on inhibition of neuronal uptake of ^3H -noradrenaline (NA) and on release of the amine from presynaptic nerve terminals.

To distinguish between the separate actions on catecholamine release and inhibition of the uptake process, two basic procedures were used. In the first, homogenate was preincubated with ^3H -NA prior to addition of the drug in order to load the nerve endings with NA so that the effects of drugs on release could be measured. The second procedure involved preincubating homogenate with the various drugs followed by addition of the ^3H -NA and further incubation in order to assess the inhibitory effects of the drugs on NA uptake.

From the former experiments, all drugs tested were found to produce some release of NA although tyramine was by far the most potent drug in this respect. Tripeleennamine and cocaine were observed to produce the least release of the twelve drugs. Of the remaining compounds, which were significantly less potent than tyramine, the tricyclic antidepressants were generally more effective in producing release than the antihistaminics. When the potencies of these compounds were correlated with their respective lipid solubilities, only tyramine deviated greatly from the established linear relationship. This

indicated that, unlike the other drugs which appeared to be causing NA release through a nonspecific mechanism related to lipophilicity, tyramine is acting by a specific mechanism, probably involving accumulation of this amine by the NA uptake mechanism followed by displacement and subsequent release of bound intracellular NA.

The studies of inhibition of NA uptake again demonstrated tyramine to be the most potent of the twelve drugs although in this case it did not differ significantly from cocaine and tripeleennamine. The remaining compounds also showed a decreased accumulation of ^3H -NA but were less potent than tyramine (although all drugs produced inhibition of uptake of NA' at a lower dose than that required for release of the amine). Tyramine again deviated from the linear relationship between inhibitory potency and partition coefficient, but so did cocaine and tripeleennamine. Chlorpheniramine and diphenhydramine also did not seem to fit the correlation although the discrepancy was less pronounced than for the other three compounds. It thus appears that drugs such as tyramine, cocaine and tripeleennamine are inhibiting accumulation of NA by a specific interaction with the neuronal uptake process, whereas the other compounds studied may be acting in a noncompetitive, nonspecific manner or with mixed effects. Only tyramine, besides blocking the uptake mechanism competitively, also appears to act as a substrate for the transport system and therefore can enter the nerve terminal to bring about direct release.

Signatures of Examiners:

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LIST OF ABBREVIATIONS

ATPase	--	adenosine triphosphatase
cAMP	--	cyclic adenosine 3',5'-monophosphate
COMT	--	catechol-O-methyltransferase
CPM	--	counts per minute
DA	--	dopamine (3,4-dihydroxyphenylethylamine)
DBH	--	dopamine- β -hydroxylase
DPM	--	disintegrations per minute
EC50	--	concentration required for half-maximal (50%) effect on release
EDTA	--	ethylenediaminetetraacetate
g	--	average gravity
ID50 or IC50	--	dose (or concentration) required for half-maximal (50%) inhibition of uptake
K _m	--	Michaelis constant
Log P	--	logarithm of the octanol/water partition coefficient
π	--	logarithm of the partition coefficient of a substituent group
MAO	--	monoamine oxidase
NA	--	noradrenaline
P	--	pellet
P/M ratio	--	particle (or particulate)/medium ratio
POBZ	--	phenoxybenzamine
POPOP	--	<u>p-bis</u> -[2-(5-phenyloxazolyl)]-benzene
PPO	--	2,5-diphenyloxazole
r	--	correlation coefficient

(continued)

rpm -- revolutions per minute

S -- supernatant

SAR -- structure-activity relationship

S.E.M. -- standard error of the mean

Vmax -- maximal rate of an enzyme reaction

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INTRODUCTION

The Transport and Storage of Noradrenaline

It has become evident in recent years that some form of mediated transport system probably exists for every proposed neurotransmitter substance and that for each neuron type, the system appears to be associated with the transmitter which is manufactured and released there. In this review, however, emphasis will be placed on the transport of only noradrenaline (NA). It is this process which has received the most attention and which has been the most thoroughly characterized because of the evidence that uptake and binding of this catecholamine without any enzymatic degradation represent the primary mechanism for termination of the action of NA after its release from the presynaptic nerve terminals. In addition, much of the information about the transport systems is a result of pharmacological research on drugs which were found to interact with the processes.

Figure 1 depicts schematically the basic events which are proposed to occur in noradrenergic synaptic transmission. The transmitter substance is believed to be stored within synaptic vesicles or granules (a) in the nerve terminal. These are highly specialized subcellular particles in which the NA appears to be concentrated in a 4:1 stoichiometric ratio with adenosine triphosphate and may be bound therein to a soluble protein

Figure 1. The basic events occurring in synaptic transmission. The various processes which may be involved are designated as follows: (a) storage within synaptic vesicles, (b) release into the synaptic cleft, (c) combination with a receptor, (d) diffusion into the blood stream, (e) metabolism extracellularly by COMT, (f) postsynaptic or extra-neuronal uptake followed by intracellular metabolism, and (g) uptake into the presynaptic nerve terminal. (adapted from Iversen, 1967)

(Shore, 1972). Depolarization in the nerve ending brings about release of these vesicular contents into the synaptic cleft (b). Proposed mechanisms for this release will be discussed in a later section. In the cleft, the NA can combine with a receptor (c) to exert its physiological or pharmacological action.

Once a transmitter is released from the presynaptic terminals, an efficient process for its removal from the region of the receptor must be available. Various mechanisms may be involved in terminating the transmitter's pharmacological action and any interference with the processes would be expected to prolong and potentiate the effects of nerve stimulation. A small amount may diffuse into the blood stream (d); another fraction may be metabolized by catechol O-methyltransferase (COMT) (e), but the greatest fraction appears to be removed from the synaptic cleft by means of a membrane transport system. This occurs most commonly into the presynaptic nerve terminal (g) although there is evidence that uptake into postsynaptic or other cells occurs, followed by intracellular metabolism (f) (Iversen, 1965c).

Neuronal Uptake of Noradrenaline

Before proceeding, it should be noted that the term "uptake" is commonly used in several different contexts but strictly speaking, only refers to the mechanism whereby extracellular amine is transferred into the intracellular space of a tissue.

In contrast, "storage" or "accumulation" may also involve transport into and retention in the storage granules as well as being affected by metabolism or release from the nerve terminal. However, since catecholamine uptake is commonly studied by measuring the accumulation of exogenous amine, the term "uptake" may also be used to refer to the more complex phenomenon, the meaning depending on the context in which the term is used.

The first suggestion that catecholamines might be taken up and stored in tissue binding sites was made by Burn in 1932. Since then, a great deal of research has been done in the area to elucidate the properties as well as the physiological and pharmacological significance of uptake processes.

Although several groups of investigators had shown that exogenous catecholamines, in large doses, could be accumulated in various peripheral tissues, it was not until labelled amines of high specific activity became available that doses comparable to normal physiological concentrations could be used. Employing relatively small doses of ^3H -adrenaline and ^3H -noradrenaline, Axelrod et al. (1959) and Whitby and coworkers (1961) were the first to demonstrate that the uptake and binding of these substances peripherally represented an important mechanism for their inactivation. This uptake was shown to occur to the greatest extent in tissues with a rich sympathetic innervation. Similarly, many other researchers have provided additional evidence that uptake and retention of NA occurs primarily in adrenergic neurons of both the peripheral and central nervous systems by using histochemical and autoradiographic methods. A

more detailed account of the evidence for neuronal uptake of NA may be found in several review papers by Iversen (1965a; 1967; 1971b).

Although early studies of uptake in brain presented problems because of the presence of a "blood-brain barrier" to catecholamines, more recently the preparation and isolation of synaptosomes have provided researchers with a new tool for studying the transport processes which occur across a neuronal membrane (De Belleruche and Bradford, 1973). Both Gray and Whittaker (1962) and DeRobertis and coworkers (1962), separately isolated brain nerve endings from the mitochondrial fraction of rat brain homogenates employing a cell fractionation technique. These endings or "synaptosomes" may be described as the pinched-off nerve terminals from brain. During tissue rupture, the presynaptic nerve terminals are torn from the post-synaptic membrane together with the postsynaptic thickening and at the same time either bud off from the axon or break away from it. In the latter case, the damaged membrane rapidly reseals to form a continuous surface. Synaptosomes contain synaptic vesicles, one or more mitochondria, and usually carry a post-synaptic thickening.

Other Types of Uptake Processes

It has become evident that other uptake processes exist in addition to neuronal uptake of NA (which is commonly denoted as "Uptake₁"). More recently, it has been demonstrated that an up-

take process exists which transports catecholamines across membranes of non-neuronal tissues and this has become known as "Uptake₂" (Iversen, 1965c). Gillespie (1973) has presented a thorough review on this topic. Another mechanism has been described which brings about redistribution of catecholamine from the axoplasm into the intracellular storage vesicles (Carlsson, Hillarp and Waldeck, 1963; von Euler and Lishajko, 1963). It is now clear that this vesicular uptake system is very much different from other characterized uptake processes. Finally, it is now known that specialized uptake processes also exist for every other proposed mammalian neurotransmitter substance. These processes and their properties have been reviewed by Iversen (1971a; 1971b).

Properties and Characteristics of Uptake₁

The neuronal uptake of NA has been studied extensively and consequently, a good deal of information is now available on this system. From the many studies it has become apparent that Uptake₁ displays almost identical properties in the NA-containing neurons of both the central and peripheral nervous systems (Iversen, 1973).

Early experiments which showed that NA in peripheral tissues was accumulated against a concentration gradient were performed by Axelrod et al. (1959) and Whitby and coworkers (1961). Similar findings using brain tissue were presented by Dengler and coworkers (1961b) who suggested that a saturable membrane

transport process was involved. A kinetic study of the uptake system in isolated, perfused rat heart (Iversen, 1963) confirmed the suggestion of Dengler et al. (1961b) that the process obeyed Michaelis-Menton kinetics by measuring initial rates of uptake at various amine concentrations. As well, he showed that the uptake process was stereochemically selective for the naturally-occurring (-)-isomer of NA and that cocaine acted as a potent competitive inhibitor of NA uptake. The kinetic constants derived from this study are given in Table I.

Table I
Kinetic Constants for Noradrenaline Uptake in Rat Heart
(from Iversen, 1963)

	Michaelis Constant, (Km) (M x 10 ⁻⁷)	Vmax $\pm$ S.E. (ng/min/g of heart)
($\pm$)-Noradrenaline	6.64	234 $\pm$ 5.1
(-)-Noradrenaline	2.66	196 $\pm$ 20.2
(+)-Noradrenaline	13.90	295 $\pm$ 8.4

Coyle and Snyder (1969b) examined the stereospecificity in different areas of rat brain and also found a marked preference of the uptake system for (-)-NA in all regions except the corpus striatum where dopamine is the major catecholamine.

The temperature-dependence of the uptake of ³H-NA was shown by Ziance and coworkers (1972a) and Colburn et al. (1968). In the perfused heart, any single metabolic inhibitor seems to

have little influence on NA uptake. In the absence of both glucose and oxygen, however, uptake of NA is inhibited but can be restored by providing either agent (Wakade and Furchgott, 1968). In comparison, in brain tissue, uptake is inhibited by metabolic inhibitors (White and Keen, 1970) and stimulated by glucose and oxygen (Dengler et al., 1962).

In both peripheral and central neurons, there is a marked dependence of the uptake system for sodium ions in the external medium, with almost complete inhibition of the process in the absence of sodium (Iversen and Kravitz, 1966; Bogdanski et al., 1968; Colburn et al., 1968; Bogdanski and Brodie, 1969; Keen and Bogdanski, 1970; Bogdanski et al., 1970). Optimum uptake was shown to occur at essentially physiological levels of both sodium (Na^+) and potassium (K^+) ions; complete absence of K^+ markedly diminished uptake while raising the external K^+ concentration also decreased the rate of NA uptake through competition with Na^+ (Iversen and Kravitz, 1966; Bogdanski et al., 1968; Colburn et al., 1968; Bogdanski and Brodie, 1969; White and Keen, 1970; Bogdanski et al., 1970). Iversen and Kravitz (1966) found that NA uptake in rat heart was not affected by the absence of calcium ions (Ca^{++}) and this was supported by Bogdanski and Brodie (1969) and Keen and Bogdanski (1970).

Ouabain, 10^{-3} M, was also shown to inhibit the uptake of ^3H -NA (Bogdanski, Tissari and Brodie, 1968; Colburn et al., 1968; Bogdanski and Brodie, 1969; White and Keen, 1971). Because of this ouabain sensitivity and Na^+ - and K^+ -dependence of NA uptake, it has been suggested that the process is carrier-mediated with

Fig. 2. A working hypothesis for the effect of inorganic ions on uptake and storage of NA by peripheral adrenergic nerve endings. "C" denotes the membrane carrier. Arrows represented by "X" indicate inhibitory factors. Brackets indicate coupled reactions. (Bogdanski and Brodie, 1969)

($\text{Na}^+ + \text{K}^+$)-dependent ATPase playing a central role to maintain the ionic gradient (Colburn et al., 1968; Bogdanski et al., 1968; Bogdanski and Brodie, 1969; Bogdanski et al., 1970). According to this theory, NA is transported across the cell membrane by a carrier with the energy for transport being provided by the inward movement of Na^+ down a concentration gradient which is maintained by ATPase (Fig. 2). However, this mechanism has been challenged. White and Keen (1971) found that low concentrations of ouabain inhibited ATPase activity to the same extent as 1 mM ouabain, yet did not depress NA uptake as the higher concentrations did. They also presented evidence that ion gradients do not provide energy for the uptake process, by altering the internal and external ion concentrations of synaptosomes (White and Keen, 1970) and a kinetic analysis by White and Paton (1972) suggested that Na^+ did not increase the affinity of a carrier for NA.

Additional evidence for a membrane carrier transport system for NA comes from the demonstrated structural specificity of the process. Many structural analogs can act as substrates for the NA uptake system and from experiments on these compounds the structure-activity relationships (SAR) for optimum binding to the uptake sites can be obtained. Similarly, various inhibitors of the uptake process have been studied and the SAR for inhibition has also been determined. It is important to differentiate between substrates and competitive inhibitors of NA uptake since the latter compounds may be able to bind with the carrier sites yet may lack the structural features necessary for transport into

the cell. To show that a substance is transported into the neuron would require direct measurement of the accumulation of this substance in the tissue. It should also be remembered that some drugs which inhibit NA uptake do not fit the SAR for inhibition and may act in a manner which is not competitive. Compounds which are taken up as substrates have the potential to affect intraneuronal binding or storage of the transmitter.

In some of the earliest experiments which demonstrated the importance of tissue uptake of catecholamines, it was observed that the uptake of NA was more important as a mechanism for the inactivation of circulating catecholamines than that of adrenaline (Axelrod et al., 1959; Whitby et al., 1961). Iversen (1965c; 1965d) showed that adrenaline was accumulated by the same mechanism as was NA and also determined kinetic constants for adrenaline uptake. From observations such as these which indicated that structural analogs of NA may also be actively accumulated by the neuron, a series of experiments resulted in the SAR for substances to serve as substrates for the NA uptake process. This has been outlined by Iversen (1971b). Briefly, the basic structural requirements for phenethylamine derivatives to serve as a substrate are (1) absence of bulky N-substituents, (2) presence of phenolic hydroxyl groups (especially in the meta-position), and (3) absence of bulky methoxyl groups on the phenyl ring.

Inhibition of Uptake

Inhibitors of NA uptake fall into two general categories, the first comprising synpathomimetic amines structurally related to NA (some of which may also act as substrates) and the second consisting of drugs not related in structure to NA and having diverse pharmacological actions.

The former group was studied extensively by both Horn (1973) in rat brain homogenates and Burgen and Iversen (1965) in rat isolated heart. The structural rules obtained for inhibition of NA uptake by the phenethylamines derivatives appeared to hold true for both central and peripheral neurons. These authors found that phenolic hydroxyl groups and α -methylation of the side chain led to an increase in affinity for the uptake sites, whereas methylation of the phenolic hydroxyl groups, β -hydroxylation of the side chain, or N-methylation led to decreased affinity. A comparison of the affinities of these analogs are shown in Table II (Burgen and Iversen, 1965).

Amphetamine is one of the structural analogs which appears to be a potent inhibitor of NA uptake but not a substrate (Thoenen et al., 1968; Iversen, 1971a, 1971b, 1973) although Thoenen's paper suggests that the uptake of amphetamine may occur yet be masked by a rapid outward diffusion, thus preventing accumulation. Azzaro et al., (1974) suggested that amphetamine does enter the neuron through the amine transport system since cocaine and desipramine both reduced its accumulation in synaptosomes of rat brain tissue. Much of the work of this research

Table II

Inhibition of Noradrenaline Uptake (Uptake₁) by Sympathomimetic Amines in the Rat Isolated¹ Heart

(Affinities were determined by direct analysis of uptake kinetics. The ID50 is the drug concentration producing 50% inhibition of noradrenaline uptake; affinities are relative to phenethylamine=100) (Burgen and Iversen, 1965)

Drug	ID50 (M)	Relative Affinity
(-)-Metaraminol	7.6×10^{-8}	1,440
Dopamine	1.7×10^{-7}	650
(+)- α -Methyldopamine	1.8×10^{-7}	610
(+)-Amphetamine	1.8×10^{-7}	610
(+)-Hydroxyamphetamine	1.8×10^{-7}	610
(-)-Nordefrin	2.0×10^{-7}	550
(-)-Noradrenaline	2.7×10^{-7}	407
(+)-Nordefrin	4.2×10^{-7}	260
Tyramine	4.5×10^{-7}	245
(+)-Amphetamine	4.6×10^{-7}	240
Metatyramine	5.1×10^{-7}	215
(+)-Methylamphetamine	6.7×10^{-7}	165
(+)-Noradrenaline	6.7×10^{-7}	165
(+)-Prenylamine	7.4×10^{-7}	149
N-Methyldopamine	7.6×10^{-7}	145
(+)-Buphenine	8.5×10^{-7}	130
(+)-Propylhexedrine	8.5×10^{-7}	130
(-)-Adrenaline	1.0×10^{-6}	110
Mephentermine	1.0×10^{-6}	110
PHENETHYLAMINE	1.1×10^{-6}	100
(+)-Octopamine	1.3×10^{-6}	85
Tranylcypromine	1.3×10^{-6}	85
(+)-Noradrenaline	1.4×10^{-6}	78
(+)-Cyclopentamine	1.4×10^{-6}	78
(+)-Adrenaline	1.4×10^{-6}	78
Noradrenalone	1.5×10^{-6}	73
2-(Naph-2-yl)ethylamine	1.5×10^{-6}	73
(+)-Phenylpropanolamine	2.0×10^{-6}	55
(+)-3,4-Dichloroisoprenaline	2.0×10^{-6}	55
(-)-Ephedrine	2.2×10^{-6}	50
Hordeine	2.5×10^{-6}	45
(+)- α -Ethylnoradrenaline	3.2×10^{-6}	34
(-)-Amphetamine	3.7×10^{-6}	30

(continued)

Table II (continued)

Drug	ID50 (M)	Relative Affinity
Phenelzine	3.8×10^{-6}	29
(+)-Phenylethanolamine	4.8×10^{-6}	23
(-)-Phenylephrine	5.6×10^{-6}	20
Tuaminoheptane	5.6×10^{-6}	20
(+)-N-Ethylnoradrenaline	9.2×10^{-6}	12
2-(p-Methoxyphenyl)ethylamine	1.0×10^{-5}	11
(+)-Methoxyphenamine	1.1×10^{-5}	10
(+)-Oxedrine	1.2×10^{-5}	9
5-Hydroxytryptamine	2.0×10^{-5}	5.5
(+)-Isoprenaline	2.5×10^{-5}	4.5
(+)-N-Butylnoradrenaline	3.5×10^{-5}	3.2
(+)-N-Isobutylnoradrenaline	4.0×10^{-5}	2.6
(+)-Metanephrine	4.3×10^{-5}	2.6
(-)-Dopa	6.0×10^{-5}	1.2
(+)-Normetanephrine	2.0×10^{-4}	0.55
6-Hydroxydopamine	2.0×10^{-4}	0.55
2-(3,4-Dimethoxyphenyl)ethylamine	2.0×10^{-4}	0.55
(+)-Methoxamine	1.0×10^{-3}	0.11
Mescaline	1.5×10^{-2}	0.007

group has been concerned with the complex actions of amphetamine (Ziance and Rutledge, 1972; Wenger and Rutledge, 1974).

Another analog of NA which is known to be a potent inhibitor of the uptake process is tyramine. Like amphetamine, it is classed as an indirectly-acting sympathomimetic agent and has been shown to displace NA from tissue stores (Hertting, Axelrod and Patrick, 1961). It has been postulated that tyramine uses the NA uptake system to gain entry to the adrenergic nerve terminals where it can bring about the release of endogenous NA (Furchgott et al., 1963). The accumulation of labelled tyramine had been demonstrated by Axelrod and coworkers (1962). In support of the theory that tyramine utilizes the same uptake system as does NA, a series of studies has shown the effects of various drug pretreatments on the uptake of NA and tyramine (McNeill and Brody, 1968; Commarato et al., 1969a; Commarato et al., 1969b; McNeill and Commarato, 1969), demonstrating that drugs which potentiate responses to NA will decrease responses to tyramine, presumably by preventing the uptake of both amines.

Among those compounds which are not structurally related to NA, cocaine is probably the most studied and best-known example and, as such, is often used as a reference compound in comparison studies. Dengler et al. (1961a) presented evidence that cocaine inhibited NA uptake and that this was related to the pharmacological potentiating effect of cocaine on the NA response. Similarly, McNeill and Brody (1966) found a relationship between cocaine and other inhibitors of NA uptake and their potentiation of the NA activation of cardiac phosphorylase. Co-

caine's competitive inhibiting effect has been shown by many other researchers including Hertting, Axelrod and Whitby (1961), and Iversen (1963; 1965b).

In addition to cocaine, other compounds of a wide variety of structures also appear to block the uptake of NA. One such group is the tricyclic antidepressants. Axelrod, Hertting and Potter (1962) demonstrated that imipramine had the ability to block the entry of ^3H -NA into storage sites. Similar findings for this and other tricyclic antidepressants have appeared throughout the literature (Hertting, Axelrod and Whitby, 1961; Iversen, 1965b; Callingham 1966; McNeill and Brody, 1968; Horn, Coyle and Snyder, 1971; Mundo et al., 1974; Squires, 1974). Mundo and coworkers (1974) found that the accumulation of labelled antidepressants by rat isolated atria seemed to be a passive and unsaturable process.

Another group of drugs which have an inhibitory effect on the uptake of NA are the antihistamines (Johnson et al., 1965; Isaac and Goth, 1965; Isaac and Goth, 1967; McNeill and Brody, 1968; Snyder, 1970; Horn, Coyle and Snyder, 1970). The adrenergic blocking agents represent yet another group of compounds which were found to act as inhibitors of uptake (Hertting, Axelrod and Whitby, 1961; Dengler et al., 1961a; Iversen, 1965b). In addition to the aforementioned drugs, inhibition of uptake of NA has been implicated as an action of the monoamine oxidase inhibitors (Iversen, 1965b; Hendley and Snyder, 1968), phenothiazines such as chlorpromazine (Axelrod, Hertting and Potter, 1962; Hertting, Axelrod and Whitby, 1961; Dengler et al., 1961a;

Iversen, 1965b; Snyder, 1970; Horn et al., 1971), the adrenergic neurone blocking drugs, guanethidine and bretylium (Hertting, Axelrod and Whitby, 1961; Dengler et al., 1961a; Iversen, 1965b), amantadine (Herblin, 1972), ethanol (Roach et al., 1973), various antiparkinsonian agents (Snyder, 1970; Horn et al., 1971), and the narcotic analgesics (Carmichael and Israel, 1973; Montel and Starke, 1973) although a recent study by Clouet and Williams (1974) suggests that the narcotic drugs do not compete with the catecholamines for the neuronal amine transport system.

Since there is such a diversity of structures and actions in these compounds, it must be emphasized that the drugs may be acting at more than one site in producing the total pharmacological response. As well, there seems to be no absolute correlation between the potencies of the various compounds as inhibitors of uptake and their potencies with respect to other pharmacological actions. Therefore, although the ability to inhibit NA uptake may aid researchers in understanding the mechanisms of action of drugs which interact with adrenergic mechanisms, it must be remembered that interactions with the uptake process may only modify the main pharmacological actions of these compounds. These other effects may obscure the expected potentiation of the NA response. For example, cocaine and imipramine possess a local anaesthetic action which may reduce the effects of sympathetic stimulation at concentrations high enough to block uptake; drugs with α -adrenergic receptor blocking action such as chlorpromazine may also fail to produce potentiation. Failure to potentiate NA may also result if the inhibitor has a much

lower affinity for the uptake sites than NA or if the concentration of drug approximates the concentration needed to saturate the uptake mechanism (Iversen 1971b). These and other factors outlined by Iversen (1971b) should be considered carefully before attempting to assess the inhibitory capacity of various drugs by measuring the potentiation of the NA response.

The Release of Noradrenaline from Nerve Endings

Just as it is important to distinguish between the uptake of NA and accumulation of this catecholamine, it is also important to differentiate between transmitter release and overflow. Release represents the actual output of amine produced by nerve stimulation whereas overflow, a much more complex parameter, refers to the increase in transmitter above control levels in the external medium of an organ or tissue preparation (Langer, 1974). This latter process may be affected by neuronal or extraneuronal uptake of NA, metabolism by MAO or COMT, or binding to receptors, as well as by an actual change in transmitter release. Therefore, any one or a combination of these effects must be considered in any study which demonstrates an increase in transmitter overflow.

In actual fact, very little is known about the detailed mechanism whereby nerve impulses and drugs bring about the release of NA from nerve terminals. Although there has been increased interest and experimentation in this area in recent years, the number of publications is small in comparison to the

profusion of papers published concerning the NA uptake process.

The mechanism of transmitter release from sympathetic nerves appears to be similar in some respects to the release of acetylcholine at cholinergic junctions, a process which has been more extensively studied. Katz (1962) has described the release of acetylcholine as a calcium-dependent unitary event of secretion in which multimolecular parcels of transmitter (the vesicle contents) are extruded suddenly from the presynaptic membrane. It has been shown that both acetylcholine and NA are stored in granules or vesicles within the nerve endings. In keeping with this, Burnstock and Holman (1966), in experiments with guinea pig vas deferens, have implied a spontaneous release of "packets" of NA and that with nerve stimulation there is an increase in the number of quanta released. Much of what is now known about NA release has resulted from experiments on "stimulus-secretion coupling" in the adrenal medulla. The main events occurring in this process, as outlined by Douglas (1966) appear to be first the reaction of the transmitter with the cell membrane resulting in increased permeability of the membrane to calcium ion (Ca^{++}). This is followed by inward movement of Ca^{++} down its electrochemical gradient to the site of action where it initiates a process which causes release of the contents of the granules. Termination of secretion occurs upon disappearance of the transmitter and/or Ca^{++} .

The dependence of NA release on Ca^{++} was demonstrated by Kirpekar and Misu (1967) in cat spleen. They concluded that depolarization of post-ganglionic sympathetic fibres may lead

to increased influx of Ca^{++} but admitted that the site of action and mechanism of the ion is unknown. From findings that barium and strontium, but not magnesium ions, could replace Ca^{++} and that sodium ions were not required for release to occur, it was suggested that the Ca^{++} was directly required for the release mechanism and not simply for nerve conduction. A review by Rubin (1970) supported the critical role of Ca^{++} in catecholamine release from central as well as from peripheral nerves whether release was due to nerve stimulation, acetylcholine, or excess potassium ions. He noted however, that tyramine-produced release does not seem to be Ca^{++} -dependent.

In attempts at defining more precisely the actual secretion mechanism, it was suggested that measurement of dopamine- β -hydroxylase (DBH) would provide information on the fate of storage vesicles since this enzyme was known to be contained within the vesicles (Viveros et al., 1969a). It was found that during nerve stimulation of the adrenal medulla, the entire soluble contents of the vesicles, including DBH, were secreted by exocytosis, leaving the vesicle membranes within the cells (Viveros, et al., 1969b). Results of a study by Weinshilboum and coworkers (1971) in sympathetic nerves were compatible with the suggested process of exocytosis. It was found that high calcium ion concentrations and the drug phenoxybenzamine enhanced the release of NA and DBH in nerves innervating guinea pig vas deferens (Johnson et al., 1971). Prostaglandin E_2 reversed this effect in this tissue but not in the adrenal medulla. The authors therefore suggested that since prostaglandin E_2 inhibited

the actions of Ca^{++} on the release process whereas phenoxybenzamine enhanced them, phenoxybenzamine may be acting by either increasing Ca^{++} influx, decreasing Ca^{++} efflux or inhibiting prostaglandin release. A report by Thoa and coworkers (1972) indicated that the integrity of the microtubule and microfilament protein complex in adrenergic nerve terminals was critical for depolarization-induced exocytosis of NA, implying that the rapid release of NA may involve a contractile mechanism similar to that occurring in muscle. Implications of the involvement of cyclic adenosine 3',5'-monophosphate (cAMP) in the release mechanism came about through the study of Wooten et al. (1973). They postulated that depolarization by an electrical stimulus may cause influx of extracellular Ca^{++} and activation of membrane-bound adenyl cyclase, producing an increase in intracellular cAMP. The cAMP may then mobilize Ca^{++} and/or act directly with Ca^{++} to bring about exocytosis.

Kinetics studies in rat heart slices were performed by Bogdanski and others in order to assess the role of various inorganic ions on the storage and release of NA from sympathetic nerves. Bogdanski and Brodie (1969) found that sodium ion (Na^+) is an absolute requirement for storage of NA and proposed that Na^+ affects storage by preventing spontaneous release. In 1970, Keen and Bogdanski reported a Ca^{++} -dependent efflux of NA in Na^+ -free media accompanied by an increased influx of Ca^{++} into the tissues, compatible with reports of Na^+ and Ca^{++} competition. They suggested that in the absence of Na^+ , Ca^{++} combines with a receptor resulting in release of NA from storage

sites; at the same time the Na^+ -dependent uptake carrier mechanism is inoperable resulting in rapid loss of amine from the cell. Further work in this area provided additional evidence that Na^+ and Ca^{++} are mutual antagonists in the release of NA from tissues (Blaszkowski and Bogdanski, 1971). They suggest several possible mechanisms to explain the competition between Na^+ and Ca^{++} . These authors then presented evidence for a Na^+ -dependent, carrier-mediated transport mechanism for the release of NA from the cell which is also inhibited by desipramine. According to their model, Ca^{++} enters the presynaptic terminal in response to depolarization. There, the free Ca^{++} participates in mobilization of stored NA by a saturable process antagonized noncompetitively by Na^+ . When mobilized, the NA could be transported across the membrane by the Na^+ -dependent carrier system. Paton (1973) also provides evidence that the efflux of NA from the cytoplasm of adrenergic nerves occurs by a cocaine-sensitive, carrier-mediated process.

Drug Effects on Noradrenaline Release

Before discussing actual drug effects on release, mention should be made of the notion of intraneuronal "compartments" or "pools" of stored NA. Although this is still an area which is poorly defined because of the profusion of complex and often conflicting evidence, many schemes have been proposed to explain the data.

One such model has been presented by Iversen (1967). Ac-

According to this scheme, most of the NA in nerve terminals is stored within particles in more than one storage form, consisting of a firmly-bound, tyramine-resistant complex and a tyramine-susceptible pool. This latter form can exchange freely with NA in the extravesicular axoplasm where it is removed mainly by metabolism by MAO. Release of NA by nerve impulses may occur from a small readily available pool which seems to be resistant to reserpine and tyramine but is replenished from the larger, reserpine-sensitive compartment. Uptake of extracellular NA occurs first into the axoplasm from which it is rapidly distributed into other intraneuronal pools.

Interest in the releasing effects of drugs gained prominence when it became evident that sympathomimetic amines could act be direct, indirect, or mixed effects. Tyramine represents the prototype of the indirectly-acting amines which produce their sympathomimetic effects by releasing NA from tissue storage sites. Evidence for a NA-releasing effect by tyramine was presented in 1958 by Burn and Rand, who found that tyramine's action was reduced by reserpine pretreatment but restored by NA and adrenaline infusions. Stjärne (1961) showed that tyramine increased NA levels in splenic blood and concluded that in the spleen, tyramine may be acting by releasing NA from intra-axonal storage granules or by making bound, extragranular NA available to receptors. In the same year, release of NA by tyramine in the rat heart was demonstrated by Hertting, Axelrod and Patrick. Inhibition of MAO in atria produced increased release of NA by tyramine, amphetamine and mephentermine but not

nerve-stimulated release (Smith, 1966). This work indicated that storage sites for NA released by tyramine differ from those affected by nerve stimulation and that substantial amounts of NA released by the indirectly-acting amines are oxidatively deaminated within the nerve terminals. The author suggested that tyramine could therefore be producing release from vesicles into the cytoplasm whereas nerve stimulation affected vesicles located adjacent to the cell membrane. However, Colburn and Kopin (1972), working with rat brain synaptosomes, indicated that tyramine releases stored NA to a site where MAO is not active since it did not release deaminated metabolites as reserpine did. This could alternatively be explained by competition between tyramine and NA for MAO since tyramine is rapidly degraded by this enzyme (with a half-life of about five minutes).

Although the exact mechanism of tyramine-induced release has not yet been resolved, it seems apparent that the process differs from nerve-stimulated release. Lindmar and coworkers (1967) demonstrated that a constant infusion of tyramine in rabbit heart released NA continuously and independently of external Ca^{++} concentration. Another paper stated that drugs such as reserpine produce a calcium-independent, non-exocytotic release of NA without concomitant release of DBH (Thoa et al., 1974). Other evidence for separate mechanisms is given by Westfall and Brasted (1974) who showed that colchicine, which prevents release of NA produced by nerve stimulation, had no effect on the release produced by tyramine. They also demonstrat-

ed that prostaglandins E_1 and E_2 decreased the release of NA induced by nerve stimulation, nicotine, potassium chloride, and aminophylline, but not tyramine.

Amphetamine-induced release of transmitters has also been widely studied since this drug is also classed as an indirectly-acting amine. In 1965, Glowinski and Axelrod demonstrated release of 3H -NA by both reserpine and amphetamine, although they acted in a different manner as indicated by the different metabolic fates of NA released by the two drugs. An increase in the concentration of normetanephrine occurred following amphetamine treatment, suggesting that this drug released NA from the neuron as unchanged amine which is then O-methylated by COMT. This study also demonstrated an inhibition of NA accumulation by amphetamine.

A significant contribution to this field of research has also been made by Rutledge and coworkers. Results of a study by Rutledge (1970) suggested that the inhibition of oxidative deamination of NA by amphetamine is primarily an indirect effect resulting from the inhibition of neuronal uptake of NA, limiting access of NA to intraneuronal MAO. Ziance and Rutledge, (1972), differentiating between actual release and inhibition of accumulation, again showed that amphetamine releases NA predominantly as unmetabolized amine because pargyline pretreatment had no significant effect on this release. They suggested that this effect was probably not due to direct inhibition of uptake of NA. Complicating the picture is evidence presented by Farnebo (1971) that amphetamine, in low concentrations,

acted primarily on extragranular catecholamines, having little releasing effect on transmitter stored within the amine storage granules.

Ziance, Azzaro and Rutledge (1972) demonstrated a marked temperature-dependence of ^3H -NA release from brain slices, chopped tissue, and synaptosomal homogenates produced by amphetamine but only partial dependence on the extracellular calcium ion concentration. In this latter respect, amphetamine-induced NA release differs from potassium-induced release which is strongly calcium-dependent, and also appears to differ from that produced by tyramine which occurs independently of Ca^{++} concentration. This suggests that all indirectly-acting amines may not be acting in the same manner to cause release. Amphetamine also exhibited a selective release from neurons containing different biogenic amines (Azzaro and Rutledge, 1973) which might explain dose-dependent behavioural changes due to this drug. Another experiment, employing cocaine and desipramine as inhibitors of neuronal NA uptake, showed that amphetamine-induced efflux was not due merely to inhibition of neuronal uptake of spontaneously-released amine (Azzaro, Ziance and Rutledge, 1974). Cocaine and desipramine also shifted the concentration-effect curve for release by amphetamine to the right but had no effect on ^3H -NA release by potassium chloride; similarly, cocaine and desipramine inhibited the uptake of low concentrations of ^3H -amphetamine into synaptosomes. These findings indicate that amphetamine also acts as a substrate for the neuronal NA uptake system, thereby entering the neurone and possibly displacing NA

from binding sites within the nerve terminal.

Among the drugs which have been shown to potentiate the actions of NA and to inhibit the uptake of ^3H -NA by adrenergic nerve terminals, both cocaine (Haefely et al., 1964; Trendelenburg, 1968; Davis and McNeill, 1973) and high doses of desipramine (Titus et al., 1966; Leitz and Stefano, 1970) also appear to possess the ability to release endogenous NA. The effect of desipramine appears to be at the level of the NA storage granule since the catecholamine released by this drug occurred primarily in the form of deaminated metabolites. (Leitz and Stefano, 1970).

An interesting concept with respect to neuronal release is the presynaptic regulation of catecholamine release, reviewed by Langer (1974). It has been known for some time that alpha-receptor blocking agents such as phenoxybenzamine (POBZ) or phentolamine increase the nerve-stimulated overflow of NA. The hypothesis of presynaptic regulation of NA release through a negative feedback mechanism mediated by alpha adrenergic receptors resulted from studies demonstrating positive changes in transmitter overflow by POBZ in tissues where beta receptors predominate. The theory proposes that released NA, above a critical concentration in the synaptic cleft, would activate presynaptic alpha receptors, inhibiting further release of the transmitter. In support of this, alpha receptor agonists inhibit, whereas the antagonists enhance nerve-stimulated release of transmitter (Farnebo and Hamberger, 1971). According to this scheme, blockade of neuronal uptake should be expected to en-

hance presynaptic inhibition of NA release and thereby reduce transmitter overflow, which could also explain the relative ineffectiveness of drugs such as cocaine or desipramine in increasing NA overflow despite their inhibition of uptake of the catecholamine. Langer also proposes that since the negative feedback mechanism is not involved in regulation of tyramine-induced NA release (which is also not calcium-dependent), the presynaptic feedback mechanism may act by modifying the availability of calcium ions for release elicited by nerve stimulation.

Thus, the effects of drugs in producing efflux of NA from nerve terminals can be very complex and may be intimately connected with their effects on the NA uptake system. For those drugs which have been shown to bring about transmitter release, the mechanisms are still unclear; for many other compounds which potentiate the response to NA, even less is known and researchers are still attempting to evaluate the relative contributions of the various drug actions on increased transmitter overflow.

Nonspecific Membrane Effects: Lipid Solubility and its Relationship to Potency

In his recent review paper, Seeman (1972) has related the membrane actions of anaesthetics and tranquilizers to their membrane concentrations (that is, lipid solubilities) and has demonstrated that a linear relationship exists between the nerve-blocking concentration of an anaesthetic and its membrane-buffer partition coefficient. In a similar manner, Carmichael and

Israel (1973) found that although there was no apparent relationship between narcotic analgesic potency and the inhibition of uptake of NA, a significant correlation existed between the lipophilic nature of these compounds and their ability to inhibit NA uptake. Also fitting this correlation were cocaine, desipramine, chlorpromazine, and benztropine, but the structural analog of NA, amphetamine, did not fit. Their results therefore suggested that inhibition of uptake of NA by many psychotropic compounds may be related to their lipid solubility rather than to a specific structure. Another study by Roach and coworkers (1973) on the effects of ethanol on transmitter uptake by rat brain synaptosomes also demonstrated a direct correlation between uptake inhibition and the oil/water partition coefficient of the inhibitor, presenting additional evidence for an interaction between the drug and membrane lipid.

Background and Objectives of the Present Study

As stated previously, pharmacologists are still trying to elucidate the precise mechanisms whereby many drugs potentiate the actions of NA and the preceeding discussion shows how the study of uptake and release of catecholamines is important pharmacologically in helping to explain the actions of drugs on adrenergic mechanisms. McNeill and coworkers have performed related experiments on the effects of various drug pretreatments on amine uptake and amine-induced phosphorylase activation in rat heart, lending support to the theory that tyramine utilizes

the same uptake system as does NA, and that drugs which potentiate the effects of NA but block the effects of tyramine do so by preventing the uptake of both amines (McNeill and Brody, 1966; McNeill and Brody, 1969; Commarato, Brody and McNeill, 1969a; 1969b; McNeill and Commarato, 1969). These experiments explored the effects of several antihistamines, tricyclic antidepressants, methylphenidate and cocaine on amine uptake. A later paper by Davis and McNeill (1973) investigated further the cardiac effects of these drugs, suggesting that a number of drugs known to potentiate the actions of noradrenaline by blocking amine uptake in the heart may also be exerting their positive inotropic effects by releasing NA from sympathetic nerve endings. However, the experiments did not positively distinguish between blockade of uptake of NA and actual release of the amine.

Therefore, it was decided to pursue this investigation of the effects of cocaine, tyramine, and several antihistamines and tricyclic antidepressants, employing a fairly simple, reproducible method involving use of catecholamine-containing nerve terminals in rat brain homogenate. A similar method has been used by other investigators to differentiate between inhibition of uptake of spontaneously-released ^3H -NA and actual release of the amine (for example, Ziance and Rutledge, 1972; Wenger and Rutledge, 1974). The specific objectives for the total period of research were as follows. First, it was desirable to study the uptake of tritiated noradrenaline by nerve terminals in rat brain homogenates in the absence of test drugs

in order to determine the time course of uptake and the effects of varying substrate concentrations. Secondly, the effects of both time and drug concentration on the efflux of ^3H -NA by the compounds were to be examined in order to compare their relative potencies regarding amine release. A third objective was to explore the effects of time and drug concentration on inhibition of ^3H -NA accumulation into nerve endings and again to determine the relative potencies of the compounds, in this case as inhibitors of uptake. Finally, a correlation of drug potency with lipophilicity of the compounds would help to distinguish between nonspecific effects on the cell membrane and specific release or interaction with an active uptake process.

MATERIALS AND METHODS

Animals

Wistar rats of either sex weighing 200 to 300 grams were used throughout the experiments. The animals were maintained on Purina Rat Chow^R and water ad libitum and were kept in a controlled environment until the time of sacrifice.

Chemicals and Drugs

The following drugs were donated: amitriptyline hydrochloride (Merck, Sharp and Dohme, Canada Ltd.), chlorpheniramine maleate (Schering Corp. Ltd.), desipramine hydrochloride (Pertofrane^R, Geigy (Canada) Ltd.), diphenhydramine hydrochloride (Benadryl^R, Parke-Davis and Co.), imipramine hydrochloride (Geigy (Canada) Ltd.), nortriptyline hydrochloride (Eli Lilly and Co. Ltd.), pargyline hydrochloride (Eutonyl^R, Abbott Laboratories Ltd.), phenindamine hydrochloride (Thephorin^R, Hoffmann-LaRoche Ltd.), promethazine hydrochloride (Poulenc Ltd.), tripeleennamine hydrochloride (Ciba Co. Ltd.), and triprolidine hydrochloride (Burroughs Wellcome and Co. (Canada) Ltd.).

Other drugs were purchased from commercial sources. These were: cocaine hydrochloride (British Drug Houses Pharmaceuticals), ($\pm$)-[7-³H]-noradrenaline (New England Nuclear Corp.),

(-)-noradrenaline (Sigma Chemical Co.), and tyramine hydrochloride (Sigma Chemical Co.). All other chemicals were used in the highest available purity.

To prepare the solution of labelled noradrenaline (a solution containing approximately 200,000 DPM as well as 200 pmoles total NA in a volume of 10 μ l to be added to 4 ml of incubation medium to give a final concentration of 0.05 μ M NA), two other solutions were required. The first of these contained ^3H -noradrenaline 0.25 mCi and 0.0045 mg in 0.25 ml (Solution "A"). (This specific activity varies with lot number but calculations are analogous for each lot received.) The second solution contained 3.248 μ g of unlabelled noradrenaline per ml to give a concentration of 19.2 M (Solution "B"). To make the desired final solution, 50 μ l of Solution "A" (containing 5319 pmoles ^3H -noradrenaline and 1.11×10^8 DPM) was added to 5269 μ l of Solution B (containing 101,061 pmoles noradrenaline) giving a labelled solution with a final volume of 5319 μ l and containing 106,380 pmoles of total noradrenaline.

Tissue Preparation

The method described below represents a modification of that used by Snyder and Coyle (1969). In a related study, these authors had pretreated rats with reserpine in order to inactivate the granular storage mechanism and deplete the endogenous catecholamines (Coyle and Snyder, 1969a). However, they later showed that kinetic data were unaltered in animals

which had received reserpine, indicating that only uptake by the neuronal membrane rather than binding within storage granules was contributing to short-term experiments on NA transport (Snyder and Coyle, 1969). Therefore animals in our study were not pretreated with reserpine.

Rats were killed by a blow to the head followed by cervical dislocation. They were immediately bled by cutting the throat and the brains were rapidly removed and transferred to a cold surface. The brains were then weighed and homogenized in eight volumes of ice-cold 0.25 M sucrose with eight to ten strokes of a motor-driven, glass homogenizing tube and teflon pestle (Potter-Elvehjem type, Kontes K-886000). The homogenate was then transferred to plastic centrifuge tubes and centrifuged at $1000 \times g$ (3000 rpm in an IEC B-20 centrifuge using rotor no. 874) for ten minutes at 0° to 4° C. Following this, the supernatant was aspirated from the large pink pellet. This pellet was discarded, the supernatant was mixed to form a uniform suspension, and an aliquot of the supernatant was heated in a test tube in a boiling water bath for ten minutes. The remaining untreated homogenate was kept in an ice bath until the incubation was begun (approximately thirty minutes after sacrificing the animal).

Incubation Procedure

Each incubation consisted of eight tubes: one boiled homogenate blank (to correct for nonspecific binding), one control (to which no drug was added), and six sample tubes.

The total volume of incubation mixture was 4.0 ml of which, typically, 0.2 ml was homogenate, 0.1 ml was drug solution, and 10 μ l was ^3H -noradrenaline solution. The remaining volume consisted of modified Krebs-Henseleit bicarbonate buffer (see Table III). This buffer was prepared fresh daily from stock solutions of each ingredient (except pargyline which is unstable in solution).

Basically, two procedures were used in order to separate release experiments from studies of uptake inhibition. For the release studies, homogenate was preincubated with ^3H -noradrenaline for 25 minutes prior to addition of the drug. This served to load the synaptosomes with ^3H -amine so that the effects of the drugs on release could be measured. For studies of uptake blockade, the homogenate was instead preincubated with drug for five minutes prior to addition of ^3H -noradrenaline. This was done to enable the drug to interact with the carrier or membrane before the catecholamine was added.

Before the actual incubation was begun, the tubes containing buffer and drug or noradrenaline were allowed to equilibrate to 37°C in a Dubnoff metabolic shaker bath for approximately ten minutes under 95% O_2 /5% CO_2 aeration. The preincubation was then begun with the addition of boiled homogenate to the first tube. Untreated homogenate was added to each of the remaining tubes at thirty second intervals. Tubes were mixed on a Vortex Mixer after each addition. At the end of the preincubation period, either drug solution or ^3H -noradrenaline was added (depending on the experimental objective as previous-

Table III
Modified Krebs-Henseleit Buffer

Chemical	g/1000 ml	concentration (mM)
NaCl*	7.07	121
KCl	0.35	4.7
CaCl ₂ **	0.183	1.4
MgSO ₄ ·7H ₂ O	0.308	1.2
NaHCO ₃	2.10	25
KH ₂ PO ₄	0.16	1.2
Glucose	2.0	11.1
Ascorbic Acid	0.20	1.1
Disodium EDTA·2H ₂ O	0.045	0.13
Pargyline HCl	0.030	0.16
Distilled Water to 1000 ml		

* since the calcium concentration was reduced (as CaCl₂), extra sodium chloride was added to give the same concentration of chloride that normal Krebs-Henseleit buffer contains

** calcium concentration was reduced by half of the original concentration found in normal (unmodified) Krebs-Henseleit buffer

ly described) at thirty second intervals and the incubation continued for the desired length of time.

The incubation was terminated by immediately transferring the incubation tubes to an ice bath. The mixture was poured into pre-chilled ultracentrifuge tubes which were then tightly capped and centrifuged at 48,000 x g for thirty minutes at 0° to 4°C (27,000 rpm in a Beckmann L2-65B ultracentrifuge using rotor type 65). Following centrifugation, tubes were again placed in an ice bath to await final processing of the supernatant and pellet fractions.

Determination of Radioactivity

Immediately following ultracentrifugation, the supernatant was decanted from the pellet fractions. Aliquots (0.1 ml) of each supernatant were transferred in duplicate to liquid scintillation counting vials and 10 ml of Bray's scintillation solvent (Bray, 1960; see Table IV) was added to each vial.

The pellets were rinsed superficially with 5 ml of ice-cold Krebs-Henseleit buffer, drained well, and suspended in 2.0 ml of absolute ethanol. Each pellet was then homogenized in a glass homogenizing tube with a glass pestle (Kontes K-885200) until a homogeneous suspension was obtained. Suspensions were centrifuged for ten minutes at 1000 x g (3000 rpm in an IEC B-20 centrifuge) and the resulting supernatant care-

Table IV
Bray's Scintillation Solvent

Chemical	Quantity	
PPO	4.0	g
POPOP	0.200	g
Naphthalene	60	g
Methanol (absolute)	100	ml
Ethylene Glycol	20	ml
Dioxane	to 1000	ml

fully decanted from the pellet which was discarded. Aliquots (0.5 ml) of supernatant were also transferred in duplicate to liquid scintillation counting vials and diluted with 10 ml of Bray's scintillation fluid.

Radioactivity of each sample was determined in a Nuclear-Chicago Isocap 300 Liquid Scintillation System by counting each sample for ten minutes using program number 1 for tritium-containing samples.

No attempt was made to separate metabolites formed in the tissue from the amine since it had been shown in similar experiments that 85% to 95% of the radioactivity in particulate fractions was unchanged catecholamine, with no difference between various brain areas (Snyder and Coyle, 1969; Coyle and Snyder, 1969b; Colburn et al., 1968) since pargyline pretreatment prevented formation of deaminated metabolites.

Calculations

Employing quench curves which had been prepared exclusively for the experimental system under study, counts per minute (CPM) for each sample were converted to disintegrations per minute (DPM) for both the extracted pellet (P) and supernatant (S) fractions. Duplicate samples were averaged at this point.

Results were calculated first as "particulate-medium ratios" (P/M ratios) of tritium, determined as disintegrations per minute per gram of pellet fraction divided by disintegrations per minute per millilitre of supernatant fluid. Using an analogous

procedure, Snyder and Coyle (1969) had determined pellet weights to be between 9 and 11 mg. Therefore, a pellet weight of 10 mg was assumed for the calculations. That is,

$$\begin{aligned} \text{P/M ratio} &= \frac{\text{DPM/g pellet}}{\text{DPM/ml supernatant}} \\ &= \frac{\text{DPM in extracted pellet sample} \times 400}{\text{DPM in supernatant sample} \times 10} \end{aligned}$$

Ratios for the blanks were subtracted from control and sample ratios. For studies of drug effects on the uptake and release processes, sample P/M ratios were compared to control ratios (with the control representing either 0% inhibition of uptake or 0% release). Therefore, calculations were expressed by:

$$\begin{array}{l} \% \text{ Uptake Inhibition} \\ \text{or} \\ \% \text{ Release} \end{array} = \frac{\text{Control ratio} - \text{Sample ratio}}{\text{Control ratio}} \times 100\%$$

for each incubation.

For Time vs Effect studies as well as Concentration vs Effect experiments, each determination was repeated at least three times so that the expressed values represent the mean $\pm$ standard error of the mean for at least three duplicate determinations.

RESULTS

Uptake in the Absence of Test Drugs

The initial experiments were conducted with the aim of establishing the basic levels of uptake of tritiated NA by the synaptosome-containing whole rat brain homogenates in the absence of drug treatment. Incubations of NA and homogenate were carried out for 2, 5, 10, 20 and 45 minutes in order to determine the time course of NA uptake under the previously-described experimental conditions. This study was performed with four different concentrations of total NA (0.05, 0.27, 0.70, and 2.0 μM) in the incubation medium, but a constant concentration of tritiated NA (0.05 μM). In order to achieve these concentrations in a total volume of 0.10 ml added to sufficient incubation medium to make 4.0 ml, the following scheme was used:

Table V

Volumes of NA Solutions Added to the Incubation Mixture

Desired NA conc. (μM)	Volume "hot" sol'n	Volume "cold"	Volume 0.01 N tartaric acid	Total volume
0.05	0.01 ml	---	0.09 ml	0.10 ml
0.27	0.01 ml	0.01 ml	0.08 ml	0.10 ml
0.70	0.01 ml	0.03 ml	0.06 ml	0.10 ml
2.0	0.01 ml	0.09 ml	---	0.10 ml

The concentration of the non-labelled ("cold") NA solution was 86.67 μM and that of the tritiated ("hot") NA solution was 20 μM , both solutions having been prepared in 0.01 N 1-tartaric acid.

From this study, the particle/medium (P/M) ratios were calculated for each sample, blank values were subtracted, and the means $\pm$ standard errors were determined for seven such determinations. The results are expressed in Table VI and Figure 3.

Table VI

Accumulation of Noradrenaline in the Absence of Drug Treatment
(Values are expressed as Particle/Medium ratios. Each value represents the mean $\pm$ S.E.M. of seven determinations.)

NA concentration	P/M ratio				
	2 min.	5 min.	10 min.	20 min.	40 min.
0.05 μM	4.27 $\pm$ 0.38	6.17 $\pm$ 0.35	7.92 $\pm$ 1.10	10.08 $\pm$ 0.58	8.19 $\pm$ 0.98
0.27 μM	4.32 $\pm$ 0.80	5.98 $\pm$ 0.74	6.80 $\pm$ 0.47	8.90 $\pm$ 0.81	6.43 $\pm$ 0.85
0.70 μM	3.70 $\pm$ 0.69	4.76 $\pm$ 0.51	5.49 $\pm$ 0.53	6.75 $\pm$ 0.62	4.88 $\pm$ 0.68
2.0 μM	2.49 $\pm$ 0.62	3.03 $\pm$ 0.36	3.31 $\pm$ 0.48	3.04 $\pm$ 0.38	2.79 $\pm$ 0.57

The graph shows that there is an increase in accumulation with time with peak uptake occurring after approximately 20 minutes incubation for all four concentrations of NA. Following this,

Figure 3. Accumulation of ^3H -noradrenaline in the absence of drug treatment, employing four concentrations of catecholamine: $0.05\ \mu\text{M}$ (O), $0.27\ \mu\text{M}$ (▲), $0.70\ \mu\text{M}$ (●), and $2.0\ \mu\text{M}$ (Δ). Each point represents the mean $\pm$ standard error of three determinations.

Figure 4. Time for peak accumulation of 0.05 μ M noradrenaline by rat brain homogenate.

a gradual decline in the level of uptake takes place. Figure 3 also shows that the lowest concentration of NA used, $0.05\ \mu\text{M}$, produces the highest P/M ratios. Because decreasing ratios with increasing substrate concentrations indicate that the uptake mechanism is becoming saturated, it was decided to use $0.05\ \mu\text{M}$ NA for all further experiments since this would demonstrate the greatest changes in accumulation with varying conditions and thus the greatest differentiation between treatments.

An experiment was also performed in which concentrations of NA above $2.0\ \mu\text{M}$ were used (5 , 10 , and $20\ \mu\text{M}$), and incubations were continued for 2 , 5 , 10 , 15 , 25 , and 40 minutes. The P/M ratios at the various times did not differ from one another for all three concentrations of catecholamine and these were all comparable with the ratio for the blank, indicating that the system was saturated by $5.0\ \mu\text{M}$ NA.

In order to more precisely locate the time of maximum uptake, more frequent time intervals were chosen in the vicinity of the 20 minutes incubation time. The homogenate was incubated with $0.05\ \mu\text{M}$ NA and the results are expressed in Figure 4. From this curve, the incubation time for peak accumulation to occur was shown to be 25 minutes. Therefore this time was chosen for later experiments on drug effects on NA release, since preincubating the homogenate with the NA for this period of time should allow maximum loading of the synaptosomes before the drug is added. Included in this last experiment was a study of the effects of both time and NA concentration on the P/M ratio for the boiled homogenate blank. Neither of these

parameters was found to have an effect on the blank values, which remained essentially constant. Thus, any convenient incubation period could be chosen for the blank tube in later time-effect studies. /

Effects of Drugs on the Efflux of Noradrenaline: Time-Effect Studies

As stated above, 25 minutes was chosen as the time for pre-incubation of ^3H -NA with the homogenate before adding the drug so that the synaptosomes would be loaded with tritiated amine and therefore the effects of the drugs on release could be measured. The concentration of drugs to be used was arbitrarily chosen as 10^{-4} M and was found to be satisfactory for differentiating between the effects of the twelve drugs. Once the drug was added to the medium, incubations were continued for 2, 5, 10, 20, and 40 minutes in order to show the time course of the drug effects. Control tubes (to which no drugs had been added) were included for each incubation time and P/M ratios for the sample (treated) tubes were compared to ratios from the control tubes (after first subtracting the blank value) and expressed as "% Release of NA". Table VII gives the means $\pm$ standard errors for triplicate determinations of the per cent of NA released by the twelve drugs at the various incubation times. These data are expressed graphically in Figures 5a, 5b, 5c, and 5d. All of the drugs which were tested produced an increased efflux of NA with time over the

Table VII

The % Release of Noradrenaline by 10^{-4} M Drugs at Various Incubation Times (Each figure represents the mean $\pm$ standard error of three determinations)

Drug	% Release of Noradrenaline				
	2 min.	5 min.	10 min.	20 min.	40 min.
Amitriptyline	22.65 $\pm$ 3.47	47.02 $\pm$ 1.40	65.16 $\pm$ 3.25	79.41 $\pm$ 3.23	92.53 $\pm$ 0.98
Chlorphenir- amine	13.34 $\pm$ 4.04	21.35 $\pm$ 2.24	26.10 $\pm$ 1.25	42.29 $\pm$ 3.93	52.46 $\pm$ 5.65
Cocaine	5.93 $\pm$ 4.28	16.24 $\pm$ 1.22	22.07 $\pm$ 2.43	36.01 $\pm$ 1.91	42.13 $\pm$ 0.79
Desipramine	17.63 $\pm$ 2.29	40.36 $\pm$ 1.29	54.15 $\pm$ 4.53	71.79 $\pm$ 3.17	91.73 $\pm$ 4.64
Diphenhydra- mine	9.11 $\pm$ 3.79	23.86 $\pm$ 7.83	25.00 $\pm$ 2.39	37.80 $\pm$ 2.63	50.43 $\pm$ 1.75
Imipramine	18.00 $\pm$ 4.38	44.44 $\pm$ 2.40	57.57 $\pm$ 1.88	72.86 $\pm$ 3.39	82.81 $\pm$ 2.08
Nortriptyline	17.05 $\pm$ 5.46	42.06 $\pm$ 3.02	59.09 $\pm$ 4.18	81.50 $\pm$ 1.09	95.92 $\pm$ 1.06
Phenindamine	6.30 $\pm$ 2.12	24.37 $\pm$ 4.47	39.40 $\pm$ 4.89	58.31 $\pm$ 1.44	72.13 $\pm$ 1.92
Promethazine	12.66 $\pm$ 3.50	44.37 $\pm$ 9.49	64.54 $\pm$ 7.83	75.97 $\pm$ 1.87	90.65 $\pm$ 4.68
Tripelenna- mine	9.78 $\pm$ 6.55	26.20 $\pm$ 11.56	26.18 $\pm$ 2.15	38.19 $\pm$ 1.77	48.37 $\pm$ 1.31
Triprolidine	20.44 $\pm$ 8.29	18.04 $\pm$ 1.50	25.92 $\pm$ 0.94	43.16 $\pm$ 5.01	48.39 $\pm$ 1.65
Tyramine	32.75 $\pm$ 5.95	43.52 $\pm$ 2.87	59.73 $\pm$ 1.23	74.62 $\pm$ 2.29	86.31 $\pm$ 0.85

Figure 5a. The time course of efflux of noradrenaline from rat brain homogenate following incubation with 10^{-4} M amitriptyline (●), imipramine (■), and tripeleennamine (▲). Each point represents the mean $\pm$ standard error of triplicate determinations.

Figure 5b. The time course of efflux of noradrenaline from rat brain homogenate following incubation with 10^{-4} M promethazine (●), chlorpheniramine (■), and cocaine (▲). Each point represents the mean $\pm$ standard error of triplicate determinations.

Figure 5c. The time course of efflux of noradrenaline from rat brain homogenate following incubation with 10^{-4} M tyramine (●), phenindamine (■), and triprolidine (▲). Each point represents the mean $\pm$ standard error of triplicate determinations.

Figure 5d. The time course of efflux of noradrenaline from rat brain homogenate following incubation with 10^{-4} M nortriptyline (●), desipramine (■), and diphenhydramine (▲). Each point represents the mean $\pm$ standard error of triplicate determinations.

control levels. Equimolar doses of tyramine, the four tricyclic antidepressants, and promethazine were the most efficacious drugs and did not differ significantly from one another ($P < 0.05$) with respect to their effects on release when compared after 10, 20, or 40 minutes of incubation (Figure 6). Similarly, at this dose, cocaine, tripeleennamine, triprolidine, chlorpheniramine and diphenhydramine appeared to be equieffective at incubations of 10 minutes or longer but produced considerably less release than did the first group. Phenindamine showed an activity which was intermediate and significantly different from the other two groups of drugs at both 20 and 40 minutes. Shorter incubations did not demonstrate this differentiation of drug effects on NA efflux.

Effects of Drugs on the Efflux of Noradrenaline: Concentration-Effect Studies

Therefore, from the above experiment, an incubation time of 20 minutes was chosen for the study of drug concentration effects on ^3H -NA release since at this time good resolution between drug effects was obtained, as well as a levelling of the response, approaching maximum efflux. The compounds were all tested in six concentrations from 10^{-8}M to 10^{-3}M . As in the time-response studies, the effects of each drug were compared to a control (which represented only spontaneous efflux after 45 minutes total incubation) and were expressed as "% Release of NA". Table VIII shows the means $\pm$ standard errors for at least three determinations of percent release of ^3H -NA by the

Figure 6. Relative efficacy, in decreasing order, for release of noradrenaline from rat brain homogenate after incubation for 20 minutes with equimolar concentrations of the twelve drugs. Brackets connect those compounds which do not differ significantly from one another.

Nortriptyline	}
Amitriptyline	
Promethazine	
Tyramine	
Imipramine	
Desipramine	
Phenindamine	
Triprolidine	
Chlorpheniramine	
Tripelennamine	
Diphenhydramine	
Cocaine	

Table VIII

The % Release of Noradrenaline after Twenty Minutes Incubation with Varying Concentrations of the Test Drugs. (Each value represents the mean $\pm$ standard error of three determinations.)

Drug	10^{-8} M	10^{-7} M	%Release of Noradrenaline 10^{-6} M	10^{-5} M	10^{-4} M	10^{-3} M
Amitriptyline	4.15 $\pm$ 3.49	11.02 $\pm$ 3.22	16.47 $\pm$ 5.69	25.04 $\pm$ 6.08	79.41 $\pm$ 3.23	107.67 $\pm$ 3.31
Chlorphenir- amine	2.64 $\pm$ 0.88	14.50 $\pm$ 6.97	19.74 $\pm$ 5.94	31.70 $\pm$ 4.70	42.29 $\pm$ 3.93	72.76 $\pm$ 2.44
Cocaine	7.20 $\pm$ 3.66	4.20 $\pm$ 4.48	25.34 $\pm$ 5.71	29.38 $\pm$ 3.36	36.01 $\pm$ 1.91	36.01 $\pm$ 2.06
Desipramine	14.72 $\pm$ 4.03	19.95 $\pm$ 1.95	13.27 $\pm$ 1.32	11.41 $\pm$ 5.54	71.79 $\pm$ 3.17	107.18 $\pm$ 4.66
Diphenhydra- mine	12.70 $\pm$ 4.32	6.89 $\pm$ 2.25	19.43 $\pm$ 1.56	33.42 $\pm$ 6.39	37.80 $\pm$ 2.63	83.38 $\pm$ 5.35
Imipramine	2.96 $\pm$ 4.19	9.38 $\pm$ 2.89	5.26 $\pm$ 3.58	8.83 $\pm$ 4.20	72.86 $\pm$ 3.39	108.95 $\pm$ 3.81
Nortriptyl- ine	13.88 $\pm$ 6.41	12.86 $\pm$ 4.66	22.42 $\pm$ 6.05	15.58 $\pm$ 7.69	81.50 $\pm$ 1.09	108.80 $\pm$ 4.47

(continued)

Table VIII (continued)

Drug	% Release of Noradrenaline					
	10^{-8} M	10^{-7} M	10^{-6} M	10^{-5} M	10^{-4} M	10^{-3} M
Phenindamine	-7.53 ± 4.44	13.73 ± 5.41	7.28 ± 2.04	24.78 ± 1.71	58.31 ± 1.44	109.84 ± 5.24
Promethazine	4.51 ± 2.36	15.37 ± 4.38	9.09 ± 6.35	11.24 ± 1.93	75.97 ± 1.87	103.08 ± 4.52
Tripelennamine	14.03 ± 4.41	25.51 ± 3.99	32.36 ± 4.54	34.31 ± 3.07	38.19 ± 1.77	62.31 ± 5.08
Triprolidine	-0.82 ± 2.02	0.46 ± 0.79	4.12 ± 3.74	9.95 ± 4.28	43.16 ± 5.01	92.52 ± 5.87
Tyramine	4.80 ± 2.39	25.65 ± 5.99	50.23 ± 3.85	69.87 ± 0.74	74.62 ± 2.29	72.32 ± 2.18

twelve compounds at six concentrations. The data for the 10^{-4} M concentration was obtained from the time-response studies (Table VII). Figures 7a, 7b, 7c, and 7d are plots of drug effect (% Release of NA) vs drug concentration in moles per litre of incubation medium. From these graphs it can be seen that tyramine was clearly effective at a dose much lower than that of any of the other test compounds, although the maximum response attained with this drug (approximately 70% release) was less than the peak responses of several of the other drugs (the tri-cyclic antidepressants, promethazine, and phenindamine) which produced essentially 100% release. The remaining compounds also failed to bring about complete release in the concentrations employed, with tripeleennamine and cocaine producing the least effect. Cocaine, in fact, failed to achieve 50% NA release, even at millimolar concentrations.

These results were used to compare the relative potencies of the twelve drugs, by calculating the concentration required to produce a half-maximal effect (EC50, or concentration for 50% effect) on efflux of ^3H -NA for each compound. These values, shown in Table IX, were derived as the mean $\pm$ standard error of the individual EC50 values from each of the three determinations. These results correspond closely to the EC50 values derived from Figures 7a to 7d. The compounds in Table IX are listed according to potency in descending order, tyramine being outstanding as the most potent of the twelve drugs and cocaine showing the least effect. For those compounds connected by brackets, the EC50 values do not differ significantly from one

Figure 7a. The effect of varying concentrations of amitriptyline (●), imipramine (■), and tripeleennamine (▲) on efflux of ³H-noradrenaline from rat brain homogenate after incubation for 20 minutes. Data points represent the means $\pm$ standard errors from three determinations.

Figure 7b. The effect of varying concentrations of promethazine (●), chlorpheniramine (■), and cocaine (▲) on efflux of ^3H -noradrenaline from rat brain homogenate after incubation for 20 minutes. Data points represent the means $\pm$ standard errors from three determinations.

Figure 7c. The effect of varying concentrations of tyramine (●), phenindamine (■), and triprolidine (▲) on efflux of ^3H -noradrenaline from rat brain homogenate after incubation for 20 minutes. Data points represent the means $\pm$ standard errors from three determinations.

Figure 7d. The effect of varying concentrations of nortriptyline (●), desipramine (■), and diphenhydramine (▲), on efflux of ³H-noradrenaline from rat brain homogenate after incubation for 20 minutes. Data points represent the means $\pm$ standard errors from three determinations.

Table IX

The Relative Potencies, in Decreasing Order, for Drugs Producing Efflux of Tritiated Noradrenaline from Rat Brain Homogenate after Incubation for Twenty Minutes

(The concentration of each drug required to produce a half-maximal effect (EC₅₀) is included, shown as the mean $\pm$ standard error of three experiments. Brackets connect those compounds which do not differ significantly from one another.)

Drug	EC ₅₀ (μ M)
Tyramine	1.13 $\pm$ 0.35
Amitriptyline	29.7 $\pm$ 6.1
Nortriptyline	32.9 $\pm$ 4.4
Promethazine	41.0 $\pm$ 2.5
Desipramine	44.0 $\pm$ 5.7
Imipramine	44.5 $\pm$ 6.3
Phenindamine	57.1 $\pm$ 3.3
Tripolidine	146 $\pm$ 31
Chlorpheniramine	184 $\pm$ 50
Diphenhydramine	192 $\pm$ 28
Tripelennamine	381 $\pm$ 131
Cocaine	>1000

another ($P < 0.05$). After tyramine, the next six drugs, shown as being equipotent, are the same as those which produced the maximum releasing effect in the time-response studies. The four remaining antihistamines, besides having a lower potency, were less efficacious.

Effects of Drugs on the Inhibition of Noradrenaline Uptake: Time-Effect Studies

In order to investigate the inhibitory effects of drugs on the uptake or accumulation of NA, it is necessary to preincubate the compound with the rat brain homogenate prior to addition of the tritiated amine to enable the drug to interact with the membrane or amine uptake mechanism. The drugs were tried initially at a concentration of 10^{-4} M as in the studies of release, but this gave almost total inhibition of uptake. Therefore 10^{-6} M was chosen as the dose to be used.

A preincubation time of five minutes had been employed by other researchers (Coyle and Snyder, 1969a; Horn, Coyle and Snyder, 1971; Horn and Snyder, 1972; Horn, 1973) and seemed a suitable time for this experiment. In addition, a preliminary study using 10^{-7} M cocaine preincubated with homogenate for 0, 5, and 25 minutes before adding the NA, showed no difference in the inhibitory effect of cocaine with the three separate preincubation times.

After the ^3H -catecholamine was added to the mixture, incubations were continued for 2, 5, 10, 20, 40, and 60 minutes to demonstrate the effect of time on the ability of the various

drugs to inhibit NA accumulation. Each assay measured the uptake of NA at one particular incubation time and included six of the drugs, a control and a blank, as previously described. After blank ratios were subtracted, sample particle-medium ratios were again compared to the control (which represented 0% inhibition of uptake) and results were expressed as "% Inhibition of NA Uptake" for each of the compounds. As in the release studies, triplicate determinations were carried out and the results are expressed as mean $\pm$ standard error of the mean (Table X).

These values for percent inhibition of uptake were plotted against the various incubation times for each drug as depicted in Figures 8a, 8b, 8c, and 8d. The graphs show that for all the drugs, the inhibition of NA accumulation was greatest within the first ten minutes of incubation, after which the effect decreased gradually and attained a relatively steady state after forty minutes. Comparing responses at forty minutes, (Figure 9) tyramine was demonstrated to have the greatest inhibiting effect, but was not significantly different from tripelennamine which, in turn, did not differ from cocaine ($P < 0.05$). A second group of compounds, consisting of the four tricyclic antidepressants and chlorpheniramine, appeared to be equieffective when tested at the same dose, but were significantly less efficacious than cocaine. Diphenhydramine, phenindamine, triprolidine, and promethazine had the least inhibitory effect of the twelve drugs. Because an incubation time of forty minutes showed a levelling of the response and also because it gave a good sep-

Table X

The % Inhibition of Tritiated Noradrenaline Uptake by 10^{-6} M Drugs at Various Incubation Times. (Each figure represents the mean $\pm$ standard error of three determinations.)

Drug	% Inhibition of Tritiated Noradrenaline Uptake					
	2 min.	5 min.	10 min.	20 min.	40 min.	60 min.
Amitriptyline	48.22 $\pm$ 4.25	29.75 $\pm$ 3.70	41.83 $\pm$ 2.86	32.71 $\pm$ 2.45	28.80 $\pm$ 4.28	28.62 $\pm$ 1.74
Chlorphenir- amine	72.19 $\pm$ 2.79	50.74 $\pm$ 2.89	56.84 $\pm$ 2.36	41.00 $\pm$ 3.91	31.05 $\pm$ 2.68	30.94 $\pm$ 3.97
Cocaine	94.71 $\pm$ 6.29	81.09 $\pm$ 1.66	77.91 $\pm$ 0.90	68.73 $\pm$ 2.31	56.55 $\pm$ 4.01	46.05 $\pm$ 1.34
Desipramine	48.25 $\pm$ 3.22	42.32 $\pm$ 5.97	37.18 $\pm$ 4.93	35.74 $\pm$ 2.65	25.60 $\pm$ 2.44	37.98 $\pm$ 3.23
Diphenhydra- mine	45.32 $\pm$ 4.10	37.49 $\pm$ 6.33	33.74 $\pm$ 2.69	31.79 $\pm$ 3.01	12.83 $\pm$ 1.80	23.69 $\pm$ 4.07
Imipramine	45.58 $\pm$ 8.70	41.42 $\pm$ 5.46	48.50 $\pm$ 4.93	33.40 $\pm$ 2.31	25.18 $\pm$ 6.36	25.57 $\pm$ 5.36
Nortriptyl- ine	57.07 $\pm$ 5.55	37.80 $\pm$ 5.80	40.66 $\pm$ 9.02	42.19 $\pm$ 1.56	29.02 $\pm$ 2.82	39.66 $\pm$ 0.70

(continued)

Table X (continued)

Drug	% Inhibition of Noradrenaline Uptake					
	2 min.	5 min.	10 min.	20 min.	40 min.	60 min.
Phenindamine	37.63 ± 4.66	24.69 ± 1.14	33.05 ± 0.85	27.82 ± 1.46	8.90 ± 3.14	13.59 ± 1.47
Promethazine	3.89 ± 9.73	19.58 ± 3.18	15.22 ± 3.59	18.10 ± 3.80	1.17 ± 4.59	6.79 ± 5.71
Tripelennamine	110.25 ± 5.67	88.65 ± 3.41	89.59 ± 1.10	82.68 ± 0.64	67.98 ± 2.45	64.85 ± 2.86
Triprolidine	20.62 ± 2.02	12.27 ± 5.04	16.22 ± 6.04	4.18 ± 5.11	4.56 ± 3.89	12.34 ± 5.18
Tyramine	100.90 ± 3.52	84.52 ± 5.71	84.62 ± 2.38	77.22 ± 1.96	74.62 ± 1.57	73.86 ± 1.09

Figure 8a. The time course of inhibition of ^3H -noradrenaline uptake into rat brain homogenate by 10^{-6} M tripelennamine (●), amitriptyline (■), and imipramine (▲). Each point represents the mean $\pm$ standard error of triplicate determinations.

Figure 8b. The time course of inhibition of ^3H -noradrenaline uptake into rat brain homogenate by 10^{-6} M cocaine (●), chlorpheniramine (■), and promethazine (▲). Each point represents the mean $\pm$ standard error of triplicate determinations.

Figure 8c. The time course of inhibition of ^3H -noradrenaline uptake into rat brain homogenate by 10^{-6} M tyramine (●), phenindamine (■), and triprolidine (▲). Each point represents the mean $\pm$ standard error of triplicate determinations.

Figure 8d. The time course of inhibition of ^3H -noradrenaline uptake into rat brain homogenate by 10^{-6} M nortriptyline (●), desipramine (■), and diphenhydramine (▲). Each point represents the mean $\pm$ standard error of triplicate determinations.

Figure 9. Relative effectiveness, in decreasing order, of equimolar concentrations of the test compounds for inhibition of uptake of tritiated noradrenaline after forty minutes incubation. (Brackets connect those compounds which do not differ significantly from one another.)

Tyramine	}	}
Tripelennamine		
Cocaine		
Chlorpheniramine	}	
Nortriptyline		
Amitriptyline		
Imipramine		
Desipramine		
Diphenhydramine	}	
Phenindamine		
Triprolidine		
Promethazine		

aration of groups of drugs which produced equivalent effects, this time was chosen for the investigation of the influence of drug concentration on uptake inhibition.

Effects of Drugs on the Inhibition of Noradrenaline Uptake: Concentration-Effect Studies

The twelve drugs were tested in concentrations from 10^{-8} M to 10^{-3} M as in the release experiments. With the five minute preincubation period, P/M ratios for the control tube represent accumulation of $^3\text{H-NA}$ by the synaptosomes after 45 minutes in the absence of an inhibitory influence. Responses to the drugs were therefore expressed relative to this 0% control as "% Inhibition of NA Uptake". A minimum of three determinations at each dose for each drug was made and these results were averaged and expressed in Table XI as the mean $\pm$ standard error. Data for the 10^{-6} M concentration was derived, in part, from the time-response studies of inhibition of NA uptake.

The results are also represented graphically in Figures 10a, 10b, 10c, and 10d by plotting % Inhibition of NA Uptake against Log Drug Concentration (in moles per litre). The graphs show that all the drugs produced essentially 100% inhibition at the highest doses, and demonstrate that tyramine, which was previously shown to be a potent releasing agent, is also an effective inhibitory agent at very low doses. In comparison, both cocaine and tripeleennamine, which had the least effects in producing release of $^3\text{H-NA}$, showed inhibition of NA uptake at doses which were comparable to that of tyramine. In order to

Table XI

The % Inhibition of Tritiated Noradrenaline Accumulation after Forty Minutes Incubation with Varying Doses of the Test Drugs. (Each point represents the mean $\pm$ standard error of a minimum of three determinations.)

Drug	% Inhibition of Noradrenaline Uptake					
	10^{-8} M	10^{-7} M	10^{-6} M	10^{-5} M	10^{-4} M	10^{-3} M
Amitriptyline	6.67 $\pm$ 4.17	7.09 $\pm$ 6.21	28.95 $\pm$ 3.03	59.04 $\pm$ 1.94	107.07 $\pm$ 2.58	103.62 $\pm$ 2.91
Chlorpheniramine	5.88 $\pm$ 5.83	0.97 $\pm$ 2.18	34.03 $\pm$ 3.53	91.65 $\pm$ 2.01	108.27 $\pm$ 2.32	108.38 $\pm$ 2.78
Cocaine	1.88 $\pm$ 8.66	13.73 $\pm$ 3.76	58.98 $\pm$ 3.74	102.23 $\pm$ 3.74	108.20 $\pm$ 2.63	104.59 $\pm$ 2.66
Desipramine	11.01 $\pm$ 1.48	23.87 $\pm$ 4.75	23.21 $\pm$ 2.95	42.84 $\pm$ 4.74	102.60 $\pm$ 3.19	104.47 $\pm$ 1.93
Diphenhydramine	1.46 $\pm$ 4.48	14.01 $\pm$ 5.88	16.65 $\pm$ 4.03	78.50 $\pm$ 0.19	102.16 $\pm$ 1.80	106.91 $\pm$ 3.36
Imipramine	3.35 $\pm$ 6.60	9.75 $\pm$ 5.46	24.51 $\pm$ 4.54	43.48 $\pm$ 7.25	106.47 $\pm$ 1.29	105.92 $\pm$ 3.01
Nortriptyline	0.09 $\pm$ 4.11	18.86 $\pm$ 2.94	26.82 $\pm$ 2.97	66.71 $\pm$ 2.57	106.25 $\pm$ 3.02	104.99 $\pm$ 0.83

(continued)

Table XI (continued)

Drug	% Inhibition of Noradrenaline Uptake					
	10^{-8} M	10^{-7} M	10^{-6} M	10^{-5} M	10^{-4} M	10^{-3} M
Phenindamine	-7.34 ± 6.39	-3.43 ± 2.21	12.10 ± 3.90	75.15 ± 2.37	104.20 ± 2.33	107.04 ± 1.75
Promethazine	-6.99 ± 1.22	-2.36 ± 1.26	2.96 ± 3.71	32.29 ± 2.45	99.64 ± 4.21	102.13 ± 0.32
Tripelennamine	4.31 ± 5.80	11.12 ± 4.75	65.07 ± 3.38	98.28 ± 1.95	105.23 ± 1.96	106.41 ± 3.57
Triprolidine	3.35 ± 3.11	-1.37 ± 7.73	4.56 ± 2.75	40.45 ± 1.86	89.94 ± 2.33	106.06 ± 2.54
Tyramine	9.56 ± 5.63	10.17 ± 4.56	74.85 ± 1.13	105.55 ± 4.09	109.12 ± 2.81	107.11 ± 2.60

Figure 10a. The effect of varying concentrations of tripelenamine (●), amitriptyline (■), and imipramine (▲) on inhibition of uptake of ^3H -noradrenaline into rat brain homogenate after 40 minutes incubation. Each point represents the mean $\pm$ standard error of at least three determinations.

Figure 10 b. The effect of varying concentrations of cocaine (●), chlorpheniramine₃ (■), and promethazine (▲) on inhibition of uptake of ³H-noradrenaline into rat brain homogenate after 40 minutes incubation. Each point represents the mean $\pm$ standard error of at least three determinations.

Figure 10c. The effect of varying concentrations of tyramine (●), phenindamine (■), and triprolidine (▲) on inhibition of uptake of ^3H -noradrenaline into rat brain homogenate after 40 minutes incubation. Each point represents the mean $\pm$ standard error of at least three determinations.

Figure 10d. The effect of varying concentrations of diphenhydramine (●), nortriptyline (■), and desipramine (▲) on inhibition of uptake of ^3H -noradrenaline into rat brain homogenate after 40 minutes incubation. Each point represents the mean $\pm$ standard error of at least three determinations.

compare the relative potencies of the twelve drugs, a statistical analysis (using the t-test for unpaired data) was made of the concentrations necessary to produce 50% inhibition of uptake (IC50), derived from the individual experiments. The compounds, listed in decreasing order according to relative potency, as well as their respective IC50 values are given in Table XII. As in the comparison of potencies derived from the time-effect study of uptake inhibition, the comparison of IC50 values shows tyramine to be the most potent inhibitor but not differing significantly from tripeleennamine ($P < 0.05$). Similarly, the potencies of tripeleennamine and cocaine were not significantly different. Promethazine appeared to be the least potent of all drugs tested in inhibiting the uptake of the tritiated amine. However, the compounds did not seem to fall into any definite groups according to therapeutic classification with respect to their relative inhibitory potencies.

Finally, a comparison of potencies of the twelve drugs with regard to both NA efflux as well as uptake inhibition is given in Table XIII in order to more easily contrast the two effects for each drug. In all cases, inhibition of accumulation is produced at a lower dose than is release of NA, although tyramine shows the least disparity in this respect since its IC50 is only approximately one-half of the EC50. For tripeleennamine and cocaine, the differences between potencies for NA release and inhibition of uptake are the greatest.

Table XII

Relative Potencies, in Decreasing Order, for Drugs Producing Inhibition of Uptake of Tritiated Noradrenaline after Forty Minutes Incubation

(The concentration of each drug required to produce a half-maximal inhibition (IC₅₀) is also given, representing the mean $\pm$ standard error of three experiments. Brackets connect those drugs which do not differ significantly from one another.)

Drug	EC ₅₀ (μ M)
Tyramine	0.405 $\pm$ 0.016
Tripelennamine	0.546 $\pm$ 0.070
Cocaine	0.571 $\pm$ 0.038
Chlorpheniramine	1.75 $\pm$ 0.18
Diphenhydramine	3.29 $\pm$ 0.28
Nortriptyline	3.62 $\pm$ 0.26
Phenindamine	3.85 $\pm$ 0.37
Amitriptyline	4.93 $\pm$ 0.04
Desipramine	11.3 $\pm$ 1.9
Imipramine	15.6 $\pm$ 1.0
Triprolidine	15.7 $\pm$ 1.3
Promethazine	18.3 $\pm$ 0.5

Table XIII

A Comparison of Drug Potencies in Decreasing Order for Effects on Both Efflux of Noradrenaline and Inhibition of Uptake of the Catecholamine

(a composite of Tables IX and XII)

Release		Uptake Inhibition	
EC50 (μ M)	Drug	Drug	IC50 (μ M)
1.13 $\pm$ 0.35	Tyramine	Tyramine	0.405 $\pm$ 0.016
29.7 $\pm$ 6.1	Amitriptyline	Tripelennamine	0.546 $\pm$ 0.070
32.9 $\pm$ 4.4	Nortriptyline	Cocaine	0.571 $\pm$ 0.038
41.0 $\pm$ 2.5	Promethazine	Chlorpheniramine	1.75 $\pm$ 0.18
44.0 $\pm$ 5.7	Desipramine	Diphenhydramine	3.29 $\pm$ 0.28
44.5 $\pm$ 6.3	Imipramine	Nortriptyline	3.62 $\pm$ 0.26
57.1 $\pm$ 3.3	Phenindamine	Phenindamine	3.85 $\pm$ 0.37
146 $\pm$ 31	Tripolidine	Amitriptyline	4.93 $\pm$ 0.04
184 $\pm$ 50	Chlorpheniramine	Desipramine	11.3 $\pm$ 1.9
192 $\pm$ 28	Diphenhydramine	Imipramine	15.6 $\pm$ 1.0
381 $\pm$ 131	Tripelennamine	Tripolidine	15.7 $\pm$ 1.3
>1000	Cocaine	Promethazine	18.3 $\pm$ 0.5

A Correlation of Drug Effects with Lipid Solubilities of the Compounds

In order to determine whether a correlation exists between the inhibition of NA uptake and/or release of tritiated amine and the lipid solubility of the drug molecule, octanol-water partition coefficients for each compound were calculated first, according to the methods of Leo et al. (1971) and are summarized in Table XIV. Graphs were then plotted with either the logarithm of the EC50 (Figure 11) or the logarithm of the IC50 (Figure 12) on the ordinate against the logarithm of the octanol/water partition coefficient on the abscissa.

In Figure 11, nine of the twelve compounds gave points which yielded a linear relationship between the two parameters ($r = -0.936$) and the slope of the resulting line was calculated to be -0.531 . The value for cocaine could not be plotted since this drug did not achieve 50% NA release; the value for phenindamine fell to the right of the line, indicating that this compound had a lower potency than would be expected based on lipid solubility alone; and tyramine showed the opposite effect since this point fell far to the left of the line, suggesting that tyramine's pronounced potency was in no way connected to its lipid solubility.

With respect to the inhibition of NA uptake (Figure 12), the linear correlation between potency and lipid solubility was not as good ($r = -0.624$) and more of the points were scattered at a distance from the computed line, although the slope (-0.535) was very close to that of Figure 11. Points for only

Table XIV

Logarithms of the Octanol/Water Partition Coefficients for the Twelve Drugs

(The partition coefficients, derived according to methods outlined by Leo et al. (1971), are shown $\pm$ "uncertainty units" which are analogous to standard deviations. Superscripts are explained following the table, giving the derivations of individual log P values.)

Drug	Log P
Amitriptyline	4.92 ± 0.02^a
Chlorpheniramine	3.88 ± 0.13^b
Cocaine	2.55 ± 0.09^c
Desipramine	4.28 ± 0.02^a
Diphenhydramine	3.34 ± 0.02^a
Imipramine	4.62 ± 0.02^a
Nortriptyline	4.60 ± 0.06^d
Phenindamine	6.12 ± 0.47^e
Promethazine	4.35 ± 0.04^f
Tripelennamine	2.59 ± 0.08^g
Triprolidine	3.92 ± 0.02^a
Tyramine	0.74 ± 0.06^h

a. experimental value, listed in Table XVII of Leo et al. (1971)

(continued)

Table XIV (continued)

- b. $\log P$ (pyridine) + $\log P$ (6-phenylpropyldimethylamine) +
 $[\log P$ (chlorobenzene) - $\log P$ (benzene)] + π (branch)
 $= 0.65 + 2.73 + 2.84 - 2.14 + (-0.20) = 3.88$
- c. $\log P$ (atropine) - $[\log P$ ($C_6H_5CH_2CH_2OH$) + π (branch)] +
 $\log P$ (benzene) + $\log P$ (acetic acid) = $1.81 - (1.36 -$
 $0.20) + 2.14 - 0.24 = 2.55$
- d. $\log P$ (amitriptyline) - $[\pi$ (methyl) + π (branch)] = $4.92 -$
 $(0.52 - 0.20) = 4.60$
- e. $\log P$ (carbazole) - π (2 double bonds) + $\log P$ (benzene) +
 $[\log P$ (N-methylpiperidine) - $\log P$ (piperidine)] =
 $3.29 - 2(-0.30) + 2.14 + (0.94 - 0.85) = 6.12$
- f. $\log P$ (promazine) + π (branch) = $4.55 + (-0.20) = 4.35$
- g. $\log P$ (N,N,N',N'-tetramethylethylenediamine) - π (methyl) +
 $\log P$ (benzene) + $\log P$ (pyridine) = $0.30 - 0.50 +$
 $2.14 + 0.65 = 2.59$
- h. $\log P$ (2-phenylethylamine) + $[\log P$ (phenol) - $\log P$ (benz-
ene)] = $1.41 + 1.47 - 2.14 = 0.74$

Figure 11. The relationship between drug potency with respect to effects on efflux of ^3H -noradrenaline (EC₅₀) and the lipid solubility of the compound (calculated as the logarithm of the octanol/water partition coefficient). The correlation coefficient for the curve was -0.936.

Figure 12. The relationship between drug potency with respect to inhibitory effects on ³H-noradrenaline uptake (IC₅₀) and the lipid solubility of the compound (calculated as the logarithm of the octanol/water partition coefficient). The correlation coefficient for this curve was -0.624.

six of the drugs (promethazine, triprolidine, and the four tricyclic antidepressants) were used in the calculations. Again, phenindamine seemed to exhibit a lower potency than its partition coefficient would predict whereas the potent effect of tyramine did not reflect its low lipid solubility. In contrast to their effects in releasing NA, both tripelennamine and cocaine gave points which fell far to the left of the calculated line, indicating that these compounds were acting to inhibit NA uptake through a mechanism (possibly the same as for tyramine) which did not depend on lipid solubility. Correlations between inhibitory potency and lipid solubility for diphenhydramine and chlorpheniramine also appeared to deviate from the calculated linear relationship; however the discrepancy was not as great as with cocaine, tyramine, or tripelennamine, possibly due to a complex mode of action.

DISCUSSION

In an attempt to differentiate between drug effects on blockade of NA uptake and release of the catecholamine, the results of this study have shown that although many substances appear to affect both processes there are significant dose-dependent differences in their activities. This observation, along with the correlation of potency with partition coefficient for some of the drugs, would imply that separation of drug effects on NA uptake inhibition and NA release can be achieved but that their actions cannot be explained by only one mechanism. These results also confirmed the observations of many other researchers that tyramine is extremely effective both in inhibiting neuronal uptake and in producing release of NA from nerve endings.

The initial studies of ^3H -NA uptake in the absence of drugs show that the time course of this uptake compares well with that demonstrated by Snyder and Coyle (1969) in which the rate of uptake declined rapidly after ten minutes incubation time. However, in their experiment, the P/M ratios did not reach a maximum after twenty minutes but instead showed an increase up to forty minutes. Our results could be explained by the observation that control ratios in the early experiments also declined as the incubation time increased beyond twenty minutes, correlating with the age of the prepared homogenate. This occurred because the assay techniques had not yet been perfected; when it was discovered that the activity of the homo-

genate decreased during the day even though it was stored in an ice-bath, the procedure was changed so that a fresh homogenate was prepared immediately before each incubation. When this was done, the P/M ratios did not reach a maximum but instead continued to increase beyond the twenty minute incubation time.

The actual P/M ratios which we obtained were also comparable to those of Snyder and Coyle (1969) although these authors showed that there were marked regional differences in this respect, with ratios in striatal tissue being approximately ten-fold higher than those in other brain areas. Since whole brain homogenate was used in our study, only a general comparison can be made. As in the kinetic study, we also showed that P/M ratios for ^3H -NA uptake decreased with increasing amine concentration, indicating a saturation of the uptake process. Initially, only 0°C -zero-time blanks and zero-tissue blanks were used. As in the study by Snyder and Coyle, at zero time the P/M ratios always exceeded unity, indicating a significant uptake of amine in the cold. Later, these two blanks were substituted by a single boiled homogenate blank since this would better represent non-specific binding than using a zero-tissue blank. As well, it would replace the 0° -zero-time blank since the boiled homogenate blank gave consistently higher P/M ratios.

Once the time course for ^3H -NA uptake had been established for the present system, the effects of the various drugs on ^3H -NA efflux showed that all drugs tested produced an increased release with time as compared to the respective control levels.

That the apparent release produced by these drugs is due simply to inhibition of uptake is unlikely since a drug such as cocaine, which is regarded as a potent inhibitor of neuronal uptake, showed the least release. However, it is obvious that both release of NA and inhibition of amine uptake may be affected simultaneously by some of the drugs (since there is no known way of inactivating one process completely without interfering with the other) and therefore it is impossible to assess the exact contribution of uptake blockade to the measured effect on NA release.

Concentration-effect studies on release illustrated that tyramine was effective at producing NA release at a much lower dose than for any of the other drugs. The apparent levelling of this curve with high doses of tyramine could be explained by conversion of tyramine to octopamine which could replace some of the noradrenaline normally released or, alternatively, by competition between tyramine with the ^3H -NA for postulated carrier sites for efflux, causing a decline in efflux of the catecholamine as suggested by Paton (1973). On the other hand, inhibition of NA uptake alone could not explain this decline since a reduction in ^3H -NA accumulation by this process would be manifested as an increase rather than a decrease in apparent efflux.

Tyramine was the only drug studied which displayed a very potent releasing ability but an extremely low octanol/water partition coefficient (Figure 11). The lack of relationship between these two properties of tyramine suggests that this substance acts specifically on the amine uptake process of the

neuronal membrane through its structural resemblance to NA, rather than depending on its lipid solubility. This is consistent with results from other experiments which have shown that tyramine, as well as being a potent inhibitor of the NA uptake system, is also accumulated by a mechanism which is sensitive to both cocaine and ouabain (Iversen, 1971b). In addition, tyramine appears to be taken up by intracellular storage particles, from which it stoichiometrically displaces endogenous catecholamine. This has been demonstrated in the adrenal medulla (Schumann and Philippu, 1962) and it is believed that particles in adrenergic neurons behave similarly. Tyramine also inhibits NA uptake into the medullary storage particles (Carlsson et al., 1963).

Neither cocaine nor tripeleennamine demonstrated any appreciable release of ^3H -NA, even at the highest doses. Similar results with cocaine were observed by Paton (1973) who interpreted them as being possibly due to a concomitant blockade of both the neuronal uptake process as well as the efflux system, so that no appreciable net release would occur. However, Paton does not offer an explanation of how cocaine (an inhibitor of NA uptake which does not appear to act as a substrate for the uptake process and therefore should not be accumulated in the nerve ending) exerts its effect on efflux, although he rules out a non-specific local anesthetic effect. In support of Paton's findings, Azarro and coworkers (1974) also provided evidence that cocaine did not inhibit spontaneous release of ^3H -NA although it inhibited amphetamine-induced release. They

suggested that cocaine may be inhibiting amphetamine-induced release by competing with amphetamine for intra-neuronal binding sites of ^3H -NA. This hypothesis was based on their evidence that cocaine was equipotent with amphetamine in effecting ^3H -NA release; a careful examination of their data, however, reveals that the two drugs are not-equipotent, but rather appear to have the same affinities for sites of efflux (since equal concentrations of the two drugs produced half of their respective maximum effects).

In 1961, Hertting, Axelrod and Patrick showed that cocaine blocked the uptake of ^3H -NA but did not release NA in the rat heart. Conversely, evidence that part of the ability of cocaine to potentiate NA occurs through release of the transmitter has been presented by Haefely, Hürlimann, and Thoenen (1964) and Trendelenburg (1968). In the former case, this conclusion was the result of the demonstration that previous treatment with reserpine greatly reduced the sympathomimetic effect of cocaine. However this result could just as easily be explained by the ability of cocaine to inhibit NA uptake. Trendelenburg (1968) expressed similar findings and also demonstrated an augmented releasing effect by cocaine when pargyline was employed to inhibit MAO. Davis and McNeill (1973) showed an increased efflux of ^3H -NA from guinea-pig atria by cocaine which was associated with a positive inotropic effect, although this efflux was considerably less than that produced by tyramine. They therefore suggested that release of the catecholamine may be contributing to the pharmacological effects of cocaine.

Cocaine was the only drug of the twelve tested in our study which could not be included in Figure 11 since the maximum release achieved by this compound, at a concentration of 10^{-3} M (the highest dose studied), did not reach 50% release of NA and therefore an EC50 value could not be calculated. Nevertheless, it appears that cocaine would not deviate greatly from the determined relationship between potency and lipophilicity because this drug possesses both a relatively low lipid solubility (only tyramine had a lower value) and a weak capacity to produce release. In contrast, a nonspecific depression of neurotransmitter release was deemed unlikely by Westfall and Brasted (1974) because cocaine and four other agents did not uniformly depress NA release by four agonists.

The failure of high doses of cocaine to show an appreciable release of ^3H -NA is interesting. If cocaine were inhibiting uptake of spontaneously-released catecholamine in a competitive manner, it might be expected to displace NA from uptake sites and reduce intracellular accumulation of the amine. Because of the experimental design used, this might be interpreted as increased NA efflux, which was observed to a limited degree. This is possible since the competitive nature of cocaine's inhibition of uptake has been shown (Iversen, 1963). An alternative explanation is that inhibition of NA uptake by cocaine might result in presynaptic regulation of release in the manner proposed by Langer (1974), thus limiting the extent of apparent release. If this were so, it may explain some discrepancies in the results of our experiments and studies employ-

ing a constant perfusion technique. In this latter case, if a neuronal uptake blocker were present, released NA would be washed away from the synaptic cleft, preventing its action on presynaptic inhibitory receptors, resulting in an increased efflux.

Desipramine has also been purported to have the ability to release NA when present in high concentrations, and, as Table IX shows, the four triptyline compounds tested in our experiments also produced release of ^3H -NA but, although equipotent, displayed a lower potency for this action than did tyramine. Figure 11 indicates that this releasing effect may be partially the result of a nonspecific action of these lipid-soluble compounds on cell membranes.

Brodie and coworkers (1968) provided evidence that desipramine, in relatively large doses, does not block cardiac accumulation of high doses of tyramine or of amphetamine, yet antagonizes the depletion of heart NA elicited by the two amines. The authors infer that the antagonism of the releasing effect on sympathomimetic amines by desipramine is due to an action within the nerve terminal, possibly on the synaptic vesicles. However, the failure to block tyramine uptake may be a dose-dependent, competitive phenomenon. The ability of desipramine to prevent the amine-induced release may be a result of its blocking effect on the granule uptake process which also accumulates tyramine (Iversen, 1971b). Iversen has suggested that the actions of indirectly-acting amines seem to be related to displacement of NA from storage particles. Leitz and Stefano

(1970) demonstrated that the released ^3H -NA was mainly in the form of deaminated metabolites indicating that desipramine exerts an effect on the amine storage granule to deplete the NA stores. At low doses of the drug, only inhibition of uptake of ^3H -NA was seen. Again, this could be explained by the inhibition by desipramine of the granule amine uptake process (rather than true release), which would prevent the NA from being protected against intraneuronal metabolism. This seems to correlate well with the observation that higher doses of desipramine are required to inhibit the particle uptake system as compared to the neuronal uptake process (Iversen, 1971b).

Titus and coworkers (1966) had also observed that very high levels of desipramine produced an appreciable release of ^3H -NA. They commented that this drug may have an effect on the storage granules, but also suggested that because many of the compounds which blocked catecholamine uptake were of diverse structures, were lipid soluble, and showed a multiplicity of effects on membranes, it is unlikely that these drugs would act on a specific carrier for NA. Thus the releasing effects of such drugs in high doses may likewise be partly due to nonspecific membrane effects.

Except for promethazine and possibly phenindamine, which seemed to have a NA releasing action equivalent to the tricyclic antidepressants, the remaining antihistamine compounds displayed a low potency for causing efflux of ^3H -NA. In addition, the antihistaminic compounds showed a close correlation between potency and lipophilicity with respect to release of

^3H -NA, again suggesting that a nonspecific mechanism of action may be responsible for the releasing effects of high doses of these drugs. The only exception is phenindamine, whose high partition coefficient did not correspond to its only moderate effect on efflux. This may be the result of an excessive affinity for the lipid portions of the membrane, sequestering the molecule and thus preventing its mobilization to possible sites of action.

Similar observations were made by Isaac and Goth (1965) in an in vivo study in rat hearts which indicated that none of the antihistamines tested (chlorpheniramine, phenindamine, promethazine, pyrilamine, and tripeleennamine) caused release of ^3H -NA. However, Davis and McNeill (1973) found that chlorpheniramine, triprolidine, and tripeleennamine produced an increased efflux of ^3H -NA from isolated guinea pig atria in conjunction with a positive inotropic effect whereas promethazine did not. These results do not agree with those of the present study.

In experiments on the inhibition of ^3H -NA uptake produced by the drugs, the time course of inhibition showed an initial peak in the first ten minutes of incubation (with slight fluctuations) followed by a gradual decline in the degree of blockade to an approximate steady-state level after forty minutes incubation time. The decrease in effect with time is probably due to competition between the NA and the drug, the extent depending on the relative affinities of each for the membrane carrier. The catecholamine would displace compounds with lower affinities until an equilibrium situation is reached. There-

fore, the initial degree of inhibition attained by each agent should represent the relative affinity of that compound for the uptake site in the absence of a significant interference by NA, whereas the level portion of the curve reflects the equilibrium between the drug and the tritiated amine.

The concentration-effect studies of NA uptake blockade after forty minutes incubation showed that all drugs studied produced an inhibition of accumulation of ^3H -NA, with essentially 100% blockade occurring when a maximum dose of 10^{-3} M was used. In contrast, the experiments of Isaac and Goth (1965, 1967) found that promethazine did not inhibit uptake significantly as compared to saline although diphenhydramine, tripelennamine, chlorpheniramine and phenindamine were effective inhibitors. However, in these studies, only a single dose of drug was used, consisting of 10 mg/kg promethazine administered intraperitoneally in in vivo studies (Isaac and Goth, 1965) and 1×10^{-6} M promethazine in the in vitro experiments (Isaac and Goth, 1967). Because it was the least potent of the twelve drugs, and this latter dose of promethazine would have shown less than 5% uptake inhibition in our investigation, it indicates that the absence of an inhibitory effect as well as a potentiating effect by promethazine may be due to an insufficient concentration of the drug. The remaining antihistamines showed a considerable inhibition of uptake in our experiments in the doses employed by Isaac and Goth (1967), corresponding to their observations.

The findings of Davis and McNeill (1973) show a greater

contrast with those of the present study. These authors found that desipramine appeared to be the most potent inhibitor of ^3H -NA uptake in isolated guinea-pig atria, producing 83% inhibition at a dose of 1×10^{-6} M. In comparison, desipramine showed only about 25% blockade of uptake at this concentration according to Figure 10d. Similarly, 3×10^{-5} M promethazine and tripeleennamine, which produced 31% and 62% inhibition of uptake respectively according to Davis and McNeill, appear to produce a much greater response in the present study as reflected in Figures 10b and 10a. These conflicting results could be explained by a number of different factors such as species and/or tissue variations (which will be discussed in more detail later), as well as differences between perfusion and incubation techniques.

Another contributing factor may be the presence of an MAO inhibitor in the incubation medium at a concentration of 1.6×10^{-4} M. Iversen (1965b) observed that of seven MAO inhibitors tested, three possessed the ability to also inhibit NA uptake in rat heart. Pargyline, which was used in our experiments, displayed no blockade of uptake at a dose of 10^{-5} M; however no other concentrations were tested to examine the effects of higher doses of the drug. Hendley and Snyder (1968) demonstrated that, in rat cortex, pargyline was moderately effective as an inhibitor of uptake of metaraminol, a phenethylamine derivative which is resistant to MAO and COMT but has a high affinity for the neuronal amine uptake system. The ID₅₀ value for uptake inhibition by pargyline was found to be 1.2×10^{-4} M,

a concentration less than that used in this study, suggesting that this MAO inhibitor may influence the measured effects of other drugs on the uptake mechanism.

Again, the releasing effects of the various drugs cannot be disregarded when comparing the compounds as inhibitors of uptake. Because of the methods employed, any substance which produces considerable efflux of NA would also appear to have a greater blocking action than actually occurs, since both effects are manifested by decreased accumulation of ^3H -NA. However, the influence of release does not appear to be important since both cocaine and tripeleennamine, neither of which produced any appreciable efflux, were highly effective inhibitors of NA uptake. In contrast, the triptyline compounds were fairly potent releasing agents but were considerably less effective than cocaine, tyramine or tripeleennamine with respect to uptake inhibition. In addition, all the compounds were more potent as inhibitors of uptake than as releasing agents, a fact which also tends to dissociate effects on efflux from effects on uptake blockade.

Table XV shows IC₅₀ values for some of the test drugs as inhibitors of catecholamine uptake, obtained by other investigators. In general, the values obtained in our study agree very well with those reported in the literature (although tripeleennamine and triprolidine could not be compared since corresponding figures for catecholamine uptake inhibition in the literature could not be found). Only in the case of the four triptyline compounds did our results show some deviation from

Table XV

Inhibition of Catecholamine Uptake by the Test Drugs in Various Tissues and Species

(Concentrations for half-maximal inhibition (IC₅₀) are given.)

Drug	IC ₅₀ (μM)	Transmitter	Species	Tissue	Reference
Amitriptyline	0.11	NA	Rat	Heart	a, b
	0.055	NA	Rat	Hypothalamus	c
	4.0	DA	Rat	Striatum	c
Chlorpheniramine	2.0	NA	Rat	Hypothalamus	d
	1.2	NA	Rat	Hypothalamus	c
	1.6	DA	Rat	Striatum	c
	2.5	DA	Rat	Striatum	d
Cocaine	0.38	NA	Rat	Heart	e, f
	0.8	NA	Rat	Cortex	g
	2.0	NA	Rabbit	Heart	b
	2.0	NA	Rabbit	Striatum	h
	1.0	NA	Mouse	Cortex	h
	0.47	NA	Mouse	Whole brain	i
Desipramine	0.007	NA	Rat	Heart	a
	0.013	NA	Rat	Heart	e, f
	0.050	NA	Rat	Hypothalamus	c, d
	0.4	NA	Rat	Cortex	g
	50	DA	Rat	Striatum	c, d, j
	0.03	NA	Rabbit	Heart	b
	50	NA	Rabbit	Striatum	h
	0.03	NA	Mouse	Cortex	h
	0.006	NA	Mouse	Whole brain	i

(continued)

Table XV (continued)

Drug	IC50 (μ M)	Transmitter	Species	Tissue	Reference
Diphenhydramine	2.70	NA	Rat	Hypothalamus	c
	4.2	NA	Rat	Hypothalamus	d
	4.6	DA	Rat	Striatum	d
	3.49	DA	Rat	Striatum	c
Imipramine	0.04	NA	Rat	Heart	a,b
	0.09	NA	Rat	Heart	e,f
	1.00	NA	Rat	Hypothalamus	c
	8.00	DA	Rat	Striatum	c
	20.0	NA	Rabbit	Striatum	h
	0.20	NA	Mouse	Cortex	h
Nortriptyline	0.02	NA	Rat	Heart	a,b
	1.300	NA	Rat	Hypothalamus	c
	5.49	DA	Rat	Striatum	c
Phenindamine	8.0	NA	Rat	Hypothalamus	c
	4.0	NA	Rat	Hypothalamus	d
	4.5	NA	Rat	Hypothalamus	j
	4.8	DA	Rat	Striatum	d,j
Promethazine	5.01	NA	Rat	Hypothalamus	d
	17.86	NA	Rat	Hypothalamus	c
	26.32	DA	Rat	Striatum	c
	15.0	DA	Rat	Striatum	d
Tyramine	0.45	NA	Rat	Heart	k
	1.0	NA	Rat	Hypothalamus	l
	0.54	DA	Rat	Striatum	l

(continued)

Table XV (continued)

References:

- a. Callingham, 1966
- b. Berti and Shore, 1967
- c. Horn, Coyle and Snyder, 1971
- d. Snyder, 1970
- e. Iversen, 1965b
- f. Iversen, 1967
- g. Azzaro et al., 1974
- h. Ross and Renyi, 1967
- i. Carmichael and Israel, 1973
- j. Coyle and Snyder, 1969a
- k. Burgen and Iversen, 1965
- l. Horn, 1973

reported IC₅₀ values, especially those obtained in heart, cortical, and hypothalamic tissues. However, these tricyclic antidepressants appear to be the only compounds studied which show a discrepancy in uptake inhibitory potencies between striatal and heart or hypothalamic tissues, with inhibition of striatal uptake requiring a considerably higher concentration than uptake blockade in the other tissues. This is most pronounced for desipramine, which shows a thousand-fold difference between the two doses, indicating significant tissue differences between the two brain regions. The IC₅₀ values for these drugs obtained in the present study correlate more closely with those reported for inhibition of catecholamine uptake in the striatum. This may have been due to the use of whole brain homogenates in our study, so that the measured effect would be a composite of the various regions.

Since the IC₅₀ values for triptyline compounds obtained in our whole brain homogenate approximated uptake inhibitory concentrations for striatal tissue although this brain region

represents only a small proportion of the total tissue, it may be that there are far more uptake binding sites per unit weight in the striatum. This could also be a possible explanation for results obtained by Snyder and Coyle (1969) who observed that P/M ratios for ^3H -NA uptake in the striatum were approximately tenfold higher than those in the hypothalamus and other brain regions, even though ^3H -NA demonstrated a higher affinity for the catecholamine uptake system in nonstriatal areas. However it may also be possible that there is a high degree of nonspecific binding of desipramine and related compounds in striatal tissue, to explain the greater concentration of drug required for uptake inhibition. In support of this, Horn, Coyle and Snyder (1971) showed through kinetic analyses that inhibition by the tricyclic compounds was competitive in the hypothalamus but noncompetitive in the corpus striatum. Failure to show competitive inhibition indicates that the inhibitory substance is probably acting at a site separate from that occupied by the substrate, suggesting that nonspecific binding could be involved. In accordance with this, Mundo et al. (1974) demonstrated that the accumulation of tritiated triptyline compounds into rat atrial tissue appeared to be a passive and unsaturable process for all the tested drugs. They also observed that drug tissue concentrations correlated with ^3H -NA uptake inhibition but not with potentiation of the chronotropic response to NA.

Another factor to be considered is that in all cases but one (Ross and Renyi, 1967) uptake into the striatum was measured with ^3H -dopamine rather than ^3H -NA. Since the two cate-

cholamines differ in their relative affinities for the amine uptake mechanism (Snyder and Coyle, 1969) with dopamine accumulation exceeding that of NA in all brain areas, care must be taken in making a comparison of inhibitory potencies of various drugs in the two brain regions. One would expect a higher concentration of drug to be required to inhibit accumulation of a transmitter displaying a greater affinity.

It is now apparent that there are marked tissue differences in the neuronal amine uptake systems, especially in the various brain regions (Snyder and Coyle, 1969). However, the properties of the system in NA-containing neurones of the central nervous system (primarily the hypothalamus) seem to be indistinguishable from those in the peripheral sympathetic neurone (Iversen, 1971b). Squires (1974) has observed the inhibition of uptake in several regions of rat brain by tricyclic compounds and has presented evidence that even the cerebral cortex does not appear to be uniform but rather, exhibits areas such as the frontal cortex with a high density of dopamine terminals (see Table XVI). As mentioned before, Horn, Coyle and Snyder (1971) found that a large number of centrally-acting drugs were competitive inhibitors of catecholamine uptake into hypothalamic synaptosomes but were noncompetitive inhibitors in the corpus striatum.

In addition to regional or tissue variations in properties of the uptake system, there also seems to be a quantitative species variation as shown in Tables XVII and XVIII (Iversen, 1971b) although the general properties appear to be similar in most species. However, Iversen states that the NA uptake sys-

Table XVI

Inhibition of Noradrenaline Uptake into Synaptosomes Prepared
from Several Brain Regions

(Concentrations for half-maximal inhibition (IC₅₀) are given.
The table is adapted from Squires, 1974.)

Inhibitor	IC ₅₀ (uM)	Tissue
Desipramine	0.001	Occipital Cortex
	10	Frontal Cortex
	0.001	Hippocampus
	10	Whole Forebrain
Imipramine	0.03	Occipital Cortex
	2.5	Frontal Cortex
	0.006	Hippocampus
	10	Whole Forebrain
Amitriptyline	0.05	Occipital Cortex
	2.5	Frontal Cortex
	0.03	Hippocampus
	3.0	Whole Forebrain

tem in guinea pig and rabbit seems to lack stereochemical specificity which suggests that some qualitative species differences may exist.

To explain the discrepancies between the results of this study and those of other authors, more than just tissue and species variation may be involved. The measurement of receptor response in evaluating the relative potencies of various inhibitors could be misleading if the drugs also produced post-synaptic effects. For example, cocaine has been shown to po-

Table XVII

Species Differences in Catecholamine Uptake in the Perfused
Hearts of Various Vertebrates (Iversen, 1971b)

Species	ID50 (μ M)	
	(-)-NA	(-)-Adrenaline
Rat	0.28	1.02
Mouse	0.65	1.08
Guinea Pig	0.98	2.72
Pigeon	1.15	2.96
Toad (<i>Bufo marinus</i>)	2.34	0.96

(The ID50 is the concentration required to produce 50% inhibition of uptake of (+)-³H-NA and is approximately equal to the K_i .)

Table XVIII

Affinity Constants for (+)-NA Uptake by Cerebral Cortex of
Various Species (Iversen, 1971b)

Species	Km (+)-NA (μ M)
Mouse	0.40
Rat	0.40
Monkey	0.40
Cat	0.75
Guinea Pig	1.10

tentiate the responses to NA in systems (such as denervated tissues) where amine uptake cannot occur, implying that cocaine has some direct effects as well as producing potentiation through inhibition of uptake (Reiffenstein, 1968; Nakatsu and Reiffenstein, 1968; Maxwell et al., 1966; Reiffenstein and Triggle, 1974). It has been suggested that the post-synaptic actions of cocaine may be due to an allosteric conformational change near the receptor to possibly alter the intrinsic activity of the receptor or the agonist-receptor binding (Reiffenstein and Triggle, 1974). Therefore this action could account for part of the potentiation produced by drugs in poorly or non-innervated tissues. Conversely, in more densely innervated tissues, where the uptake system has been shown to play an important role in terminating the responses to NA, inhibition of this uptake and the degree of potentiation may be more closely correlated.

As noted in the Results section, the correlation between inhibitory potency and lipid solubilities of the tested compounds was not as well-defined as for the releasing effects. This may reflect multiple sites or modes of action of many of these drugs. Drugs such as cocaine, tyramine, or tripeleptamine, which do not show a correlation between potency and solubility, may inhibit uptake through a specific interaction with the transport process. In fact, cocaine and tyramine have been shown to act as competitive uptake inhibitors (Iversen, 1963; Iversen, 1971b).

The inhibitory activity of the tricyclic antidepressants

relates more closely to their lipid solubilities, which may imply a nonspecific rather than a competitive effect, in contrast to the mechanism described by Iversen, (1971b). But since the NA uptake inhibition produced by these compounds in our study resembles their action in the striatum where they have been shown to act noncompetitively (Horn, Coyle, and Snyder, 1971), this could explain their apparent nonspecific effect.

The antihistamines which, like cocaine, were included by Seeman (1972) in his general classification of anesthetics, did not seem to fit the relationship shown in Figure 12. Only promethazine and triprolidine fell close to the computed curve. Thus other factors besides nonspecific membrane effects may contribute to the measured inhibitory response.

Structurally nonspecific drugs may exert their action as a result of such physicochemical properties as degree of ionization, solubility, thermodynamic activity and surface tension effects rather than through an interaction with a specific receptor (Korolkovas, 1970). For most drugs which lack a structural resemblance to actively transported compounds, their rate of passage across lipoprotein cellular membranes will depend to a great extent on their liposolubility, the lipophilic compounds crossing membrane barriers rapidly. Similarly, such drugs could accumulate at some point of vital importance to a cell and thereby disrupt certain metabolic processes.

For example, Seeman (1972) summarizes the membrane actions of the lipid-soluble anesthetics and tranquilizers as electrical stabilization of the membrane along with fluidization and

disordering of the membrane components. As a result of these conformational changes and membrane expansion, either stimulation or inhibition of the associated enzymes and proteins may occur, depending also on the electrical charge of the drug, by modifying the permeability of membranes to ions or solutes. Seeman also postulates that enhanced neurosecretion of bound substances may occur during membrane fluidization through expansion and subsequent fusion of two membranes. However, since lipid-soluble drugs such as the anesthetics displace Ca^{++} , which is required for stimulus-secretion coupling, inhibition of secretion may possibly mask any enhanced neurosecretion. In the same way, such drugs could theoretically diminish the uptake of neurotransmitters indirectly through interference with membrane permeability to ions, whether these fluxes be passive, active or facilitated.

Therefore, the results of these experiments have provided evidence that the actions of certain drugs in causing release of NA from nerve endings may be differentiated from the inhibitory effects of these drugs on NA uptake using the methods employed. However, the mechanisms whereby these drugs act may be complicated and in most cases can only be speculative since, for most of the processes involved, the knowledge is still incomplete.

SUMMARY AND CONCLUSIONS

In this in vitro study, a homogenate of rat whole brain tissue was employed to examine the effects of twelve drugs on the uptake and release of ^3H -noradrenaline from nerve terminals since this preparation has been previously used by other investigators and has been shown to contain synaptosomes, isolated nerve endings which were pinched off from their axons during the homogenization process. To differentiate between releasing effects of the drugs and inhibition of NA uptake, the incubation procedure was varied. That is, to investigate NA release, the homogenate was first preincubated with ^3H -NA to load the synaptosome prior to addition of the drug; to study uptake inhibition, the homogenate was instead preincubated with drug and then ^3H -NA was added and the incubation continued.

The results of this study may be summarized as follows:

1. In the absence of drug treatment, the accumulation of ^3H -NA increased with time (showing a peak accumulation after 25 minutes incubation) and diminished with increasing concentrations of NA (indicating saturation by $5.0\ \mu\text{M}$ NA).
2. Employed in a concentration of $10^{-4}\ \text{M}$, all twelve drugs studied demonstrated a releasing action which increased with time. Although simultaneous inhibition of uptake could have contributed to the measured effect, it was felt that this would be minimal or unlikely since cocaine, a potent inhibitor of neuronal NA uptake, showed the least release.

3. Following incubation for 20 minutes, all the drugs showed increasing releasing effect with increasing dose although tyramine was effective at a much lower dose than for any of the remaining compounds. From these data, concentrations required to produce half-maximal effects on release (EC50) were determined, demonstrating tyramine to be by far the most potent drug in this respect, whereas cocaine and tripeleennamine showed the least release of NA.

4. A plot of the logarithm of the EC50 value vs the logarithm of the octanol/water partition coefficient showed a linear relationship for nine of the twelve drugs indicating that a non-specific mechanism of action based on lipid solubility may be responsible for the releasing effects of high doses of these drugs. The failure of tyramine to fit this correlation suggests that this drug may be acting by competing for the neuronal catecholamine uptake process to gain entry into the nerve terminal where it can displace bound NA and thus produce release of the amine. This evidence supports that of many other investigators who have implied a similar mechanism of action for tyramine. The failure of cocaine to produce any appreciable release of NA despite its reported competitive inhibition of uptake may be due to postulated presynaptic inhibition of release; or it may instead be indicative that cocaine is not a substrate for the uptake process so that, although it might block the uptake system, it could not gain entry to the nerve terminal to displace the bound catecholamine.

5. At a concentration of 10^{-6} M, all twelve drugs demonstrated an inhibitory effect on NA uptake which showed an initial decrease with time and then levelled after approximately 40 minutes incubation. Again, the effects which these drugs may have on efflux did not seem to influence the measured response on uptake inhibition since cocaine and tripeleennamine which seemed to possess a minimal releasing action were highly effective inhibitors of NA uptake.

6. When the drugs were incubated for 40 minutes with the ^3H -NA, increasing concentrations of the compounds were observed to produce increasing inhibition of uptake reaching 100% with maximum concentrations of all twelve drugs. From these data, concentrations required to produce 50% inhibition (IC_{50}) were calculated, allowing relative potencies of the drugs to be compared. As in studies of release, tyramine was the most potent compound, with its IC_{50} and EC_{50} values being comparable. In contrast, tripeleennamine and cocaine were approximately equipotent with tyramine as inhibitors of NA uptake although their potencies with respect to release were far lower. This indicates that cocaine and tripeleennamine, like tyramine, are probably acting specifically on the NA uptake mechanism to produce their blockade. The remaining drugs, although less potent than tyramine, also showed a greater potency as inhibitors of NA uptake than as releasing agents, and their IC_{50} values compare well with reported values in the literature. These compounds could be acting by a noncompetitive mechanism to produce their inhibition.

7. Because the correlation between inhibitory potency and lipid solubility was not very good, it may be that the tested compounds are acting through several possible mechanisms or with mixed effects. Those pdrugs which fall on or close to the line are probably acting in a nonspecific manner which is related to their lipid solubilities; compounds which deviate greatly from the established relationship probably are inhibiting through a specific interaction with the uptake process; and those drugs which fall between may be acting through a combination of these effects or at yet another site.

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