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TRICYCLIC ANTIDEPRESSANTS AS UPTAKE BLOCKERS

Girolamo A. Ortolano
University of Rhode Island

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TRICYCLIC ANTIDEPRESSANTS

AS

UPTAKE BLOCKERS

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OF

GIROLAMO A. ORTOLANO

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Major Professor _____

_____

_____

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ABSTRACT

The biogenic amine hypothesis of affective disorders
ascribes a functional depletion of neurotransmitters within
critical regions of the brain as either the cause or, at the
very least, a concomitancy of the depressive syndrome. Much
indirect evidence has accummulated to suggest that the ther-
apeutic effect of tricyclic antidepressants (TCA's) is med-
iated by their ability to block the re-uptake of physiologi-
cally released neurotransmitter leaving more neurotransmitter
available within the synapse to interact with the post-synap-
tic receptor, thereby correcting the posited functional de-
pletion.

The evidence upon which the proposed mechanism of TCA's
therapeutic activity is based is largely indirect. Most of
the evidence comes from in-vitro studies of the ability of
tricyclics to block neurotransmitter uptake into either
brain slices, minces or preparations of pinched-off nerve
endings (synaptosomes). The therapeutic response, however,
cannot be demonstrated upon acute administration of these
drugs and clinically effective activity is usually not ob-
served until after several weeks of chemotherapy. The up-
take inhibitory characteristics which have been demonstrated
in-vitro, therefore, cannot explain the latency to the ther-
apeutic effect.

A time-course for the effect of protriptyline administra-
tion upon H^3-1-norepinephrine (H^3-1-NE) uptake into hypothal-
amic crude synaptosomal homogenate (CSH) was determined

after acute, sub-acute and chronic treatment. In addition, the ability of a number of TCA's to block the uptake of three biogenic amines, the catecholamines, norepinephrine and dopamine, and the indoleamine, serotonin, was assessed using a CSH in-vitro test system.

The results support the biogenic amine hypothesis as well as the proposed mechanism of action of TCA's. Uptake blockade could be demonstrated after acute and chronic administration but not after sub-acute administration. An attempt to describe some of the characteristic effects of individual TCA's as well as effects common to the class of drugs was approached on the basis of predominant neurotransmitter influences.

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For Mom, Dad, Brett, Keith and Kathy

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CHAPTER I: INTRODUCTION

The biogenic amine hypothesis of affective disorders, admirably and extensively reviewed by Baldessarini (1975), was first proposed by Jacobsen in 1959 and popularized by Schildkraut and Kety (1967). The hypothesis developed from observations of the effects of drugs upon emotionality and the resultant biochemical changes produced by these drugs in experimental animals. Reserpine, once used widely in the treatment of essential hypertension, produces depression in a significant proportion of patients necessitating withdrawal of the medication. Reserpine has also been shown to drastically deplete levels of physiologically active biogenic neurotransmitter amines within the brains of experimental animals. The biogenic amine hypothesis attributes lowered levels or turnover of these amines as either the cause or, at the very least, a concomitancy of the depressive syndrome.

Further support for the hypothesis comes from the noted clinical antidepressant properties of a class of drugs referred to as monoamine oxidase inhibitors (MAOI). Iproniazid, an early member of the class, produced mood elavation in patients being treated for tuberculosis. Inhibition of MAO, an enzyme which degrades biogenic amines within the central nervous system (CNS), results in increased levels of the amines. Reviews of the support for the biogenic amine hypothesis abound (Davis, 1970; Schildkraut, 1973; Leonard, 1975; Weil-Malherbe, 1972) and even some dealing selectively with either norepinephrine (Schildkraut, 1965), dopamine (McClure, 1973) or serotonin (Lapin and Oxenkrug, 1969) as

the critical neurotransmitter exist.

Tricyclic antidepressants have been shown to be effect-
ive inhibitors of the primarily neurotransmitter-specific,
"hi-affinity", uptake process which many investigators
believe is responsible for the termination of activity of
physiological concentrations of neurotransmitter at the
synapse. The ability of these antidepressants to block the
uptake of neurotransmitter from the synapse may result in
more neurotransmitter available within the synapse to inter-
act with the post-synaptic receptor. This property of the
drug would logically tend to reverse the effect of a lowered
level or lowered potency or, in terms of the biogenic amine
hypothesis, a functional depletion of neurotransmitter and
can account for a reversal of the depressive symptoms.

The onset of clinical antidepressant activity, however,
cannot be explained by correlations with antidepressant
plasma levels (Oswald, 1972). A lag of several weeks of
treatment before antidepressant activity becomes apparent
has been reported in clinical trials of tricyclic anti-
depressant therapy (Hammer, et al., 1962; Asberg et al.,
1970). Uptake studies to date deal primarily with in-vitro
effects of antidepressants and in doing so cannot uncover
any significant alterations in the drug effects on the uptake
systems which might be coincident with and possibly related
to the clinical antidepressant activity. Although one
recent study (Ross and Renyi, 1975b) reports the effect of
uptake blockade in animals treated for one day orally, there

has not been a study reporting the effect of chronic admini-
stration of tricyclics on the hi-affinity uptake system of
biogenic amines in the central nervous system. The design
of many antidepressant drugs and the animal screening models
for potential new antidepressants relies heavily upon the
uptake inhibitory property of the tricyclics. Although the
property of uptake inhibition fits the heuristic biogenic
amine hypothesis, it has yet to be established that the
therapeutic effect is mediated by this mechanism either
entirely or in part.

A test of the hypothesis that tricyclic antidepressants
mediate this therapuetic activity by inhibiting the uptake
of biogenic amines can be approached experimentally using
isolated pinched off nerve endings (synaptosomes) as a
model of nerve endings functioning in-situ (Kuhar, 1973;
Bradford, 1975). Characterization of the model and optimi-
zation of the parameters for unidirectional transport are
the required preliminary experiments. The classical pharma-
cological approach necessitates establishing a time course
for the effect as well as a dose-response curve. Treatment
effects may then be compared with that of vehicle injected
controls to conclude the test of this hypothesis.

To confirm that uptake inhibition occurs upon repeated
administration of tricyclics would lend support to the bio-
genic amine hypothesis and further substantiates the concept
that tricyclics may mediate their effect by virtue of this
well documented property.

CHAPTER II: REVIEW OF THE LITERATURE

A. Neurotransmitter inactivation by uptake processes: An historical background.

Interest in measuring the uptake of catecholamines and the physiological significance of this process came about as a result of a number of experiments designed to illustrate the mechanism by which catecholamines are inactivated under physiological conditions. Monoamine oxidase (MAO) was then considered the enzyme responsible for inactivating the catecholamines at sympathetic nerve endings in much the same way in which acetylcholinesterase was thought to act at cholinergic neurons (Burn, 1953). Koelle and Valk (1954) reported that unlike acetylcholinesterase in cholinergic neurons, MAO is not selectively associated with adrenergic neurons. Kamijo et al. (1955), Balzer and Holtz (1956) and Corne and Graham (1957) all reported the inability of a variety of MAOI's to significantly potentiate the actions of catecholamines either administered exogenously or released by stimulation of the sympathetic nerve.

Catechol-O-methyltransferase (COMT) has been shown to play a role in the metabolism of catecholamines (Axelrod, 1957, 1959; Hertting and Axelrod, 1961; Rosell et al., 1963), but Bacq (1959) reported only a slight potentiation of the effects of endogenous catecholamines produced by stimulation of sympathetic nerves after COMT was inhibited by pyrogallol. Wylie et al. (1960) reported similar results upon administration of exogenous catecholamines as did Murnaghan and Mazurkiewicz (1963) using another COMT inhibitor, 4-methyltropolone.

Burn (1932) first suggested that exogenously admin-
istered catecholamines might be taken up into storage sited
of certain peripheral tissue, but it was not until fifteen
years later that Raab and Humphreys (1947) reported an
increase in catecholamine content of cat and dog hearts
after the administration of large doses of epinephrine and
norepinephrine.

Acceptance of the importance of tissue uptake in the
inactivation of catecholamines awaited the development of
radioactively labeled catecholamines with high specific
activity and this uptake was demonstrated by Axelrod et al.
(1959). ^{3}H-Epinephrine (0.1 mg/kg) administered to mice
was found to disappear in two phases. The first phase was
a rapid metabolism of approximately 70% of the injected
dose. The remaining 30%, however, disappeared more slowly.
The interpretation of these results was that exogenously
administered catecholamines are accumulated in tissues and
may serve to inactivate catecholamines under normal physio-
logical conditions. Whitby et al. (1961) drew the same
conclusions from the results of similarly designed experi-
ments. Further support for the concept of uptake being
responsible for inactivation of catecholamines came from
Muscholl (1961) using a combination of bioassay and flouri-
metric techniques and from the laboratory of Stromblad and
Nickerson (1961) as well. Numerous reports substantiating
this conclusion followed and are reviewed by Iversen (1965).

Considerable evidence has accumulated to suggest that

the uptake of catecholamines occurs into post-ganglionic nerve endings. Kopin et al. (1964) maintained that if allowances are made for differences in regional blood flow through various tissues studied, there is a good correlation between the accumulation of norepinephrine and the level of endogenous norepinephrine. The levels of endogenous norepinephrine may be taken to reflect the extent of innervation of the organ or tissue by sympathetic post-ganglionic terminals, although turnover may be judged a better indicator by some (Costa, 1969). Additional evidence that the uptake of norepinephrine occurs primarily into sympathetic post-ganlionic neuronal endings became available when Hertting et al. (1961) reported almost complete disappearance of the ability of norepinephrine and epinephrine to accumulate in tissues in which the sympathetic post-ganglionic nerves innervating the tissue had been cut and the terminals allowed to degenerate. This approach was modified by Zaimis et al. (1965), Iversen et al. (1966) and Sjoqvist et al. (1967) whereby sympathetic innervation was obliterated by immunosympathectomy at birth; the results obtained were similarly interpreted.

A number of techniques have been employed to conclude that localization of accumulated radiolabeled norepinephrine occurs predominantly in neuronal endings include studies using density gradient centrifugation (Potter and Axelrod, 1962; 1963), autoradiography and electron microscopy (Worle et al., 1962; Marks et al., 1962; Samorajski et al., 1964)

and histochemical flourescence techniques (Hamberger *et al.*, 1964). Although the evidence implicates the neuronal ending as the site of uptake, methodology at that time was not available to accurately separate and describe the component of uptake into tissue other than neuronal endings. Fischer and Kopin (1964), however, did present evidence to indicate that the uptake of catecholamines was not limited to neuronal tissue in their report describing uptake into denervated salivary glands of the rat.

Further elaboration of the characteristics of uptake systems of peripheral tissues came about as a result of attempts to study the effects of drugs. Macmillan (1959) proposed the "nerve uptake hypothesis" ascribing the potentiating properties of cocaine to its ability to interfere with the catecholamine uptake mechanism. Adrenergic blocking agents were found to inhibit the uptake of ^{3}H-norepinephrine including phenoxybenzamine and dicholro-isoproterenol (Axelrod *et al.*, 1962b). Tricyclic antidepressants, neuroleptics of the phenothiazine class as well as guenethidine, bretylium and ouabain were shown to be effective inhibitors of the noradrenergic uptake mechanism (Axelrod *et al.*, 1961b; Herhitting *et al.*, 1962; Dengler *et al.*, 1962) as well as the sympathomimetic amines tyramine, amphetamine and ephedrine (Axelrod and Tomchick, 1960; Axelrod *et al.*, 1961a, Heritting *et al.*, 1962; Dengler *et al.* 1961).

Muscholl (1960), Axelrod *et al.* (1961b) and Heritting

_et al._ (1962) all reported that reserpinized tissues failed
to accumulate epinephrine and norepinephrine. Dengler (1961)
found that the accumulation of epinephrine and norepin-
ephrine in tissue slices was prevented when reserpine was
present in the incubation medium. As a result of these
experiments there was relatively wide acceptance that reser-
pine inhibited the uptake of catecholamines (Brodie and
Beaven, 1963). After an extensive review of the literature,
however, Shore (1963) concluded that the actions of reser-
pine were predominantly centered around the intracellular
uptake and storage mechanism and not the neuronal membrane
uptake system. Direct evidence to support this hypothesis
came from Lindmar and Muscholl (1964) where in both normal
and reserpinized isolated rat heart preparations, they
infused 0.5 ug of norepinephrine over a 10 minute period
and collected and analyzed the perfusate for unchanged
norepinephrine. Analyzing the norepinephrine content of
the heart after the perfusion interval, they determined that
in normal hearts the catecholamine taken up from the medium
was almost entirely accounted for as unchanged norepinephrine
accumulated into the tissue. In reserpinized hearts, how-
ever, the amount of norepinephrine in the perfusate was the
same as in the perfusate of normal hearts, but tissue anal-
ysis revealed very much lower levels of catecholamine with
very high levels of MAO metabolites in the perfusate. These
These results suggested that norepinephrine accumulation was

not affected but its subsequent storage was.

Iversen (1965a, 1965b) reported characteristics of the uptake of norepinephrine into isolated rat hearts. At low concentrations of epinephrine or norepinephrine (.07 - 7 uM) the uptake followed classical Michaelis-Menten kinetics and Km and V_{max} values where calculated showing a greater affinity of the stereoisomer, l-norepinephrine, for the uptake mechanism than d-norepinephrine. The racemate, dl-norepinephrine, exhibited a higher V_{max} and lower Km than dl-epinephrine.

Similar experiments were performed using high concentrations of catecholamines (5-230 uM) in the perfusion medium (Iversen 1965a, 1965b). The accumulation of catecholamines under these conditions could not be explained in terms of the uptake system described when using low catecholamine concentrations in the perfusate, however, the characteristics of this uptake process could be described using classical enzyme kinetic models. This second uptake system, a process for which the term Uptake 2 (U_2) has been applied, showed a much lower affinity than could be ascribed to the initial uptake system described (referred to as Uptake 1, abbreviated U_1).

Several properties of U_2 serve to distinguish it from U_1. Catecholamines accumulated by U_2 disappeared rapidly from the tissue if the perfusion was continued using a catecholamine-free medium, although 2 ug. of catecholamine per gram of tissue remained even after prolonged periods of

washout. A comparison of the kinetic constants showed that unlike U_1, U_2 exhibited no stereospecificity; also, although U_2 accumulated norepinephrine more rapidly, epinephrine exhibited a greater affinity for uptake with the Km being about five times lower than that for norepinephrine. Although U_2 appeared inoperative at low substrate concentrations and thought to be activated where a critical threshold of substrate concentration is reached (Iversen, 1965a), a more recent study reported evidence to conclude that both systems operate at all substrate concentrations with U_1 having the greater influence at low and U_2 at high substrate concentrations (Lightman and Iversen, 1969).

Another property serving to distinguish between U_1 and U_2 includes substrate specificity and the effect of drugs on inhibiting the transport effected by each system. Burgen and Iversen (1965) reported the results of a variety of substituted B-phenylethylamines on the U_2 system. Interestingly, O-methylation as well as N- substitution greatly enhanced that affinity for U_2; whereas the effect of these compounds on U_1 are exactly opposite in nature (Horn, 1973). Cocaine, imipramine and desipramine while potent inhibitors of U_1 were found to be only weakly active with respect to U_2 (Iversen 1965a; Salt, 1972). Phenoxybenzamine is a potent inhibitor of U_2 as is a variety of steroids including B-estradiol, corticosterone, testerone, deoxycorticosterone and androsterone (Iversen and Salt, 1972).

Originally it was suggested that U_2 might represent a

separate neuronal membrane transport system at sites along
the entire neuron; that is, cell body, axon and nerve
terminal. Evidence for this concept was provided by the
histochemical studies of Norbert and Hamberger (1964) and
Hamberger et al. (1964) who reported that the accumulation
of norepinephrine was localized to terminal regions of post-
ganglionic sympathetic neurons when low doses of norepin-
ephrine were administered and amine throughout the cell
body and axon as well as the terminals when high doses were
administered. This hypothesis, however failed to explain
the washout phenomenon reported to exist for U_2.

Another possibility offered was that U_2 may represent
an uptake of catecholamines by storage granules within
adrenergic neuronal terminals (Iversen, 1965a). Evidence
for this hypothesis included the indisputable similarity of
properties between U_2 and either isolated adrenergic nerve
granules or adrenal medullary granules incubated in-vitro
(Euler and Lishajko, 1964; Carlsson et al., 1963). The
particle uptake system is saturable at amine concentrations
of 80 ug/ml. which is very close to that described for U_2,
also, both are relatively insensitive to inhibition by
cocaine, desipramine or metraminol and both are inhibited by
tyramine and phenoxybenzamine. Granular uptake lacks
specificity for the optical isomers of epinephrine and nor-
epinephrine. Finally, the uptake of catecholamines in
isolated granules occurs at concentrations of 1 ug/ml. or
higher in the absence of magnesium and adenosine triphos-

phate and U_2 is known to begin to exert substantial influence at this concentration of amine. One important difference between U_2 and the isolated particles is reflected by the fact that no difference in affinity can be shown for epinephrine and norepinephrine as can be observed in the U_2 system.

The hypothesis which has gathered the most support by far is that U_2 represents an extra-neuronal uptake mechanism (Iversen, 1975). Support has come from the results of histochemical studies of the distribution of catecholamines in various tissues after exposure to high concentrations of exogenously administered norepinephrine. The work of Fisher and Kopin (1964) cited earlier may have provided that needed impetus to investigate the association between non-neuronal or extra-neuronal uptake and U_2. After perfusion of rat hearts with medium containing high concentrations of norepinephrine, intense flourescence was observed in cardiac muscle cells and unidentified connective tissue cells as well as nerve terminals (Ehinger and Sporrong, 1968; Farnebo and Maimfors, 1969; Clarke et al., 1969; Jacobowitz and Brus, 1971). Extraneuronal uptake, like U_2, was found to be sensitive to normetanephrine and relatively insentive to desimpramine (Farnebo and Malfors, 1969; Clarke et al., 1969). Numerous reports of uptake of catecholamines into nonneuronal tissue including salivary gland parenchyma (Hamberger, 1967), smooth muscle of cat spleen (Gillespie 1968, 1973), vas deferens (Gillespie et al., 1970), chick

amnion (Burnstock et al., 1971), perfused rabbit ear artery
(Avakian and Gillespie, 1968), cat nictitating membrane
(Draskoczy and Trendlenburg, 1970) and guinea pig trachea
(O'Donnell and Saar, 1973), appeared. Kinetic constants for
the extra-neuronal uptake into arterial cells was shown to
have characteristics similar to U_2 (Gillespie and Towart,
1973). The uptake of norepinephrine into smooth muscle
also showed susceptibility to phenoxybenzamine and washout
was inhibited by normetanephrine (Gillespie, 1968).

Although U_1 has been considered the principal mechanism
responsible for the inactivation of physiological concentra-
tions of norepinephrine (Iversen, 1971), the preponderance
of sympathetic innervation is clearly a consideration in
determining the importance of either uptake mechanism in
isolated tissue preparations, as is the concentration of
catecholamine in the medium. Smooth muscle of the vas-
culature contains relatively little sympathetic innervation
and it would seem reasonable to conclude that U_2 is most
important in terminating the action of circulating catechol-
amines in this tissue. The evidence to support the
hypothesis that neuronal uptake is the principal inactivating
mechanism of depolarize-released neurotransmitter comes
from reports that organ response to sympathetic nerve stim-
ulation is greatly increased by drugs shown to be effective
inhibitors of the neuronal uptake mechanism (Thoenen, 1964a,
1964b; Brown, 1965). Based on results reported by Langer
(1970), U_1 may account for the recapture of as much as 70-

90% of the transmitter released by low frequency sympathetic nerve stimulation in the cat nictitating membrane.

B. Characterization of kinetically distinct neuronal uptake
    mechanisms.

    i) Norepinephrine, dopamine and tyrosine:

Norepinephrine uptake as an active process described
using Michaelis-Menten kinetics was dissociated from the
contribution of passive diffusion by using low substrate
concentrations (10^{-7}M) in the medium of brain slice prepara-
tions (Dengler, 1962). The active component of uptake into
brain slices was shown to be temperature dependent, occur
against a concentration gradient, be dependent upon Na^+ in
the medium, require oxygen and glucose, can be lowered by
metabolic inhibitors (Dengler et al., 1962; Dengler, 1965;
Ross and Renyi, 1964, 1966), and does not require calcium
or magnesium ions (Hamberger, 1967). Limited accumulation
occurs when incubation is carried out in a sucrose medium
(Mirkin et al., 1963).

Similar results have been reported with the use of a
crude synaptosomal homogenate. Davis et al. (1967),
Bogdanski and Tissari (1967), Bogdanski et al. (1968)
Colburn et al. (1968) and White and Keen (1971) all showed
an absolute requirement for 143 mM Na^+ concentrations and
inhibitory effects of increasing K^+ concentrations above
5mM, as well as temperature dependence and reduction in
uptake by metabolic inhibitors. Conclusions of these
studies suggest that uptake by brain slices, at low sub-
strate concentrations similar to that used by Dengler (1962),
occurs primarily into neuronal endings.

Harris and Baldessarini (1973) showed similar require-

ments for dopamine uptake into rat corpus striatum with one notable exception. Whereas Snyder and Coyle (1969) reported no substantial effect on initial uptake of catecholamines using reserpine pretreatment, Harris and Baldessarini (1973) reported a considerable effect.

Additional support for the hypothesis that amine accumulation occurs into nerve endings comes from the work of Snyder et al. (1965) who showed that intraventricular injection of labeled norepinephrine was found localized to a great extent in the synaptosomal fraction upon differential centrifugation. Histochemical techniques confirmed that the uptake of monoamines into rat cerebral cortex was confined to varicosities indistinguishable from those containing endogenous norepinephrine (Hamberger and Masuoka, 1965; Hamberger, 1967). Henn (1967) using electron microscopic autoradiography reported that 80% of the H^3-norepinephrine accumulated by brain slices was localized to neurons. More recently Lidbrink and Jonsson (1974) showed a reduction of uptake of norepinephrine into cerebral cortices after noradrenergic terminals were destroyed by lesion of the dorsomedial reticular formation with 6-hydroxy-dopamine.

Snyder and Coyle (1969) studied the stereospecificity of norepinephrine and dopamine uptake in different areas of the rat brain. The Michaelis constant of 0.4 uM for nor-epinephrine uptake into extrastriatal areas is in good agreement with the 0.56 uM Km reported by Colburn et al.

(1968) using whole rat brain. Snyder and Coyle (1969)
extended the range of catecholamine concentration from 0.05
uM using 1.2 uM as the upper limit. They reported that the
striatum had a higher affinity for dopamine than norepin-
ephrine and both neurotransmitters display only a single
uptake component over the range of substrate concentrations
studied. All other brain areas showed two components of
dopamine uptake, one considered a high affinity the other a
low-affinity transport mechanism, whereas norepinephrine
continued to be described adequately by a straight line
using the Lineweaver-Burk method suggesting only one trans-
port system exists for this amine. In a later paper (Coyle
and Snyder, 1969), they reported the Ki values for mutual
inhibition of dopamine and norepinephrine uptake in all
regions of rat brain to be identical with their respective
Km values and concluded that these two catecholamines use
the same transport systems (presumably dopaminergic) and
would not require such a characteristic.

The uptake of precursors of norepinephrine and dopamine,
namely tyrosine and dopa, have been described (Naeme, 1961;
Guroff et al., 1961; Dengler et al., 1962; Yoshida et al.,
1963a, 1963b, 1963c, 1965) but delineation of two transport
systems has not been the focus of attention and whether
these systems exist remains to be shown. Chirigos et al.,
(1960) have reported stereospecificity of 1-tyrosine for
brain tissue and illustrated its uptake inhibition by the
amino acids leucine, isoleucine, valine, histadine and

cysteine. They also noted that stereospecificity was lacking in muscle preparations. Interestingly, Ross and Renyi (1966) studied the uptake of tyramine and l-dopa at concentrations similar to those reported for high affinity uptake of dopamine (Snyder and Coyle, 1969) and reported Km's for dopamine and tyrosine of 0.7 uM and 0.4 uM, respectively, in slices of whole brain. These values are similar to those reported for extra-striatal norepinephrine uptake (Coyle and Snyder, 1969; Snyder and Coyle, 1969). It would appear that these Km values adequately describe a high affinity component of uptake when compared with other amino acids with Km's in the millimolar range (Cohen and Lajtha, 1972). In addition, Wofsey et al. (1971) present evidence to indicate that tyrosine may become selectively incorporated into a population of synaptosomes that can be separated from those which accumulate norepinephrine by a technique known as incomplete equilibrium centrifugation.

ii) Serotonin and tryptophan:

Fuxe and Ungerstedt (1967) described the localization of intraventricularly administered serotonin to serotonergic neurons using histochemical flourescence techniques. Although a number of attempts were made to illustrate serotonin accumulation in brain slices (for a review see Katz and Chase, 1970) most attempts were unsucessful in separating out an active transport process from simple diffusion until Blackburn et al. (1967) incubated brain slices with C^{14}-serotonin at a concentration of 2×10^{-8}M and

reported considerable accumulation of the amine with 60%
recovered in the synaptosomal fraction upon subcellular
fractionation using differential centrifugation techniques.
Similarly, Kuhar et al. (1970) studied the subcellular
localization of serotonin after incubation of brain slices
with the amine in concentrations ranging from 10^{-5} to 10^{-8} M
and found the major portion of accumulated serotonin local-
ized to synaptosomes.

The kinetics of serotonin accumulation into slices of
rat striatum and hypothalamus revealed two distinct trans-
port processes with $Km_H=1.7x10^{-7}M$ and $Km_L=8.0x10^{-6}M$ in the
striatum and $Km_H=1.4x10^{-7}M$ and $Km_L=8.0x10^{-6}M$ in the hypo-
thalamus (Shaskan and Snyder, 1970). The Ki values for the
competitive inhibition of norepinephrine uptake by serotonin
approximated the Km for low affinity serotonin into catechol-
aminergic neurons. These kinetically distinct components
were confirmed by Wong et al. (1973).

Kuhar et al. (1974) review the results of a number of
studies illustrating the reduction of serotonin uptake
associated with the selective destruction of serotonin nerve
terminals by midbrain raphe lesions suggesting that uptake
occurs predominantly into serotonergic nerve endings when
low substrate concentrations are employed. Histochemical
flourescence as well as the measurement of tryptophan
hydroxylase activity and endogenous serotonin levels support
the hypothesis that high affinity uptake process is specific
for neurotransmitter inactivation upon depolarized release

(Kuhar et al., 1972). Massari and Sanders-Bush (1975) have utilized this concept of high-affinity neurotransmitter-specific uptake to compare the effects of para-choloro-amphetamine or B-9 lesions with the distribution of serotonergic neuronal endings in different areas of the brain.

Initial studies of tryptophan transport into synaptosomal preparations utilized substrate concentrations limited to the low affinity component of transport (Grahame-Smith and Parfitt, 1970). In this study the concentrations of sodium ions in the media were varied from 0 to 112 mM by osmotic replacement with either choline or sucrose and resulted in no effect on tryptophan uptake, however, cyanide and ouabain did significantly reduce the uptake of substrate. Interestingly, preloading the synaptosomes with 1-tryptophan resulted in an increase in the uptake of either 1-tryptophan or 1-phenylalanine. L-Tryptophan preloaded synaptosomes were also shown to greatly accelerate the rate of efflux of tryptophan by either 1-tryptophan or 1-phenylalanine implying that a faciltated exchange process was occuring. A number of amino acids were reported very active in stimulating the efflux of tryptophan from preloaded synaptosomes. It would appear that this low affinity transport system represents the same mechanism by which other amino acids gain entry into the neuron and is not specific for tryptophan.

A more recent study revealed a high affinity component of the uptake of 1-tryptophan as well as a low affinity component (Parfitt and Grahame-Smith, 1974). Although the

affinity Michaelis constant (Km_L) originally reported by
Grahame and Parfitt (1970) as $1.0 \times 10^{-3}M$ is considerably
disparate from their more recent data (Km_L $3.2 \times 10^{-4}M$); use
of different tissue preparation, i.e. whole brain vs.
cerebral cortex, can account for the results. The Km_H,
however, is only lower than the the Km_L by a factor of
three reported in this study, (Parfitt & Grahme-Smith, 1974),
whereas, other investigators have reported differences
between high and low affinity uptake systems differing from
a minimum of 21 (in cerebral cortex) to over 300 (glycine in
the spinal cord) orders of magnitude which would appear to
represent physiologically more distinguishable processes
(Snyder et al., 1973).

iii) Other neurohumors:

a) Acetylcholine and choline:

Unlike the catecholamines and serotonin, acetylcholine
appears to be inactivated largely by the intrasynaptic
degradative enzyme acetylcholinesterase and diffusion rather
than an uptake mechanism (for a review see Katz and Miledi,
1973). Early work aimed at characterizing an uptake process
for acetylcholine did not prove fruitful (Guth, 1962;
Burton, 1964). Even in the presence of the reversible chol-
inesterase inhibitor physostigmine, negative results were
reported (Elliot and Henderson, 1951; Schuberth and Sundwall,
1967) although it was suggested that physostigmine may have
strong inhibitory actions on the uptake mechanisms (Polak
and Meeuws, 1966). Use of the organophosphate cholinester-

ase inhibitors including paraoxon (Diethyl-4-nitrophenyl phosphate) have resulted in significant accumulations of acetylcholine into brain tissue (Polak and Meeuws, 1966; Schuberth and Sundwall, 1967; Liang and Quastel 1969; Heilbronn, 1969) with the uptake requirement for oxygen, reduced by metabolic inhibitors and described using Michaelis-Menten kinetics (Schuberth and Sundwall, 1967; Polak and Meeuws, 1966; Liang and Quastel, 1969).

Acetylcholine uptake seems unimportant physiologically for it has been shown that acetylcholine uptake into synaptosomes is neither readily incorporated into vesicles (Marchbanks, 1968) nor released by electrical stimulation or potassium depolarization (Katz et al., 1973). Further evidence leading to a conclusion that acetylcholine uptake is not physiologically important comes from the results of Marchbanks (1969b) and Adamic (1970) suggesting that both molecules utilize the same carrier. Similarly autoradiographic techniques have been employed using slices of rat cortex incubated in labeled acetylcholine and the distribution of label was reported as diffuse with no obvious preference for specific cell types or structures (Polak, 1969). Finally, Katz et al. (1973) attempted to collect the excess acetylcholine which might be produced by brain stimulation in the presence of an acetylcholine uptake inhibitor if the acetylcholine transport process were physiologically active. They report that no additional acetylcholine is collected in the presence of uptake inhibitor

and conclude that acetylcholine uptake may not be physio-
logically operational. These results may be explained by
considering the transport mechanism responsible for acetyl-
choline uptake to be identical with the choline carrier;
the low affinity choline transport mechanism being common
to all cells requiring choline as substrate for synthesis
of phasphatidyl- or phosphorycholine.

Hodgkin and Martin (1965) first described the ability
of choline to be transported into excitable tissue, the
squid axon. A year later Schuberth et al. (1966) reported
the presence of choline in cerebral cortex tissue slices
and localization of choline transport within nerve endings
was established by Marchbanks (1968b) using synaptosomal
preparations with a reported Km of 100uM. A number of
similar studies reported Km's varying from as low as 80 uM
(Potter, 1968) to 440 uM (Hensworth and Bosmann, 1971).

Green and Haubrich (1971) using synaptosomal prepara-
tions incubated with low concentrations of choline, reported
a significant amount of acetylcholine formed and suggested
that more than one uptake system may exist for choline
accumulation in the brain. Yamamura and Snyder (1973) and
Dowdall and Simon (1973) reported two kinetically distinct
carrier-mediated systems for choline transport having a Km_H
of 1-5 uM and Km_L less than 25 uM. The two systems showed
clearly distinguishable effects of Na concentration (Haga
and Noda, 1973) with the high affinity component more dep-
endent on alterations in sodium concentration in the media.

Although it has been suggested that the high affinity
component represents a mechanism of cholinergic neurons
which may serve to recapture the product of intrasynaptic
acetylcholinesterase for the synthesis, storage and sub-
sequent reutilization of acetylcholine, conclusive evidence
of this is not available as yet (Kuhar and Murrin, 1978).

Kuhar, et al. (1973) have provided evidence to indicate
that choline uptake occurs into cholinergic neurons primar-
ily by the high affinity uptake process. Septal lesions,
known to result in reductions of acetylcholine in the
cerebral cortex and hippocampus, resulted in a reduction of
choline uptake by 60% in synaptosomes prepared from these
cholinergically innervated areas, whereas, tryptophan and
glutamatic acid accumulation were unaffected although GABA
and norepinephrine uptake were affected slightly.

b) gamma-Aminobutyric acid (GABA)

GABA was shown to be taken up by slices of rat cerebral
cortices when a low concentration (0.05uM) of substrate was
used in the medium (Iversen and Neal, 1963). The electron
microscopic autoradiographic experiments of Bloom and
Iversen (1971) suggest that more than 70% of the GABA
taken up into brain slices is localized to the nerve term-
inals. It has also been suggested that at this low concen-
tration, reminiscent of that used to detect high affinity
uptake, a distinct population of nerve terminals are res-
ponsible for the uptake of labeled GABA (Iversen and Snyder,
1968; Iversen and Johnston, 1971). Clearly distinguishable

high and low affinity components for GABA uptake into
synaptosomes and brain slices have been reported (Levi and
Raiteri, 1973a, 1973b).

As with other high affinity transport systems there is
an absolute requirement for physiological concentrations of
electrolytes and uptake is inhibited strongly by low con-
centrations of Na^+ (Iversen and Neal, 1968). Inhibition of
GABA uptake has been reported with 2,4-dinitrophenol, oua-
bain and malonate (Kuriyama et al., 1969; Snodgrass et al.,
1973).

c) Glutamate, aspartate and glycine:

Logan and Snyder (1972), Balcar and Johnston (1972a,
1972b) and Bennett et al. (1972) have studied the uptake of
glutamate and aspartate and have reported that transport
occurs by both high and low affinity mechanisms with the
high affinity system having an absolute dependence on
sodium ions. Furthermore, the metabolic requirements of
both glutamate and aspartate uptake are indistinguishable
with azide, cyanide and omission of glucose from the media
having minimal effects and it appears as though glutamate
and aspartate share the same transport carrier. Wofsey
et al. (1971), using subcellular distribution studies, have
associated the high affinity uptake with a unique popula-
tion of nerve terminals suggesting that the high affinity
mechanism may be directly related to the function of
replenishment of neurotransmitter.

A high affinity uptake system specific for glycine has

been shown in slices of rat spinal cord, pons and medulla
(Neal, 1971; Johnston and Iversen, 1971), slices of cat
spinal cord (Balcar and Johnston, 1973) as well as in crude
synaptosomes of rat spinal cord (Logan and Snyder, 1972;
Bennett et al., 1972) and in purified synaptosomes of
cerebral cortex of the rat (Peterson and Raghupathy, 1973).
Localization of glycine uptake into nerve endings by the
high affinity system has been shown by autoradiographic
analysis (Matus and Dennison, 1972; Iversen and Bloom, 1972;
Ehinger, 1972; Hosli and Hosli, 1972; Ljungadhl and Hokfelt,
1973) as well as subcellular fractionation techniques
(Johnston and Iversen, 1971; Arregui et al., 1972).

Glick (1972) has shown inhibitors of Na^+/K^+ dependent
ATP-ase to be effective inhibitors of glycine uptake; how-
ever, no inhibitors has yet been described as being more
effective in inhibiting either the high or the low affinity
uptake. The low affinity mechanism of glycine uptake seems
to be relatively non-specific in that l-alanine, l-serine
and other small neutral amino acids compete for the trans-
port site (Cohen and Lajtha, 1972).

d) Histamine and histadine:

Although the probable mechanism of histamine inactiva-
tion is via the degradative enzyme histamine methyltrans-
ferase, subcellular distribution studies show the incorpor-
ation into the P_2 (synaptosomal) fractions and after sub-
sequent treatment in a hypo-osmotic medium histamine was
shown to be localized to the vesicular fraction (Snyder

et al., 1974). Young et al. (1971) reported an ontogenic related change in the distribution of histamine among sub-cellular fractions. Older animals (21 days) showed greatest accumulation in the synaptosomal fraction whereas younger animals appeared to concentrate histamine in the nuclear pellet.

A stereospecific uptake of L-histidine into brain slices has been demonstrated (Neame, 1964) although the substrate concentrations were not low enough to differentiate a high affinity component and in all probability the low affinity system is shared by other basic amino acids such as trypto-phan.

C. Effects of tricyclic antidepressants on the hi-affinity
    uptake process of serotonergic noradrenergic and
    dopaminergic neurons.

   i) in-vitro

The ability of tricyclic antidepressants to block the
uptake of norepinephrine into brain slices was described
by Dengler et al. (1961) using concentrations of radio-
actively labeled norepinephrine ($2.8 - 14.0 \times 10^{-8}$M) well
within the concentration range where hi-affinity transport
is responsible for a large part of the uptake of norepin-
ephrine into nerve endings. The concentration at which the
uptake of norepinephrine into nerve endings is inhibited by
fifty percent, (IC_{50}), varies depending upon the prepara-
tion used, i.e. brain slices versus synaptosomes, as well
as by brain region. The IC_{50} for desmethylimipramine (DMI)
varies from 0.003 uM in rat brain synaptosomes (Ross and
Renyi, 1975a) to 0.11 uM in rat brain slices (Salama et al.,
1971). Buus Lassen et al., (1975) reported an IC_{50} for DMI
of 10 uM in rat forebrain synaptosomes as opposed to 0.001
uM in synaptosomes prepared from rat hippocampal tissue.
The large difference is believed to be due to the concentra-
tion of noradrenergic neuronal endings within brain regions
(Squires, 1974; Buus Lassen et al., 1975).

It is also true that studies utilizing different spe-
cies as well as different preparations reported identical
IC_{50} values for the tricyclics tested. Ross and Renyi
(1967a) utilizing mouse cortex slices reported an IC_{50}
value of 0.03 uM for the inhibition of norepinephrine uptake

by DMI whereas an identical value was reported by Shaskan and Snyder (1970) using a crude synaptosomal homogenate of rat hypothalamus. A good correlation between the partition coefficient and the IC_{50} values for a number of chemically different uptake inhibitors was found to exist in brain slices (Carmichael and Israel, 1973).

All studies report DMI to be the most potent tricyclic tested with respect to its ability to block norepinephrine uptake (Ross and Renyi, 1967a; Shaskan and Snyder, 1970; Horn et al., 1971; Salama et al., 1971; Carmichael and Israel, 1973; Squires, 1974; Maxwell et al., 1974; Ross and Renyi, 1975a; Buus Lassen et al., 1975). Many studies indicate that the demethylated form of the tertiary amines, i.e. the secondary amines, are more potent with respect to their ability to inhibit norepinephrine uptake whereas the tertiary amines are more efficacious in its effect on serotonin uptake (Blackburn et al., 1967; Ross and Renyi 1967b; 1969; Carlsson, 1970; Shaskan and Snyder, 1970; Kannengiesser et al., 1973; Horn and Trace, 1974; Ross and Renyi, 1975a and Buus and Lassen et al., 1975).

Structure activity relationships have been studied with respect to both the noradrenergic (Horn and Snyder, 1972; Ferris et al., 1972; Horn, 1973a; Maxwell et al., 1974) and serotonergic (Horn, 1973b) transport system in the central nervous system. It is on the basis of these studies that evidence can be found to substantiate the concept that there exists a chemically distinguishable transport

mechanism for each neurotransmitter.

The uptake inhibitory characteristics of tricyclics as they apply to dopamine have been studied (Ross and Renyi, 1967a). Their effect on this transport system has not generally been considered as important due to the relatively large IC_{50} values reported (Horn et al., 1971). There is no correlation with the inhibition of uptake of dopamine into either rat hypothalamus or whole brain synaptosomes with respect to methyl substitution on the side chain nitrogen of any of the tricyclic antidepressants (Horn et al., 1971; Halaris et al., 1975). Alternatively, antiparkinsonian drugs have been shown to be very potent in their ability to block dopamine uptake in the central nervous system (Coyle and Snyder, 1969b).

ii) in-vivo

Imipramine was shown to reduce the amount of labeled epinephrine found in homogenates of whole mice when compared with saline treated controls (Axelrod and Tomchick, 1959). Similarly, imipramine increased the rate of disappearance of labeled norepinephrine in the whole animal (Axelrod and Tomchick, 1960). Hertting et al. (1961) reported reduced levels of H^3-norepinephrine in heart, spleen, adrenals, liver and muscles with elevated normetanephrine found in muscle tissue after treatment with imipramine. Glowinski and Axelrod (1964) administered imipramine DMI or amitriptyline to rats and after 1 hour administered H^3-norepinephrine by an intra-cerebroventricular (ICV) injection. All three

compounds were reported to be effective in inhibiting the
uptake of H^3-NE into rat brain. It is interesting to note
that Schanberg (1963) could not show imipramine to be
effective in inhibiting the uptake of 5-hydroxytrptophan,
the precursor of serotonin, into brain slices when the drug
was administered i.p. 1 hour prior to sacrifice with sub-
sequent testing in-vitro.

As with the in-vitro data, many studies measuring the
effectiveness of tricyclics on either norepinephrine or
serotonin uptake report tertiary amines as being more
effective then the secondary amines in blocking the uptake
of 5HT in the central nervous system (Ross and Renyi, 1975b).
Conversely, a number of studies conclude the secondary
amines to be more effective than tertiary amines in blocking
NE uptake (Ross and Renyi, 1967; Schildkraut et al., 1969a;
Carlsson et al., 1969b; Ross and Renyi, 1975b).

Palaic et al., (1967) reported no uptake inhibitory
effect of DMI 10mg/kg; injected i.p. 20 minutes prior to ICV
H^3-5-HT injection. Similarly, Carlsson (1970) could not show
an effect of DMI on the release of serotonin by H75/12 into
rat brain. Schubert et al., (1970) failed to demonstrate
an effect on the rate of disappearance of H^3-5-HT after
intravenous injection of H^3-tryptophan of either secondary
amine, DMI or nortriptyline, whereas both imipramine and
amitriptyline retarded the rate of disappearance of H^3-5-HT.
Histochemical evidence is available to suggest that imip-
ramine blocks serotonin uptake (Carlsson et al., 1968;
Corrodi and Fuxe, 1968).

D. Effects of tricyclics on the metabolism of biogenic amines:

i) Serotonin

Da Prada and Pletscher (1966) reported a significant reduction in the levels of 5-hydroxyindoleacetic acid, (5-HIAA) the major metabolite of serotonin in the CNS, for both imipramine and amitriptyline. The ratio of product to precursor indicates that imipramine is slightly more efficacious in reducing the turnover of serotonin and this is reflected in the in-vitro data as well (Ross and Renyi, 1969; 1975; Kannengiesser, 1973). Palaic et al., (1967), using an intracerebroventricular perfusion of H^3-5HT at 55 uM concentration for 1 hour, demonstrated a reduction in endogenous levels of 5-HIAA but not of H^3-5HIAA. At this concentration the predominant idoleamine inactivation mechanism is presumably a low affinity transport system. It seems possible that the effect of DMI on inhibiting the uptake of 5HT may have been overshadowed by the low affinity transport mechanism as well as diffusion. A reduction in endogenous levels of 5HIAA imply decreased activity of the neuron which may come about as a result of feedback mechanisms in the event DMI is inhibiting 5HT uptake. Subsequently increases in the intrasynaptic amine concentration and hence its interaction with post-synaptic receptors is the postulated mechanism for feedback inhibition (Costa and Meek, 1974).

C^{14}-Serotonin was administered intracisternally 15 minutes prior to an i.p. injection of either DMI or IMI

(25mg/kg) and 2 hours after the intracisternal injection
brains were assayed for H^3-5HT and deaminated metabolites
(Schildkraut et al., 1969b). The results indicate impr-
amine as being more effective in increasing levels of
radioactive amine. These results are consistent with the
idea that the tricyclics reduce the turnover of 5HT by the
postulated inhibitory feedback mechanism. Similar results
were obtained in the study of Schubert et al., (1970) in
which both imipramine and amitriptyline reduced the turn-
over of 5HT as measured by a reduction in the ratio of H^3-
tryptophan to H^3-5HT. Bruinvels (1972) and Alpers and
Himwich (1972) reported reduced turnover of 5HT as a result
of tricyclic treatment. More recently, Leonard and Kafoe
(1976) showed a decreased turnover of serotonin with both
acute and chronic imipramine as well as DMI treatments.
Buus Lassen et al., (1975) tested the effects of tricyclics
administered 3 hours prior to sacrifice and all resulted in
lowering levels of 5HIAA.

ii) Norepinephrine

Schanberg et al., (1967) administered either IMI or
DMI 90 minutes prior to the intraventricular administration
of H^3-NE and demonstrated increased levels of H^3-normeta-
nephrine in the brains of drug treated rats. When the
drugs were given 90 minutes after the intraventricular
injection of H^3-NE and animals were sacrificed 3 hours
after the antidepressant, the levels of labeled norepin-
ephrine increased. Normetanephrine increased and deaminated

catechol metabolites decreased reflecting the anticipated response of agents which block the uptake mechanism. Schildkraut et al. (1969a) demonstrated greater decreases in levels of H^3-NE by treatment with secondary amines DMI and PRO than by the tertiary amines IMI and AMI. In fact AMI did not result in altering the levels of H^3-NE found in the brains of drug treated rats when compared with saline treated controls. These results are similarly reflected in the in-vitro data of Ross and Renyi (1967a) and Shaskan and Snyder (1970). Schildkraut et al. studied the effects of chronic administration of the tertiary amine, imipramine (1970), and the secondary amine protriptyline (1971), on the levels of norepinephrine and its metabolites. In these experiments, tricyclic antidepressants were administered by intra-peritoneal injection and H^3-NE followed 6 hours later by intracisternal injection. The animals were sacrificed either 6 minutes or 3 1/2 hours after the labeled compound was administered. Upon acute administration, imipramine significantly reduced the levels of H^3-NE when animals were sacrificed 6 minutes after the label whereas the reduction by protriptyline was not statistically significant. Normetanephrine levels were increased whereas deaminated catechols and O-methylated deaminated metabolites were decreased for both drugs studied. When the animals were sacrificed 3 1/2 hours after the label was administered, a trend towards increased values of norepinephrine was noted but did not achieve statistical significance. These

results could be explained within the framework of the proposed mechanism of action of these drugs as transport inhibitors. It seems reasonable that after 9 1/2 hours the drug was nearly completely metabolized and the neurons responded to the effect of decreased levels in the presence of drug to increasing their levels by facilitating the uptake of amines. Normetanephrine levels remained somewhat elevated and deaminated catechol metabolites were not nearly as depressed as after the 6 minute sacrifice period. Chronic administration of either drug resulted in decreased levels of H^3-norepinephrine when studied either 6 minutes or 3 1/2 hours after administration of the labeled amine. Similarly, normetanephrine was increased and deaminated catechol metabolites were severely depressed. The levels of endogenous amines were reduced below control for both drugs. The data suggests that both the secondary and tertiary amines have the same effect on norepinephrine metabolism when administered chronically; that of inhibiting uptake and increasing turnover as measured by the rate of disappearance of H^3-NE from the brain (levels of H^3-NE at 6 minutes vs. levels at 3 1/2 hours after label).

In a more recent study of the effect of chronic administration of IMI and DMI, turnover of the biogenic amines including norepinephrine was reported as being decreased although the decrease was not statistically significant (Leonard and Kafoe, 1976). In these experiments turnover was measured as the ratio of specific activity of labeled

product to labeled precursor after administration of H^3-tyrosine and H^3-tryptophan. The results are obviously at variance with that reported by Schildkraut et al. (1970, 1971) with respect to the turnover of norepinephrine, and the authors could not offer a reason for the apparant discrepancy. The effects of chronic administration of tricyclics on the turnover of norepinephrine awaits further study before a conclusion can be drawn.

iii) Dopamine

Dopamine has been the least studied of the catecholamines with respect to the effects of tricyclic antidepressants on its metabolism. Da Prada and Pletscher (1966) reported a slight but insignificant decrease of homovanillic acid (HVA) after an acute administration of imipramine whereas amitriptyline caused a significant increase in HVA. Similar results were obtained by Nielsen (1975) in a study of the affect of acutely administered amitriptyline. Levels of dopamine were shown to be increased although the increase did not achieve statistical significance. DMI, however, did increase the levels of dopamine significantly.

Chronic administration of both imipramine and DMI decreased the turnover of dopamine (Leonard and Kafoe, 1976).

CHAPTER III: EXPERIMENTAL WORK

A. Introduction:

The use of any experimental system necessitates an evaluation of its performance characteristics or characterization. The rigor applied to characterization is often goverened by the investigators knowledge and understanding of the system as acquired through a review of the literature and personal experience. In this regard, the following characterization experiments were performed to establish some of the important parameters pertinent to the experimental protocol as well as some characteristics believed to be indicative of a properly functioning system.

Following the characterization are experiments designed to directly test the hypothesis that protriptyline blocks the uptake of tritiated l-norepinephrine in an in-vitro hypothalamic synaptosomal test after acute, subacute and chronic administration of the drug. Since the methodology employed in the characterization differs little with that used for the test of the hypotheses, the methods section contains a "general methods" section and the deviations from the general methods are noted in the subsequent numbered subsections corresponding to each individual experiment.

Subsection i through ix can be considered characterization experiments and x through xii are specifically directed towards the objective of this thesis.

B. Methods:

General: Male Long Evans rats (Charles River Breeding

Labs) weighing 200-450 gms. were group housed with food and water given ad libitum in a windowless room in which an artificial photoperiod was provided (light: 0800-1600 E.S.T.). Animals were sacrificed by decapitation at approximately the same time each day (11:00-12:00 noon) to eliminate the possible source of error attributable to chronobiological variations in the uptake of neurotransmitters as reported by Lew and Quay (1974). The latency from decapitation to immersion of the dissected brain tissue into ice cold 0.32M sucrose never exceeded 1.0 minute and was usually accomplished within 45 seconds.

Brains were dissected using a modification of the method developed by Glowinski and Iversen (1966). The brain was removed from the skull and placed with its ventral side upon a previously cooled glass plate positioned on ice (generally within 20 seconds from decapitation). An icecold stainless steel spatula was used to make a medialsaggital incision to the level of the corpus callosum. With the lateral ventricles as the starting point the cerebral cortex was rolled back laterally to expose the hemisphere of tissue containing the characteristic striae of the striatum. The medial aspect of the striatal tissue was teased free with the blade of the spatula. After removal of both striatal tissue masses the cortex was folded closed and placed on its dorsal surface. The end of a curved spatula was inserted at the posterior portion of the Circle of Willis and maneuvered in such a way as to emerge at its

anterior portion. The tissue was dissected free along the side edges of the spatula with a dissecting needle. Although referred to here as hypothalamic this tissue mass is predominantly hypothalamic in origin with some mid-brain tissue as depicted in the diagram provided by Glowinski and Iversen (1966). The remaining tissue was placed on either lateral side and the hemisphere partially separated to observe the approximate location of the genu of the corpus callosum. The brain was then sliced in a coronal plane with the genu as its posterior boundary. This tissue mass was termed forebrain and contained cerebral cortex and some limbic structures.

The dissected tissue was immediately placed into ice-cold 0.32 sucrose (10% w/v) and allowed to sit on ice until all dissections were completed. The tissue was then homogenized in a Teflon[R] -glass tissue grinder with a clearance of 0.030 cm using 10 up and down strokes at about 1,000 r.p.m. The supernatant fraction of a 1,000 x g x 10 minute centrifugation carried out at $4^{o}C$ in a Sorvall RC-2B refrigerated centrifuge was used as the crude synaptosomal homogenate (CSH).

The incubation medium (IM) originally used in preliminary experiments was Kreb's bicarbonate (Umbreit et al., 1964), as modified by Snyder and Coyle (1969) and contained in millimoles (mM): NaCl (118.5), KCl (4.8) $CaCl_2$ (1.2) KH_2PO_4 (1.2) $MgSO_4 7H_2O$ (1.2), $NaHCO_3$ (24.8), d-glucose (11.1), ascorbic acid (1.1), disodium ethylenediamine tetra-

acetic acid (EDTA) (0.13) and the monoamine oxidase inhib-
itor (MAOI), nialamide (0.01). The ascorbic acid and EDTA
were added to increase the stability of the neurotrans-
mitters by preventing auto-oxidation shown to occur in
oxygenated media (Iversen, 1972).

One milliliter (ml.) of CSH was introduced into 6-8 ml.
of incubation medium (IM) resulting in a concentration of
0.4 to 0.9 mg. protein per ml. IM. Protein was determined
by the method of Lowry et al. (1951). Usually, six aliquots
were equally divided among $0^{\circ}C$ and $37^{\circ}C$ conditions. In
some experiments the number of replicates per condition
reached 8.

The radio-labeled neurotransmitter (2uCi/ml) was added
to the IM in a volume of 5 microliters (ul.) per ml. of IM
resulting in an IM which was 0.1 uM with respect to neuro-
transmitter. One ml. aliquots (minimum of 3 and maximum
of 8 determinations per condition) were incubated at $37^{\circ}C$
in a Dubnoff metabolic shaker (with no agitation) and an
equal number were kept on ice in glass test tubes (10 mm
O.D. x 75 mm). After incubation for the specified time
interval (norepinephrine and dopamine, 10 minutes; serotonin
5 minutes) the tubes were placed into a Sorvall RC-2B ref-
rigerated centrifuge to spin at 13,000 x g x 60 minutes to
form a pellet containing synaptosomes, mitochondria and
microsomes or the P_2 fraction described by Gray and
Whittaker (1962).

One-tenth ml. of the supernatant was placed into

scintillation vials containing 2 ml. of absolute ethanol prior to the addition of toluene phosphor scintillation fluid. Pellets were solubilized in 1.0 ml. of absolute ethanol with the aid of a bent dissecting needle used to break up the pellet and the solution was poured into a scintillation vial. The tubes were washed with 1.0 ml. of absolute ethanol and this wash was added to the scintillation vial prior to the addition of scintillation fluid.

Ten ml. of toluene phosphor containing 5.5 g/liter of 2,5-diphenyloxazole (PPO) and 125 mg/liter of B-bis-(2-phenyloxazole)-benzene (POPOP) in toluene. The radioactivity was counted in a Packard Tri-Carb Model 3335 for 10 minutes. The efficiency for tritium was optimum (45%) at a gain setting of 57.5 with the window virtually wide open. Quenching between samples was not found to be significant and therefore no correction was made. Background counts was subtracted from all samples with counts less than ten times the value obtained for background.

The data are expressed as either Tissue (or Particulate): Medium ratios or uptake in nanograms/mg. protein/10 minutes (or 5 minutes where applicable). In preliminary experiments calculations were performed with the aid of a computer. Two programs were used the more complex of which is presented in the appendix, Figure A-1 is a flowchart and table A-1 is the program listing. The less complicated program was a modification of VIVO to allow for a single value of protein concentration per neurotransmitter (or

brain region) condition.

Mean values obtained by incubating at $0-4^{\circ}C$ were routinely subtracted from values obtained at $37^{\circ}C$ as a control for non-specific adsorption of neurotransmitter to a variety of binding sites including plasma proteins and presumably the transport carrier itself. Uptake was not found to be saturable at $0-4^{\circ}C$ over the concentration range 0.04-2.0 uM. An untreated sample was used as a value representing maximal uptake with which drug treated samples were compared. Hypothalamic homogenate was used for nor-epinephrine uptake studies striatal for dopamine and fore-brain for serotonin.

i) Determination of neurotransmitter integrity:

The radioactive neurotransmitters used in this study were obtained from New England Nuclear Co. as H^3-1-nor-epinephrine (H^3-1-NE: 5.85 Ci/mM), 5-hydroxytryptamine binoxalate (H^3-5-HT: 2.2 Ci/mM), and dopamine (DA: 1.0 Ci/mM). The corresponding unlabeled compounds were obtained from Sigma Chemical Company as well as the O-methylated and deaminated metabolites of the neurotransmitters. The unlabeled compounds were spotted on cellulose NM300 thin layer chromatographic plates in 10 ul. volumes containing 1-5 ugms. of the test substance as originally described by Giese et al. (1967). The plates were allowed to dry after spotting and were equilibrated in a TLC tank for 30-60 minutes prior to immersion into the solvent (butanol: 5N acetic acid=100:35). The solvent travel was spotted after

approximately 15 cm. Clear separation of all the neuro-
transmitter was obtained and a test system for neurotrans-
mitter integrity was established by spotting the labeled
neurotransmitter. After developing, the visualizable spot
was scraped off and placed into a scintillation vial and
counted. All adsorbent below the spot was placed into a
scintillation vial as was all adsorbent after the spot.
High counts found only directly on the visualized spot were
interpreted to mean the neurotransmitter remained intact.
Once radioactivity either before or after the visualized
spot was apparent, the labeled neurotransmitter was no
longer used.

ii) Time course for incubation

All samples incubated at $37^{o}C$ were placed in the hot
water bath and removed sequentially over the time intervals
reported in the results. Another set of tubes identically
prepared except that the labeled neurotransmitter was
omitted, were placed in an ice-water bath. The labeled
neurotransmitter was added at various times during the
course of the total incubation time beginning with longest
time interval sample (prepared just prior to introducing
the $37^{o}C$ samples into the heated water bath). All tubes
were vortexed after addition of the labeled neurotrans-
mitter. Results are expressed as Tissue:Medium ratio
calculated as ngms H^{3}-l-NE gm^{-1} brain:ngms. H^{3}-l-NE $ml.^{-1}$
supernatant. This experiment was repeated 6 times with the
brains of 2-6 animals pooled in different experiments.

iii) Synaptosomal concentration versus uptake

The synaptosomal concentration was varied by delivering different amounts of the CSH to the IM and adjusting to an identical final sucrose concentration in each tube by the addition of an appropriate volume of 0.32M sucrose. The tubes were kept in an ice-water bath until the start of the experiment at which time half were transferred to the hot water bath for the previously specified periods of time. The brains of two to six animals were pooled for each experiment and the experiment was repeated five times. The results are expressed as a Tissue (Particulate): Medium ratio calculated ngms. H^3-1-NE/gm brain wet weight: ngms H^3-1-NE/ml and supernatant in an attempt to demonstrate the concentrative property of the synaptosomal preparation.

iv) Effect of calcium ions and ethylenediamine-tetra-acetic acid

An attempt to evaluate the contribution of facilitated exchange diffusion in the synaptosomal preparation was made by varying the calcium concentration in the media and employing ethylenediamine tetra-acetic acid (EDTA) to chelate endogenous calcium. Six determinations were made at both 0 and 37°C. The mean of the six 0°C samples was subtracted from each of the 37°C values followed by the transformation to uptake expressed as ngms H^3-1-NE/mg ptotein/ 10 minutes. The media of Coyle and Snyder (1969) was used as a control to which greater or lesser concentrations of calcium were compared.

The brains of six aminals were pooled and aliquots

were placed in media that was identical to the media of
Snyder and Coyle (1969) except that calcium and EDTA were
modified as follows. For the first condition, 1.2mM cal-
cium was replaced with 10mM Ca^{++} and EDTA was omitted. The
calcium concentration was then lowered to 2.5mM in the
second condition. The third condition employed was that of
Snyder and Coyle (1969) as a control. In the fourth tube
the medium was modified by deleting calcium and adding 0.1
mM EDTA. The EDTA concentrations were increased logarith-
mically in subsequent tubes to 1.0 and 10mM. The pH was
checked in each and found to require adjustment in the 10
mM EDTA solution by the drop-wise addition of 0.1N NaOH to
bring the pH from 6.6 to 7.4. All other solutions were in
the range 7.35-7.45.

v) Kinetics

Preliminary studies of the kinetics for the transport
of biogenic amines into the CSH included all three neuro-
transmitters, l-norepinephrine, serotonin and dopamine.
Seven substrate concentrations used during preliminary
investigations ranged from 2.0 to 0.01 uM (2-10uCi/ml.).
An aliquot of the supernatant was used as a measure of the
total radioactivity in the media since the difference
between IM and supernatant counts did not differ by more
than 7% at any time. The brains of 2 animals were pooled
and the media of Coyle and Snyder (1969) was used in the
studies using serotonin and dopamine as substrate.

l-Norepinephrine kinetics was performed with a calcium-

free media supplemented with 0.1 uM EDTA. The substrate
concentrations ranged from 2.0 to 0.01 uM (2-10 uCi/ml.).
Three determinations were made for each condition and the
data was transformed to uptake expressed in ngms H^3-1-NE/
mg protein/10 minutes. The experiment was repeated three
times.

vi) Subcellular distribution of H^3-1-norepinephrine

Continuous sucrose gradients were prepared using a
Beckman Density Gradient former resulting in a linear
gradient from 0.38 to 1.4M sucrose in a 12 ml. volume. The
gradients were prepared 3 hours prior to their use and
stored in an ice-water bath until ready for use. The CSH
was prepared as an outlined above and after incubation and
subsequent centrifugation the pellet was resuspended in a
1.0 ml. volume and layered onto the sucrose gradients.

An identical aliquot of the CSH was prepared in media
in which the monoamine oxidase inhibitor, nialamide, was
omitted. Unlabeled 1-NE was added to a final concentration
of 0.1 uM and processed in an identical manner as the radio-
active sample.

The gradients were centrifuged for 90 minutes at
130,000 x g in an International ultracentrifuge using a
swinging bucket rotor. Fractions were obtained by piercing
the bottom of polyallomar centrifuge tubes and collecting
0.4 ml. fractions.

The samples containing radioactive neurotransmitter
was assayed for tritium by placing 0.1 ml. samples into

scintillation vials containing 2 ml. of absolute ethanol to which 10 ml. of toluene phosphor was subsequently added.

Unlabeled neurotransmitter samples were assayed for monoamine oxidase activity by a modification of the method of Wurtman and Axelrod (1963). A 0.1 ml. aliquot of the sample was added to 0.25 ml. of phosphate buffer (pH 7.4) in a test tube. C^{14}-tryptamine was added and the tubes were incubated at $37^{\circ}C$ for twenty minutes. The reaction was stopped by the addition of 0.2 ml of 2N HCl and the indole acetic acid formed was extracted into 6 ml. of toluene by shaking. Four ml. aliquots of the toluene were placed into scintillation vials and 10 ml. of toluene phosphor added prior to counting. The results obtained are expressed as percent of radioactivity in each fraction compared with the sum of all fractions.

vii) Regional distribution of neurotransmitter uptake

The uptake of each neurotransmitter into each brain region employed in this study was investigated. The method employed was similar to that outlined above in methods except that all these brain regions were studied for each neurotransmitter. The media used was that described by Snyder and Coyle (1969).

The results are expressed as Particulate:Medium ratio calculated as CPM/mg protein/Incubation time: CPM/ml. supernatant.

viii) Effect of calcium in l-norepinephrine release
induced by hi-potassium depolarization

The role of calcium in the release of norepinephrine

and its consequent effect on uptake was studied. The hypothalami of ten animals were pooled and divided into equal aliquots. Preincubation media used were as follows: (1-2) hi-K^+ (45mM) plus Ca^{++} (2.5 mM), (3) normal Kreb's (2.5 mM Ca^{++}) and (4) hi-K^+ less Ca^{++}. The hi-K^+ media was prepared by equimolar replacement of Na^+ by K^+. The CSH aliquots were incubated in twice the normal incubation medium volume resulting in a protein concentration of 0.24 mg/ml. Incubation was carried out at $37^{o}C$ for 15 minutes with continuous oxygenation of the media with $O_2:CO_2$ (95:5). The tubes were then centrifuged at 15,000 x g for 15 minutes and the resultant pellets reconstituted with modified Kreb's (i.e. no Ca^{++} + 0.1 mM EDTA) in half the volume used for the preincubation. To tube #1 was added the diluent for the neurotransmitter. To the second tube was added a calculated amount of unlabeled neurotransmitter required to completely refill the synaptosomes (to 1 ugm/g tissue wet weight). Tube 3 received neurotransmitter diluent as did #4 and all tubes were incubated for 30 minutes at $37^{o}C$. Following centrifugation at 15,000 x g for 15 minutes the pellets were resuspended in modified Kreb's media to which H^3-1-NE was added to achieve a resultant concentration of 0.1 uM and four one ml. aliquots of each tube were incubated at 0° and eight one ml. aliquots were incubated at $37^{o}C$ for 10 minutes. All tubes were then centrifuged at 13,000 x g for 60 minutes in a Sorvall RC-2B centrifuge. The pellets were solubilized in the usual manner described in the gen-

eral methods. The data was calculated as ngm H^3-1-NE/mg protein/10 minutes and expressed as percent of control.

ix) IC_{50} for some representative tricyclic anti-depressants

The ability of tricyclic antidepressants to block the uptake of labeled neurotransmitter by 50 percent (i.e. IC_{50}) was studied by varying the concentration of antidepressant using 4 concentrations increasing progressively by one order of magnitude. The uptake of neurotransmitter calcu-lated as ngms H^3-1-NE/mg protein in the drug treated samples were then compared with an untreated control and expressed as a percent of control. The concentration of drug resulting in uptake representing 50% of control was determined graphically and described in the results.

x-xi) Protriptyline - acute and subacute time course

Protriptyline was administered intraperitoneally either acutely (20 mg/kg) or subacutely (10mg/kg) at varying times prior to sacrifice as stated in the results. Acute refers to a single injection prior to sacrifice, whereas, subacute refers to 6 consecutive days of injections. On any given day two animals from each treatment condition was represented to randomize day to day experimental variations. The animals were sacrificed at approximately the same time each day necessitatingaschedule of injection running through-out the day (i.e. 5:00, 9:00, 11:00 A.M. and 11:00 P.M.).

Three determinations were made for each condition (i.e. $0^{\circ}C$ and $37^{\circ}C$). Incubations were performed in modified Kreb's

media (no Ca^{++} + 0.1mM EDTA).

xii) Protriptyline - chronic time course

One hundred animals were randomly assigned into five groups of twenty animals each. These five groups were administered either saline or drug beginning on five consecutive days. Within each group of twenty, four groups of five animals each represented either saline injected controls or 2, 6 and 24 hour post-protriptyline (10 mg/kg i.p.) injected treatments. The brains of 5 animals in each group were pooled prior to homogenization and the uptake study was carried out over the course of 5 days after 18 consecutive days of injections for each group of twenty animals.

C. Results and discussion:

Included in the results sections are discussions pertaining to the individual experiment which serve to develop a sense of continuity and an appreciation for the course of investigation taken in this thesis. The general discussion to follow considers the results of the research presented here as it relates to more general considerations of the possible mechanism(s) of action of tricyclic antidepressants.

The terminology used in the literature with respect to neurotransmitter transport studies is governed largely by the methodology employed (Graefe, 1976). Although the definitions reviewed here are not strictly adhered to in the literature, it is useful to describe the differences and establish the terminology used in this report. "Uptake" of amines can be defined as transmembranous flux into the cell regardless of whether or not intracellular accumulation of the substrate occurs. Many studies attempt to measure "net uptake" which takes into account the accumulation of unchanged amine by the tissue preparation. The tissue preparation can lose accumulated amines by MAO metabolism and a common approach is to inhibit MAO in an attempt to obtain an estimate of net uptake. The use of amines which do not undergo metabolism by MAO (e.g. metaraminol) is an alternate approach to obtain a measure of net uptake. In either case the measurement only approaches the true value of net uptake because exchange processes are

usually not considered by investigators. There is suffi-
cient evidence to suggest that both hetero-exchange as well
as homo-exchange processes do occur (Cohen & Lajtha, 1972;
Levi et al., 1976). Many studies use short incubation times
to maximize the magnitude of unidirectional flow of sub-
strate and prolonged incubation usually results in a devia-
tion from linearity. "Re-uptake" refers more to the process
in-situ and is inappropriately used as a synonym for uptake.
This thesis utilized the term uptake to represent an appar-
ent accumulation of amines and assumes that exchange pro-
cesses are not significant over the short incubation times
used.

General:

The reproducibility of the brain dissection procedure
is reported in table 1 and compares favorably with the values
reported by Glowinsky and Iversen (1966). Since the bound-
aries differ some, the difference in values between those
published and reported here appear to be reasonable. The
weighed brain regions were carried through the homogeniza-
tion procedure and the functional relationship between the
wet weight of brain tissue for a given brain region and the
resultant concentration of protein in the IM was determined
as shown in figure 1 for the hypothalamus. From the inter-
section of two regression lines (i.e. y on x and x on y) the
mean values of both variables are obtained. From these
values corrections for dilution can be made and the appro-
priate relationships between mg protein (as a crude measure
of synaptosomal concentration) and a gram of tissue wet weight

Table 1. Reproducibility of the brain dissection procedure

Brain region	Tissue wet weight ($\bar{X}$ ± s.e.m.)
Hypothalamus	133 ± 8
Forebrain	199 ± 8
Striatum	85 ± 4

Mean weights for various brain regions dissected from 24 animals.

Figure 1. Functional relationship between brain tissue wet weight and resultant protein concentration in the incubation medium.

$X_y = 12.1 + 415.4\ Y$

$P < 0.005$

$Y_x = 0.076 + 0.00162\ X$

$P < 0.005$

INCUBATION MEDIUM PROTEIN CONCENTRATION (mg/ml)

TISSUE WET WEIGHT (mg)
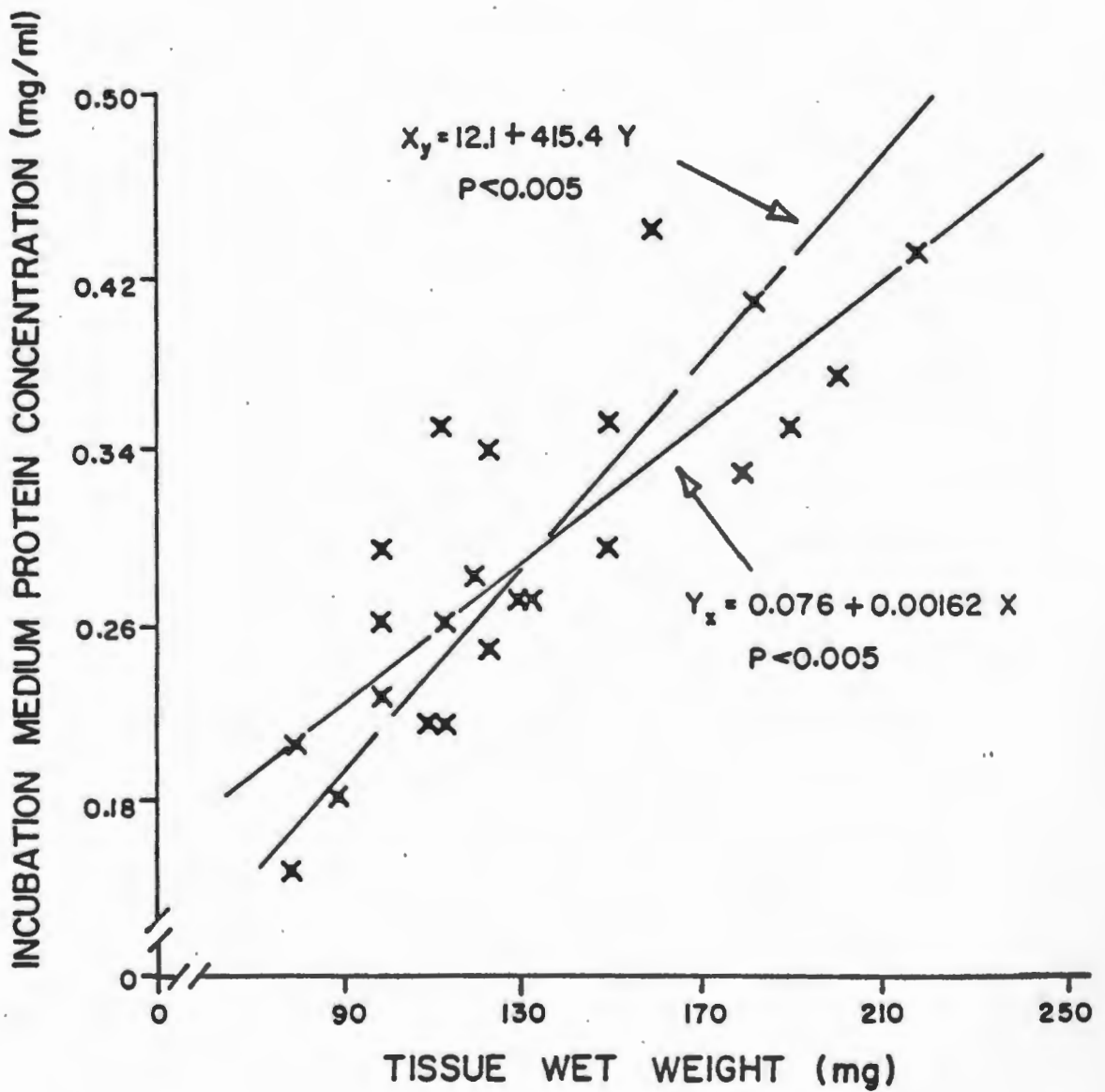
can be formulated. One gram of forebrain, hypothalamus and striatum are equivalent to 460, 300 and 285 mg protein respectively. Conversions from mg protein to grams wet tissue weight were made using these relationships.

i) Neurotransmitter integrity

The functional integrity of the substrates used in biochemical systems of the type reported here is an important consideration with respect to interpreting the results. Structual changes of the substrate molecule can result in drastically altering the observed kinetic constants as well as possess inhibitory properties themselves as outlined in the literature review. Monitoring the integrity of the neurotransmitters is important and can be accomplished in at least two ways. A simple method is to use chromatography to identify the substance by separating it from closely related molecular species. The procedure outlined by Giese et al. (1967) and reported here clearly resolved all three neurotransmitters with virtually no overlap in the resultant radio-chromatogram shown in figure 2. De-aminated and O-methylated metabolites were shown to possess clearly distinguishable spots on the developed chromatogram. The thin layer chromatographic (TLC) procedure was performed between series of experiments on both the unlabeled stock solution as well as the radioactive neurotransmitter.

A method for the detection of specifically inhibitory contaminants in radioactive substrates is described by Timpton et al. (1977). The advantage of this procedure is

Figure 2. Integrity of radioactive neurotransmitter sub-
strates determined by thin layer chromatography.
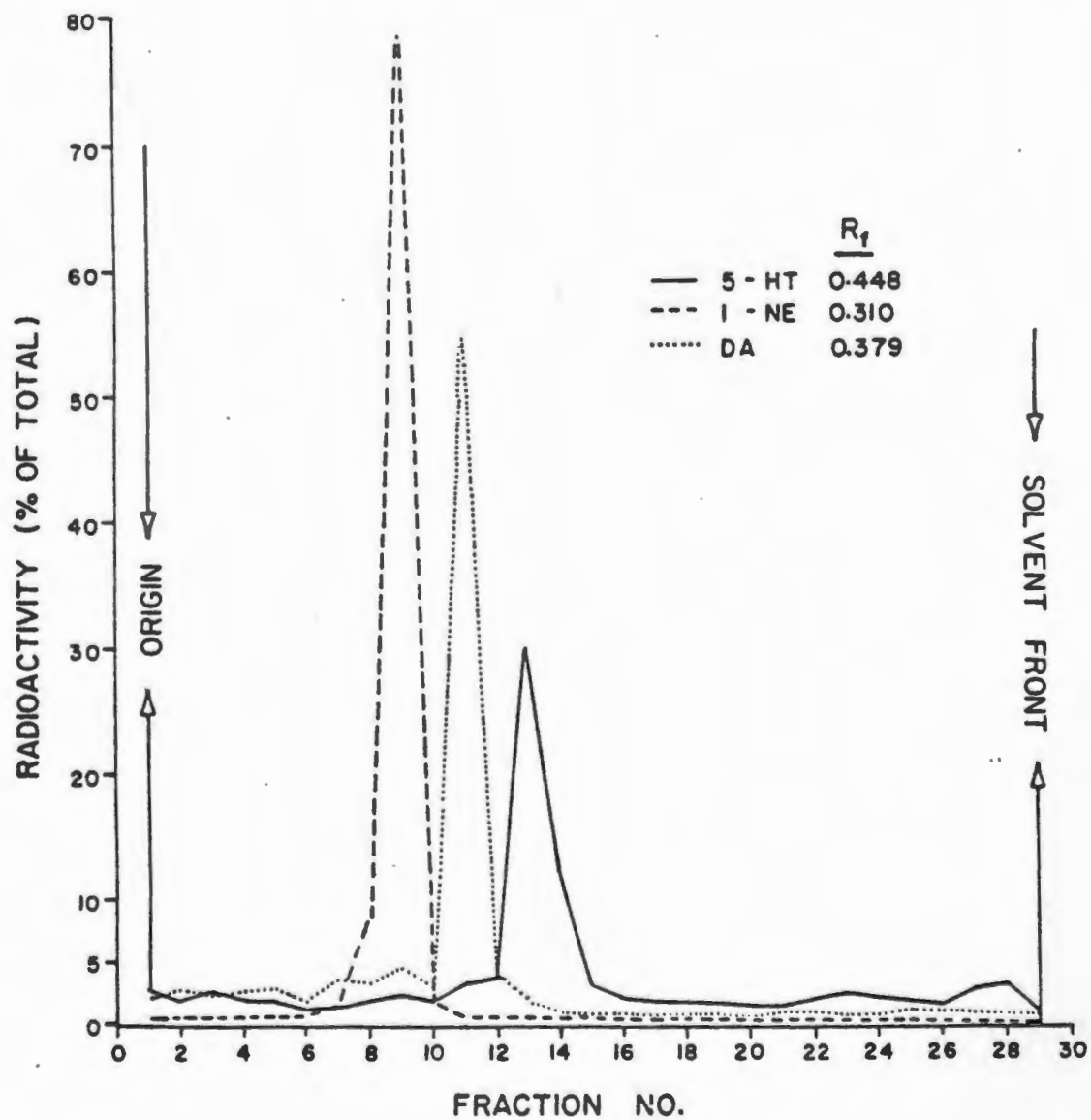
Figure 3. Effect of incubation time upon the uptake of
H^3-l-NE into hypothalamic crude synaptosomal
homogenate.
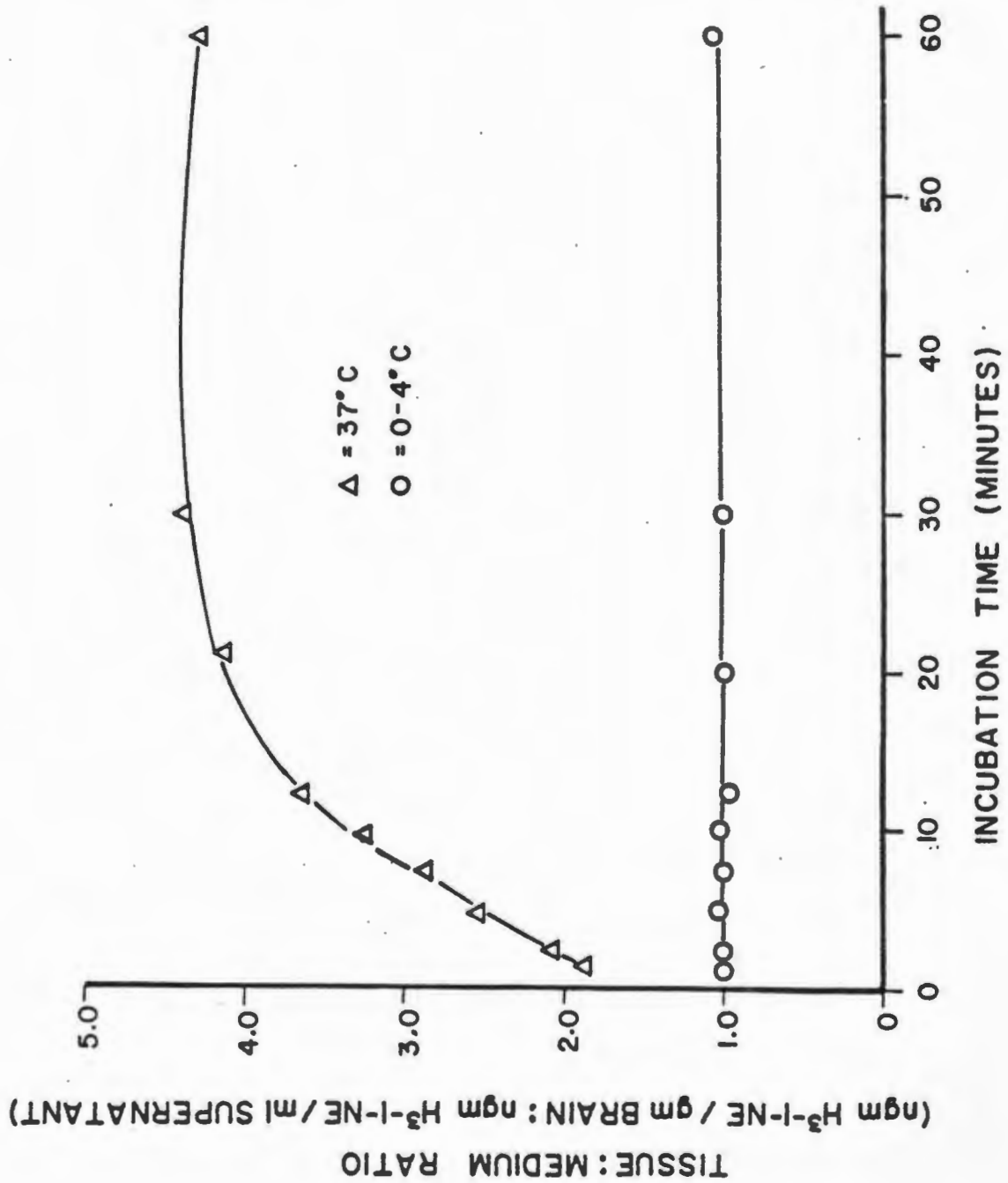
minutes). Similar results were obtained for DA uptake into striatal CSH. These results agree with those published (Coyle and Snyder, 1969; Snyder and Coyle, 1969). Serotonin uptake was linear for six minutes and this value agrees exactly with that reported by Shaskan and Snyder (1970). Wong et al. (1973) demonstrated linearity for 5HT uptake for at least 3 minutes and the difference may be accounted for by the use of a 5 minute pre-incubation or equilibration of the IM prior to introducing the radioactive neurotransmitter and as well as a lower incubation temperature ($30^{o}C$). One more complicating factor includes the use of a velocity term as the ordinate of the time course curve presented by these investigators. The velocity term (nM/mg protein/3 min) requires each value for uptake at each time point to be corrected for uptake over 3 minutes. Making the appropriate correction from the velocity term to a concentration term (nM/mg protein) results in a linear function for at least 5 minutes. As a result of these experiments, incubation times for the uptake of NE and DA of 10 minutes were established and 5 minutes for 5-HT. The Tissue:Medium ratio shown in figure 3 demonstrates that the process at $0^{o}C$ seems to be one of adsorption or diffusion whereas at $37^{o}C$ a concentrative phenomenon appears operative with ratios as high as 4. Dopamine uptake gave ratios one order of magnitude higher than those of 5-HT or NE. The tremendous concentrative power of dopaminergic nerve endings in the striatum may be as a result of finer nerve terminals

resulting in increased surface area for uptake as suggested
by Glowinski and Iversen (1966).

iii) Synaptosomal concentration versus uptake

The concentration of tissue in the incubation medium
was shown to vary considerably as a result of the variation
in the dissection procedure. Another source of this type of
variation is the physical size of the brain which varies
with the age or weight of the animal. Differences in pro-
tein concentration (assumed to be a measure of synaptosomal
concentration) was studied in order to understand the nature
of this variable and apply whatever corrections seemed nec-
essary. The results shown in figure 4 for H^3-1-NE are
representative of the results found for the uptake of
labeled 5-HT and DA. The points represent the mean of 3
determinations with the coefficient of variation less than
10% for values above 0.5 mg protein per ml. of IM. Values
below 0.5 mg had a much larger degree of variability assoc-
iated with their means. The resultant curves were calculated
using the original data points by polynomial regression.
The data was shown to be satisfied best by the second degree
polynomial equations shown in the figures with their atten-
dent levels of probability.

Two interesting points can be made by observation of
figure 4. First, the ordinate is expressed as uptake in
ngms H^3-1-NE corrected for mg protein. Theoretically, if
increasing synaptosomal concentrations results in an increase
in uptake described by a linear function, one might expect

Figure 4. Effect of synaptosomal concentration in the incubation medium upon the uptake of H^3-l-NE into hypothalamic crude synaptosomal homogenate.

$$-\text{x}-\ Y_{37°C} = 4.09 - 7.06X + 4.08X^2 \quad P<0.01$$

$$-\text{O}-\ Y_{0°C} = 3.67 - 8.25X + 4.89X^2 \quad P<0.01$$
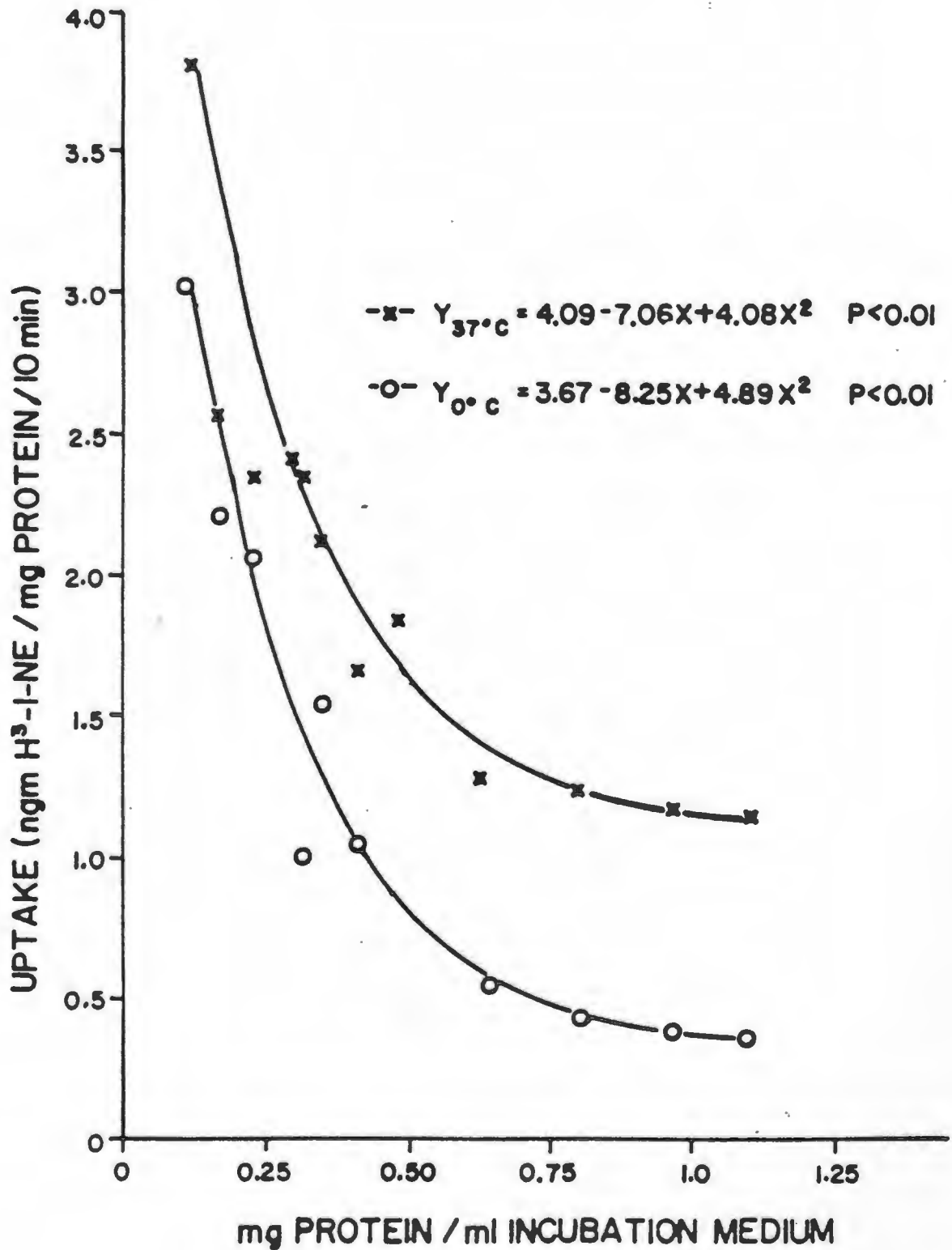
that uptake corrected for mg protein would be a straight line with a slope close to zero. At concentrations of protein greater than 0.5 mg/ml. this theoretical curve appears to be approximated fairly well.

The tremendous increase in uptake demonstrated at low synaptosomal concentrations may be explained by an increase in membranous surface area. Since the uptake is increased for both 0 and $37^{o}C$ one might conclude that the increase is due to adsorption to the membranous particles whose surface area increases tremendously as a result of dilution. Concentrations above 0.5 mg/ml. may reflect a state where by a certain degree of clumping occurs resulting in minimizing the surface area exposed to media substrate. This clumping effect appears not to increase tremendously over the range 0.5 - 1.1 mg protein/ml. IM although a slight decline in uptake may be detected with increasing concentrations of protein.

Secondly, it may be noted that the difference between uptake at $37^{o}C$ and $0^{o}C$ is constant for any synaptosomal concentration. This finding is important for it implies there is no effect of packing upon the measure of protein. Interestingly, the relationship holds for low synaptosomal concentrations where, admittedly, the variability is high. An attempt was made to stay within the specified range where variability was lowest. Similar relationships for striatal and forebrain tissue were found and the critical values obtained were 0.16 and 0.25 mg protein per ml. IM respectively.

iv) The effect of calcium and ethylenediamine-tetra acetic acid

The results of manipulating the calcium concentration in the media are presented in figure 5. A one-way analysis of variance of this data resulted in rejection of the hypothesis that the means were equal at $P < .005$ ($F_{obs} = 33.9$ compare with $F(5, 26; \alpha = .005) = 4.38$). An F_{max} test for homogenity of variance demonstrated no violation of the assumption. A Student-Neumen-Keuls test for differences between treatment means indicated that the Snyder and Coyle (1969) medium resulted in uptake significantly different from that obtained with normal Kreb's medium(Snedecor and Cochran, 1967). When calcium was removed from the medium and 0.1 mM EDTA added, the uptake into hypothalamic synaptosomes was shown to be significantly greater than the value obtained using the medium of Snyder and Coyle (1969). Higher concentration of EDTA resulted in attenuation of the response.

Much mystery still surrounds the role of calcium in stimulus-secretion coupling mechanisms of neurotransmitter release (Rubin, 1970; Phillis, 1974; Baldessarini, 1975). As has been pointed out earlier in this report, preliminary experiments were performed using the medium described by Snyder and Coyle (1969). The rationale for its use was never made clear in the literature. In 1968, Snyder et al. reported the results of kinetic experiments of racemic H^3-NE in brain slices. The medium described was both normal Kreb's and an ascorbate, EDTA and pargyline supplemented

Figure 5. Effect of altering calcium concentrations in the incubation media upon H^3-l-NE uptake into hypothalamic crude synaptosomal homogenate.
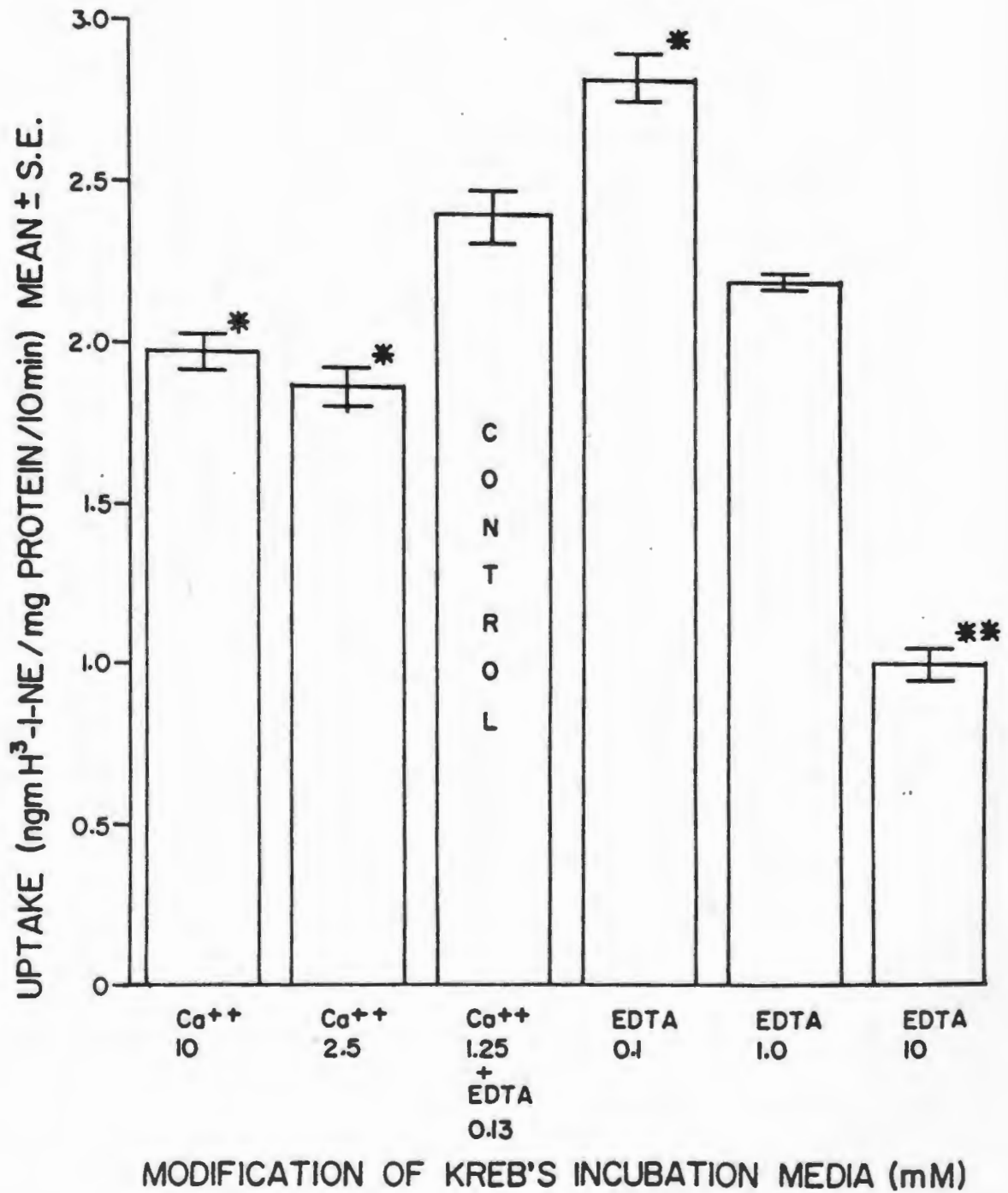
Kreb's. The authors suggested that EDTA and ascorbate
reduced auto-oxidation and pargyline prevented the formation
of deaminated metabolic products of NE, but calcium levels
were not reduced. Subsequently, Snyder and Coyle (1969)
published the use of their calcium reduced medium and it has
been used in subsequent studies with no attendant discussion.

EDTA is a chelator of divalent cations. The molar
ratio of divalent cation to chelate molecule is, at the
theoretical limit, equal to two. Since EDTA possesses four
carboxylic acid groups it is theoretically possible for two
calcium ions to electrically neutralize one EDTA molecule.
EDTA, however, is not specific for chelating calcium, Martell
and Sillen (1964, 1971) report the metal chelate stabilities
of EDTA to be greater for Cu (ll), Zn (ll) and Fe (ll) by 70,
49 and 30 % than for calcium. Magnesium has a stability
constant 23% lower than calcium.

Complex ion-chelator formations may also occur in acidic
media by protonation of the amine groups. Therefore, it is
with considerable conserative bias that the theoretical
stoichiometric limit of 2 moles of Ca^{++} required to form a
complex with one mole of EDTA is used to calculate divalent
ion equivalency in the various media employed. Theoretically
then, 0.13 mM EDTA would complex 0.26 mM Ca^{++} rendering the
media of Snyder and Coyle equivalent to 0.99 mM Ca^{++} Kreb's.
In consideration of the conservativeness eluded to above, a
Ca^{++} concentration slightly higher than this might be more
equivalent to the Snyder and Coyle medium.

In a separate experiment the uptake of l-NE into hypo-
thalamic CSH was measured using the Snyder and Coyle medium
compared with 1.25 mM Ca^{++} Kreb's. The results, analyzed
using a two-tailed t-test did not show the means to be
significant at $P < .05$ although the 1.25 mM Ca^{++} mean value
obtained was approximately 9% below the value obtained
using the Snyder and Coyle medium. Acceptance of the null
hypothesis suggests that the two media may be equivalent
with respect to the availability of Ca^{++} ions.

The media of Snyder and Coyle was chosen in an attempt
to duplicate the experimental protocol and compare the
results as an aid to characterization of the system. Sub-
sequent to these preliminary studies use of the medium was
questioned and prompted this experiment. The choice of
deleting calcium and adding EDTA was made using purely
theoretical considerations. Since uptake studies attempted
measuring unidirectional flux it was reasoned that efflux
of endogenous amines in calcium enriched media might con-
found the results. Indeed both homo-and hetero-exchange
processes are known to occur in transport systems of neuro-
transmitter amino acids (Cohen and Lajtha, 1972). Pre-
venting the calcium dependent process would serve to reduce
the accumulation of label by reducing its specific activity
in the IM. Controlling dilution by calcium-independent or
spontaneous release of endogenous neurotransmitter is not
possible and should be considered as a source of error in
any estimate of unidirectional transmembranous flux of

neurotransmitter molecules.

The results indicate that removal of calcium, up to a point, does result in an increase in the value of uptake. Increasing the EDTA concentration beyond 0.1 mM results in a dose dependent decline in uptake. Interestingly, Rajan et al. (1977) reported a dose dependent inhibition of H^3-NE binding by metal chelators in mouse cerebral vesicles in vitro. It is possible then that high concentrations of chelating agent interfere with metal coordination complex formation within vesicles. It is also possible that since the chelating agents bind to copper and iron, high concentrations of chelators may interfere with cell respiratory processes requiring these metal ions. Either mechanism may result in the attenuation of uptake demonstrated at high EDTA concentrations.

Blaustein et al. (1972) demonstrated a 37% decrease in synaptosomal norepinephrine release from a calcium-free normal Kreb's medium compared with 2.5 mM Ca^{++} normal Kreb's. The magnitude of this release, calculated from the data provided in the paper and making the assumption that one rat brain weighs approximately 2 grams, results in a value of 2.2 ng NE/g wet weight/min released into the normal Kreb's medium and 1.45 ng NE/g wet weight/min in Ca^{++}-free Kreb's. The data presented in figure 5 demonstrates a 54% increase in uptake using a Ca^{++}-free EDTA-enriched medium when compared with normal Kreb's. The value of uptake obtained in the experiments described in this thesis are

5.5 and 8.5 ng/NE/g/min in normal Kreb's and calcium free EDTA enriched Kreb's respectively. These figures indicate that Ca^{++} independent or spontaneous neurotransmitter release may contribute significantly to homoexchange processes confounding a measurement of unidirectional flux into synaptosomes.

Kant and Meyerhoff (1977) studied the effect of calcium on norepinephrine release from hypothalamic tissue minces. These authors compared the release of NE in a normal Kreb's versus a calcium free Kreb's media at $0^{o}C$ and reported a seemingly insignificant increase (5%) of release in normal Kreb's. Lane and Aprison (1977) reported all three neurotransmitters (i.e. NE, DA & 5-HT) to behave similarly. Since the release of endogenous neurotransmitter can be minimized by modifying the media all subsequent experiments reported here have been performed with calcium-free EDTA enriched (0.1 mM) Kreb's media referred to herein as "modified Kreb's".

v) Kinetics

Characteristics of transport systems of the type used in this study have been determined using relationships described by enzyme kinetics. Linear transformations of the Michaelis-Menten equation leads to graphical techniques for the determination of kinetics constants (i.e. Km and V_{max}). A number of linear transformations are possible and most are reviewed in standard texts on enzyme kinetics (Roberts, 1977). In recent years a growing number of investigators have become concerned with the validity of the methods employed.

The most popular method by far is that of Lineweaver-Burk
(1934), although Dowd and Riggs (1965) demonstrated that
this method led to least accurate determinations of kinetic
constants whether the curve was plotted by eye or using the
method of least squares. The Hofstee plot (S/v vs. S) was
considered the most favorable since it was viewed that of
the two variables S had less error associated with it than
v. Estimates of kinetics constants can be obtained using
non-linear statistical techniques (Cleland, 1967; Wentworth,
1965a, b) but these methods require iterative procedures and
have not demonstrated popularity among biochemists. Elmore
et al. (1963) applied a linear regression technique to the
Lineweaver-Burk transformation which accounted for the
variability associated with each data point. Wilkinson
(1961) applied a similar technique except that a weighting
factor was used with the assumption that the distribution
of the observations of the velocity about a given substrate
concentration was directly proportional to the magnitude of
the velocity. This assumption can be shown to be true in
many biological systems where analysis using Michaelis-
Menten kinetics apply. Probably the best method from the
standpoint of a mathematical approach to the determination
of kinetic constants is that of Wilkinson (1961) although
it requires the assumption of normally distributed data as
any parametric method does.

Recently, a graphical method for the analysis of kin-
etic data called the direct linear plot (DLP) has been

reported by Eisenthal and Cornish-Bowden (1974). Non-para
metric statistical considerations supporting the use of this
method have been described (Cornish-Bowden and Eisenthal,
1974; Trager and Porter, 1977). The advantage to the direct
linear plot over the other methods as pointed out by the
authors is that it is simple to construct with kinetic con-
stants read directly off the graph without calculations as
is done with reciprocal plots. It is extremely sensitive
to outliers or abberant observations and provides accurate
determinations of kinetics constants.

The kinetics data for 1-NE uptake into hypothalamic
CSH was analyzed using a number of different methods, some
graphical and some statistical. The results obtained by
various methods are shown in table 2. The easiest to pre-
pare. The kinetics constants obtained were compared and the
results obtained indicate the direct linear plot to be as
good an estimator of the kinetics constants obtained by
either Wilkinson (1961) regression or the standard para-
metric linear regression model described by Elmore et al.
(1963).

The DLP was used in the calculation of all kinetics
data reported here and is described briefly to acquaint the
reader with the technique. The interester reader is refered
to the original references of Cornish-Bowden and Eisenthal for
the theory and a more thorough discussion of techniques and
applications. The DLP is so named "...because it represents
each observation by a straight line, ..." and utilizes the
concept of plotting data in parameter space rather than

Table 2. Comparison of methods used to determine kinetics constants for H^3-1-NE uptake into rat hypothalamic CSH

Km (uM)	V_{max} (ng/mg prot/10min)	Method	Reference
0.308	2.85	1/v vs. 1/S	Lineweaver-Burk, 1934
0.239	2.434	S/v vs. S	Woolf plot (Haldane & Stern, 1932)
0.236	2.40	v/S vs. v	Augustinson (Hofstee)
0.262 $\pm$.018	2.539 $\pm$.098	reduced weighted regression assuming constant coefficient of variation	Johansen & Lumry, 1961; Wilkinson, 1961
0.276	2.628	weighted linear regression	Steel and Torrie, 1960; Elmore, Kingston & Shields, 1963
0.235	2.340	Direct Linear Plot	Eisenthal & Cornish-Bowden, 1974

observation space. The ordinate is labeled V_{max} and the abscissa Km and the corresponding set of observations, i.e. v and S, are represented as points on the abscissa and ordinate, respectively. A line is drawn through each set of data points and is continued as far as possible into the first quandrant. The data for l-NE kinetics is shown in figure 6 using the DLP. It can be demonstrated that for n pairs of observations there exists 1/2 n(n-1) intersections of the lines extended into the first quandrant. The non-parametric theory specified the most likely unbiased estimator of the parameters to be determined is given by the point associated with the median of all interecting lines. That point can be taken from the associated values of the axes labeled Km and V_{max}. For 6 data points there are 6/2 (6-1)= 15 intersections with the median being the point described between the points designating the seventh and eighth intersecting lines. Two intersecting lines yields one point whereas three intersecting lines coming close to the same place can be shown to be represented by three points. Counting may begin from the low left corner of quadrant 1 and proceed to the upper right. The first circle represents 4 intersecting lines or 5 intersections. Since we are interested in that intersection which falls between 7 and 8, that intersection must be in the second circle (i. e. between intersections 4 and 9).

Comparisons of the kinetic data obtained using the DLP with reported values are presented in tables 3, 4 and 5 for

Figure 6. 'Direct Linear Plot' of H^3-1-NE uptake into hypothalamic crude synaptosomal homogenate.

Table 3. Kinetic constants obtained for norepinephrine uptake using substrate concentrations in the high affinity range.

K_m (uM)	V_{max} (nM/g × min)	Reference	Remarks
.0.4	0.11	Ross and Renyi, 1964	d, l-NE mouse cortex slices
.0.48	0.71	Salama et al., 1971	d, l-NE rat brain slices
0.4	1.66	Snyder et al., 1968	d,l-NE rat brain slices
0.2	N/A	Ross and Renyi, 1975	l-NE rat brain synaptosomes
0.5	N/A	Ross and Renyi, 1975	l-NE rat brain slices
0.2 - 0.4	0.33 - 0.40	Shaskan and Snyder, 1970	d, l-NE synaptosomes hypothalamus
0.227	0.413	This study	calcium-free plus EDTA enriched medium

Table 4. Kinetic contants obtained for serotonin uptake using substrate concentrations in the high affinity range.

Km (uM)	V_{max} (nM/g X min)	Reference	Remarks
0.7	0.23	Ross and Renyi, 1969	mid-brain mouse slices
0.1	1.13	Wong et al., 1973	rat brain synaptosomes
0.14-0.31	0.66-0.82	Shaskan and Snyder, 1970	rat brain synaptosomes
0.08	N/A	Ross and Renyi, 1975	rat brain synaptosomes
0.6	N/A	Ross and Renyi, 1975	rat brain slices
0.15	0.84	This study	Medium of Snyder and Coyle (1969)

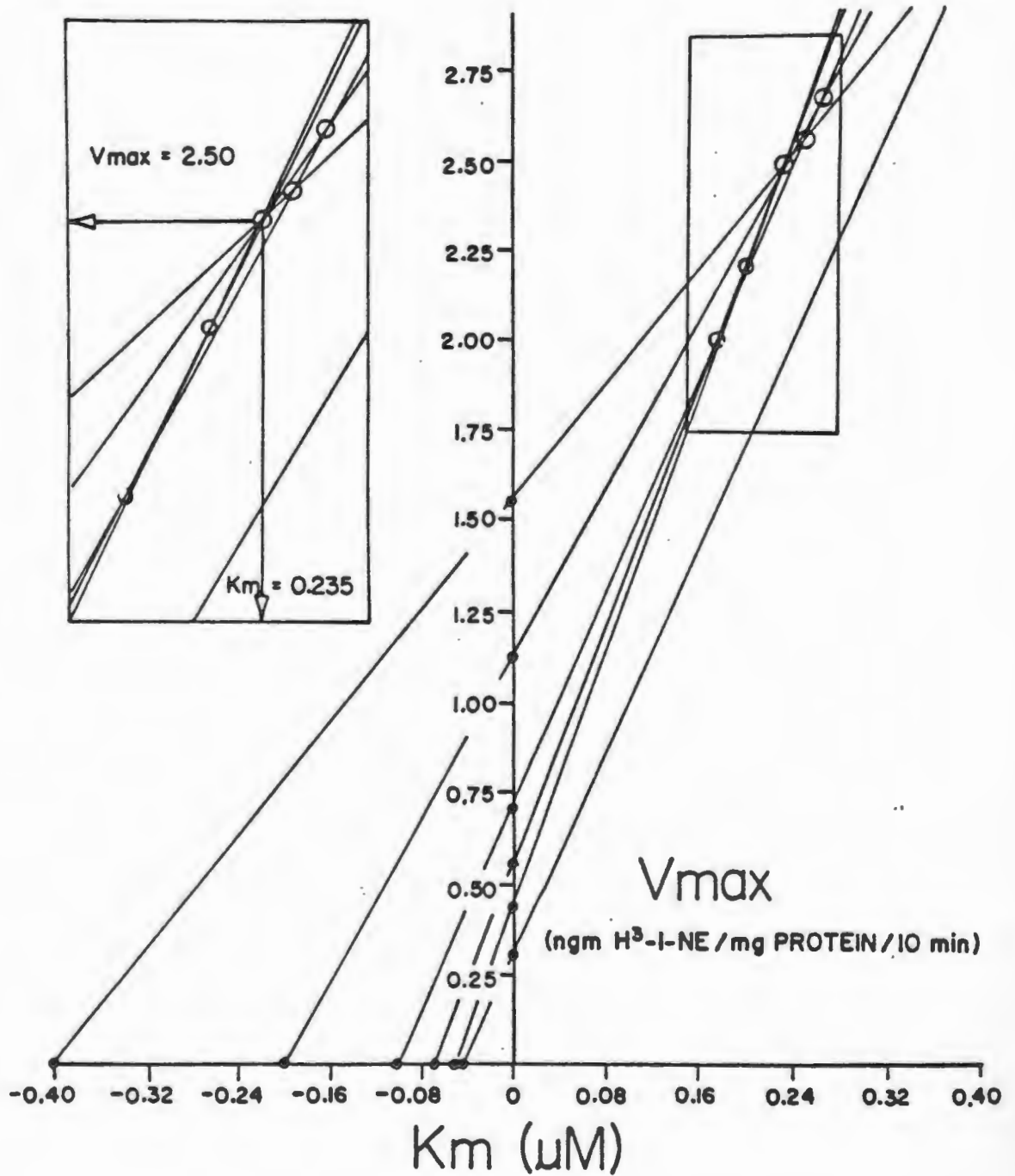N/A The information was not available in the cited paper.

Table 5. Kinetic constants obtained for dopamine uptake
using substrate concentrations in the high
affinity range.

K_m (uM)	V_{max} (nM/g x min)	Reference
0.31-0.4	4.08-5.0	Shaskan and Snyder, 1970
0.13	5.08	This study

NE, 5-HT and DA respectively. The data appears in good agreement with the values reported by other investigators. Small differences may be due to the tissue preparation used (slices vs. synaptosomes) as well as the duration of incubation which can result in larger differences in the V_{max} values. These results suggest that the experimental protocol as used here yields results that are similar to those obtained in other laboratories. Further support for this conclusion is offered in a review of the in-vitro data of section ix.

vi) Subcellular distribution of H^3-norepinephrine uptake

An attempt to characterize the functional integrity of the synaptosomal transport system was made by separating some of the constituents of the CSH. The synaptosomes are known to be confined to a region on the sucrose gradient corresponding to 1.1 molar (Gray and Whittaker, 1962). Mitochondria are known to sediment below 1.2 M sucrose and monoamine oxidase activity is taken to be a marker for mitochondria (Laduron, 1977). In figure 7 it can be observed that the radioactive neurotransmitter concentration is highest at the synaptosomal fraction and some label can be seen associated with myelin and microsomes as might be expected due to adsorption. The supernatant possesses a large degree of label but little or no MAO activity. This evidence suggests that the CSH is selectively incorporating radioactive neurotransmitter within the synaptosomal fraction and there is little contamination of uptake into either

Figure 7. Subcellular distribution of previously accumu-
lated H^3-1-NE into hypothalamic crude synapto-
somal homogenate.
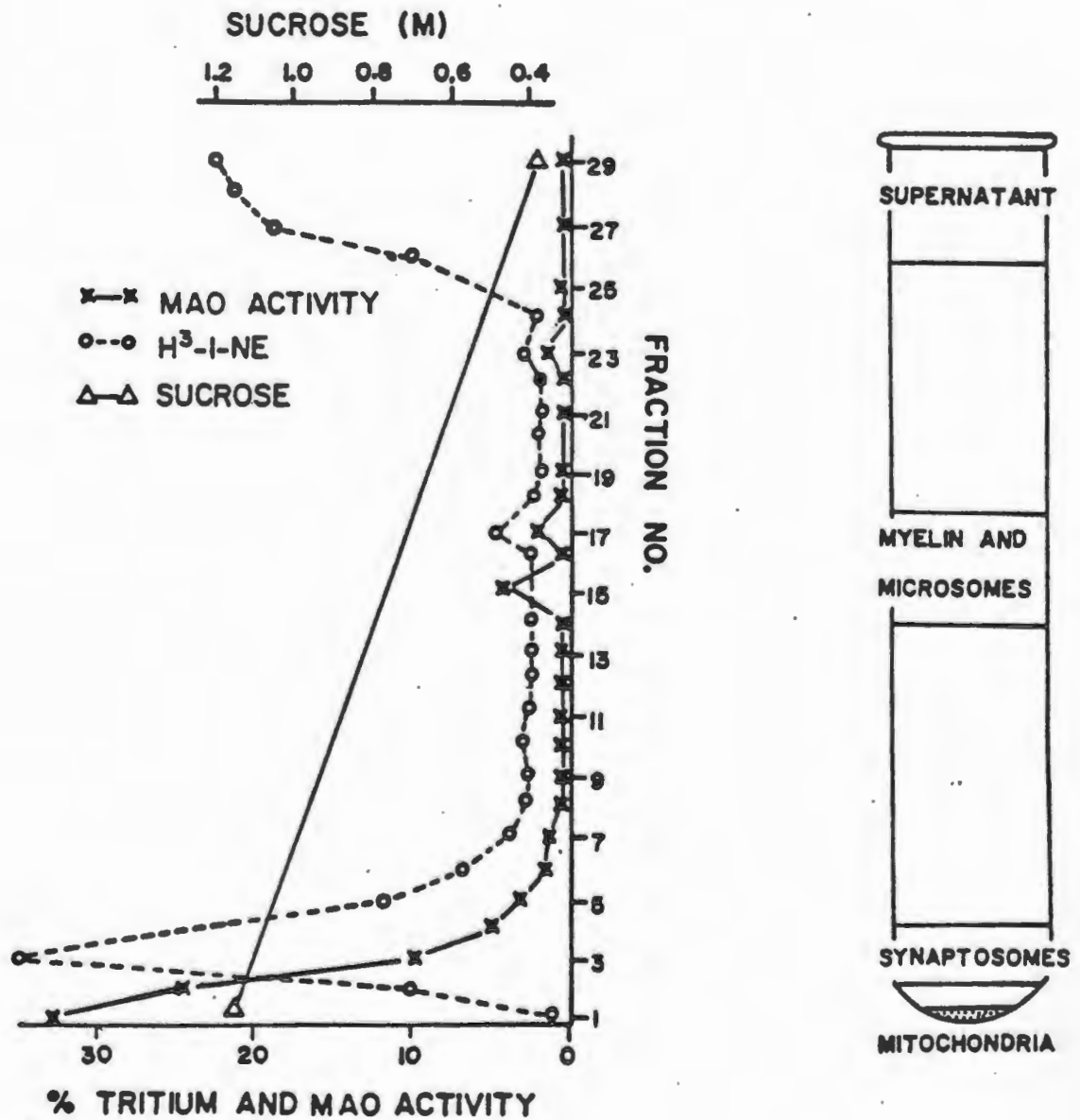
mitochondrial or microsomal components.

vii) Regional distribution of neurotransmitter uptake

The selection of brain regions for studying the uptake
of different neurotransmitters was based on the rationale
that selection of brain regions with a high concentration
of nerve terminals would result in a greater degree of
uptake compared with non-specific binding. Snyder and Coyle
(1969) used hypothalamus for norpeinephrine and striatum for
dopamine uptake studies. Kuhar et al. (1972) used forebrain
for serotonin uptake studied. Confirmation of this selection
of brain regions was attempted by studying the uptake of each
neurotransmitter in each brain region. Figure 8 depicts the
results. Each value and its associated variation represents
the results obtained from 3 animals. Dopamine uptake is
unquestionably greatest into the striatal tissue although a
fair amount of dopamine can be taken up into hypothalamic
tissue presumably into noradrenergic as well as dopaminergic
neurons to some degree (Coyle & Snyder, 1969). Norepin-
phrine uptake, although large in the striatum, may represent
uptake into dopaminergic and other nerve terminals.
Hokefelt (1970) estimated the noradrenergic contribution
towards total catecholamines in the striatum to be 5% based
on histochemical observations. The selection of tissue for
uptake is similarly clear with greatest values obtained for
serotonin uptake into forebrain tissue.

The choice of NE into hypothalamus appears to be
governed more by tradition and concentrations of all three

Figure 8. Regional distribution of high affinity neuro-
transmitter uptake.
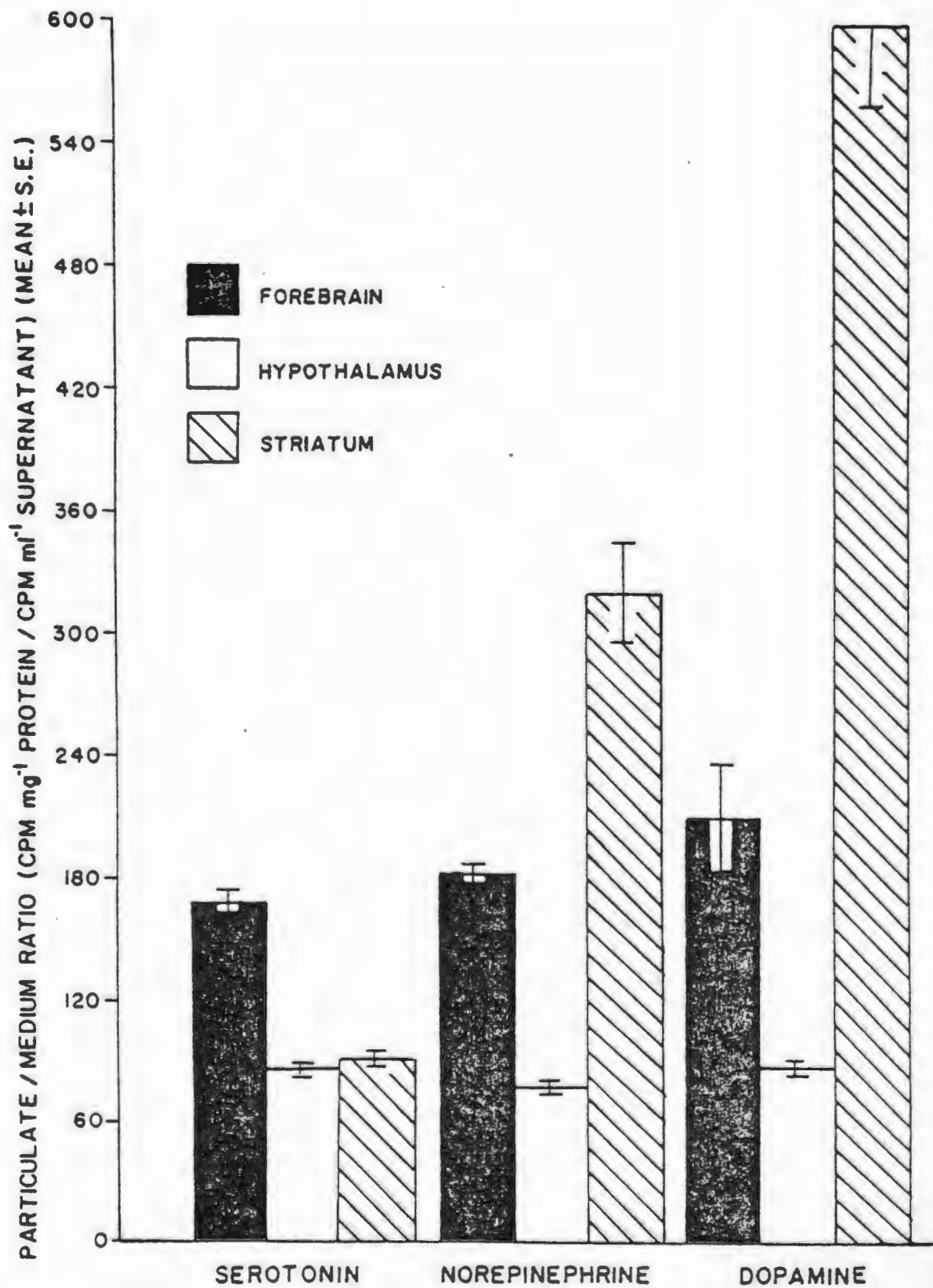
neurotransmitters are shown to be equivalent in the hypo-thalamus (Anden et al., 1966).

viii) Effect of calcium in l-norepinephrine induced
release by hi-potassium depolarization

This study was prompted by the observation that a tremendous degree of variability occured in the in-vitro measurement of uptake after intraperitoneal administration of protriptyline. In an attempt to uncover the source of this variability it was reasoned that endogenous neurotrans-mitter may influence uptake. The results of this experiment are presented in table 6. Although the data presented here could not be shown to differ using an analysis of variance, the data can be taken to suggest a trend. Stimulation of release of endogenous neurotransmitter by hi-K^+ depolar-ization resulted in greater uptake when compared with tissue identically processed in normal Kreb's. Depleting the synaptosomes by hi-K^+ in the absence of Ca^{++} results in a value of uptake intermediate between what can be considered a greater and lesser degree of emptying. Refilling the synaptosomes with unlabeled NE and subsequently incubating with labeled neurotransmitter results in a value higher than that obtained for the same treatment except for refill-ing.

The results appear predictable. Removing neurotrans-mitter from the synaptosomes may reasonably result in greater uptake by increasing the physical capacity to accumulate neurotransmitter taken up. Kant and Meyerhoff (1977) demonstrated at 17.5% increase in NE released by

Table 6. Effects of Hi-K$^+$ depolarization pretreatment upon
subsequently measured H^3-l-NE uptake

Tissue:Medium ratio (CPM/gm. brain:CPM/ml. supernatant)
Percent of Control (Means ± s.e.m.)

Normal Kreb's	Hi-K$^+$ with Ca^{++}	Hi-K$^+$ without Ca^{++}	NE prefilling Hi-K$^+$ with Ca^{++}
100 ± 27	160 ± 43	139 ± 18	177 ± 49

Hi-K^+ in the absence of Ca^{++} which can be used to explain the reason for an increase in uptake over normal Kreb's. The fact that uptake is increased in normal Ca^{++} Hi-K^+ as opposed to Ca^{++} free Hi-K^+ media suggests that exogenously administered NE may equilibrate with both neurotransmitter compartments (i.e. vesicular and cytoplasmic).

The increase in uptake measured in the refilling condition appear inconsistent with notions regarding the distribution of neurotransmitter within the synaptosome. Classical theory considers norepinephrine to be bound with a protein, chromagranin A, ATP and Mg^{++} (Cooper, Bloom & Roth, 1974). It is also postulated that a mobile pool exists within the vesicle (Kirshner, 1962). The exact relationship between the uptake of exogenously administered norepinephrine and subsequent compartmentalization between vesicles and cytoplasm is not known. Release studies using preloading techniques make the assumption of equal distribution inherently. It might appear then that previously accumulated norepinephrine distributes itself to some extent in both compartments and in doing so may result in inhibiting any further uptake of exogenously applied neurotransmitter by mass action. The results indicate quite the contrary and may be interpreted to reflect an uneven distribution within compartments. Although admittedly speculative, one explanation may be that recently accumulated neurotransmitter is inactivated or bound by vesicular complexing components. This binding would reduce the outward

flow of previously accumulated neurotransmitter and may
result in a priming effect on intra-vesicular Mg-ATP-protein
complex. Alternatively, high cytoplasmic concentrations of
accumulated NE may result in a greater rate of exchange so
that uptake may appear to increase overall but net uptake
does not change. Answers to these questions are experiment-
ally approachable and in view of the fact that Grahame-Smith
and Parfitt (1970) report similar results with l-tryptophan,
a study of this type may help to elucidate the compartment-
alization of exogenously administered neurotransmitter.

ix) IC_{50} for some representative tricyclic anti-
depressants

There are, as in obtaining kinetics constants, differ-
ent methods used to calculate the IC_{50} for a compound with
respect to particular response. Ross and Renyi (1967)
introduced the concept of an uptake blocker standard (1.0 x
10^{-4}M cocaine) by which other compounds of varying doses
could be compared. The data is reported as percent of total
inhibition and the IC_{50} calculated by graphic observation of
the resultant log dose-response curve. Another alternative
is to use a vehicle-treated (or untreated) control and com-
pare the value obtained for drug treated samples with those
obtained for controls and represent the data graphically as
in the log dose-response curve of figure 9. The results in
this figure are the means obtained in four separate experi-
ments when drug treated samples where compared with an un-
treated control. The resultant points were connected by a
straight line using visual methods and the point at which

Figure 9. Graphical determination of IC_{50}'s.

$IC_{50} = 4.2 \times 10^{-8} M$

log [PROTRIPTYLINE, (molar)]
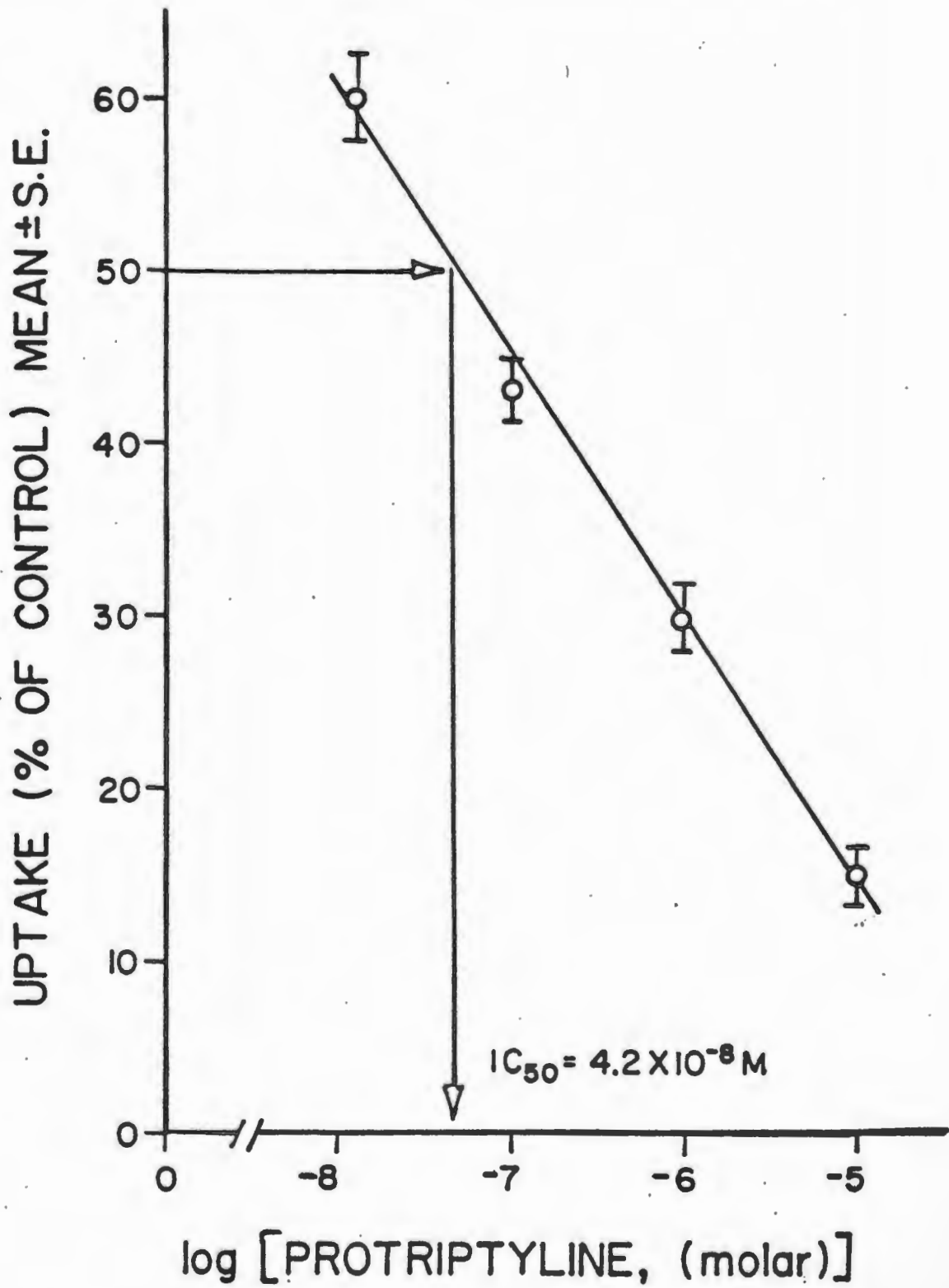
uptake is equivalent to 50% of control was taken directly by dropping a perpendicular line to the log dose axis.

Use of the logarithmic transformation for biological data has statistical considerations as its basis. Finney (1971) demonstrates how biological data of dose versus response are typically skewed and a logarithmic transformation results in a normal frequency distribution thereby rendering analysis of the data to the application of linear regression and correlation models. The assumption of linearity over a large portion of the dose-response curve is unjustified except for rough approximations (Goldstein, 1967). The use of more accurate methods of determining values of inhibitory constants such as probit analysis can be used for critical experiments. The objectives of these experiments are most conveniently served by the graphic method described.

The results of calculated IC_{50}'s are compared with published results for serotonin, norepinephrine and dopamine in tables 7, 8 and 9 respectively. Review of these results indicates that the obtained values correlate well with published values. The generalizations described in the literature review for the uptake blocking characteristics of tertiary versus secondary amines is supported by the data summarized in tables seven through nine. Tertiary amines appear more effective in blocking serotonin uptake whereas secondary amines are potent inhibitors of norepinephrine uptake. There appears to be no obvious separation of effects

Table 7. Norepinephrine uptake IC_{50}'s* for representative secondary and tertiary amines of the tricyclic antidepressant class of drugs.

secondary		tertiary			
PRO	DMI	IMI	AMI	DOX	Reference
	0.03	0.2			Ross and Renyi, 1967a
	0.03	0.15			Shaskan and Snyder, 1970
	0.05	1.0	0.055	0.65	Horn et al., 1971
	0.006				Carmichael and Israel, 1973
0.06	0.008				Maxwell et al., 1974
	0.003	0.028	0.054		Ross and Renyi, 1975
0.043 (4)	0.075 (2)	2.4 (4)	0.1 (2)	0.51 (2)	This study (number of experiments)

*IC_{50}'s expressed in micromolar (uM)

Table 8. Serotonin uptake IC_{50}'s* for representative secondary and tertiary amines of the tricyclic antidepressant class of drugs.

secondary		tertiary			
PRO	DMI	IMI	AMI	DOX	References
	0.285		0.49		Horn and Trace, 1974
	1.1	0.14	0.18		Ross and Renyi, 1975
2.3	2	0.5	0.5		Squires, 1974
	4				Ross and Renyi, 1969
	5.8	0.5			Carlsson, 1970
8.3	2.7				Shaskan and Snyder, 1970
2.7	3.3	0.3	0.67		Buus Lassen et al., 1975
4	6.0	0.6	0.8		Kannengiesser et al., 1973
9.1 (4)	4.5 (2)	0.1 (4)	---	2.5 (2)	This study (number of experiments)

*IC_{50}'s expressed in micromolar (uM)

Table 9. Dopamine uptake IC_{50}'s* for representative seconday and tertiary amines of the tricyclic antidepressant class of drugs.

secondary		tertiary			
PRO	DMI	IMI	AMI	DOX	References
	80				Ross and Renyi, 1967
6	50	8	4	25	Horn et al., 1971
5 (4)	50 (2)	16 (4)	4 (2)	---	This study (number of experiments)

*IC_{50}'s expressed in micromolar (uM)

of either secondary or tertiary amines with respect to the inhibition of dopamine uptake.

x) Protriptyline - acute time course

The results of 6 animals in each treatment group given 20 mg/kg protriptyline i.p. are presented in figure 10. A one-way analysis of variance applied to the data could not demonstrate the differences seen in this figure to be statistically significant. The data from an additional twelve animals were combined for the control and 6 hour treated groups. The results of a comparison of treatment means using a two-tailed t-test demonstrated significance at the P .05 level. Variability in this system has been shown to be high throughout preliminary investigations of intraperitoneal administration of these drugs with subsequent testing in-vitro. It is important to note that the number of subjects in the acute as well as the subacute study represent individual animals. The variability of the steady-state levels of tricyclic antidepressants in plasma has been shown to be high (Asberg et al.,1971) and this phenomenon may be reflective of the high variability observed in these results. In any event, there appears to be an effect of blocking the uptake of l-NE on acute administration of the drug after six hours.

xi) Protriptyline - subacute time course

Preliminary investigations using a dosage schedule of 20 mg/kg daily resulted in the death of a number of animals within 8 to 14 days. In virtually all cases of death,

Figure 10. Time course for the acute administration of protriptyl-
ine upon the uptake of H^3-1-NE into hypothalamic crude
synaptosomal homogenate.

S.I.C. - Saline Injected Controls; ( ) - no. animals

94
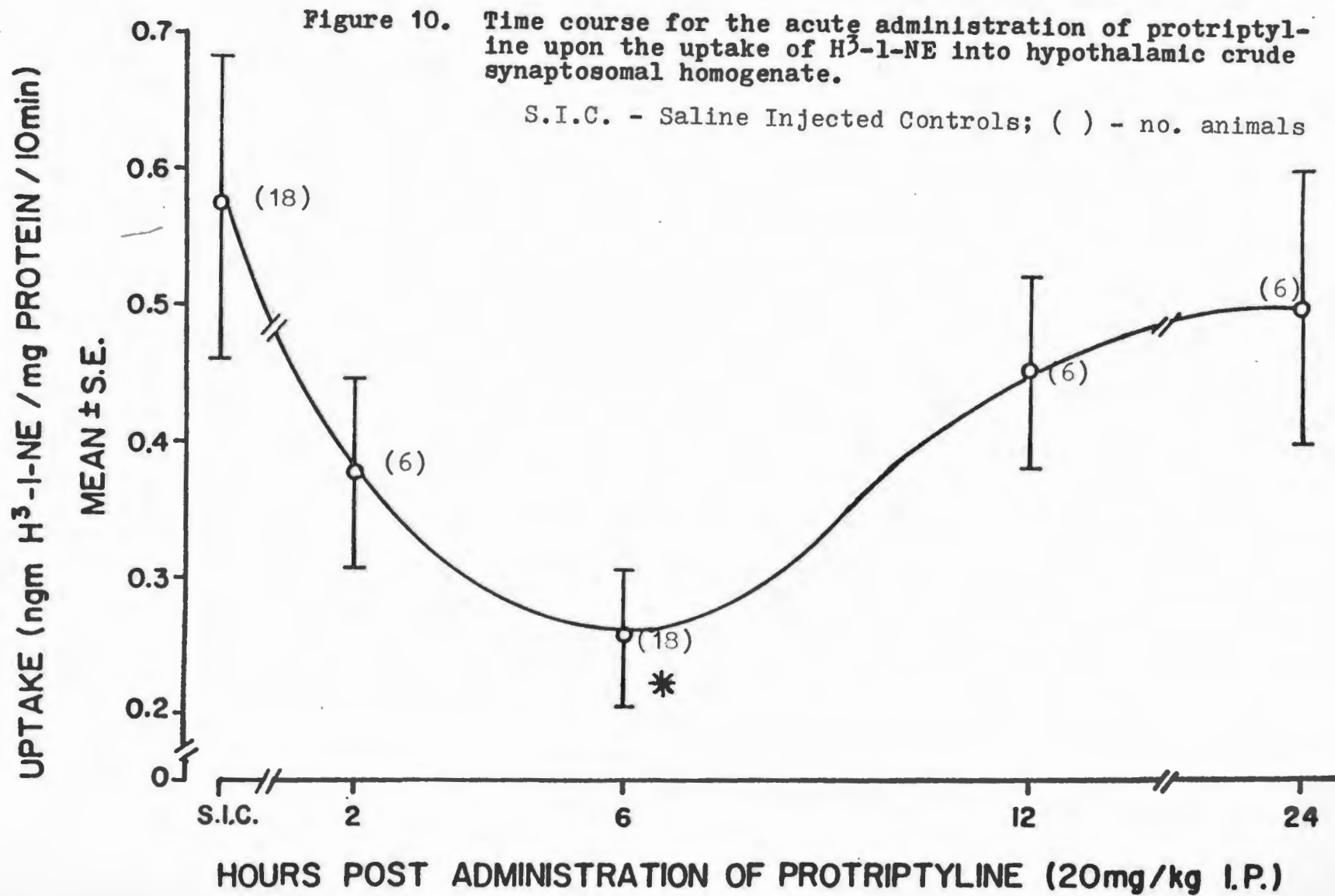
distension of the abdomen was obvious. Autopsies performed
on the animals revealed the lower intestine to be filled
with gas whereas the upper intestine and stomach was filled
with partially digested food. It was concluded that pro-
nounced anticholinergic activity of the drug resulted in
paralysis of peristalsis with death due to starvation.
Gross behavioral observations of the animals prior to death
showed progressive weakening with diminished locomotor
activity. The dose employed for the subacute and chronic
study was reduced from 20 to 10 mg/kg to insure the survival
of the animals during the chronic study and provide a source
for comparison to the subacute study.

Figure 11 illustrates the results obtained from 10
animals per condition administered 10 mg/kg protriptyline
i.p. Once again application of an analysis of variance to
the data did not result in statistically significant results.
The reduction of uptake observed at 2 hours was 18% of
saline injected controls (S.I.C.). Application of a number
of statistical methods including Winsorizing and Trimming
(Winer,1971) did not result in establishing differences
between treatment means. The results then can only be taken
as suggestive of an effect at 2 hours.

Ten metabolites for the tertiary amine, imipramine,
have been identified and the degree of catabolism of all
but the conjugated species have been studied in-vitro using
a rat liver microsomal preparation (Bickel,1966). The
tertiary amine is catabolized to its corresponding secondary

Figure 11. Time course for the sub-acute administration of protriptyline upon the uptake of H^3-1-NE into hypothalamic crude synaptosomal homogenate.

S.I.C. - Saline Injected Controls

N = 10
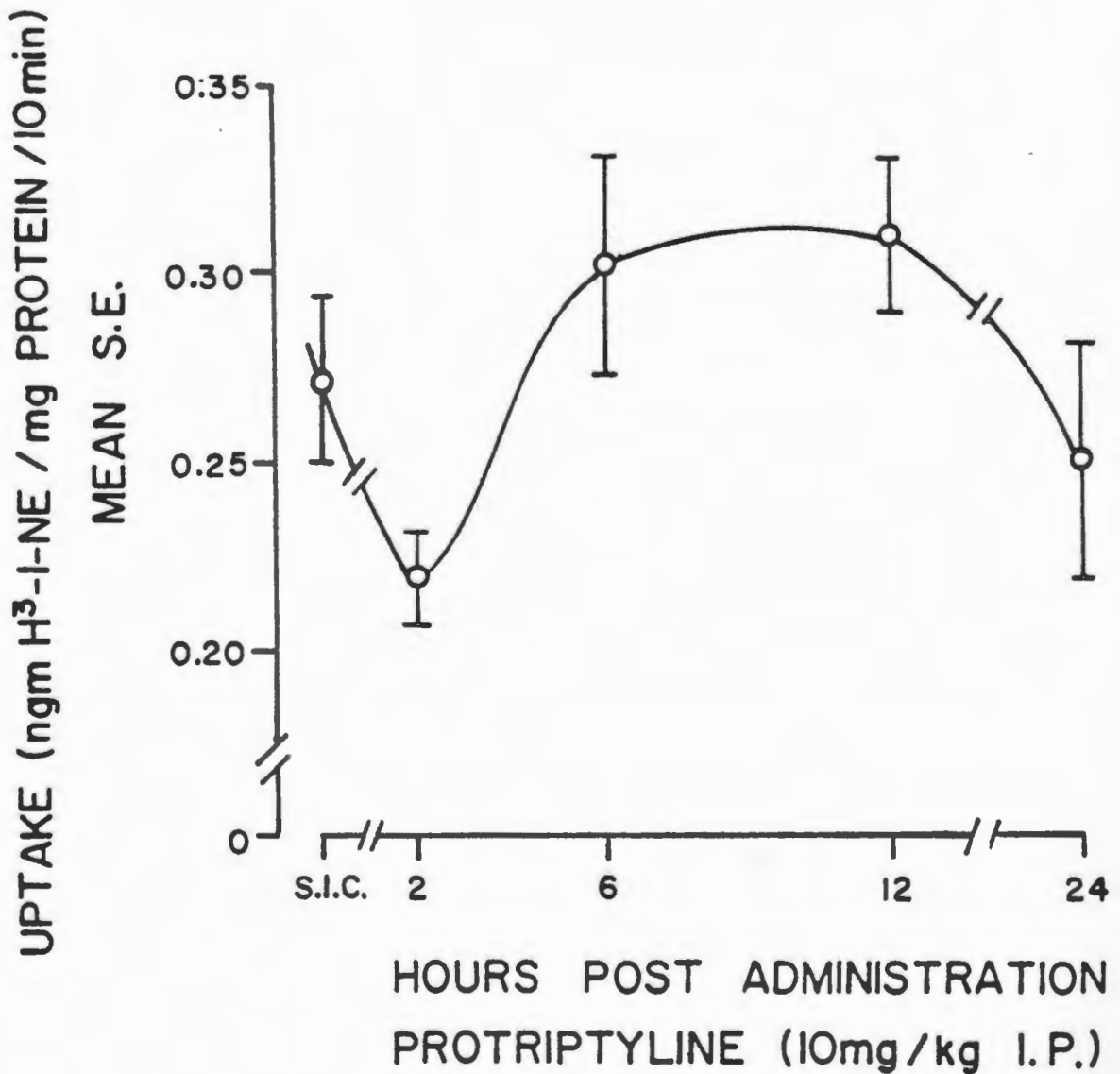
amine and this represents the only major metabolite formed in-vitro. The secondary amine attains peak plasma levels within two hours. Subsequent metabolism of the secondary amine to a primary amine or N-oxidation or ring hydroxylation is less than 10% of the total substrate concentration within the parameters of the in-vitro test. These results may reflect the metabolism in-vivo and may account for the time course observed in the acute study.

Subacute administration, however, does not lead to a clearly interpretable time course for the effect of uptake inhibition. In fact, variability is extremely high at this point with noticeable, although not statistically significant, increases in uptake above controls which may suggest a rebound phenomenon of the neuronal ending to re-capture lost neurotransmitter. The subacute data of figure 11 can be interpreted in another way. Instead of postulating a clear-cut but relatively ineffective decrease in uptake at two hours as well as a small rebound increase above controls at six and twelve hours, it is possible to view the results as simply no effect after six days of drug. The former view has much conjecture associated with it, the latter is more nearly that necessarily obtained by strict statistical interpretation of the results.

xii) Protriptyline - chronic time course

The results of chronic (18 days) administration of protriptyline upon the uptake of l-NE into hypothalamic CSH are depicted in figure 12. Application of a one-way analysis

Figure 12. Time course for the chronic administration of protriptyline upon the uptake of H^3-1-NE into hypothalamic crude synaptosomal homogenate.

S.I.C. - Saline Injected Controls

N = 5
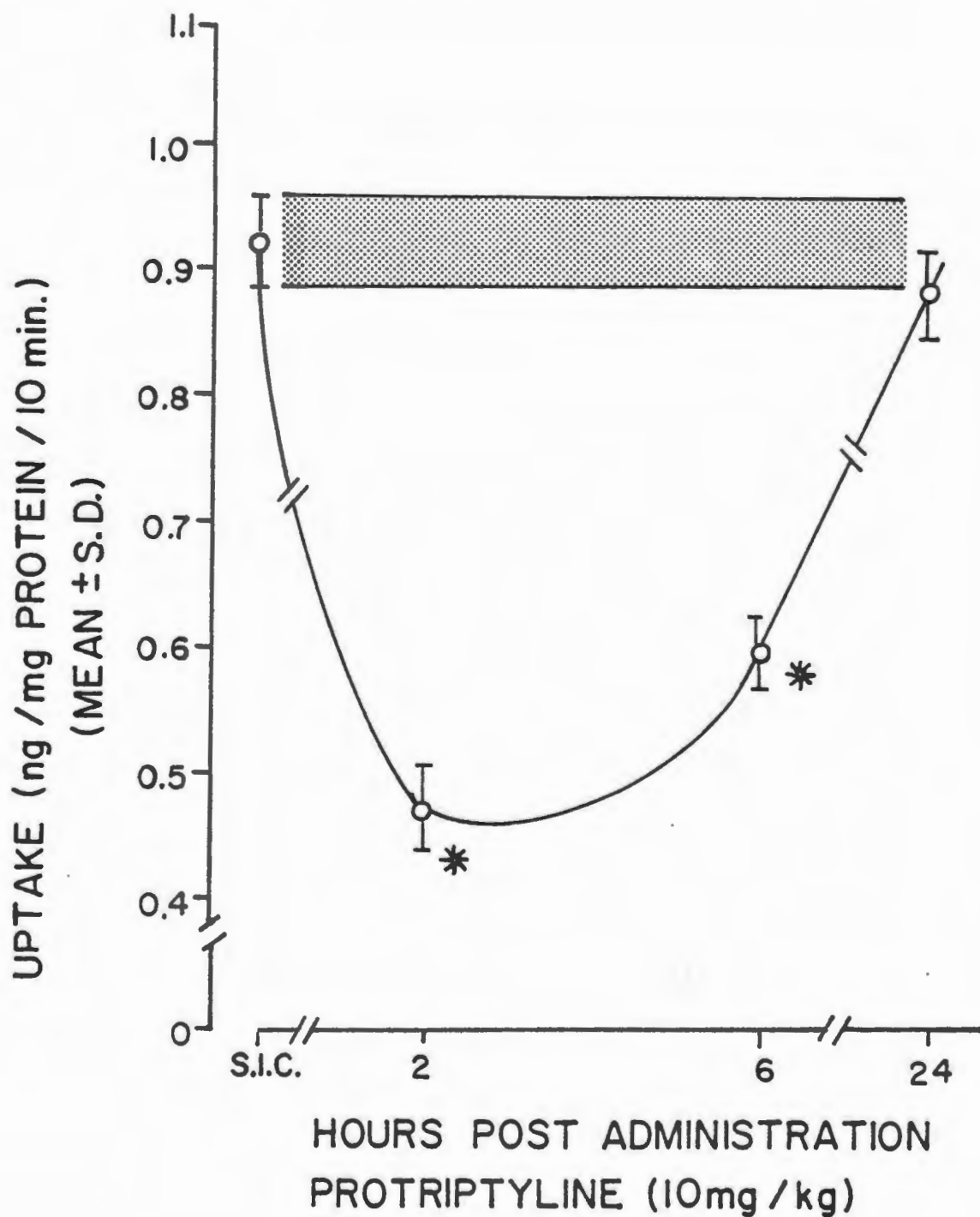
of variance resulted in rejection of the hypothesis of equal means ($F_{3,16}$ = 10.52, P .01) and the assumption of homogeneity of variance was not violated as determined by an F_{max} test. Subsequent comparison of the treatment means was performed using the Student-Neuman-Keuls multiple range test and demonstrated statistically significant differences at P .01 for control versus two hours, control versus six hours and twenty-four versus two hours. Treatment at twenty-four versus six hours was shown to be significant at P .05, whereas, no statistically significant differences could be demonstrated for either the two versus six hour or control versus twent-four hour treatment groups.

The measure of dispersion about the means represented in figure twelve are standard deviations and not standard errors of the means as used in the acute and subacute figures. Although twenty-five animals were used in each treatment condition, the effect of pooling allows them to be represented only as an n_i = 5. Even with relatively low n_i compared with previous studies, the data obtained are so precise that it may be concluded that the effect of pooling serves to mask the individual variability considerably.

One additional point worthy of discussion is that of the variation observed between the control groups of the acute, subacute and chronic studies. The weights (and ages) of the animals used in each of these studies varied considerably because selection of the animals used in these studies was based primarily upon animal availability. In the acute

group the animals weighed approximately 300 grams and the
uptake value for the controls in that group was approximately
0.6 as indicated in figure 10. The subacute study employed
animals weighing 450 grams and the control uptake value of
0.3 can be observed from figure 11. Finally, the chronic
group employed 200 gram animals and their control values
were 0.9 as depicted in figure 12. It is easy to conclude
that uptake is inversely proportional to the age or weight
of the animal. Although each group had its own control, a
good argument can be made for the inappropriateness of
comparing the results between groups because two variables
are known to differ i.e. treatment and the age of the animals.
The argument is difficult to refute on theoretical grounds
and no attempt to do so will be persued. Instead, these
experiments will be repeated with post-pubertal animals prior
to publication.

A comparison of the maximal percent of uptake inhibition
with respect to their own saline injected controls in the
acute, subacute and chronic studies are 56, 18 and 48 percent,
respectively. If the consideration noted in the preceding
paragraph turns out to be unjustified, then these values
alone suggest that uptake inhibition is prominant during
either acute or chronic administration and not nearly as
effective during subacute administration of protriptyline.
The results may suggest that chronic adm nistration of pro-
triptyline results in a long lasting inhibition of the
transport carrier for l-NE uptake into the hypothalamic CSH,

unlike the results of the subacute experiment. Since uptake
after subacute administration may be considered neither
prolonged nor unidirectional, it appears that therapeutic
effectiveness would not be anticipated during subacute ad-
ministration and this reflects the clinical picture very
accurately. The prolonged effect upon chronic administra-
tion appears predictive of a therapeutic response and is
clearly temporally related to the therapeutic effect observ-
ed clinically. These results support the hypothesis that
tricyclic antidepressants may work by blocking re-uptake
of endogenous neurotransmitter in-situ.

CHAPTER IV: GENERAL DISCUSSION

A. Interpretive constraints

One of the principal responsibilities of a scientist is to attempt to remove or at least counter-balance the bias which permeates his or her line of investigation. A good deal of bias is corrected for with the proper use of controls but much bias remains at the level of assumptions made prior to the experimental design. Three assumptions have been made and are discussed here to alert the reader to the limitations of the interpretations made both in the preceding chapter and the conclusions to follow. The first deals with the assumption of unidirectional transmembranous flux of neurotransmitter into synaptosomes and how this phenomenon may confound the measurement of uptake. The second assumption is that of no effect of endogenous neurotransmitter levels upon uptake. Finally the assumption of equivalent protein binding of tricyclic antidepressants to physiological proteins present in the incubation medium is discussed.

Much of the experimental work reported here is concerned with the characterization of the synaptosomal system used to study the effects of intraperitoneally administered drugs by subsequent in-vitro testing. The characterization lends credibility to the proper functioning of the system in that it clearly demonstrates the degree to which this system compares with those used by other investigators. However, in no way does it resolve critical issues with respect to the transport of l-norepinephrine into synaptosomes.

One such issue remaining unresolved is that regarding

the extent to which neurotransmitter exchange processes
occur and may confound the measurement of an estimate of net
uptake. An approach to resolving this question may be to
measure the specific activity of neurotransmitter located
in the incubation medium, the synaptosomal cytoplasmic as
well as vesicular fractions separately both before and
after incubation at physiological temperatures. The results
of these experiments may indicate whether endogenous neuro-
transmitter stored within vesicles, the cytoplasm or that
resulting from de novo synthesis somehow contributes to-
ward an exchange process.

Another unresolved question is that of the relationship
between endogenous neurotransmitter content and the uptake
of exogenously applied neurotransmitter. A review of the
literature suggests that endogenous biogenic amine levels
are lowered upon chronic administration and turnover
appears to be increased. These effects appear intuitively
concordant with what one might expect of uptake inhibitors,
however, this interpretation presupposes that the effect of
uptake inhibition is responsible for the lowered levels.
The evidence comes from demonstrating the uptake inhibitory
characteristics of the tricyclic antidepressants as measured
in-vitro. Lowered levels of endogenous amines may be re-
sponsible for a decrease in uptake and if this is so, the
inhibition of uptake observed may be an indirect effect of
the drug. This important question seems experimentally
approachable by comparing the effects of biogenic amine

synthesis inhibitors upon uptake measured both in-vitro and after parenteral administration with subsequent testing in-vitro. The data presented in this thesis with respect to neurotransmitter refilling of previously emptied synapto-somes suggests that levels of neurotransmitters can affect uptake. Snyder and Coyle (1969) reported a decrease in up-take of norepinephrine in animals previously treated with reserpine as opposed to saline injected controls. In addition it is important to realize that compartmentation of neurotransmitter within the neuronal ending may affect uptake differentially. Investigation of the pharmaco-dynamics of the tricyclics with respect to uptake inhibition should consider the effect of pool sizes as well as total levels of endogenous neurotransmitters.

The potencies of the tricyclics as uptake inhibitors are taken from concentration terms in-vitro whereas it has been shown that the binding of tricyclic antidepressants to plasma proteins is variable and does not reflect a clear separation of effects between secondary and tertiary amines (Borga et al., 1969). The in-vitro experiments therefore necessitate an assumption of equivalent binding to plasma proteins for a direct domparison of potency to be considered legitimate. Protriptyline and desmethylimipramine are bound to plasma proteins leaving only 8.0 and 8.4 percent free in the plasma, respectively. Imipramine and amitrip-tyline are free in plasma to the extent of 4.2 and 3.6 per-cent, respectively. Nortriptyline however, is 94.5 percent

bound. Since free drug is considered to be pharmacological-
ly active whereas that bound to plasma proteins are inactive,
the relative degree of binding may be an important variable
in ascertaining meaningful potency ratios in-vitro and in-
vivo (Glassman et al., 1977). Although much work has been
done in an attempt to correlate blood plasma levels with
the therapeutic effect (Alexanderson et al., 1973), little
has been reported on the relationship between the concentra-
tion of free drug and clinical outcome (Glassman et al.,
1973, Muscettola et al., 1978).

B. Alternative mechanisms

Caution must be taken at this time to refrain from
concluding that tricyclics mediate their therapeutic ef-
fects by uptake inhibition or blockade. The spectrum of
pharmacological activity of the tricyclic antidepressants
is not limited to uptake blockade. In fact, two additional
biochemical effects are being suggested as possible deter-
minants of the therapeutic effect of the tricyclics.
Edwards and Burns (1974) demonstrated the ability of tri-
cyclics to block MAO found in human platelets and Roth and
Gillis (1974) reported an MAO inhibitory effect of tricyc-
lics in rabbit brain mitochondria.

Alternatively, tricyclics possess reasonable strong
anticholinergic effects demonstrated both peripherally,
using antagonism of carbachol contraction of the guinea pig
ileum, and centrally by displacements of H^3-quinuclidinyl
benzylate, a potent muscarinic antagonist, from homogenates

of rat brain (Sayers and Burki, 1976; Snyder and Yammamura, 1977). Janowsky et al. (1972) review the evidence for the efficacy of anticholinergics in the treatment of depression.

In addition to the effects suggested as components of the thymoleptic effect, tricyclics possess other therapeutic effects when administered in doses below that required for the treatment of depression. Wilkerson (1978) reported o the ability of tricyclics to reverse ouabain induced ventricular arrhythmias in dogs at doses below that used for the treatment of depression. Similarly, protriptyline has been demonstrated to be effective in the treatment of hypersomnia and the narcolepsy-catalepsy syndrome after a single bedtime dose of 10 mg (Schmidt et al., 1977).

Other clinically effective antidepressants exhibit different pharmacological profiles than the tricyclics. Mianserin is not a very potent inhibitor of catecholamine or serotonin uptake (Goodlet et al., 1977). Nomifensine possesses weak anticholinergic activity but is very potent inhibitor of dopamine uptake and has clinical psychostimulatory properties (Hunt et al., 1974).

Comparisons of the performance of tricyclics as well as other clinically effective antidepressants in a number of biochemical systems may serve to demonstrate the extent to which each property (i.e. anticholinergic, uptake inhibition, receptor binding affinity, MAO inhibition, etc.) may either contribute to or be predictive of either the thymoleptic effect or side effects. These comparisons may suggest a

role for specific neurotransmitters or a balance of neuro-
transmitters in depression or any of the other clinical
entities in which tricyclics are or can be used.

One type of comparative technique is demonstrated in
table 10. The table was compiled from all in-vitro data
reviewed in tables 7 - 9 as well as the anticholinergic data
of Shein and Smith (1978). Inclusion of the anticholiner-
gic potencies was based upon availability of comparative
data for the particular tricyclics investigated and reported
in this thesis. Unfortunately, comparative data across a
number of seconday and tertiary amines are not available
for the other properties mentioned.

Before proceeding it is important to point out that a
similarly compiled table may possess a great deal more
predictive value if each of the biochemically determined
potencies were measured after chronic parenteral administra-
tion of the drug. As Inch __et al__. (1973) point out, the com-
parison of drug effects in-vivo can only be considered valid
if the time course for the effect of each drug is deter-
mined and the time representing the optimal effects are used
to determine potencies. This would necessitate performing
a time course for each drug after chronic administration.
The in-vitro comparisons then are performed with the under-
standing that they might not reflect the actual relative
potencies which may occur upon chronic administration of
the drug, however, it represents a starting point and a
source of comparison for data which will no doubt become

Table 10. Summary of relative and absolute potencies of representative secondary and tertiary amines of the tricyclic antidepressant (TCA) class of drugs.

Subclass of TCA	DRUG	POTENCIES UPTAKE IC_{50}'s (uM) *			** R Ach P	RELATIVE POTENCY RATIOS					
		5-HT	NE	DA		$\frac{5\text{-}HT}{NE}$	$\frac{DA}{NE}$	$\frac{5\text{-}HT}{DA}$	$\frac{Ach}{5\text{-}HT}$	$\frac{Ach}{NE}$	$\frac{Ach}{DA}$
SECONDARY	PRO	5.3±1.4 +(5)	.015±.01 (2)	5.5±.5 (2)	2.18	104	108	.964	.411 ‡(0.9%)	42.7 (98.1%)	.396 (0.9%)
	DMI	3.3±.67 (9)	.025±.01 (7)	60±10 (3)	0.33	132	2600	.055	.100 (0.7%)	13.2 (99.2%)	.006 (0.04%)
TERTIARY	IMI	.33±.09 (5)	.75±.45 (5)	12±4 (2)	1.00	.440	16.0	.028	3.03 (68.6%)	1.30 (29.5%)	.083 (1.8%)
	AMI	.53±.1 (5)	.07±.01 (4)	4 ± 0 (2)	4.17	7.57	57.0	.133	7.86 (11.4%)	59.6 (87.1%)	.959 (1.4%)
	DOX	2.9±.4 (2)	.58±.7 (2)	25 (1)	0.42	5.00	43.0	.116	.145 (16.3%)	.724 (81.7%)	.017 (1.9%)

* mean ± s.e.m. calculated from individual investigators' results presented in tables 7-9.
+=#of independent studies. ‡=% of total anticholinergic influence described in the text.
** R Ach P = Relative Anti-cholinergic Potency obtained from Shein & Smith, 1978.
PRO=protriptyline   IMI=imipramine   DOX=doxepin   NE=norepinephrine
DMI=desmethylimipramine   AMI=amitriptyline   5-HT=serotonin   DA=dopamine

available with future research.

To the left of the vertical double line in table 10 are the uptake inhibitory potencies of the tricyclics with respect to three neurotransmitters as well as the relative (to imipramine) anticholinergic potencies. The relative potency ratios for serotonin, norepinephrine and dopamine, referred to as the biogenic amine ratios, can be compared with one another but the anticholinergic:biogenic amine ratios, in the last three columns of table 10, cannot be compared directly with the biogenic amine ratios simply because a different response measure was used in determining the anticholinergic influence. The values obtained for the anticholinergic:biogenic amine ratios become more meaningful when viewed as a percent of the total anticholinergic influence with respect to each of the biogenic amines. The values placed within parentheses directly below the anticholinergic: biogenic amine ratios were obtained by summing the three ratios for a single drug and expressing each ratio as a percent of the total.

Several portions of this table demonstrate interesting correlations with known effects of tricyclics. Based upon the biogenic amine comparisons in the first three columns to the right of the double line, there appears to be no difference between the effects of amitriptyline and doxepin. Clinical studies do not demonstrate any clear differences between doxepin and amitriptyline as antidepressants (Beck, 1973). Doxepin has been reported to be well tolerated by

older patients because of fewer incidences of anticholiner-
gic side effects. This difference is clearly reflected in
the potencies of anticholinergic effects where amitriptyline
is ten times more potent than doxepin. This may suggest
that the anticholinergic properties are not important for
the clinical therapeutic effect. Cairncross et al. (1963)
suggested that the anticholinergic effect was important for
therapeutic activity but Blackwell et al. (1972) concluded
that the anticholinergic effect was related more to the
sedative properties of the drugs. This is difficult to
reconcile since stimulation of locomotor activity, a
behavioral response measure which could be viewed as an
inverse indicator of sedation, has been shown to be centrally
mediated by anticholinergic drugs (Aquilonius et al., 1972).
It may be that the difference in anticholinergic potency is
only reflected in the periphery with these two drugs. The
particular proportional indluences upon the biogenic amine
systems may serve to mask any psychostimulatory properties
these drugs possess if they were purely anticholinergic.
This may occur as a result of the lack of proportional
influences within the periphery that these neurotransmitter
systems may possess within the central nervous system. A
look at the anticholinergic:biogenic amine ratio "percent"
figures of table 10 suggests that there are no differences
in the relative effects of anticholinergic to biogenic
amine influences between amitriptyline and doxepin. It
may be as a result of this balancing influence that doxepin

and amitriptyline are sedative.

On the other end of the pharmacological spectrum is the psychostimulatory effects of protriptyline. Protriptyline is the only tricyclic antidepressant recommended by a manufacturer for use in anergic patients. Since dopaminergic influences are believed to be responsible for stimulating effects on locomotor activity (Fibiger et al., 1973; Creese and Iversen, 1973; 1975; Tseug and Loh, 1974; Tseug et al., 1974) and cholinergic-dopaminergic interactions are important as well (Klawans, 1968; Hornykiewicz, 1966; Friehoff & Alpert, 1973), it seems reasonable to conclude that a cholinergic-dopaminergic interaction may mediate the psychostimulatory effect of this drug. Compared with desmethylimipramine, the percent of anticholinergic-dopaminergic interaction is 22 times greater for protriptyline whereas the effects with respect to anticholinergic-norepinephrine or anticholinergic-serotonin influences are norepinephrine or anticholinergic-serotonin influences are comparable. This may reflect the mechanism of psychostimulation occuring in-vivo.

This approach may offer a reasonable, although admittedly speculative, interpretation for the different effects observed with the clinical use of tricyclics, but most important is the therapeutic effect. Review of the relative potency ratios for biogenic amines demonstrates the common factors which may suggest how all these drugs may work with respect to the neurotransmitters studied. All

dopamine:norepinephrine ratios are greater than one and all serotonin:dopamine ratios are less than one. Considering only the biogenic amines, this is the only factor common to all tricyclics reflected in table 10. It may be suggested that the relative effects of serotonin with respect to dopamine and norepinephrine with respect to DA are involved in the antidepressant effect of these drugs.

Continuing with the concept that a balance of effects upon critical neurotransmitters may be responsible for the therapeutic effect, these observations are reminisent of the elements of the "permissive hypothesis of depression" (Prange et al., 1974). This hypothesis states that serotonin acts as a gating or controlling influence for the expression of either mania or depression by increased or decreased levels of norepinephrine, respectively. Agents affecting these systems perhaps with respect to a third buffer or reference system such as the dopaminergic system may account for the common dimension required for a unified neurotransmitter hypothesis of affective disorders.

An attempt to explain the mechanism of action of these drugs using only four of possibly scores of neurotransmitters is admittedly premature. Certainly other mechanisms may be responsible for the therapeutic effect both within as well as outside the frame work of the biogenic amine hypothesis.

It is important to nore that the development of the tricyclics came out of investigations with antihistaminic

drugs are tricyclics possess antihistaminic properties.
Research directed towards evaluating these properties may
reflect more light on even more complex interactions then
we dare to expect. Knowledge of the role of neurotrans-
mitters in behavior is still at its infancy (Baldessarini,
1975). Until more data is accumulated with respect to the
pharmacokinetics, physical properties and pharmacodynamics
of tricyclic antidepressants, table like that of table 10
represent a way in which leads toward a particular line of
investigation may be obtained. A glimmer of hope in unrav-
elling the complex interactions of a number of variables in
biological systems is the increasing applications of statis-
tical methods. Techniques of utilizing parametric and non-
parametric statistical methods are becoming more accessible
and easier to apply. Pattern recognition techniques are
available to aid in the analysis of complex interactions
using cluster analysis, path analysis, principal components
and other multivariate approaches (Harper, et al., 1977).
C. Conclusion

Should the limitations noted above prove to be neglig-
ible, then it would appear that protriptyline blocks the
uptake of norepinephrine upon chronic administration whereas
when administered sub-acutely uptake blockade is not clearly
discernable. These findings demonstrate a temporal corre-
lation of uptake blockade with clinical effectiveness in
humans and lends support to both the catecholamine hypothesis
and the proposed mechanism of action of tricyclics as uptake

blockers. In addition it is interesting to note that there is a risk of attempted suicide in depressives during the initial days of chemotherapy (Beck, 1973) and sub-acute administration displays highly variable effects and perhaps even some potentiation of uptake thereby enhancing the functional depletion of neurotransmitter at the level of the post synaptic receptors. This effect could explain the clinical observation of increased suicidal tendency during initiation of tricyclic chemotherapy.

In support of the sub-acute data obtained is the evidence that twelve hours post-protriptyline using either serotonin or tryptamine as substrate, MAO activity is elevated (Ed Kaiser-personal communication). An increase in uptake provides more substrate for MAO therefore these results appear corroborative of the subacute data presented here.

It appears that the relative anticholinergic properties can be predictive of the psychostimulatory effects of protriptyline. Comparisons of the effects of drugs using in-vitro biochemical techniques may be useful in formulating hypotheses regarding the mechanism of action of the tricyclic antidepressants. The lack of psychostimulatory action of the most potent anticholinergic of the tricyclics, amitriptyline, may be explained by a relative interaction between anticholinergic and dopaminergic influences.

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APPENDIX

Figure A-1. Flowchart for "VIVO": A program used to
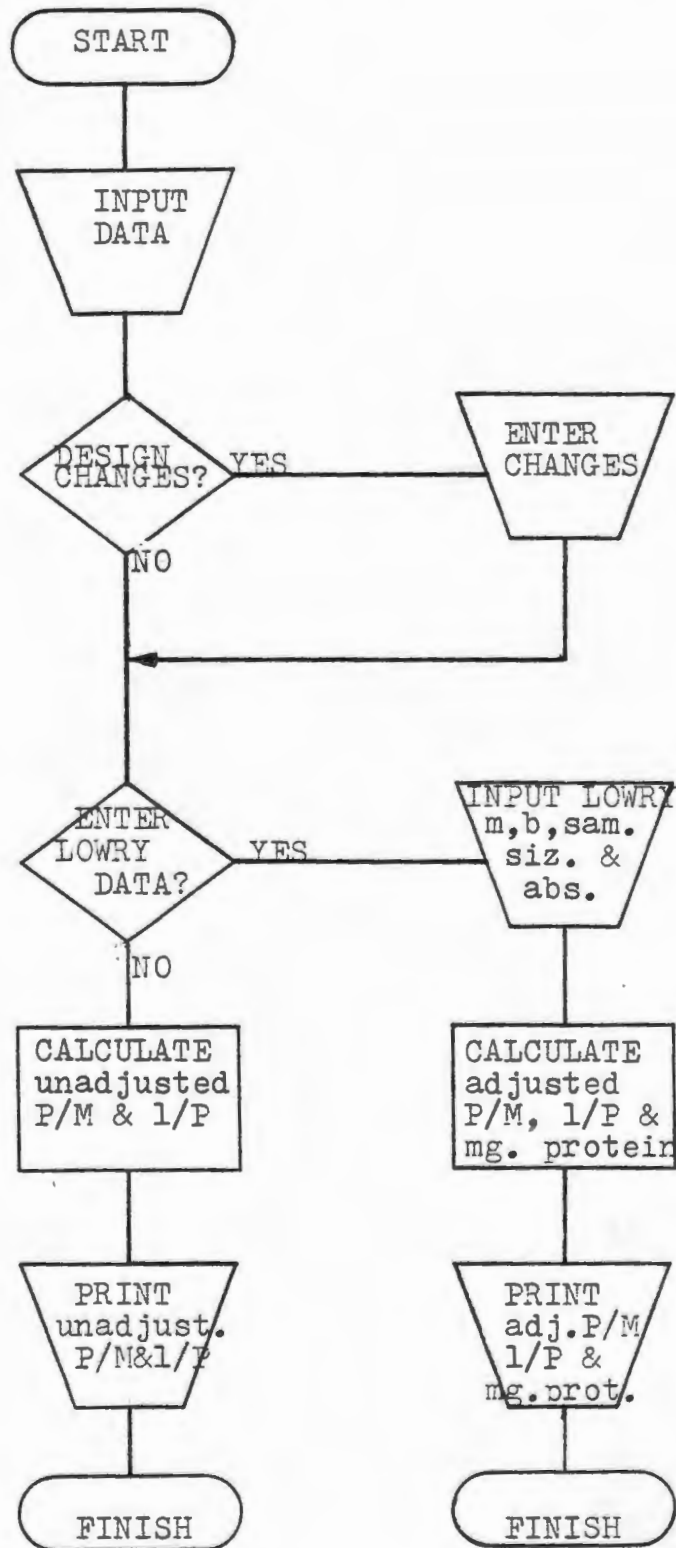calculate data for synaptosomal uptake studies

Table A-1. Listing of 'VIVO': A program used to cal-
culate data for synaptosomal uptake studies.

```
vivo          15:47    sun   23 jan 77

100 dim z(100), l(100), m(100), n(100), q(100)
110 dim .r(100), g(50), h(50), j(100), y(100)
120 dim i(100), s(100), w(100), p(100), e(100)
130 dim a(100), b(100), c(100), d(100), f(100), t(100)
140 print 'experiment number?'
150 input n$
160 print
170 print 'specify n (where n = # of reaction vessels):',
180 input n
190 print
200 print 'enter cpm data, in order, from the liquid'
210 print 'scintillation spectrometer printout.'
220 print 'enter each value individually by pressing'
230 print 'the return key after each entry.'
240 print
250 print '37 degrees; serotonin; incubation media values'
260 for k = 1 to n/6
270 input i(k)
280 next k
290 print
300 print '37 degrees; norepinephrine; incubation media values'
310 for k = n/6+1 to n/3
320 input i(k)
330 next k
340 print
350 print '37 degrees; dopamine; incubation media values'
360 for k = n/3+1 to n/2
370 input i(k)
380 next k
390 print
400. print '37 degrees; serotonin; supernatant values'
410 for k = 1 to n/6
420 input s(k)
430 next k
440 print
450 print '37 degrees; norepinephrine; supernatant values'
460 for k = n/6+1 to n/3
470 input s(k)
480 next k
490 print
500 print '37 degrees; dopamine; supernatant values'
510 for k = n/3+1 to n/2
520 input s(k)
530 next k
540 print
550 print '37 degrees; serotonin; wash values'
560 for k = 1 to n/6
570 input w(k)
```

```
580 next k
590 print
600 print '37 degrees; norepinephrine; wash values'
610 for k = n/6+1 to n/3
620 input w(k)
630 next k
640 print
650 print '37 degrees; dopamine; wash values'
660 for k = n/3+1 to n/2
670 input w(k)
680 next k
690 print
700 print '37 degrees; serotonin; pellet values'
710 for k = 1 to n/6
720 input p(k)
730 next k
740 print
750 print '37 degrees; norepinephrine; pellet values'
760 for k = n/6+1 to n/3
770 input p(k)
780 next k
790 print
800 print '37 degrees; dopamine; pellet values'
810 for k = n/3+1 to n/2
820 input p(k)
830 next k
840 print
850 print 'zero degrees; serotonin; incubation media values'
860 for k = n/2+1 to 2*n/3
870 input i(k)
880 next k
890 print
900 print 'zero degrees; norepinephrine; incubation media values'
910 for k = 2*n/3+1 to 2*n/3+n/6
920 input i(k)
930 next k
940 print
950 print 'zero degrees; dopamine; incubation media values'
960 for k = 2*n/3+n/6+1 to n
970 input i(k)
980 next k
990 print
1000 print 'zero degrees; serotonin; supernatant values'
1010 for k = n/2+1 to 2*n/3
1020 input s(k)
1030 next k
1040 print
1050 print 'zero degrees; norepinephrine; supernatant values'
1060 for k = 2*n/3+1 to 2*n/3+n/6
1070 input s(k)
1080 next k
1090 print
1100 print 'zero degrees; dopamine; supernatant values'
1110 for k = 2*n/3+n/6+1 to n
```

```
1120 input s(k)
1130 next k
1140 print
1150 print 'zero degrees; serotonin; wash values'
1160 for k = n/2+1 to 2*n/3
1170 input w(k)
1180 next k
1190 print
1200 print 'zero degrees; norepinephrine; wash values'
1210 for k = 2*n/3+1 to 2*n/3+n/6
1220 input w(k)
1230 next k
1240 print
1250 print 'zero degrees; dopamine; wash values'
1260 for k = 2*n/3+n/6+1 to n
1270 input w(k)
1280 next k
1290 print
1300 print 'zero degrees; serotonin; pellet values'
1310 for k = n/2+1 to 2*n/3
1320 input p(k)
1330 next k
1340 print
1350 print 'zero degrees; norepinephrine; pellet values'
1360 for k = 2*n/3+1 to 2*n/3+n/6
1370 input p(k)
1380 next k
1390 print
1400 print 'zero degrees; dopamine; pellet values'
1410 for k = 2*n/3+n/6+1 to n
1420 input p(k)
1430 next k
1440 print
1450 print 'were there any changes in the experimental design?'
1460 input a$
1470 if a$ = 'no' then 1640
1480 if a$ = 'yes' then 1490
1490 print
1500 print 'enter the following values:'
1510 print ;'incub. med. vol. (ml.), its sample aliquot (ml.):'
1520 input #1, #2
1530 print ;'wash vol. (ml.), its sample aliquot (ml.):'
1540 input #3, #4
1550 print ;'pellet (sol. vol./sam. vol.):'
1560 input #5
1570 print ;'sup. sample aliquot (ml.):'
1580 input #6
1590 print ;'brain homogenate sample volume (ml.):'
1600 input #8
1610 #7 = #1 - #2
1620 print
1630 go to 1730
1640 #1 = 4.0
1650 #2 = 0.1
```

```
1660 #3 = 6.0
1670 #4 = 0.1
1680 #5 = 1.575
1690 #6 = 0.1
1700 #7 = 3.9
1710 #8 = 0.2
1720 print
1730 def fna(k) = p(k) * #5
1740 def fnr(k) = int(10**6/fna(k))/100
1750 def fnb(k) = w(k) * #3/#4
1760 def fnc(k) = s(k) * #7/#6
1770 def fnd(k) = (fnc(k) + fnb(k))/#7
1780 def fne(k) = int(-10**4*(i(k)*#1/#2 - (fna(k)+fnb(k)+fnc(k
)))/(i(k)*#1/#2))/100
1790 def fnf(k) = int(10**5*fna(k)/fnd(k))/100
1800 print
1810 print 'do you have the results of the protein assay?'
1820 input c$
1830 if c$ = 'yes' then 2220
1840 print 'pause for paper insertion-press return key when rea
dy'
1850 pause
1860 print ,'experiment #', n$
1870 print
1880 print
1890 print ,,;;;;'unadjusted'
1900 print ;;'sample no.';;;'% error';;;;;'p/m','1/p'
1910 print ,,'(exp-3)','(exp-4)'
1920 print
1930 print ,'37 degrees'
1940 print
1950 for k = 1 to n/6
1960 print ;;;k;;;fne(k);;;fnf(k);;;;fnr(k)
1970 next k
1980 for k = n/6+1 to n/3
1990 print ;;;k;;;fne(k);;;fnf(k);;;;fnr(k)
2000 next k
2010 print
2020 for k = n/3+1 to n/2
2030 print ;;;k;;;fne(k);;;fnf(k);;;;fnr(k)
2040 next k
2050 print
2060 print ,'zero degrees'
2070 print
2080 for k = n/2+1 to 2*n/3
2090 print ;;;k;;;fne(k);;;fnf(k);;;;fnr(k)
2100 next k
2110 print
2120 for k = 2*n/3+1 to 2*n/3+n/6
2130 print ;;;k;;;fne(k);;;fnf(k);;;;fnr(k)
2140 next k
2150 print
2160 for k = 2*n/3+n/6+1 to n
2170 print ;;;k;;;fne(k);;;fnf(k);;;;fnr(k)
2180 next k
2190 print
```

```
2200 go to 3410
2210 print
2220 print 'enter the results of the protein assay in the form'
2230 print '(m,b,a1,s1,a2,s2,...an,sn) where m and b are the'
2240 print 'lowry regression coefficient and intercept, respect
ively;'
2250 print 'a1 to an are the absorbances and s1 to sn are their
 respective'
2260 print 'sample sizes (ml.) used to obtain valid absorbance
readings'
2270 print 'beginning with 1=forebrain control, 2=low dose, 3=1
ow-med,'
2280 print '4=hi-med and 5=hi dose; 6-10 represent hypothalamus
 control to'
2290 print 'hi dose samples and finally 11-15 are striatal valu
es.'
2300 print ;;'(enter 2 values per line)'
2310 print
2320 input m,b
2330 print
2340 for k = 1 to n/2
2350 input g(k),h(k)
2360 next k
2370 def fnj(k) = lgt(g(j))
2380 def fny(k) = (fnj(k)-b)/m
2390 dim k(100)
2400 def fnk(k) = int(1000*(fny(k)*#8/h(j)/10**3))/1000
2410 def fnl(k) = fna(k)/fnk(k)
2420 def fnm(k) = fnl(k)/fnd(k) ·
2430 def fnn(k) = 10**5/fnl(k)
2440 print 'pause for paper insertion-press return key when rea
dy'
2450 pause
2460 print ,'experiment #',n$
2470 print
2480 print
2490 print ,;;;;'  lowry adjusted'
2500 print 'sample no.';;;'% error';;;;'p/m';;;'  1/p','mg. pro
t.'
2510 print ,;;;;' (exp-3)';;' (exp-5)'
2520 def fnq(k) = int(10**5*fnm(k))/100
2530 def fnt(k) = int(10**2*fnn(k))/100
2540 dim o(30)
2550 dim u(30)
2560 print
2570 print ,'37 degrees'
2580 print
2590 for k = 1 to n/6
2600 let j = k
2610 print ;k;;;fne(k);;;fnq(k);;;fnt(k);;;fnk(k)
2620 let o(k) = fnq(k)
2630 let u(k) = fnt(k)
2640 next k
2650 print
2660 for k = n/6+1 to n/3
2670 let j = k
```

```
2680 print ;k;;;fne(k);;;fnq(k);;;fnt(k);;;fnk(k)
2690 let o(k) = fnq(k)
2700 let u(k) = fnt(k)
2710 next k
2720 print
2730 for k = n/3+1 to n/2
2740 let j = k
2750 print ;k;;;fne(k);;;fnq(k);;;fnt(k);;;fnk(k)
2760 let o(k) = fnq(k)
2770 let u(k) = fnt(k)
2780 next k
2790 print
2800 print ,'zero degrees'
2810 print
2820 for k = n/2+1 to 2*n/3
2830 let j = k-15
2840 print ;k;;;fne(k);;;fnq(k);;;fnt(k);;;fnk(k)
2850 let o(k) = fnq(k)
2860 let u(k) = fnt(k)
2870 next k
2880 print
2890 for k = 2*n/3+1 to 2*n/3+n/6
2900 let j = k-15
2910 print ;k;;;fne(k);;;fnq(k);;;fnt(k);;;fnk(k)
2920 let o(k) = fnq(k)
2930 let u(k) = fnt(k)
2940 next k
2950 print
2960 for k = 2*n/3+n/6+1 to n
2970 let j = k-15
2980 print ;k;;;fne(k);;;fnq(k);;;fnt(k);;;fnk(k)
2990 let o(k) = fnq(k)
3000 let u(k) = fnt(k)
3010 next k
3020 print
3030 print 'do you wish to print p/m corrected for'
3040 print 'non-specific absorption?'
3050 input d$
3060 if d$ = 'yes' then 3080
3070 if d$ = 'no' then 3410
3080 print 'pause for paper insertion-press return key when rea
dy'
3090 pause
3100 print ,'experiment #',n$
3110 print
3120 print 'values reported are adjusted for non-specific absor
ption'
3130 print
3140 print ;;;,'p/m'
3150 print ;;;'(exp-3)(cpm/mg. protein)/(cpm/ml. sup.)'
3160 print
3170 print 'serotonin'
3180 print
3190 for k = 1 to n/6
3200 let j = k+15
```

```
3210 print ,;;o(k)-o(j)
3220 print
3230 next k
3240 print
3250 print 'norepinephrine'
3260 print
3270 for k = n/6+1 to n/3
3280 let j = k+15
3290 print ,;;o(k)-o(j)
3300 print
3310 next k
3320 print
3330 print 'dopamine'
3340 print
3350 for k = n/3+1 to n/2
3360 let j = k+15
3370 print ,;;o(k)-o(j)
3380 print
3390 next k
3400 print
3410 end
```