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N-Methylation of Beta-Carbolines as a Potential Bioactivation Route in Parkinson's Disease

Debra Ann Gearhart
Loyola University Chicago

LOYOLA UNIVERSITY CHICAGO

N-METHYLATION OF BETA-CARBOLINES AS A POTENTIAL BIOACTIVATION ROUTE IN PARKINSON'S DISEASE

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DEBRA ANN GEARHART

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DEDICATION

This accomplishment is especially dedicated to my son, Joel.
His love, sensitivity, unique humor, intelligence, and unending curiosity
have always provided me with welcome relief,
and an essential balance in my life.

To Mom, Kent, Kim, family and friends
for their unconditional love, support, and continued inspiration.

The highest reward for a person's toil is not what they get for it, but what they become by it.

by John Ruskin

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ABBREVIATIONS

2-MeßC(s)+	a generic 2-N-methylated ß-carboline(s)
2,9-Me_2ßC(s)+	a generic 2,9-N,N-dimethylated ß-carboline(s)
2-MeH+	2-methylharmanium
2-MeHi+	2-methylharminium
2-MeHl+	2-methylharmol
2-MeNH+	2-methylnorharmanium ion
9-MeNH	9-methylnorharman
2,9-Me_2H+	2,9-N,N-dimethylharmanium
2,9-Me_2NH+	2,9-N,N-dimethylnorharmanium ion
ACN	acetonitrile
ANOVA	Analysis of Variance
ßC-2-NMT	ßC-2N-methyltransferase
ßC-9-NMT	ßC-9N-methyltransferase
ßC(s)	ß-carboline(s)
CON	Control
CM	carboxymethyl
DEAE	diethylaminoethyl
DMF	dimethylformamide
DTT	dithiothreitol
EAS	epoxy-activated sepharose

EDTA	ethylenediaminetetraacetic acid
FC	frontal cortex
HCl	hydrochloric acid
$HClO_4$	perchloric acid
HPLC	high performance liquid chromatography
HIC	hydrophobic interaction chromatography
IC_{50}	inhibition constant 50%
IEC	ion-exchange chromatography
K_2HPO_4	potassium phosphate dibasic
K_i	inhibition constant
K_M	Michaelis-Menten constant
KCl	potassium chloride
MeßC+	2N-monomethylated ß-carboline and/or 2,9-N,N-dimethylated ß-carboline
MeOH	methanol
MPP+	1-methyl-4-phenylpyridinium ion
MPTP	1-methyl-4-phenyltetrahydropyridine
$NaHCO_3$	sodium bicarbonate
NH	norharman
Na_2HPO_4	sodium phosphate dibasic
$NaCO_3$	sodium carbonate
NaOH	sodium hydroxide
NH_4OH	ammonium hydroxide
$(NH_4)_2SO_4$	ammonium sulfate
PC12	pheochromocytoma cells

PD	Parkinson's disease
PMSF	phenylmethylsulfonyl fluoride
PNMT	phenylethanolamine N-methyltransferase
PUT	putamen
RP-HPLC	reverse-phase high performance liquid chromatography
SAH	S-adenosyl-L-homocysteine
SAM	S-adenosyl-L-methionine
SCX-HPLC	strong-cation exchange high performance liquid chromatography
s.e.m.	standard error
s.d.	standard deviation
SN	substantia nigra
SPE	solid-phase extraction
v	velocity
V_{max}	maximal velocity

INTRODUCTION

Background

It is hypothesized that ß-carboline-N-methyltransferases (ßC-NMT) and the neurotoxic products of their catalytic activities, N-methylated ß-carbolinium cations (MeßCs+), may play a role in the pathogenesis of idiopathic Parkinson's disease (Collins 1994; Collins et al. 1985). This hypothesis was originally based on the structural similarity between MeßCs+ and neurotoxic 1-methyl-4-phenylpyridinium ion (MPP+). In the central nervous system of human and nonhuman primates, MPP+ selectively destroys the dopaminergic nigrostriatal system of the basal ganglia, resulting in a parkinsonian condition (Burns et al. 1983; Burns et al. 1984; Chiueh et al. 1984; Langston 1995; Langston et al. 1983). In support of this hypothesis, previous work indicates that MeßCs+ share several toxic properties with MPP+ (Albores et al. 1990; Cobuzzi et al. 1994; Collins 1994; Collins et al. 1996; Collins et al. 1992; Collins et al. 1995; Drucker et al. 1990; Fields et al. 1992; Neafsey et al. 1995; Neafsey et al. 1989). In addition, MeßCs+ have been detected in postmortem human brain tissue from normal subjects (Matsubara et al. 1993), but such studies have not been done in Parkinson's cases. Notably, MeßCs+ are elevated in the lumbar cerebrospinal fluid of Parkinson's patients relative to control values (Matsubara et al. 1995).

Relevant to this dissertation was the finding that mammalian brain possesses enzymatic activities capable of generating neurotoxic MeßCs+ from simple ß-carbolines (ßCs) (Collins et al. 1992). In tissue homogenates from

guinea pig and rat brain, various ßCs are N-methylated at both their pyridyl (2N-) and indole (9N-) nitrogens by S-adenosyl-L-methionine-dependent N-methyltransferases (Collins et al. 1992; Matsubara et al. 1992b). These activities have been designated as ßC-2N-methyltransferase (ßC-2-NMT) and ßC-9N-methyltransferase (ßC-9-NMT), for N-methylation occurring at the pyridyl and indole nitrogens of ßCs, respectively. Matsubara et al. (1993) detected ßC-2-NMT activity, but not ßC-9-NMT activity, in postmortem normal human brain tissue.

The primary focus of this dissertation is a more detailed investigation of the properties of these neurotoxin-producing ßC-N-methyltransferases, particularly the 2N-methylation activity.

Specific Aims

Aim 1

Characterization of optimal assay conditions used in the assessment of brain ß-carboline-2N-methyltransferase (ßC-2-NMT) activity. Enzyme characterization is a prerequisite to the partial purification of bovine brain ßC-2-NMT (Aim 2), and to comparison of its activity in human postmortem brains from control and Parkinson's disease subjects (Aim 3).

Aim 2

Partial purification of bovine brain ß-carboline-2N-methyltransferase (ßC-2-NMT) using conventional protein purification techniques. Results from this aim should lay the foundation for subsequent studies on ßC-2-NMT, including determination of its protein sequence, cloning of the gene, and perhaps speculation regarding its physiological function. Related to the physiological function of ßC-2-NMT is the finding that ßC-2-NMT activity may be due to phenylethanolamine-N-methyltransferase (PNMT). Evidence in support of the equivalency of ßC-2-NMT and PNMT or a PNMT-like activity is presented in this dissertation.

Aim 3

Measurement of ß-carboline-2N-methyltransferase (ßC-2-NMT) and ß-carboline-9N-methyltransferase (ßC-9-NMT) activities in postmortem human brains from Parkinson's disease (PD) and control individuals. Brain regions evaluated include frontal cortex, substantia nigra, and putamen. This study expands upon Matsubara's original work by including PD brain and two additional brain regions in the assessment of both ßC-NMT activities.

CHAPTER 1

REVIEW OF RELEVANT LITERATURE

Parkinson's Disease

Clinical Symptoms and Pathogenesis

Parkinson's disease (PD) is a chronic disorder of the extrapyramidal motor system. The disease onset is insidious and progresses slowly, with the time from the beginning of the pathological process to manifestation of clinical symptoms being several years (Berkow 1977; Koller et al. 1991). Clinically, Parkinson's disease is primarily characterized by the following motor symptoms: bradykinesia, akinesia, pill-rolling tremor, muscular rigidity, postural instability, shuffling gait, lack of facial expression, and infrequent blinking. However, nonmotor symptoms occur variably in PD, including loss of the sense of smell, depression, dementia, and sensory disturbances. In addition, numerous autonomic nervous system disturbances have been noted, such as orthostatic hypotension, excessive sweating, dermatitis, gastrointestinal symptoms, sexual dysfunction, and urological problems (Koller et al. 1995).

Neuropathologically, PD affects the basal ganglia system, where there is specific loss of the dopaminergic, pigmented substantia nigra (pars compacta) (Berkow 1977; Koller and Pahwa 1995). Cell bodies in the substantia nigra pars

compacta project to the striatum (caudate and putamen), and degeneration of the nigrostriatal pathway results in a profound decrease in the neurotransmitter dopamine in the striatum. The nigral projections to the putamen are apparently more affected in PD than projections to the caudate (Kish et al. 1988). Motor symptoms of PD do not present clinically until striatal dopamine decreases to about 20% of control levels (Kollerand Pahwa 1995). In addition to nigrostriatal degeneration, neuronal loss frequently occurs in the locus ceruleus, nucleus basalis of Meynert, peripeduncular nucleus, and in the dorsal motor nucleus of the vagus (Forno 1995, 1996).

Small intraneuronal inclusions, known as Lewy bodies, are the pathological hallmark of PD. Lewy bodies may contain lipofuscin, neuromelanin, mitochondria, and other organelles. Common to all Lewy bodies are biochemically-altered neurofilament proteins -- Lewy body neurofilaments are abnormally phosphorylated, ubiquitinated, proteolyzed, and cross-linked (Forno 1996). Definitive pathologic diagnosis of PD must include the presence of Lewy bodies in the substantia nigra; however, several other dopaminergic, as well as nondopaminergic, brain nuclei often exhibit these cytoplasmic inclusions (Forno 1995, 1996). The dopaminergic ventral tegmentum may also demonstrate Lewy bodies, both in the presence and absence of nerve cell loss. Lewy bodies are frequently present in noradrenergic neurons of the locus ceruleus and sympathetic ganglion. Several cholinergic nuclei may also exhibit Lewy bodies, including the nucleus basalis of Meynert, the peripeduncular nucleus, the Edinger-Westphal nucleus, the intermediosacral nucleus, and intestinal autonomic ganglion (Forno 1995). The serotonergic dorsal raphe and superior central sulcus often display these inclusions. The posterior and lateral cell groups of the

hypothalamus, the dorsal motor nucleus of the vagus, the cerebral cortex, as well as the olfactory tubercle may demonstrate Lewy bodies in PD. Forno (1995) states that "the widespread affection of these selected nuclei is such an integral part of the disease process that it [presence of Lewy bodies] deserves to be considered in relation to the etiology of the disorder."

Epidemiology

Epidemiology examines the interrelationships between host, agent, and environment in disease. Epidemiologic data related to Parkinson's disease are often difficult to interpret due to the long latency period preceding the clinical presentation PD (Koller et al. 1991). Roman's recent review of the neuroepidemiology of PD addresses the numerous problems associated with interpretation of epidemiologic data: specifically, adaptation of universal diagnostic criteria in PD, and improved design of epidemiological studies would reduce the problems associated with data interpretation (Roman et al. 1995).

Parkinson's disease is a disease of aging in that clinical manifestations usually are not observed until after age fifty, and it affects 1.5% of the population between 70 and 79 years of age (Martilla 1987). Both the prevalence and incidence of PD increases with advancing age. The prevalence of PD varies geographically -- from 18 cases per 100,000 population in China, to 182 per 100,000 in Iceland. Incidence rates worldwide vary between 2 to 24 cases per 100,000 population. There is apparently a slightly higher preponderance of PD in males than females (Roman et al. 1995).

In epidemiologic terms, "agent" has application primarily in the study of infectious diseases and may have some significance with a subset of Parkinson's cases. Fazzini et al. (Fazzini et al. 1992) reported the presence of

elevated levels of antibodies to coronovirus in the cerebrospinal fluid of PD patients. Kohbata et al. (Kohbata et al. 1993) demonstrated the presence of antibodies to the soil-pathogen, *Nocardia asteroides*, in the serum of PD patients; moreover, this organism causes a parkinson-like syndrome in mice (Kohbata et al. 1991). Zack and Tanner (1995) recently reviewed infectious diseases and their relevance to PD. The authors conclude that, although there is not strong evidence to support a primary role for a single pathogen in PD, hypotheses that an infectious process may increase the susceptibility of predisposed individuals to the disease merit further investigation.

Preliminary data regarding the factors and lifestyles associated with the risk for developing PD include living in an industrialized nation, rural living and drinking of well-water, a tendency toward less use of alcohol and tobacco, less physical activity, and a more rigid preclinical personality (Koller et al. 1990; Kondo et al. 1993; Tanner 1989). Tanner and Zack (1995) have reviewed comorbid conditions proposed to have a relationship with PD; the comorbid conditions were endocrine disorders (thyroid disease, diabetes mellitus); metabolic abnormalities (cytochrome P450 enzymes, mitochondrial dysfunction); mood disorders (depression, anxiety); stroke; atherosclerosis; cancer; physiologic stress; head trauma; and drug use. They conclude that "none [of these associations] likely contribute to more than a few of all cases of Parkinson's disease..... but [these associations] may provide pieces to the etiologic puzzle."

As mentioned above, metabolic biomarkers that have been studied in Parkinson's disease include those enzymes involved in the metabolism of xenobiotics. In patients diagnosed with PD sulfur metabolizing enzymes were underactive, and monoamine oxidase-B activity was variable. Most pertinent

to this dissertation is the demonstration of an impaired metabolism of pyridine derivatives in Parkinson's disease, as evidenced by accumulation in the urine of N-methylated derivatives of nicotinamide following oral administration of the compound. Notably, levels of N-methylated nicotinamide were 100-fold greater in PD compared to control subjects -- suggesting either increased N-methylation of nicotinamide, or decreased catabolism of N-methylated nicotinamide in PD (Williams 1995; Williams et al. 1993).

Etiology

General Hypotheses

Etiology refers to the cause of a disease. Parkinsonian symptoms may occur during treatment with dopamine antagonists, as a consequence of carbon monoxide or manganese poisoning, with cerebral trauma or tumor, and following encephalitis (Berkow 1977; Koller and Pahwa 1995). However, the cause which underlies idiopathic Parkinson's disease remains a mystery, although roles for heredity, infection, metabolic dysfunction, and the environment have been proposed (Barbeau 1984). As reviewed by Jenner et al. (1992), study of the substantia nigra after death in PD patients reveals the following changes, which may provide clues regarding the mechanisms that underlie the disease: mitochondrial complex I deficiency, depleted levels of reduced glutathione as well as high levels of lipid peroxidation products (indicators of oxidative stress), high iron, and low ferritin levels. These authors summarize by stating that current views predict an interplay between environmental or endogenous agents (toxins) and a genetic predisposition to the disease -- an interplay that ultimately manifests in the neurochemical and

clinical features of Parkinson's disease.

The environmental toxin theory of the etiology of PD is central to this dissertation, and will therefore be addressed in more detail.

An Environmental Cause for PD?

The theory implicating a role for an environmental toxin in PD gained impetus with discovery of the selective nigrostriatal toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Langston et al. 1983). MPTP is a contaminant in meperidine that was illicitly synthesized for "street-drug" concoctions. Drug-users that injected the meperidine mixtures presented in hospital emergency rooms with classic parkinsonian symptoms (Langston et al. 1983). Further research showed that MPTP produces a parkinsonian-like condition in nonhuman primates (Burns et al. 1983; Davis et al. 1979; Langston et al. 1984a; Langston et al. 1983). Furthermore, following MPTP administration, postmortem pathological findings included the characteristic degeneration of the nigrostriatal pathway was observed in nonhuman primates (Burns et al. 1983; Burns et al. 1984; Langston et al. 1984a), as well as one human case (Davis et al. 1979). Positron emission tomography of ^{18}F-labeled dihydroxyphenylalanine (DOPA) uptake appears to be useful in assessment of striatal dopamine deficiency, since the rank order of ^{18}F-DOPA uptake was: MPTP-induced parkinsonian subjects < Parkinson's patients < asymptomatic MPTP-exposed subjects < normal control individuals (Calne et al. 1985).

MPTP is a lipophilic molecule that crosses the blood brain barrier; it is nontoxic prior to bioactivation to its active metabolite, neurotoxic 1-methyl-4-phenylpyridinium ion (Langston et al. 1984b; Markey et al. 1984). Monoamine

oxidase (MAO) catalyzes the oxidation of MPTP to an intermediate, 1-methyl-4-phenyl-2,3-dihydropyridinium ion (MPDP+); MPDP+ is then further oxidized to MPP+ (Chiba et al. 1985; Trevor et al. 1988). Current literature indicates that MPTP is a substrate for both MAO-A and MAO-B, but that in brain, glial MAO-B preferentially forms MPP+ (Di Monte et al. 1992; Salach et al. 1984). MPP+ is actively taken up into dopaminergic neurons via the dopamine transporter (Javitch et al. 1985); therein, MPP+ accumulates in mitochondria where it inhibits Complex I (NADH dehydrogenase) of the respiratory chain (Nicklas et al. 1985; Salach et al. 1984). Inhibition of this complex ultimately results in an inability of the mitochondria to carry out oxidative phosphorylation and synthesize adenosine triphosphate (ATP) (Chan et al. 1991). As a consequence of decreased ATP production, cellular homeostasis is disrupted, and neuronal cell death ensues (Mizuno et al. 1988; Nicklas et al. 1985; Salach et al. 1984). Inhibition of mitochondrial respiration also places the cell in a state of oxidative stress (Takeshige et al. 1979; Zeevalk et al. 1996; Zoccarato et al. 1988). The cationic nature of MPP+ is believed to be an important structural feature required for toxicity since it likely facilitates pyridinium ion transport into dopaminergic cells, and accumulation of the cation down the electrochemical gradient into mitochondria (Davey et al. 1992).

The discovery of MPTP advanced the hypothesis that an environmental agent may play a causative, or at least a contributory role in the pathogenesis of PD. Although MPTP has not been detected in the environment, several chemicals exist both endogenously and exogenously which are structurally similar to MPTP or its neurotoxic metabolite MPP+. One such group of compounds are the N-methylated ß-carbolines (MeßCs+) (Collins and Neafsey 1985).

N-Methylated ß-Carbolines: Structure, Toxicity, and Formation

Structure of N-Methylated ßCs

N-methylated ß-carbolines (MeBCs+), particularly the 2N-methylated derivatives are structural analogues of MPP+ (Figure 1) (Collinsand Neafsey 1985).

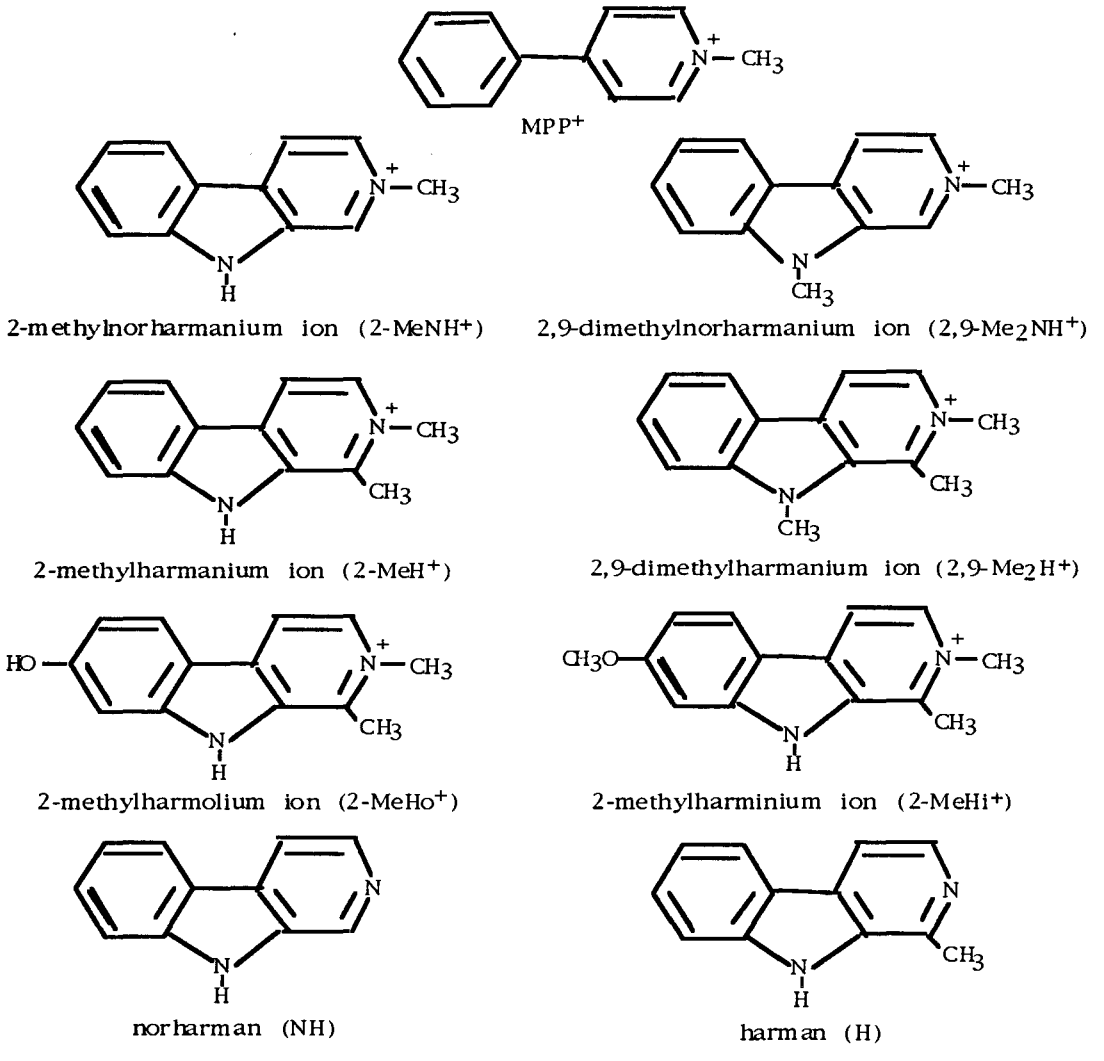


Figure 1. N-methylated ßCs are structural analogues of neurotoxic MPP+.

Toxicity of N-Methylated ß-Carbolines

N-methylated ß-carbolines (MeßCs+) are also functional analogues of neurotoxic MPP+, because like MPP+, MeßCs+ exert cytotoxic effects both in vitro and in vivo.

Inhibition of Mitochondrial Respiration

Like MPP+, MeßCs+ inhibit respiration in isolated liver mitochondria (Albores et al. 1990; Fields et al. 1992). Several MeßCs+ inhibit mitochondrial respiration at Complex I (NADH dehydrogenase) almost as effectively as MPP+, and inhibition at Complex II (succinate dehydrogenase) is greater by MeßCs+ than by MPP+ (Table 1). In contrast to MPP+, MeßCs+ also weakly inhibit mitochondrial respiration at Complex III (coenzyme Q-cytochrome C reductase) (Fields et al. 1992).

Table 1.--Inhibition of Mitochondrial Respiration by MPP+ and MeßCs+

	Site of Inhibition	
	Complex I	Complex II
Compound	IC50 (μM)	IC50 (μM)
MPP+	171	>1200
2-MeNH+	1145	n.d.
2-MeH+	2850	n.d.
2-MeHi+	186	225
2-MeHo+	209	225
2,9-Me_2NH+	90-100	n.d.
2,9-Me_2H+	100	250

Table modified from Albores et al. (Albores et al. 1990) and Fields et al. (Fields et al. 1992). Key to abbreviations: n.d.=not done, MPP+=1-methyl-4-phenylpyridinium ion, 2-MeNH+=2-methylnorharmanium ion, 2-MeH+=2-methylharmanium ion, 2-MeHi+=2-methylharminium ion, 2-MeHo+=2-methylharmolium ion, 2,9-Me_2NH+=N,N-2,9-dimethylnorharmanium ion, 2,9-Me_2H+=N,N-2,9-dimethylharmanium ion, and IC_{50}=concentration of compound that inhibited respiration to 50% of control. See Figure 1 for structures.

Inhibition of Dopamine Uptake

Both ßCs and MeßCs inhibit dopamine uptake into striatal synaptosomes isolated from rat brain, albeit more weakly than MPP+ (Drucker et al. 1990). Nonmethylated ßCs (e.g, norharman, harman, harmine, and harmol) inhibited dopamine uptake with IC_{50}s in the range 12-33 μM. Their 2N-methylated derivatives demonstrated IC50s ranging from 15-88 μM. These values are an order of magnitude higher than the IC_{50} for inhibition of dopamine uptake by MPP+ (0.4 μM). Inhibition of dopamine uptake by 2,9-Me_2ßCs+ has not been

evaluated. These data suggest that ßCs and MeßCs are substrates for the dopamine transporter. A representative MeßC+, 2($^{14}CH_3$)-harmine, has been shown to be a substrate for the dopamine transporter as evidenced by its partially nomifensin-inhibitable, Na^+-dependent accumulation into striatal synaptosomes (Drucker et al. 1990).

Toxicity to PC12 Cells in Culture

Several N-methylated ßCs are toxic to PC12 cells in culture as indicated by the release of lactate dehydrogenase into the culture media (Cobuzzi et al. 1994; Collins et al. 1992). In low energy media (glucose=0.22 mM) the rank order toxicity of these proposed neurotoxins was: MPP^+ = 2,9-Me_2NH^+ > 2-$MeHi^+$ = 2,9-Me2H^+ > 2-MeH^+. In high energy media (i.e., containing 25x more glucose) toxicity due to these compounds was abolished. It was suggested that in the presence of high glucose the effects of mitochondrial inhibition (decreased ATP synthesis) are compensated for by increased ATP synthesis from anaerobic glycolysis (Cobuzzi et al. 1994). In addition, evidence indicates that these compounds are toxic to rat primary mesencephalic cultures, although not as selectively as MPP^+ (Collins et al. 1996; Collins et al. 1995). The mechanisms which underlie MeßC+ toxicity have not be clearly elucidated, but inhibition of mitochondrial respiration is likely to contribute.

Nigrostriatal Degeneration

Like MPP^+, several MeßCs+ cause nigrostriatal damage following unilateral stereotaxic injection of the compounds into the substantia nigra of rats (Neafsey et al. 1995; Neafsey et al. 1989). Injected MeßCs+ produced nigral

lesions that ranged in size from 25 - 94% of the lesion-size produced by MPP+. In addition, several compounds reduced ipsilateral striatal dopamine content to 23 - 64% of control dopamine levels. The most effective MeßCs+ were 2,9-Me_2NH^+ and 2,9-Me_2H^+; both compounds produced nigral lesions that were comparable to those produced by MPP+, and reduced striatal dopamine to 37-42% of control values.

MeßCs+ in CSF From Control and Normal Cases

Matsubara et al. (1995) recently detected simple ßCs and their N-methylated derivatives in the lumbar cerebrospinal fluid (CSF) of control subjects and parkinsonian patients. Results show a significantly higher quantity of total N-methylated ßCs (2-MeßCs+ plus 2,9-Me_2ßCs+) in the CSF of persons with idiopathic PD versus controls; measurements were 85 ± 18 fmol/ml and 20 ± 4 fmol/ml in PD and controls, respectively. Moreover, 2,9-Me_2NH^+ was present in the CSF from 12 of 22 PD patients, but absent in control CSF.

MeßCs+ in Normal Human Brain

Matsubara detected MeßCs+ in the substantia nigra and parietal cortex of postmortem normal human brain tissue (Matsubara et al. 1993, 1996). In the substantia nigra 2-$MeNH^+$ (3.14 ± 1.47 pmol/g tissue) and 2,9-Me_2NH+ (0.77 ± 0.12 pmoles/g tissue) were present in all samples (N=4). In the cortex 2-$MeNH^+$ (0.17 ± 0.02 pmoles/g tissue) was present in each sample (N=13), and 2,9-Me_2NH^+ (0.10 ± 0.02 pmoles/g tissue) was detected in 11 of 13 cases. Neither 2-MeH^+ or 2,9-Me_2H^+ was detected in the substantia nigra (N=4), but 2 of the 13

cases demonstrated 2-MeH$^+$ (0.02 ± 0.02 pmoles/g tissue) and 2,9-Me_2H^+ (0.03 ± 0.02 pmoles/g tissue) in the parietal cortex.

Formation of N-Methylated ßCs in Brain

Most pertinent to this dissertation is the discovery that MeßCs$^+$ are formed from simple ßCs via an S-adenosyl-L-methionine (SAM)-dependent enzymatic process in the presence of guinea pig and rat brain homogenates (Collins et al. 1992; Matsubara et al. 1992b). Moreover, it was later shown that normal human brain exhibits ßC-2N-methyltransferase activity, but not ßC-9N-methyltransferase activity (Matsubara et al. 1993). These ßC-N-methyltransferase activities will be discussed in further detail below (Figure 2).

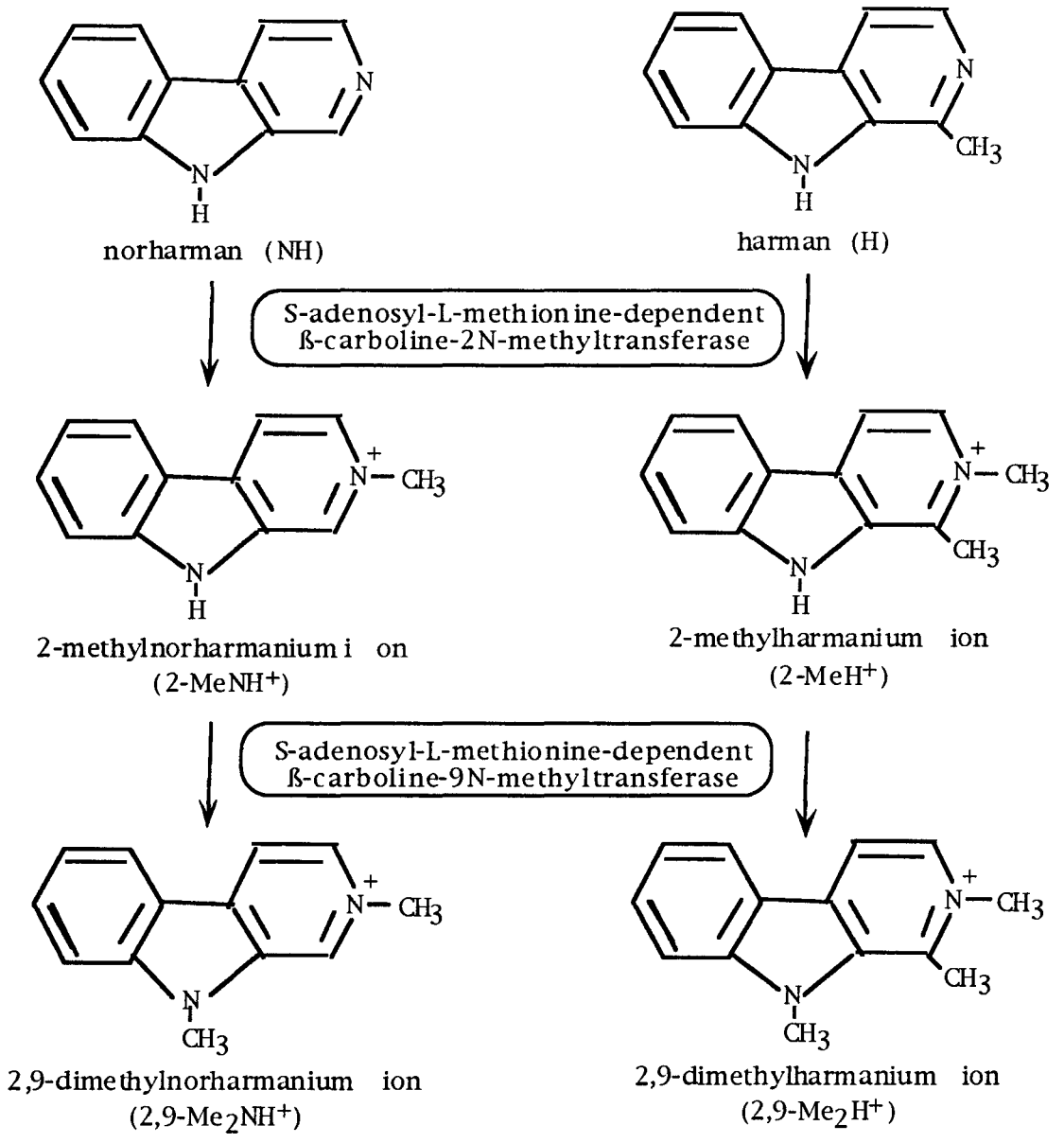


Figure 2. Representative ß-carboline-N-methyltransferase reactions.

ßC-N-Methyltransferases and Their Substrates

Substrates

ß-carboline 2N-methyltransferase (ßC-2-NMT) forms 2-MeßCs+ from simple ßCs, such as norharman, harman, and harmine (Collins et al. 1992; Matsubara et al. 1992b). Norharman and harman substrates are present in mammalian tissues, including normal human brain (Airaksinen et al. 1981; Bosin et al. 1989; Collins 1983; Fekkes et al. 1992; Matsubara 1996; Matsubara et al. 1993; Schouten et al. 1986; Shoemaker et al. 1980). Norharman and harman levels are higher in the substantia nigra relative to the parietal cortex of normal human brains (Matsubara 1996). In the parietal cortex norharman and harman were present at 0.58 ± 0.11 and 0.24 ± 0.04 pmoles/g tissue, respectively (N=13). In the substantia nigra norharman and harman were detected at 16.00 ± 8.00 and 1.04 ± 0.11 pmoles/g tissues, respectively. Interestingly, Kuhn et al. (1995) reported significantly elevated (p=0.01) plasma norharman in PD patients (30.0 ± 52.7, range 0-192 pg/ml) compared to control subjects (4.9 ± 7.9, range 0-26.5 pg/ml). Mean plasma values for harman were elevated in PD but were not significantly different than control values.

The presence in brain of simple ßCs, which can serve as substrates of the ßC-2-NMT, is not surprising given their widespread availability from exogenous as well as endogenous sources. Blood-brain barrier penetration of representative ßCs and 2-MeßCs+ was recently demonstrated by Matsubara et al. (1996). He utilized striatal microdialysis to show that NH, 9-MeNH, and 2-MeNH+ are present in the brain following intraperitoneal (i.p). injection of these compounds into C57/black mice. Blood-brain barrier penetration of

nonmethylated simple ßCs (NH and 9-MeNH) was expected, due to their presumably hydrophobic structure; however, entry of the charged MeßCs+ (2-MeNH+) was quite surprising. Simple ßCs may be formed endogenously by the condensation of indoleamines and aldehydes to form tetrahydro-ß-carbolines, which may be subsequently oxidized to the corresponding ßC (Melchior et al. 1982; Susilo et al. 1987). Exogenous sources of simple ßCs include: plants, tobacco smoke, industrial waste, alcoholic beverages, soy sauce, and pyrrolyzed tryptophan-rich foods such as meat (Adachi et al. 1991; Bosin et al. 1988; Felton et al. 1990; Gross et al. 1993; Holmstedt 1982; Rommelspacher et al. 1985a; Rommelspacher et al. 1985b).

ß-Carboline-N-Methyltransferase(s)

Two SAM-dependent N-methyltransferase (ßC-NMT) activities have been detected in guinea pig and rat brain homogenates (Collins et al. 1992; Matsubara et al. 1992b).

The first activity, ßC-2N-methyltransferase (ßC-2-NMT), catalyzes the 2N-methylation of simple ßCs to form 2-MeßCs$^+$. This activity has also been measured in normal postmortem human brain (Matsubara et al. 1993), where activity was 1.30 ± 0.42 pmol/h/mg protein in the substantia nigra, and 1.06 ± 0.26 pmol/h/mg protein in the parietal cortex. Substrates that have been identified for ßC-2-NMT include: norharman, harman, harmine, 9-methylnorharman, and 9-methylharmine (Matsubara et al. 1992b). Curiously, the soil-residing bacteria, *Kitasatosporia setae,* possesses an enzyme activity that is also able to 2N-methylate norharman (Peczynska-Czoch et al. 1987). Whether this N-methyltransferase and ßC-2-NMT are the same enzyme requires further investigation.

The second activity, ßC-9N-methyltransferase (ßC-9-NMT), catalyzes the 9N-methylation of 2-MeßCs$^+$ to form 2,9-Me_2ßCs$^+$. Non-methylated ßCs do not appear to be substrates for ßC-9-NMT. It is yet to be determined if ßC-2-NMT activity and ßC-9-NMT activity are due to a single enzyme, or represent distinct enzymatic activities. N-methylation of ßCs appears to occur sequentially, with N-methylation at the pyridyl (2N-) nitrogen preceding indole (9N-) methylation. This sequence is predicted because no 9-MeNH was detected in an incubation in which norharman was the substrate; 2-MeNH$^+$ and 2,9-Me_2NH$^+$ were the only detectable products. Substrates identified for ßC-9-NMT include: 2-methylnorharmanium, 2-methylharmanium, and 2-methylharminium ions (Matsubara et al. 1992b).

The pH optimum for ßC-9N-methyltransferase activity from guinea pig whole-brain homogenate was in the range 8.2-8.3 using a phosphate buffer system. The pH optimum for ßC-2N-methyltransferase activity was not determined. Kinetic parameters for methylation of norharman derivatives at a fixed concentration of SAM (8 µM) in crude guinea pig brain homogenates were as follows (Matsubara et al. 1992b):

Activity	Substrate	K_m (µM)	V_{max} (pmol/h/mg protein)
ßC-9-NMT	2-MeNH+	17.8	4.9
ßC-2-NMT	9-MeNH	17.8	2.2

Previous studies from this laboratory indicate that guinea pig brain ßC-2-NMT activity resides primarily in the nuclear (236.1 pmol/h total activity) and microsomal (14.3 pmol/h total activity) subcellular fractions. ßC-9-NMT activity was found in the nuclear (233.8 pmol/h total activity), microsomal (82.7 pmol/h total activity), and mitochondrial (58.6 pmol/h total activity) fractions. Neither ßC-methyltransferase activity was detected in undialyzed cytosol (Matsubara et al. 1992b).

The regional distribution of both ßC-NMT activities was assessed in guinea pig brain (Matsubara et al. 1992b). ßC-2-NMT specific activity was highest in the pons/medulla (6 pmol/h/mg protein) and lowest in the frontal cortex (0.5 pmol/h/mg protein). Intermediate ßC-2-NMT activity was found in the hippocampus, hypothalamus, striatum, and midbrain. Similarly, ßC-9-NMT specific activity was also highest in the pons/medulla (9 pmol/h/mg protein) and lowest in the frontal cortex (2 pmol/h/mg protein). Intermediate ßC-9-NMT activity was detected in the hypothalamus, hippocampus, midbrain, and

striatum. Overall, the specific activity of ßC-9-NMT was higher than that of ßC-2-NMT activity in every brain region tested, particularly in the hypothalamus.

Matsubara et al. (1996) recently reported the presence of cerebral 2-MeNH+ following i.p injection of norharman or 9-MeNH in C57/black mice. The author speculated that injected 9-MeNH is demethylated in the liver to norharman, and that the norharman is then available for methylation by ßC-2-NMT to 2-MeNH+. In the same study 2,9-Me_2NH^+ was detected in mouse brains following i.p injection of norharman, 9-methylnorharman, or 2-MeNH+. A most interesting finding is that subchronic treatment with norharman or 2-MeNH+ not only selectively reduced nigrostriatal dopamine, but also produced bradykinesia in these mice. These experiments are consistent with the hypothesis that in vivo formation of MeßCs+ by ßC-2-NMT and ßC-9-NMT reduces striatal dopamine, which produces a parkinsonian condition.

S-Adenosyl-L-Methionine-Dependent Methyltransferases

General

Enzymes that catalyze S-adenosyl-L-methionine (SAM)-dependent methylation of a substrate belong to the E.C. 2.2.1 class as defined by the International Enzyme Commission (Barman 1969). A generic SAM-dependent methyltransferase reaction is diagrammed in Figure 3.

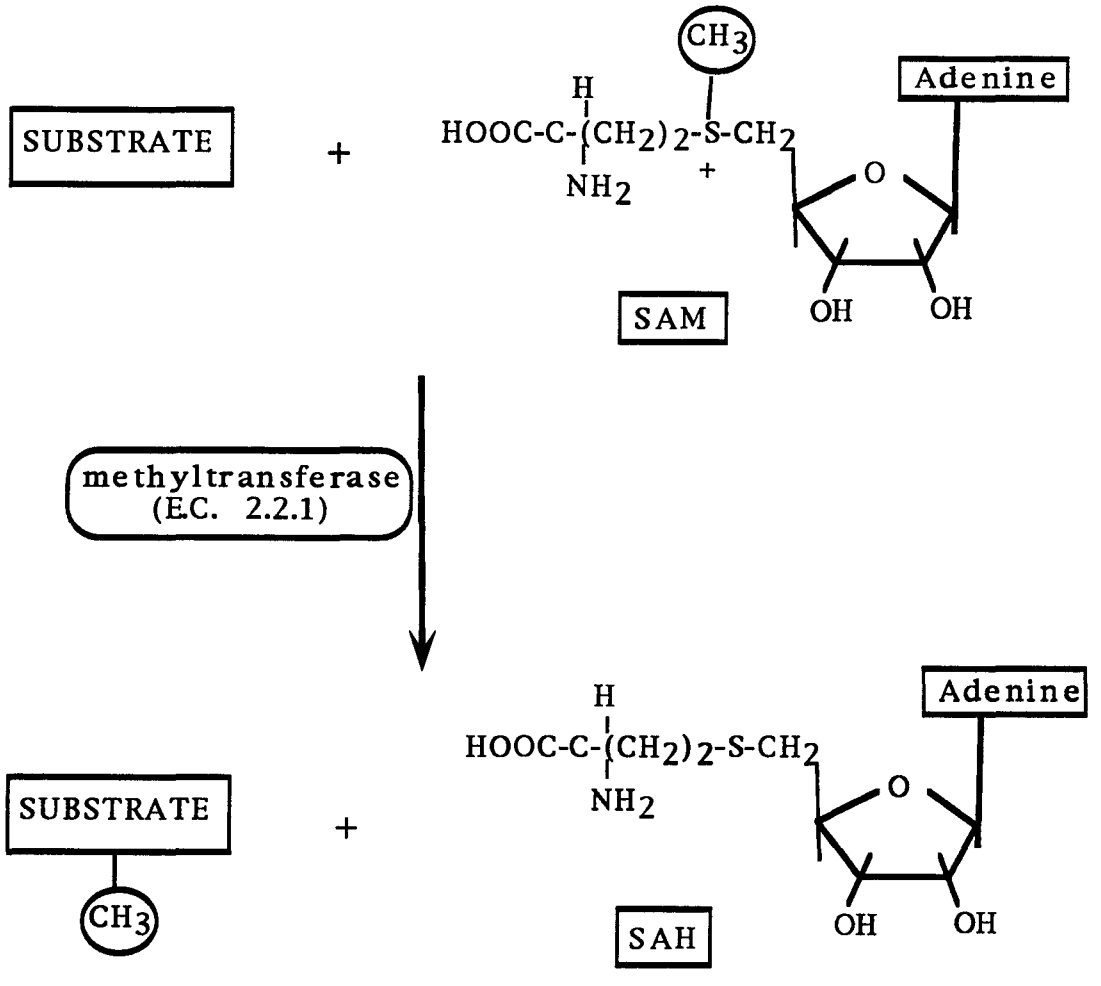


Figure 3. Generic SAM-dependent methyltransferase reaction.

All SAM-dependent methyltransferases studied to date are inhibited by S-adenosyl-L-homocysteine (SAH) in a competitive manner. Inhibition by SAH is not surprising since products often inhibit the enzymes responsible for their formation, and SAH is a co-product of SAM-dependent methylation reactions. Inhibition by SAH varies, depending upon the enzyme affected, with K_i's falling into the 100 nM - 12 μM range (Deguchi et al. 1971; Lawrence et al. 1990). Therefore, if an enzyme under study is truly a SAM-dependent methyltransferase, inhibition by SAH should be a property of that enzyme. ßC-NMT inhibition by SAH was not investigated by Matsubara.

In addition, a peptide inhibitor of methyltransferase reactions has also been reported; this peptide is proposed to inhibit many methyltransferases via a noncompetitive mechanism (Park et al. 1993). This inhibitor was purified from rat liver cytosol, and exhibited the following characteristics: 29 amino acids, 52% glycine with no basic amino acid residues, and a molecular weight of 2584. The authors noted that extensive dialysis is required for removal of the inhibitor from tissue homogenates.

Enzymatic N-Methylation of Azaheterocycles

The presence of a SAM-dependent N-methyltransferase activities have been detected in dialyzed cytosolic fractions from rabbit and guinea pig lung, kidney, spleen, and brain. These rather nonspecific activities have been assigned a number of names including amine-N-methyltransferases A and B, indolethylamine-N-methyltransferase, and arylamine-N-methyltransferase (Ansher et al. 1986a; Ansher et al. 1986b). The nonspecificity of these enzymes is typified by their ability to N-methylate a variety of azaheterocycles at the

pyridyl nitrogen. N-methylation results in the formation of a quaternary pyridinium ion; recall that this cationic feature is essential for the neurotoxicity due to MPP^+.

Interestingly, pyrido-N-methyltransferase activities (e.g., amine N-methyltransferases A and B) -- present in human, monkey, mouse, and rabbit brain -- are capable of forming MPTP from precursor 4-phenyl-1,2,3,6-tetrahydropyridine. These enzymes also act on 4-phenylpyridine to generate MPP^+. As summarized in Table 2, the specific activities of these N-methyltransferase activities in mammalian brain homogenates (Ansher et al. 1986a) are comparable to the N-methylation activities reported toward ßCs (Matsubara et al. 1993) and tetrahydro-ß-carbolines by Matsubara et al. (Matsubara et al. 1992a) .

Table 2.--N-Methylation of Various Azaheterocycles

	N-Methyltransferase Activity (pmole/h/mg protein)	
Substrate	Human Brain	Rat Brain
4-phenylpyridine (a)	3.7	2.7
4-phenyltetrahydropyridine (a)	0.17	6.8
9-methylnorharman (b)	1.30 (cortex)	0.4
	1.06 (nigra)	
tetrahydro-ß-carboline (c)	-----	2.6

Table 2 summarizes data from three investigators: (a) Ansher et al. (1986a) , (b) Matsubara et al. (1993), and (c) Matsubara et al. (1992a) . Note that assays were not run under identical conditions.

Studies indicate that these amine-N-methyltransferases have the following properties: (a) reside in the cytosolic fraction of tissue homogenates, (b) usually unmeasurable prior to dialysis, and (c) higher in fresh vs. frozen tissue (Ansher et al. 1986a; Ansherand Jakoby 1986b). Ansher and Jakoby purified these amine-N-methyltransferases A and B from rabbit liver; the enzymes have very broad and overlapping specificity for a number of primary, secondary and tertiary amines (Ansher et al. 1986a; Ansherand Jakoby 1986b). The azaheterocycles tested included the above-mentioned pyridine derivatives, as well as isoquinoline, but ßCs were apparently not evaluated as substrates. Rabbit liver cytosolic activities exhibited pH optima of 6.2 and 7.8 for transferase A and B, respectively.

Saavedra et al. (1973) described the distribution of a cytosolic nonspecific N-methyltransferase present in the brain of several species, including human postmortem brain tissue. That SAM-dependent enzyme utilizes tryptamine as well as several other amines as substrates. Similar to ßC-2-NMT, the enzyme exhibited a K_M with respect to SAM of 52 µM and a tissue-dependent V_{max} in the range 2 - 15 pmol/h/mg protein.

Naoi et al. (Naoi et al. 1989a) reported the presence of a cytosolic N-methyltransferase activity in human brain homogenates that forms potentially neurotoxic N-methyl-1,2,3,4-tetrahydroisoquinoline from tetrahydroisoquinoline (TIQ). Likewise, Matsubara identified a cytosolic N-methyltransferase activity present in rodent brain that catalyzes methylation at the 2N-nitrogen of tetrahydro-ß-carbolines (Matsubara et al. 1992a). It is conceivable that ßC-NMT activity is due to any of the above-mentioned enzymatic activities.

General Enzyme Properties and Terminology

This portion of the literature review contains information directly related to the content of this dissertation, and therefore is not intended to cover enzyme properties and kinetics in detail (Barman 1969; Lehninger 1975).

General Characteristics of Enzymes

The International Enzyme Commission has numerically classified enzymes according to the type of reactions that they catalyze (Barman 1969). Each characterized enzyme is assigned a number comprised of four figures: the first figure places the enzyme in a main group (e.g., group 2 = transferases), the second figure identifies the subclass (e.g., subclass = methyltransferases), the third figure represents the sub-subclass, and finally the fourth figure is the serial number of the specific enzyme within its sub-subclass. As noted previously, SAM-dependent methyltransferases belong to E.C.2.2.1.

Nearly all enzymes are proteins with a primary structure composed of amino acids linked covalently by peptide bonds; interactions between amino acid side-chains give rise to secondary structure which then participates in higher order structure. Amino acid side-chains, as well as the α-amino and α-carboxyl termini, impart acid-base, polar, and nonpolar properties to regions of the protein. These properties are exploited in the purification techniques that are described in a subsequent section of this chapter.

Enzymes catalyze biological reactions, and as catalysts are not consumed during the reactions in which they participate. Analogous to other chemical

catalysts, enzymes affect reaction rate but not the equilibrium of the reactions that they catalyze.

Properties unique to enzyme catalysts are detailed below and include: sensitivity to extremes of temperature, pH, ionic strength, other solvent conditions, and specificity toward its substrate(s).

Sensitivity to Temperature, pH, Ionic Strength, Solvents

Changes in these parameters affect amino acid side-chains of the enzyme, leading to alterations in ionization state which affects hydrogen bonding and hydrophobic interactions. These changes are often detrimental to the physical, chemical, and catalytic stability of the enzyme.

Substrate Specificity

Substrate specificity of an enzyme is divided into four categories.

(1) Absolute specificity. This type of specificity implies that an enzyme will act on a single substrate.

(2) Group specificity. Group specificity applies to enzymes that act on more than one substrate, but require certain atomic groupings within those substrates.

(3) Reaction specificity. This type of enzyme requires the least amount of specificity, and describes enzymes that do not require strict atomic groupings in the vicinity of the reactions that they catalyze.

(4) Stereochemical specificity. This kind of specificity implies that an enzyme will only effect a reaction on one stereoisomer of a given substrate.

Other Characteristics

Enzyme catalyzed reactions should exhibit linear formation of product as a function of time and protein concentration, assuming that the enzyme remains active and substrates are not limiting during the assay.

Effect of Additives

Miscellaneous additives may stabilize or destabilize enzymes during their assay and purification, as discussed by Scopes (1994a) and Deutscher (1990).

Antioxidants. When enzymes are removed from their intracellular environment (reducing conditions) an antioxidant such as dithiothreitol or mercaptoethanol is often needed to maintain enzyme stability and activity.

Protease Inhibitors. Tissue disruption may result in the release of cellular proteases; therefore protease inhibitors are often utilized, especially during the early steps of enzyme isolation. Commonly used protease inhibitors are shown in Table 3.

Table 3.--Commonly Used Protease Inhibitors
[adapted from Scopes (1994a) and Deutscher (1990)]

Protease Inhibitor	Protease Class Inhibited	Useful Concentration
phenylmethylsulfonyl fluoride (PMSF)	serine proteases	0.1-1 mM
benzamidine	serine proteases	1 mM
aprotinin	serine proteases	5 µg/ml
leupeptin	thiol proteases	1 µg/ml
antipain	thiol proteases	1 µg/ml
pepstatin A	acid proteases	1 µg/ml
EDTA/EGTA	metalloproteases	0.1-1 mM

Metals. Many enzymes require metals for activity, while others are inhibited in presence of metals. Transition elements that may affect enzyme activity include: Mn^{2+}, $Fe^{2+/3+}$, Co^{2+}, Ni^{2+}, $Cu^{1+/2+}$, Zn^{2+}, Ca^{2+}, and Mg^{2+} (Gray 1971).

Enzyme Terminology

Miscellaneous terms associated with enzymes, particularly enzyme kinetics, are listed in the GLOSSARY of this dissertation (Barman 1969; Dixon et al. 1979b; Lehninger 1975). Other concepts are presented below.

Michaelis-Menten equation and kinetics. The Michaelis-Menten equation mathematically describes -- for a single-substrate enzyme or often a two-substrate enzyme (when one of the substrates is fixed at a saturating concentration) -- the relationship between the following parameters: initial rate of the reaction (v), substrate concentration [S], and kinetic constants (K_M and V_{max}).

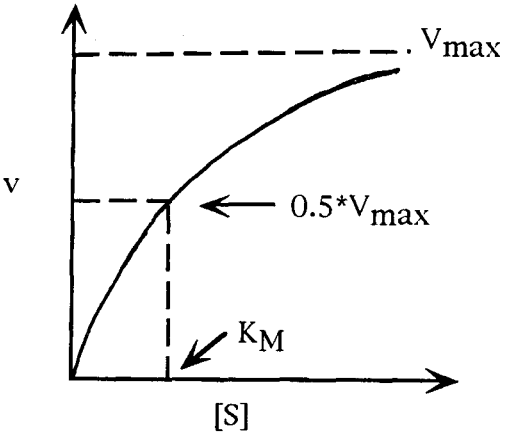
Reaction:

$$[E] + [S] \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [ES] \underset{k_{-2}}{\overset{k_{cat}}{\rightleftharpoons}} [E] + [P]$$

Equation: $v = V_{max}\,[S] / ([S] + K_M)$

Assumptions: [P] and [E] interaction is negligible, therefore k_{-2} is negligible, k_{cat} is first-order, [S], [E], and [ES] in equilibrium (steady-state)

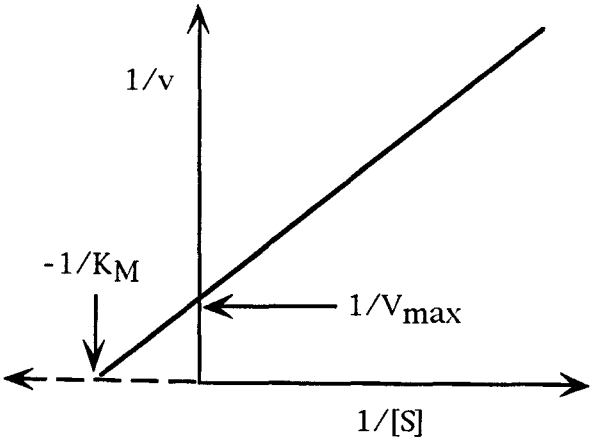
Graphically:



Lineweaver-Burk equation. This equation is a commonly used linear transformation of the Michaelis-Menten equation.

Equation: $1/v = (K_M/V_{max})(1/[S]) + 1/V_{max}$

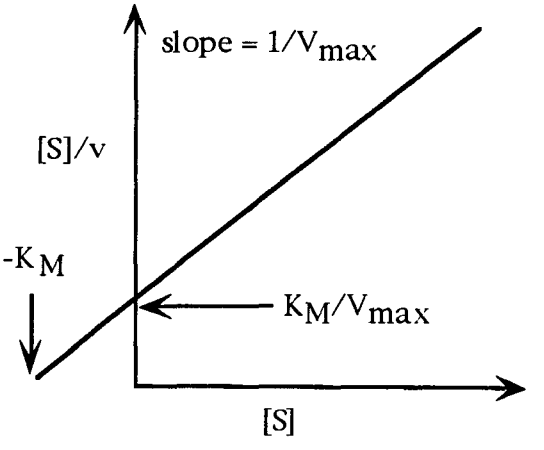
Graphically:



Hanes equation. This is another linear transformation of the Michaelis-Menten equation.

Equation: $[S]/v = (K_M/V_{max}) + ([S]/V_{max})$

Graphically:



Dixon Equations and Plots for Inhibition

Dixon Equation and Plot for Competitive Inhibition

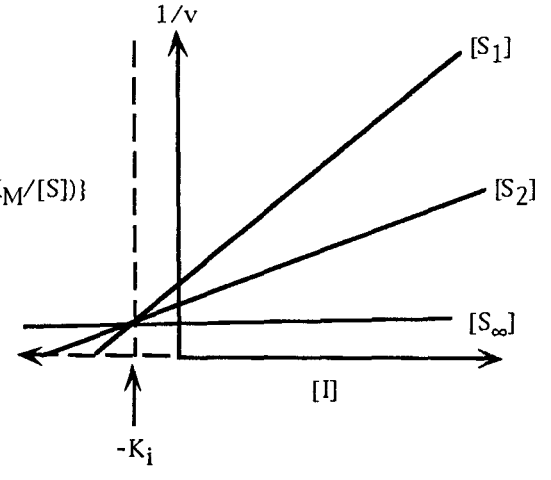
Substrate: $[S_\infty] > [S_2] > [S_1]$

Inhibitor: [I]

$$1/v = \{K_M/(V_{max}[S]K_i)\}[I] + (1/V_{max})\{1 + (K_M/[S])\}$$

$$\text{slope} = K_M/(V_{max}[S]K_i)$$

$$K_i = K_M/(V_{max}[S]\text{slope})$$



Dixon Equation and Plot for Noncompetitive Inhibition

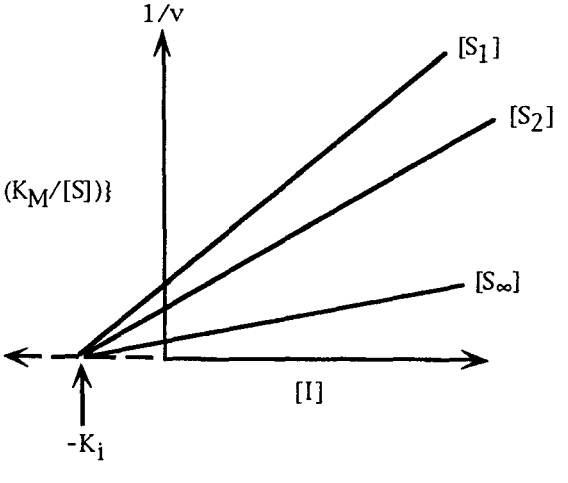
Substrate: $[S_\infty] > [S_2] > [S_1]$

Inhibitor: [I]

$$1/v = \{1 + (K_M/[S])/(V_{max}K_i)\}[I] + (1/V_{max})\{1 + (K_M/[S])\}$$

$$\text{slope} = \{1 + (K_M/[S])\}/(V_{max}K_i)$$

$$K_i = \{1 + (K_M/[S])\}/(V_{max}\text{slope})$$



Dixon Equation and Plot for Uncompetitive Inhibition

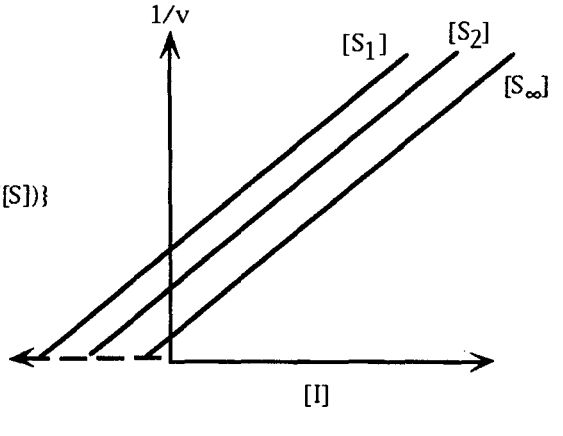
Substrate: $[S_\infty] > [S_2] > [S_1]$

Inhibitor: [I]

$$1/v = \{1/(V_{max}K_i)\}[I] + (1/V_{max})\{1 + (K_M/[S])\}$$

$$\text{slope} = 1/(V_{max}K_i)$$

$$K_i = 1/(V_{max}\text{slope})$$



Literature Related to Protein Purification

General

This section of the literature review is intended to be a simple overview of applicable protein purification techniques. Enzyme purification may include the following general sequence of steps: preparation of tissue extract, subcellular fractionation, ammonium sulfate or other bulk precipitation techniques, miscellaneous chromatographic methods, and usually polyacrylamide gel electrophoresis (Deutscher 1990).

Total Protein Assays

UV Absorption at 280 nm and 260 nm

This is a nondestructive method that is frequently used to monitor protein in crude extracts or column effluents. The primary disadvantage of this method is its lack of sensitivity. Absorbance at 280 nm is due to the presence of tryptophan and tyrosine residues in proteins, as well as nucleic acids. Proteins absorb little at 260 nm, a wavelength at which absorbance is due primarily to the pyrimidine and purine residues of nucleic acids. Therefore, a reasonable estimate of the quantity of protein present in a relatively clear sample may be obtained by measuring the absorbance of the sample at 280 nm and 260 nm for use in the following equation:

$$\text{protein concentration (mg/mL)} = 1.55A_{280} - 0.76A_{260}$$

Lowry Protein Assay

This method is based upon the interaction of Cu^{2+} with peptide bonds to form a complex; under basic conditions the complex is reduced to Cu^{1+}. Folin Reagent then interacts with both the complexed Cu^{1+} and the side-chains of tyrosine, tryptophan, and cysteine. This interaction slowly imparts a blue color to the mixture, which is detected at 500 nm to 750 nm using UV spectroscopy. Absorbance at a set wavelength in this range correlates with protein concentration in the original sample. Solutions required in this assay are described in Scopes (Scopes 1994c) and in Appendix A of this dissertation.

Coomassie Blue Dye Binding Assay

This method is based on an observed shift in absorbance from 465 nm to 595 nm under acidic conditions when protein interacts with the negatively charged dye. Side chain groups in proteins that facilitate this interaction include those of arginine, histidine, lysine, tyrosine, tryptophan, and phenylalanine. Solutions required in this assay are described in Scopes (1994c) and in Appendix A of this dissertation.

Subcellular Fractionation

This technique could be considered a gross means of protein fractionation in that it allows for the separation of many undesirable proteins and nonproteinaceous materials from the enzyme of interest based on their location in distinct subcellular compartments. Subcellular fractionation methodology has been recently reviewed by Storrie and Madde (Deutscher 1990). Subcellular fractionation is routinely done using modifications of the differential centrifugation protocol (Table 4) (Alberts et al. 1989).

Table 4. -- Subcellular Fractionation Protocol
[adapted from Alberts (1989)]

	Centrifugation	
Steps	Parameters	Pellet Contains
low speed	1000*g*, 10 min.	whole cells, nuclei, cytoskeletons
medium speed	20,000*g*, 20 min.	mitochondria, lysosomes, peroxisomes
high speed	80,000*g*, 1 hr.	microsomes, small vesicles
very high	150,000*g*, 3 hrs.	ribosomes, viruses, large macromolecules

Bulk Precipitation Techniques

This technique exploits the effects of salts or organic solvents on protein conformation and consequently protein solubility. Proteins with variable amino acid compositions can be separated based on their behavior at different electrolyte or solvent concentrations. Commonly used precipitants are the neutral salt, ammonium sulfate, or organic solvents such as polyethylene glycol, ethanol, or acetone. Bulk precipitation techniques are discussed in detail by Scopes (1994a), as well as Englard and Seifer in the Methods in Enzymology series (Deutscher 1990). In practice, the precipitant is added in small aliquots while the protein extract is stirred at low temperature. The mixture is stirred for a defined period and then centrifuged to pellet and isolate precipitated proteins. Additional precipitant may then be added to the resulting supernatant, the mixture again centrifuged, and so on. Proteins are therefore fractionated (precipitated) based on their individual solubilities in progressively more concentrated salt solution or organic solvent.

Chromatographic Methods

General

A series of laboratory guides published by Pharmacia are suitable primary references for chromatographic isolation of proteins (Pharmacia 1991; Pharmacia 1993a; Pharmacia 1993b; Pharmacia 1994). These guides describe in detail the practical aspects of preparing, using, and maintaining various chromatographic resins. The books "Protein Purification: Principles and Practice" by Scopes (1994a) and "Guide to Protein Purification" by Deutscher (1990) are also invaluable resources during the development of a protein purification protocol. Each of these references was utilized in the preparation of the remainder of this literature review.

In general, all chromatographic methods exploit the properties of the protein in solution by differentially promoting or preventing binding of proteins to chromatographic resins. Chromatographic resins are commercially available that have been chemically modified to possess the numerous functional groups and characteristics described below.

Both ion exchange and hydrophobic interaction chromatographic methods are considered high capacity methods because they allow for fractionation of samples composed of larger quantities of proteins, as compared to lower capacity methods such as gel filtration and affinity chromatography. High capacity methods are typically utilized early in purification procedures, while lower capacity methods follow later, in order of decreasing capacity and/or specificity for the protein to be isolated.

Ion Exchange Chromatography

Ion exchange chromatography (IEC) allows for separation of sample molecules as a function of the charge of the exchanger and proteins. Anion exchangers contain positively charged groups such as quaternary amines or diethylaminoethyl (DEAE); these exchangers interact with negatively-charged protein molecules. Cation exchangers, possess negatively charged groups such as a sulfonic acid or carboxymethyl (CM) side-chains; these resins interact with positively-charged protein molecules. Both pH and ionic strength are manipulated in order to affect the interaction of protein with the exchanger. For example, proteins are typically applied to an anion exchange column under conditions of low salt in buffer at pH 7-9; under these conditions many proteins have a net negative charge, and therefore bind to the positively-charged exchanger. Proteins are routinely eluted from ion-exchange matrices using an increasing salt gradient, or less commonly a pH gradient. Fractions from the column are collected and assayed for total protein content and protein of interest.

Hydrophobic Interaction Chromatography

Hydrophobic interaction chromatography (HIC) enables the separation of proteins based on their hydrophobicity. HIC matrices contain hydrophobic moieties such as simple ethyl to dodecyl alkyl chains or phenyl groups. Hydrophobic interactions between proteins and the HIC matrix are favored under conditions of high salt and low pH. Temperature and additives may also affect HIC interactions, albeit in a less straightforward manner. Therefore, samples are usually applied to HIC columns in a high salt buffer, with the same buffer being utilized to pre-equilibrate the column. Successive elution of

bound protein is often achieved using a gradient of decreasing salt concentration. Occasionally organic solvents or other additives are utilized in elution buffers for HIC. Column effluent is collected in fractions that are subsequently assayed for total protein and proteins of interest.

Affinity Chromatography

In general, affinity chromatographic methods take advantage of the specificity of an interaction between an enzyme and its substrates or inhibitors. A substrate or inhibitor that is specific for the enzyme of interest is covalently attached to a suitable chromatographic matrix by conventional methods. Dye chromatography is a subset of affinity chromatography, in which dye molecules are covalently attached to the matrix. The affinity column is equilibrated with sample buffer and then the sample is applied to the column. Elution of bound protein may be accomplished using salt or pH gradients. Tightly bound proteins are often eluted by a solution containing either the substrate/inhibitor or a substrate/inhibitor analogue. Column fractions are assayed for total protein and proteins of interest.

Gel Filtration Chromatography

Gel filtration chromatography (GFC) is synonymous with size-exclusion chromatography (SEC). In this method molecules are separated on the basis of size; GFC matrices are available with a range of pore sizes which either exclude or allow passage of sample molecules. Excluded molecules elute from the column first, while molecules that are able to enter matrix pores are eluted from the column as a function of decreasing molecular weight. This method not only serves to fractionate proteins based on their size, but also has utility

in the determination of the approximate molecular weight of proteins and peptides. These gels are also used to desalt protein and peptide solutions, as well as to carry-out buffer exchange on sample solutions.

Sequence of Chromatographic Steps

Ion exchange chromatography is routinely used early in a purification procedure depending upon the method used to bulk precipitate proteins following subcellular fractionation. If ammonium sulfate is used as the precipitant, hydrophobic interaction chromatography (HIC) should follow, since application to HIC columns is typically achieved under conditions of high salt concentration, such as the solutions generated following ammonium sulfate precipitation. If polyethylene glycol is used as the precipitant, ion exchange chromatography should follow the PEG step, since solutions are usually applied to IEC columns under low salt conditions and PEG may interfere with HIC.

Additional Comments

Reference to other relevant literature, not cited in this chapter ("Review of Relevant Literature"), is more appropriately included in the "Discussion" sections of the individual chapters.

CHAPTER 2

ß-CARBOLINE-9N-METHYLTRANSFERASE

Background

This chapter is included for completeness and historical purposes. It is not presented in as much detail as subsequent chapters, and therefore only key studies are considered. Due to ßC-9-NMT's novel activity -- methylation of an indole nitrogen -- this enzyme was originally the proposed focus of this dissertation project. Unfortunately, ßC-9-NMT activity could not be consistently measured in animal brain, but this activity was consistently detected in postmortem human brain (Chapter 5). The ßC-9-NMT project was eventually abandoned in favor of investigations pertaining to brain ßC-2N-methyltransferase activity; however certain several ßC-9-NMT studies are described in this chapter.

Studies presented in this chapter include: (a) determination of kinetic constants for ßC-9-NMT with respect to S-adenosyl-L-methionine; (b) detection of ßC-9-NMT activity in guinea pig brain and various rat organs; (c) comparison of ßC-9-NMT activity in phosphate and TRIS buffer; (d) ßC-9-NMT activity as a function of time; (e) the effect of homogenate dialysis on detectable activity; (f) evaluation of ßC-9-NMT activity in bovine brain and yeast -- these sources were evaluated as possible starting materials for purification purposes; and (g) subcellular localization of ßC-9-NMT activity from bovine brain.

Materials and Methods

Reagents

ß-Carboline substrate, 2-methylnorharmanium iodide (2-MeNH+), was synthesized in our laboratory according to Matsubara (1992b). Unlabeled S-adenosyl-L-methionine (chloride, iodide, or toluenesulfonate salt) was purchased from Sigma Chemical Company. Tritiated S-[methyl-^{3}H]-adenosyl-L-methionine (60-85 Ci/mmol) was bought from American Radiolabeled Chemicals or Dupont NEN. All other reagents were appropriately pure for their intended use and were utilized as purchased.

Preparation of ßC-9-NMT Source

Guinea Pig Brain

Frozen guinea pig brain was purchased from either Rockland or Harlan. Extract preparation was done at 0-5 °C. Brain was thawed slightly, cut into small pieces, and homogenized in a glass homogenizer by a motor-driven teflon pestle (5-10 up-and-down strokes @ 100 rpm). In indicated experiments, homogenation was accomplished using a Tissuemizer® set at its lowest setting. Brain (1 g) was homogenized in 2-10 ml of homogenation buffer, consisting of 10 mM Na_2HPO_4 (pH 7.4) or 100 mM TRIS (pH 8.3).

Yeast Extract

Red Star active dry yeast (5 g) was added to a polypropylene or glass centrifuge tube containing 15 ml of 100 mM TRIS buffer (pH 8.3). The mixture was vortexed until the powder was completely wetted to form a slurry. Small glass beads (0.5-1 mm in diameter, 15 ml in all) were added to the slurry, and

the mixture was vortexed vigorously in order to disrupt yeast cell walls. Six vortexing cycles (30 seconds per cycle) were carried-out. Between each cycle the tube was placed on ice for 2-3 minutes to dissipate heat generated during the disruption process. Cell debris was removed from the extract by passage through glass wool or centrifugation.

Bovine Brain

Bovine brain was utilized in some experiments. Tissue procurement and preparation are described in detail in subsequent sections.

Protein Determination

A Coomassie blue dye-binding method was utilized as described in Scopes (Scopes 1994c). Details are in Appendix A.

The ßC-9-NMT Assay

This activity catalyzes the transfer of the tritiated methyl moiety from S-adenosyl-L-methionine (SAM) to 2-methylnorharmanium ion (2-MeNH+) to form 2-methyl-9(^{3}H-methyl)-norharmanium ion (2,9-Me_2NH^+) and S-adenosyl-L-homocysteine (Figure 4).

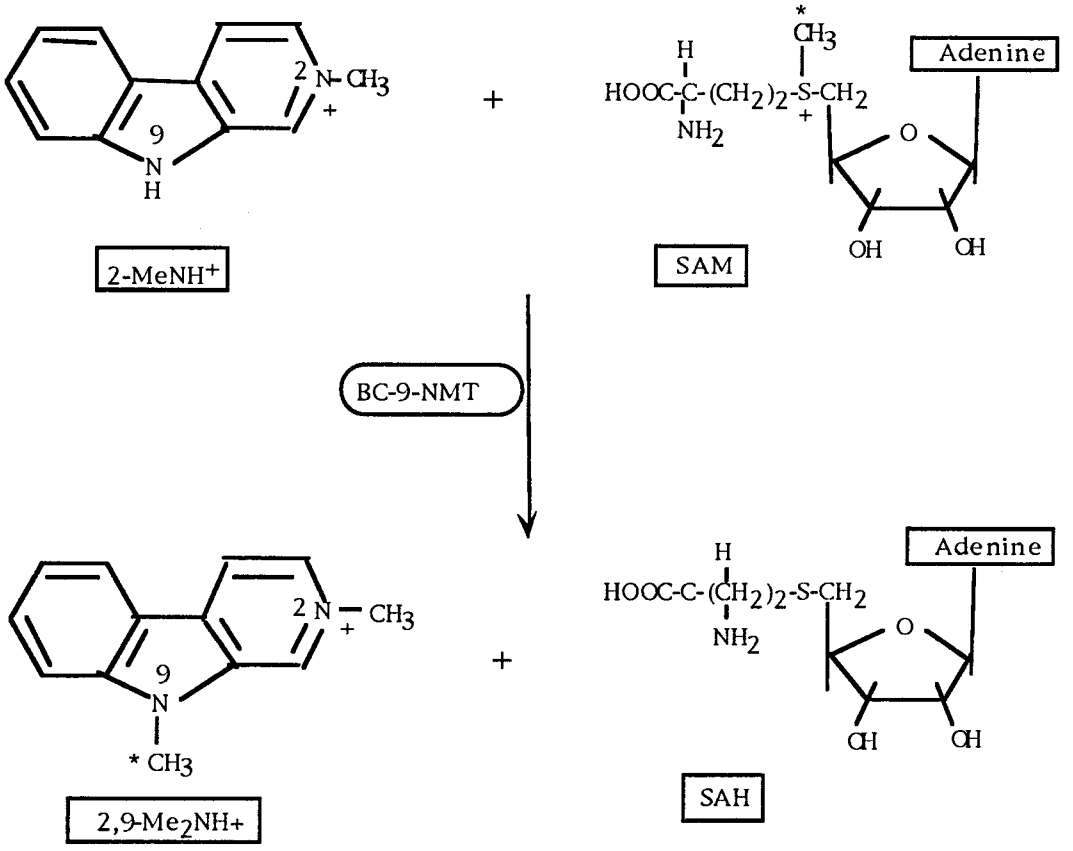


Figure 4. ßC-9-NMT assay. Site of tritium label *. Abbreviations: 2-methylnorharmanium ion (2-MeNH+), 2,9-dimethylnorharmanium ion (2,9-Me_2NH^+), S-adenosyl-L-methionine (SAM), S-adenosyl-L-homocysteine (SAH).

The ßC, 2-MeNH+, was chosen over other substrates (e.g. norharman) since its pyridyl position (2N-) is already methylated, allowing for measurement of 9N-methylation exclusively. This substrate is most like a physiological substrate for ßC-9-NMT. The assay was performed as previously reported by Matsubara et al. (1993) with modifications. Concentrated stock solutions of 2-MeNH+ and SAM were combined with organ fraction or yeast extract in 1.5 ml polypropylene microcentrifuge tubes. The SAM was usually dissolved in 10 mM Na_2HPO_4 buffer (pH 7.4), 2-MeNH+ was prepared in 100 mM Na_2HPO_4 buffer (pH 9.5), and tissue homogenate or yeast extract was present in 10 mM Na_2HPO_4 buffer (pH 7.4). However, in indicated studies, substrates and enzyme extract were made in 100 mM TRIS buffer (pH 8.3). Stock solution volumes usually remained constant across studies, while stock solution concentrations and final pH varied depending upon the experiment. Assay volume was typically 370 µl, comprised of the following stock solutions: 50 µl ^{3}H-SAM, 20 µl non-radioactive SAM, 200 µl 2-MeNH+, and 100 µl enzyme solution. In assays that excluded radioactive SAM, total volume was routinely 200 µl: 50 µl each of 2-MeNH+ and SAM stocks, plus 100 µl enzyme extract. Final concentrations of substrates are indicated in individual experiments. The assay was gently vortexed and subsequently maintained at 37 °C in a shaking water bath for 15-60 minutes.

The reaction was then treated in one of two ways: with or without the use of solid-phase extraction (SPE) cartridges. When SPE was excluded from the reaction work-up, the assay was terminated by placing the tubes on ice and adding 8.6 µl of concentrated perchloric acid ($HClO_4$). Precipitated protein was pelleted by centrifugation at 3000*g* for 5 minutes. If noted, the pellet was

resuspended in 0.5-1.0 ml of 0.1 N $HClO_4$, centrifuged, and the resulting supernatant combined with the first. Combined supernatant was transferred to a weighed 1.1 ml conical glass HPLC vial, and the vial plus contents was re-weighed. After injection of this solution onto the HPLC column, vial plus contents were weighed again so that injection weight could be calculated.

Solid-phase extraction (SPE) cartridges are prefabricated devices that contain chromatographic resins (packing material) packaged in polypropylene housings. These housings have luer fittings at each end to facilitate connection to either a syringe or a vacuum manifold with appropriate connectors, which allows for passage of solution through the cartridge. Cartridges may contain various quantities (100 mg-1 g) of packing material. Packing material is typically silica-based and may be derivatized with ion-exchange or reverse-phase (e.g., C18) functional groups. In general, samples are applied to pretreated cartridges; pretreatment often includes the same solutions in the subsequent elution of sample components. Use of SPE cartridges allows for the separation or enrichment of sample components based on the affinity of these components for the packing material under various solvent conditions. In the studies reported in this dissertation the rationale for the use of ammonium hydroxide, methanol (acetonitrile), and acidified methanol (acetonitrile) is discussed in detail in the "Discussion" section of this chapter.

When the SPE procedure was included in the work-up, the ßC-9-NMT reaction was terminated by placement on ice and addition of 200 µl 0.5 N $HClO_4$. The mixture was centrifuged to pellet precipitated protein (3000*xg* for 5 minutes), and supernatant was transferred to a glass culture tube containing

100 µl concentrated ammonium hydroxide (NH_4OH) and 1.0 ml Milli-Q water. The protein pellet was vigorously resuspended in 500 µl 0.01 N $HClO_4$ and centrifuged (3000*g* for 5 minutes); the resulting supernatant was mixed with 100 µl 1 N potassium hydroxide and then combined in the glass tube with the initial supernatant.

Contents of the glass tube were slowly applied to a Sep-Pak® C18 Plus solid-phase extraction cartridge (Millipore-Waters part no. 20515, 360 mg). The SPE cartridge was previously pretreated with 5 ml each of methanol (or acetonitrile):5 N acetic acid (3:1, v/v), methanol or acetonitrile, and 1% NH_4OH (w/v) in that sequence. Slow application of all solutions to the cartridge (2 to 5 drops per second) was achieved using a syringe or a vacuum manifold. Following sample application, the extraction cartridge was washed with 5 ml of 1% (w/v) NH_4OH followed by 5 ml methanol or acetonitrile. These two SPE fractions and the sample application fraction were analyzed for 2,9-Me_2NH^+. The final eluent (2*x*2 ml) -- containing 3:1 (v/v) methanol (or acetonitrile): 5 N acetic acid -- promoted elution of 2,9-Me_2NH^+ from the cartridge into a clean glass culture tube. This eluate was evaporated to dryness at 30-40 °C using a Speed Vac concentrator (Savant Instruments Inc., Hicksville NY).

Reverse-Phase High Performance Liquid Chromatography (RP-HPLC)

ßC-9-NMT activity was quantitated by measuring 2-9(^{3}H-methyl)-Me_2NH^+ present in the reaction work-up using RP-HPLC. Details of the HPLC method are described in Appendix B. Acidified supernatant -- the solution

resulting from the work-up that excluded SPE -- was assayed "as-is". When the SPE protocol was used the resulting Speed Vac residue was dissolved in 200 µl HPLC mobile phase or 0.01 N $HClO_4$ by sonication and vortexing and then filtered. Samples were filtered through a 0.45 µm nylon membrane filter (Micro-Spin, Chrom-Tech Inc.) prior to injection (10-150 µl) onto the HPLC system. Isocratic HPLC analysis was carried out under ambient conditions on a Bondclone 10 µm C18 column (Phenomenex™, 300 mm *x* 3.9 mm). Mobile phase consisted of 17% (v/v) HPLC grade acetonitrile mixed with 83% (v/v) aqueous buffer. Buffer contained 1 M sodium phosphate monobasic adjusted to pH 3.5 with phosphoric acid (final mobile phase pH 4.0 ± 1). The HPLC system was a Shimadzu SIL-9A programmable autosampler, a Beckman 110A pump or BAS PM40 (0.8 ml/minute), and an in-line radioactive flow detector (Radiomatic Instruments Flo-One\ß). Ultima-Flo™ AP (Packard Instruments) liquid scintillation cocktail was used at a flow rate of 2.8 ml per minute, resulting in a scintillation fluid to mobile phase mixing ratio of 7:2. The radioactive flow detector was equipped with a 2.5 ml flow cell and an automatic stream splitter. It was necessary to bypass the radioactive flow detector by splitting the HPLC column effluent to waste until about 17-18 minutes into the run to reduce interference due to tritiated materials (e.g., ^{3}H-SAM) present in the sample. Radioactive peak area corresponding to 2,9-Me_2NH^+ (retention time 19-20 minutes) was integrated using Radiomatic Flo-One\ß system software. Peak area was reported as total counts per minute (cpm) under the 2,9-Me_2NH^+ curve. Flo-One\ß counting efficiency and reaction specific activity were determined routinely; this allowed for calculation of pmoles tritiated 2,9-Me_2NH^+.

A representative chromatogram ("radiogram") is presented in Figure 5.

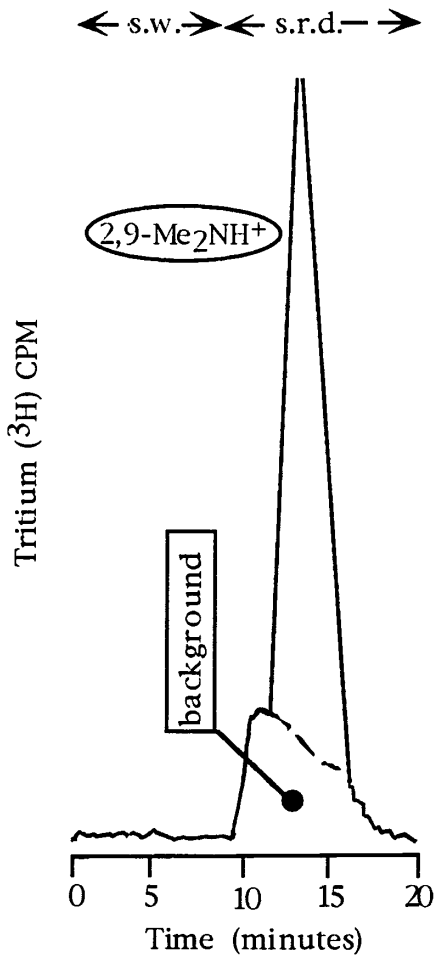


Figure 5. Rendition of representative RP-HPLC radiogram. Peak represents radioactivity due to 2,9-dimethylnorharmanium ion (2,9-Me_2NH^+) formed at pH 8.5 by ßC-2-NMT in the presence of 9-methylnorharman (9-MeNH), S-adenosyl-:methionine (SAM), and ^{3}H-SAM. Background represents radioactivity in the absence of 9-MeNH. Chromatographic separation was performed using RP-HPLC as described in text. Column effluent was split to waste (s.w.) from 0-9 minutes into analysis, and then split to radioactive detector (s.r.d.) from 9-20 minutes.

Strong-Cation Exchange High Performance Liquid Chromatography (SCX-HPLC)

An alternate HPLC method was SCX-HPLC with fluorescence detection (see details in Appendix B). Sample preparation was the same as described for RP-HPLC. Isocratic SCX-HPLC analysis was carried out under ambient conditions on a strong cation exchange column (Vydac catalog #401TP104, 250 mm*x* 4.6 mm). Mobile phase consisted of 50% (v/v) HPLC grade acetonitrile mixed with 50% (v/v) aqueous buffer. Buffer contained 300 mM ammonium chloride and 200 mM ammonium acetate, adjusted to pH 3.4 with glacial acetic acid (final mobile phase pH 4.5 ± 1). The HPLC system included a Shimadzu SIL-9A programmable autosampler, a Beckman 110A or BAS PM40 pump (0.8 ml/minute), and a Perkin-Elmer LC240 fluorescence detector. Excitation and emission wavelengths for detection of 2,9-Me_2NH^+ were 310 nm and 450 nm, respectively. Data reporting and integration were achieved with a Shimadzu C-R1B Chromatopac reporting integrator. Retention times for relevant ßCs were: NH @ 6.5-7.5 minutes, 2-$MeNH^+$ @ 9.5-10.5 minutes, 9-MeNH @ 10.5-11.5 minutes, and 2,9-Me_2NH^+ @ 14-15 minutes (Figure 6). Peak areas for 2,9-Me_2NH^+ standard solutions of known concentration were analyzed in order to plot a standard curve. The resulting linear equation was used to calculate the concentration of 2,9-Me_2NH^+ present in ßC-9-NMT reactions (Figure 7).

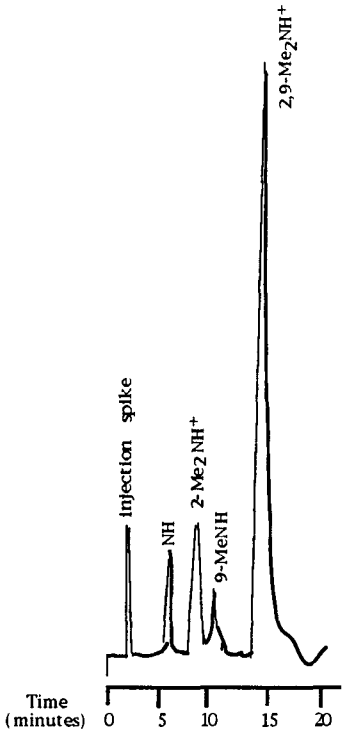


Figure 6. Rendition of representative SCX chromatogram. Compounds: NH=norharman, 2-Me2NH+=2-methylnorharmanium ion, 2,9-Me2NH+=2,9-dimethylnorharmanium ion. Column (SCX): Vydac 401TP104, 250 mm x 4.6 mm. Mobile phase (0.8 ml/min): 50% acetonitrile + 50% pH 3.4 buffer, buffer=300 mM ammonium chloride + 200 mM ammonium acetate. Fluorescence detection: excitation at 310 nm, emission at 450 nm.

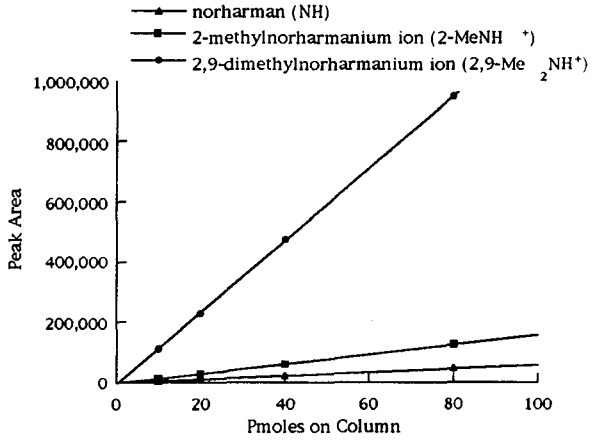


Figure 7. SCX chromatography standard curve. Refer to Figure 6 for details.

First Experiment Replicating the ßC-9-NMT Activity Reported by Matsubara

Assays (370 µl) contained guinea pig brain homogenate (0.636 mg protein), 1.08 mM 2-MeNH+, and SAM (8.1 µM, 4.28 µCi) in sodium phosphate buffer at a final pH of 8.55. Assays were maintained at 37 °C for one hour. The SPE procedure was not utilized in the work-up.

Determination of Apparent Kinetic Constants With Respect to the Methyl Donor (SAM)

Assays included guinea pig brain homogenate (0.716 mg protein), 2-MeNH+ (1.08 mM), ^{3}H-SAM (7.1 or 14.2 µCi), and SAM at a final concentration ranging from 10 - 400 µM. Total assay volume was 370 µl and final pH was 8-8.1 in sodium phosphate buffer. No SPE work-up was done following assay incubation at 37 °C for one hour. ßC-9-NMT activity -- expressed as pmoles of 2,9-Me_2NH^+ formed per hour per mg protein (pmol/h/mg protein) -- was determined at each SAM concentration. Due to a technical problem the "10 µM SAM" sample was not evaluated. Plotting and curve-fitting were accomplished using Kaleidagraph™ data analysis/graphics application software (Abelbeck Software). The Lineweaver-Burk analysis was used to plot data and determine the kinetic constants, K_M and V_{max} (Lehninger 1975).

TRIS Buffer Compared to Phosphate Buffer and ßC-9-NMT Activity in Yeast Extract

All substrates were dissolved in 100 mM TRIS buffer (pH 8.3) instead of sodium phosphate buffer; concentrations were 1.08 mM 2-MeNH+ and 500 µM

SAM (16.8 μCi). Guinea pig brain was homogenized in the same TRIS buffer using a Tissuemizer®; 100 μl of the resulting homogenate (1.132 mg protein) was added to each ßC-9-NMT assay. Yeast extract, 100 μl per assay, contained 1.338 mg protein. Reactions were prepared in duplicate and maintained at 37 °C for one hour. One set of incubations was subjected to the SPE procedure and the other was not.

Measurement of ßC-9-NMT Activity in Various Rat Organs

Organs were quickly dissected from an ether-anesthetized, adult-female Sprague-Dawley rat. Tissues were placed on foil on dry-ice, wrapped, and then stored at -80 °C. Rat tissues analyzed for ßC-9-NMT activity included: lung, liver, kidney, spleen, and thymus. Guinea pig brain was also evaluated and served as a comparative control. Tissue homogenates (5 ml buffer per gram tissue) were prepared in 100 mM TRIS buffer (pH 8.3) using a Tissuemizer®. ßC-9-NMT assays contained 1.08 mM 2-MeNH$^+$ and SAM (8.1 μM, 7.5 μCi) in TRIS buffer at a final pH 8.3. Assay volume was 185 μl due to a limited supply of ^{3}H-SAM. Samples were maintained at 37 °C for one hour. The SPE procedure was included in this experiment, and tritiated 2,9-Me_2NH^+ was measured in each SPE fraction. Syringes were used to apply sample and eluents to the SPE cartridges.

Fluorescence Detection of 2,9-Me_2NH^+ Product (Rather Than Radioactive Detection)

Reactions consisted of guinea pig brain homogenate (100 μl, 1.28 mg protein), 100 μM 2-MeNH$^+$, and SAM (500 μM) in 100 mM TRIS buffer (pH 8.3).

Assays (200 µl) were maintained at 37 °C for one hour. Note that the following changes were incorporated into this experiment: no radioactive SAM was used, 2-MeNH+ concentration was reduced about 10-fold, and assay volume was reduced from 370 µl to 200 µl. The SPE procedure was not included in the assay work-up. Three distinct types of "blank" assays were evaluated: one lacking 2-MeNH+, one without SAM, and one with boiled homogenate. Replicate reactions (N=3-5) were prepared and analyzed at each condition.

Fluorescence detection of 2,9-Me_2NH^+ was achieved using a Perkin-Elmer LC240 detector: excitation wavelength was 310 nm and emission wavelength was 450 nm. These wavelengths were optimal for detection of 2,9-Me_2NH^+.

Dialysis of Guinea Pig Brain Homogenate

Guinea pig brain was homogenized in 5 volumes of 10 mM Na_2HPO_4 (pH 7.4) containing the protease inhibitor phenylmethylsulfonylfluoride (PMSF). A 1.2 ml aliquot of this homogenate was dialyzed against 1 L of 10 mM Na_2HPO_4 (pH 7.4). Dialysis was done at 0-5 °C using SpectraPor 3 (Scientific Products) dialysis tubing with a 3500 molecular weight cutoff (m.w.c.o.).

Assays contained boiled homogenate or undialyzed homogenate (0.640 mg protein) or dialyzed homogenate (0.524 mg protein). Substrates included 500 µM 2-MeNH+ and 500 µM SAM in 100 mM Na_2HPO_4 buffer at pH 9.5. Volume totaled 200 µl and final pH was 8.5. Six replicates were prepared for each condition, and assays were maintained at 37 °C for one hour. Reactions were terminated by the addition concentrated $HClO_4$. Strong-cation exchange

chromatography with fluorescence detection was utilized to measure 2,9-Me_2NH^+ in the reaction work-up. ßC-9-NMT activity was expressed as pmoles of 2,9-Me_2NH^+ formed per hour per mg protein (pmol/h/mg protein).

ßC-9-NMT Activity in Frozen Bovine Brainstem

Frozen bovine brain was purchased from Pel-Freez and stored at -80 °C. Homogenation and subcellular fractionation was similar to the Mizoguchi et al. procedure (Mizoguchi 1989). Brainstem (pons and medulla oblongata) was thawed, cut into small pieces, and homogenized at 0-5 °C in 4 volumes of buffer at 0-5 °C in a glass homogenizer by 6 up-and-down strokes of a motor-driven teflon pestle. Homogenation buffer consisted of 0.32 M sucrose, 1 mM $NaHCO_3$ (pH 7.2), 0.5 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mM PMSF. Homogenate was diluted to 10% (w/v) with homogenation buffer and filtered through 4 layers of gauze to yield crude whole brainstem homogenate.

Homogenate was centrifuged at 1400*g* for 10 minutes, and the pellet was resuspended in homogenation buffer to 10% (w/v) and re-homogenized. Centrifugation at 710*g* for 10 minutes afforded pellet "P_1", comprised of nuclei and cell debris. The "P_1" supernatants were pooled and centrifuged at 13,800*g* for 10 minutes. The resulting pellet was washed and centrifuged at 13,800*g* for 10 minutes, yielding "P_2" -- theoretically a mixture myelin, mitochondria, and synaptosomes. Combined "P_2" supernatants were centrifuged at 105,000*g* for 60 minutes to produce "P_3" and "S_3", equivalent to microsomal and cytosolic (soluble) fractions, respectively. Pellets were diluted in 10 mM Na_2HPO_4 (pH

7.4) prior to use in ßC-9-NMT assays. Dialysis of crude homogenate or subfraction was accomplished in SpectraPor 3 (3500 m.w.c.o.) dialysis tubing against 1 L of 10 mM Na_2HPO_4 (pH 7.4). Dialysis was carried-out overnight at 0-5 °C; dialysis buffer was changed 3-4 times.

For ßC-9-NMT assays, 2-MeNH+ (500 µM) and SAM (370 µM) were prepared in 100 mM Na_2HPO_4 (pH 9.5). Final assay pH was 8.3-8.5 and volume was 200 µl. Blank assays contained all substrates and boiled homogenate or boiled subfraction. Replicates (N=5-7) were prepared at each assay condition. Reactions were maintained at 37 °C for one hour and then terminated by $HClO_4$ addition. Product 2,9-Me_2NH+ was quantitated using SCX-HPLC and fluorescence detection.

ßC-9-NMT Activity in Fresh Bovine Brainstem

Fresh bovine brain was obtained from Aurora Packing Company (Aurora, IL). Brainstem (medulla, midbrain, and pons) was cut into pieces and homogenized briefly in a Waring blender at 0-5 °C with 0.32 M sucrose, 1 mM $NaHCO_3$ (pH 7.2), 0.5 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM PMSF, and 1 µg/µl leupeptin. Homogenation was subsequently continued in a glass homogenizer using 12 up-and-down strokes of a motor-driven teflon pestle (1000 rpm). Crude homogenate was stirred for 15 minutes in a plastic beaker and then centrifuged at 1400*g* for 10 minutes. The pellet was resuspended in buffer and centrifuged at 600*g* for 10 minutes to yield the crude nuclear pellet ("P_1"). The combined supernatants were saved but not assayed in this experiment. Crude homogenate or nuclear extract was dialyzed so that ßC-9-NMT activity could be

compared in undialyzed and dialyzed fractions.

Reactions (200 µl) at pH 8.3 in phosphate buffer, contained 500 µM 2-MeNH+, 370 µM SAM, and 100 µl of dialyzed or undialyzed bovine brain fraction. Assays were maintained for 1 hour at 37 °C and then terminated by the addition of concentrated $HClO_4$. ßC-9-NMT activity was calculated by measuring 2,9-Me_2NH^+ in the work-up using SCX-HPLC with fluorescence detection. Note that this experiment was repeated three times with variable results.

Results

First Experiment Replicating the ßC-9-NMT Activity Reported by Matsubara

ßC-9-NMT activity was 4.6 pmol/h/mg protein in guinea pig whole-brain homogenate. This value is consistent with the activity (4.7 pmol/h/mg protein) reported by Collins et al. (1992) under identical experimental conditions.

Effect of S-Adenosyl-L-Methionine Concentration on ßC-9-NMT Activity

Figure 8 represents the Lineweaver-Burk plot derived for guinea pig whole-brain homogenate ßC-9-NMT activity as a function of SAM concentration. Calculated kinetic constants with respect to SAM were: V_{max}, 33 ± 12 pmol/h/mg protein and K_M, 997 ± 113 µM.

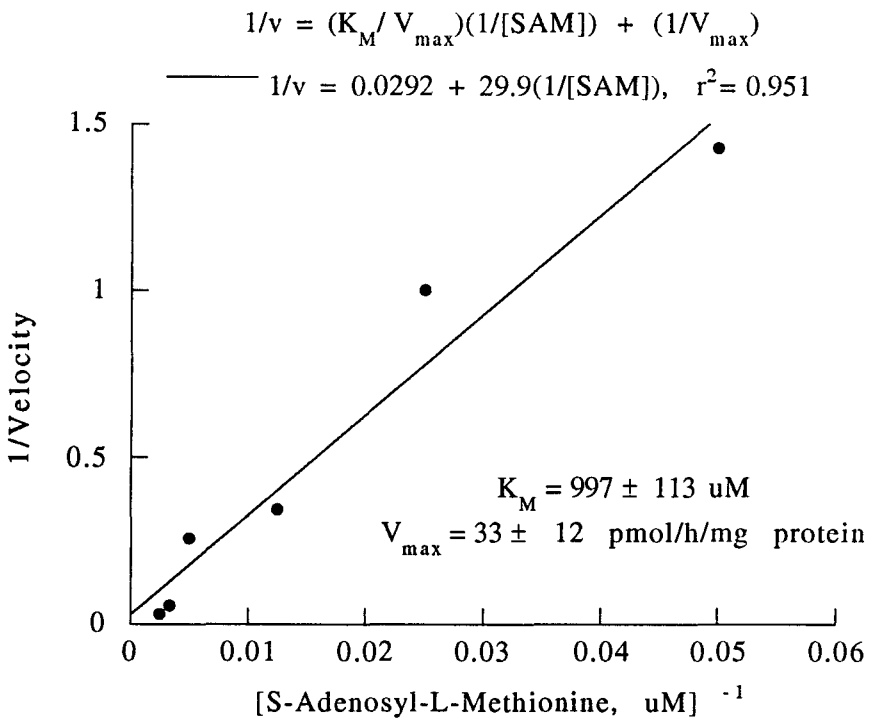


Figure 8. Lineweaver-Burk plot for ßC-9-NMT activity as a function of SAM concentration. Enzyme activity (pmol 2,9-Me_2NH^+ formed/h/mg protein) was determined in guinea pig whole-brain homogenate. SAM concentration varied from 20 to 400 µM while ßC substrate (2-$MeNH^+$) was fixed at 1.08 mM. Assay was carried out for one hour at 37 °C at pH 8.05 in phosphate buffer. The Lineweaver-Burk equation is shown as well as the equation and coefficient of determination (r^2) resulting from linear curve-fitting of the data. Values for K_M and V_{max} are calculated values ± standard errors.

Effect of TRIS Buffer on ßC-9-NMT Assay, and ßC-9-NMT Activity in Yeast

Brain ßC-9-NMT activity was comparable in TRIS buffer and phosphate buffer. Activity was detected in both brain and yeast extracts (Table 5). This table also compares the results obtained with and without the SPE work-up. "Sample" SPE fraction represents the flow-through from the cartridge during application of the initial basified sample to the SPE cartridge and "NH4OH" is the 1% (w/v) ammonium hydroxide wash fraction. Unexpectedly, 2,9-Me_2NH^+ was detected in "Sample" and "NH_4OH" fractions. As expected, no product was found in the methanol fraction off the SPE cartridge. "H+/ACN" is the acidified acetonitrile used to elute tritiated 2,9-Me_2NH^+ from the cartridge -- but no product was measured here. "Acid" represents the acidified supernatant resulting from the reaction work-up that excluded SPE.

Table 5.--Brain and Yeast ßC-9-NMT Activity in Solid-Phase Extraction Cartridge Fractions and Acidified Supernatant

	ßC-9-NMT Activity (pmol /h/mg protein)				
	Fraction				
	Sample	NH4OH	H+/ACN	Total	Acid
Brain	8.6	12.4	0	21.0	39.8
Yeast	0	15.0	0	15.0	5.0

Values are the number of pmoles of 2,9-Me_2NH^+ in each fraction from the solid-phase extraction cartridge. Following ßC-9-NMT assay, proteins were precipitated by $HClO_4$, resulting supernatant was basified with KOH/NH_4OH, and then applied to SPE cartridge. "Sample" = fraction collected during sample application to the cartridge, "NH4OH" = fraction collected during ammonium hydroxide wash, "H+/ACN" = fraction collected during the acidified acetonitrile wash, and "Total" is the sum of the 3 fractions. "Acid" is the acidified supernatant from assay that did not include the SPE work-up.

ßC-9-NMT Activity in Rat Organs Compared to Guinea Pig Brain

Guinea pig brain and each of the rat organs tested has ßC-9-NMT activity (Table 6). Rat thymus exhibited the highest ßC-9-NMT activity at 44.6 pmol/h/mg protein. Note that in liver assays the chromatograms were difficult to interpret due to "background" radioactivity; therefore, activity was not reported in those fractions. Total ßC-9-NMT activity in guinea pig brain (22 pmol/h/mg protein) is consistent with the brain "Total" in the previous experiment (21 pmol/h/mg protein). Consistent with previous experiment, these data suggest that a substantial quantity of tritiated 2,9-Me_2NH^+ was not retained by the cartridge during sample application ("Sample") and that product was present in SPE fractions other than the acidified methanol eluate ("H+/MeOH"). No product was found in the methanol wash. The percentage of total product recovered in all fractions is represented in parentheses (Table 6).

Table 6.--ßC-9-NMT Activity in Guinea Pig Brain and Rat Organs

	ßC-9-NMT Activity (pmol/h/mg protein)			
	Fraction			
	Sample	NH4OH	H+/MeOH	Total
Organ				
Brain	4.2 (19%)	8.9 (40%)	9.1 (41%)	22.3
Lung	1.3 (7%)	8.8 (43%)	10.3 (50%)	20.4
Thymus	4.6 (10%)	23.7 (53%)	16.3 (37%)	44.6
Liver	---	---	2.8	---
Kidney	0.9 (5%)	9.6 (56%)	6.7 (39%)	17.2
Spleen	0 (0%)	7.5 (45%)	9.1 (55%)	16.6

Values are the number of pmoles of 2,9-Me_2NH^+ in each fraction from the solid-phase extraction cartridge. Following ßC-9-NMT assay, proteins were precipitated by $HClO_4$, resulting supernatant was basified with KOH/NH_4OH, and then applied to SPE cartridge. SPE fractions, in order of collection were: "Sample" = fraction collected during sample application to the cartridge, "NH4OH" = fraction collected during ammonium hydroxide wash, "H+/ACN" = fraction collected during the acidified acetonitrile wash, and "Total" is the sum of the 3 fractions.

Fluorescence Detection of 2,9-Me_2NH^+ Product (Rather Than Radioactive Detection)

Fluorescence detection of 2,9-Me_2NH^+ in ßC-9-NMT assays is possible. Figure 9 shows that ßC-9-NMT activity in "blank" assays is statistically different than complete assays when fluorescence detection is utilized to measure 2,9-Me_2NH^+ formed. "Activity" in blanks is due to 2,9-Me_2NH^+ present in the substrate 2-$MeNH^+$ or other fluorescent compounds unrelated to 2,9-Me_2NH^+, but with an identical retention time. Additionally, 2,9-Me_2NH^+ product present in the assays lacking 2-$MeNH^+$ substrate may be due to endogenous 2,9-Me_2NH^+ present in the homogenate itself. Net ßC-9-NMT activity (complete minus boiled) is 9.6 ± 3.1 pmol/h/mg protein.

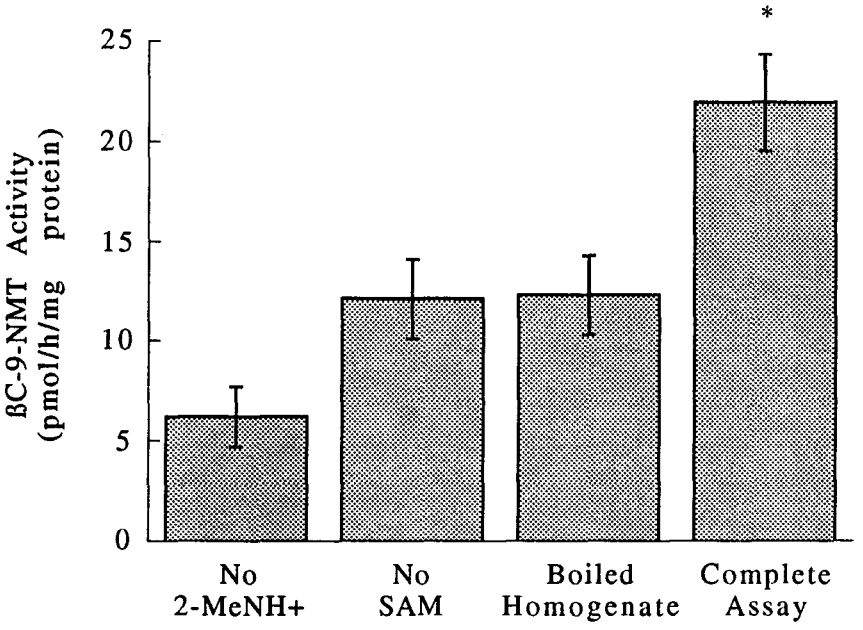


Figure 9. ßC-9-NMT activity in guinea pig brain homogenate with 2,9-Me_2NH^+ measurement by fluorescence detection. Error bars are s.e.m. Complete assay is significantly different than assay containing boiled homogenate or assays lacking substrate (* $p<0.05$). All assays were done at 37 °C for 1 hour at pH 8.3, and included guinea pig brain homogenate (1.28 mg protein) that was not boiled, except where noted. All assays contained 100 µM 2-MeNH+, except for the "No 2-MeNH+" assay. All assays contained 500 µM SAM except for the "No SAM" assay.

Dialysis of Guinea Pig Brain Homogenate

It appears that dialysis increases ßC-9-NMT activity in guinea pig brain homogenate, perhaps due to the removal of a low molecular weight inhibitor (Figure 10).

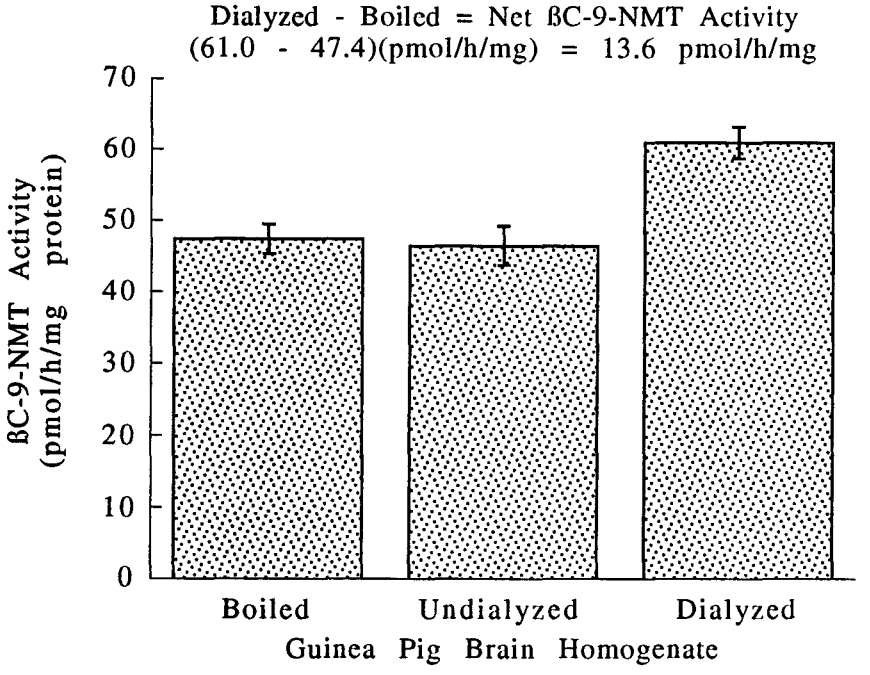


Figure 10. ßC-9-NMT activity in dialyzed guinea pig brain homogenate. Error bars are s.e.m. Dialyzed (3500 m.w.c.o.) homogenate is significantly different than assay containing undialyzed homogenate or boiled undialyzed homogenate ($p<0.0005$). All assays were done at 37 °C for 1 hour at pH 8.5, and included undialyzed guinea pig brain homogenate that was boiled or not boiled (0.640 mg protein), or dialyzed/non-boiled homogenate (0.524 mg protein). All assays contained 500 µM 2-MeNH+ and 500 µM SAM.

ßC-9-NMT Activity in Frozen Bovine Brainstem

ßC-9-NMT specific activity in undialyzed bovine brainstem homogenate was 14.6 pmol/h/mg protein. Dialysis increased the activity 3-fold, to 46.8 pmol/h/mg protein. ßC-9-NMT activity in undialyzed crude nuclear fraction was 121.3 pmol/h/protein. Dialysis of the crude nuclear fraction resulted in a complete loss of enzyme activity. No ßC-9-NMT activity was detected in undialyzed or dialyzed crude mitochondrial, microsomal, or cytosolic subfractions.

ßC-9-NMT Activity in Fresh Bovine Brainstem

ßC-9-NMT activity in the crude homogenate from fresh bovine brainstem was very high at 125.1 pmol/h/mg protein. This activity is equivalent to that found in the nuclear extract for the preceding experiment. Inconsistent with the previous experiment, undialyzed nuclear fraction exhibited 5.9 pmol/h/mg protein ßC-9-NMT activity. Samples derived from dialyzed crude homogenate or nuclear extract could not be assayed due to an autosampler malfunction.

Repeats of this experiment produced inconsistent results. In the second experiment no ßC-9-NMT activity was found in either undialyzed crude homogenate or nuclear extract. In third experiment, 14.2 pmol/h/mg ßC-9-NMT activity was found in undialyzed crude homogenate; dialysis increased activity to 126.0 pmol/h/mg protein. ßC-9-NMT activity was not assessed in nuclear extract. In the fourth attempt, no activity was detected in undialyzed or dialyzed crude homogenate. However, the undialyzed crude nuclear extract exhibited a ßC-9-NMT specific activity 25.5 pmol/h/mg protein. Dialysis of the

nuclear extract abolished ßC-9-NMT activity.

Discussion

ßC-9-NMT activity was detected in guinea pig and bovine brain, several rat organs, and even yeast extract. Increasing SAM concentration or extract dialysis increased ßC-9-NMT activity. Activity in the TRIS buffer was comparable to activity in phosphate buffer.

As will be discussed below, it is speculated that ßC-9-NMT activity is quite variable from animal to animal. There are indications that this enzyme is temperature-labile and that its activity is modified by endogenous dialyzable substances. These speculations would explain (in part) the several failed attempts, aimed at reproducing Matsubara's work, which preceded the first experiment in which ßC-9-NMT activity was successfully measured. Difficulties in assessment of ßC-9-NMT activity are summarized below, beginning with a discussion of the solid-phase extraction procedure work-up.

Solid-Phase Extraction Protocol

The solid-phase extraction (SPE) protocol includes four steps: sample application, NH_4OH wash, methanol or acetonitrile wash, and finally an acidified methanol (acetonitrile) eluate. The SPE procedure was originally developed as a means of increasing the sensitivity for detection of 2,9-Me_2NH^+. Use of SPE decreases non-ßC tritiated compounds in the sample that contribute to background during HPLC analysis. Lower apparent ßC-9-NMT activity in samples subjected to the SPE procedure versus non-SPE samples is probably

due to a reduction tritiated background as a consequence of the SPE work-up. The majority of this background radioactivity is ^{3}H-SAM and its metabolites.

The three ring structure of ßCs or MeßCs+ predicts that hydrophobic forces drive the interaction between these compounds and the C18 SPE cartridge. Since the cartridge is a silica-based packing-material, under basic conditions the matrix silanol groups will be negatively charged. It is suspected that a secondary interaction occurs between the negatively charged matrix and positively charged MeßCs+ (@ pH > 10). This interaction is apparently an important interaction, because when the column or sample is not basified, MeßCs+ are not retained by the cartridge (data not shown). The NH_4OH wash is intended to remove unwanted compounds from the cartridge, while MeßCs+ are retained. However, if this wash is not basic, MeßCs+ are eluted from the column during this step (data not shown). In experiments reported here in which 2,9-Me_2NH+ was measured in the "Sample" and "NH_4OH" fractions, it is speculated that either the column or the sample was at a pH less than 10. It is also possible, but not as likely, that the radioactive compounds detected in these fractions are not 2,9-Me_2ßCs+, but rather compounds the exhibit a HPLC retention time similar to that of 2,9-Me_2NH^+.

The methanol or acetonitrile wash (i.e., third step in the elution protocol) disrupts hydrophobic interactions and causes elution of ßCs (not MeßCs+). In addition, this solvent serves as a transition solution between the NH_4OH and the acidified methanol (acetonitrile) wash. It is surmised that acidification of the final methanol or acetonitrile eluent is necessary in order to protonate silanol groups on the matrix, thereby disrupting the proposed electrostatic interaction between the silica matrix and MeßCs+. The

acetonitrile or methanol is included to further disrupt hydrophobic interaction between the matrix and MeßCs+. This eluate effectively promotes elution of 2,9-Me_2NH^+ from the C18 SPE cartridge.

Effect of SAM Concentration on ßC-9-NMT Activity

Matsubara evaluated the kinetic constants, K_M and V_{max}, with respect to the ßC substrate, but these parameters were not ascertained regarding SAM. In the present study the K_M for SAM was 560 µM, a concentration that is 70-fold higher than the SAM concentration (8 µM) routinely utilized in ßC-9-NMT assays. Consider the Michaelis-Menten (M-M) equation and the calculated velocity under conditions of 8 µM SAM, using K_M = 560 µM and V_{max} = 20 pmol/h/mg protein.

M-M equation: $v = V_{max} [S] / (K_M + [S])$

v = reaction velocity

V_{max} = maximal velocity

[S] = substrate concentration

K_M = Michaelis constant, experimentally the substrate concentration at which velocity is $V_{max}/2$

The calculated velocity when SAM is 8 µM is 0.3 pmol/h/mg protein, a low activity that may not be consistently detectable. Therefore, in subsequent experiments SAM concentration was increased to 300-500 µM, ^{3}H-SAM was also

increased. Problems associated with increasing assay ^{3}H-SAM will be addressed in a subsequent paragraph.

Buffer Comparison

TRIS buffer was evaluated as an alternative to phosphate buffer in the ßC-9-NMT assay. TRIS is expected to be a better buffer than phosphate since the TRIS pK_a (8.2) is within the pH range (pH 8-8.5) of the ßC-9-NMT assay, while the pK_a for phosphate buffer (pK_a 7.2) is not within the desired range for the ßC-9-NMT assay. Results indicate that ßC-9-NMT activity was higher in pH 8.3 TRIS buffer (39.8 pmol/h/mg protein) compared to previous experiments in Na_2HPO_4 at pH 8.05. This suggests that ßC-9-NMT activity may be inhibited somewhat in the presence of phosphate ions and/or the enzyme is more active at pH 8.3 compared to pH 8.05. These issues were not addressed any further, and TRIS buffer was employed in several of the experiments presented below.

Activity in Yeast and Rat Organs

ßC-9-NMT activity was detected in several rat organs as well as yeast cell extract. Enzyme activity was assessed in these preparations in an effort to identify a potential starting material for enzyme purification purposes.

Rationale for Use of Fluorescence Detection of 2,9-Me_2NH^+

An unfortunate drawback to increasing SAM in these reactions is the concomitant increase in ^{3}H-SAM required so that tritiated 2,9-Me_2NH^+ could be

detected. Excessive use of tritiated SAM is quite expensive and contributes significantly to background during HPLC analysis. Therefore fluorescence detection was investigated as an alternate method for measuring 2,9-Me_2NH^+ formed by the action of ßC-9-NMT. ß-Carbolines, particularly N-methylated ßCs, exhibit fluorescent properties -- especially at acidic HPLC conditions -- and are consequently amenable to fluorescent detection. In contrast SAM is very weakly fluorescent under these same conditions, so a large excess of non-radioactive SAM could be used in the ßC-9-NMT assay with little effect on the quality of the chromatogram.

The advantages of fluorescence detection of 2,9-Me_2NH^+ are that it circumvents the requirement for the SPE protocol, eliminates ^{3}H-SAM from the ßC-9-NMT assay, and allows for the analysis of a greater number of samples per day (use of an autosampler is possible). Because of these advantages, the fluorescent assay is much more efficient and more economical than the radioactive assay. One disadvantage of the fluorescent assay derives from the discovery that the substrate 2-$MeNH^+$ contains traces (<0.1%, w/w) of product 2,9-Me_2NH^+. This "background" 2,9-Me_2NH^+ is bothersome since its level often exceeds that of the 2,9-Me_2NH^+ formed by ßC-9-NMT. Because of this background, 5-7 replicates each of blank and complete assays are required in order to achieve statistically significant differences between these two conditions. This large number of replicates obviously precludes the use the fluorescent assay during the proposed purification of ßC-9-NMT. Another disadvantage of fluorescent detection is that when a large amount of 2-$MeNH^+$ is used in the ßC-9-NMT assay, the 2,9-Me_2NH^+ product peak appears as a small shoulder peak on the 2-$MeNH^+$ substrate peak. This poor resolution

occasionally makes 2,9-Me_2NH^+ quantitation difficult. Attempts at purification of 2-$MeNH^+$ to remove trace 2,9-Me_2NH^+ were fruitless.

ßC-9-NMT Activity as a Function of Time

ßC-9-NMT activity as a function of time offered some interesting clues regarding the stability of the enzyme or its product. Recall that activity increased up to about 15 minutes and then decreased to below detection. This suggests that ßC-9-NMT is either labile or that another enzyme system present in the assay is catabolizing the 2,9-Me_2NH^+ formed by ßC-9-NMT. However, a similar time profile was not observed for ßC-2-NMT activity (see Chapter 3), implying that 2,9-Me_2NH^+ is not catabolized by endogenous enzymes under similar conditions. Unfortunately this ßC-9-NMT time-study could not be replicated; i.e., ßC-9-NMT activity was often measured at the 30 or 60 minute time-points in subsequent experiments.

Inconsistent Detection of Activity

Over an 8 month period of study, ßC-9-NMT activity became rather elusive and could not be measured on a consistent basis. The effects of several factors on ßC-9-NMT activity were assessed. Factors evaluated included: characteristics of guinea pig brain (vendor, sex and age of animal); SAM vendor; reaction work-up; and removal of endogenous SAH by SAH hydrolase plus adenosine deaminase. Often ßC-9-NMT activity could not be detected or at least distinguished statistically from blanks (except occasionally in yeast extracts). Matsubara indicated that he experienced similar difficulties in

assessment of ßC-9-NMT activity upon his return to Japan. He suggested using brain tissue harvested from older animals, increasing SAM concentration, and dialysis of brain extracts (to remove endogenous ßC-9-NMT inhibitors). As reported in this Chapter, increasing SAM concentration did increase ßC-9-NMT activity; however, increased SAM concentration had no effect on the consistency of detection of ßC-9-NMT activity. Subsequent evaluation of brain harvested from old and young guinea pigs did not affect ßC-9-NMT activity. Dialysis of brain homogenate was the only strategy that enhanced detection of ßC-9-NMT activity, and this effect was not dramatic. This is consistent with the presence of a dialyzable inhibitor of ßC-9-NMT; there are reports of dialyzable low-molecular-weight inhibitors of methyltransferases (Park et al. 1993).

Bovine Brain ßC-9-NMT Activity

ßC-9-NMT activity was also evaluated in bovine brain -- a potential inexpensive and abundant source of enzyme for purification purposes. Bovine brainstem was initially tested since Matsubara et al. (1992b) reported highest ßC-9-NMT activity in guinea pig brainstem. Initial studies, using frozen bovine brainstem, confirmed these published results, which showed a primarily nuclear localization for ßC-9-NMT activity. It is intriguing that dialysis of bovine-brainstem whole-homogenate increased ßC-9-NMT activity, while dialysis of nuclear extract reduced or abolished enzyme activity. This suggests that ßC-9-NMT may exist in two forms: a non-nuclear form that is inhibited by some dialyzable substance and a nuclear form that requires a dialyzable factor.

In contrast to the results for frozen bovine brainstem, in the first experiment using fresh bovine brainstem, very little nuclear activity was

measured and there was a high level of ßC-9-NMT activity in whole-homogenate. It is possible that subtle differences in homogenation between this and the previous study may account for these results. Perhaps in the present study, nuclei were damaged or disrupted thereby releasing ßC-9-NMT into the whole-homogenate.

Similar to activity from guinea pig brain, bovine brainstem ßC-9-NMT activity could not be measured consistently. It was suspected that freezing may adversely affect ßC-9-NMT activity, but this suspicion was not evaluated systematically.

ßC-9-NMT activity was aggressively pursued for at least a year. After that, ßC-9-NMT and ßC-2-NMT activities were re-evaluated in bovine and guinea pig brain using RP-HPLC with radioactive detection (data not shown). ßC-9-NMT activity was undetectable in both samples, while ßC-2-NMT activity was present in both. Consequently, the investigation of ßC-2-NMT began (see Chapter 3).

Noteworthy is the fact that ßC-9-NMT activity was later consistently measured in postmortem human brain tissue (see Chapter 5). Briefly, ßC-9-NMT activity in human brain was higher in the 10,000xg supernatant fraction than it was in the corresponding particulate fraction. In control tissue particulate fraction activity was 7.8 pmol/h/mg protein (range 0-45.2 pmol/h/mg protein) across the brain regions tested, while supernatant activity averaged 14.3 pmol/h/mg protein (range 0-67.7 pmol/h/mg protein). Consistent measurement of ßC-9-NMT in human brain suggests that it may be a better source than bovine brain for study of ßC-9-NMT activity.

CHAPTER 3

CHARACTERIZATION OF BOVINE BRAIN ß-CARBOLINE-2N-METHYLTRANSFERASE

Background

This chapter describes experiments designed to characterize the following properties of bovine brain ß-carboline-2N-methyltransferase (ßC-2-NMT): (a) subcellular localization; (b) apparent kinetic constants, Michaelis constant (K_M) and maximal velocity (V_{max}) with respect to its cosubstrates 9-methylnorharman (9-MeNH) and S-adenosyl-L-methionine (SAM); (c) dependence of activity on protein concentration; (d) pH optimum for enzyme assay; (e) activity as a function of assay time; (f) inhibition by S-adenosyl-L-homocysteine (SAH), a competitive inhibitor of SAM-dependent methyltransferases; and (g) the effect of various metals on its activity. Delineation of these properties was essential to the development of a sensitive and reproducible enzyme assay for use in future studies, including the purification of ßC-2-NMT from bovine brain (Chapter 4) and comparison of ßC-2-NMT activity in parkinsonian and normal human brain (Chapter 5).

Materials and Methods

Reagents

ß-Carboline substrate, 9-methylnorharman, was synthesized in our laboratory using two different methods: according to Matsubara (Matsubara et al. 1992b) or a modification of the method described by Rubottom (Rubottom et al. 1974). The modified Rubottom method is described in Appendix C. Unlabeled S-adenosyl-L-methionine (toluenesulfonate salt) and tritiated S-[methyl-^{3}H]-adenosyl-L-methionine (60-85 Ci/mmol) were purchased from Sigma Chemical and Dupont NEN, respectively. All other reagents were of appropriate purity for their intended use and were utilized as purchased.

Subcellular Fractionation

Bovine brain (including brainstem) was obtained fresh (Aurora Packing Co., Aurora IL), divided sagittally into equal halves, and frozen at -80 °C for three months. Brain was quickly thawed in a 40 °C water bath, and subsequently homogenized in 2 volumes (2 ml/g tissue) of cold buffer "H1" using a chilled Waring blender at low speed (3 cycles x 30 seconds per cycle). Buffer '"H1" contained 150 mM potassium chloride (KCl), 1 mM dithiothreitol (DTT), 1 mM ethylenediaminetetraacetic acid disodium (EDTA), and 20 mM sodium phosphate dibasic (Na_2HPO_4) at pH 7.4. The homogenate was filtered through two to four layers of gauze prior to centrifugation. Centrifugation was accomplished at 5-10 °C in a Sorvall RC5B Superspeed centrifuge (SS-34 rotor) or a Beckman L8-M Ultracentrifuge equipped with a SW28 rotor. Centrifugation at 900*g* (10 minutes), 9000*g* (15 minutes), and 100,000*g* (one hour) afforded crude nuclear, mitochondrial, and microsomal plus cytosolic

fractions, respectively. Crude particulate fractions were diluted with 150 mM KCl prior to inclusion in assays; crude cytosol was not diluted.

For experiments other than subcellular fractionation, bovine brain (including brainstem) was obtained fresh (Aurora Packing Co., Aurora IL), divided sagittally into equal halves, and frozen at -80 °C for seven months. Brain was quickly thawed in a 40 °C water bath, and subsequently homogenized in 2 volumes (2 ml/g tissue) of cold "H2" using a chilled Waring blender at low speed (3 cycles x 30 seconds per cycle). Solution "H2" contained 150 mM KCl and 1 mM DTT. The crude cytosolic supernatant was obtained from filtered homogenate following sequential centrifugation at 15,000*g* (30 minutes) and 100,000*g* (one hour). Cytosolic supernatant was stored at -80 °C in polypropylene tubes in 2-3 ml aliquots. Frozen aliquots were thawed as needed for use in ßC-2-NMT assays. For some studies, fresh bovine brain was homogenized in buffer "H3" which contained 20 mM K_2HPO_4, 150 mM KCl, 1 mM DTT, 1 mM EDTA, 1 mM phenylmethylsulfonylfluoride, and 1 µg/ml each leupeptin and pepstatin A. Cytosol was obtained and stored as described for buffer "H2". Composition of the homogenation buffer had no obvious effect on ßC-2-NMT stability or assay.

Determination of ßC-2-NMT Activity

This activity catalyzes the transfer of the tritiated methyl moiety from SAM to 9-MeNH resulting in the formation of 2-(^{3}H-methyl)-9-MeNH$^+$ and SAH (Figure 11) .

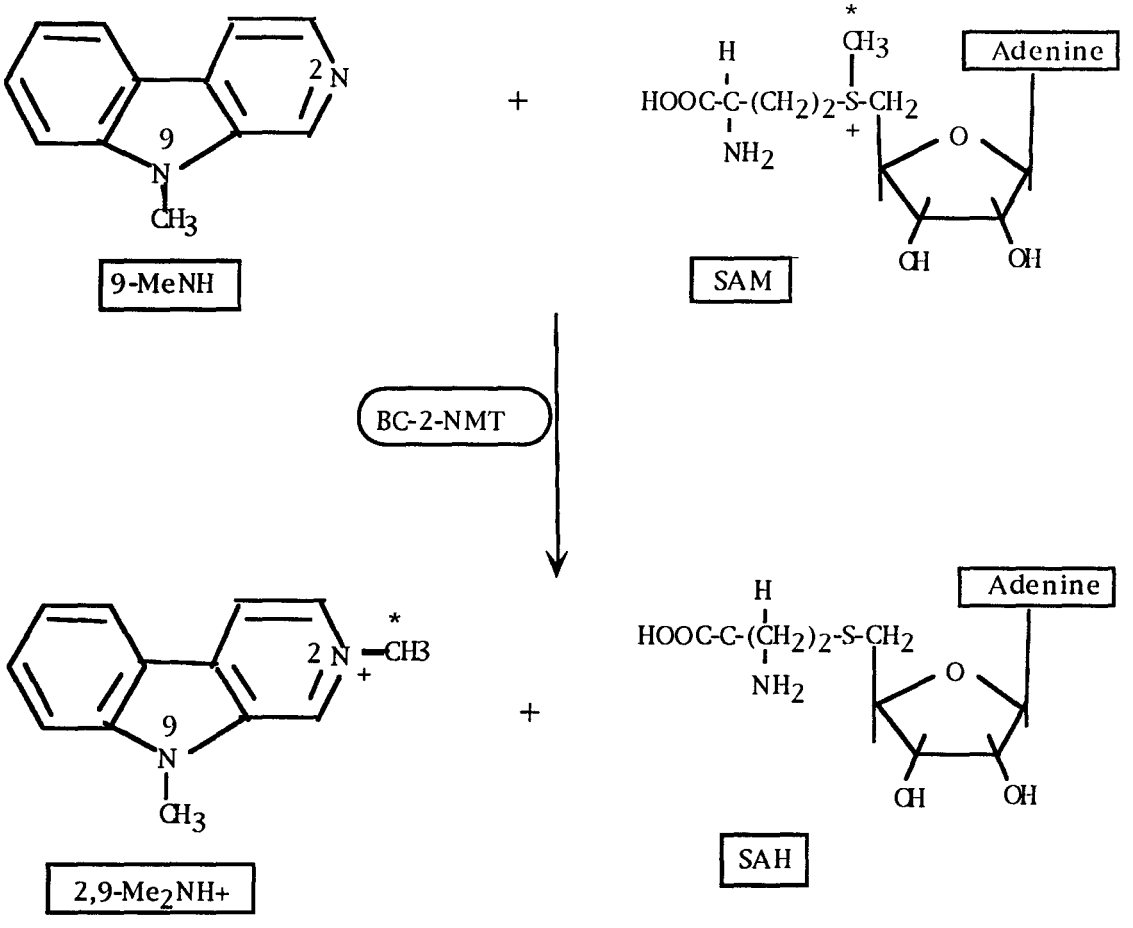


Figure 11. ßC-2-NMT assay. Site of tritium label in molecule marked with *. Abbreviations: 9-methylnorharman (9-MeNH), 2-[^{3}H-methyl]-9-methylnorharmanium ion (2,9-Me_2NH^+), S-adenosyl-L-methionine (SAM), S-adenosyl-L-homocysteine (SAH).

Use of the norharman, a presumed physiological substrate, in the assessment of ßC-2-NMT activity would have been confusing since the reaction product, 2-MeNH+ (refer to Figure 2) is subsequently 9N-methylated by ßC-9-NMT; i.e., it would be difficult to quantitate the product 2-MeNH+ since it converted to 2,9-Me_2NH^+. Therefore, the ßC substrate 9-MeNH was used to assess ßC-2-NMT activity since its indole position (9N-) is already methylated, allowing for measurement of methylation occurring exclusively at the 2N- position of the ßC. It was assumed that 9-MeNH and norharman are similarly 2N-methylated by ßC-2-NMT. This assumption was made based on similar enzyme activities reported by Collins et al. (1992) for guinea pig brain catalyzed 2N-methylation of 9-MeNH and norharman. ßC-2-NMT activity was 2.17 ± 0.17 pmol/h/mg protein with 9-MeNH as the substrate. Total ßC-2-NMT activity with norharman as the substrate was 7.85 pmol/h/mg protein) -- the total was calculated by summing the 2-MeNH+ (4.80 ± 0.06 pmol/h/mg protein) and 2,9-Me_2NH^+ (3.05 ± 0.05 pmol/h/mg protein) present in the assay.

The assay was carried out as previously reported by Matsubara et al. (1993) with modifications as detailed below. Enzyme assays were prepared in 1.5 ml polypropylene microcentrifuge tubes by mixing concentrated stock solutions of 9-MeNH and SAM with whole brain homogenate or subfraction. Stock solutions of SAM were prepared in water, and 9-MeNH was dissolved in a buffer mixture consisting of 50 mM sodium phosphate (Na_2HPO_4) and 50 mM bicine at pH 6 to 9. Brain homogenate or subcellular fraction containing 0.014 to 3.33 mg protein per assay tube was present in buffer "H1", "H2", or "H3". Stock solution volumes remained constant across studies, whereas stock solution concentrations and final pH varied depending upon the experiment.

Assay volume was 370 µl, comprised of the following stock solutions: 50 µl ^{3}H-SAM, 20 µl non-radioactive SAM, 200 µl 9-MeNH, and 100 µl enzyme solution. The final assay pH, as well as the final substrate and protein concentrations are indicated below for each experiment. The assay was gently vortexed and subsequently maintained at 37 °C in a shaking water bath for one hour, except in the time study.

Following incubation at 37 °C the assay tube was placed on ice and protein precipitated by the addition of 200 µl 0.5 N perchloric acid ($HClO_4$). The mixture was centrifuged to pellet precipitated protein (7000*g* for 5 minutes); supernatant was transferred to a glass culture tube containing one ml 1% (w/v) ammonium hydroxide (NH_4OH, pH 10 ± 0.2) and 100 µl 1 N potassium hydroxide. The protein pellet was vigorously resuspended in 500 µl 0.01 N $HClO_4$ and centrifuged (10,000*g* for 5 minutes); the resulting supernatant was combined in the glass tube with the initial supernatant. Contents of the glass tube were slowly applied to a Sep-Pak® C18 Plus solid-phase extraction cartridge (Millipore-Waters part no. 20515, 360 mg). The cartridge was pretreated with 5 ml each of methanol:5 N acetic acid (3:1, v/v), methanol, and 1% NH_4OH (w/v) in that sequence. Slow application of all solutions to the cartridge (2 to 5 drops per second) was achieved using a vacuum manifold. Following sample application, the extraction cartridge was washed with 8 ml of 1% NH_4OH followed by 5 ml methanol; these washes were discarded since they contained undesired compounds that contributed significantly to background radioactivity during subsequent analysis. The final wash (5 ml), containing 3:1 (v/v) methanol:5 N acetic acid, promoted elution of tritiated 2,9-Me_2NH^+ product from the cartridge into a clean glass culture tube. This eluate was

evaporated to dryness at 30 - 40 °C using a Speed Vac concentrator (Savant Instruments Inc., Hicksville NY).

Reverse-Phase High Performance Liquid Chromatography (RP-HPLC)

Activity of ßC-2-NMT was quantitated by RP-HPLC analysis of 2-(^{3}H-methyl)-9-MeNH$^+$ present in the Speed Vac residue. Residue was dissolved in 200 µl HPLC mobile phase by sonication and vortexing. Samples were filtered through a 0.45 µm nylon membrane filter (Micro-Spin, Chrom-Tech Inc.) prior to injection (150 µl) onto the HPLC system. Isocratic HPLC analysis was carried out under ambient conditions on a Bondclone 10 µ C18 column (Phenomenex™, 300 mm *x* 3.9 mm). Mobile phase consisted of 17% (v/v) HPLC grade acetonitrile mixed with 83% (v/v) aqueous buffer. The buffer contained 50 mM triethylamine in 250 mM sodium phosphate monobasic adjusted to pH 3 with phosphoric acid (final mobile phase was pH 3.4 ± 1). The HPLC system consisted of a Shimadzu SIL-9A programmable autosampler, a Beckman 110A pump (0.8 ml/minute), and an in-line radioactive flow detector (Radiomatic Instruments Flo-One\ß). Ultima-Flo™ AP (Packard Instruments) liquid scintillation cocktail was used at a flow rate of 2.8 ml per minute resulting in a scintillation fluid to mobile phase mixing ratio of 7:2. The radioactive flow detector was equipped with a 2.5 ml flow cell and an automatic stream splitter. It was necessary to bypass the radioactive flow detector by splitting the HPLC column effluent to waste until about ten minutes into the run in order to reduce interference due to tritiated materials (e.g., ^{3}H-SAM) present in the sample. Radioactive peak area corresponding to 2,9-Me_2NH^+

(retention time 12-13 minutes) was calculated using Radiomatic Flo-One\ß system software and resulting integrated peak area was reported as total cpm under the 2,9-Me_2NH^+ curve (see Figure 12 for representative radiogram).

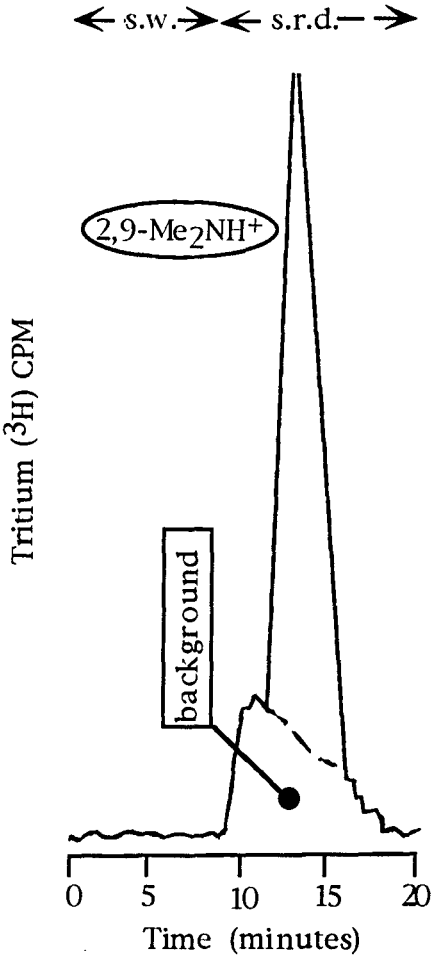


Figure 12. Rendition of representative HPLC radiogram. Peak represents radioactivity due to 2,9-dimethylnorharmanium ion (2,9-Me_2NH^+) formed at pH 8.5 by ßC-2-NMT in the presence of 9-methylnorharman (9-MeNH), S-adenosyl-:methionine (SAM), and ^{3}H-SAM. Background represents radioactivity in the absence of 9-MeNH. Chromatographic separation was performed using RP-HPLC as described in text. Column effluent was split to waste (s.w.) from 0-9 minutes into analysis, and then split to radioactive detector (s.r.d.) from 9-20 minutes.

Determination of pH Optimum for ßC-2-NMT Assay

Buffers composed of 50 mM bicine (pK_a 8.3) plus 50 mM Na_2HPO_4 (pK_a 7.2) spanning the range pH 6.6-9.0 were utilized to prepare 9-MeNH stock solutions (1 mM); final 9-MeNH concentration in the assay was 540 µM. Duplicate samples were prepared to contain 0.6 mg protein from bovine brain cytosol (in "H2"), and SAM at a final concentration of 105 µM (3.6 µCi) in addition to 9-MeNH stock at the indicated pH. Final assay pH was determined to be identical to the pH of the buffer used to prepare 9-MeNH stock solutions. ßC-2-NMT activity, expressed as pmoles of 2,9-Me_2NH^+ formed per hour per mg protein (pmol/h/mg protein), was plotted as a function of assay pH.

Assessment of ßC-2-NMT Activity as a Function of Protein Concentration

Bovine brain cytosol in "H2" was concentrated using a Centricon® 30 concentrator (Amicon ®, Beverly MA). Concentrated cytosol was then serially diluted with 150 mM KCl to provide solutions containing various protein concentrations for use in the ßC-2-NMT assay. Assays were prepared to contain: protein (0.014 -3.33 mg per assay), 9-MeNH (540 µM), and SAM (105 µM, 4.5 µCi) at pH 8.5. ßC-2-NMT-catalyzed formation of 2,9-Me_2NH^+ (pmol/h) was plotted as a function protein concentration in the assay.

The Effect of Length of Assay Time on ßC-2-NMT Activity

Assays contained bovine brain cytosol (0.687 mg protein in buffer "H3"), 540 µM 9-MeNH, and 105 µM SAM (2.88 µCi) at pH 8.5. Duplicate samples

were maintained at 37 °C for 0, 15, 30, 60, 120, and 180 minutes prior to termination of assay. The ßC-2-NMT-catalyzed formation of 2,9-Me_2NH^+ was plotted as a function of time at 37 °C.

Determination of Apparent Kinetic Constants With Respect to the Methyl Donor SAM

Assays contained bovine brain cytosol in "H2" (0.79 mg protein), 9-MeNH (540 µM, pH 8.5), ^{3}H-SAM (2.3 or 6.0 µCi), and SAM at a final concentration ranging from 2.6 - 520 µM. ßC-2-NMT activity -- expressed as pmoles of 2,9-Me_2NH^+ formed per hour per mg protein (pmol/h/mg protein) -- was plotted as a function of SAM concentration. Plotting and curve-fitting were accomplished using Kaleidagraph™ data analysis/graphics application software (Abelbeck and Synergy Software, Reading PA). Nonlinear curve fitting was employed to fit the data to the Michaelis-Menten equation for the determination of apparent kinetic parameters, the Michaelis constant (K_M) and maximal velocity (V_{max}). This program uses the Levenberg-Marquardt algorithm to fit data to a user-defined nonlinear equation. Data were also analyzed using a Hanes plot, a linear transformation of the Michaelis-Menten equation.

Determination of Apparent Kinetic Constants With Respect to the ß-Carboline 9-MeNH

Assays included bovine brain cytosol (0.79 mg protein), SAM (260 µM, 5.6 µCi), and 9-MeNH at a final concentration ranging from 5 µM to 1 mM at pH 8.5. Data analysis was achieved as described above.

Effect of S-Adenosyl-L-Homocysteine (SAH) on ßC-2-NMT Activity

The effect of SAH on ßC-2-NMT activity was determined in preparations containing bovine brain cytosol in "H3" (1.33 mg protein), 540 µM 9-MeNH, 105 µM SAM (2.77 µCi), and SAH at pH 8.5. Cytosol was concentrated, and endogenous SAH theoretically removed by ultradiafiltration using a Centricon® 30 concentrator; ultradiafiltration was carried out against 10 mM Na_2HPO_4 (pH 7) containing 150 mM KCl. Assays were prepared in duplicate and contained SAH ranging from 1 µM to 1 mM. Results are reported as percent of control ßC-2-NMT activity in the absence of added SAH. Only the linear range of the curve is shown (1 µM - 100 µM) for ease in determination of the IC_{50} following linear curve-fitting of the data.

Effect of Various Metals on ßC-2-NMT Activity

The effect of a number of transition metals on ßC-2-NMT activity was determined by adding chloride salts of metals or EDTA at a final concentration of 1 mM. Activity was measured in bovine brain cytosol in "H2" or "H3" (0.721 mg protein) at pH 8.5; substrates were present at 540 µM and 105 uM (3.4 µCi) for 9-MeNH and SAM, respectively. Results are reported as percent of control ßC-2-NMT activity, control activity is activity in the absence of added EDTA or metal chloride. Data were obtained from two different experiments, each with its own control. Composition of homogenation buffer ("H2" or "H3") did not appear to affect results between studies.

Protein Measurement

Protein was determined using a modified Lowry method as described in Scopes (1994a). Details of this method are provided in Appendix A.

Results

Subcellular Distribution of ßC-2-NMT Activity

Bovine brain ßC-2-NMT activity (Table 7) resides predominantly in the cytosolic supernatant (72% of total activity), although there is also notable nuclear activity (26% of total activity). The cytosolic subfraction also exhibits the highest specific activity. Comparable results -- with respect to fraction specific activity and subcellular distribution of total activity -- were obtained upon subcellular fractionation of guinea pig brain (data not shown).

Table 7.--Subcellular Localization of ßC-2-NMT Activity in Bovine Brain

Subcellular Fraction	% Recovered Activity	Specific Activity (pmol/hr/mg protein)
Crude	--------	4.1
Nuclear	26.5	1.4
Mitochondrial	0.7	1.6
Microsomal	0.5	3.7
Cytosolic	72.3	21.9

Bovine brain was homogenized in 2 volumes (2 ml/g tissue) of cold buffer containing 150 mM potassium chloride (KCl), 1 mM dithiothreitol (DTT), 1 mM ethylenediaminetetraacetic acid disodium (EDTA), and 20 mM sodium phosphate dibasic (Na_2HPO_4) at pH 7.4. Homogenate was filtered through 2-4 layers of gauze prior to centrifugation. Centrifugation at 900*g* (10 minutes), 9000*g* (15 minutes), and 100,000*g* (one hour) afforded crude nuclear, mitochondrial, and microsomal plus cytosolic fractions, respectively. Crude particulate fractions were diluted with 150 mM KCl prior to inclusion in assays; crude cytosol was not diluted.

pH Optimum for ßC-2-NMT Assay

Under the assay conditions utilized, ßC-2-NMT activity increased linearly from pH 6.6 to approximately pH 8.0, where activity reached a plateau that extended to pH 9.0 (Figure 13).

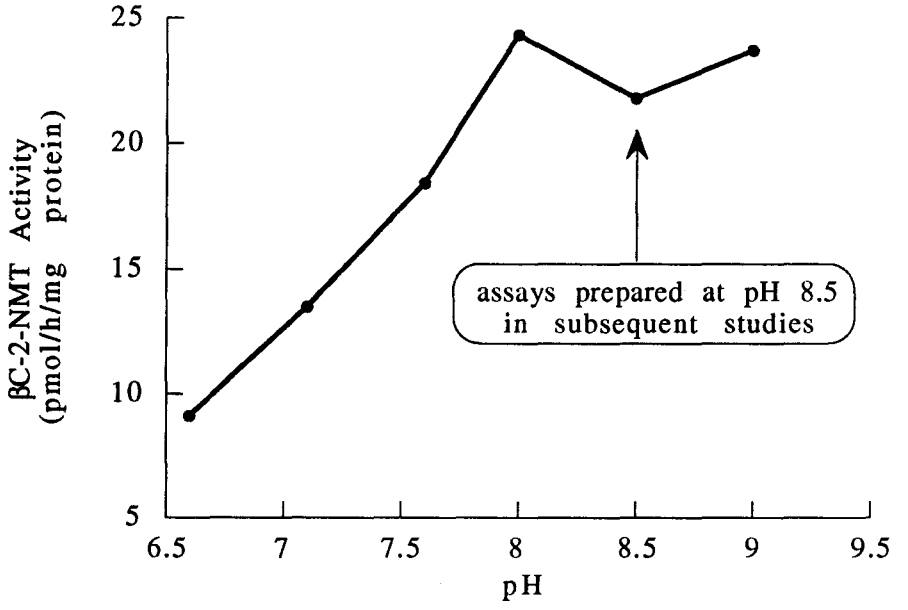


Figure 13. ßC-2-NMT activity as a function of assay pH. Buffer system was 50 mM bicine plus 50 mM Na_2HPO_4. Components present included: 0.6 mg protein from bovine brain cytosol, 9-MeNH (540 µM), and SAM (105 µM, 3.6 µCi).

Linearity of ßC-2-NMT Activity as a Function of Time and Protein Concentration

Under assay conditions in which product formation was much less than substrate concentration, ßC-2-NMT activity increased linearly with respect to time (Figure 14) and protein concentration (Figure 15). Least squares curve-fitting to a linear equation of activity as a function of total protein or time showed a highly linear relationship (r=0.996 and 0.980 respectively).

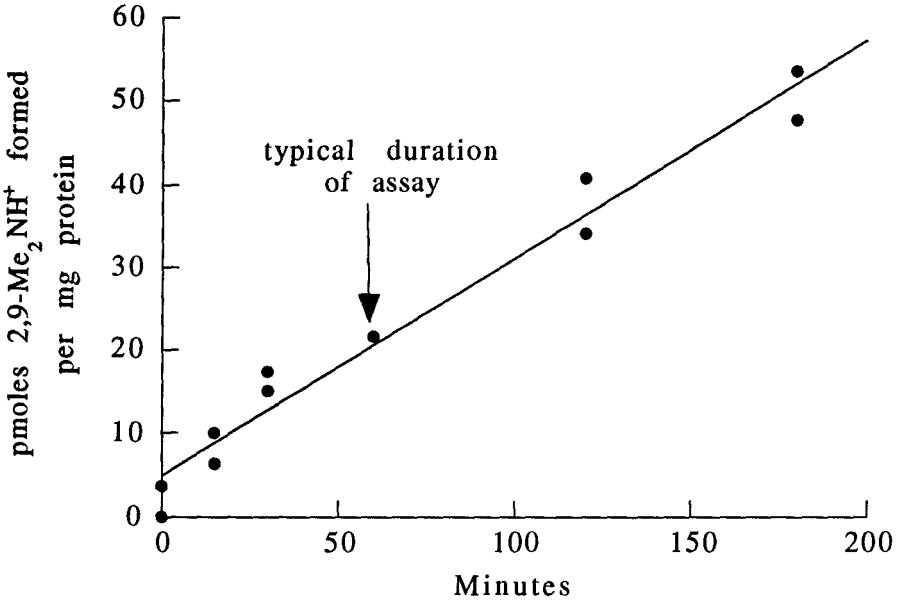


Figure 14. ßC-2-NMT-catalyzed formation of 2,9-Me_2NH^+ as a function of assay time. Assays contained bovine brain cytosol (0.687 mg protein in bicine-phosphate buffer), 540 µM 9-MeNH, and 105 µM SAM (2.88 µCi) at pH 8.5.

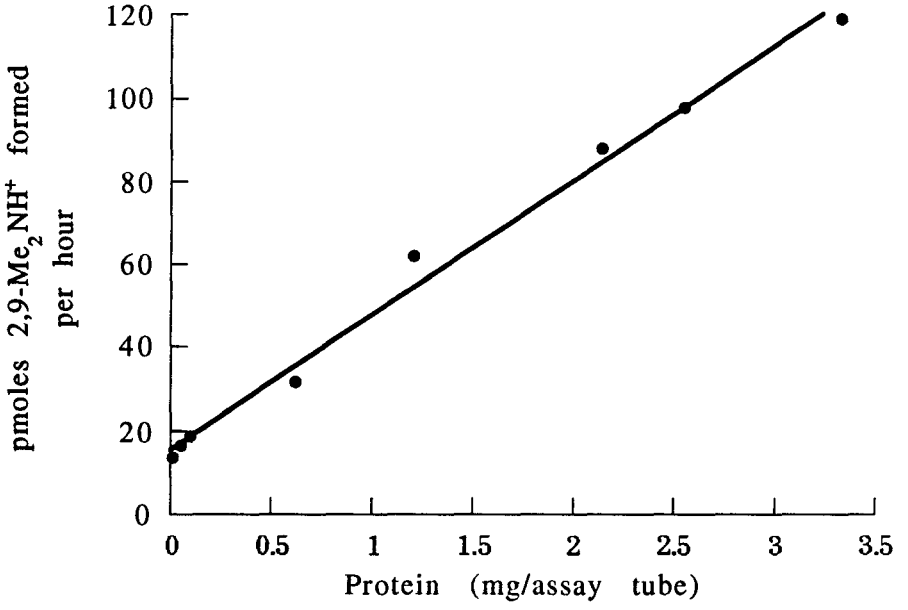


Figure 15. ßC-2-NMT-catalyzed formation of 2,9-Me_2NH^+ as a function of protein concentration. Assays (37 °C) contained bovine brain cytosol (0.014 - 3.33 mg protein per assay), 9-MeNH (540 µM), and SAM (105 µM, 4.5 µCi) at pH 8.5. Data not corrected for background therefore line does not pass through origin.

Apparent Kinetic Constants With Respect to Substrates, SAM and 9-MeNH

The high correlation coefficients ($r>0.96$) obtained following nonlinear curve fitting of the data to the Michaelis-Menten equation indicate that ßC-2-NMT obeys Michaelis-Menten kinetics with respect to both 9-MeNh (Figure 16) and SAM (Figure 17) when the non-varied substrate is fixed near saturation. Values for K_M and V_{max} are reported as parameter value ± standard error of that parameter value. Graphical analysis of the data calculates K_M and V_{max} values of 75 ± 20 µM and 48 ± 3 pmol/h/mg protein with respect to 9-MeNH (Figure 16). Regarding SAM, estimates of K_M and V_{max} are 81 ± 21 µM and 53 ± 4 pmol/h/mg protein (Figure 17). As shown in Figures 18 and 19, linear transformation of the Michaelis-Menten equation and data analysis in the form of a Hanes plot generates K_M and V_{max} estimates similar to those obtained following nonlinear regression analysis of the data.

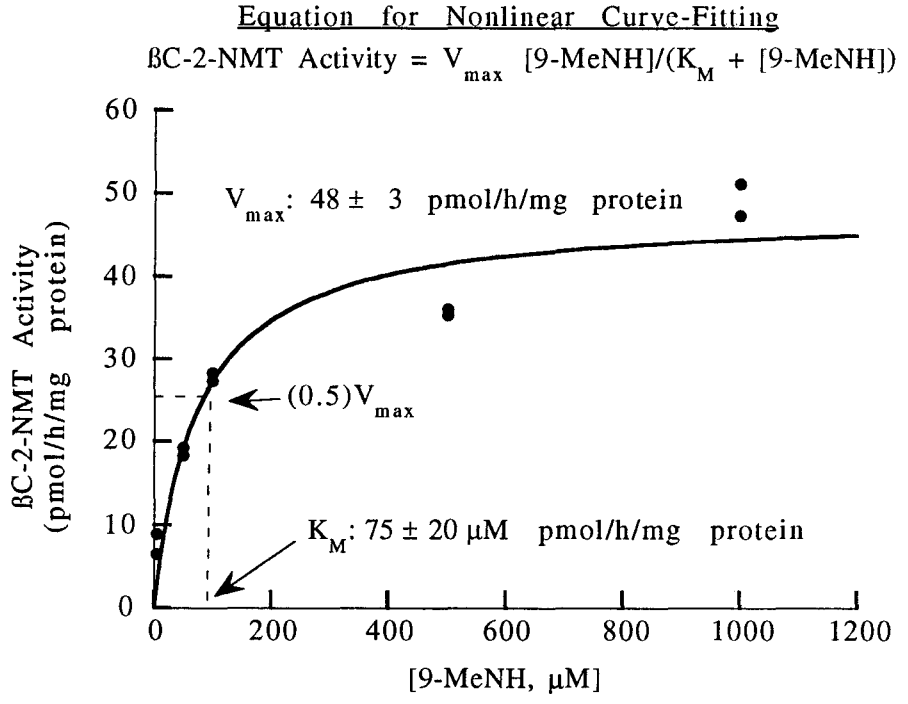


Figure 16. ßC-2-NMT activity as a function of 9-MeNH concentration. Assays included bovine brain cytosol (0.79 mg protein), SAM (260 µM, 5.6 µCi), and 9-MeNH at a final concentration ranging from 5 µM to 1 mM at pH 8.5.

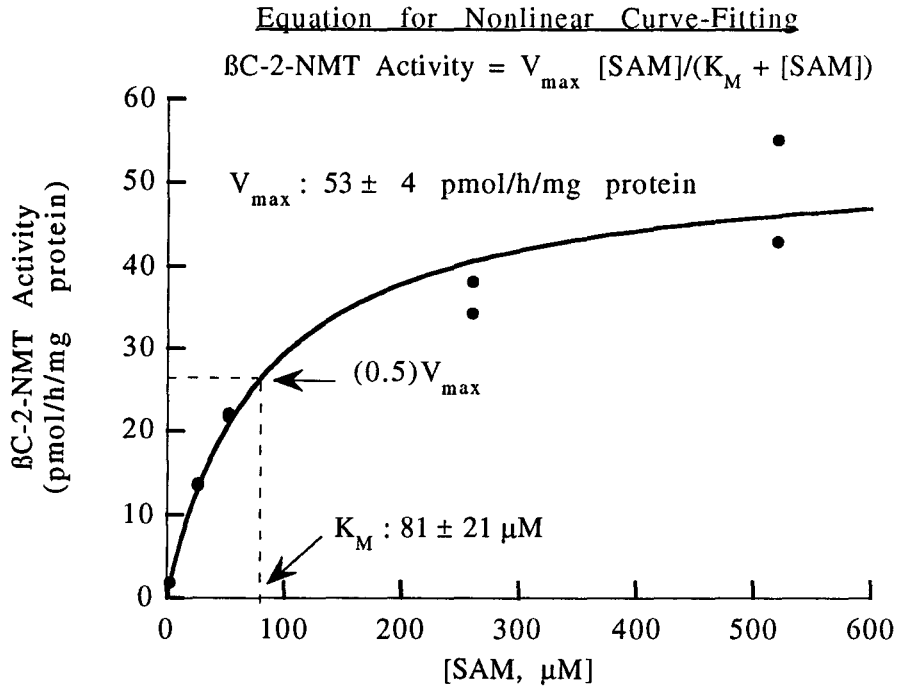


Figure 17. ßC-2-NMT activity as a function of SAM concentration. Assays at pH 8.5 contained bovine brain cytosol in (0.79 mg protein), 9-MeNH (540 µM), ^{3}H-SAM (2.3 or 6.0 µCi), and SAM at a final concentration ranging from 2.6 - 520 µM.

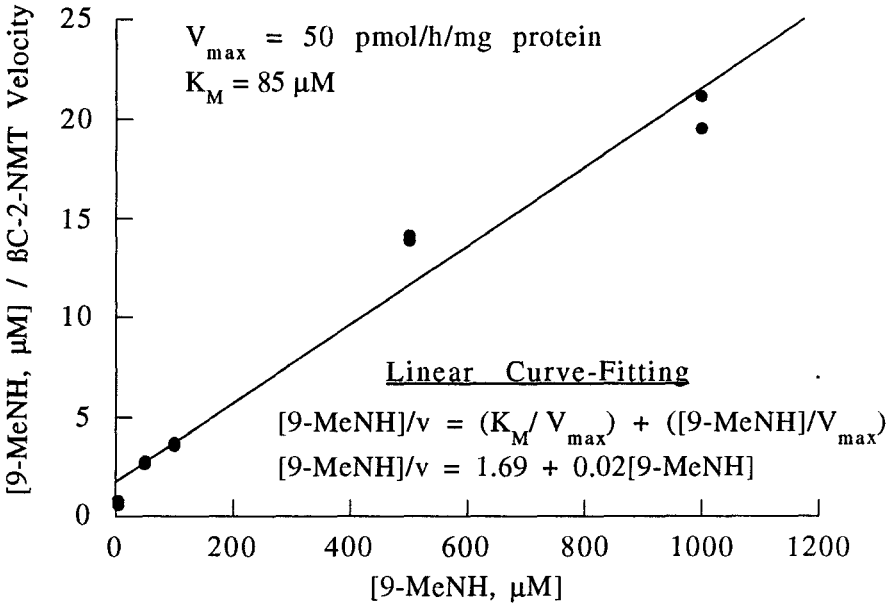


Figure 18. Hanes plot of kinetic data with respect to 9-MeNH concentration. Assays included bovine brain cytosol (0.79 mg protein), SAM (260 μM, 5.6 μCi), and 9-MeNH at a final concentration ranging from 5 μM to 1 mM at pH 8.5.

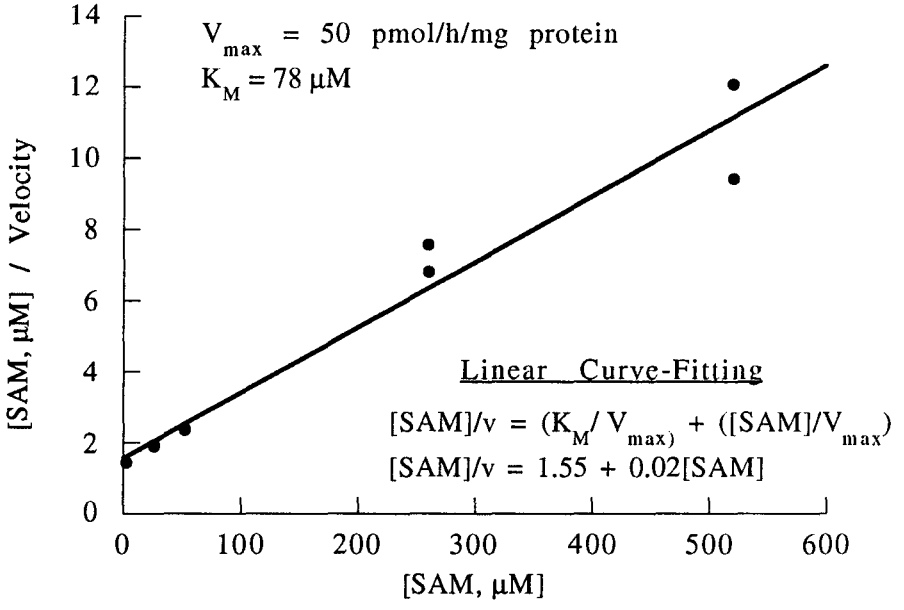


Figure 19. Hanes plot of kinetic data with respect to SAM concentration. Assays (pH 8.5) contained bovine brain cytosol (0.79 mg protein), 9-MeNH (540 μM), ^{3}H-SAM (2.3 or 6.0 μCi), and SAM at a final concentration ranging from 2.6 - 520 μM.

S-Adenosyl-L-Homocysteine (SAH)
Inhibits ßC-2-NMT Activity

The activity of bovine brain ßC-2-NMT is inhibited by SAH in a concentration-dependent manner (Figure 20). The IC_{50} for SAH, i.e., the concentration of inhibitor producing a 50% reduction in ßC-2-NMT activity compared to control, is 14.8 µM.

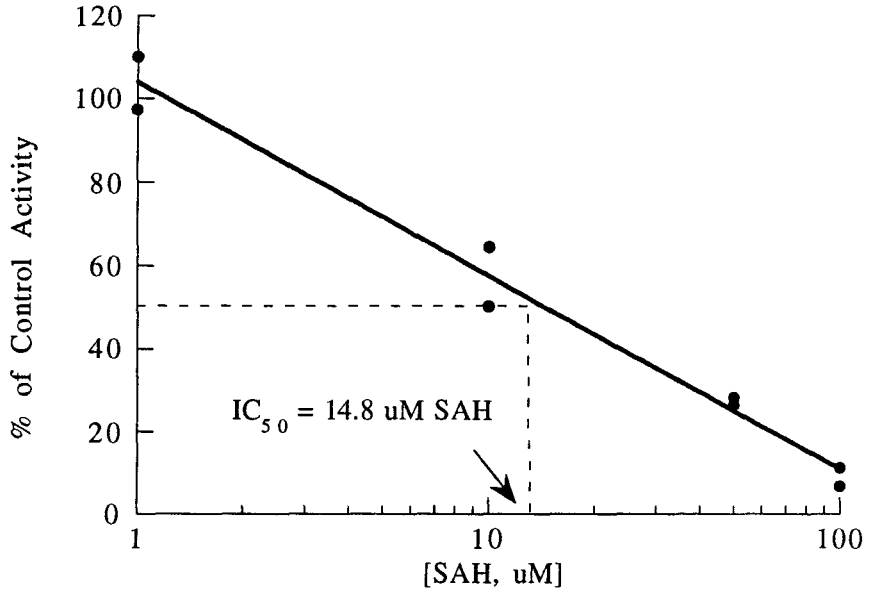


Figure 20. SAH inhibition of ßC-2-NMT activity. Assays (pH 8.5) contained bovine brain cytosol (1.33 mg protein), 540 µM 9-MeNH, 105 µM SAM (2.77 µCi), and SAH.

Effects of Transition Metals on ßC-2-NMT Activity

Certain transition metals affected ßC-2-NMT activity (Table 8). ßC-2-NMT activity was increased 2-fold in the presence of iron ($p<0.05$) and manganese ($p<0.05$). Increased ßC-2-NMT activity in the presence of calcium did not reach statistical significance. Nickel, magnesium and copper did not appear to affect ßC-2-NMT activity, but zinc completely inhibited formation of 2,9-Me_2NH^+. ßC-2-NMT activity was 139% of control in the presence of the chelating agent EDTA (1 mM).

Table 8.--Effect of Transition Metals on ßC-2-NMT Assay

Metal[a]	% of Control[c]
Iron	209 ± 0.7 (2) [d]
Iron [b]	231 ± 13 (2) [d]
Manganese	192 ± 13 (2)
Cobalt	146
Calcium	121 ± 0 (2)
Nickel	116
Magnesium	101
Copper	101
Zinc	0 ± 0 (2)

[a] As chloride salt, 1 mM, 2^+ oxidation state

[b] 3^+ oxidation state

[c] Values are mean ± s.d. for (N) replicates

[d] Significantly different than control ($p<0.05$), ANOVA followed by Tukey-HSD post-hoc test

Discussion

Enzyme Kinetics

Fitting of kinetic data to the Michaelis-Menten (M-M) equation indicates that ßC-2-NMT obeys M-M kinetics with respect to both 9-MeNH and SAM, when one substrate is fixed and the other is varied. For guinea pig brain, previous studies from this laboratory reported kinetic constants with respect to 9-MeNH of 17.8 μM and 2.2 pmol/h/mg protein, for K_M and V_{max} values, respectively (Matsubara et al. 1992b), results that differ from those reported here. Kinetic parameters with respect to SAM were not determined in previous studies.

In addition to species differences (guinea pig vs. bovine), two factors may contribute to the difference in kinetic constants reported by Matsubara (1992b) and those reported here: (a) SAM concentration utilized between studies, and (b) the method of data analysis utilized in each investigation. Regarding the effect of SAM concentration, Matsubara determined kinetic parameters with respect to 9-MeNH with SAM fixed at 8 μM. The present study indicates that 8 μM SAM is well below its K_M of 81 μM. In fact, if 8 μM SAM is inserted into the M-M equation using the present K_M and V_{max} values for SAM, the calculated activity is about 5 pmol/h/mg protein, a value comparable to the V_{max} described by Matsubara (1992b). In the present study, SAM concentration was fixed at 260 μM, equivalent to a concentration three times its K_M; the literature suggests (Dixon et al. 1979a) that a higher SAM concentration is more appropriate for testing the adherence of ßC-2-NMT to M-M kinetics with 9-MeNH as the variable substrate. Increasing SAM concentration from 8 μM (the concentration used by Matsubara) to 260 μM (the

concentration used in this dissertation), while 9-MeNH is varied, increased the V_{max} estimate approximately 20-fold, from 2.2 pmol/h/mg protein for guinea pig brain (Matsubara et al. 1992b) to the value reported in this dissertation, 48 pmol/h/mg protein for bovine brain.

In addition to the effect of SAM concentration discussed above, data analysis methods employed between studies may contribute to the discrepancy in the K_M for 9-MeNH reported by Matsubara (1992b) and the value reported in this dissertation. These researchers utilized a non-weighted Lineweaver-Burk linear transformation (double-reciprocal plot) of the M-M equation in data analysis. Though commonly used, this plot does not reflect experimental error accurately, a point discussed by Cornish-Bowden (Cornish-Bowden 1979). In fact, when kinetic data presented here are fitted to the Lineweaver-Burk equation, the estimated K_M with respect to 9-MeNH was nearly identical to the 17.8 μM value obtained by Matsubara. It is noteworthy that close scrutiny of a Lineweaver-Burk plot of these data revealed a marked downward curvature of data points near the y-axis (data not shown). This observed deviation from linearity in the double-reciprocal plot prompted an investigation of other options regarding data analysis.

Nonlinear curve- fitting, which fits the data directly to the Michaelis-Menten equation, i.e., without linear transformation of the original equation, was therefore employed. Use of nonlinear cure-fitting circumvents certain problems associated with the use of linear transformation and subsequent graphical analysis of nonlinear data. For a review of the applicability of nonlinear regression in the analysis of nonlinear data (e.g., enzymatic data) the reader is directed to papers by Wilkinson et al. (1961) and Motulsky et al.

(1987). Hanes plots of the kinetic data were generated, following the more the more familiar linear transformation of the M-M equation, as referenced in Cornish-Bowden (1979). As shown in Figures 13 and 14, Hanes plots yield kinetic parameters that are reasonably close to those obtained with nonlinear curve-fitting.

The V_{max} of bovine brain ßC-2-NMT with respect to both substrates is quite small. Since V_{max} is derived from the product of enzyme concentration and k_{cat}, the small magnitude of V_{max} may indicate that ßCs are poor substrates for this N-methyltransferase (i.e., low turnover number), or that the amount of ßC-2-NMT present in brain tissue is quite small. ßC-2-NMT-catalyzed formation of neurotoxic products in relatively low amounts due to the enzyme's low V_{max}, would be consistent with the hypothesis that this N-methyltransferase could participate in the neuropathogenesis of Parkinson's disease -- a disease that takes years to manifest symptoms related to the underlying neurodegeneration. It is postulated that the low activity of ßC-2-NMT could result in the generation of small quantities of neurotoxic 2-MeßCs$^+$, which over a period of many years would cause the death of sufficient neurons to elicit the clinical characteristics of PD.

Other Properties of ßC-2-NMT

Linearity of ßC-2-NMT activity as a function of time and protein concentration is fundamental to reliable monitoring of enzyme activity during protein purification. Note that enzyme activity was not linear, but instead plateaued, with respect to time or protein concentration in preliminary experiments which utilized SAM at 8 µM instead of 105 µM (data

not shown). The linearity of ßC-2-NMT activity through 180 minutes at 37 °C is also an indication that the enzyme is thermally stable under these conditions.

All SAM-dependent methyltransferases studied to date are inhibited by S-adenosyl-L-homocysteine (SAH) in a competitive manner (Deguchiand Barchas 1971; Lawrence and Robert-Gero 1990). This is not surprising since products often inhibit the enzyme responsible for their formation, and SAH is a product of SAM-dependent methylation reactions. Inhibition by SAH varies depending upon the specific methyltransferase affected, with K_i's falling into the 100 nM to 12 µM range. Fifty percent inhibition of ßC-2-NMT activity is observed when added SAH is present at about 14.5 µM, which corresponds to a K_i of 4.5 µM (data not shown), consistent with the behavior of other SAM-dependent methyltransferases.

The manganese and iron effects, although not dramatic, are intriguing since both metals are associated with parkinsonism (Irwin et al. 1995). Manganese miners occasionally display parkinsonian symptoms such as bradykinesia. Neuropathologically, the globus pallidus shows extensive cell loss but other basal ganglia components, including the substantia nigra may also be affected (Mena 1979); however, manganese poisoning is not clinically classified as PD. In contrast, aberrant iron metabolism has been suggested to play a role in Parkinson's disease (PD); persons afflicted with PD exhibit higher than normal free iron (Dexter et al. 1989), and decreased ferritin protein in the substantia nigra (Dexter et al. 1990). Hypothetically, if iron is indeed elevated in the substantia nigra of persons with PD, this high iron concentration could conceivably increase ßC-2-NMT activity in that brain

region -- leading to local formation of neurotoxic N-methylated ß-carbolinium compounds and subsequent cell death.

Previously Characterized N-Methyltransferases

It is possible that ßC-2-NMT represents a previously characterized enzyme for which ß-carbolines were not evaluated as substrates. Saavedra et al. (1973) described the distribution of a non-specific N-methyltransferase present in the brain of several species, including human postmortem brain tissue. That SAM-dependent enzyme utilizes tryptamine as well as several other amines as substrates. Similar to ßC-2-NMT, the enzyme exhibited a K_M with respect to SAM of 52 µM and a V_{max} in the range 2 -15 pmol/h/mg protein, depending upon the tissue. The subcellular distribution and specific activity of the non-specific N-methyltransferase described by Saavedra are similar to those reported here for ßC-2-NMT. However, the regional distribution of this enzyme differed from that for ßC-2-NMT described by Matsubara et al. (1992b).

Ansher and Jakoby (1986b, 1987) purified amine N-methyltransferases A and B from rabbit liver. The enzymes exhibited broad, overlapping N-methylating activities toward numerous amines, including several azaheterocycles. The azaheterocycles tested included derivatives of pyridine and isoquinoline, but ß-carbolines were not tested as substrates. It is noteworthy that both N-methyltransferases A and B were purified from cytosol and both exhibited pH profiles with respect to desmethylimipramine and tryptamine that were similar to that of ßC-2-NMT toward

9-MeNH. Ansher et al. (1986a) also found these amine N-methyltransferase activities in human, monkey, and rodent cytosolic brain extracts; substrates included 4-phenyltetrahydropyridine and 4-phenylpyridine, potential precursors of neurotoxic MPP^+. Specific activities toward these substrates were in the range 0.17-23 pmol/h/mg protein, similar to the values reported in the present study.

In addition, Naoi, et al. (1989a) reported the presence of a cytosolic N-methyltransferase activity in human brain homogenates that forms potentially neurotoxic N-methyl-1,2,3,4-tetrahydroisoquinoline from tetrahydroisoquinoline (TIQ). The TIQ-methylating activity exhibited kinetic parameters and a pH optimum similar to those of ßC-2-NMT. Likewise, Matsubara et al. (1992a) identified a cytosolic N-methyltransferase activity present in rodent brain that catalyzes methylation at the 2N-nitrogen of tetrahydro-ß-carbolines. It is conceivable that ßC-2-NMT and any of the N-methyltransferases described above are the same or least similar enzymes.

Interestingly, evidence presented in Chapter 6 of this dissertation indicates that ßC-2-NMT may be due to the epinephrine-synthesizing enzyme, phenylethanolamime-N-methyltransferase (PNMT).

Cytosolic or Nuclear Localization of ßC-2-NMT Activity?

The cytosolic location of ßC-2-NMT disagrees with the primarily nuclear location of the enzyme previously reported from this laboratory, in which guinea pig brain cytosolic activity was undetectable (Matsubara et al. 1992b). However, subcellular fraction of guinea pig brain (data not shown) showed a subcellular distribution and specific activities that were similar to those

reported here for bovine brain. This discrepancy between studies could be due to variation in the intensity of homogenation preceding subcellular fractionation. Since subcellular enzymatic markers were not utilized to monitor fractionation, the cause of the discrepancy remains unknown. However, cytosolic ßC-2-NMT activity is consistent with the subcellular distribution of the N-methyltransferases previously described by Saavedra et al. (1973), Ansher et al. (1986a), Naoi et al. (1989a), and Matsubara et al. (1992a).

Conclusions

The studies presented in this chapter define experimental conditions to be utilized in subsequent investigations; specifically ßC-2-NMT purification from bovine brain cytosol (Chapter 4) and measurement of its activity in normal and parkinsonian human brain (Chapter 5). For the proposed studies, cosubstrates 9-MeNH and SAM will be present at 540 µM and 105 µM (2-6 µCi) respectively; assays will be maintained at 37 °C for 60 minutes at pH 8.5.

CHAPTER 4

PARTIAL PURIFICATION OF ß-CARBOLINE-2N-METHYLTRANSFERASE FROM BOVINE BRAIN

General Comments

Difficulties

The assay at present is very labor- and time-intensive and is not amenable to utilization in the purification of ßC-2-NMT. Only 12 samples may reasonably be prepared and analyzed per day. This number is incompatible with the number of potential column fractions generated in a typical column chromatography procedure, even if alternate fractions are analyzed. Moreover, the low activity of ßC-2-NMT in combination with the dilute nature of column fractions necessitates concentration of each fraction prior to incorporation into the BC-2-NMT assay. Concentration using Centricon concentrators requires 1-4 hours depending upon the composition of the fraction. Once the concentrated fraction is prepared, the time required to obtain ßC-2-NMT activity results for those fractions is detailed below.

For the maximum of 12 samples:

• preparation of substrate solutions	0.5 h
• prepare tubes and ßC-2-NMT assays	0.5 h
• incubation at 37 °C	1.0 h
• protein precipitation and centrifugation	0.5 h
• solid-phase extraction procedure	0.5 h
• Speed Vac drying	2.0 h
• HPLC analysis (baseline, ^{3}H-SAM, 12 samples)	5.0 h
• protein assays (another day)	1.0 h
Total time required (minimum)	11 h

Recommendations

It is recommended that a new work-up of the ßC-2-NMT assay be developed. The incubation of substrates with enzyme is acceptable; however, the subsequent work-up and analysis of 2,9-Me_2NH^+ should be modified. The new work-up/analysis should be simple, e.g., spectrophotometric analysis of 2,9-Me_2NH^+ formed or liquid scintillation counting of tritiated 2,9-Mc_2NH^+ product. This can only be accomplished if radioactive substrates and products can be completely separated either physically (using SPE, liquid extraction) or "analytically" based on differences in spectral properties. Separation based on spectral characteristics is probably not possible unless a coupled assay is developed since ßCs, MeßCs+, and SAM exhibit overlapping ultraviolet and fluorescent spectra.

Purification Overview

General Background

A series of laboratory guides published by Pharmacia will be the primary references utilized during the chromatographic isolation of ßC-2-NMT (Pharmacia 1991; Pharmacia 1993a; Pharmacia 1993b; Pharmacia). These guides describe in detail the practical aspects of preparing, using, and maintaining various chromatographic resins. The books "Protein Purification: Principles and Practice" by Scopes (1994a) and "Guide to Protein Purification" by Deutscher (1990) were utilized throughout ßC-2-NMT partial-purification.

Summary of Results

A graphical summary of the experimental results to be discussed further in this chapter is presented in Figure 21. The starting material for ßC-2-NMT purification was the 100,000*xg* supernatant from whole bovine-brain homogenate. This cytosolic supernatant generally exhibited a ßC-2-NMT specific activity of 15-20 pmol/h/mg protein, using the assay conditions defined in the "Conclusions" of Chapter 3.

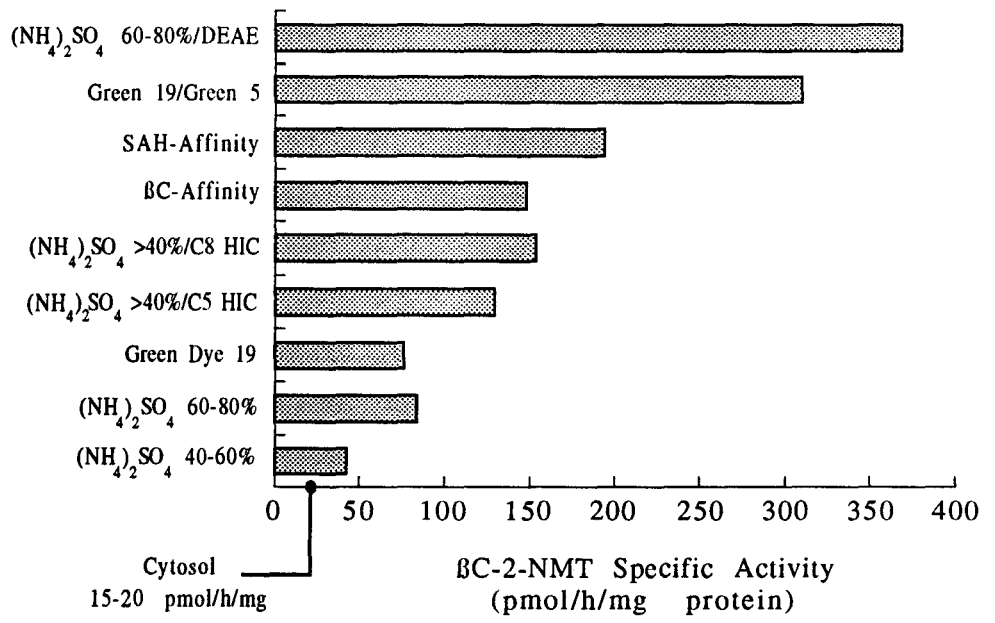


Figure 21. Summary of ßC-2-NMT purification results (details in chapter).

Key: each bar represents 1-2 steps in sequence

$(NH_4)_2SO_4$ 60-80%/DEAE: 60-80% ammonium sulfate fraction from bovine brain cytosol dissolved in pH 7 imidazole buffer, then subjected to batch chromatography on DEAE-Sephadex (anion exchange matrix). ßC-2-NMT activity (represented by bar) was eluted from the matrix with pH 7 imidazole buffer containing 0.3 M NaCl.

Green 19/Green 5: Bovine brain cytosol was applied to a Green 19 dye column, eluate from this column was applied to a Green 5 dye column. ßC-2-NMT activity (represented by bar) was eluted from Green 5 dye column using pH 7.4 Na_2HPO_4 containing 3 M NaCl.

DEAE/SAH-Affinity: DEAE-Sephadex batch chromatography elute (above) applied to an S-adenosyl-L-homocysteine affinity column. ßC-2-NMT activity (represented by bar) was eluted from the column with pH 7 imidazole buffer containing 1 M NaCl.

ßC-Affinity: Bovine brain cytosol in 20 mM Na_2HPO_4 (pH 7.3)/150 mM KCl was applied to a ß-carboline affinity column. ßC-2-NMT activity (represented by bar) was eluted from the column using a 1 mM buffered norharman solution.

$(NH_4)_2SO_4$/C8: supernatant from 40% ammonium sulfate fractionation was applied to a octyl-agarose (C8) hydrophobic interaction chromatography column. ßC-2-NMT activity (represented by bar) was measured in application flow-through.

$(NH_4)_2SO_4$/C5: supernatant from 40% ammonium sulfate fractionation was applied to a pentyl-agarose (C5) hydrophobic interaction chromatography column. ßC-2-NMT activity (represented by bar) was measured in application flow-through.

Green 19: column chromatography (same conditions as above) on a Green 19 dye column

60-80% $(NH_4)_2SO_4$: the 60-80% ammonium sulfate fraction (desalted)

40-60% $(NH_4)_2SO_4$: the 40-60% ammonium sulfate fraction (desalted)

Cytosol: bovine brain cytosol, typically the starting material for each step above

Ammonium Sulfate Precipitation Experiments

Theory

This technique exploits the effect of salt on protein conformation and therefore solubility; proteins with variable amino acid compositions can be separated based on their behavior at different electrolyte concentrations. This method is discussed in detail by Scopes (1994b). In practice, ammonium sulfate is added at low temperature and in small portions. The mixture is centrifuged to pellet precipitated proteins and additional precipitant is added to the resulting supernatant; the mixture again centrifuged, and so on. Proteins are fractionated (precipitated) based on their individual solubilities in progressively more concentrated salt solutions or organic solvents.

Materials and Methods

Preparation of Fractions

Bovine brain cytosol (12 ml) -- containing 20 mM Na_2HPO_4 (pH 7.4), 150 mM KCl, 1 mM DTT, 1 mM EDTA, 1 mM PMSF, leupeptin (1 µg/ml), and pepstatin A (1 µg/ml) -- was thawed from -80 °C. The aliquot had been stored at -80 °C for 2 months prior to use. A sample, representing "control cytosol", was retained for protein and ßC-2-NMT assays. The starting material was 11.0 ml of thawed cytosol. Ammonium sulfate precipitation of cytosolic proteins was accomplished at 5-10 °C with constant stirring, by adding solid $(NH_4)_2SO_4$ to either cytosol ("0-40% cut") or the supernatant resulting from the previous cut. Supernatant and pellet (cut) were separated by centrifugation at 10,000 *xg*

for 10 minutes at 0-5 °C. The following ammonium sulfate fractions were prepared: 0-40%, 41-60%, and 61-80% saturation with respect to $(NH_4)_2SO_4$. The resulting $(NH_4)_2SO_4$ pellets were dissolved in 2.0 ml of 150 mM KCl containing 10 mM Na_2HPO_4 at pH 7. If necessary, fraction pH was adjusted to pH 7. Solutions were filtered through a 0.2 µm polysulfone syringe filter to remove particulates and denatured protein. The turbid solution resulting from reconstitution of the 0-40% pellet could not be filtered, probably due to the presence of large particles or protein aggregates that clogged the filter; therefore, the sample could not be desalted and assayed as such. The filtered solutions derived from the 41-60% and 61-80% samples (0.5 ml of each) were desalted using ultradiafiltration against buffer (150 mM KCl, 10 mM Na_2HPO_4 at pH 7) on a Centricon 30 concentrator. Total protein and ßC-2-NMT activity were determined in desalted and non-desalted samples, except as noted.

ßC-2-NMT Assay

Details of the assay and work-up are described in Chapter 3. In this experiment reactants were present at the following concentrations: 9-McNH (540 µM), SAM (105 µM, 2.98 µCi), and 100 µl of indicated fraction containing ßC-2-NMT activity.

Protein Determination

Protein was assessed using a modification of the Lowry method as described in the Appendix A.

Results

"Control cytosol" contained 81 mg of total protein and 2146 pmol/h of ßC-2-NMT activity -- therefore, its specific activity was 26.6 pmol/h/mg protein. Figure 22 depicts ßC-2-NMT specific activity present in cytosol and indicated $(NH_4)_2SO_4$ fraction prior to desalting the fraction. The sum of the protein in each $(NH_4)_2SO_4$ fraction (excluding the 60-80% supernatant) was 41 mg; this represents 51% of the protein present in the original cytosol. With respect to ßC-2-NMT activity, 1023 pmol/h (48% of the original cytosolic activity) was recovered in the summed $(NH_4)_2SO_4$ fractions.

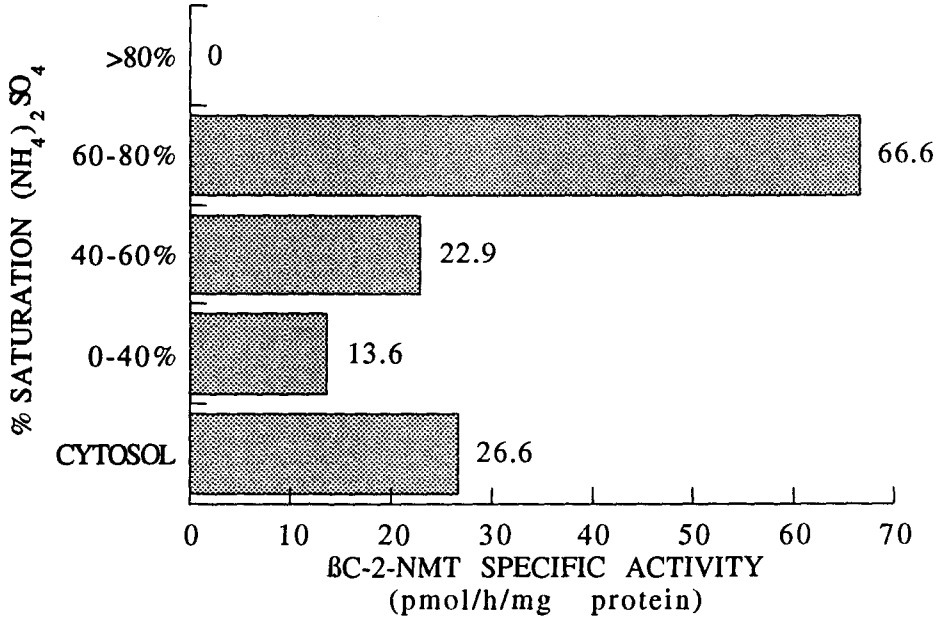


Figure 22. ßC-2-NMT activity in crude cytosol and fractions resulting from precipitation with ammonium sulfate. Fractions were not desalted prior to assays. Values next to bars represent ßC-2-NMT activity in that fraction.

The results presented in Figure 23 indicate that desalting $(NH_4)_2SO_4$ fractions prior to use in ßC-2-NMT assays increases the measured ßC-2-NMT activity. Total protein was affected less drastically by desalting.

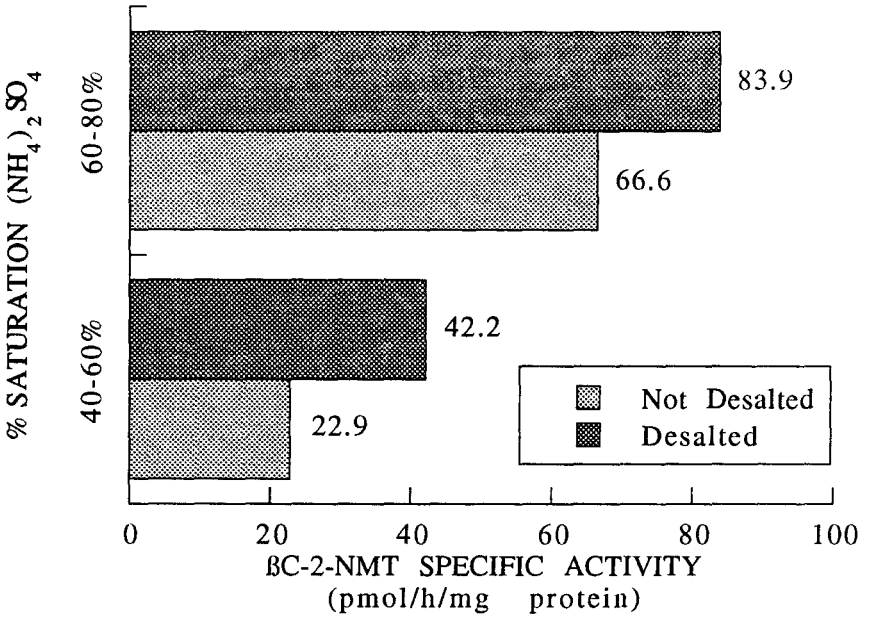


Figure 23. The effect on ßC-2-NMT activity of desalting ammonium sulfate fraction. Fractions were desalted using ultradiafiltration on a Centricon 30 concentrator.

Discussion

The presence of ammonium sulfate affects both the Lowry protein and the ßC-2-NMT assays. The Bradford assay would be a better choice regarding protein measurement in these samples since it is not affected by the presence of $(NH_4)_2SO_4$ (Petersen 1983). This experiment shows that removal of $(NH_4)_2SO_4$ from the fraction prior to its utilization in the ßC-2-NMT assay increases the measured specific activity 1.2 to 1.8-fold.

Screening Experiment for Binding of ßC-2-NMT to Ion-Exchange Chromatography Matrices in Batch Mode as a Function of Buffer pH

Theory

Ion exchange chromatography allows for separation of molecules based on charge; anion exchangers contain positively charged groups such as quaternary amines or diethylaminoethyl (DEAE), which interact with sample molecules possessing negative charges. Cation exchangers, possess negatively charged groups such as a sulfonic acid or carboxymethyl (CM) side-chains; these resins interact with sample molecules that are positively charged. Both pH and ionic strength are manipulated in order to affect the interaction of sample molecules with the anion exchanger. In general, low ionic strength conditions favor binding to ion exchangers. Lower pH buffers (pH 4-8) are used in cation chromatography, and higher pH buffers (pH 5-9) are used in anion chromatography. Proteins are then eluted using an increasing salt gradient. Fractions from the column are collected and assayed for total protein content and protein of interest.

Materials and Methods

Ion-Exchange Resins

Ion-exchange resins were obtained in the dry-state from Pharmacia. Technical information pertaining to each of the Sephadex resins is summarized in Table 9.

Table 9.--Properties of Evaluated Ion-Exchange Resins

	Ion-Exchange Resin	
	DEAE Sephadex A-50	CM Sephadex C-50
Description	weakly basic anion exchanger	weakly acidic cation exchanger
Functional group	diethylaminoethyl	carboxymethyl
Matrix	cross-linked dextran	cross-linked dextran
Particle size	40 - 120 μ	40 - 120 μ
Capacity	3.5 ± 0.5 mmoles/g	4.5 ± 0.5 mmoles/g
Swelling Ratio	varies with pH and ionic strength	varies with pH and ionic strength

Sephadex resins (10 g) were swelled in about 500 ml of 10 mM Na_2HPO_4 (pH 7.4) and subsequently stored in a capped jars at room temperature. These resins were equilibrated in other buffers as indicated in subsequent experiments.

Ion-Exchange Buffers

Buffers were prepared as 1 M stock solutions that were diluted to the required strength for individual procedures. The respective buffer salt was dissolved in Milli-Q water, the pH was adjusted using either NaOH or HCl, and the solution was diluted with Milli-Q water to the final volume. The buffer and corresponding pH utilized in anion and cation exchange chromatography protocol are summarized in Table 10.

Table 10.--Ion-Exchange Chromatography Buffers

Anion Exchange Buffers	pH	Supplier
Piperazine chloride	5	Sigma
Bis-tris chloride	6	Sigma
Bis-tris chloride	7	Sigma
Tris hydrochloride	8	Sigma
Ethanolamine chloride	9	Sigma
Cation Exchange Buffers		
Sodium acetate	5	Merck
Sodium malonate (dibasic)	6	Eastman
Sodium phosphate (monobasic)	7	Fisher
HEPES (sodium salt)	8	Sigma

Batch Chromatography

Bovine brain cytosol (6 ml) -- containing 20 mM Na_2HPO_4 (pH 7.4), 150 mM KCl, 1 mM DTT, 1 mM EDTA, 1 mM PMSF, leupeptin (1 µg/ml), and pepstatin A (1 µg/ml) -- was thawed from -80 °C. The aliquot had been stored at -80 °C for 3 days prior to use. The sample was concentrated 4-fold to 1.25 ml using a Centricon 30 concentrator.

Ion-exchange resins were equilibrated at room temperature using the buffers (diluted to 0.05 M) listed in Table 11. Approximately 1.5 ml of each Sephadex slurry was transferred to a 1.5 ml microcentrifuge tube. The tubes were centrifuged briefly in order to pack the gel, and the resulting supernatant was discarded. The appropriate buffer (100 µl) and 100 µl of concentrated cytosol were added to each of the tubes containing packed resin. The contents of the tubes were mixed on a lab rocker for 15 minutes at room temperature. Sephadex gel was settled with a brief centrifugation, and 150 µl of the resulting supernatant was transferred to a clean microcentrifuge tube. Supernatants were analyzed for ßC-2-NMT activity and total protein.

ßC-2-NMT Assay

Assay components were present as follows: 9-MeNH (540 µM), SAM (150 µM, 3.72 µCi), and 100 µl of concentrated cytosol or 100 µl of supernatant derived from one of the nine ion-exchange chromatography conditions described above. Assay volume was 370 µl, pH was 8.5, and assay was maintained at 37 °C for one hour. Details of the ßC-2-NMT assay may be found in Chapter 3.

Protein Determination

Protein was determined as described in the Appendix A.

Results

DEAE-Sephadex Chromatography

As depicted in Figure 24, ßC-2-NMT activity is retained by the anion exchange resin more extensively than total protein, and retention of ßC-2-NMT activity increases as pH increases. The largest difference between ßC-2-NMT activity bound and total protein bound occurs at pH 7.

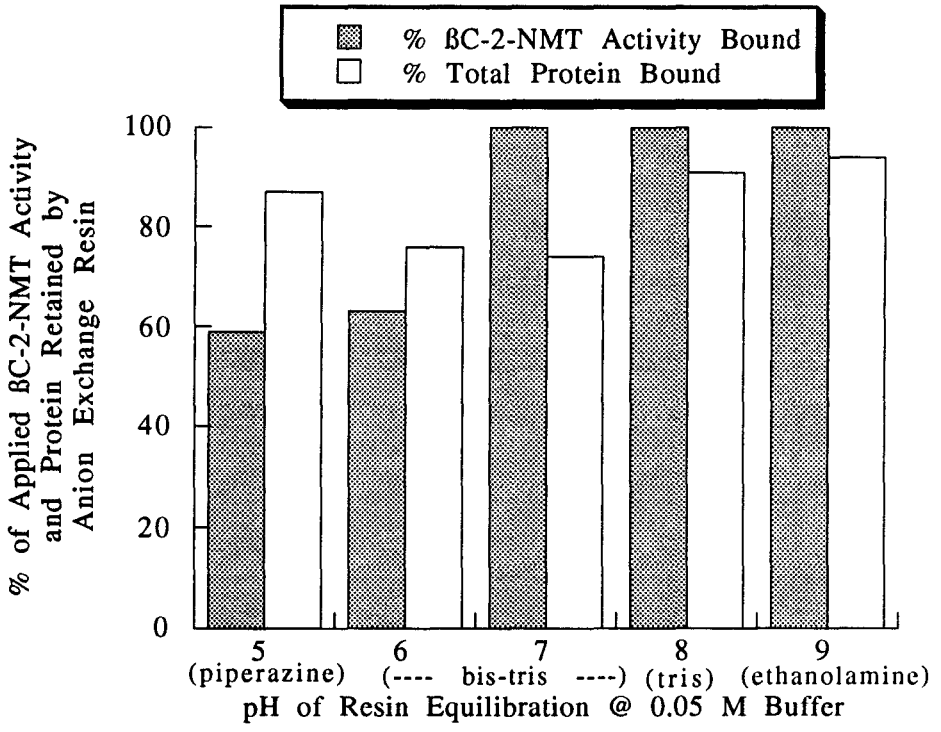


Figure 24. Binding of total protein and ßC-2-NMT to DEAE-Sephadex as a function of equilibration buffer pH.

CM-Sephadex Chromatography

As shown in Figure 25, ßC-2-NMT activity binds to the cation exchanger to a lesser degree than total protein. ßC-2-NMT binding appears to increase as a function of increasing equilibration pH. The greatest difference between percentage of ßC-2-NMT activity and total protein bound occurs at pH 5.

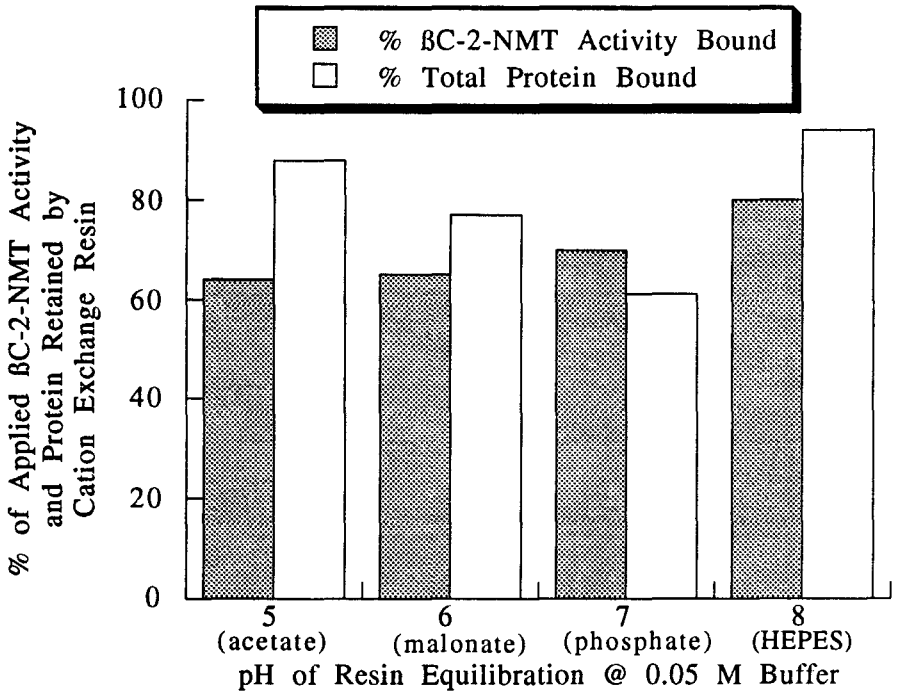


Figure 25. Binding of total protein and ßC-2-NMT to CM-Sephadex as a function of equilibration buffer pH.

Discussion

ßC-2-NMT generally sticks to both cation and anion exchange resins, suggesting that this enzyme is relatively hydrophilic and probably possesses both positively and negatively charged patches in its structure. The ideal circumstance would be chromatographic conditions in which most of the ßC-2-NMT activity is bound along with very little total protein, or conversely, a condition that binds ßC-2-NMT activity inefficiently and total protein to a high degree. In the screening experiment, at least 60% of applied total protein and ßC-2-NMT activity are bound by these resins at each pH evaluated. However, the greatest difference between ßC-2-NMT activity and total protein bound was observed at pH 7 for the anion exchanger, and at pH 5 for the cation exchange resin. Since a higher percentage of applied ßC-2-NMT activity was retained by the anion-exchange resin than by the cation-exchange resin, anion exchange chromatography at pH 7 was investigated in subsequent experiments.

Screening Experiment for Binding of ßC-2-NMT to DEAE-Sephadex in Batch Mode at pH 7 as a Function of Ionic Strength

Materials and Methods

Batch Chromatography

Bovine brain cytosol (8 ml) -- containing 20 mM Na_2HPO_4 (pH 7.4), 150 mM KCl, 1 mM DTT, 1 mM EDTA, 1 mM PMSF, leupeptin (1 µg/ml), and pepstatin A (1 µg/ml) -- was thawed from -80 °C. The aliquot had been stored at -80 °C for 1.5 months prior to use. Prior to concentration on a Centricon 30, the aliquot was filtered through a 0.22 µ polysulfone syringe filter (13 mm diameter). The filtered sample was concentrated to 25-30 µl overnight in the

coldroom. The Centricon retentate was diluted and quantitatively transferred to a clean microcentrifuge tube using 600 µl of equilibration buffer (0.05 M BIS-TRIS buffer at pH 7).

Equilibration buffers #2 -#10, of increasing ionic strength (I), were prepared as detailed in Table 11 by mixing 5.0 ml of 0.5 M BIS-TRIS buffer (pH 7) with the listed volume of NaCl stock solution (0.45 M). All solutions were brought to a final volume of 50 ml using Milli-Q water. The pH of each buffer solution was between pH 6.9-7.

Table 11.--Preparation of NaCl-BIS-TRIS Buffer Solutions for DEAE-Sephadex Batch Chromatography

Buffer #	0.45 M NaCl (ml)	I = Final Ionic Strength (M)
1	0	0.05
2	5	0.10
3	10	0.15
4	15	0.20
5	20	0.25
6	25	0.30
7	30	0.35
8	35	0.40
9	40	0.45
10	45	0.50

Initially 25 ml of DEAE-Sephadex was equilibrated in buffer #1 and then 2 ml of this slurry was transferred to each of ten 13 x 75 mm glass culture tubes. The tubes were centrifuged briefly in order to pack the gel (1 ml), and the resulting supernatant was discarded. The gel in each of the ten tubes was then equilibrated using one of the ten buffers listed in Table 11, transferred to a 1.5 ml microcentrifuge tube, centrifuged briefly, and the supernatant from gel was discarded. The appropriate buffer (80 µl) and 40 µl of concentrated cytosol were added to each of the tubes containing packed resin. The contents of the tubes were mixed on a lab rocker for 15 minutes at room temperature. The resin in each tube was packed by brief centrifugation, and then 50 µl of the resulting supernatant was transferred to a clean microcentrifuge tube. Supernatants were analyzed for ßC-2-NMT activity and total protein.

ßC-2-NMT Assay

Assay components were present as follows: 540 µM 9-MeNH (275 µl of a 727 µM stock), 150 µM SAM (20 µL of a 1.928 mM stock), 3.35 µCi ^{3}H-SAM (in 50 µL), and 25 µl of control cytosol or supernatant derived from one of ten anion-exchange chromatography conditions described above. Control cytosol was prepared by mixing 40 µl of concentrated cytosol with 80 µl of 150 mM KCl. Total assay volume was 370 µl, pH was 8.5, and assay was maintained at 37 °C for 75 minutes. Details of the ßC-2-NMT assay and subsequent analysis described in Chapter 3.

Protein Determination

Total protein was determined using a modified Lowry method as detailed in Appendix A.

Results

The sample equilibrated at 0.05 M ionic strength could not be analyzed due to a technical problem; therefore results begin with the 0.1 M condition. As Figure 26 shows, 70% of the applied ßC-2-NMT activity and 45% of total protein was retained by DEAE-Sephadex at pH 7 when the ionic strength was 0.1 M (0.05 M bis-tris buffer plus 0.05 M NaCl). ßC-2-NMT binding generally decreased as ionic strength increased (except at I = 0.25 M) up to I = 0.4 M, where ßC-2-NMT retention by the matrix appeared to decrease.

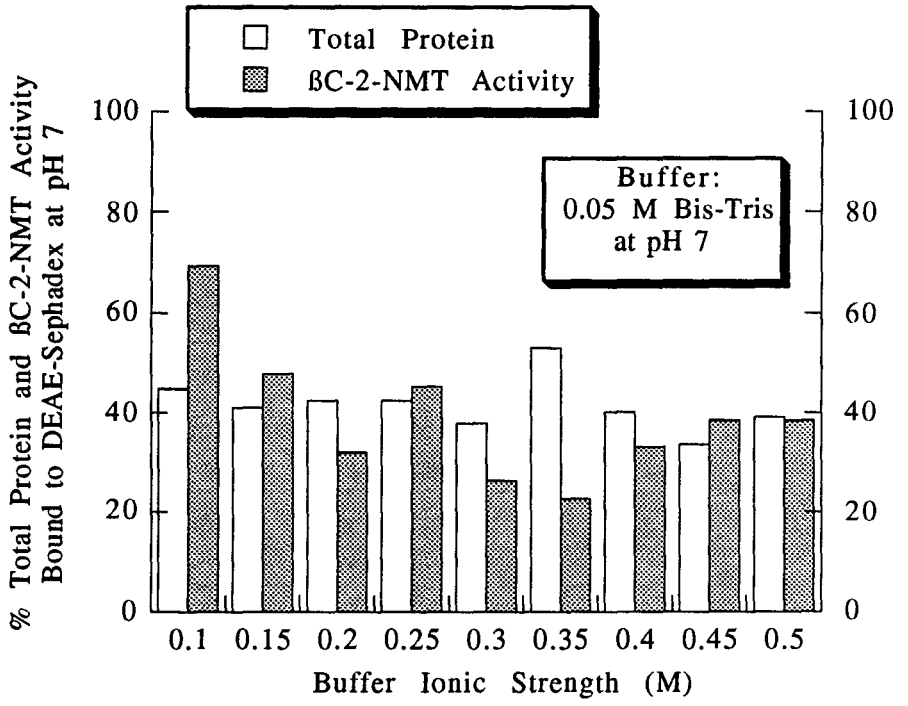


Figure 26. Binding of total protein and ßC-2-NMT activity to DEAE-Sephadex at pH 7 as a function of equilibration buffer ionic strength.

Discussion

A high salt concentration is required to disrupt the electrostatic interaction between ßC-2-NMT and the positively charged chromatography matrix. About 25% of the applied ßC-2-NMT activity remains bound to the anion-exchange chromatography matrix at I=0.3 M, this suggests that ßC-2-NMT is a relatively hydrophilic, negatively-charged protein. Increased binding of ßC-2-NMT to the matrix at I > 0.35 M may be due to a change on ßC-2-NMT structure at the higher ionic strength resulting in an exposure of ionic groups that may then interact with the matrix. The increased binding could also be the result of experimental error. Based on these results a proposed DEAE-Sephadex batch chromatography protocol might begin with equilibration of matrix and cytosol application at pH 7 in 0.05 M bis-tris buffer containing 0.05 M NaCl. The resin would then be washed with equilibration buffer to remove loosely bound proteins. Elution of ßC-2-NMT could be accomplished using 0.05 M BIS-TRIS buffer (pH 7) containing 0.25-0.30 M NaCl (total I = 0.30 - 0.35 M). This idea was evaluated in the subsequent experiment.

DEAE-Sephadex Batch Chromatography Scale-Up

Materials and Methods

Bovine brain cytosol (55 ml) in 20 mM Na_2HPO_4 (pH 7.4), 150 mM KCl, 1 mM DTT, 1 mM EDTA, 1 mM PMSF, leupeptin (1 µg/ml), and pepstatin (1 µg/ml) was used in this study; the aliquot had been stored at -80 °C for 2 months prior to thawing. An 1 ml aliquot was retained for control assays, and the remaining 54 ml was stirred magnetically in a polypropylene beaker in an ice-water

bath. A 60 - 80% ammonium sulfate pellet was obtained according to fractionation procedures previously described. The pellet was dissolved in 8 ml 0.05 M imidazole buffer at pH 7. The solution was filtered through a 0.2 µm polysulfone syringe filter prior to simultaneous ultradiafiltration and concentration on a Centriprep 30 concentrator to a final volume of 1.85 ml. An 150 µl aliquot was retained for assays, and the remaining 1.7 ml was mixed with about 20 ml of DEAE-Sephadex that had been previously equilibrated in 0.05 M imidazole buffer (pH 7). The BIS-TRIS buffer used in previous studies was not available in sufficient quantity for this study, so imidazole buffer was substituted. The mixture was agitated gently on a lab rocker for 20-30 minutes at room temperature. Resin was recovered by vacuum filtration on a Buchner funnel and the resulting filtrate saved (9.9 ml). The resin was washed while on the funnel with 5 ml of equilibration buffer; filtrate volume was 4.4 ml. The resin was transferred to a polypropylene tube containing 3 ml of 0.05 M imidazole buffer (pH 7) plus 3 M NaCl. The mixture was agitated for 30 minutes as described above. The slurry was filtered to recover eluate (7 ml). Initial cytosol and subsequent fractions were analyzed for total protein and ßC-2-NMT activity.

ßC-2-NMT Assay

Assays were done according to details presented elsewhere (Chapter 3). Duplicate assays were prepared for each fraction evaluated; assays contained 9-MeNH (540 µM), SAM (105 µM, 3.65 µCi) at pH 8.5 plus 100 µl concentrated column fraction or initial cytosol. In addition, blank assays were prepared which lacked 9-MeNH, but contained all other components.

Protein Determination

The modified Lowry method was used in the assessment of total protein in cytosol and column fractions (Appendix A).

Results

The overall yield of ßC-2-NMT activity from this procedure was poor (7%). The purification from cytosol to the final 0.3 M NaCl eluate was 14.4-fold. Results are summarized in Table 12 and include initial cytosol, desalted 60-80% $(NH4)_2SO4$ fraction ("60-80%"), and high salt eluate from the DEAE-Sephadex ("0.3 M NaCl"). The percentages in parentheses in Table 13 represent the fraction yield from initial cytosol. The purification factor is the increase in specific activity from the previous step.

Table 12.--Purification Results for Ammonium Sulfate Fractionation Followed by DEAE-Sephadex Batch Chromatography

Fraction	Protein (mg)	ßC-2-NMT (pmole/h)	Specific Activity (pmol/h/mg)	Purification Factor
Cytosol	396	10098	25.5	----
60 - 80%	28	1056 (10%)	37.7	1.5
0.3 M NaCl	1.96	721 (7.1%)	368	9.8

A 60%-80% $(NH_4)_2SO_4$ fraction prepared from bovine brain cytosol (100,000 *x g* supernatant) was dissolved in pH 7 imidazole buffer (equilibration buffer), and applied in batch-mode to DEAE-Sephadex resin. After washing the resin with equilibration buffer, ßC-2-NMT was eluted with pH 7 imidazole containing 0.3 M NaCl.

Discussion

Excessive loss in total ßC-2-NMT (90%) suggests that the 60-80% $(NH_4)_2SO_4$ fractionation-step should be excluded from subsequent studies. These results are encouraging with respect to purification using batch chromatography on DEAE-Sephadex. A nearly 10-fold purification was achieved during batch chromatography, and the yield for this step alone was reasonable at 68%. Suggestions for future studies include: DEAE-Sephadex batch chromatography under these conditions except exclude the $(NH_4)_2SO_4$ fractionation step, and to use dialysis to bring bovine brain cytosol to resin equilibration conditions (0.05 M imidazole buffer, pH 7) prior to mixing with resin.

Binding of ßC-2-NMT Activity to Dye Chromatography Columns

Background

Dye chromatography is a subset of affinity chromatography, in which polycyclic-aromatic dyes are covalently attached to a chromatography matrix such as agarose. Interaction between dye and enzyme may occur biospecifically as a result of the structural similarity between certain dyes and nucleotide cofactors required by the enzyme. Conversely, interaction between the immobilized dye and protein of interest may be nonspecific, via ionic or hydrophobic mechanisms.

Materials and Methods:
Dye Screening Experiment

Column Chromatography

Dye resin Test Kit No. RDL-9 was purchased from Sigma Chemical Company (St. Louis, MO). Column bed volumes were pre-packed at 2.5 ml; the multi-dye test kit contained nine columns with different dye-agarose substitutions. Characteristics of the nine columns supplied in the kit are listed in Table 13.

Table 13.--Components of Multi-Dye Test Kit

Catalog #	Dye Substitution	Identification
CB-5	Cibacron Blue	Cib. Blue
RG5-5	Reactive Green	Green 5
RB10-5	Reactive Brown	Brown 10
RB72-5	Reactive Blue 72	Blue 72
RB4-5	Reactive Blue 4	Blue 4
RY86-5	Reactive Yellow 86	Yellow 86
RY3-5	Reactive Yellow 3	Yellow 3
RR-120-5	Reactive Red 120	Red 120
RG-19-5	Reactive Green 19	Green 19

Each dye column was equilibrated with 5-10 column volumes of equilibration buffer in the coldroom at 0-5 °C; buffer contained 10 mM Na_2HPO_4 at pH 7.3. It was necessary that bovine cytosol be present in the same buffer prior to application to dye columns; therefore, solvent exchange on a PD10 desalting column (Sephadex G-25M, Pharmacia) was utilized to prepare cytosol in equilibration buffer.

Bovine brain cytosol in 20 mM Na_2HPO_4 (pH 7.4), 150 mM KCl, 1 mM DTT, 1 mM EDTA, 1 mM PMSF, leupeptin (1 µg/ml), and pepstatin (1 µg/ml) was used in this study; the aliquot had been stored at -80 °C for 5 months prior to use. Details of solvent exchange procedure follow: each of three Pharmacia PD10 columns was equilibrated with equilibration buffer. Cytosol (2.5 ml) was added to each desalting column, and protein was eluted using 3.5 ml of equilibration buffer. Eluates (10.5 ml total) from these columns were collected and combined; 1.5 ml was retained and represented "control cytosol".

One ml (3.2 mg protein) of "control cytosol" was applied to each of the nine equilibrated dye columns at 0-5 °C. Flow-through from each column was collected independently into 13 x 100 mm polypropylene tubes. Columns were washed with 6.0 ml of equilibration buffer; this wash was combined with the previous column flow-through (total volume 7.0 ml). A control sample was prepared by mixing 1.0 ml of "control cytosol" with 6.0 ml of equilibration buffer to yield 7.0 ml total sample volume. Samples were evaporated to dryness using a Speed Vac concentrator at ambient temperature. Each residue was reconstituted using 500 µl of Milli-Q water. The pH of each sample was in the range 7-8 as expected. These solutions were assayed for total protein and ßC-2-NMT activity as described below.

The goal of the screening experiment was to identify one "positive (+)" column and one "negative (-)" column. By definition the "positive" column binds most of the ßC-2-NMT activity and less total protein, whereas the "negative" column binds very little ßC-2-NMT activity and a relatively high quantity of total protein.

ßC-2-NMT Assay

Assay components were present as follows: 9-MeNH (540 µM), SAM (150 µM, 3.69 µCi), and 100 µl of concentrated cytosol or column fraction. Assay volume was 370 µl, pH was 8.5, and assay was maintained at 37 °C for one hour. Details of the ßC-2-NMT assay are in Chapter 3.

Protein Determination

Total protein was determined using a modified Lowry method as detailed in the Appendix A.

Results: Dye Screening Experiment

As Figure 27 shows, the "Green 19" column retained approximately 60% of the total protein applied and did not bind any detectable ßC-2-NMT activity; therefore, the "Green 19" column was defined as the "negative" column. The "Green 5" column bound all of the applied ßC-2-NMT activity and 60% of the applied protein; the "Green 5" column was therefore designated the "positive" column. "Yellow 86" and "Yellow 3" dye columns retained little if any protein or ßC-2-NMT activity.

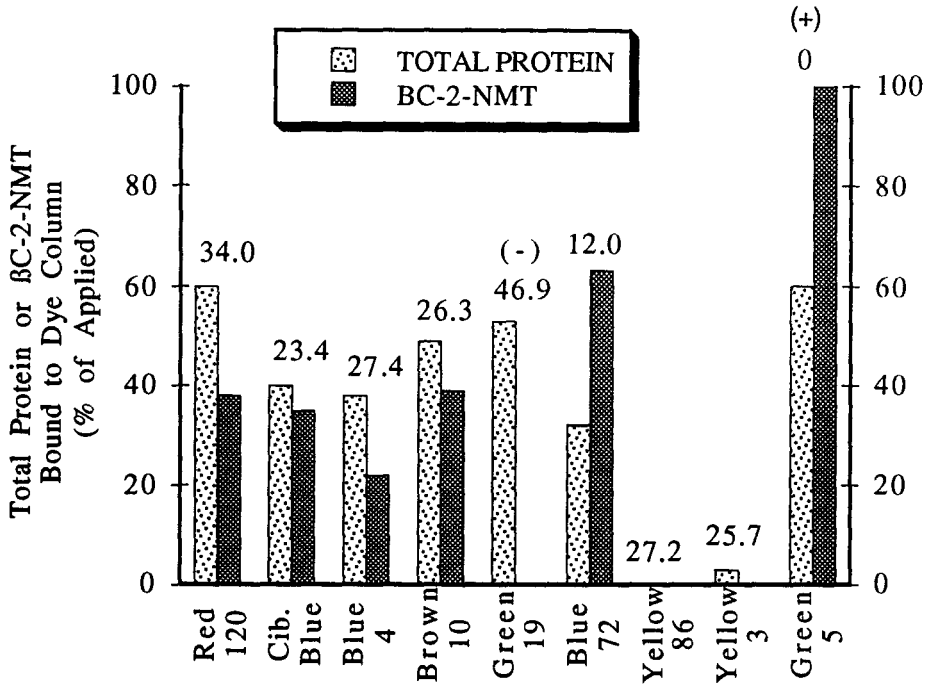


Figure 27. Total protein and ßC-2-NMT activity retained by various dye chromatography columns. Values above the bars represent ßC-2-NMT activity (pmol/h/mg protein) of column eluate. "Green 19" is designated "negative" since it does not bind ßC-2-NMT. "Green 5" is designated "positive" since it retains 100% of the applied activity.

Discussion: Dye Screening Experiment

These results suggest that the "Green 19" and "Green 5" dye chromatography columns may be used in sequence for the purification of ßC-2-NMT activity from bovine brain cytosol. Specifically, bovine brain in equilibration buffer could be applied to the "Green 19" column. The "Green 19" column flow-through combined with the column wash could subsequently be applied to a "Green 5" column. ßC-2-NMT activity could then be eluted from the "Green 5" column using a high salt buffer or other eluent. The proposed experiment is presented below.

Materials and Methods:
Chromatography on Green Dye Columns
Experiment 1

Column Chromatography

Cytosol used in this experiment contained 20 mM Na_2HPO_4 (pH 7.4), 150 mM KCl, 1 mM DTT, 1 mM EDTA, 1 mM PMSF, leupeptin (1 µg/ml), and pepstatin (1 µg/ml); the aliquot had been frozen at -80 °C for 14 months. PD10 desalting columns were utilized to bring bovine brain cytosol to equilibration buffer conditions (10 mM Na_2HPO_4 at pH 7.3). All subsequent steps were carried-out in the coldroom at 0-5 °C. "Green 19" and "Green 5" columns (2.5 ml) were equilibrated using 5 column volumes of equilibration buffer. A portion of the PD10 column eluate was reserved for control assays.

The PD10 column eluate (5.4 ml) was applied to the "Green 19 (negative)" column and the column was washed with 5 ml of equilibration buffer. Sample application flow-through and wash were combined (10.0 ml total). A 2.0 ml aliquot of this solution was concentrated using a Centricon 30 concentrator. The remaining solution (8.0 ml) was applied to the "Green 5 (positive)" column. This column was washed with 5 ml of equilibration buffer, and the wash was combined with flow-through from sample application (12.0 ml total). A 2.0 ml aliquot was concentrated completely using a Centricon 30 concentrator. ßC-2-NMT activity was eluted from the "Green 5 (positive)" column with 5 ml 10 mM Na_2HPO_4 containing 2 M NaCl (pH 7.3). Eluate was collected (5.0 ml) and a 2.0 ml aliquot was concentrated as described above. Centricon 30 concentration was achieved overnight in the coldroom. Centricon retentates (25-30 µl) were diluted in 300 ul of equilibration buffer; these concentrated solutions were used in the ßC-2-NMT and protein assays.

ßC-2-NMT Assay

Assay components were present as follows: 9-MeNH (540 µM), SAM (105 µM, 3.01 µCi), and 100 µl of concentrated cytosol or column fraction. Assay volume was 370 µl, pH was 8.5, and assay was maintained at 37 °C for one hour. Details of the ßC-2-NMT assay are in Chapter 3.

Protein Determination

Total protein was determined using modified Lowry method as detailed in the Appendix A.

Results:
Chromatography on Green Dye Columns
Experiment 1

Results are shown in Table 14.

Table 14.--Recovery of ßC-2-NMT Activity and Total Protein During Reactive Green Dye Column Chromatography

Fraction	Volume (ml)	ßC-2-NMT (pmol/h)	Protein (mg)	Specific Activity (pmol/h/mg)
Cytosol (applied to "Green 19" column)	5.4	426	13.46	31.6
"Green 19" wash plus flow-through (W/FT)	10	583	7.68	75.9
"Green 19" W/FT applied to "Green 5"	8	466	6.14	75.9
""Green 5" wash plus flow-through	12	301	4.09	73.6
"Green 5" eluate (2 M NaCL/buffer)	5	82	0.63	130

These data are summarized graphically in Figure 28. "Green 19 (negative)" column results were similar to the results obtained in the screening experiment: in the present study 43% of the total protein applied to the column was retained by the "Green 19" column, since 57% of applied protein was measured in the flow-through plus wash from this column. As predicted from the screening experiment, none of the applied ßC-2-NMT activity was bound by the "Green-19" column. However, the "Green 5 (positive)" column results differed from those in the screening experiment: in this study 67% of applied protein (compared to 40% previously) and 64% of applied ßC-2-NMT activity (0% previously) were present in the flow-through plus wash from the "Green 5" column. In other words, in this experiment only 36% of the ßC-2-NMT activity that was applied to the "Green 5" column was retained by that column, whereas in the screening experiment 100% retention was observed. Most of the ßC-2-NMT activity retained by the "Green 5" column was effectively eluted from that column by the buffered 2 M NaCl wash, while 18% of the applied activity remained bound to the "Green 5" column after 2 M NaCl elution.

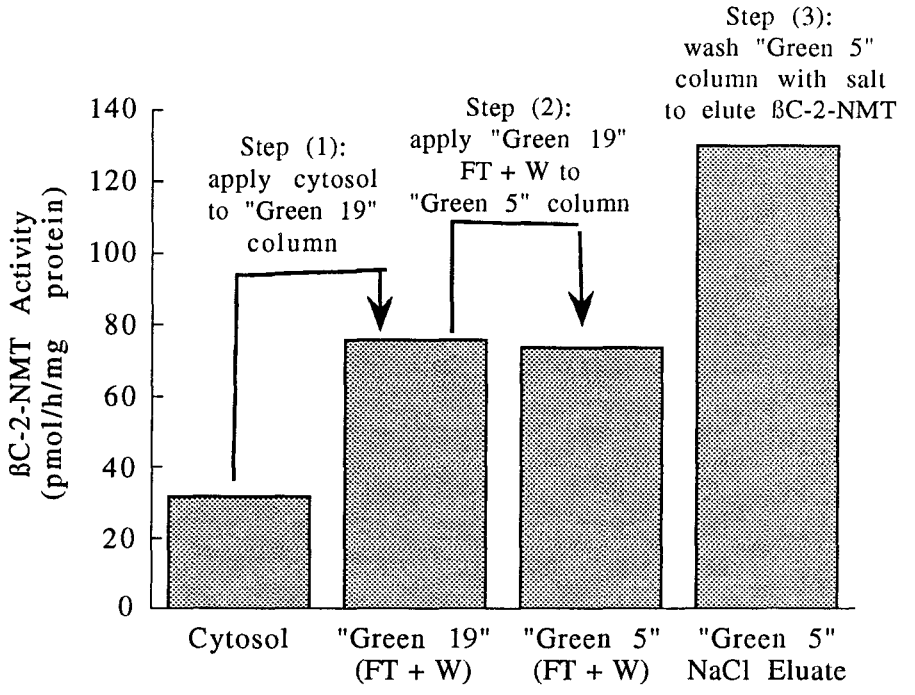


Figure 28. ßC-2-NMT activity in fractions from reactive green dye columns. Chromatographic steps: (1) Cytosol was applied to the "Green 19" column. (2) Flow-through and wash from the "Green 19" column was applied to the "Green 5" column. (3) "Green 5" column was washed with buffered 2 M NaCl to elute ßC-2-NMT activity.

Discussion:
Chromatography on Green Dye Columns
Experiment 1

The major difference in from this study compared to the screening experiment is the lack of complete retention of ßC-2-NMT by the "Green (positive)" column. There are three possible explanations for this observation: (1) the previous experiment is not reproducible, (2) the "Green 5" column capacity was exceeded in this study with respect to protein and specifically ßC-2-NMT binding, or (3) the column was contaminated from previous study and binding efficiency consequently decreased. A new "Green 5" column with an 8-fold greater bed volume will be utilized in "Experiment 2". If the steps beginning with the "Green 5" sample application are considered: 77% of the protein applied to the column is accounted for in either the "Green 5" flow-through/wash (67%) and salt elution (10%) from the column. ßC-2-NMT activity recovered in those two fractions were 82%; i.e., 64% of the ßC-2-NMT activity applied to the "Green 5" column was found in the column flow-through plus wash, while 18% of the applied activity resided in the high salt eluate from the column. This suggests that 23% of the total protein and 18% of the ßC-2-NMT activity, respectively -- actually applied to the "Green 5" column -- remained bound to that column after the high salt wash.

A modification of this experiment incorporated the use of clean Reactive Green Dye columns, each with an 8-fold greater bed volume. In addition, the final NaCl wash was increased from 2 M to 3 M NaCl. The details of that experiment are described below.

Materials and Methods:
Chromatography on Green Dye Columns
Experiment 2: Scale-Up

Column Chromatography

Reactive Green 19 on 4% beaded agarose (catalog no. R-2882) and Reactive Green 5, crosslinked on 4% beaded agarose were obtained from Sigma Chemical Company (St. Louis, MO). Slurries of each dye chromatography matrix were filled into each of two empty 12 cm x 1.5 cm EconoPrep polypropylene columns (BioRad); each bed volume was approximately 20 ml. Dye columns were equilibrated with 5-10 column volumes of equilibration buffer in the coldroom (0-5 °C); buffer contained 10 mM Na_2HPO_4 at pH 7.3. It was necessary that bovine cytosol be present in the same buffer prior to application to dye columns; therefore, solvent exchange on a PD10 desalting column (Sephadex G-25M, Pharmacia) was utilized to prepare cytosol in equilibration buffer. Bovine brain cytosol in 20 mM Na_2HPO_4 (pH 7.4), 150 mM KCl, 1 mM DTT, and 1 mM EDTA was used in this study; the aliquot had been stored at -80 °C for 1 month prior to use. The solvent exchange procedure was identical to previous experiments.

Brain cytosol (9.4 ml) was applied to the equilibrated "Green 19" dye column at 0-5 °C. The column was washed with 20 ml of equilibration buffer. Flow-through from sample application was combined with column wash (27 ml), and a 5.0 ml aliquot was concentrated to 500 µl using a Centricon 30 concentrator. The concentrate was used for total protein and ßC-2-NMT assays. The remaining unconcentrated eluate from the "Green 19" column (22 ml) was applied to the equilibrated "Green 5" column. This column was washed with 20 ml of equilibration buffer, and this wash was combined with the sample

application flow-through (41 ml total). A 5.0 ml aliquot was concentrated to 500 µl using a Centricon 30 concentrator. ßC-2-NMT activity was eluted from the "Green 5" column with 10 mM Na_2HPO_4 (pH 7.4) containing 3 M NaCl, and a 5.0 ml sample of this eluate was concentrated to 500 µl.

ßC-2-NMT Assay

Assays were done according to details presented elsewhere (Chapter 3). Duplicate assays were prepared for each fraction evaluated; assays contained 9-MeNH (540 µM), SAM (105 µM, 3.65 µCi) at pH 8.5 plus 100 µl concentrated column fraction or initial cytosol. In addition, blank assays were prepared which lacked 9-MeNH, but contained all other components.

Protein Determination

The modified Lowry method was used in the assessment of total protein in cytosol and column fractions (Appendix A).

Results:
Chromatography on Green Dye Columns
Experiment 2: Scale-Up

As summarized in Table 15, this 2-step procedure resulted in a 17-fold purification from cytosol and an overall 9% yield of ßC-2-NMT activity.

Table 15.--Purification Results: Green Dye Chromatography

Fraction	ßC-2-NMT (pmol/h)	Protein (mg)	Specific Activity (pmol/h/mg)	Purification	Yield (%)
Cytosol	846	45.3	18.7	-------	100
"Green 19"	859	11.3	76.0	4	102
"Green 5" (wash)	16	5.2	3.1	-------	----
"Green 5" (NaCl)	62	0.2	310	17	9

Bovine brain cytosol in equilibration buffer (10 mM Na_2HPO_4 at pH 7.3) was applied to a "Green 19" column. The column was washed with equilibration buffer. This wash combined with initial eluate was applied to a "Green 5" column, the column was washed. ßC-2-NMT was eluted using 10 mM Na_2HPO_4 containing 3 M NaCl.

Note that since a 5 ml aliquot was removed from the "Green 19" column flow-through, actual ßC-2-NMT activity applied to "Green 5" column was 700 pmole/h (9.2 mg protein). Very little ßC-2-NMT activity (16 pmole/h or 2% of applied) was removed from the "Green 5" column as a consequence of washing the column with equilibration buffer. In contrast, 56% (5.2 mg) of the total protein applied to the "Green 5" column was eluted from the column during

the wash step. The final wash, composed of 20 mM Na_2HPO_4 (pH 7.4) and 3 M NaCl, eluted 62 pmoles/h ßC-2-NMT activity (9% of applied) in 0.2 mg of protein. This indicates that 622 pmole/h ßC-2-NMT activity and 3.8 mg protein remained bound to the "Green 5" column.

Discussion:
Chromatography on Green Dye Columns
Experiment 2: Scale-Up

This study indicates that dye chromatography on Reactive Green 19 and Reactive Green 5 matrices may be useful in the purification of ßC-2-NMT. As expected, none of the ßC-2-NMT activity was retained by the "Green 19 (negative)" column. Total protein bound to the "Green 19" column was 34 mg, equivalent to 75% of applied protein; this value is higher than the expected 40-60%. The increased retention of total protein is likely due to the ratio of applied protein to column bed volume between studies. In this study this ratio was 2.3 (mg protein:column volume) and in the previous investigations the ratio of applied protein to column volume was greater.

The larger column volume probably accounts for the poor yield of ßC-2-NMT activity from the "Green 5" column. Improved recovery from the "Green 5 (positive)" column may be possible by using (1) a larger wash volume, (2) a "Green 5" column with a smaller bed volume, (3) an alternate eluent (e.g., dilute acetic acid, reactive green dye 5).

Hydrophobic Interaction Column Chromatography of ßC-2-NMT

Background

Hydrophobic interaction chromatography (HIC) enables the separation of molecules based on their hydrophobicity. Hydrophobic interactions between sample components and the HIC matrix are favored under conditions of high salt. Therefore, samples are usually applied to HIC columns in a high salt buffer, with the same buffer being utilized to equilibrate the column. Successive elution of bound protein is achieved using a either a step or continuous gradient of decreasing salt concentration. Collected fractions are assayed for total protein and protein of interest.

Materials and Methods: Screening Experiment at Low Ionic Strength

Column Chromatography

A "Hydrophobic Interaction Chromatography Test Kit" (kit no. MAA-8) containing eight pre-packed 2.5 ml columns was obtained form Sigma Chemical Company (St. Louis, MO). Columns were manufactured to contain hydrophobic ligands of a discrete carbon number covalently attached to agarose (agarose--$(CH_2)_nCH_3$) as indicated in Table 16.

Table 16.--Components of HIC Test Kit

Carbons (#)	Catalog #	HIC Matrix
2	E 1879	ethyl agarose
3	P 5268	propyl agarose
4	B 6882	butyl agarose
5	P 5393	pentyl agarose
6	H 1882	hexyl agarose
8	O 6376	octyl agarose
10	D 3013	decyl agarose
12	D 2264	dodecyl agarose

Columns were conditioned with 10 column volumes of equilibration buffer at room temperature and then stored in the coldroom. Equilibration buffer contained: 150 mM KCl, 20 mM Na_2HPO_4 (pH 7.4), 1 mM EDTA, 1 mM DTT, and 10% glycerol. A 50 ml aliquot of bovine brain cytosol (in equilibration buffer) was thawed from storage at -80 °C (1 month). In the coldroom, a 2.5 ml sample of cytosol was applied to each HIC column. The sample application flow-through ("S") from each column was collected into polypropylene tubes and placed on ice. Each column was subsequently washed with 3 column volumes (7.5 ml) of cold equilibration buffer. The washes ("W") were collected into polypropylene tubes and saved on ice. Finally, each column was eluted with 2.5 ml of dilute buffer containing 20 mM Na_2HPO_4 (pH 7.4), 1 mM EDTA, and 1 mM DTT. Eluates ("E") were saved as above. All fractions ("S", "W", and "E")

were analyzed for total protein content, but only "S" and "E" fractions were assayed for ßC-2-NMT activity.

ßC-2-NMT Assay

Assays were done according to details presented elsewhere (Chapter 3). Assays contained 9-MeNH (540 µM), SAM (105 µM, 3.208 µCi) at pH 8.5 plus 100 µl column fraction or original cytosol.

Protein Determination

The modified Lowry method was used in the assessment of total protein in cytosol and column fractions (Appendix A).

Results: Screening Experiment at Low Ionic Strength

Figures 29 and 30 show the percentage of applied protein or ßC-2-NMT that was not retained by each HIC column; i.e., the plot shows the percentage of applied protein or ßC-2-NMT found in the sample application flow-through ("S") or final eluate ("E") fractions for each column.

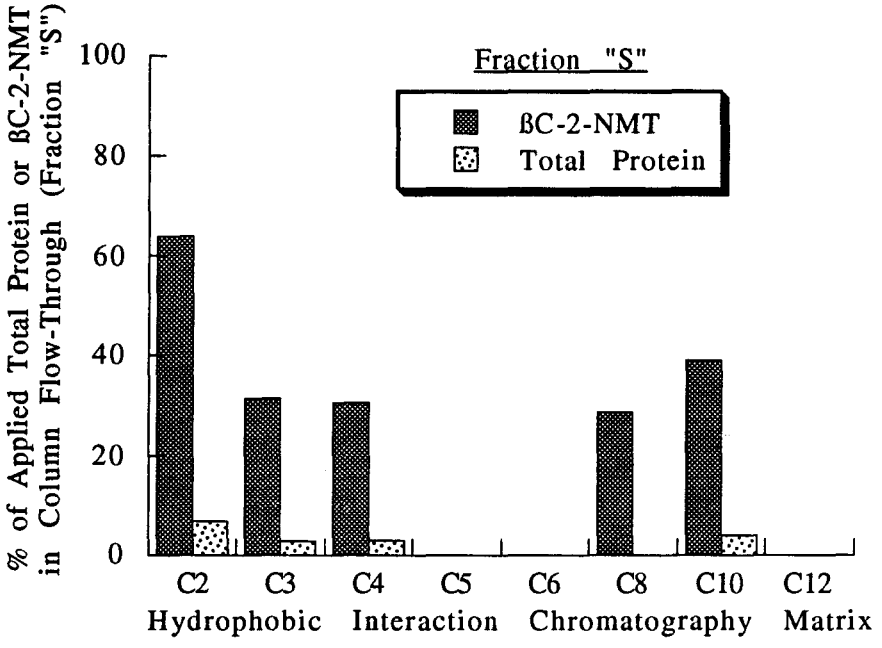


Figure 29. Total protein and ßC-2-NMT activity not retained by HIC columns during sample application under low ionic strength (I = 0.17 M) conditions.

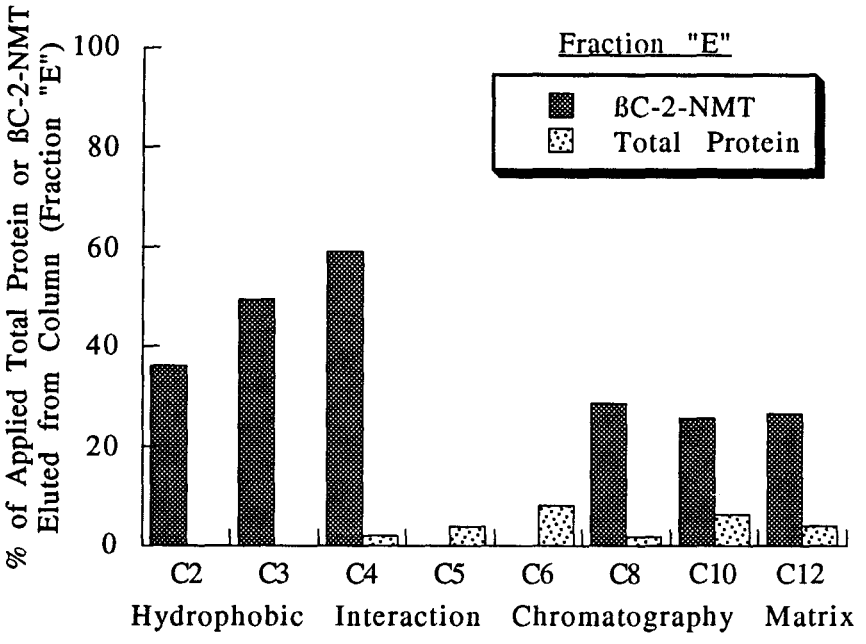


Figure 30. Total protein and ßC-2-NMT activity eluted from HIC columns using a buffer of lower ionic strength (I = 0.02 M) than application buffer.

Table 17 shows the percentage of applied total protein that was recovered in column fraction ("S", "W", and "E"), the sum total of those fractions, and the calculated amount of total protein remaining on the column.

Table 17.--Total Protein Recovered in Each Fraction From HIC Columns

	% of Applied Total Protein in Fraction				
Column	"S"	"W"	"E"	Total	On Column
C-2	7	97	0	104	0
C-3	3	110	0	113	0
C-4	3	96	2	101	0
C-5	0	0	4	4	96
C-6	0	123	8	131	0
C-8	0	6	2	8	92
C-10	4	8	6	18	82
C-12	0	3	4	7	93

Bovine brain cytosol in 150 mM KCl, 20 mM Na_2HPO_4 (pH 7.4), 1 mM EDTA, 1 mM DTT, and 10% glycerol (equilibration buffer) was applied to each column, and the column flow-through collected ("S"). Columns were washed with equilibration buffer ("W"). Final eluate ("E") consisted of equilibration buffer lacking 150 mM KCl.

Discussion: Screening Experiment at Low Ionic Strength

This experiment would have been more meaningful and complete if ßC-2-NMT activity had also been measured in the wash ("W") fraction, since without those values it is impossible to conclude if this activity was in the wash fraction or was retained by the column after all treatments. As Table 17 indicates much of the total protein was removed from the shorter-carbon chain HIC columns by the initial wash, whereas the longer-chain HIC columns retained total protein quite efficiently. It is possible that substantial ßC-2-NMT activity may have been removed from the columns during the wash. The cytosolic samples were applied under relatively low ionic strength conditions because doing so would allow for cytosol to be used directly after subcellular fractionation, without the need for solvent exchange, thereby eliminating one step. The results with this approach were discouraging since much of the ßC-2-NMT activity did not bind to the HIC columns under these conditions, and that loosely bound protein (and possibly ßC-2-NMT) was removed from several of the columns during the wash step. Therefore, the experiment was repeated under conditions which favor hydrophobic interactions, namely sample application under high ionic strength conditions.

Materials and Methods: Screening Experiment at High Ionic Strength

Column Chromatography

HIC columns were equilibrated in the coldroom with buffer composed of 2 M $(NH_4)_2SO_4$, 150 mM KCl, and 20 mM Na_2HPO_4 (pH 7.3). An aliquot of bovine brain cytosol (30 ml, 89 mg protein, 2296 pmol/h ßC-2-NMT) in 150 mM KCl, 20

mM Na_2HPO_4 (pH 7.4), 1 mM EDTA, 1 mM DTT, and 10 % (v/v) glycerol was thawed from -80 °C; the aliquot had been frozen for 2 months. The cytosol was stirred magnetically and maintained in an ice-water bath while solid ammonium sulfate was added to the cytosol to make it 2 M with respect to $(NH_4)_2SO_4$ -- equivalent to 45% saturation with respect to $(NH_4)_2SO_4$. Subsequent steps were conducted as described previously in this chapter. The supernatant resulting from the 0-45% $(NH_4)_2SO_4$ fraction was applied to equilibrated HIC columns (2 ml per column), and sample application flow-through was collected into polypropylene tubes on ice. Columns were then washed with 6 ml of equilibration buffer. This wash was combined with sample application flow-through (8 ml total). A "control" sample was prepared by mixing 2 ml of the 0-40% $(NH_4)_2SO_4$ supernatant with 6 ml of equilibration buffer. These solutions were concentrated completely on Centricon 30 overnight. Control solution did not concentrate completely (volume = 750 µl); therefore 750 µl of 10 mM Na_2HPO_4 (pH 7.4) was added to the other Centricon retentates. The concentrated samples were utilized in total protein and ßC-2-NMT assays.

ßC-2-NMT Assay

Assays were done according to details presented previously (Chapter 3). Assays contained 9-MeNH (540 µM), SAM (105 µM, 3.325 µCi) at pH 8.5 plus 100 µl concentrated column fraction or concentrated control cytosol.

Protein Determination

The modified Lowry method was used in the assessment of total protein in cytosol and column fractions (Appendix A).

Results: Screening Experiment at High Ionic Strength

Figure 31 reflects the quantity of total protein and ßC-2-NMT activity, represented as percent of applied, present in the combined sample application flow-through plus wash fractions -- that is total protein and ßC-2-NMT not bound by the HIC columns.

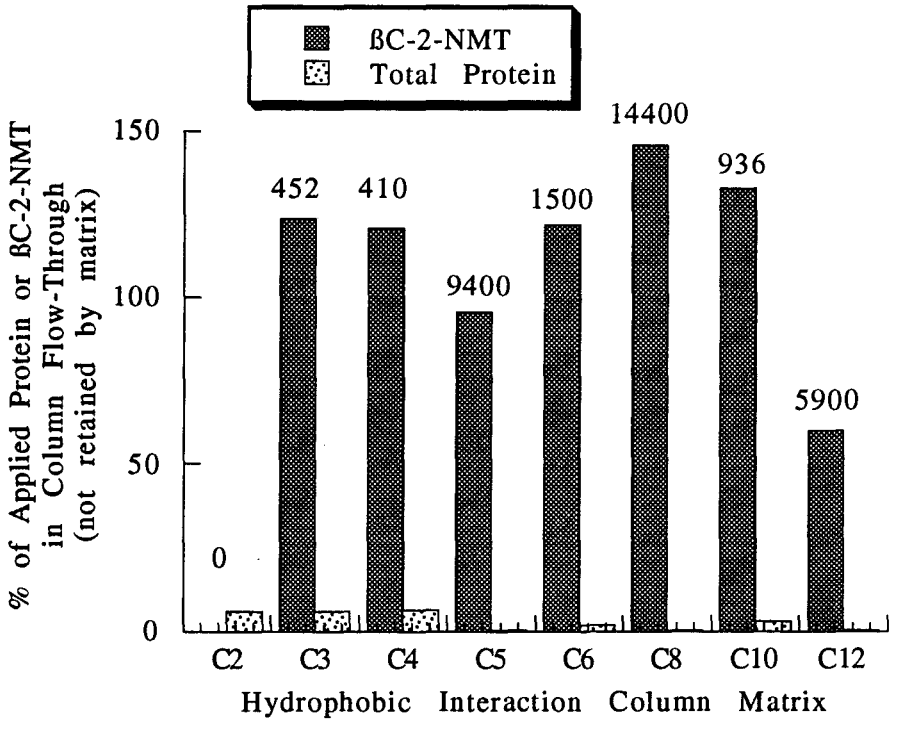


Figure 31. Total protein and ßC-2-NMT activity not bound by HIC columns at high ionic strength conditions. Values above bars represent ßC-2-NMT activity in a given fraction.

Discussion: Screening Experiment at High Ionic Strength

Since the control solution did not concentrate completely (750 µl remained), 750 µl of 10 mM Na_2HPO_4 (pH 7.4) was added to the other retentates. The control should have been concentrated completely like the other samples, because the control was really no longer a true control since it contained a high concentration of ammonium sulfate compared to other samples. As a consequence, "control ßC-2-NMT activity" was low, due to presence of the high salt concentration. Therefore, based on previous experience, an assumption was made that about 50% of original cytosolic activity was recovered in the 0-46% $(NH_4)_2SO_4$ supernatant (31 ml), giving 1150 pmol/h ßC-2-NMT (calculated) and 52.4 mg protein (measured). This is equivalent to 3.4 mg protein and 74 pmole/hr ßC-2-NMT activity applied to each column (per 2 ml). These values were used to calculate the percentage of applied protein or ßC-2-NMT residing in respective column fractions.

Data indicate that total protein was more effectively retained by the HIC matrices than ßC-2-NMT in nearly all cases. At high salt conditions, greater than 100% of the applied ßC-2-NMT activity did not stick to the C3, C4, C6, C8, and C10 columns, suggesting that ßC-2-NMT is not a very hydrophobic protein. The observation that more than 100% recovery was obtained in these fractions suggests that: the assumptions in the preceding paragraph are incorrect (i.e., applied activity was higher than calculated) or that an inhibitor of ßC-2-NMT was retained by these columns. No blank ßC-2-NMT assays were analyzed in this study, therefore it is also possible that the reported ßC-2-NMT activity is inflated due to a high amount of nonspecific background. Furthermore it is possible that column capacity for ßC-2-NMT may have been exceed under these

conditions. The next section describes experiments in which blank assays were prepared, and chromatography repeated under these conditions using C5 and C8 columns with larger bed volumes.

Materials and Methods: C5 and C8 HIC Column Chromatography Scale-Up

Column Chromatography

Pentyl-agarose (catalog #P-5393) and octyl-agarose (catalog #O-6376) HIC matrices were obtained from Sigma and filled into each of two 20 ml polypropylene EconoPrep columns (BioRad). Columns were equilibrated in the coldroom with buffer containing: 2 M $(NH_4)_2SO_4$, 150 mM KCl, 20 mM Na_2HPO_4, 1 mM EDTA, and 1 mM DTT.

A 35 ml aliquot of bovine brain cytosol in 150 M KCl, 20 mM Na_2HPO_4, 1 mM EDTA, and 1 mM DTT was thawed from -80 °C (stored 1 month). The aliquot was stirred and chilled in an ice-water bath during the addition of 9.178 g of solid $(NH_4)_2SO_4$ (equivalent to 45% saturation). The pH of the mixture was adjusted to pH 7.3. Ammonium sulfate precipitation was accomplished as previously described in this chapter. A two ml aliquot of the supernatant (38 ml total) was ultradialyzed and concentrated on a Centricon 30 (final volume = 300 µl).

The remaining 0-45% $(NH_4)_2SO_4$ supernatant was applied to each of the two HIC columns (18 ml per column). Sample application flow-through was collected and saved on ice in a 150 ml polypropylene container with lid. Each column was washed with 40 ml (2 column volumes) of equilibration buffer, and this wash was combined with the original column fraction (total volume =

55 ml). A 4 ml aliquot of each column fraction was ultradialyzed and concentrated on a Centricon 30 concentrator. Final volume of each concentrate was 350 µl in 10 mM Na_2HPO_4 buffer at pH 7.3. Total protein and ßC-2-NMT activity were measured in each concentrated column fraction, desalted 0-45% $(NH_4)_2SO_4$ supernatant, and original cytosol.

ßC-2-NMT Assay

Assays were carried out according to details presented previously (Chapter 3). Assays were prepared in duplicate to contain 9-MeNH (540 µM), SAM (105 µM, 2.445 µCi) at pH 8.5 plus 100 µl concentrated column fraction, desalted/concentrated $(NH_4)_2SO_4$ fraction, or initial cytosol.

Protein Determination

The modified Lowry method was used in the assessment of total protein in cytosol and column fractions (Appendix A).

Results:
C5 and C8 HIC Column Chromatography Scale-Up

These results are inconsistent with those in the previous experiment where a tremendous increase in specific activity was observed with HIC. Purification from cytosol through HIC column eluate was 4.4-fold for the C5 column eluate, and 5.2-fold C8 column eluates. ßC-2-NMT and total protein present at each step of purification is summarized in Table 18. Fractions include initial cytosol, the supernatant resulting from the 0-45% $(NH_4)_2SO_4$ "precipitation step ("0-45%), the material applied to each HIC column, and the

quantity of protein and ßC-2-NMT measured in the column flow-through plus wash ("FT/W") fractions from each of the HIC columns ("C5" and "C8).

Table 18.--Hydrophobic Interaction Chromatography Purification Results

Fraction	Protein (mg)	ßC-2-NMT (pmol/h)	Specific Activity (pmol/h/mg)
Cytosol	331.45	9800	29.6
0 - 45%	86.42	4236	50.1
HIC applied	40.93	2049	50.1
C5 (FT/W)	2.743	355	129.4
C8 (FT/W)	1.829	281	153.7

Supernatant ("0-45%") obtained following 0-45% $(NH_4)_2SO_4$ fractionation of bovine brain cytosol was divided in half and applied to equilibrated pentyl (C5) and octyl (C8) columns ("HIC applied"). Composition of equilibration buffer was 2 M $(NH_4)_2SO_4$, 150 mM KCl, 20 mM Na_2HPO_4, 1 mM EDTA, and 1 mM DTT. The column flow-through and subsequent wash were combined and analyzed ("C5 FT/W" and "C8 FT/W").

As expected, ammonium sulfate precipitation resulted in a 1.7-fold purification of ßC-2-NMT activity from bovine brain cytosol; yield of enzyme activity was 44%. For the "C5" column, approximately 83% of the applied ßC-2-NMT activity, and 93% of the total protein remained in association with the column following the wash-step. For the C8 column 86% of the applied ßC-2-NMT, and 96% of applied total protein remained bound to the column.

Discussion: C5 and C8 HIC Column Chromatography Scale-Up

The enhanced binding of ßC-2-NMT activity relative to total protein may be due to the increase in bed volume utilized in this study compared to the previous experiment. Based on this experiment, which incorporated better controls, it is concluded that the previous experimental results which showed a tremendous increase in ßC-2-NMT using this protocol were in error.

Preparation and Evaluation of S-Adenosyl-L-Homocysteine-Sepharose Affinity Chromatography Column

Materials and Methods: SAH-CH-Sepharose Affinity Chromatography

Coupling Reaction

The goal was to prepare an affinity chromatography matrix in which SAH was covalently attached to CH-Sepharose 4B. This was accomplished at acidic conditions by reacting CH-Sepharose-4B with SAH in the presence of a carbodiimide (Figure 32).

SAH-CH-Sepharose column was prepared according to Harvima et al. (1985) and Pharmacia technical literature (Pharmacia 1993a). The primary reactants were purchased from Sigma Chemical Company (St. Louis, MO):

Reactant	FWT	Catalog #
S-adenosyl-L-homocysteine	384.4	A-9384
1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (HCl salt)	191.7	E-1769
6-Aminohexanoic acid-Sepharose 4B (CH-Sepharose 4B)	-----	A-8769

The supplier provided the following information regarding the CH-Sepharose 4B chromatography matrix:

Matrix: Sepharose 4B

Activation: cyanogen bromide

Spacer: provides an 8-atom spacer when ligands are coupled to free carboxy groups using a carbodiimide coupling agent

Extent of Activation: 10-14 µmoles per ml of gel (40-56 µmoles/g dry)

Form: lyophilized powder stabilized with lactose and dextran

Swelling Ratio: 1 g swells to approximately 4 ml of gel

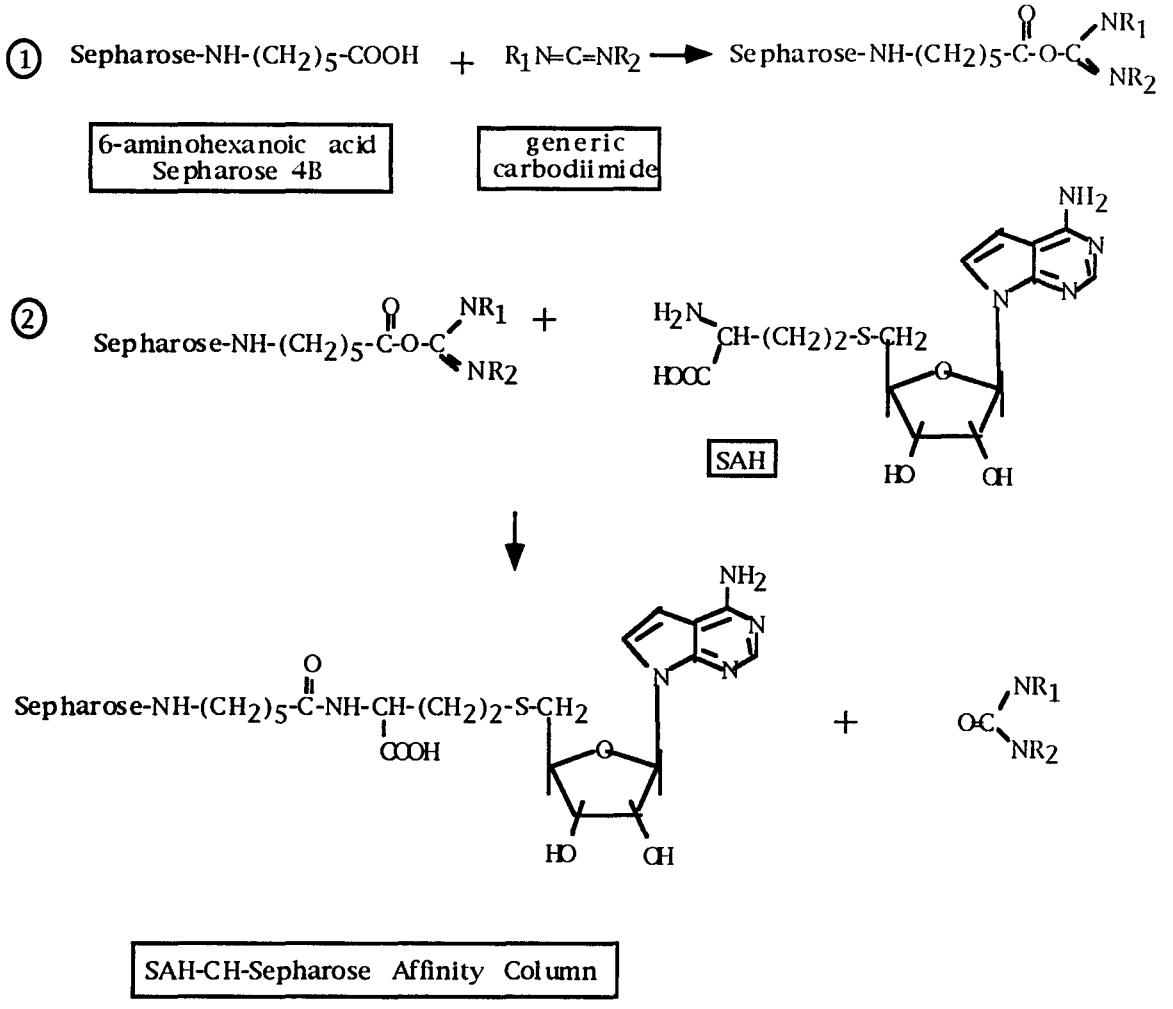


Figure 32. Preparation of S-adenosyl-L-homocysteine (SAH) Sepharose affinity chromatography column.

The CH-Sepharose 4B (2 g, 80-112 μmoles active groups) was swollen in 20 ml of 0.5 M NaCl, and then washed on a scintered glass funnel with 500 ml 0.5 M NaCl followed by 100 ml Milli-Q water at pH 6. SAH (100 mg, 260 μmoles) was dissolved in 15 ml of Milli-Q water at pH 4.6-6. The SAH solution was added to the CH-Sepharose 4B with minimal stirring on a magnetic stir plate. The carbodiimide (235 mg, 1.23 mmoles) was added as a solid in small amounts to the stirred SAH and CH-Sepharose mixture. The mixture was transferred to a tube, capped, and mixed by rocking on a lab rocker plate. The pH of the coupling reaction was checked during the first hour to assure that it remained between pH 4.6 and pH 6. The reaction was rocked at room temperature overnight (15-20 hours). No blocking reaction was done, and the affinity gel was washed extensively with Milli-Q water to remove urea product, unreacted SAH, and carbodiimide. The gel was stored at 0-5 °C in 2 M KCl and 10 mM K_2HPO_4 (pH 6.5). The extent of coupling of SAH to CH-Sepharose 4B was not determined.

Column Chromatography

Approximately 1 ml of the SAH-CH-Sepharose 4B affinity gel was filled into a polypropylene 1 cc tuberculin syringe fitted with a stopcock and silanized glass wool plug at the outlet. The column was equilibrated with 10 ml of 0.05 M imidazole buffer (pH 7) containing 0.3 M NaCl. The sample (3.5 ml) that was applied to the affinity column was the 0.05 M imidazole buffer (pH 7)/ 0.3 M NaCl eluate derived from DEAE-Sephadex batch chromatography protocol described previously in this chapter. The sample application flow-through was collected, and a 1.5 ml portion concentrated to 150 μl on a Centricon 30 concentrator. The column was washed with 2 ml of 0.05 M imidazole buffer (pH 7) containing 1 M NaCl. This wash was collected and a 1.5 ml aliquot

concentrated to 150 µl. The concentrated solutions were analyzed for ßC-2-NMT activity and total protein.

ßC-2-NMT Assay

Details of the assay, work-up, and RP-HPLC analysis are discussed in Chapter 3. Assays (pH 8.5) were maintained at 37 °C for one hour. The reactions consisted of 9-MeNH (540 µM), SAM (105 µM, 3.30 µCi), and 100 µl of respective concentrated column fraction.

Protein Determination

Protein measurements were carried-out using a modified Lowry method as detailed in Appendix A.

Results: SAH-CH-Sepharose 4B Affinity Chromatography

Total protein and ßC-2-NMT activity recovered in each fraction are summarized in Table 19.

Table 19.--SAH-CH-Sepharose Affinity Chromatography

Fraction	Protein (mg)	ßC-2-NMT (pmol/h)	Specific Activity (pmol/h/mg)
Applied	0.756	102	135
Flow-through	0.525	12.3	23.4
1 M NaCl wash (buffered)	0.166	32.2	194

SAH column equilibrated in 0.05 M imidazole buffer containing 0.3 M NaCl. Sample "Applied" to column was the 0.3 M NaCl eluate from DEAE-Sephadex chromatography discussed in a previous section of this chapter. Column "Flow-through" was collected, and then ßC-2-NMT was eluted from the column with 0.05 M imidazole buffer containing 1 M NaCl ("1 M NaCl wash").

Regarding total protein: 91% of the applied protein was accounted for in the initial flow-through fraction (0.525 mg, 69%), and following the buffered 1 M salt wash (0.166 mg, 22%). These results indicate that 0.065 mg (9%) of the total protein applied to the column remained bound to the SAH-CH-Sepharose 4B affinity matrix.

In contrast, 55% (57 pmol/h) of the applied ßC-2-NMT activity remained in association with the SAH affinity column, since 12% (12.3 pmol/h), and 32% (32.2 pmol/h) of the applied activity was recovered in the flow-through and wash fractions, respectively.

Discussion: SAH-CH-Sepharose 4B Affinity Chromatography

These results are discouraging since a mere 1.4-fold purification was achieved with respect to ßC-2-NMT activity, beginning with application to the SAH column (specific activity, 135 pmol/h/mg protein) and ending with the buffered 1 M NaCl elution (specific activity, 194 pmol/h/mg protein). Typically purification by ligand-specific affinity methods is much greater. The final eluate did not remove all of the remaining ßC-2-NMT activity. In fact, 57 pmol/h ßC-2-NMT activity and 0.068 mg of total protein remained bound to the affinity column. This is equivalent to 838 pmol/h/mg protein ßC-2-NMT specific activity still retained by the SAH-CH-Sepharose column.

These results indicate that a more efficient means of eluting ßC-2-NMT activity from the SAH-CH-Sepharose 4B affinity column is required, such as dilute acetic acid, or affinity elution with SAH or SAM. Due to the high cost of SAH and SAM, affinity elution would be quite expensive compared to elution with acid. In addition, since ßC-2-NMT exhibits a higher affinity for SAH (IC_{50} = 14 µM) than SAM (K_M = 85 µM), it may be impractical to elute ßC-2-NMT activity from the SAH column using affinity elution with SAM.

Regarding the preparation and use of SAH-CH-Sepharose affinity columns in future studies, the following suggestions are made:

(1) preparation of a new affinity matrix, since literature indicates that efficiency of an SAH-column decreases as a function of storage time (Harvima et al. 1985)

(2) utilization of more SAH in the coupling reaction

(3) evaluation of the efficiency of coupling using the method described by Harvima et al. (1985), in which a small aliquot of

coupled gel was hydrolyzed and the liberated SAH analyzed using HPLC

(4) "affinity" elution using an adenosine solution.

Preparation and Evaluation of ß-Carboline-Sepharose Affinity Chromatography Column

Materials and Methods: Experiment 1

Coupling Reaction

The goal was to prepare an affinity chromatography matrix in which a ßC was covalently attached to Sepharose 6B. This was done by reacting harmol with epoxy-activated Sepharose 6B (EAS) under basic conditions (Figure 33). The methods were similar to those described by Hermanson et al. (1992) and in the Pharmacia technical literature (Pharmacia 1993a).

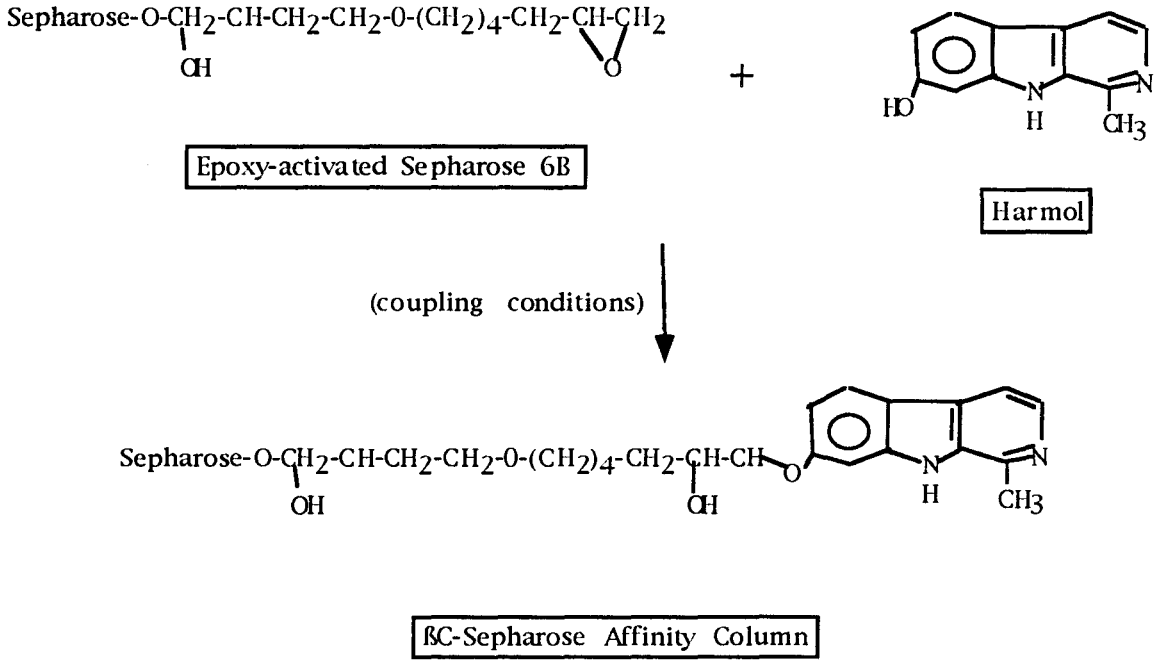


Figure 33. Scheme for preparation of ßC-affinity chromatography column.

Epoxy-activated Sepharose 6B was purchased as a lyophilized powder from Sigma Chemical Company, St. Louis, MO (Catalog # E-6754). The following information was provided by the manufacturer:

Activated group: Sepharose-(1,4-bis(2:3-epoxypropoxy)butane

Extent of activation: 19 - 40 μmole per ml of gel

Spacer-arm length: 12 atoms

Swelling properties: 1 g swells to 3 ml of gel

Harmol (1-methyl-9H-pyrido[3,4-b]indol-7-ol) hydrochloride dihydrate was purchased from Sigma (FWT 270.7, Catalog #H-1000).

Sodium carbonate buffer (100 ml, 0.1 M, pH 10) was prepared by dissolving 1.06 g of sodium carbonate (Na_2CO_3, FWT 105.99, Sigma Chemical Co.) in 80 ml of Milli-Q water. The solution pH was adjusted to pH 10 and the solution brought to 100 ml using Milli-Q water.

Lyophilized epoxy-activated-Sepharose 6B (1 g) was suspended in 10 ml of Milli-Q water and then transferred to a scintered glass funnel. The gel was washed with 200 ml of Milli-Q water, exercising care to keep the gel moist. The rinsed gel (3 ml) was then washed with 20 ml of Na_2CO_3 buffer and subsequently transferred as a slurry (5 - 6 ml) to a small amber vial.

Due to the limited solubility of ßCs under alkaline conditions, harmol was dissolved in dimethylformamide (DMF) prior to addition to Na_2CO_3 buffer. Harmol (162 mg, 600 μmoles) was dissolved in 2.5 ml of DMF followed by the slow addition of Na_2CO_3 buffer (2.5 ml). The pH of the mixture (pH 7-8) was adjusted to pH 10 using 8 N and 1 N sodium hydroxide. Harmol (600 μmole) was present in a 5-fold excess relative to the maximum number of epoxy-activated sites (120 μmole on 3 ml of gel).

Harmol solution was combined with the EAS gel slurry in the amber vial and capped. The vial contents (10-12 ml total) were maintained for 18 hours at 40 °C in a shaking water bath. The gel settled to the bottom of the vial during this procedure. Ideally the gel should have been maintained as a suspension in order to improve the efficiency of the coupling reaction.

Determination of Coupling Efficiency

The maximum theoretical quantity of harmol that could be covalently attached via the epoxy-activated groups was 40 µmoles of harmol per ml of gel (40 nmoles harmol per µl gel).

A 100 ul aliquot of the coupled gel slurry was transferred to a 1.5 ml microcentrifuge tube, and then washed repeatedly with Milli-Q water to remove free harmol. The gel was washed with 0.1 M HCl, Milli-Q water, and finally 10 mM Na_2HPO_4 (pH 7.4). The rinsed gel (30 µl of settled gel) was suspended in 970 µL 10 mM Na_2HPO_4 (pH 7.4). Assuming 100% efficiency of the coupling reaction, maximally 1200 nmole (1.2 µmoles) of harmol would be covalently bound to the 30 µl of gel. Therefore a standard solution was prepared to contain 1.2 µmole/ml of harmol in 10 mM Na_2HPO_4 (pH 7.4). This standard was serially diluted with buffer to yield standards ranging from 0.00012 - 0.12 µmole/ml harmol. The wavelength of maximal absorbance of the harmol solution was determined by scanning from 220-500 nm using the Gilford Response spectrophotometer. This wavelength was used to measure the absorbance for each standard, and linear curve-fitting of the data was used to define the equation for the standard curve. Samples were also analyzed at this wavelength in small volume quartz cuvettes with a 1 cm path, where buffer

was used as in the reference cuvette. Absorbance of the coupled gel was determined spectrophotometrically, and coupling efficiency subsequently calculated. Coupling efficiency appeared to be low, and therefore in an effort to improve coupling, the reaction pH was increased from 10.2 to pH 12.7 by the addition of 8 N NaOH. The coupling reaction was mixed at room temperature on a rocker plate until absorbance values for samples of the mixture reached a plateau and then began to decline.

Blocking of Unreacted Activated Sites

The coupled gel was transferred to a scintered glass funnel and washed extensively with Milli-Q water (200 ml) in order to remove free harmol. In addition, the gel was washed with 50 ml each 0.5 N HCl and Milli-Q water. Blocking of any remaining epoxy-activated sites was accomplished by shaking the gel at 40 °C for 14 hours with 20 ml 1 M ethanolamine (pH 9.4). The gel was washed with 100 ml each Milli-Q water and 150 mM KCl. Gel was stored in the refrigerator in 150 mM KCl until it was filled into a column (2-3 days).

ßC-Sepharose Column Chromatography

The ßC-Sepharose affinity matrix was filled into an empty PolyPrep chromatography column (BioRad); this polypropylene column had a 10 ml reservoir on top of a bed volume of about 2.5 ml (0.8 cm x 4 cm bed). The column was equilibrated with 50 ml of 10 mM Na_2HPO_4 containing 1 M NaCl (pH 7.4).

A 2 ml aliquot of bovine brain cytosol was thawed and ultradialyzed on a Centricon 30 concentrator against equilibration buffer. The cytosol had been stored at -80 °C for 1 month in 150 mM KCl, 1 mM DTT, and 10% (w/v) glycerol.

Ultradialyzed cytosol (2.5 ml) was applied to the equilibrated ßC-Sepharose column by gravity flow. The column flow-through was collected and a 1.0 ml aliquot concentrated to 300 µl on a Centricon 30 concentrator. The column was washed (with the add of a vacuum) using 25 ml of equilibration buffer. The wash was collected and a 2.0 ml sample concentrated to 300 µl. ßC-2-NMT elution was attempted using 2 ml of 10 mM Na_2HPO_4 buffer (pH 7.4) containing 2 M NaCl and 3 M NaCl in succession. Aliquots (2.0 ml) of these eluates were concentrated to 300 µl. Total protein and ßC-2-NMT activity were measured in each column fraction.

ßC-2-NMT Assay

Details of the assay, work-up, and RP-HPLC analysis are discussed in Chapter 3. Assays, at pH 8.5, were maintained at 37 °C for 45 minutes; reactions consisted of 9-MeNH (540 µM), SAM (105 µM, 2.194 µCi), and 100 µl of the concentrated column fractions.

Protein Determination

Protein measurements were carried-out using a modified Lowry method as detailed in Appendix A.

Results: ßC Affinity Chromatography
Experiment 2

Coupling Procedure

The coupled and rinsed gel was yellow under alkaline conditions and colorless in an acidic environment. This suggests that at least some ßC is bound to the gel, since free harmol exhibits similar properties.

The harmol standards exhibited an intense absorbance maxima at 320.5 nm ($A_{320.5}$). Therefore, this wavelength was utilized to determine the concentration of harmol present in the standards and samples. The harmol standard curve is represented in Figure 34.

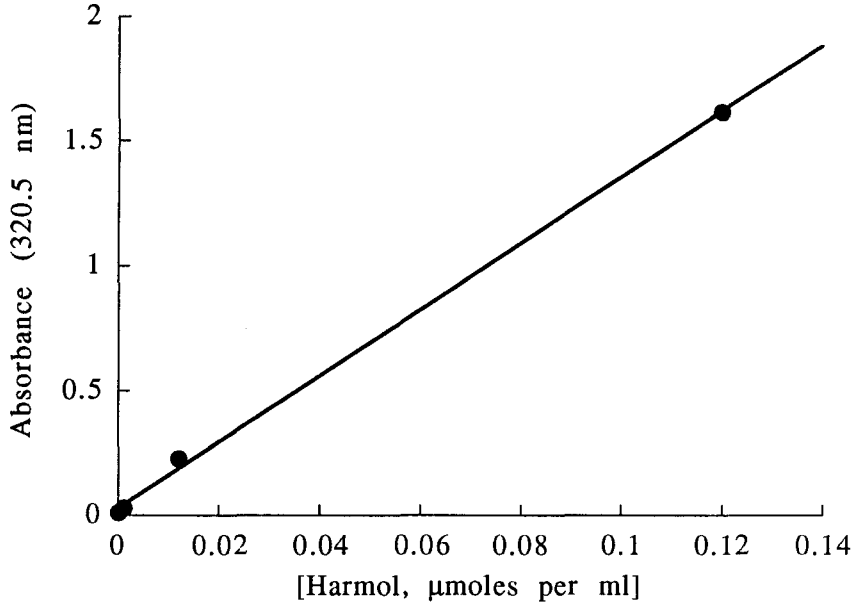


Figure 34. Standard curve for harmol in 10 mM Na_2HPO_4 (pH 7.4).

Analysis of these data resulted in the following equation:

$$A_{320.5} = 0.03 + 13.251(\ \mu moles/ml\ \ harmol).$$

The 30 µl sample of coupled gel (in 1 ml total volume) exhibited an $A_{320.5}$ of 0.2107, corresponding to 0.013 µmoles harmol per 30 µl gel. Therefore, the quantity of harmol coupled per ml of gel was 0.45 µmoles. This value represented only 1-2% of theoretical quantity. The reaction pH was increased from pH 10.2 to pH 12.7 in an effort to improve coupling efficiency. After mixing for one hour at room temperature a 30 µl aliquot was analyzed for harmol bound to gel as described above. For this sample, $A_{320.5}$ = 0.6802, which corresponds to 2.33 µmoles harmol per ml gel. This value represents 6-12% of theoretical value of 19-40 µmoles/ml. The reaction was allowed to proceed 20 hours at room temperature. Analysis showed a decrease in $A_{320.5}$ to 0.3956 (i.e., 0.92 µmoles harmol/ml gel), therefore coupling reaction was terminated and the blocking reaction was initiated.

ßC-Sepharose Column Chromatography

The results of this experiment were very difficult to interpret because the HPLC traces appeared similar--much like background, with no discernable peaks. Approximately 10.6 mg of total protein was applied to the column; 9.9 mg (93%) of that was retained by the column initially and subsequent washing removed 4.2 mg protein (40% of applied). This indicates that 5.7 mg of total protein (54% of applied) remained bound to the ßC-Sepharose column after washing with equilibration buffer. No additional protein was eluted by subsequent buffer solutions containing 2 M or 3 M NaCl .

Discussion: ßC-Sepharose Affinity Chromatography Experiment 1

It is possible that the ßC-2-NMT activity was removed from the column during the first wash step, but that its presence there was undetectable due to dilute nature of the wash. It is more reasonable to speculate that the ßC-2-NMT activity remained bound to the ßC-Sepharose column, since 54% of the protein applied remained in association with the column following extensive washing. It is conceivable that the high salt buffers used in this experiment -- a condition that favors hydrophobic interaction -- may have actually strengthened the interaction between the hydrophobic ßC (attached to the matrix) and protein or ßC-2-NMT -- rather than lessened the interaction as planned. Follow-up experiments were carried out using buffers of lower ionic strength in order to address this concern.

Materials and Methods: ßC-Sepharose Affinity Chromatography Experiment 2

Column Chromatography

The ßC-Sepharose column prepared in "Materials and Methods: ßC-Sepharose Affinity Chromatography (Experiment 1) was utilized in "Experiment 2". The major differences in this experiment compared to "Experiment 1" were, (1) sample was applied under lower ionic strength buffer conditions, (2) wash volume was decreased, and (3) an attempt was made to elute ßC-2-NMT activity from the column with norharman ("affinity elution"). The column was equilibrated in buffer containing 20 mM Na_2HPO_4

(pH 7.3) and 150 mM KCl. Bovine brain cytosol (5 ml), in the same buffer was applied to the column by gravity flow. Column flow-through was collected and filtered through a 0.22 µm polysulfone syringe filter (13 mm diameter). The entire fraction was concentrated completely on a Centricon 30 concentrator. The column was washed with 6 ml of equilibration buffer, and the wash was collected and treated like previous fraction. Norharman (2.0 ml, 1 mM in equilibration buffer) was used to elute ßC-2-NMT from the column. The norharman (NH) wash was repeated three additional times. These fractions were ultradialyzed (three solvent exchanges) to remove norharman, and simultaneously concentrated on a Centricon 30 concentrator.

Centricon 30 concentration proceeded overnight at 0-5 °C. Equilibration buffer (300 µl) was added to each retentate; buffer was not added to the initial column flow-through since its volume measured 450 µl "as-is". These concentrated solutions were used in the ßC-2-NMT assay and for protein determination.

ßC-2-NMT Assay

Details of the assay, work-up, and RP-HPLC analysis are discussed in Chapter 3. Assays, at pH 8.5, were maintained at 37 °C for one hour; reactions consisted of 9-MeNH (540 µM), SAM (105 µM, 3.112 µCi), and 100 µl of respective concentrated column fraction.

Protein Determination

Protein measurements were carried-out using a modified Lowry method as detailed in Appendix A.

Results: ßC-Sepharose Affinity Chromatography Experiment 2

The results of this experiment are summarized in Table 20.

Table 20.--ßC-Sepharose Affinity Chromatography Results

Column Fraction	Protein (mg)	ßC-2-NMT (pmol/h)	Specific Activity (pmol/h/mg)
Cytosol	34.0	671	19.7
Flow-through	6.8	65.7	9.5
Wash	4.3	145	33.8
NH (1^{st})	0.37	38.8	101
NH (2^{nd})	0.27	32.5	122
NH (3^{rd})	0.14	40.8	283
NH (4^{th})	0.09	16.6	178

The ßC-Sepharose column was equilibrated in 20 mM Na2HPO4 (pH 7.3) containing 150 mM KCl. Bovine brain cytosol ("Cytosol") in same buffer was applied to the column and "Flow-through" collected. Column was washed with equilibration buffer ("Wash"), and ßC-2-NMT subsequently eluted using four sequential volumes of 1 mM norharman solution (NH 1^{st} - 4^{th}).

Approximately 35% (12.0 mg) of the total applied protein was accounted for in all fractions, indicating that 65% of total protein remained on the column. With respect to ßC-2-NMT activity, 60% of the applied activity was accounted for (405 pmol/h), with 31% of the applied activity in the combined flow-through and wash fractions (211 pmol/h), suggestive of poor binding of ßC-2-NMT under these conditions. Affinity elution of ßC-2-NMT activity with

norharman solution resulted in the release of 19% of the applied activity (129.0 pmol/h), and approximately 50% of the applied ßC-2-NMT activity remained in association with the ßC-Sepharose column (339 pmol/h). The specific activity of the combined norharman fractions was 148 pmol/h/mg protein; therefore, the overall purification from cytosol to norharman eluates was 7.5-fold with a 19% yield.

Discussion: ßC-Sepharose Affinity Chromatography Experiment 2

More total protein remained bound to the column in this experiment compared to "Experiment 1", 65% compared to 54%, respectively. This may indicate that non-specific binding of protein is increased at lower ionic strength used in "Experiment 2" or that other interactions -- such as ionic interaction with the ethanolamine used to block reactive sites -- may be occurring. The lack of ßC-2-NMT binding during cytosol application to the column and subsequent washing indicates that binding of ßC-2-NMT may be favored under conditions of higher ionic strength utilized in "Experiment 1". The incomplete elution of bound ßC-2-NMT by a 1 mM NH solution was unexpected, and the 7.5-fold purification atypical for a true affinity column. These data suggest that a more concentrated NH solution may be required for complete ßC-2-NMT displacement from the column. It is also possible that a pH change may facilitate complete elution of ßC-2-NMT activity. These experiments have not been done.

Discussion: Affinity Chromatography
General Comments

Purification of ßC-2-NMT activity using SAH and ßC affinity columns in sequence would likely be a powerful approach to ßC-2-NMT purification. In theory the SAH-column could be used bind SAM-dependent methyltransferases, including ßC-2-NMT. Obviously it is necessary to identify an efficient means of eluting ßC-2-NMT activity from the SAH-CH-Sepharose 4B affinity column. The ßC-2-NMT-containing fraction from the SAH-column could subsequently be applied to the ßC affinity column under conditions that bind this activity efficiently -- perhaps at a pH similar to pH optimum for enzyme assay (pH 8.5). In theory a tremendous purification of ßC-2-NMT could be achieved at this step, since a relatively small number of proteins are presently known to specifically interact with ßCs. A few proteins expected to bind to a ßC-affinity column include, (1) those that catabolize ßCs, (2) monoamine oxidase (ßCs inhibit MAO), and (3) the benzodiazepine receptor to which ßCs bind. As with the SAH-column, an effective procedure for elution of ßC-2-NMT from the ßC-column is fundamental to the success of this method. Consideration should also be given to the possibility that the ßC-column may act as a nonspecific hydrophobic matrix due to the hydrophobic nature of the ßC molecule.

Overall Purification Summary

Ammonium sulfate fraction followed by DEAE-Sephadex batch chromatography resulted in a nearly 20-fold purification of ßC-2-NMT activity -- from a cytosolic activity of 20 pmol/h/mg protein to nearly 400 pmol/h/mg protein. Purification of ßC-2-NMT varied depending upon the purification protocol utilized (Figure 35).

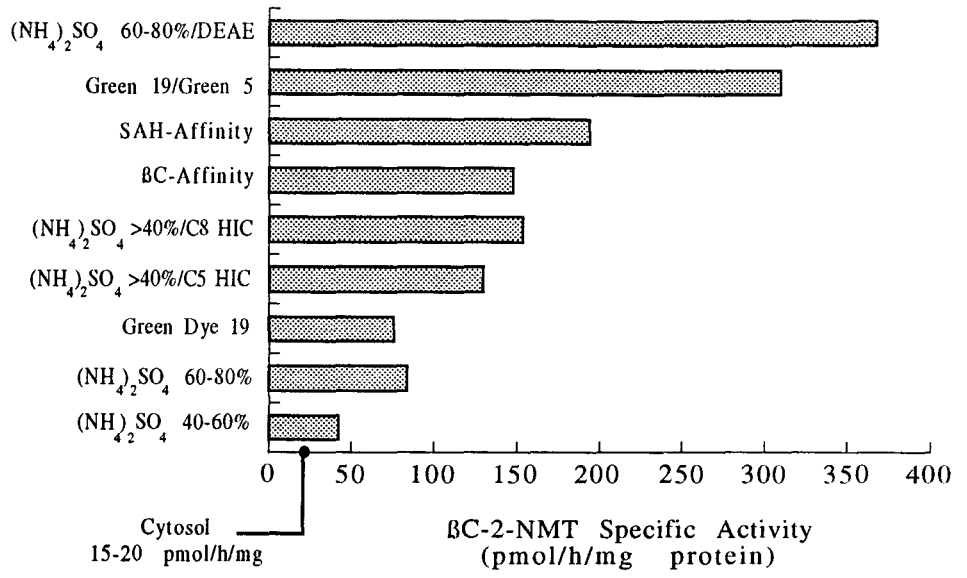


Figure 35. Summary of ßC-2-NMT purification (see this chapter for details).

Key: each bar represents 1-2 steps in sequence

$(NH_4)_2SO_4$ 60-80%/DEAE: 60-80% ammonium sulfate fraction from bovine brain cytosol dissolved in pH 7 imidazole buffer, then subjected to batch chromatography on DEAE-Sephadex (anion exchange matrix). ßC-2-NMT activity (represented by bar) was eluted from the matrix with pH 7 imidazole buffer containing 0.3 M NaCl.

Green 19/Green 5: Bovine brain cytosol was applied to a Green 19 dye column, eluate from this column was applied to a Green 5 dye column. ßC-2-NMT activity (represented by bar) was eluted from Green 5 dye column using pH 7.4 Na_2HPO_4 containing 3 M NaCl.

DEAE/SAH-Affinity: DEAE-Sephadex batch chromatography elute (above) applied to an S-adenosyl-L-homocysteine affinity column. ßC-2-NMT activity (represented by bar) was eluted from the column with pH 7 imidazole buffer containing 1 M NaCl.

ßC-Affinity: Bovine brain cytosol in 20 mM Na_2HPO_4 (pH 7.3)/150 mM KCl was applied to a ß-carboline affinity column. ßC-2-NMT activity (represented by bar) was eluted from the column using a 1 mM buffered norharman solution.

$(NH_4)_2SO_4$/C8: supernatant from 40% ammonium sulfate fractionation was applied to a octyl-agarose (C8) hydrophobic interaction chromatography column. ßC-2-NMT activity (represented by bar) was measured in application flow-through.

$(NH_4)_2SO_4$/C5: supernatant from 40% ammonium sulfate fractionation was applied to a pentyl-agarose (C5) hydrophobic interaction chromatography column. ßC-2-NMT activity (represented by bar) was measured in application flow-through.

Green 19: column chromatography (same conditions as above) on a Green 19 dye column

60-80% $(NH_4)_2SO_4$: the 60-80% ammonium sulfate fraction (desalted)

40-60% $(NH_4)_2SO_4$: the 40-60% ammonium sulfate fraction (desalted)

Cytosol: bovine brain cytosol, typically the starting material for each step above.

CHAPTER 5

MEASUREMENT OF ß-CARBOLINE-2N-METHYLTRANSFERASE AND ß-CARBOLINE-9N-METHYLTRANSFERASE ACTIVITIES IN POST-MORTEM HUMAN BRAIN FROM CONTROL AND PARKINSON'S DISEASE SUBJECTS

Background

ß-carboline-N-methyltransferases (ßC-NMTs) and the products of their catalytic activities, neurotoxic N-methylated ß-carbolinium cations (McßCs$^+$), may play a role in the pathogenesis of idiopathic Parkinson's disease (Collins 1994; Collins and Neafsey 1985). To explore this hypothesis, supernatant and particulate fraction ßC-2N-methyltransferase and ßC-9N-methyltransferase activities were measured in human postmortem brains. These activities were assessed in substantia nigra, putamen, and frontal cortex from control (N=11) and Parkinson's disease (N=12) cases. If ßC-2N- and/or ßC-9N-methyltransferase contributes to the development of PD, the hypothesis predicts that these activities may be increased relative to control brain activities -- probably early in the disease process. In addition, it is predicted that these activities may be elevated in brain areas primarily affected in PD, namely the substantia nigra and/or putamen. However, if these enzyme activities are indeed elevated early in pre-symptomatic PD, that elevation may be undetectable since the tissue used here was obtained from patients with well-developed PD.

Materials and Methods

Brain Tissue

Frozen sections of postmortem human brain were obtained from three different brain banks: (1) National Neurological Research Specimen Bank, VAMC Wadsworth Division, Los Angeles, CA 90073 (supported by NINDS/NIMH, National Multiple Sclerosis Society, Hereditary Disease Foundation, Comprehensive Epilepsy Program, Tourette Syndrome Association, Dystonia medical Research Foundation, and Veterans Health Services and Research Administration, Department of Veterans Affairs); (2) The Rush Brain Bank, Chicago, IL (supported by NIA AG09466 and AG10161); and (3) The Loyola University/Hines VA Brain Bank, Loyola University Medical Center, Maywood, IL (supported by the Department of Pathology).

Sections evaluated in this study included substantia nigra, putamen, and frontal cortex obtained at autopsy from control subjects and patients diagnosed with Parkinson's disease. Table 21 shows pertinent information regarding these tissues by region, including: subject age, postmortem interval, and sex. Due to the limited availability of Parkinson's tissue it was not possible to obtain samples that were strictly age-matched to control tissue, although the age ranges were roughly equivalent for the two groups.

Table 21.--Characteristics of Postmortem Brain Tissue

	Group	
Brain Region	Control	Parkinson's
Substantia nigra		
N	6	8
Male/Female	2/4	4/4
Age	73±9 (64-88)	76±4 (69-81)
PMI	16.6±6.9 (8-25.5)	12.9±5.0 (6.5-23.5)
Putamen		
N	7	7
Male/Female	6/1	5/2
Age	68±3[a] (64-73)	76±5 (69-83)
PMI	14.9±7.1[b] (7-25.5)	8.6±2.8 (5.5-13)
Frontal cortex		
N	7	7
Male/Female	6/1	5/2
Age	67±3[c] (64-73)	77±4 (72-83)
PMI	13.3±5.5 (7.0-20.0)	10.2±6.3 (5.5-23.5)

Note: Values for age (years) and PMI (hours) represent mean ± s.d., with the range in parentheses. Superscripts indicate (a) significantly different than PD ($p<0.005$), (b) significantly different than PD ($p<0.05$), (c) significantly different than PD ($p<0.0005$).

A total of 48 different samples were evaluated in random order; each day three tissue samples were prepared for the ßC-NMT assays. The frozen sections were thawed slightly, and approximately 100 mg of tissue was removed using a clean tissue punch. In sections of substantia nigra or putamen this sample was taken from the center of the structure. In coronal sections of frontal cortex, samples were removed from the superficial surface of the middle frontal gyrus. The tissue sample was weighed, placed in a 2 ml glass homogenizer on ice, and homogenized at 0-5 °C in 5 volumes of homogenation buffer (5 µl buffer per mg tissue). Homogenation buffer (pH 7.2) consisted of 150 mM potassium chloride, 20 mM sodium phosphate (Na_2HPO_4), 1 mM dithiothreitol, and 1 mM ethylenediaminetetraacetic acid disodium. Homogenation was achieved with five up and down strokes of a motor-driven (300 rpm) teflon pestle. Homogenate was transferred to a 1.5 ml microcentrifuge tube where centrifugation at 10,000*g* for thirty minutes yielded crude particulate and supernatant fractions. Particulate fraction was diluted with 200 - 400 µl of homogenation buffer prior to use in ßC-NMT assays, and supernatant was used "as-is".

Reagents

ß-Carboline substrates, 9-methylnorharman (9-MeNH) hydrochloride or 2-methylnorharmanium iodide (2-MeNH+), were synthesized in our laboratory according to Rubottom (Rubottomand Chabala 1974) or Matsubara (Matsubara et al. 1992b), respectively. Unlabeled S-adenosyl-L-methionine (toluenesulfonate salt) was purchased from Sigma Chemical (St. Louis, MO), and tritiated S-[methyl-^{3}H]-adenosyl-L-methionine (60-85 Ci/mmol) was obtained from Dupont (New England Nuclear, Boston, MA).

The ßC-N-Methyltransferase Assays

These activities catalyze the transfer of the tritiated methyl moiety from S-adenosyl-L-methionine (SAM) to 9-MeNH or 2-MeNH+ to form SAH and 2(^{3}H-methyl)-9-MeNH+ or 2-9(^{3}H-methyl)-NH+ (Figure 36).

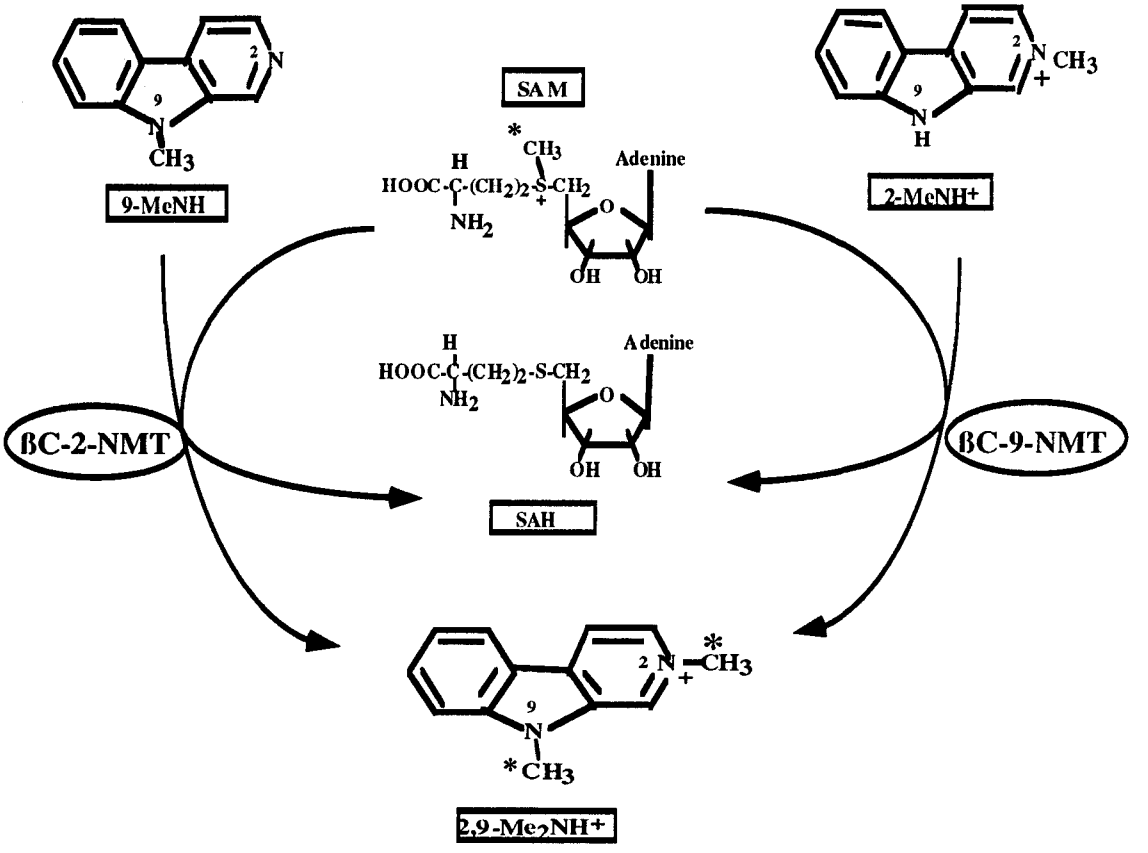


Figure 36. Human brain ßC-NMT assay scheme.

The ßC substrates, 9-MeNH and 2-MeNH+, were used to assess ßC-2-NMT and ßC-9-NMT activities, respectively, in both particulate and supernatant fractions from each brain region. Enzyme assays were done in 1.5 ml polypropylene microcentrifuge tubes by mixing concentrated stock solutions of ß-carboline (540 µM final), SAM (105 µM final), and ^{3}H-SAM (4.6 µCi) with supernatant or diluted particulate fraction. Stock solutions of SAM were prepared in water, and ßC was dissolved in a buffer mixture consisting of 50 mM Na_2HPO_4 plus 50 mM bicine at pH 8.5. Assay volume was 370 µl (pH 8.5), comprised of the following stock solutions: 50 µl ^{3}H-SAM (4.6 µCi), 20 µl non-radioactive SAM (1.943 mM), 200 µl ßC (1 mM), and 100 µl of supernatant or particulate fraction.

Each tube was gently vortexed and incubated at 37 °C in a shaking water bath for one hour. Following this incubation each sample was placed on ice and protein precipitated by the addition of 200 µl 0.5 N perchloric acid ($HClO_4$). The mixture was centrifuged to pellet precipitated protein (7000*g* for 5 minutes), with the supernatant transferred to a glass culture tube containing 1 ml of 1% (w/v) ammonium hydroxide (NH_4OH) and 100 µl of 1 N potassium hydroxide. The protein pellet was vigorously resuspended in 500 µl 0.01 N $HClO_4$ and centrifuged at 10,000*g* for 5 minutes, with the resulting supernatant combined with the initial supernatant. This solution was then slowly applied to a Sep-Pak® C18 Plus solid-phase extraction (SPE) cartridge (360 mg, Millipore-Waters part no. 20515). The cartridge was pretreated with 5 ml of methanol:5 N acetic acid (3:1, v/v), methanol, and 1% (w/v) NH_4OH in that sequence. Slow application (2 to 5 drops per second) of all solutions to the SPE cartridge was achieved using a vacuum manifold. Following the initial sample

application, the SPE cartridge was washed with 8 ml of 1% (w/v) NH_4OH and then by 5 ml methanol; these washes were discarded since they contained compounds that could contribute to the background radioactivity during subsequent analysis. The final wash (5 ml), consisting of 3:1 (v/v) methanol:5 N acetic acid, promoted elution of tritiated 2,9-Me_2NH^+ from the cartridge into a clean glass culture tube. This eluate was evaporated to dryness at 30-40 °C using a Speed Vac concentrator (Savant Instruments Inc., Hicksville NY).

Reverse-Phase High Performance Liquid Chromatography

Activities of ßC-NMTs were quantitated by measuring tritiated 2,9-Me_2NH^+ product using reverse-phase HPLC. The residues from the Speed Vac concentration step were dissolved in 200 µl HPLC mobile phase by sonication and vortexing. Samples were filtered through a 0.45 µm nylon membrane filter (Micro-Spin, Chrom-Tech Inc.) prior to injection (150 µl) onto the HPLC system. Isocratic HPLC analysis was carried out under ambient conditions on a Bondclone 10 µm C18 column (Phenomenex™, 300 mm *x* 3.9 mm). The mobile phase was a mixture of 17% (v/v) HPLC grade acetonitrile and 83% (v/v) aqueous buffer. The buffer contained 50 mM triethylamine in 250 mM sodium phosphate adjusted to a pH of 3 with phosphoric acid (final mobile phase pH 3.4). The HPLC system included a Shimadzu SIL-9A programmable autosampler, a Beckman 110A pump (0.8 ml/minute), and an in-line radioactive flow detector (Radiomatic Instruments Flo-One\ß). Ultima-Flo™ AP (Packard Instruments) liquid scintillation cocktail was used at a flow rate of 2.8 ml per minute, resulting in a scintillation fluid to mobile phase mixing ratio of 7:2.

The radioactive flow detector was equipped with a 2.5 ml liquid flow cell and an automatic stream splitter. It was necessary to bypass the radioactive flow detector by splitting the HPLC column effluent to waste until about ten minutes into the run. Splitting to waste reduced interference due to tritiated materials, primarily ^{3}H-SAM and its degradation products, present in the samples. Radioactive peak area corresponding to 2,9-Me_2NH^+ (retention time 12 - 13 minutes) was calculated using Radiomatic Flo-One\ß system software; the resulting integrated area was reported as total cpm under the 2,9-Me_2NH^+ peak. ßC-NMT activities are reported as pmoles 2,9-Me_2NH^+ formed per hour per mg protein (pmol/h/mg protein) and were calculated using experimentally defined values for detector counting efficiency and the quantity of ^{3}H-SAM added to the ßC-NMT assay. Counting efficiency and ^{3}H-SAM added to ßC-NMT assays were determined on each day of HPLC analysis. Total protein was determined in particulate and supernatant fractions using a modified Lowry method as described by Scopes (1994d) and in Appendix A.

Data Analysis

Kaleidagraph™ (Abelbeck/Synergy Software, Reading, PA) was used for graphical analysis, and for determination of correlation coefficients for each ßC-NMT activity as a function of age or PMI. Statistical analysis of data was done using SPSS for Windows. A three-way factorial ANOVA was used to compare ßC-2-NMT or ßC-9-NMT activity as a function of fraction (particulate vs. supernatant), disease-state (control vs. PD), and brain region (substantia nigra vs. putamen vs. frontal cortex).

Results

Analysis of Variance (ANOVA)

The detailed results of the factorial ANOVA for ßC-2-NMT activity and ßC-9-NMT activity as a function of fraction, brain region, and disease-state are presented in Appendix D.

ßC-2-NMT Activity

The three-way ANOVA was significant ($F(4,79)=4.037$, $p=0.005$), but of the three factors tested only the fraction factor had a significant main effect ($F(1,79)=11.997$, $p=0.001$), consistent with the generally higher level of activity in the supernatant fraction that can be seen in Figures 32-34. No significant F-value was found for disease-state or brain region factors with respect to ßC-2-NMT activity. A one-way ANOVA run on the 12 distinct "conditions" (fraction*disease-state*brain region; see Figure 37) failed to reach statistical significance ($F(11, 79)=1.7427$, $p=0.0824$), and Bonferroni post-hoc multiple comparisons tests revealed that no two groups were significantly different with respect to ßC-2-NMT activity. Thus the higher ßC-2-NMT activity in the supernatant fraction appears to be a general effect.

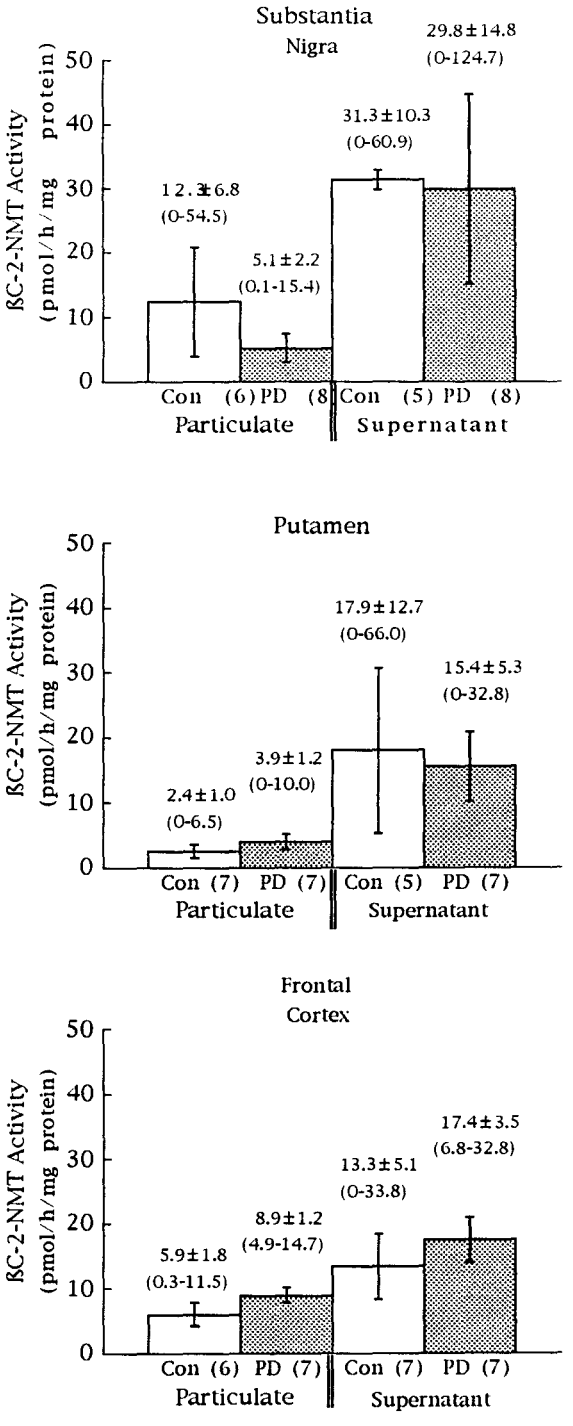


Figure 37. ßC-2-NMT activity in control and Parkinson's disease brain regions. Fraction (10,000 *xg*) = particulate or supernatant. "Con" = Control and "PD" = Parkinson's with replicates per group (N). Values above bars represent mean ± s.e.m. with the range in parentheses.

ßC-9-NMT Activity

The three-way ANOVA was significant overall ($F(4,83)=4.947$, $p=0.001$), with a significant main effect for fraction ($F(1,83)=14.354$, $p=0.0003$) and a significant interaction effect for brain region and disease-state ($F(2,83)=5.020$, $p=0.009$). No significant main effects were found for disease-state or brain region regarding ßC-9-NMT activity. A one-way ANOVA run on the 12 "conditions" (fraction*disease-state*brain region; see Figure *) was significant ($F(11, 83)=3.4806$, $p=0.0006$). Post-hoc Bonferroni multiple comparison tests found that frontal cortex supernatant ßC-9-NMT activity in Parkinson's disease was significantly higher than control frontal cortex ßC-9-NMT activity ($p<0.05$) as can be seen in Figure 38. In fact, Parkinson's frontal cortex supernatant ßC-9-NMT activity was significantly higher than ßC-9-NMT activity for all other groups ($p<0.05$), except for control substantia nigra and control putamen activity.

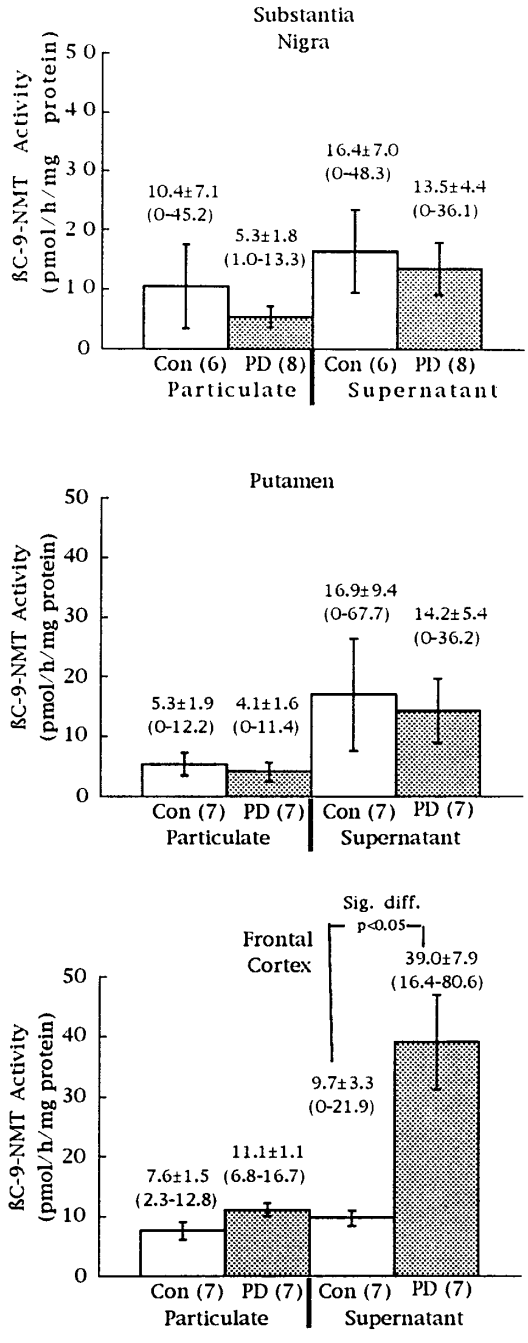


Figure 38. ßC-9-NMT activity in control and Parkinson's disease brain regions. Fraction (10,000 *xg*) = particulate or supernatant. "Con" = Control and "PD" = Parkinson's with replicates per group (N). Values above bars represent mean ± s.e.m. with the range in parentheses.

ßC-NMT Activities as a Function of Age and Postmortem Interval

Within each brain region, unpaired Student's t-tests were utilized to compare mean age and mean postmortem interval (PMI) between PD and control groups (refer to Table 21). In addition, the Pearson correlation coefficient (r) was calculated to determine if any of the four ßC-N-methyltransferase activities, within each brain region, correlated with either age or postmortem interval.

In substantia nigra samples, there was no statistical difference between control and PD groups with respect to mean patient age or postmortem interval. Furthermore, in the substantia nigra, ßC-NMT activity did not correlate with either patient age or tissue PMI.

In the putamen specimens, control cases were significantly younger than PD patients ($p<0.005$), but ßC-NMT activity did not correlate with subject age. The PMI for control tissue from putamen exceeded that for PD samples ($p<0.05$). Only particulate fraction ßC-9-NMT activity in control putamen correlated with PMI, specifically, this activity decreased as a function of increasing PMI ($r=-0.93$, $p<0.005$).

Particulate fraction ßC-9-NMT activity from control frontal cortex increased as a function of increasing PMI ($r=0.76$, $p<0.05$); this was considered unimportant since frontal cortex samples were PMI-matched for control and PD tissue. Regarding samples of frontal cortex, the mean age of the control group was significantly lower than that of the PD group ($p<0.0005$); however, ßC-NMT activity did not correlate with subject age. Supernatant ßC-9-NMT activity in frontal cortex -- the only ßC-NMT activity that was different in PD vs control subjects -- is plotted as a function of age in Figure 39.

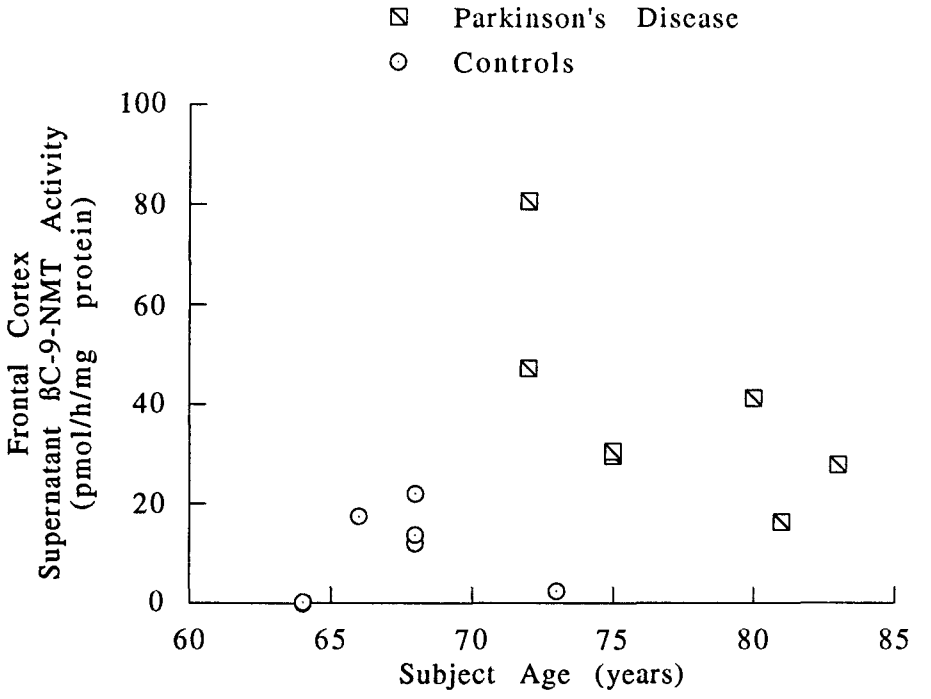


Figure 39. Frontal cortex supernatant ßC-9-NMT activity as a function of subject age. No significant correlation was found for PD or Control group or for the two groups combined with respect to enzyme activity as a function of age.

Discussion

This is the first description of ßC-9N-methyltransferase activity in human brain; only rat and guinea pig brain activity was previously described (Collins et al. 1992; Matsubara et al. 1992b). The 13-fold higher SAM concentration used in the ßC-9-NMT assay in this study likely accounted for the detection of ßC-9-NMT activity; i.e., Matsubara et al. (1993) use of 8 µM SAM may have been insufficient for detection of ßC-9-NMT activity. These data confirm the presence of ßC-2N-methyltransferase activity in postmortem human brain, a finding originally reported by Matsubara et al. (Matsubara et al. 1993). The study presented here expands upon Matsubara's work by examining ßC-NMT activities in Parkinson's disease brain, as well as distinguishing between particulate and supernatant fraction activities. It was found that both ßC-NMT activities were higher in the supernatant compared to the particulate fraction, indicating a predominantly cytosolic localization for these enzymes. Higher supernatant ßC-2-NMT activity is consistent with the results reported in Chapter 3 for bovine brain ßC-2-NMT activity. Most importantly, the data reported here indicate that frontal cortex supernatant ßC-9-NMT activity is significantly elevated in Parkinson's disease compared to controls ($p<0.05$).

Age and Postmortem Interval Effects

The significant age differences between the control and Parkinson's groups for putamen and frontal cortex specimens are unfortunate, but likely not important since ßC-NMT did not vary with subject age. In contrast, PMI could be considered a confounding variable in our study, since particulate fraction ßC-9-NMT activity varied with PMI in control tissue from putamen

and frontal cortex. This suggests that there may in fact be a difference between control and PD cases for pellet ßC-9-NMT activity in putamen, but that a significant difference was not found due to the effect of PMI this activity (i.e., a Type II statistical error may have been committed).

Basal Ganglia Findings

No assumptions were made *a priori* that ßC-NMT activities would be the same across the brain regions evaluated. However, since the nigrostriatal system is most dramatically affected in PD, the hypothesis logically predicts that ßC-2-NMT and/or ßC-9-NMT activity may be higher in the substantia nigra and/or putamen relative to the frontal cortex. These data do not directly support this prediction, because although mean ßC-2-NMT activity in the substantia nigra of control tissue is higher than in PD tissue, the difference is not statistically significant. It is possible that in the PD brain, regional distribution of measurable ßC-NMT activity is confounded by the MeßC$^+$-induced death of the neurons that, before death, possessed high ßC-NMT activity. Note that selective elevation of ßC-NMT activity in the nigrostriatal system is not a requirement in this model since selective uptake of neurotoxic MeßCs$^+$ into this system would also confer neurotoxic selectivity to the nigrostriatal system. Consistent with the idea of selective uptake, MeßCs$^+$ do modestly inhibit dopamine uptake into rat synaptosomes (Drucker et al. 1990), implying that these compounds are substrates for the dopamine transporter.

Frontal Cortex Findings

The significant elevation of supernatant ßC-9-NMT activity in the frontal cortex of PD brain compared to controls ($p<0.05$) was unexpected.

Furthermore, considering only Parkinson's tissue, frontal cortex supernatant ßC-9-NMT activity was significantly higher compared to substantia nigra or putamen activity. It is believed that these differences are real and are not accounted for by differences between group age or PMI, since supernatant ßC-9-NMT activity did not correlate with PMI or age in any tissue. It is speculated that this observation may reflect both a general elevation of supernatant ßC-9-NMT activity in PD brain, and a selective, local vulnerability of the nigrostriatal neurons to the effects of resultant 2,9-Me_2ßCs$^+$. Death of the nigrostriatal neurons, originally containing the ßC-9-NMT activity, would lead to a commensurate loss of detectable ßC-9-NMT activity in the nigrostriatal nuclei. Perhaps early in the disease process, well before profound cell-death, ßC-NMT activities are higher in PD relative to controls. Cortical neurons that exhibit increased supernatant ßC-9-NMT activity, but are resistant to the toxic effects of resultant Meßcs$^+$, would remain viable and consequently make measurement of enzyme activity possible. Consistent with the postulate that cortical neurons may be resistant to these toxins, it is interesting to note that dopaminergic neurons projecting to the frontal cortex from the ventral tegmental area are spared in PD.

However, it is equally conceivable that elevated supernatant ßC-9-NMT activity in the frontal cortex is a compensatory response to the death of adjacent dopaminergic nigral neurons occurring in Parkinson's disease.

Parkinson's Disease

The hypothesis in this lab implies that ßC-2-NMT and/or ßC-9-NMT activity is higher in Parkinson's disease compared to controls, resulting in the formation of excessive neurotoxic Meßcs$^+$ in PD brain and consequent

neuronal death. Furthermore, one would expect that ßC-NMT activity would be elevated during the subclinical or early stages of PD -- a hypothesis that can not be readily investigated. As discussed above the original hypothesis is partially supported by elevated supernatant ßC-9-NMT activity in the frontal cortex of PD tissue. Since MeßCs+ are able to penetrate the blood-brain barrier, these compounds probably cross plasma membranes. Therefore, MeßCs+ formed in the frontal cortex could enter the CSF, and subsequently exert toxic effects on the nigrostriatal system.

Conversely, if elevated ßC-9-NMT activity in the frontal cortex is truly a local effect it is proposed that formation of MeßCs+ in the frontal cortex may result in the death of a discrete population of vulnerable cortical neurons at the site of MeßC+ formation. Perhaps cells that exhibit high ßC-9-NMT activity are resistant to resultant MeßCs+, while other cells in close proximity are vulnerable. It follows that the death of these vulnerable cells could manifest clinically as dementia or depression -- two nonmotor symptoms documented in many PD patients (Koller and Pahwa 1995).

While these data certainly do not provide compelling evidence for a global elevation of ßC-NMT activity in Parkinson's brain, a role for MeßCs+ in the neuropathogenesis of idiopathic PD remains viable considering the following parallel hypothesis. If MeßCs+ are elevated in PD, it is conceivable that their ßC-NMT-catalyzed formation is similar to that of controls, but that catabolism of MeßCs+ is impaired in PD. In PD brain a deficiency in the catabolism of MeßCs+, to nontoxic compounds, could also lead to elevated levels of these compounds -- culminating in cell death in brain regions where this hypothetical catabolic deficiency occurs. Indeed, studies have been reported

which indicate that abnormalities in xenobiotic metabolism may contribute to the development of PD (Williams 1995).

It is also possible that persons afflicted with PD are exposed to higher levels of substrates of ßC-NMT, and consequently form higher levels of toxic MeßCs+ throughout life, until sufficient neurons are killed to manifest in the clinical symptoms of PD. This is consistent with a recent report indicating that plasma levels of norharman and harman -- substrates of the ßC-2-NMT activity investigated in this report -- are elevated in individuals diagnosed with Parkinson's disease (Kuhn et al. 1995). Studies are underway in our laboratory in which levels of MeßCs+ will be compared in postmortem human brain tissue from PD and control individuals. Those experiments, in combination with the results reported here, should clarify the picture regarding the contribution of N-methylated-ß-carbolines to the pathogenesis of Parkinson's disease.

CHAPTER 6

EVIDENCE THAT ß-CARBOLINE-2N-METHYLTRANSFERASE ACTIVITY IS DUE TO A PHENYLETHANOLAMINE N-METHYLTRANSFERASE-LIKE ENZYME

It was hypothesized that ßC-NMT activity may be due to a previously characterized CNS-residing N-methyltransferase -- such as phenylethanolamine-N-methyltransferase (PNMT) -- this enzyme catalyzes the formation of epinephrine from norepinephrine. PNMT is a cytosolic SAM-dependent enzyme that is found in the adrenal medulla, brain, and other tissues (Kennedy et al. 1995). To test this hypothesis that ßC-NMT activity may be due to PNMT several experiments were performed.

The first experiment was designed to determine if purified bovine-adrenal PNMT catalyzes N-methylation of either 9-MeNH or 2-MeNH+, for the assessment of ßC-2-NMT and ßC-9-NMT activity, respectively.

The second experiment confirmed the presence of PNMT catalytic activity in bovine-brain cytosol by measuring the conversion of norepinephrine to epinephrine in the presence of tritiated-SAM.

The third experiment evaluated the effect of LY134046 on ßC-2-NMT activity; LY134046 is a selective inhibitor of brain and adrenal PNMT. If ßC-2-NMT is the same as PNMT, one would expect an inhibition of ßC-2-NMT in the presence of LY134046.

In the fourth and fifth experiments several PNMT substrates were evaluated for their ability to inhibit ßC-2-NMT activity. If ßC-2-NMT is PNMT, then substrates of PNMT should inhibit ßC-2-NMT activity, since these substrates would compete with the ßC for PNMT.

The sixth experiment assessed ßC-2-NMT activity in human medulla oblongata and human adrenal medulla. Both tissues exhibit documented PNMT activity, particularly the adrenal medulla. If ßC-2-NMT is PNMT one would expect ßC-2-NMT activity in these tissues.

In the final experiment PNMT was immunoprecipitated from bovine-brain cytosol. The resulting supernatant was evaluated for ßC-2-NMT activity, with the expectation that activity would be reduced or absent following PNMT immunoprecipitaton.

Experiment 1:
ßC-N-Methylation by PNMT

Materials and Methods

This experiment was repeated twice, and in each study replicate samples were prepared at each condition. Assay conditions were as follows:

9-MeNH or 2-MeNH+	540 µM
SAM	105 µM
^{3}H-SAM	3.60 µCi (study 1)
	6.74 µCi (study 2)
PNMT	3.3 units

Stock solutions used were:

9-MeNH or 2-MeNH+ (1 mM stock in buffer "A")	200 µl
SAM (1.943 mM stock in Milli-Q water)	20 µl
^{3}H-SAM (3.60 or 6.74 µCi in Milli-Q water)	50 µl
PNMT Solution (0.033 units/µl)	100 µl
Total volume	370 µl

Buffer "A" contained 50 mM bicine and 50 mM Na_2HPO_4 dibasic at pH 8.5. Sources of substrates are described in Chapter 5. Replicate assays were prepared to contain either 2-MeNH+, 9-MeNH, or no ßC (blank assay). Final assay pH was 8.5. PNMT (25 units per vial) was obtained as a lyophilized powder in a rubber-stoppered vial from Sigma Chemical Company (St. Louis, MO). The contents of the vial were dissolved in 800 µl Milli-Q water. Label information regarding this enzyme is listed in Appendix E. ßC-2-NMT assays were maintained at 37 °C for one hour. The assay work-up and measurement of 2,9-Me_2NH^+ by RP-HPLC were performed as described in Chapter 5.

Results

Bovine adrenal PNMT catalyzes the 2N-methylation of 9-MeNH, but does not carry-out 9N-methylation of 2-MeNH+ (Table 22). Within each experiment, a 2-tailed t-test was used to compare the assay results without βC (blank) to assays containing βC. The reaction with 2-MeNH+ as substrate did not differ from the blank reaction with respect to 2,9-Me_2NH^+ formation. However, the reaction with 9-MeNH was statistically different than the blank assay (Study 1 **$p<0.0005$, Study 2 *$p<0.005$).

Table 22.-- Bovine Adrenal PNMT-Catalyzed 2N- and 9N-Methylation of βCs

	βC-N-Methylation Activity (mean ± s.e.m., N=2)	
Conditions	2,9-Me_2NH^+ Formed (pmol/h)	2,9-Me_2NH^+ Formed (pmol/h/unit PNMT)
Study 1		
Blank (no βC)	2.7 ± 0.9	0.8 ± 0.3
plus 2-MeNH+	1.4 ± 0.7	0.4 ± 0.2
plus 9-MeNH	58.8 ± 1.4**	17.8 ± 0.4**
Study 2		
Blank (no βC)	18.1 ± 5.6	5.7 ± 1.7
plus 2-MeNH+	26.6 ± 0.5	8.1 ± 0.2
plus 9-MeNH	80.1 ± 0.4 *	24.3 ± 0.1*

Recall that 2N-methylation is activity measured when 9-MeNH is used as the substrate, and that 9N-methylation represents activity determined with 2-MeNH+ as the substrate. Net 2N-methylation activity was calculated for each experiment by subtracting 2N-methylation in the blank assay from 2N-methylation in the presence of 9-MeNH. Net 2N-methylation of 9-MeNH in Study 1 was 17.0 ± 0.5 pmol/h/unit PNMT, and net 2N-methylation in Study 2 was 18.6 ± 1.7 pmol/h/unit PNMT.

Conclusion

These data show that PNMT can 2N-methylate a ßC-substrate and therefore do not refute the hypothesis that ßC-2-NMT and PNMT are the same or similar enzymes.

Experiment 2:
PNMT Activity in Bovine Brain Cytosol

Materials and Methods

PNMT activity in bovine brain cytosol was assessed using L-norepinephrine as the substrate. Similar studies were not done with octopamine and phenylethanolamine due to difficulties encountered with the extraction procedure for their N-methylated derivatives. The final substrate concentrations were:

SAM	105 µM
^{3}H-SAM	1.034 µCi
L-norepinephrine	0, 10, 40, 100, and 250 µM

The total volume of the assay was 370 µl and the final pH was 8.5. Duplicate assays were prepared in 1.5 ml polypropylene microcentrifuge tubes as follows:

SAM (1.943 mM stock in Milli-Q water)	20 µl
^{3}H-SAM (1.034 µCi in Milli-Q water)	50 µl
L-norepinephrine (stock in buffer "A")	200 µl
Bovine brain cytosol (1.156 mg protein)	100 µl

L-norepinephrine bitartrate stock solutions utilized were: 18.5 µM, 74 µM, 185 µM, 462.5 µM. A blank assay was prepared that lacked L-norepinephrine. Assays were incubated at 37 °C for one hour.

The assay work-up was done according to the method described by Chatelain et al. (1990). The PNMT assay was terminated by its transfer to a 13 x 100 mm glass culture tube containing 250 µl of 1 N HCl. Solid NaCl (1 g) was added, and the mixture was extracted with 6 ml of 1 N HCl-washed butanol. A 5 ml portion of the butanol layer was evaporated to dryness in a Speed Vac concentrator at 40 °C. The residue was dissolved in 5 ml of EconoSafe liquid scintillation cocktail, and subsequently transferred to 20 ml polypropylene scintillation vial. The culture tube was rinsed with 5 ml of EconoSafe, and this rinse was added to the scintillation vial. Additional EconoSafe (10 ml) was added to each scintillation vial, and the contents were mixed on a lab vortexer. Tritium was measured in these vials using a Beckman LS 6500 liquid scintillation counter. Background radioactivity (represented by the blank assay) was subtracted from the results for each assay that contained norepinephrine. Results are presented as pmoles of epinephrine formed per hour (pmol/h/mg protein).

Results

Bovine brain cytosol exhibits PNMT activity as evidenced by the conversion of norepinephrine to epinephrine (Figure 40). The maximal velocity (V_{max}) was 1.8 nmol/h/mg protein, and the K_M for norepinephrine was 63 μM.

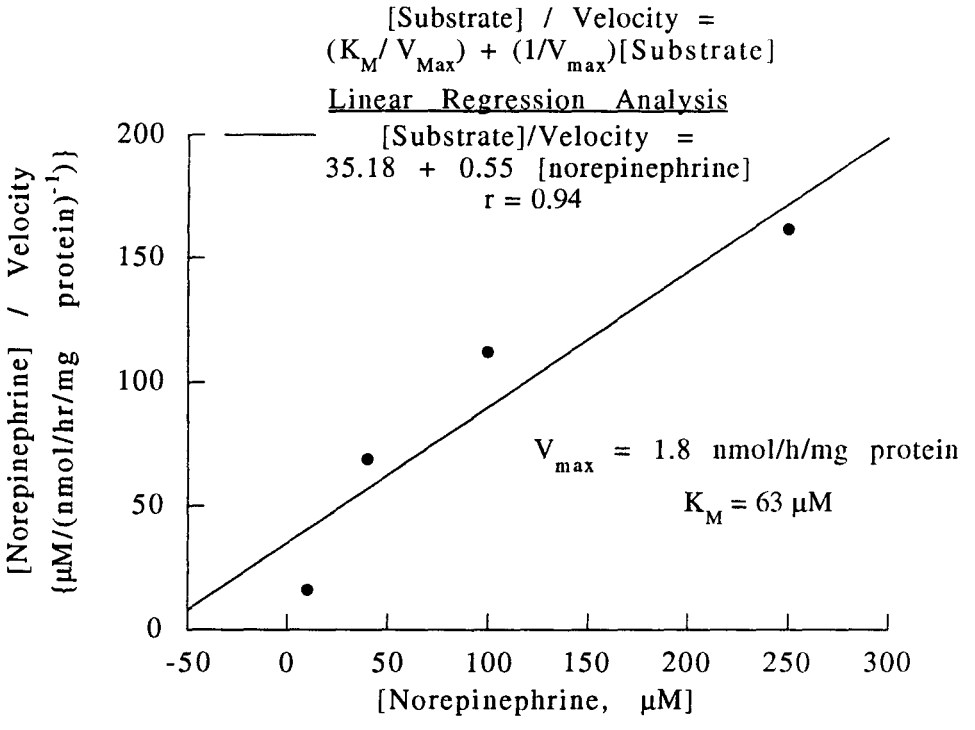


Figure 40. Hanes plot for formation of epinephrine from norepinephrine in bovine brain cytosol. Substrates were SAM (105 μM, 1.034 μCi) and L-norepinephrine (0, 10, 40, 100, and 250 μM) at pH 8.5.

Conclusion

This study simply confirms the presence of PNMT in the same bovine-brain preparation that was utilized in experiments that evaluated ßC-2-NMT activity, indicating that ßC-2-NMT and PNMT exist in the same cytosol.

Experiment 3:
Inhibition of ßC-2-NMT Activity by LY134046

Materials and Methods

The final assay concentrations of substrates and the PNMT-inhibitor LY134046 were:

9-MeNH	100 µM
SAM	105 µM
^{3}H-SAM	3.54 µCi
LY134046	1 nM, 1 µM, 500 µM, 1 mM, or 5 mM

Assay volume was 370 µl at a final pH of 8.5. Assays were prepared in 1.5 ml polypropylene microcentrifuge tubes as follows:

9-MeNH (740 µM stock in buffer "A")	50 µl
SAM (1.943 mM stock in Milli-Q water)	20 µl
^{3}H-SAM (3.54 µCi in Milli-Q water)	30 µl
LY134046 (stock in buffer "A")	170 µl
Bovine brain cytosol (0.711 mg protein)	100 µl

LY134046 stock solution concentrations utilized were: 2.176 nM, 2.176 µM, 1.088 mM, 2.176 mM, or 10.882 mM. A control ßC-2-NMT assay, in the absence of LY134046, contained 170 µl buffer "A" in place of LY134046. Two distinct kinds of blank assays were prepared in which each blank lacked 9-MeNH: one blank contained 1 mM LY134046 ("inhibitor-control"), whereas the other blank lacked LY134046. LY134046 was purchased from Research Biochemicals International (Natick, MA); information regarding the inhibitor is listed in Appendix E. ßC-2-NMT assays were incubated at 37 °C for one hour. The assay work-up and measurement of 2,9-Me_2NH^+ by RP-HPLC were performed as described in Chapter 5. The appropriate blank N-methylation activity was subtracted from values obtained in complete assays.

The resulting data were plotted in three ways:

(1) percent of control ßC-2-NMT activity as a function of LY134046 concentration.

(2) percent of control ßC-2-NMT activity as a function of log LY134046 concentration. The IC_{50} was determined from this plot.

(3 a Dixon plot, 1/ßC-2-NMT activity as a function of LY134046 concentration. The K_i for LY134046 was determined from this plot.

Results

LY134046 inhibited ßC-2-NMT in a concentration-dependent manner. Enzyme activity was nearly completely inhibited at 500 µM LY134046, and complete inhibition of activity was observed at LY134046 concentrations greater than or equal to 1 mM (Figure 41).

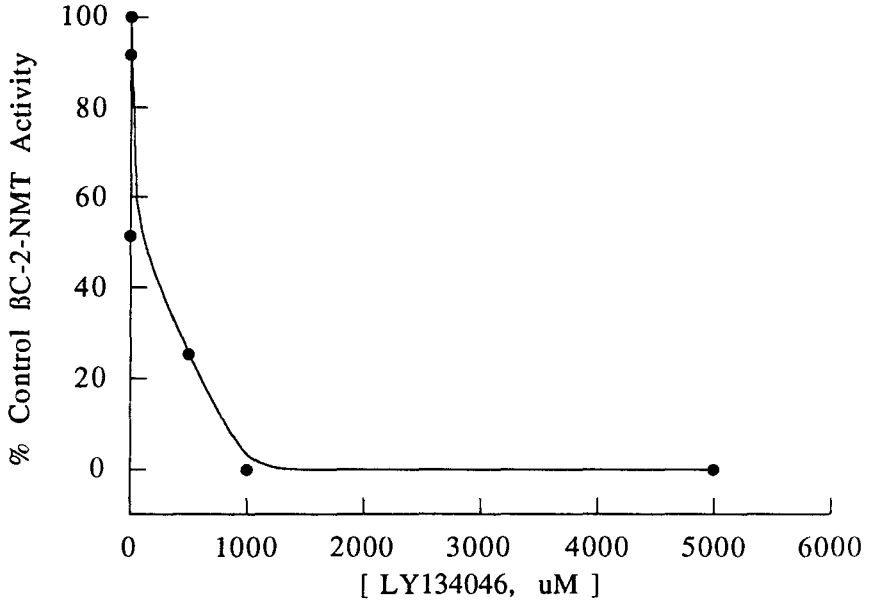


Figure 41. Inhibition of bovine brain cytosol ßC-2-NMT activity by LY134046. Substrates were 9-MeNH (100 µM), SAM (105 µM, 3.54 µCi), and inhibitor LY134046 (0-5 mM) at pH 8.5.

A linear relationship exists between percent of control ßC-2-NMT activity and LY134046 concentration when inhibitor concentration (range 1 nM-1 mM) is plotted on a log scale. The IC_{50} value -- the concentration of LY134046 required to inhibit ßC-2-NMT activity by 50 percent -- was 1.2 µM LY134046 (Figure 42).

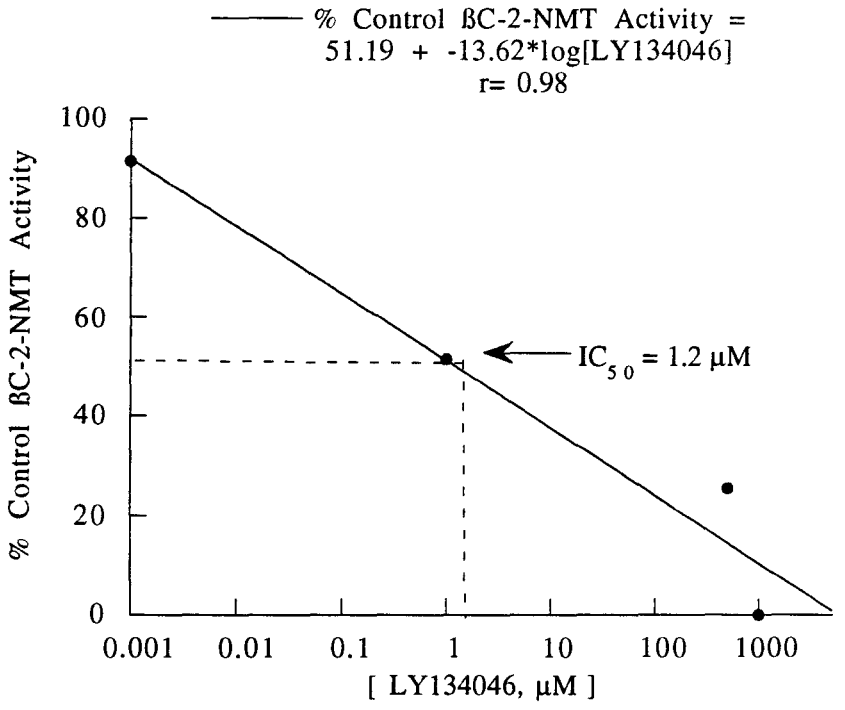


Figure 42. Determination of IC_{50} for LY134046 dependent inhibition of ßC-2-NMT activity. Assay at pH 8.5 for 1 hour at 37 °C. Substrates: 100 µM 9-MeNH, 0-1 mM LY134046, 105 µM (3.54 µCi) SAM.

The Dixon plot for competitive inhibition is defined by the following equation:

$$1/v = (K_M/V_{max}*[S]*K_i)[I] + (1/V_{max})\{1 + (K_M/[S])\}$$

This equation predicts that slope = $(K_M/V_{max}*[S]*K_i)$, when 1/Velocity is plotted against inhibitor concentration. In Chapter 3, V_{max} and K_M for ßC-2-NMT with respect to 9-MeNH were determined to be 48 pmol/h/mg protein and 75 µM, respectively. Graphical analysis of inhibition of ßC-2-NMT activity by LY134046 predicts a K_i = 0.4 µM (Figure 43).

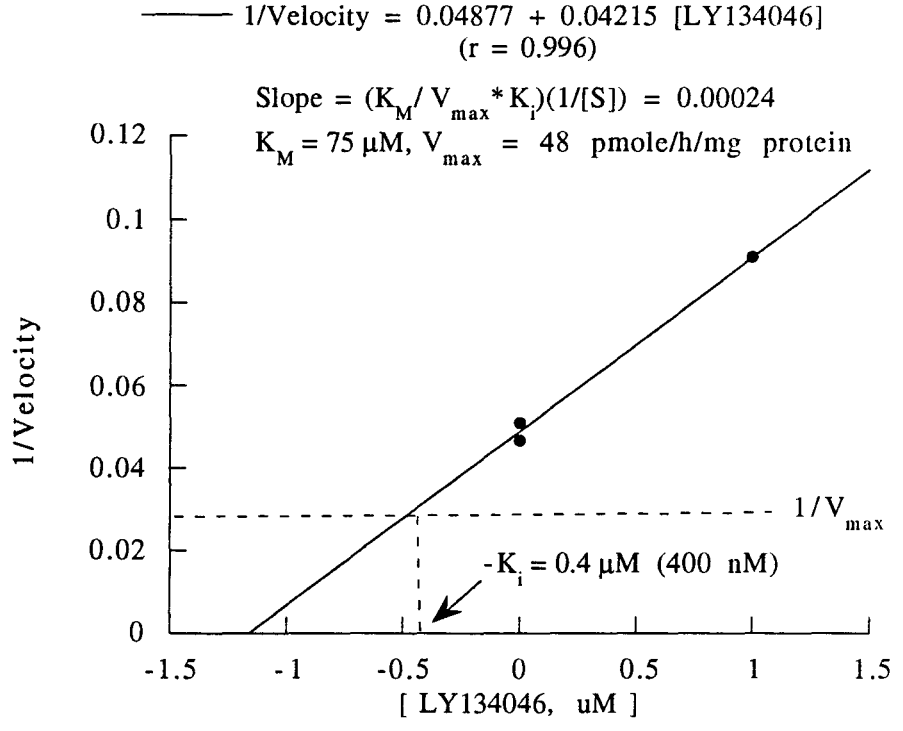


Figure 43. Dixon plot for LY134046 inhibition of ßC-2-NMT activity. Assay at pH 8.5 for 1 hour at 37 °C. Substrates: 100 µM 9-MeNH, 0-1.5 µM LY134046, 105 µM (3.54 µCi) SAM.

Conclusion

These results support the hypothesis that ßC-2-NMT is PNMT or a PNMT-like enzyme. However, the K_i for LY134046 inhibition of ßC-2-NMT is higher than its K_i (24 nM) for inhibition of brainstem PNMT (Fuller et al. 1981).

Experiment 4: Inhibition ßC-2-NMT by PNMT Substrates

Materials and Methods

PNMT N-methylates norepinephrine, octopamine and normetanephrine to form epinephrine, synephrine, and metanephrine, respectively. If ßC-2-NMT and PNMT are equivalent, one would expect ßC-2-NMT activity to be reduced in the presence of these PNMT substrates. Final concentrations of substrates in this study were:

9-MeNH	540 µM
SAM	105 µM
^{3}H-SAM	3.54 µCi
PNMT substrate	1 mM

Assay volume was 370 µl at a final pH of 8.5. Assays were prepared in 1.5 ml polypropylene microcentrifuge tubes using the stock solutions listed:

9-MeNH (1.332 mM stock in buffer "A")	150 µl
SAM (3.89 mM stock in Milli-Q water)	10 µl
^{3}H-SAM (3.02 µCi in Milli-Q water)	10 µl
PNMT substrate (stock in buffer "A")	120 µl
Bovine brain cytosol (0.525 mg protein)	80 µl

Blanks assays were prepared for each PNMT substrate as described in Experiment 2 above. ßC-2-NMT assays were maintained at 37 °C for one hour. The assay work-up and measurement of 2,9-Me_2NH^+ by RP-HPLC were performed as described in Chapter 5. Results are reported as percent of control ßC-2-NMT activity. Control activity represents ßC-2-NMT activity in the absence of PNMT substrate.

Results

The three PNMT substrates evaluated inhibited ßC-2-NMT activity to varying degrees. ßC-2-NMT activity was inhibited to 26% of control by octopamine, to 74% of control by normetanephrine, and to 80% of control by norepinephrine.

Conclusion

These data indicate that PNMT substrates are competing with 9-MeNH for ßC-2-NMT, consistent with the hypothesis that ßC-2-NMT and PNMT are the same enzyme.

Experiment 5: Concentration-Dependent Inhibition of ßC-2-NMT Activity by PNMT Substrates

In this series of experiments a range of PNMT substrate concentrations was utilized -- this range bracketed the literature K_M of the respective substrate for PNMT. Note that the concentration of 9-MeNH was reduced to 100 µM, from the 540 µM concentration used in Experiment 3, since an inhibitor (i.e., the PNMT substrate) is more effective when the substrate (9-MeNH) is present at or below its K_M (75 µM). For each of the PNMT substrates evaluated, the data were plotted in two ways:

(1) percent of control ßC-2-NMT activity as a function of PNMT substrate concentration

(2) a Dixon plot, 1/ßC-2-NMT activity as a function of PNMT substrate concentration -- the K_i for the PNMT substrate (i.e, inhibitor) was determined from this plot.

Materials and Methods: ßC-2-NMT Inhibition by L-Norepinephrine

Final concentrations of substrates and inhibitor (norepinephrine) in the assays were:

9-MeNH	100 µM
SAM	105 µM
^{3}H-SAM	2.31 µCi
L-norepinephrine	0, 10, 40, 100, and 250 µM

The total volume of the assay was 370 µl and the final pH was 8.5. Assays were prepared in 1.5 ml polypropylene microcentrifuge tubes as follows:

9-MeNH (370 µM stock in buffer "A")	100 µl
SAM (1.943 mM stock in Milli-Q water)	20 µl
^{3}H-SAM (2.31 µCi in Milli-Q water)	50 µl
L-norepinephrine (stock in buffer "A")	100 µl
Bovine brain cytosol (1.156 mg protein)	100 µl

L-norepinephrine stock solutions utilized were: 37 µM, 148 µM, 370 µM, 925 µM. Blank assays were prepared containing no 9-MeNH, without and with norepinephrine (250 µM final). Background N-methylation was subtracted from values obtained in complete assays. L-norepinephrine bitartrate was obtained from Sigma Chemical Company (St. Louis, MO); information regarding this reagent is listed in Appendix E.

Results:
ßC-2-NMT Inhibition by Norepinephrine

Norepinephrine inhibited 2N-methylation of 9-MeNH in a concentration-dependent manner. ßC-2-NMT activity was inhibited 50% in the presence of 48 µM norepinephrine (Figure 44). The biphasic nature of the curve suggests the presence of two enzymes that are differentially inhibited by norepinephrine.

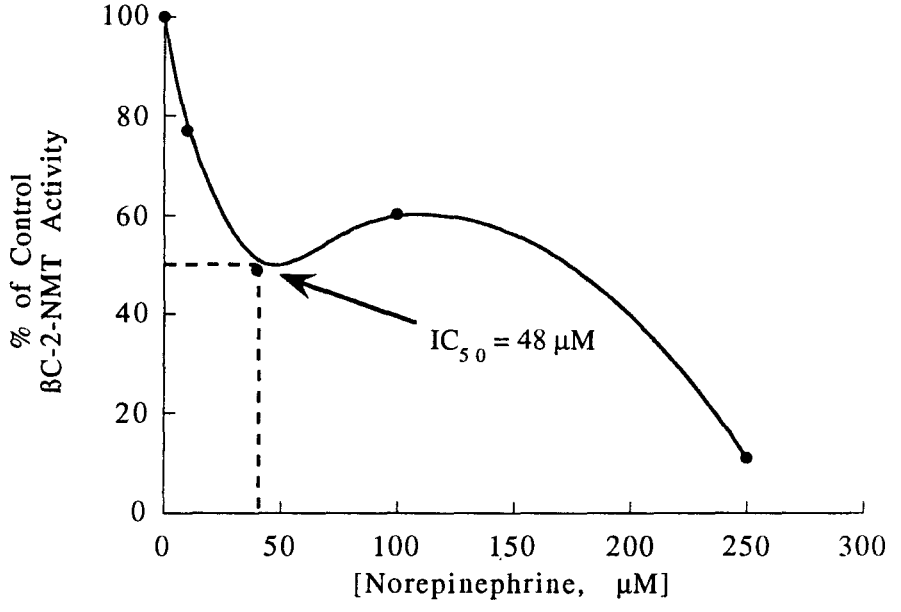


Figure 44. Concentration-dependent inhibition of ßC-2-NMT activity by norepinephrine. Assay at pH 8.5 for 1 hour at 37 °C. Substrates: 100 µM 9-MeNH, 0-250 µM norepinephrine, 105 µM (2.31 µCi) SAM.

The Dixon plot (Figure 45) confirms a concentration-dependent inhibition of ßC-2-NMT activity by norepinephrine. From this experiment alone, the type of inhibition could not be determined. Using the equations for competitive, noncompetitive, and uncompetitive inhibition, the calculated K_i for norepinephrine is probably in the range 21-36 µM.

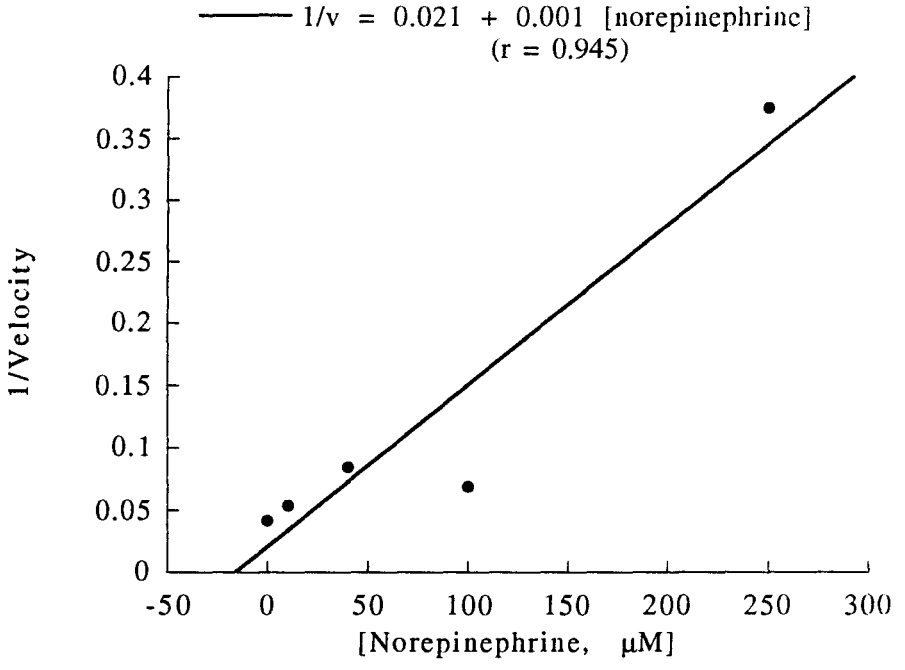


Figure 45. Dixon plot for norepinephrine inhibition of ßC-2-NMT activity. Assay at pH 8.5 for 1 hour at 37 °C. Substrates: 100 µM 9-MeNH, 0-250 µM norepinephrine, 105 µM (2.31 µCi) SAM.

Materials and Methods: ßC-2-NMT Inhibition by DL-Octopamine

The final concentrations of substrates and inhibitor in the assays were:

9-MeNH	100 µM
SAM	105 µM
^{3}H-SAM	3.33 µCi
DL-octopamine	0, 3, 12, 30, and 75 µM

The total volume of the assay was 370 µl and the final pH was 8.5. Assays were prepared in 1.5 ml polypropylene microcentrifuge tubes as follows:

9-MeNH (370 µM stock in buffer "A")	100 µl
SAM (1.943 mM stock in Milli-Q water)	20 µl
^{3}H-SAM (3.33 µCi in Milli-Q water)	50 µl
DL-octopamine (stock in buffer "A")	100 µl
Bovine brain cytosol (1.156 mg protein)	100 µl

DL-octopamine stock solutions utilized were: 11.1 µM, 44.4 µM, 111 µM, 277.5 µM. Blank assays were prepared containing no 9-MeNH, without and with DL-octopamine (75 µM final). Background N-methylation was subtracted from values obtained in complete assays. DL-octopamine hydrochloride was obtained from Sigma Chemical Company (St. Louis, MO); information regarding this reagent is listed in Appendix E.

Results:
ßC-2-NMT Inhibition by DL-Octopamine

Octopamine inhibited 2N-methylation of 9-MeNH in a concentration-dependent manner. ßC-2-NMT activity was inhibited 50% in the presence of 31 µM octopamine (Figure 46). The biphasic curve indicates the presence of two enzymes.

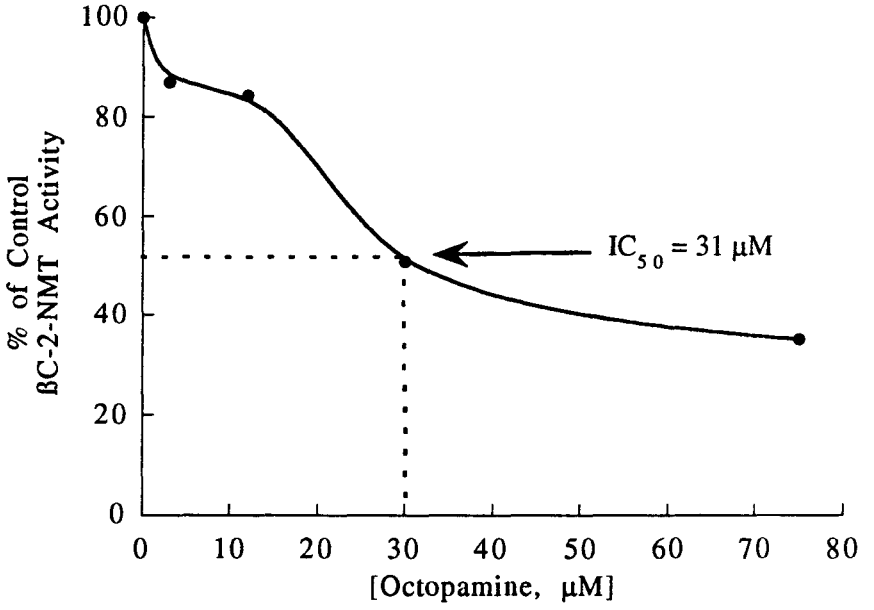


Figure 46. Concentration-dependent inhibition of ßC-2-NMT activity by octopamine. Assay at pH 8.5 for 1 hour at 37 °C. Substrates: 100 µM 9-MeNH, 0-75 µM octopamine, 105 µM (3.33 µCi) SAM.

The Dixon plot also reveals a concentration-dependent inhibition of ßC-2-NMT activity in the presence of octopamine (Figure 47). From this experiment alone, the type of inhibition could not be determined. Using the equations for competitive, noncompetitive, and uncompetitive inhibition, the calculated K_i for octopamine is probably in the range 9.2-21.4 µM.

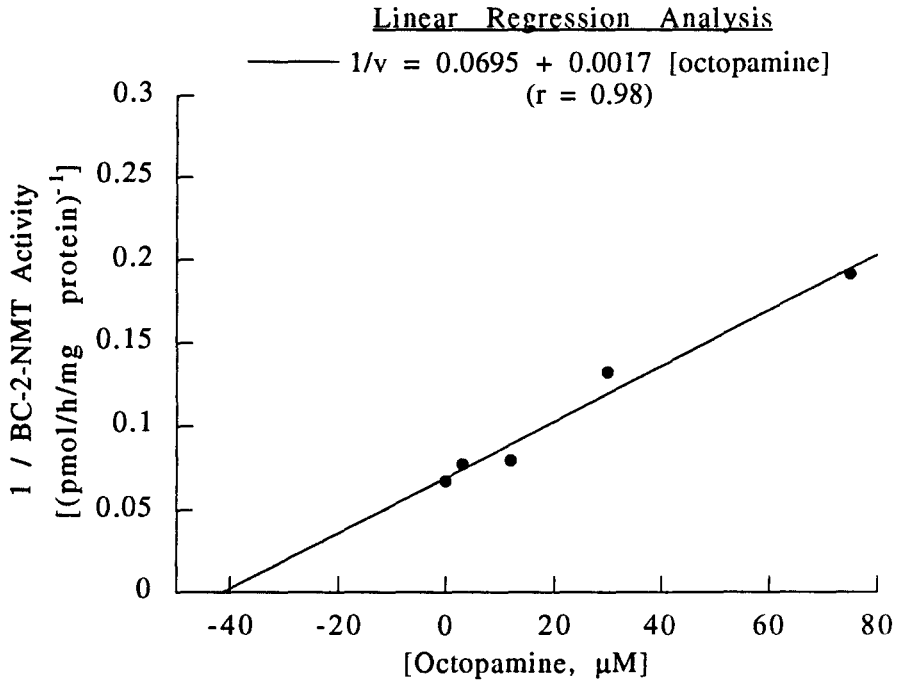


Figure 47. Dixon plot for octopamine inhibition of ßC-2-NMT activity.

Materials and Methods: ßC-2-NMT Inhibition by DL-Phenylethanolamine

The final concentrations of substrates and inhibitor in the assays were:

9-MeNH	100 µM
SAM	105 µM
^{3}H-SAM	2.78 µCi
DL-phenylethanolamine	0, 40, 160, 400, and 1 mM

The total volume of the assay was 370 µl and the final pH was 8.5. Assays were prepared in 1.5 ml polypropylene microcentrifuge tubes as follows:

9-MeNH (370 µM stock in buffer "A")	100 µl
SAM (1.943 mM stock in Milli-Q water)	20 µl
^{3}H-SAM (2.78 µCi in Milli-Q water)	50 µl
DL-phenylethanolamine (stock in buffer "A")	100 µl
Bovine brain cytosol (1.156 mg protein)	100 µl

DL-phenylethanolamine stock solutions utilized were: 148 µM, 592 µM, 1.48 mM, 7.7 mM. Blank assays were prepared containing no 9-MeNH, without and with DL-phenylethanolamine (1 mM final). Background N-methylation was subtracted from values obtained in complete assays. DL-phenylethanolamine was obtained from Sigma Chemical Company (St. Louis, MO); information regarding this reagent is listed in Appendix E.

Results:
ßC-2-NMT Inhibition by Phenylethanolamine

Phenylethanolamine inhibited the 2N-methylation of 9-MeNH in a concentration-dependent manner. ßC-2-NMT activity was inhibited 57% in the presence of 1000 µM phenylethanolamine (Figure 48); concentrations higher than 1000 µM were not evaluated. Linear curve-fitting of these data after plotting of ßC-2-NMT activity as a function of the log of the phenylethanolamine concentration predicts an IC_{50} value of 1750 µM.

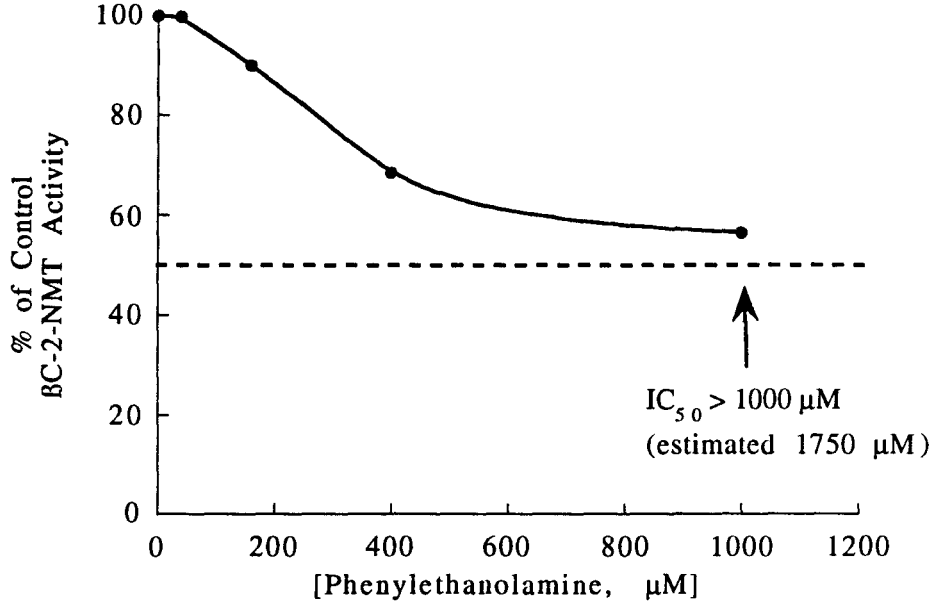


Figure 48. Concentration-dependent inhibition of ßC-2-NMT by phenylethanolamine. Assay at pH 8.5 for 1 hour at 37 °C. Substrates: 100 µM 9-MeNH, 0-1 mM phenylethanolamine, 105 µM (2.78 µCi) SAM.

The Dixon plot confirms a concentration-dependent inhibition of ßC-2-NMT activity in the presence of phenylethanolamine (Figure 49). From this experiment alone, the type of inhibition could not be determined. Using the equations for competitive, noncompetitive, and uncompetitive inhibition, the calculated K_i for octopamine is probably in the range 156-365 µM.

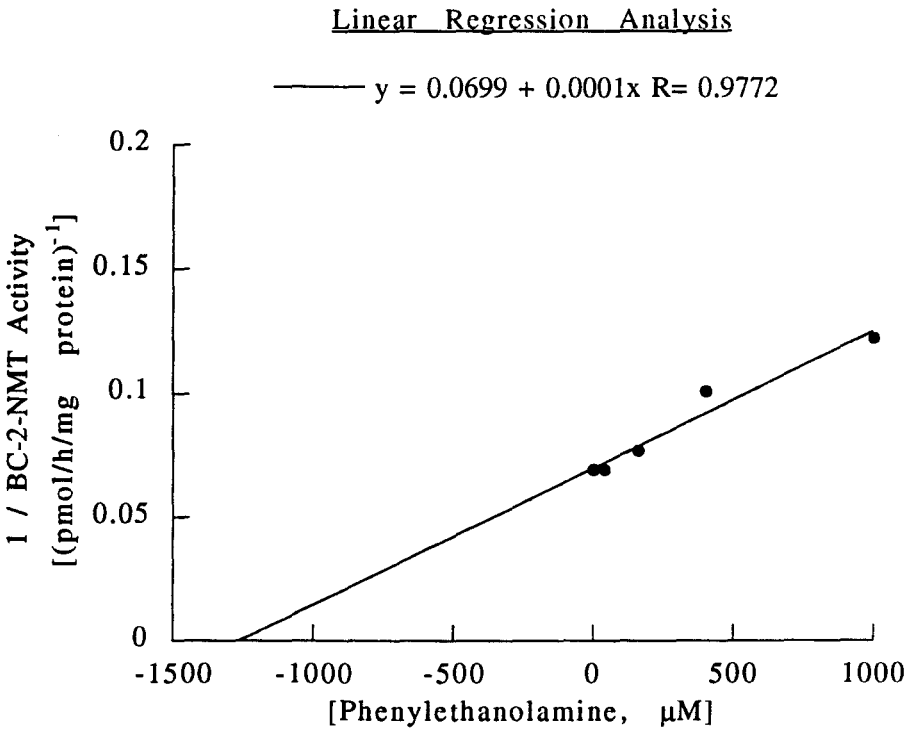


Figure 49. Dixon plot for phenylethanolamine inhibition of ßC-2-NMT activity. Assay at pH 8.5 for 1 hour at 37 °C. Substrates: 100 µM 9-MeNH, 0-1 mM phenylethanolamine, 105 µM (2.78 µCi) SAM.

Conclusion

All three PNMT substrates inhibited ßC-2-NMT activity in a concentration-dependent manner. The rank order Ki's of the three PNMT substrates for ßC-2-NMT inhibition were:

octopamine < norepinephrine < phenylethanolamine

(9-21 μM) (21-36 μM) (156-365 μM)

This rank order is consistent with the K_M's of these same substrates for monkey brainstem PNMT (Lew et al. 1977):

octopamine ≤ norepinephrine < phenylethanolamine

(21 μM) (22 μM) (200 μM)

The hypothesis that ßC-2-NMT and PNMT are the same enzyme is supported by these data. The PNMT substrates with the lowest K_M (norepinephrine and octopamine) exhibit the lowest K_i for inhibition of ßC-2-NMT. These data show inhibition of ßC-2-NMT by PNMT substrates with a rank order that is comparable to the K_M's of these substrates for brain PNMT.

Experiment 6:
ßC-2-NMT Activity in Human Medulla Oblongata and Human Adrenal Medulla

Materials and Methods

Frozen postmortem human medulla oblongata or adrenal medulla was obtained from the Loyola University/Hines VA Tissue Bank, Loyola University Medical Center, Maywood, IL 60253. Frozen tissue was thawed slightly and approximately 100 mg of tissue was removed using a clean tissue punch. Tissue was weighed and placed in a 2 ml glass homogenizer on ice, and was homogenized in 5 volumes (5 µl per mg) of homogenation buffer. Homogenation buffer (pH 7.2) consisted of 150 mM potassium chloride, 20 mM sodium phosphate (Na_2HPO_4), 1 mM ethylenediaminetetraacetic acid disodium, and 1 mM dithiothreitol. Homogenation was accomplished with five up and down strokes of a motor-driven (300 rpm) teflon pestle. Homogenate was transferred to a 1.5 ml microcentrifuge tube, where centrifugation at 10,000*g* for thirty minutes afforded crude particulate and supernatant fractions. Particulate fraction was diluted with about 200 - 400 µl of homogenation buffer prior to use in βC-NMT assays; supernatant was not diluted prior to assay. ßC-NMT activity was not determined in the particulate fraction from medulla oblongata.

The βC-NMT assay, work-up, and RP-HPLC analysis was done according to the methods described in Chapter 5. Prior to plotting these data, background N-methylation (in the absence of ßC) was subtracted from the corresponding data obtained in the presence of ßC.

Results

ßC-2-NMT or ßC-9-NMT activity was not discernable from background when the supernatant fraction from human medulla oblongata was used as the enzyme source.

ßC-2-NMT was activity was highest in the supernatant fraction derived from human adrenal medulla. Very little particulate fraction ßC-2-NMT activity was detected. Particulate and supernatant ßC-9-NMT activity was approximately 4-fold lower than supernatant ßC-2-NMT activity (Figure 50).

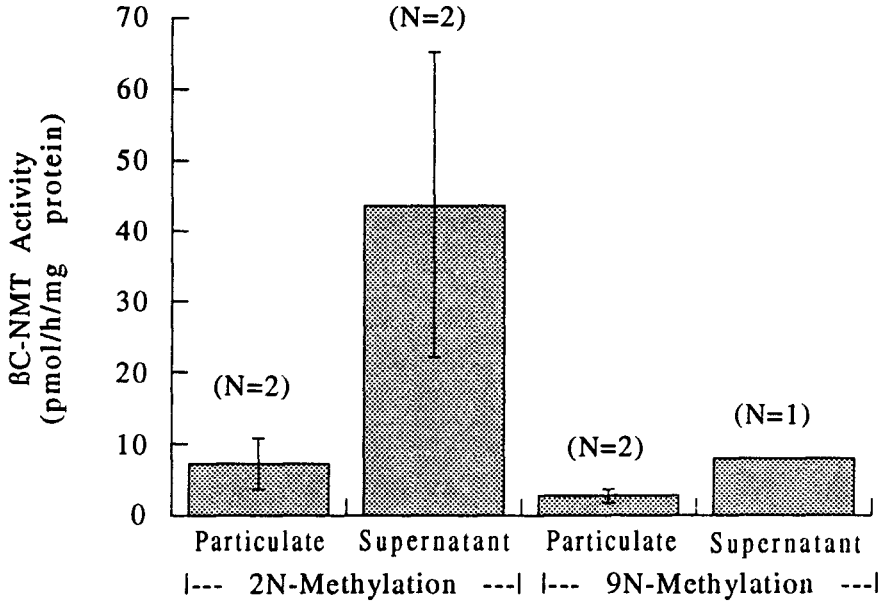


Figure 50. ßC-NMT activities in particulate and supernatant fractions from human adrenal medulla.

A large tritiated peak was present in blank particulate fraction assays; this peak was not observed when ßC-substrates were included in the incubations. The retention time of the unknown peak was approximately 2 minutes later than the retention time for 2,9-Me_2NH^+. The peak corresponded to the incorporation 61.6 ± 17.6 pmol of tritiated methyl group/h/mg protein.

Conclusions

PNMT catalytic activity is present in the medulla oblongata and the adrenal medulla (Axelrod 1971; Kitabchi et al. 1969; Kopp et al. 1979). The absence of ßC-2-NMT activity in the human medulla oblongata is therefore inconsistent with the idea that ßC-2-NMT is PNMT.

However, the presence of supernatant ßC-2-NMT activity in the adrenal medulla -- a tissue that exhibits high PNMT activity -- supports the hypothesis that ßC-2-NMT and PNMT are the same enzyme.

Experiment 7: Measurement of ßC-2-NMT Activity in Bovine Brain Cytosol After Immunoprecipitation of PNMT

Materials and Methods

If ßC-2-NMT is synonymous with PNMT, one would predict a loss in ßC-2-NMT after immunoprecipitation of PNMT from bovine brain cytosol. The protocol for this study was a modification of the method described by Anderson and Blobel (1983).

PNMT antibody was purchased from Incstar Corporation (Catalog #22572, Stillwater, MN). This polyclonal antisera was obtained from rabbits immunized with bovine adrenal PNMT. According to Incstar, this anti-PNMT cross-reacts with brain PNMT from various species. ImmunoPure® Immobilized Protein A/G Gel was obtained from Pierce (Catalog #20421, Rockford, IL). This gel has protein A (isolated from *Staphylococcus aureus*) and protein G (from Streptococcus species) covalently attached to agarose gel. Proteins A and G bind the Fc portion of the constant region on immunoglobulins derived from numerous species. Bovine brain cytosol was concentrated using a Centricon 30 concentrator. The theoretical interaction of PNMT from bovine brain cytosol, anti-PNMT, and protein A/G is diagramed in Figure 51.

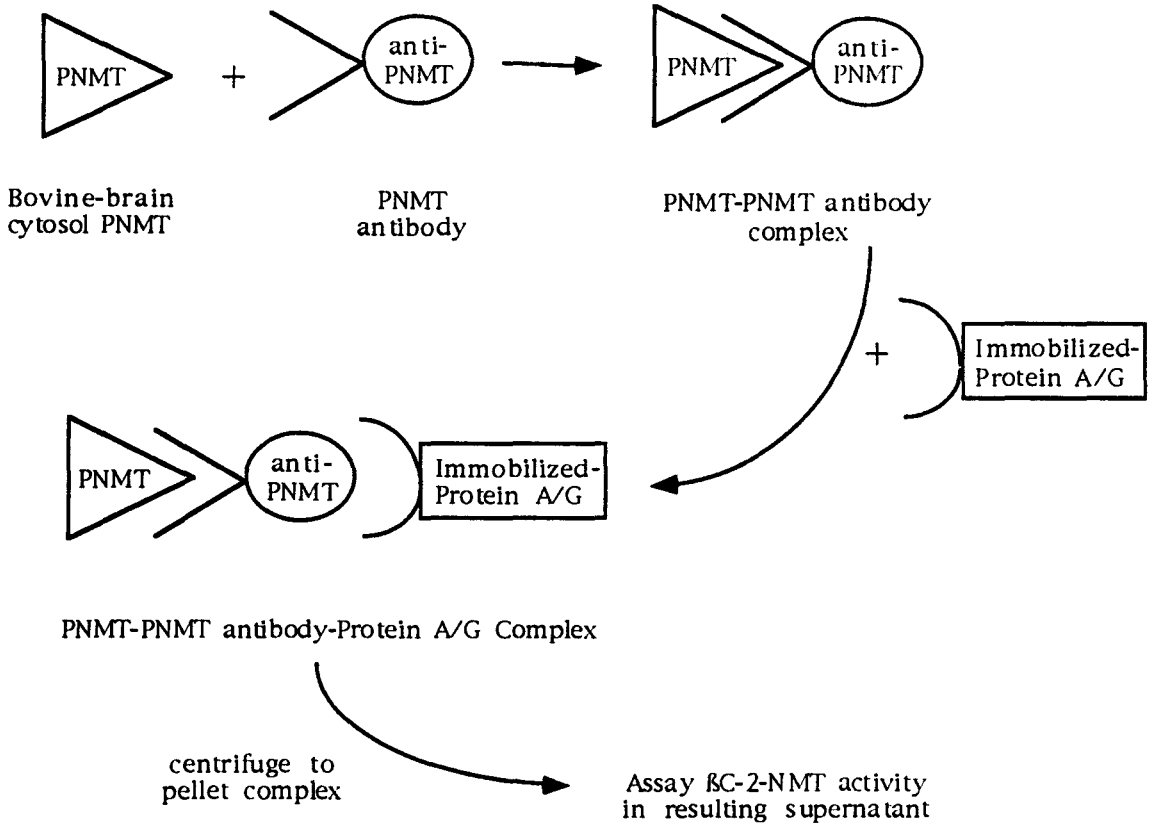


Figure 51. Proposed immunoprecipitation of PNMT from bovine-brain cytosol.

The contents of each of two vials containing lyophilized anti-PNMT were reconstituted using 100 µl Milli-Q water, and the resulting solutions combined. Concentrated bovine brain cytosol (60 µl) was added to each of seven 1.5 ml polypropylene microcentrifuge tubes. Tubes designated as "control-cytosol" also received 80 µl of 10 mM Na_2HPO_4 buffer (pH 7.3); total volume was therefore 140 µl. Anti-PNMT was added to the remaining four tubes -- 10, 30, 50, or 80 µl of antisera were added -- final volume of these four tubes was also brought to 140 µl with buffer. The tubes were gently vortexed and then set at room temperature for one hour, prior to refrigeration for 19 hours. The samples were centrifuged at 12,000*g* for 15 minutes, and portion of the resulting supernatants (130 µl) was transferred to clean 1.5 ml microcentrifuge tubes.

Immobilized Protein A/G was equilibrated in 100 mM Na_2HPO_4 (pH 7.3) and then diluted with buffer to yield a 1:1 suspension of gel:buffer. This suspension (800 µl) was added to each of five clean 1.5 ml microcentrifuge tubes. Tubes were centrifuged to pack the gel (400 µl), and resulting supernatant was discarded. Equilibration buffer (50 µl) was added to each of these tubes, as well as to two empty tubes. "Control-cytosol" from the previous step -- i.e., cytosol not exposed to anti-PNMT -- was added to each of the 2 tubes containing only equilibration buffer; these controls were never exposed to anti-PNMT or Protein A/G. The final control was designated "A/G-Control": it was prepared by adding "control-cytosol" to Protein A/G gel. To the remaining four tubes that contained Protein A/G gel, the supernatants resulting from anti-PNMT incubation were added. All tubes were agitated gently on a lab rocker for 3 hours at room temperature. The mixture was

centrifuged at 12,000*g* for 15 minutes to pack the gel. In theory the packed gel should contain the PNMT - PNMT antibody - Protein A/G agarose complex. A portion of the resulting supernatant (150 µl) was transferred to clean microcentrifuge tubes, and 100 µl of each of these solutions were analyzed for ßC-2-NMT activity. ßC-2-NMT assays and work-up were done as described in Chapter 5.

Results

ßC-2-NMT activity was apparently unaffected by immunoprecipitation of PNMT, because supernatant from bovine-brain cytosol that was exposed to anti-PNMT and immobilized protein A/G exhibited the same ßC-2-NMT activity as supernatant from the protein A/G control incubation.

Conclusion

Assuming that PNMT was precipitated from the cytosol, this experiment does not support the hypothesis that ßC-2-NMT and PNMT are the same enzyme.

Summary of Results

The results of experiments (a) through (e), to be discussed further in detail, are consistent with the hypothesis that ßC-2-NMT and PNMT are the same enzyme:

(a) purified bovine adrenal PNMT 2N-methylates 9-MeNH

(b) LY134046 inhibits ßC-2-NMT in concentration-dependent manner

(c) PNMT substrates inhibit ßC-2-NMT in a concentration-dependent fashion

(d) supernatant fraction derived from human adrenal medulla catalyzes the 2N-methylation of 9-MeNH

(e) demonstration of PNMT activity in bovine-brain cytosol as determined by the conversion of norepinephrine to epinephrine in the presence of SAM

However, the results of experiments (f) and (g), taken at face value, do not support the hypothesis that ßC-2-NMT and PNMT are equivalent enzymes:

(f) the apparent absence of ßC-2-NMT activity in the supernatant fraction isolated from human medulla oblongata

(g) the lack of effect on ßC-2-NMT activity of immunoprecipitation of PNMT from bovine-brain cytosol

Discussion

PNMT 2N-Methylates 9-MeNH

The most compelling evidence to support the equality of ßC-2-NMT and PNMT is that purified bovine adrenal PNMT catalyzes the 2N-methylation of 9-MeNH, but not 2-MeNH+. Similarly, 9-MeNH is 2N-methylated 4-fold more effectively than 2-MeNH+ is 9N-methylated in the presence of the crude supernatant fraction derived from human adrenal medulla -- a tissue with high PNMT activity. In addition, these data suggest that ßC-2N-methylation and ßC-9N-methylation may be catalyzed by two different enzymes. The specific activity of bovine adrenal PNMT toward 9-MeNH was 1.8% (17.8 pmol/h/unit PNMT) of PNMT's labeled activity toward a more standard substrate, normetanephrine (1 nmole/h/unit PNMT). A comparable ratio is obtained for bovine brain cytosol when the V_{max} for PNMT (1.8 nmol epinephrine/h/mg protein) is compared to the V_{max} for ßC-2-NMT (48 pmol/h/mg protein) under identical assay conditions -- 9-MeNH methylation is 2.7% of that for norepinephrine. These observations imply that 9-MeNH is a poor substrate for PNMT, but that PNMT can nonetheless 2N-methylate a ßC.

ßC-2-NMT Activity in the Adrenal Medulla

Supernatant ßC-2-NMT activity in human adrenal medulla was 43.7 pmol/h/mg protein. Data from Chapter 5 of this dissertation show that supernatant ßC-2-NMT activity in human brain varied regionally from 13.3 - 31.3 pmole/h/mg protein. Taken together these experiments indicate that ßC-2-NMT activity in the human adrenal medulla is 140 - 328% of ßC-2-NMT

activity in human brain. These results are consistent with those reported for human PNMT, where adrenal medulla activity was 450% of cerebrum activity (Kitabchi and Williams 1969). The presence of the large unknown peak in the blank assay (without ßC substrate) from the particulate fraction of adrenal medulla was surprising. It is postulated that this peak represents the methylated product of an endogenous ßC or catecholamine -- an endogenous substrate whose methylation is inhibited in the presence of 9-MeNH or 2-MeNH+.

Inhibition of ßC-2-NMT by PNMT Substrates and a PNMT Inhibitor

The concentration-dependent inhibition of ßC-2-NMT by the specific PNMT inhibitor, LY134046, supports the hypothesis that the ßC-2-NMT and PNMT are the same enzyme. The K_i for inhibition of rat brain PNMT by LY134046 is 24 nM (Fuller et al. 1981), lower than the 400 nM K_i value for LY134046 inhibition of ßC-2-NMT reported here. The K_i for an inhibitor is dependent upon assay conditions, and those conditions differed between this study and that of Fuller et al. (1981). Specifically, Fuller utilized partially-purified PNMT derived from rat brainstem supernatant, and an assay pH of 7.0. The present study used crude supernatant fraction from whole bovine brain, and an assay pH of 8.5. It is reasonable to speculate that differences in species and assay pH between experiments could account for the differences in K_i values for LY134046 determined in the two studies

Three distinct PNMT substrates -- norepinephrine, octopamine, and phenylethanolamine -- inhibited ßC-2-NMT activity in a concentration-dependent manner, indicating a competition between 9-MeNH and those PNMT

substrates for the same enzyme. The biphasic nature of the inhibition plots suggests the presence of two different enzymes, or isozymes of the same enzyme, that are differently inhibited in the presence of the PNMT substrates. Dixon plots of reciprocal ßC-2-NMT activity as a function of PNMT substrate (ßC-2-NMT inhibitor) concentration were each linear. The mechanism of inhibition by these substrates cannot be confirmed without further experiments done at various 9-MeNH concentrations.

Recall that when SAM donates its methyl group to a substrate during a N-methyltransferase-catalyzed reaction, the products are the N-methylated substrate and SAH. SAH inhibits most SAM-dependent methyltransferases in a competitive manner. It is therefore reasonable to consider that ßC-2-NMT inhibition in the presence of PNMT substrates could be due to the SAH that is formed as PNMT methylates its substrates. When norepinephrine alone was utilized in the PNMT assay, the calculated V_{max} for epinephrine formation was 1.8 nmoles/h/mg protein -- an equivalent amount of SAH would be the co-product of this reaction. This amount of SAH (1.8 nmoles/h/mg protein) corresponds to a concentration of 4.9 µM SAH in the ßC-2-NMT assay. ßC-2-NMT activity would only be inhibited to 72% of control activity at this SAH concentration (refer to Chapter 3 for SAH inhibition curve), quite different than the 10% of control ßC-2-NMT activity observed in the norepinephrine inhibition experiment. Moreover, SAH probably does not accumulate during a methyltransferase reaction, since it may be removed by the action of cytosolic SAH hydrolase and adenosine deaminase to form inosine (Chiang et al. 1996). Therefore it is improbable that SAH accounts for the observed inhibition of ßC-2-NMT in the presence of norepinephrine.

ßC-2-NMT Activity in the Medulla Oblongata

The lack of ßC-2-NMT activity in the medulla oblongata is disturbing since this brain region ("C1 area", dorsal to the mid portion of the inferior olive) has a high level of PNMT activity (Fuller 1982; Kopp et al. 1979; Lew et al. 1977; Mefford 1988), and one would expect a high level of ßC-2-NMT activity in this brain region if PNMT and ßC-2-NMT are the same enzyme. The supernatant used in my experiment was not dialyzed, and it is possible that dialyzable endogenous compounds (e.g., epinephrine or other catecholamines) may have been present that inhibited ßC-2-NMT activity. PNMT is inhibited at high concentrations of endogenous catecholamines, including epinephrine and norepinephrine (Pohorecky et al. 1973). Unfortunately a positive control for PNMT activity -- conversion of norepinephrine to epinephrine -- was not included in this study. This experiment should be repeated using 2-3 different medulla oblongatas in which the supernatant fraction is dialyzed to remove endogenous inhibitors. In addition, positive controls should be included in each case to assure that PNMT activity with norepinephrine as the substrate is detectable under the assay conditions.

Immunoprecipitation of PNMT

Immunoprecipitation of PNMT, followed by the assessment of ßC-2-NMT activity in the resulting supernatant, is a practical approach to addressing the equivalency of PNMT and ßC-2-NMT. It is possible that the results reported here are false-negative results due to technical problems such as insufficient precipitation of PNMT. Therefore, the experiment should be repeated with a positive control that would assure that adequate anti-PNMT is utilized.

Overall Conclusions

In summary, taken together these data provide strong evidence that ßC-2-NMT activity is due to PNMT. Purification of ßC-2-NMT and comparison of its properties and primary sequence to that of PNMT would provide definitive proof of their equivalency. Assuming that ßC-2-NMT is equivalent to PNMT, there is substantial circumstantial evidence that PNMT-catalyzed formation of cytotoxic 2-MeßCs+ may play a key role in the pathogenesis of PD. This evidence is discussed in subsequent text. A new hypothesis regarding the etiology of Parkinson's disease -- a hypothesis that incorporates a role for PNMT -- is presented at the end of that discussion.

Evidence That is Consistent With a Role For PNMT in the Pathogenesis of Parkinson's Disease

PNMT Co-Localization With PD Pathology

PNMT catalytic activity and immunoreactivity are present in regions of human brain that are affected by PD. Primary pathological findings in PD include nerve cell loss and the presence of Lewy bodies in the substantia nigra, locus ceruleus, and peripeduncular nucleus (Forno 1995). These same human brain nuclei exhibit PNMT activity ranging from trace activity in the interpeduncular nucleus to an activity of 2.6 pmole/h/mg protein in the locus coeruleus (Kopp et al. 1979). In addition, several other loci that frequently demonstrate pathology in PD also express PNMT, such as the hypothalamus, dorsal motor nucleus of the vagus, raphe nuclei, and the olfactory tubercle. It is conceivable that PNMT-catalyzed formation of neurotoxic 2-MeßCs+ in these nuclei contributes to the specific neuronal loss in PD. Further investigation

may reveal a high degree of correlation between PNMT (ßC-2-NMT) activity and brain pathology in PD.

Consistent with this speculation, Gai et.al (1993) reported a selective loss of PNMT-positive neurons in PD. Specifically, in PD medulla oblongata the number of neurons in the C1 cell group was reduced to 47% of control, and in the C3 area numbers were 12% of control values. It is plausible that this decrease in cell number could be a result of PNMT-catalyzed formation of neurotoxic 2-MeßCs+ within the C1 and C3 neurons early in the disease process. Cell death induced by 2-MeßCs+ would manifest as a loss in PNMT-positive cells. In addition, PNMT activity is reduced in the hypothalamus and pallidum of PD brains (Nagatsu et al. 1977, 1984), areas that express PNMT, and consequently could generate 2-MeßCs+. Epinephrine in the CSF from PD patients is either unchanged (Tohgi et al. 1990) or decreased (Eldrup et al. 1995), the later being consistent with decreased brain PNMT activity resulting from loss of PNMT-positive neurons.

Adrenal Medulla Pathology in PD

The adrenal medulla exhibits high PNMT activity compared to other tissues. Evidence presented in this chapter demonstrates the presence of substantial ßC-2-NMT activity and trace ßC-9-NMT activity in human adrenal medulla. The sequential action of ßC-2-NMT (PNMT) followed by ßC-9-NMT activity would form 2-MeßCs+ and then 2,9-Me_2ßCs+ in the adrenal medulla -- toxins that may kill those cells in which they are formed. Interestingly, many PD patients demonstrate pathology, including Lewy bodies, in the adrenal medulla (den Hartog Jager 1970; Stoddard 1994). In a recent review article addressing the condition of the adrenal medulla in PD (Stoddard 1994),

Stoddard concluded that adrenal tissue, plasma, and urinary catecholamines are decreased in PD cases relative to control subjects. These results would be consistent with those expected subsequent to PNMT-catalyzed formation of toxic MeßCs+ in the adrenal medulla. As the PNMT-positive cells die due to the toxicity of MeßCs+, the capacity of the medulla to synthesize epinephrine would also be expected to decrease. Perhaps this inability to synthesize epinephrine contributes to the hypotension observed in idiopathic PD. It is noteworthy that PC12 cells -- an immortalized cell-line derived from adrenal tissue -- are susceptible to the toxicity induced by MeßCs+ (Cobuzzi et al. 1994).

Peripheral ßC-2-NMT (PNMT) Activity

Demonstration of ßC-2-NMT and ßC-9-NMT activity in the adrenal medulla is interesting because it implies that MeßCs+ could be formed peripherally following exposure to ßCs (substrates for ßC-2-NMT). Furthermore, Matsubara recently reported that 2-MeNH+ and 2,9-Me_2NH^+ are able to penetrate the blood-brain barrier (Matsubara et al. 1996). Taken together these observations suggest that 2-MeßCs+ formed in adrenal medulla have access to the brain. Within the brain 2-MeßCs+ could be selectively transported into dopaminergic neurons via the dopamine transporter, and therein exert their toxic effects (Drucker et al. 1990).

PD, Stress, and PNMT

Physiological stress results in increased brain and adrenal PNMT activity (Turner et al. 1979; Turner et al. 1978). Assuming that ßC-2-NMT is PNMT, then in theory during a stressful situation 2-MeßC+ formation would be concomitantly increased (if substrates were present). Eatough et al. (1990)

reported that PD patients experienced significantly greater extreme stress during life, especially in childhood, compared to control individuals. Perhaps also relevant is the report which indicates a higher incidence of PD in survivors of prisoner of war camps (Gibberd et al. 1980). It is possible that the association between extreme physiological stress and PD is due to excessive formation of MeßCs+ resulting from increased PNMT activity.

The PNMT Hypothesis of Parkinson's Disease

This expanded hypothesis includes an early, and possibly precipitating, role for PNMT in the pathogenesis of Parkinson's disease (Figure 52). This hypothesis states that physiological stress, which is suggested as a predisposing factor to PD, increases PNMT (ßC-2-NMT) activity both in the adrenal medulla and brain (Turner et al. 1978, 1979). Norharman, a substrate for ßC-2-NMT, is elevated in the plasma of PD patients (Kuhn et al. 1995). PNMT (ßC-2-NMT) may catalyze the formation of cytotoxic 2-MeßCs+ (e.g., 2-MeNH+) peripherally and centrally in PNMT-containing cells. The MeßCs+ could remain in the cells in which they were formed, or may exit and enter dopaminergic neurons via the dopamine transporter (Drucker et al. 1990). These 2-MeßCs+ and/or their N,N-dimethylated metabolites (2,9-Me_2ßCs+) inhibit mitochondrial respiration (Albores et al. 1990; Fields et al. 1992), thereby depleting ATP and causing neuronal death. Mitochondrial inhibition may also increase oxidative stress within affected neurons (Takeshige and Minakami 1979; Zeevalk and Bernard 1996; Zoccarato et al. 1988) -- neurofilaments may become oxidatively damaged, and consequently prone to aggregation and Lewy body formation (Hill 1996; Montine et al. 1995; Troncoso et al. 1995).

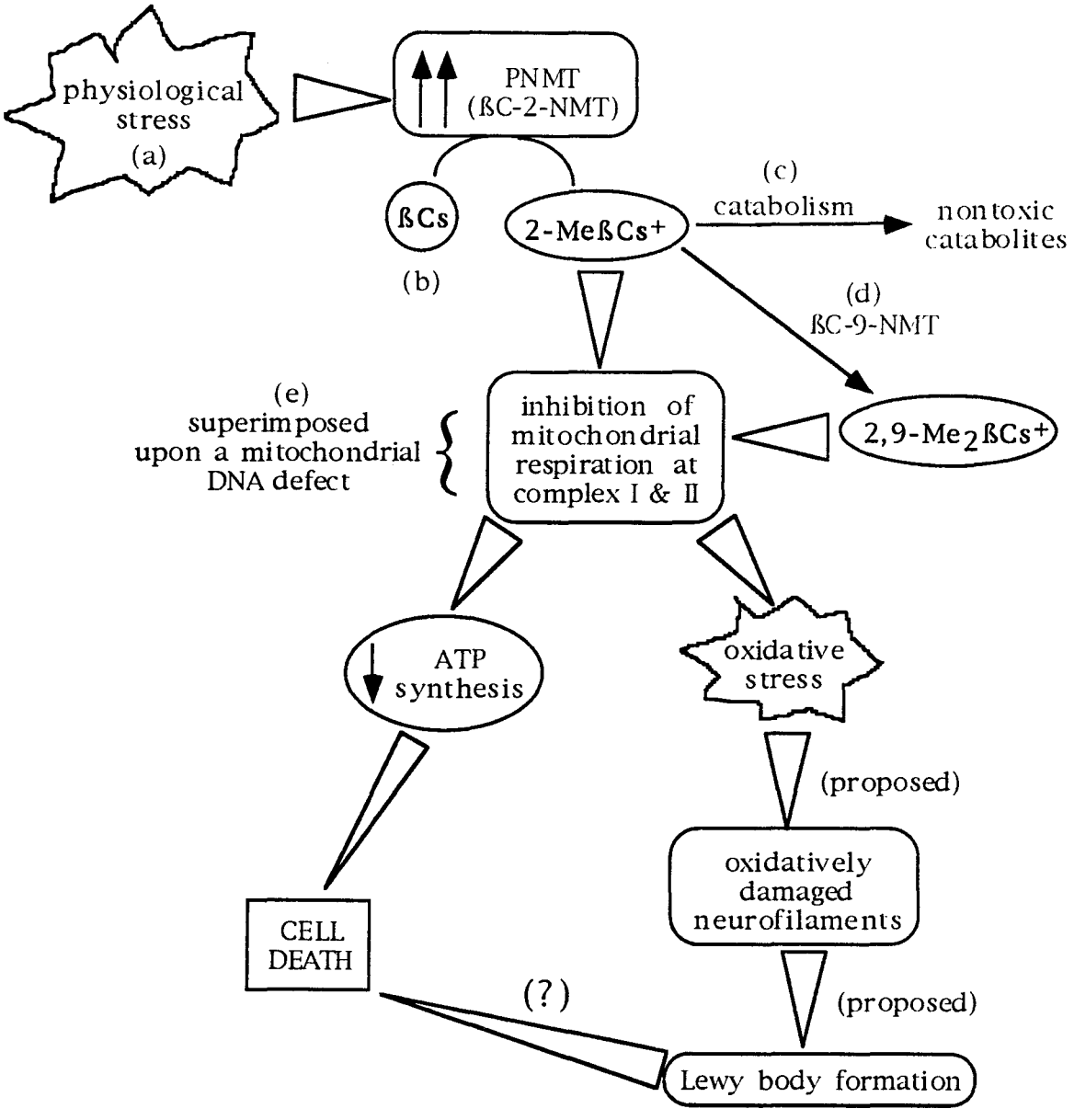


Figure 52. The PNMT hypothesis for Parkinson's disease: a proposal that the risk for development of PD increases as the number of factors (a-e) present increases. Each of this factors has an association with PD (see test for details). Factors are: (a) increased stress, (b) increased load of ßC substrates, (c) decreased xenobiotic metabolism, (d) increased ßC-9-NMT activity, (e) decreased mitochondrial function due mtDNA defect.

CHAPTER 7

SUMMARY AND SPECULATIONS

Summary

It is hypothesized that ß-carboline-N-methyltransferases (ßC-NMTs) and/or the neurotoxic products of their catalytic activities, N-methylated ß-carbolinium cations (MeßCs$^+$), may play a role in the pathogenesis of idiopathic Parkinson's disease (Collins 1994; Collins and Neafsey 1985). The primary focus of this dissertation was ß-carboline-2N-methyltransferase activity (ßC-2-NMT), but ß-carboline-9N-methyltransferase (ßC-9-NMT) activity was also studied, although much less rigorously.

The most important finding was that in the frontal cortex of Parkinson's disease (PD) brains, supernatant ßC-9-NMT activity is increased relative to activity in control tissue. This implies that higher levels of 2,9-Me_2ßCs$^+$ may be formed in PD brain compared to normal brain. It is possible that brain ßC-9-NMT activity is globally elevated in PD -- particularly early in the disease process -- but that with disease progression, elevated ßC-9-NMT activity becomes undetectable due to MeßC+-induced death of the cells that originally contained the enzyme.

An equally important and exciting finding is the detection of relatively high ßC-2-NMT activity in human adrenal medulla, suggesting that neurotoxic 2-MeßCs+ may be formed in this tissue. There are at least four possible fates for adrenal-derived 2-MeßCs+: (1) liver metabolism to nontoxic compounds, (2) toxic effects on adrenal cells, (3) entry into the central nervous system, and (4) conversion to more toxic 2,9-Me_2ßCs+ by the action of adrenal and brain ßC-9-NMT. Notably, this dissertation is the first report describing ßC-9-NMT activity in human brain, as well as the measurement of ßC-2-NMT and ßC-9-NMT activities in human adrenal medulla.

The presence of ßC-2-NMT activity in the adrenal medulla is consistent with a new hypothesis proposed in this dissertation (Figure 52). A fundamental aspect of this hypothesis is that ßC-2-NMT activity is due to the epinephrine-synthesizing enzyme, phenylethanolamine-N-methyltransferase (PNMT) or an enzyme very similar to PNMT. This hypothesis is supported by three key observations: (1) purified bovine adrenal PNMT catalyzes the 2N-methylation of 9-methylnorharman, (2) LY134046, a selective inhibitor of PNMT, also inhibits ßC-2-NMT, and (3) substrates of PNMT inhibit ßC-2-NMT activity in a concentration-dependent manner.

Another observation that may have relevance to PD is that ßC-2-NMT activity is increased 2-fold in the presence of iron (Fe^{2+} or Fe^{3+}). Iron concentration is high in the substantia nigra compared to other brain regions, and its level appears to be higher in PD brain compared to control tissue. Perhaps elevated nigral iron in PD brain increases ßC-2-NMT activity, which results in increased local formation of neurotoxic 2-MeßCs+ there.

Only a partial purification of ßC-2-NMT was achieved in this study. However, the observations and suggestions presented here, regarding ßC-2-NMT purification, should be useful if this project is continued by another investigator -- however the development of less labor-intensive assay should be a priority. The inhibition experiments presented in Chapter 6 indicate that ßC-2-NMT activity may be due to two enzyme activities; this would also complicate purification of ßC-2-NMT activity.

Speculations

As summarized in Figures 52 and 53, it is hypothesized that PNMT may play a key role in the pathogenesis of PD. Stress-induced elevation of PNMT (presumably ßC-2-NMT) could result in excessive formation of 2-MeßCs$^+$ both in the adrenal medulla and brain. Resultant 2-MeßCs$^+$ could then be 9N-methylated by ßC-9-NMT -- the activity that is elevated in PD brain -- to the more toxic 2,9-Me_2ßCs$^+$. Local formation of MeßCs$^+$ may culminate in cell death due to neurotoxin-induced mitochondrial inhibition. In addition, 2-MeßCs$^+$ may cross the plasma membrane and subsequently enter other cells either passively or actively via the dopamine transporter. Active transport into dopaminergic cells may explain why nigrostriatal neurons are more severely affected in PD. It is interesting to consider that ßC-2-NMT (PNMT) may also catalyze the formation of other proposed N-methylated dopaminergic neurotoxins, such as N-methylated derivatives of tetrahydroisoquinolines (Naoi et al. 1989b). Perhaps an early event associated with the pathogenesis of PD is N-methylation of several distinct classes of azaheterocycles (including ßCs) to form mildly to moderately neurotoxic derivatives. Chronic exposure to these compounds over several years could eventually kill sufficient

dopaminergic neurons to manifest the clinical symptoms of Parkinson's disease.

It is fascinating to consider the role that microbe-catalyzed formation of 2-MeßCs+ may play in the pathogenesis of PD. Both *Kitasatosporia setae* and *Nocardia asteroides* are Actinomycetes, which are vegetable parasites that reside in soil. *Kitasatosporia setae* cultures transform norharman to 2-MeNH+ (Peczynska-Czoch et al. 1987), and mice inoculated with *Nocardia asteroides* develop a parkinsonian-syndrome (Kohbata and Beaman 1991). It is plausible that *Nocardia asteroides* may also be able to produce neurotoxic 2-MeßCs+ from simple ßCs. Thus human ingestion or inhalation of these soil pathogens could provide "additional enzymatic machinery" able to produce neurotoxic 2-MeßCs+. Perhaps the association between rural living and PD is closely related to exposure to these soil (groundwater?) pathogens. Regarding this speculation, two questions arise: (1) does *Nocardia asteroides* possess ßC-2-NMT activity, and/or (2) in PD, is there evidence of *Kitasatosporia setae* infection?

Overall Model of Parkinson's Disease

An overall model for the etiology of idiopathic Parkinson's disease is presented in Figure 53 -- with ßC-NMT-catalyzed formation of MeßCs+ playing a key role. Steps that are marked with an asterisk (*) were either discovered or studied during the development of this dissertation. Steps marked with a question-mark (?) have been postulated by the author or proposed by other investigators.

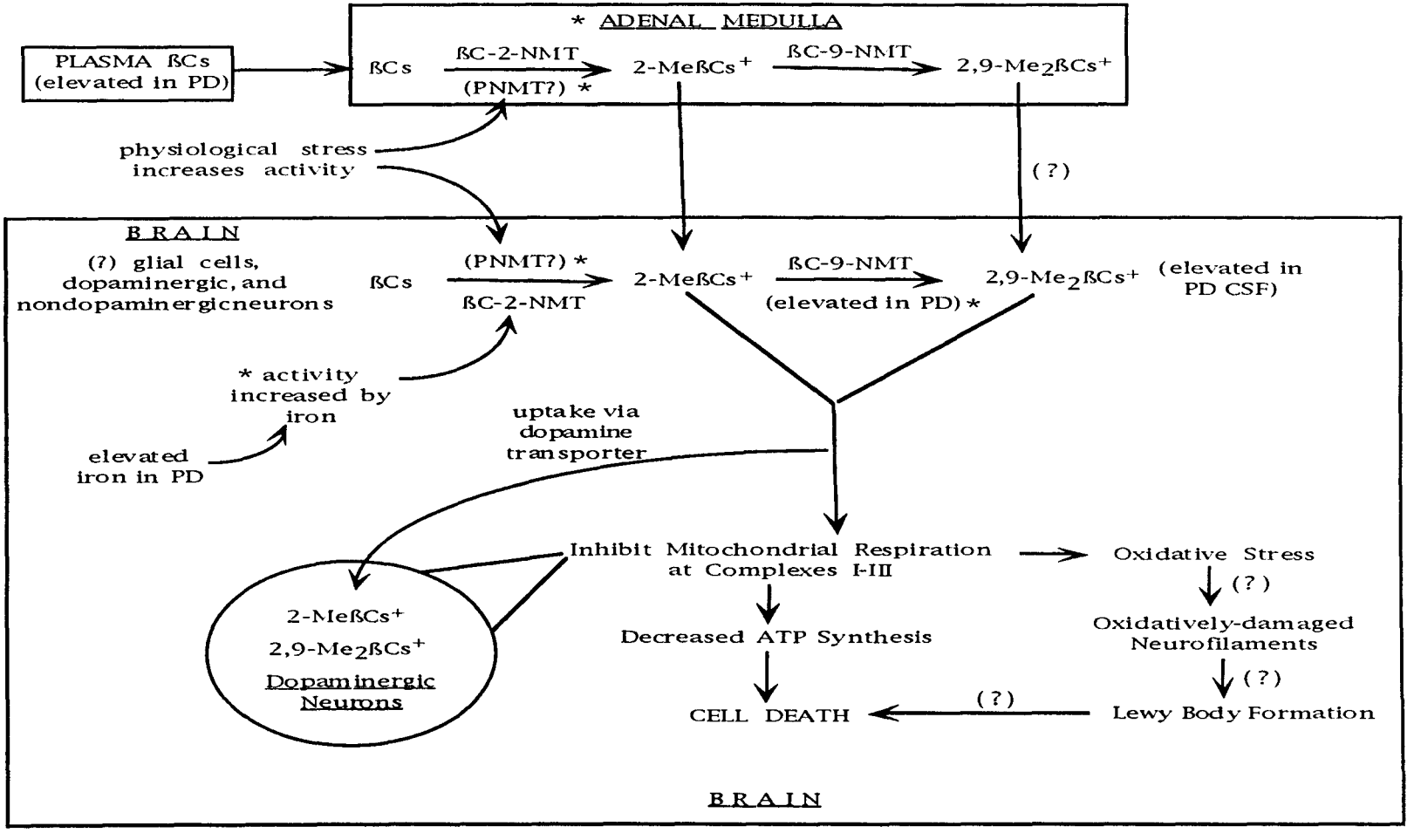


Figure 53. N-methylated-ß-carboline hypothesis of Parkinson's disease.

APPENDICES

APPENDIX A

APPENDIX A:
PROTEIN ASSAYS

Solution preparation was modified from Scopes (1994d).

Lowry Reaction

Solution A: Copper Sulfate Solution
Dissolve 500 mg copper sulfate heptahydrate ($CuSO_4 \cdot 5H_2O$) and 1 g sodium citrate tribasic in 100 ml of Milli-Q water. Scopes states that is more stable than the commonly used copper tartrate solution and may be stored indefinitely.

Solution B: Sodium Carbonate Solution
Dissolve 10 g of sodium carbonate (Na_2CO_3) and 2 g sodium hydroxide (NaOH) in 500 ml Milli-Q water

.

Solution C: Make fresh the day of protein assay, by mixing 50 ml (50 parts) of solution B and 1 ml (1 part) of solution A. This solution will be very basic (≥ p H 10). Use 2 ml of this solution per protein assay.

Solution D: Dilute Folin-Ciocalteau reagent with an equal volume of Milli-Q water. Prepare a total volume that is sufficient to carry out all assay -- use 200 µl per protein assay.

Standard and Sample Preparation

Standard Preparation:
Bovine serum albumin (BSA, Fraction V) was dissolved in 1 mM sodium dodecyl sulfate (SDS) to a final concentration of 1 µg/µl (BSA stock)). Tubes for generation of the protein standard curve were prepared according to Table 23. Standards were prepared in triplicate. The reference (blank) sample was the same as the standard that did not contain BSA.

Table 23.--Preparation of BSA Standards

Tube	BSA/tube (µg)	BSA Stock (µl)	1 mM SDS (µl)
1	0	0	200
2	20	20	180
3	50	50	150
4	100	100	100
5	150	150	50
6	200	200	0

Sample Preparation:
Concentrated samples (homogenates, extracts, or column fractions) were diluted in 1 mM SDS; the dilution usually varied from 1:5 to 1:50. Dilute samples were not diluted prior to analysis for protein. Diluted or undiluted samples were assayed in triplicate if sufficient sample was available. A predetermined volume of sample was added to a tube, and the volume was brought to 200 µl using 1 mM SDS.

Assay:
To each standard and sample tube added 2.0 ml of "Solution C". Tubes were vortexed and set at room temperature for 5-10 minutes. Then 200 µl of "Solution D" was added to each tube. It is important that tubes were mixed quickly after addition of "Solution D", therefore tubes were vortexed in sets of 10-15 following the addition of "Solution D". Tubes remained at room temperature for 20-30 minutes. Solutions developed a blue color, the absorbance of which was determined at 700 nm using a Gilford Response spectrophotometer. Polystyrene disposable semi-micro cuvettes were used (1.5 ml, 1 cm light path). Standards were analyzed using the standard curve program on the spectrophotometer; this program automatically generates the curve and the corresponding linear equation with correlation coefficient. The samples are run following standard analysis according to the prompts on the spectrophotometer monitor. The program uses the standard curve information to calculate the protein (µg) per sample cuvette. Protein in the original sample is then calculated taking into account sample dilution prior to assay.

Coomassie Blue Dye Binding

Mix 600 mg of Coomassie Brilliant Blue G-250 with 1000 ml of a 2% perchloric acid solution. This solution is stable indefinitely. Filter to remove undissolved material. Use 2-3 ml of this solution per protein assay. this solution will color blue glassware and plasticware. This color can be removed with either methanol or a detergent solution.

Standard, Sample Preparation, and Assay

These steps were identical to the Lowry method with two exceptions. Following standard or sample preparation, 3 ml of Coomassie blue dye solution as added to each tube. Tubes were vortexed and maintained at room temperature for between 2 and 30 minutes. Standard and sample absorbance was determined at 595 nm.

APPENDIX B

APPENDIX B:
HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

Preparation of HPLC Mobile Phase

RP-HPLC Mobile Phase Composition

83% (v/v) 0.25 M NaH_2PO_4 plus 50 mM TEA in water (pH 3)
17% (v/v) Acetonitrile

To make 2 L of mobile phase: In a 2 L beaker equipped with magnetic stir bar, add 49.8 g sodium phosphate monobasic (NaH_2PO_4, fwt 120), 1500 ml Milli-Q water, and 7.0 ml of triethylamine (TEA). Stir until all solids are dissolved (15-30 minutes). Adjust pH to 3.0±0.1 using concentrated phosphoric acid. Transfer this solution to 2 L graduated cylinder an dilute to a final volume of 1660 ml using Milli-Q water. Transfer back to the 2 L beaker and mix. Add 340 ml of HPLC grade acetonitrile while mixing. Vacuum filter through a 0.2 µm nylon membrane filter (47 mm diameter). Stir under house vacuum in order to degas the mobile phase. The final mobile phase pH is approximately pH 3.4.

SCX-HPLC Mobile Phase

60% (v/v) 0.3 M NH_4Cl plus 0.2 M NH_4OAc in water (pH 4)
40% (v/v) Acetonitrile

In a 2 L beaker equipped with magnetic stir bar, add 19.4 g ammonium chloride (NH_4Cl, fwt 53.9), 18.5 g ammonium acetate (NH_4OAc, fwt 77.08), and 1000 ml of Milli-Q water. Stir for 15-30 minutes to dissolve solids. Adjust the pH to 4.0±0.1 using glacial acetic acid (90-120 ml required). Transfer this solution to a 2 L graduated cylinder and dilute to 1200 ml with Milli-Q water. Transfer back to the 2 L beaker, mix, and add 800 ml HPLC-grade acetonitrile. Mix, filter, and degas as described above. The final pH of the mixture is approximately pH 5.

Fluorescence Detector

Model: Perkin-Elmer LC240
Wavelengths: Excitation 310 nm, Emission 450 nm (optimal for 2,9-Me_2NH^+)
AF: usually set at 1024 (decreasing this value will increase sensitivity)
Response: usually set at 5
Note: after turning instrument on, press "58 control" to prevent lamp from automatically turning off after 30 minutes

Integrator (used with fluorescence detection)

Model: Shimadzu C-R1B Chromatopac Reporting Integrator
Usual settings are:
Width = 20 (width is set to peak width at 0.5*peak height for narrowest peak in trace)
Slope = 800-1000 (decreasing slope will increase detection of small changes from baseline -- if slope is too low, noise in baseline will be reported as peaks)
Speed (of chart) = 2
Att (attenuation) = 5-6 for normal ßC-NMT assays (decreasing attenuation value will increase sensitivity)
Min Area (minimum area) = variable (represents the minimum area for peaks that are to be included in the integrating report -- if this value is too low, the report will include many small peaks that really represent only noise
Stop Time = 15-20 minutes (the time that data collection is terminated -- depends upon compounds that are present)

Radioactive Detector

Model: Radiomatic Flo-One\ßeta (supported by Packard Instruments)
Program Disk is needed to boot system and change operating parameters, while the Run (data) disc is needed to store runs.

Determination of background is done with mobile phase and scintillation cocktail flowing through flow cell. The settings below were used for the background determination prior to the beginning of sample analysis:
Condition = H-3
Update Time = 10
Mode = Scaler
Results = Net cpm
Cell type = liquid
Cell size = 2.5
Auto pump = yes
Pump flow rate = 2.8
HPLC Flow rate = 0.8
Scaler time = 1
Spitter Ratio = 100
Fraction collector channel = 0

Discriminator settings =	Channel 1	Channel 2	Channel 3
	1-40	5-100	

Background subtract = 0

the background check was run for 10 minutes. Total cpm reported is divided by 10 to give the average cpm in minute (usually 40 cpm). this value is used in the "background subtract" field during sample analysis.

Typical instrument settings for sample analysis were:
Condition = H-3
Update Time = 10
Mode = Integrate
Results = Net cpm
Cell type = liquid
Cell size = 2.5
Auto pump = yes
Pump flow rate = 2.8
HPLC Flow rate = 0.8
Scaler time = 1
Spitter Ratio = 100
Fraction collector channel = 0

Discriminator settings =	Channel 1	Channel 2	Channel 3
	1-40	5-100	

Background subtract = 40
Minimum peak area: 80 (i.e., 2x background subtract)

APPENDIX C

APPENDIX C
SYNTHESIS OF 9-METHYLNORHARMAN HCL

The synthesis was a modification of that described by Rubottom and Chabala (1974).

Reaction

The reaction was carried-out in an oven-dried 25 ml round-bottom single-necked flask equipped with a magnetic stir bar. Norharman free base (NH, 678.5 mg, 3.95 mmole) was dissolved in 14 ml of hexamethylphosphoramide (HMPA) that had been stored over molecular sieves at least overnight. Sodium amide ($NaNH_2$, 1.04 g, 3.4 equivalents with respect to NH) was washed into the reaction flask with the aid of about 2 ml HMPA. The flask headspace was purged with nitrogen and immediately stoppered to minimize exposure of the reaction to moisture. The contents of the flask were stirred for five hours at room temperature. The mixture was light yellow, with a blue-tint at the air-mixture interface.

After the five hour stir, the reaction was chilled to 5-10 °C. Methyl iodide (CH_3I, 4.35 mmole, 271 µl, 1.1 equivalents with respect to NH) was quickly added. The mixture became blue and then green. The mixture was sampled after 15 minutes so that the extent of the reaction could be determined by SCX-HPLC (see Chapter 1 and Appendix B). After 15 minutes the reaction was green and 50% of the NH remained. After 30 minutes the reaction was brownish-green and 30% of the NH was still unreacted. An additional 61 µl CH_3I (0.98 mmole) was added and the reaction was stirred for 10 minutes at 5-10 °C. Little to no NH was detected by SCX-HPLC.

Reaction Work-Up

Milli-Q water (16 mL) was added to the reaction mixture; the reaction pH was 13.5. Reaction pH was adjusted to pH 7 using concentrated HCl. mixture was mixed vigorously and then transferred to a separatory funnel. Diethyl ether (Et_2O, 25 ml) was added to the contents of the separatory funnel. The funnel was inverted several times in order to extract the aqueous layer with the Et_2O. The two layers were allowed to separate and the Et_2O (upper) layer added to a 250-ml flask containing anhydrous magnesium sulfate ($MgSO_4$); the $MgSO_4$ served to remove water from the Et_2O extract. The lower aqueous layer was extracted four more times using 25 ml of Et_2O each time. All ether extracts were combined with the original extract over $MgSO_4$. The $Et_2O/MgSO_4$ mixture was filtered to remove $MgSO_4$. The Et_2O filtrate was evaporated to an oil using a Rotovap evaporator. Methanolic- HCl (2.7 ml) was added to the oil, followed by the addition of about 5-10 ml of Et_2O. The mixture was placed at -20 °C to expedite crystallization of 9-methylnorharman hydrochloride. The pale yellow crystals were filtered through Whatman 40 filter paper, and the crystals were washed with cold Et_2O. The crystals were dried overnight in a vacuum oven at 40-50 °C.

Yield: 495 mg (2.27 mmoles based on a molecular weight of 218)
Percent yield = (2.27 mmole/3.95 mmole)*100 = 57%
Purity: >95% by SCX-HPLC; structure confirmed by NMR (Dr. David Crumrine, Department of Chemistry, Loyola University Chicago)

APPENDIX D

APPENDIX D
Table 24.--ANOVA RESULTS

Enzyme Activity = f(F + R + G + F*R + F*G + R*G + F*R*G)

F = Fraction = Particulate or Supernatant
R = Brain Region = Substantia nigra or Putamen or Frontal Cortex
G=Group = Control or Parkinson's disease

ßC-2N-Methyltransferase Activity

Source	SS	df	MS	F-ratio	p-value
Main Effects	5488.349	4	1372.087	4.037	0.005
Fraction (F)	4077.090	1	4077.090	11.997	0.001
Region (R)	1421.680	2	710.840	2.092	0.131
Group (G)	3.665	1	3.665	0.011	0.918
2-way interactions	869.735	5	173.947	0.512	0.766
F*R	648.584	2	324.292	0.954	0.390
F*G	4.799	1	4.799	0.014	0.906
R*G	208.268	2	104.134	0.306	0.737
3-way interactions	73.634	2	36.817	0.108	0.897
F*R*G	73.634	2	36.817	0.108	0.897
Explained	6515.019	11	592.274	1.743	0.082
Residual	23110.105	68	339.854		
Total	29625.124	79	375.002		

ßC-9N-Methyltransferase Activity

Source	SS	df	MS	F-ratio	p-value
Main effects	3477.492	4	869.373	4.947	0.001
Fraction (F)	2522.520	1	2522.520	14.354	0.0003
Region (R)	719.763	2	359.882	2.048	0.136
Group (G)	253.178	1	253.178	1.441	0.234
2-way interactions	2357.925	5	471.585	2.683	0.028
F*R	217.100	2	108.550	0.618	0.542
F*G	408.632	1	408.632	2.325	0.132
R*G	1764.280	2	882.140	5.020	0.0091
3-way interactions	770.573	2	385.287	2.192	0.119
F*R*G	770.573	2	385.287	2.192	0.119
Explained	6728.328	11	611.666	3.481	0.001
Residual	12653.152	72	175.738		
Total	19381.480	83	233.512		

APPENDIX E

APPENDIX E
MATERIALS USED IN CHAPTER 6

Purified Bovine-Adrenal PNMT

Catalog # / lot #	P-8924 / 15H3891
Enzyme Commission #	EC 2.1.1.28
Source	bovine adrenal medulla
Activity	"one unit will convert 1.0 nanomoles of normetanephrine to metanephrine per hr at pH 8.5 at 37 °C"
Contents	"lyophilized powder containing approx. 70% protein (Biuret); balance is primarily phosphate buffer salts, pH 7.5: 1.1 mg solid, 24 units/mg solid, 32 units/mg prot."

LY134046 (PNMT Inhibitor)

Catalog # / lot no.	L-105 / GG-389A
Molecular weight	252.57
Chemical name	8,9-dichloro-2,3,4,5-tetrahydro-1H-2-benzazepine, hydrochloride salt

Norepinephrine (PNMT substrate)

Catalog # / lot no.	A-9512 / 36H0770
Molecular weight	319.3
Chemical name	(-)-arterenol, bitartrate salt, or 2-amino-1-(3,4-dihydroxyphenyl) ethanol, bitartrate salt

Octopamine (PNMT substrate)

Catalog # / lot no.	O-0250 / 128F0347
Molecular weight	189.6
Chemical name	1-(p-hydroxyphenyl)-2-aminoethanol, hydrochloride salt

DL-phenylethanolamine (PNMT substrate)

Catalog # / lot no.	H-1754 / 36H2514
Molecular weight	137.2
Chemical name	DL-β-hydroxyphenethylamine

GLOSSARY

Allosteric effector: a compound that binds to the enzyme at a site other than the active site, and affects the reaction catalyzed by the enzyme -- enzyme activity may be increased or decreased

Allosteric site: a site or sites on an enzyme, distinct form the active site, where compounds bind, and consequently affect the catalytic activity of the enzyme

Catalytic constant: k_{cat}, experimentally-defined the rate constant for formation of product [P] from the enzyme-substrate complex [ES] -- equivalent to turnover number

Catalytic site: equivalent to active site -- the site on an enzyme where substrate [S] binds and the actual formation of product [P] occurs

Competitive inhibitor: a compound that binds to free enzyme [E] at the active site in competition with the substrate [S] -- the inhibition may be overcome by increasing [S] -- inhibitor presence increases the K_M of the substrate, while V_{max} is unaffected

Enzyme-substrate complex: [ES], the concentration of enzyme [E] in a complex with the substrate [S]

Free enzyme: [E], the concentration of free enzyme, i.e., the concentration of enzyme not in a complex with substrate [S]

Inhibitor: [I], the inhibitor concentration, an inhibitor decreases the activity of an enzyme

k_1: rate constant for the formation of the enzyme-substrate complex from free enzyme [E] and substrate [S]

k_{-1}: rate constant for the dissociation of the enzyme-substrate complex [ES] to free enzyme [E] and substrate [S]

k_{-2}: rate constant for the association of free enzyme [E] and product [P] to form the enzyme-substrate complex [ES] -- this constant assumed to be negligible in simple Michaelis-Menten kinetics

K_i: the inhibitor constant, the dissociation constant for the inhibitor-enzyme complex

Michaelis constant: K_M, a function of kinetic constants experimentally equal to the substrate concentration at which the reaction velocity (v) is proceeding at one-half maximal velocity ($0.5*V_{max}$)

Maximum velocity: V_{max}, a kinetic constant which represents the maximum velocity for product [P] formation under stated reaction conditions -- at V_{max} the enzyme is said to be saturated with substrate [S]

Noncompetitive inhibitor: binds to the enzyme and inhibitor presence decreases V_{max}, but does not affect substrate K_M

Product: [P], the product concentration, i.e., the compound formed by action of an enzyme on a substrate, in an enzyme-catalyzed reaction

Substrate: [S], the substrate concentration in an enzyme-catalyzed reaction

Specific activity: the amount of product [P] formed by an enzyme per unit time per mass of protein (e.g., pmol/h/mg protein) -- more precisely the units of enzyme per mg protein

Turnover number: equivalent to k_{cat} -- the number of substrate molecules transformed per minute by a single enzyme molecule when the enzyme concentration is the rate limiting factor, formerly called the molecular or molar activity

Uncompetitive inhibitor: binds to enzyme and inhibitor presence decreases both K_M and V_{max}

Unit: U, the amount of an enzyme that will catalyze the transformation of one μmole of substrate [S] to product [P] per minute at a specified temperature (usually 25 °C), at a defined substrate concentration

Velocity: v, the rate at which substrate [S] is converted to product [P] in an enzyme-catalyzed reaction, units are in concentration per unit time (e.g., pmoles/hour)

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VITA

The author, Debra Ann Gearhart, was born in Markle, Indiana on August 18, 1958 to Fredrick and Kathryn Gearhart; she is the eldest of four children with brothers Jeffrey, Kentin, and Jay.

Debra was a 1976 graduate of Huntington North High School located in Huntington, Indiana. In the Fall of that year she entered the pharmacy program at Purdue University in West Lafayette, Indiana. Debra was initiated into the National Chapter of the Rho Chi Society Pharmaceutical Honor Society, Alpha Zeta Chapter there in November 1979. In May 1982 she obtained a Bachelor of Science degree in Pharmacy from Purdue.

She was employed by Eli Lilly and Company from January 1981 to July 1991. Her experience there was diverse as she held three different positions, including: a technician in an organic synthesis lab; an operator in the antibiotic production facility; and finally her primary position as a Pharmaceutical Chemist in Parenteral Product Research and Development.

In August 1991 Debra entered the Neuroscience Graduate Program at Loyola University Medical Center. In the Fall of 1992 she joined Dr. Michael Collins' laboratory in the Department of Molecular and Cellular Biochemistry where she began studies related to the brain-dependent N-methylation of ß-carbolines. While at Loyola, Debra was a teaching assistant in the Medical Neuroscience course, as well as a tutor in the Medical Biochemistry course.

Debra and her son, Joel, will relocate to Augusta, Georgia, where she will begin a postdoctoral fellowship in Dr. William David Hill's laboratory at the Medical College of Georgia. She will continue to pursue interests related to mechanisms underlying neurodegeneration in the central nervous system. Dr. Hill's main focus pertains to the study of neurofilament proteins, such as: (1) the role that biochemical modification of neurofilaments may play in the formation of Lewy bodies, and (2) identification of proteins that interact with neurofilaments.

DISSERTATION APPROVAL SHEET

The dissertation submitted by Debra Ann Gearhart has been read and approved by the following committee:

Michael A. Collins, Ph.D., Director
Professor, Department of Molecular and Cellular Biochemistry
Stritch School of Medicine, Loyola University Chicago

John M. Lee, M.D., Ph.D.
Assistant Professor, Departments of Pathology and Pharmacology
Stritch School of Medicine, Loyola University Chicago

Edward J. Neafsey, Ph.D.
Associate Professor, Department of Cell Biology, Neurobiology and Anatomy
Stritch School of Medicine, Loyola University Chicago

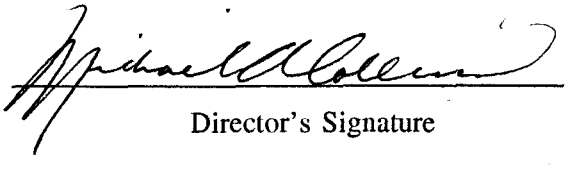
Richard M. Schultz, Ph.D.
Professor, Chairman, Department of Molecular and Cellular Biochemistry
Stritch School of Medicine, Loyola University Chicago

William H. Simmons, Ph.D.
Professor, Molecular and Cellular Biochemistry
Stritch School of Medicine, Loyola University Chicago

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the committee with reference to content and form.

The dissertation is, therefore, accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

3/25/97
Date


Director's Signature